Improvement of Endoglucanase Activity in *Penicillium oxalicum* ID10-T065 by Ultra Violet Irradiation and Ethidium Bromide Mutation

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Abstract

Penicillium sp. is known as a filamentous fungus that produces complete cellulase. This study aims to improve endoglucanase activity of *Penicillium oxalicum* ID010-T065 by mutation with ultra violet irradiation (with dose of 0.1 J/cm^2, 15 cm), ethidium bromide (10 µg/mL, 1 hour) and combination of both mutagens. The endoglucanase activity of all mutants was higher than that of the wild type (1.03 U/mL). Mutant UVEB-42 exposed to combine mutation showed the highest endoglucanase activity (2.76 U/mL) with a 2.70 fold increase. Mutant EB-45 (1.83 U/mL) exposed to ethidium bromide solution showed a 1.8 fold increase. Mutant UV-13 (1.72 U/mL) exposed to UV irradiation for 3 minutes showed a 1.7 fold increase. All mutants have optimum endoglucanase activity at 50 °C. Mutant UVEB-53 showed the highest thermostability by retaining 86 % of endoglucanase activity at 90 °C. The gene analysis of the endoglucanase I gene (*eg1*) showed 3 bases mutation in mutant UV-13 and UVEB-53 that changed proline to serine. Mutant EB-45 showed 4 mutated bases that changed valine to glysine and proline to serine. Two bases mutation in Mutant UVEB-53 changed proline to serine. Bases mutation which is occured in *eg1* gene could influence the enhance of endoglucanase activity in mutant.

Keywords: endoglucanase, mutation, ultra violet, ethidium bromide, *Penicillium*

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Introduction

Cellulases are ones of hydrolytic enzyme that can break β-1,4 glucan linkages. Based on the region of the substrate and hydrolysis products, cellulase is divided into three major groups, endoglucanase (E.C.3.2.1.4), exoglucanase or cellobiohydrolase (E.C.3.2.1.91), and β-glucosidase (E.C. 3.2.1.21) (Lynd *et al*., 2002). Complete cellulase consists of the three major enzymes that act synergistically to hydrolyse cellulose into glucose (Sukumaran *et al.,* 2005).

Endoglucanase is a key enzyme involved in cellulose hydrolysis. It cuts cellulose chain internally, in the main amorphous regions to produce oligosaccharides. Endoglucanase decreases polymerization degree of cellulose that consist of about 8,000-12,000 glucose units (Lynd *et al.,* 2002). The molecular mass of endoglucanase is around 30-55 kDa (Syed *et al.,* 2013). The cellulolytic system of *Penicillium* sp. is mainly constituted by endoglucanase and showed broad substrate specifity such as towards carboxymethyl cellulose, avicel, lichenan, and laminarin (Jeya *et al*., 2010). Fungal crude enzyme generally contains multiple cellulases, which support the complete depolymerisation of cellulose (Kim *et al.,* 2014).

Filamentous fungi secrete a wide range of cellulases. The enzymes are secreted outside the cells and robust (Seiboth *et al.,* 2011). *Penicillium* has shown strong ability to produce more balanced native lignocellulolytic enzyme system than *Trichoderma reesei*. Some unique features have been found in *Penicillium oxalicum,* including higher β-glucosidase activity, higher numbers of lignocellulolytic enzyme gene, and different

response of cellulase gene expression to some dissacharides (Liu *et al.,* 2013). The endoglucanases of *Penicillium* have been reported to have an extensive thermal stability with optimum temperature 50-70 °C (Meera *et al.,* 2010; Wei *et al.,* 2010). It is considered that thermophilic cellulases have commercial applications, as they are robust and resistant to high temperatures (Juture *et al.,* 2014).

Production of cellulase is a major factor in hydrolysis of cellulosic material. It is important to make the process economically feasible. For efficient cellulase production, strains can be improved by mutagenesis which is reported to be successful method. When fungus is exposed to mutagens at sublethal concentration, the rate of enzyme productions increase (Chand *et al.,* 2005; Li *et al.,* 2010). Syafriana *et al*., (2014) described that mutation by UV irradiation could improve β-glucosidase activity better than ethyl methyl sulfonate (EMS). Meanwhile, combined mutation (UV-EMS) showed a decrease activity of β-glucosidase.

The aims of this work were to screen the collection of *Penicillium* sp. for production of cellulose in carboxy methyl cellulose (CMC) medium, improve endoglucanase activity of selected isolate by exposing its spores to ultra violet (UV) irradiation and ethidum bromide (EtBr), and conduct sequence analysis of endoglucanase I encoding gene (*eg1*).

Materials and Methods

Chemicals and Microorganisms. CMC was purchased from Sigma, Potato Dextrosa Agar (PDA) was purchased from Oxoid. Potato Dextrosa Broth (PDB) were purchase from BD. EtBr, Congo Red, Tween-80, Dinitrosalyclic acid (DNS) reagents and all other reagents of analytical grade were purchased from Merck. The isolates of *Penicillium* sp. were obtained from Applied Microbiology Laboratory of The Research Center for Biotechnology, Indonesia Institute of Sciences (LIPI).

