Enhancement of β-Glucosidase Activity in *Penicillium* sp. by Random Mutation with Ultraviolet and Ethyl Methyl Sulfonate

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Abstract

The genus *Penicillium* has a potential ability to produce β-glucosidase. The aim of the study was to improve the β-glucosidase activity of *Penicillium* sp. ID10-T065 with physical (Ultraviolet = UV), chemical (Ethyl Methyl Sulfonate = EMS), and combined mutation (UV-EMS). The spores of *Penicillium* sp. ID10-T065 were exposed into UV irradiation for 3 minutes with dose of 0.1 J/cm^2 and 13 cm of distances. Chemical mutation was done by treated spores into 3% of EMS solution for an hour. Combined mutation of UV and EMS were also performed by UV for 3 minutes (0.1 J/cm^2, 15 cm) and continued with soaking into 2-3% of EMS solution. The developed mutants were screened, selected and assayed. Comparison of enzyme activities with the wild- type (1.78 U/ml), mutant UV13 (5.53 U/ml) showed a 3.1 fold increase; mutant EM31 (4.26 U/ml) showed a 2.4 fold increase. Meanwhile, mutant UM23 obtained from the multiple exposures showed a decreased activity (1.75 U/ml). Mutant UV13 showed the best enzyme activity to be considered as a potential strain for β-glucosidase producer. This result needs to be further elaborated especially on its genetic stability studies in order for the ascertained as a stable mutant.

Keywords: β-glucosidase, UV, EMS, *Penicillium*

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Introduction

Cellulases are ones of the largest industrial enzymes worldwide due to its application in food, textiles, feed, detergents, paper and bioethanol industry (Bhat, 2000; Wilson, 2009). Cellulases are responsible for the conversion of cellulose into glucose by hydrolysis β-1,4 glycosidic linkage (Bayer *et al.*, 1998). There are three types of cellulase enzymes: first is endoglucanase (EG) which hydrolyzes the internal glycosidic linkage and releasing new terminal ends; second is cellobiohydrolase (CBH) which hydrolyzes the new cellulose chain and releases cellobioses as the main products; and the third is β-glucosidase (BGL) which hydrolyzes cellobiose into glucose. These three types of enzymes act synergistically (Sukumaran *et al.*, 2005; Mathew *et al.*, 2008; Singhania *et al.*, 2013). Among them, BGL plays a key role for complete hydrolysis of cellulose to glucose because it is involved in the final step of cellulose degradation to glucose (Iwashita *et al.*, 1999; Bai *et al.*, 2013; Sørensen *et al.*, 2013).

BGL can be found in microorganisms such as bacteria and fungi (Lynd *et al.*, 2002). BGL from fungi are preferable than bacteria because its high activity also its thermal and pH stability (Ramani *et al.*, 2012). Among other fungi, *Aspergillus* is a well known fungus which produces high BGL activity (Adsul *et al.*, 2009). In the last few years, BGL activity from other fungi such as that found in the *Penicillium* has been extensively studied because of its potential ability in producing complete types of cellulase enzyme, especially in producing higher BGL than other fungi (Ramani *et al.*, 2012; Sørensen *et al.*, 2013). Syed *et al.* (2013) reported that strain *Penicillium* sp. CPF2 has the BGL activity about 2.8 IU/ml. That amount of activity is 9 times higher than that from BGL activity of

Rut C30 strain (0.3 IU/ml), the most potential mutant of *Trichoderma reesei* (Peterson & Nevalainen, 2012).

Production costs of enzymes have become major problems in industrial application. Thus we need to improve the efficiency and cost effective production of enzymes. According to Vu *et al.* (2009), and Yao *et al.* (2012), one of the ways to increase enzyme activity is by doing random mutation whether physically or chemically. UV irradiation is known as one of the most effective physical agent to enhance enzyme activities in fungi (Prabakaran, *et al.*, 2009). The specific action of UV to cell is on adjacent pyrimidine bases on the same strand to form a dimer which called pyrimidine dimer. Pyrimidine dimer can cause a lesion in DNA helix structure and inhibit the next replication process (Hogg, 2005; Pfeifer, *et al.*, 2005). One of the effective chemical agent in induced mutation to fungi is EMS. EMS is an alkylating agent which acts by alkylating guanine or thymine at the oxygen atom involved in hydrogen bonding. This action will caused impairment in DNA base-pairing (Sega, 1984; Hogg, 2005).