Screening Cellulase Activity of *Penicillium* sp. Twelfth isolates of *Penicillium* sp. were initially screened based on their ability to produce halozone on CMC agar medium containing 1 % CMC as the sole carbon source. The composition of the CMC Agar medium was as follow (g/L): 10 g CMC, 0.2 g $MgSO_4.7H_2O$, 0.75 g KNO_3, 0.5 g K_2HPO_4, 0.02 g $FeSO_4.7H_2O$, 0.04 g $CaCl_2$, 2 g yeast extract, 1 g glucose, and 18 g agar. The isolates were grown on CMC agar medium for 5 days at 30 °C. The cellulase activity was indicated as clear orange halos around the colony after staining with 0.5 % Congo Red solution for 30 minutes and washing three times with 2 % NaCl (Teather & Wood, 1982). Enzymatic Index (EI) was calculated by comparing the diameter of halozone to diameter of colony (Florencio *et al*., 2012).

Identification of Fungal Strain by 18S rRNA gene. The DNA of selected isolate was extracted using the method of Cenis *et al*. (1992). Polymerase chain reaction (PCR) was conducted using forward primer 18 F (5'-ATC TGG TTG ATC CTG CCA GT-3') and reverse primer 18 R (5'-GAT CCT TTC GCA GGT TCA CC-3'). Specifically, 1 µL extract DNA was added to the 24 µL PCR master mixture which consisted of 12 µL Go Taq Green Master Mix, 10 µL dH_2O, 1 µL forward primer and 1 µL reverse primer. PCR was conducted using a Thermal Cyclers (Techne Tc-5000, UK), undertaking the reaction mix using the following program: an initial denaturation step at 96 °C for 5 minutes, 30 cycles of denaturation at 96 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute, followed by final extension for 7 minutes at 72 °C to ensure full extension of the products. The amplified products were then purified and sequenced, after which the sequence was aligned using BLAST (Altschul *et al*., 1998) with similar well-known sequences obtained from GeneBank/DDBJ/EMBL database.

Strain Improvement by Mutation. Potential strain for cellulase production was selected for strain improvement by mutation. Isolate was grown on PDA slant for 7 days at 30 °C. The slant was added with 10 mL dH_2O contained 0.1 % Tween-80, and the suspension was made in which the number to 10^4 spores/mL as described by Syafriana *et al*. (2014). The spore suspension was subjected to mutagen. Three methods of mutation were applied for strain improvement, (1) UV mutation: 1 mL spore suspension ($53\text{-}66\times10^4$ CFU/mL) was exposed to UV irradiation (with dose of 0.1 J/cm, 15

cm) for 0, 3, 6, 9, 12, and 15 minutes. The duration of UV exposure was adjusted to get sublethal dose. (2) EtBr mutation: EtBr stock solution (10 µg/mL) was added to 200 µL spore suspension and kept at rotary shaker for 1 hour. The volume of EtBr was adjusted from 0.5, 1.0, 1.5, 2. 2.5 µL to get sublethal dose, (3) Combination mutation: 1 mL spore suspension was exposed to UV irradiation followed by incubated in EtBr solution for 1 hour. Optimal dose from UV and EtBr mutation was used for combination mutation. The treated spores (100 µL) was inoculated onto PDA and incubated for 3-7 days at 30 °C.

Isolation and Selection of Mutants. Mutant isolates were screened and chosen to be inoculated into CMC Broth medium. Mutants were selected on the basis of the diameter of hydrolytic zones surrounding the colonies.

Enzymes Production. A 100 mL of culture medium was inoculated with selected mutant from 5 days old culture of PDA plates and cultivation was performed at 26-28 °C with shaking at 60 stroke/minutes. After 5 days cultivation, the mycelium was filtered off, the culture filtrate was centrifuged at 8,000 rpm for 20 minutes at 4 °C, and supernatant was used as crude enzyme and stored at -20 °C.

Endoglucanase Assay. Endoglucanase assay was done using 1 % CMC as substrate according to Haggett *et al*. (1979) with slight modification. Enzymes activity was measured at pH 7.0. Specifically, 0.5 mL of substrate in 20 mM phosphate buffer pH 7.0 was put in a test tube, then 0.5 mL of crude enzyme was added into the tube and incubated at 30-90 °C for 30 minutes. After hydrolysis, the enzyme reaction was stopped by adding 1 mL of DNS. The absorbance of solution was read using spectrophotometer with λ 540 nm. The reducing sugar was estimated as glucose by Miller method (1959). One unit of endoglucanase activity is defined as the amount of enzyme required to liberate 1 µmol of glucose from appropriated substrate per minute under standard assay.

Optimum temperatures and pH of mutant crude enzyme. Endoglucanase activity was assayed under different temperatures (30, 40, 50, 60, 70, 80, and 90 °C) to determine the optimum temperature of endoglucanase activity.

Amplification, Sequencing, and Analysing of *eg1* gene. The *eg1* gene of wild type *Penicillium* sp. and its mutants were amplified using PCR from their genomic DNA with two flanking primers, pF (GCG TCT CCC CGT GTC TAC CTC CTC) and pR (AGA CTT GGC GTT GGC GAT GAC CTT). The PCR was conducted under following condition: 96 °C, 5 minutes; 30 cycles of 96 °C, 30 seconds; 58.5 °C, 30 seconds; 72 °C, 1 minute; and 72 °C, 7 minutes. The expected PCR products size is around 543 bp, which were then purified and sequenced. After that, the sequences of wild type and its mutants were alligned using software BioEdit Sequence Alignment (Jeya *et al*., 2010).

Results

Screening of Cellulase Using the Congo Red Test

Initial screening of twelve *Penicillium* sp. isolates was carried out using Congo Red test. The endoglucanase activity easily detected on agar plates by staining with Congo Red because dyes are adsorbed only by long chain polisaccharide. This test is based on observation of growth and measurement of clear zone that is used for calculation of the enzymatic index (EI). The strain that showed an EI higher than 1.50 was considered to be potential producer of celullases (Florencio *et al*., 2012).

Table 1. Enzymatic Index of isolates *Penicillium* sp.

No	Isolates	Diameter (cm)		EI (a/b)
		Halozone (a)	Colony (b)	
1	ID10-T001	-	-	-
2	ID10-T006	0.69	0.63	1.10
3	ID10-T009	0.84	0.45	1.87
4	ID10-T017	0.20	0.76	0.26
5	ID10-T018	2.25	0.75	3.00
6	ID10-T035	0.80	1.50	0.53
7	ID10-T043	0.30	1.80	0.17
8	ID10-T045	0.60	1.90	0.31
9	ID10-T047	0.89	0.80	1.11
10	ID10-T051	-	-	-
11	ID10-T058	0.50	1.00	0.50
12	ID10-T065	1.66	0.34	4.88

Table 1 shows results of enzymatic index obtained from the cultivation of the fungi in CMC Agar medium for 3 days incubation at 30 °C. Screening on solid substrate was considerably advantages because the natural habitats of filamentous fungi are solid media. The isolates that showed highest EI was isolate ID10-T065 (EI = 4.88) followed by isolate ID10-T018 (EI = 3.00). Eight isolates of *Penicillium* had EI lower than 1.5 indicated the strain was not considered as potential cellulase producer. The EI of isolate ID10-T001 and ID10-T051 unsuccesfully calculated because its colony growth covered whole plate and formed irregular colony.

Identification of the Isolate

Among 12 isolates, isolate ID10-T065 (EI = 4.88) was selected for molecular identification based on best enzymatic index. Sequencing of the 18S rRNA gene region of the isolate ID10-T065 was performed. The blast sequence of 18S rRNA gene showed a 99 % match with *Penicillium oxalicum* strain KF1529421 recorded in the GeneBank/DDBJ/EMBL database.

The sequence lengths of isolat ID10-T065 range is between 804-2,392 bp and the alignment range was 1,590 bp. Previously, Wu *et al.* (2003) reported the sequence length of 31 species of fungi by 18S rRNA gene was 1,744. The 18S rRNA gene sequences are suitable for defining species or genus. Their relatively slow rate of molecular evolution makes 18S rRNA gene a good candidate for finding consensus-conserved regions suitable for genus or higher taxonomical level detections.

The morphological characteristic of isolate ID10-T065 is the off-white color of aerial mycelium during initial stage of culture, turned green thereafter, and finally become deep green in color. The colony appeared flocculent, with smooth edge, and had a white or grayish yellow underside. While strains were observed under the microscope, it showed a mycelium of septate hyphae and stipes fruiting in *Penicilli* structures. The molecular and morphological characteristic indicated the strain belonged to *Penicillium* (Tiwari *et al*., 2011).

Mutagenesis

Penicillium oxalicum ID10-T065 was found to be the most potent fungal strain for cellulase production. Therefore, the isolate was chosen for strain improvement by random mutagenesis. Isolate ID10-T065 with the highest EI was subjected to mutagen. After that, mutants were picked up and isolated in PDA and CMC Agar medium. The selection of hypercellulase mutants was based on the ability to grow on sublethal dose and the higher of EI. It was reported that when fungi were grown with mutagens at sublethal concentrations, enzyme production increased. Sublethal dose enable a high frequency of mutation (Chand *et al*., 2005).

Spores viability of *P. oxalicum* ID10-T065 exposed to UV irradiation under different exposure times is illustrated in Figure 1A. The result indicates that survival rates declined with the extending of the irradiation time. With 3 minutes exposure to UV irradiation, the lethality of spores became 96 % with total colonies of 7×10^4 CFU/mL. Spores of *P. oxalicum* ID10-T065 were very sensitive to UV irradiation and no colony was found at 6 minutes of exposure. A three minutes UV exposure was chosen for combination mutation.

Spores viability of *P. oxalicum* ID10-T065 exposed to EtBr solution under different concentrations is illustrated in Figure 1B. Addition of 2 µL of EtBr solution into 200 µL spores and incubated for 1 hour, led to the lethality of spores, which became 88 % with total colonies 4×10^4 CFU/mL. Addition of higher volume of EtBr (more than 2 µL) resulted 100 % lethality of spores. EtBr of 2 µL was chosen for combination mutation.

Spores viability of *P. oxalicum* ID10-T065 exposed to combination of 3 minutes UV irradiation and EtBr under different concentrations is illustrated in Figure 1C. UV irradiation followed by soaking in 2 µL of EtBr resulted in the lethality of spores became 68% with total colonies of 10×10^4 CFU/mL. The use of 2.5 µL of EtBr solution caused the lethality of spores became 71 % and total colony 9×10^4 CFU/mL.

After mutagenesis, mutant colonies were picked up and screened. Table 2 shows the EI of mutants after 3 days cultivation in CMC Agar medium. Mutant EtBr showed the best EI followed by mutant UV. UV irradiation induced two adjacent pyrimidines (thymine and cytosine) to combine forming a pyrimidine dimer that resulted in error pairs in

DNA (Cupples, 2001). While EtBr caused stretching in DNA duplex by insertion between the DNA that induces frameshift mutation (Ennis, 2001)

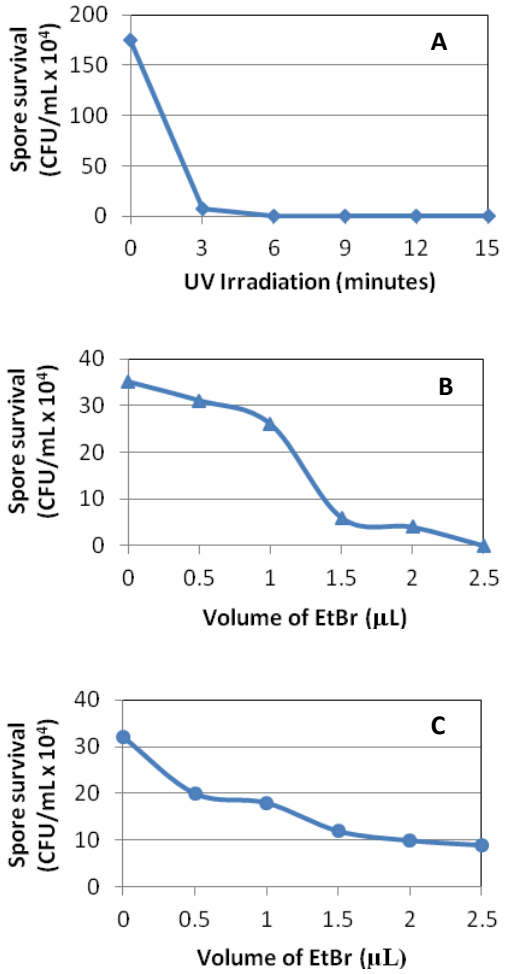

Figure 1. *P. oxalicum* ID10-T065 spores viability. (A) UV irradiation treatment at different exposure times, (B) EtBr treatment for different concentrations, and (C) Combination of UV irradiation for 3 minutes and EtBr for different concentration

Table 2. Enzymatic Index of Mutants *P. oxalicum* ID10-T065

No	Mutant	Diameter (cm)		EI (a/b)
		Halozone (a)	Colony (b)	
1	Control	2.50	0.80	2.13
2	UV-11	1.53	0.48	2.19
3	UV-12	1.63	0.49	2.33
4	UV-13	3.40	1.00	2.40
5	UV-14	1.73	0.53	2.26
6	UV-15	1.77	0.54	2.28
7	EB-42	2.80	0.80	2.50
8	EB-45	2.68	0.75	2.57
9	UVEB-42	2.13	0.85	1.50
10	UVEB-53	2.61	1.025	1.55

Mutants of *P. oxalicum* ID10-T065 show EI value around 1.50-2.57. The average EI of the ninth mutant was 2.17 and high EI was obtained by EB-45 (2.57) and EB-42 (2.50) (Table 2).

Selected mutant from UV treatment was UV-13 (EI = 2.40). Selected mutant from EtBr treatment was EB-45 (EI = 2.57) and EB-42 (EI= 2.50). Selected mutant from combination mutation were UVEB-42 (EI = 1.50) and UVEB-53 (EI = 1.55). Mutant combination showed the lowest EI than that of mutant UV and EtBr. However the highest endoglucanase activity was found in mutant UVEB-42 (Table 2). The contrast results may due to use of solid substrate (CMC Agar medium) for cellulase screening meanwhile for endoglucanase assay used liquid substrate (CMC Broth medium).

Optimum temperature

Optimum temperature of selected mutants is shown in Figure 2. The endoglucanase of mutants shows broad range activity from 30-90 °C and optimum at 50 °C. The endoglucanase activity of all mutants was higher than that of the wild type (1.03 U/mL). Increasing of endoglucanase activity of mutants may be due to enhancement of enzyme secretion because of the overexpression of cellulase genes. Enhanced of cellulase activity generally caused by increasing of enzyme secretion. Overexpression of gene increased enzyme secretion (Li *et al*., 2010).

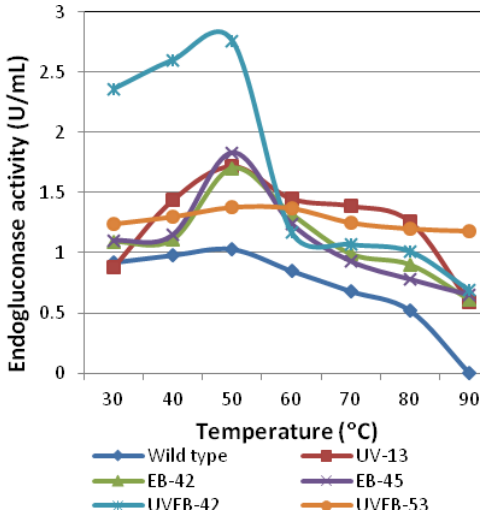

Figure 2. Endoglucanase activity profile of *P. oxalicum* ID10-T065 and its mutants under different temperatures.

The highest endoglucanase activity (2.76 U/mL) is obtained in mutant UVEB-42 that showed a 2.7 fold increase. Mutant UVEB-53 has the lowest endoglucanase activity (1.38 U/mL) but showed the best thermostability by retaining 86 % of its activity at 90 °C. These finding indicate that sequential treatment of UV and EtBr is suitable for the improvement of thermostability and endoglucanase production in *P. oxalicum* ID10-T065.

Mutant EB-45 (1.83 U/mL) showed a 1.8 fold increase of enzyme activity. Mutant UV-13 (1.72 U/mL) showed a 1.7 fold increase. Mutation has improved the endoglucanase activity 1.7-2.7 fold.

The pH of Mutants Crude Enzyme

Fungal crude enzyme generally contains multiple cellulases, which assist during complete depolymerisation of cellulose. Syed *et al*. (2013) reported that *Penicillium* sp. produced multiple cellulases consist of β-glucosidase (2.8 IU/mL), endocellulase (19 IU/mL), FPase (1.2 IU/mL) and xylanase (40 IU/mL).

Our research shows that mutagenesis influences pH of the mutant crude enzyme. Mutagenesis drives pH variation between wild type and mutant crude enzyme (Figure 3). The pH of mutants crude enzyme were 4.0-5.5 lower than wild type (6.0). Crude enzyme of mutant UVEB-42 with pH 4.0, which has the highest endoglucanase activity. Crude enzyme of mutant EB-42 dan EB-45 showed pH 5.5, while mutant UV-13 showed pH 5.0. Cellulase activity is sensitive to the pH of the culture. The effect of culture pH on the enzyme activity is significant. Presetyo *et al*. (2010) reported that CMCase activity was highest in a pH 6.0 culture. In the pH 4.5 the cellulase activity was similar to 4.0 and 5.5 (10.5 FPU/mL), meanwhile the cellulase production was significantly lower in the pH 6.5.

The drop in pH of mutant crude enzyme may arise as a result of formation of cellobiose, oxidized to cellobionolactone by cellobiose dehydrogenase. Cellobionolactone is subsequently hydrolysed to carboxylic acid (Beguin & Aubert, 1994).

Sequence Analysis of *eg1* gene

In a desire to further understand the enhancement of endoglucanase activity, the *eg1* gene of wild type *P. oxalicum* ID10-T065 and its mutant (UV-13, EB-45, UVEB-42, UVEB-53) were detected by amplification using PCR then sequenced. Analysis of *eg1* gene at position 373-915 confirmed 4 bases mutations in mutant EB-45, two bases altered in UVEB-53, meanwhile UV-13 and UVEB-42 showed three base alterations (Table 3).

Another factor that potentially can have profound affect on mutagenesis is the possible lesion of gene. Mutagenesis will affect the enzyme activity if mutation occurs in coding region. There are many opportunities for mutagenic DNA repair and replication for a damaged gene that is about to be replicated (Korogodin *et al*., 1991).

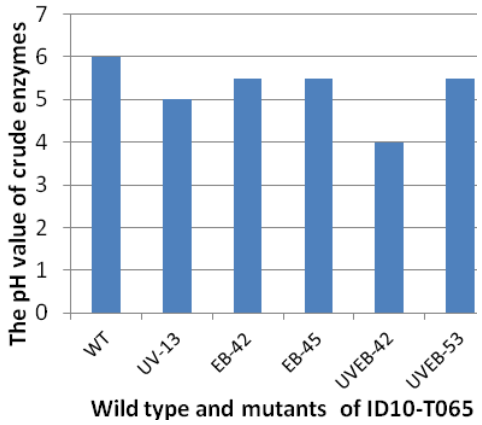

Figure 3. The pH value of crude enzyme in *P. oxalicum* ID10-T065 and mutants

Table 3. Bases mutation at *eg1* gene sequence of mutants *P. oxalicum* ID10-T065

Mutan	Base position	Base subtitution	Codon alteration
UV-13	463	T → G	CCT → TCG
	464	C → T	
	466	T → G	
EB-45	462	T → G	GTT → GGG
	463	T → G	CCT → TCG
	464	C → T	
	466	T → G	
UVEB42	463	T → G	CCT → TCG
	464	C → T	
	466	T → G	
UVEB53	462	T → G	GTT → GGT
	466	T → G	CCT → CCG

As shown in Table 3, in UV-13 the mutation of CCT to TCG in base position 463, 464, and 466 changed the amino acid of proline to serine. In EB-45, the mutation of GTT to GGG changed valine to glycine and mutation of CCT to TCG changed proline to serine. In UVEB-42, the mutation of CCT to

TCG changed proline to serine. In UVEB-53, the mutation of GTT to GGT changed valine to glysine meanwhile the mutation of CCT to CCG did not change amino acid (silent mutation). Silent mutation would have had little or no effect on the measured phenotype. Amino acid alteration of enzyme may increase or decrease the enzyme activity of mutant (Ennis, 2001). Base subtitutions are the most common form of mutation in bacteria and yeast (Smith *et al*., 1992)

Mutated bases of *eg1* gene mutants were marked with color (Figure 4). Specific primer was used to detect *eg1* gene of wild type and mutant of *P. oxalicum* ID10-T065 (UV-13, EB-45, UVEB-42 and UVEB-53). Based on reference strains (*Penicillium decumbens*), fragment size of *eg1* gene was 1429 bp (Wei *et al*., 2010). The segment of *eg1* gene that successfully amplified was 415 bp, which was about one third of *eg1* total fragment. The *eg1* gene alignment showed that mutation was in position 462-466 of the sequence, and there were no bases mutation in other position of sequence (467-920). The base alteration (2-4 base) may have important role to enhance the EG activity of mutant.

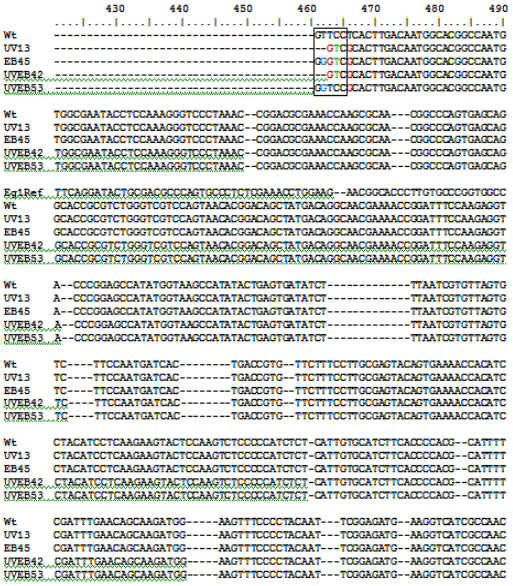

Figure 4. Sequence alignment of endoglucanase I (*eg1*) gene between wild type *P. oxalicum* ID10-T065 and mutants (UV-13, EB-45, UVEB-42 and UVEB-53). Mutated bases are shown in color.

Discussion

Strain improvement for cellulase production has been suggested as a key to reduced cost of bioconversion cellulose into glucose. *Penicillium* is known as potential cellulase producer that efficiently degrades cellulose (Picart *et al.,* 2007). Extracellular enzymes such as β-glucosidase, endocellulase, lipase, pectinase, protease, and xylanase can be secreted by *Penicillium* (Syed *et al.,* 2013). Screening of cellulase activity was done by incubating isolates in solid medium containing 1 % CMC. Congo Red dye was used for chromogenic reaction due to its better performance in extracellular enzyme activity detection with various fungal species (Yoon *et al*., 2007).

Potential cellulase producer is indicated by enzymatic index more than 1.5 (Li *et al.,* 2010). The isolates that had IE more than 1.5 were ID10-T065 (4.88) and ID10-T018 (3.00), even higher than RUT C30 (2.98) and *Trichoderma koningii* (1.90) (Florencio *et al*., 2012), *Aspergillus tereus* (1.32) and *Trichoderma* sp. (1.71) (Kader & Omar, 1998). Isolate ID10-T065 is found to be the most potent fungal strain for cellulase production based on the highest enzymatic index.

The degree of enzyme activity in *Penicillium* is expressed as strong, moderate, and weak by measuring the size of clear zone. When the clear zone was over 0.5 cm, the activity was treated as strong. Moderate activity was recorded when clear zone was 0.1-0.4 cm. When clear zone size was less than 0.1 cm, the activity was treated as weak (Yoon *et al.,* 2007). Based on the classification, eight isolates have strong activity and two isolates have moderate activity. Strong enzymatic activity was owned by ID10-T058 (0.50 cm), ID10-T045 (0.60 cm), ID10-T006 (0.69 cm), ID10-T035 (0.80 cm), ID10-T009 (0.84 cm), ID10-T047 (0.89 cm), ID10-T065 (1.66 cm), and ID10-T018 (2.25 cm), whereas moderate activity was shown by ID10-T017 (0.20 cm) and ID10-T043 (0.30 cm).

Based on the sequence of 18S rRNA gene analysis, ID10-T065 showed the highest similarity (99 %) to *Penicillium oxalicum* strain 114-2 (KF152942). Slow molecular evolution of 18S rRNA gene makes it a good candidate for finding consensus conserved

regions suitable for genus or higher taxonomical level detections (Wu *et al.*, 2003)

Spore of *P. oxalicum* ID10-T065 was subjected to mutagens at sublethal concentration. Survived spore of *P. oxalicum* ID10-T065 was declined by extending of the UV irradiation time and EtBr concentration. Shafique *et al.* (2011) reported the increasing cellulase activity of mutant *T. viride* by increasing of UV duration and EMS concentration. Killing rate of *Trichoderma viride* spores was nearly 100 % by 1.5 minutes UV exposure (Li *et al.,* 2010) and killing rate of *Humicola insolens* was 95 % after 75 minutes UV exposure (Javed *et al.,* 2013).

The endoglucanase activity of mutant and wild type *P. oxalicum* ID10-T065 was recorded over a broad range of temperature (30-90 °C) with the optimal activity at 50 °C and declined thereafter. Temperature affects the kinetic energy of molecules, including biomolecules such as proteins, the collision and reaction rates, and the strength of molecules interactions (Elias *et al.,* 2014).

In general, Fungi have their optimum temperature for enzyme activity at 50 °C (De Castro *et al*., 2010). Endoglucanase activity was increased by elevating of temperature up to 50 °C. Similar optimum temperature (50 °C) was found in *Penicillium occitans* (Chaabouni *et al.,* 2005), *Penicillium notatum* (Das *et al*., 2012), *Chaetomium thermophile* (Naim & Jamil, 2007), and *Neurospora crassa* (Yazdi *et al*., 1990).

The highest endoglucanase activity was obtained in mutant UVEB-42 (2.76 U/mL) from combination mutation. These finding indicates that the simultaneous treatment of UV and EtBr are suitable for improvement endoglucanase activity of *Penicillium*. Javed *et al*. (2011) reported that mutation based on the alternative UV and chemical treatment (EMS, EtBr, NTG) in *Humicola insolens* has resulted in highly stable mutant passed through generation by evaluating the cellulase production for one year. The best mutant has endoglucanase activity 1.7 U/mL.

Ultra violet irradiation induces both substitution and deletion mutation of the bases, EtBr solution which might have caused permanent change in DNA sequence. Strain improvement using UV mutagenesis developed mutant with 57.4% higher endoglucanase (Kumar, 2015). Ultra violet mutation improved endoglucanase activity of *Aspergillus niger* to 1.4 fold (Irfan *et al*., 2011).

Mutant *Aspergillus* sp. XTG-4s that was improved for endoglucanase production by sequential treatments by two repeated round of ϒ-irradiation, UV treatment, and four repeated rounds of treatment with nitrosoguanidine (NTG) increased 2.03 fold from 18.73 U/ml to 37.86 U/ml. Mutant XTG-4s was stable after subculture for 19 times (Vu *et al*., 2009).

Mutant UVEB-53 showed an extensive thermal tolerant by retaining 86 % of endoglucanase activity at 90 °C for 30 minutes. Increasing thermostability in mutant UVEB-53 may be due to genetic alteration after mutagenesis. Fawzi and Hamdy (2011) reported the partially purified mutant CMCase of *Chaetomium cellulolyticum* was more stable than the wild type. Mutant CMCase retained its original activity after heating up to 60 °C for 1 hour and 50 °C for 1.5 hour. Meanwhile, wild type CMCase retained its original activity at 50 °C only for 30 minutes. The cellulases were found to be highly thermostable with no loss in enzyme activities at 80 °C and 90 °C (Kumar, 2015).

The factors which are known to affect thermostability of enzyme are electrostatic and hydrophobic interaction, hydrogen and disulphide bonds, overall rigidity, compactness, glycosilation and methal binds (Voutilainen, 2011). Single mutation can significantly increase the thermostability of cellulase and their optimal activities. A cystein to serine mutation of cellobiohydrolase resulted in an increased thermostability by 8 °C and a 10 fold increase in expression (Heinzelman *et al*., 2009). Increasing of enzyme thermostability is estimated as consequences of many small cumulative changes in the protein structure. Elias *et al.* (2014) mentioned that thermophilic enzyme exhibits a higher structural packing and lower configurational flexibility than mesophilic enzymes. Because of its ability to hydrolyze cellulose at temperatures above 50 °C, endoglucanase was regarded as thermophilic enzyme and potentially used as excellent tool for industrial hydrolysis of cellulose.

To determine mutation in *eg1* gene, sequence of wild type and mutants of *P. oxalicum* ID10-T065 were aligned and compared. The *eg1* gene alignment shows that mutation was in position 462-466 of sequence, and there were no mutation in other position of

sequence (467-920). Base subtitution changed codon which then changed amino acid of *eg1* gene.

Ultra violet and ethidium bromide mutagenesis caused base mutation in *eg1* gene. UVC rays can be entirely absorbed by DNA. When UVC rays passed through DNA, the atoms of DNA molecule are excitated. Losing of electrons caused covalent bonds between DNA bases changed and induced two adjacent pyrimidines (thymine and cytosine) to combine forming a pyrimidine dimer. If DNA damage is not repaired immediately, DNA polymerase would not replicate the damaged strand that induced error in pairs DNA. DNA repair mechanism plays an important role to prevent mutation (Cupples, 2001).

The insertion of EtBr molecules causes a stretching of the DNA duplex so the DNA polymerase made mistake into inserting an extra base opposite an intercalated molecule. The result is that intercalating agents cause frameshift mutation (Ennis, 2001).

The consequences of base mutations in protein coding regions of a gene depend on the substitution and its locations. Substitution which is occured in the active site of enzyme could deactivate enzyme. Substitution in the outside of gene did not affect transcription, considered as a silent mutation (Madigan *et al.* 2012).

Alteration in the amino acid of enzyme may increase or decrease the enzyme activity of mutant (Ennis, 2001). This reseach shows that bases mutation induced changes on amino acid. Li *et al*. (2010) reported that base mutation in *eg1*gene of mutant *Trichoderma viride* that change amino acid lead to enhanced cellulase production.

Mutation in *eg1* gene of mutant *P. oxalicum* ID10-T065 have enhanced EG activity up to 1.3-2.7 fold increase. The increase of EG activity related to protein secretion. Ribeiro *et al*. (2013) reported that increasing activity of endoglucanase, β-glucosidase, and α-amilase in mutant *Ashbya gossypi* was along with enhanced of protein secretion. Mutant *P. decumbens* produces more secreted protein especially cellulase and hemicellulase. Mutant also showed enhanced supply of amino acid and decreased synthesis of secondary metabolites (Liu *et al*., 2013).

Genes are segments of DNA that consist of a number of codons encoding specific protein. Generally, gene length is about 1000 bp. The endoglucanase gene of *P. oxalicum* itself sized 1400 bp (Leylaie *et al.* 2013), whereas *P. decumbens* sized 1425 bp (Wei *et al*. 2010), *Volvariella volvacea* sized 1167 bp (Ding *et al*. 2001), *A. Fumigatus* sized 993 bp (Meera *et al*. 2011), and *A. Nidulans* sized 1228 bp (Chikamatsu *et al*. 1999). In this study, the segment of *eg1* gene that succesfully amplified was 415 bp, which was about one third of *eg1* total fragment.

Mutation that induces EG activity may have occured in other endoglucanase genes of mutant *P. oxalicum* ID10-T065. Martinez *et al.* (2013) reported the total endoglucanase genes in some fungi includes *Aspergillus nidulans* (14), *A. Fumigatus* (16), *A. Oryzae* (13), *T. Reesei* (8), and *P. crysogenum* (19). Therefore, gene analysis of mutants needs to be conducted in other segments that have not covered by specific primer. Analysis of the promoter area also needed to be conduct due to mutation in promoter could affect the transcription of cellulase significantly. Mutation in promoter enhanced the rate transcription by increasing the chances of RNA polymerase binds to the promoter (Doelle, 1994).

Le Crom *et al*. (2009) performed sequencing to identify mutation in the genomes of two hyper-producing strains (NG14 and RUT30). They detected surprisingly high number of mutagenic events: 233 single nucleotides variants, 15 small deletions, and 18 large deletion, leading to the loss of more than 100 kb of genomic DNA. Li *et al.* (2010) reported that enhancement enzyme secretion in mutant *T. viride* was caused by the increase of enzyme secretion and not related to the speed of growth. Liu *et al.* (2013) reported that increase of cellulase activity caused by alteration in the composition of enzyme secretion. The proportion of cellulases and hemicellulase in mutant *P. decumbens* increased, while the production of amylases, protease, and other proteins decreases.

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