Some studies using UV irradiation to enhance enzyme activities in fungi has been reported by Dillon *et al.* (1992), Kuhad *et al.* (1994), Ike *et al.* (2010), and Javed *et al.* (2011). Another study about EMS effect in enzyme activities has been reported by Duarte *et al.* (2011), Ramzan *et al.* (2013), and Ribeiro *et al.* (2013). Besides mutation with single mutagen, the combined mutation of both UV and EMS can induce wide genetic variabilities in fungi (El-Bondkly & Keera, 2007). Adsul *et al.* (2009) and Liu *et al.* (2011), have investigated ultraviolet (UV) and ethyl methyl sulfonate (EMS) mutation effects against cellulase enzymes in *Penicillium janthinellum* NCIM 1171 and *Penicillium decumbens* strain ML-017. Their results showed that cellulase activities in each strain were improved around 1-1.5 times compared to their wild-type.

The aim of the present study was to improve the BGL activity of *Penicillium* sp. ID10-T065 through physical (UV), chemical (EMS), and combined mutation (UV-EMS). We also examined the BGL activities from mutants and wild-type when inoculated in two different substrate concentrations. In this work we find out that different concentrations of substrate can increase enzyme activities largely. In this report we focused on the amount of mutation that affect the BGL activity.

Materials and Methods

Microorganisms. *Penicillium* sp. ID10-T065 was obtained from Laboratory of Applied Microbiology, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI).

Media and chemicals. The media used were *Potato Dextrose Agar* (PDA) [Difco], *Carboxy Methyl Cellulose* (CMC) [Sigma] Agar, and Liquid Basal medium which contain 0.1% and 1% cellobiose [Merck]. The chemicals used were cellobiose, glucose, EMS, tween-80, DNS, and phosphate buffer pH 7.0.

Preparation of spore suspension. The *Penicillium* sp. ID10-T065 isolate was grown in a PDA slant until sporulated (approximately 7-12 days). Ten ml of distilled water containing 0.1% tween-80 was added onto a PDA slant culture as spore suspension. The spore suspension was scraped using a loop needle and homogenized with vortex. The spore suspension was then diluted to 10^{-4}.

UV mutagenesis. UV mutagenesis was modified from Rajeshkumar & Ilyas (2011) and Radha *et al.* (2012) methods. One ml of spore suspension (10^4 CFU/ml) was transferred into an empty Petri dish. Spore suspension was exposed to UV light by using UV Crosslinker which emitted 0.1 J/cm^2 radiation in 13 cm of distance from the lamp. Variation of the incubation time was done in 3 minutes increments, i.e. 0, 3, 6, 9, 12, and 15 minutes until killing rate reached 90-99%.

Treated spore from each mutant was transferred into an Eppendorf tube and after that centrifuged for 5 minutes at 13,000 rpm. A hundred µl of spore sediment was spread into a Petri dish which contained PDA and incubated for 7 days at 30ºC.

EMS mutagenesis. EMS mutagenesis was based on Radha *et al.* (2012) with modification. A hundred µl spore suspension (10^4 CFU/ml) in PDA slant was transferred into 6 Eppendorf tubes. Each tube contained different concentration of EMS solution (v/v)

(0%, 1%, 2%, 3%, 4%, and 5%). Spore suspension and EMS solution were homogenized and incubated for 1 h in a rotary shaker (60 strokes/minutes) at 26-28^{o}C.

Treated spore was washed twice with 500 µl distilled water and centrifuged for 5 min at 13,000 rpm. Spore sedimentation as much as 100 µl furthermore was transferred into a Petri dish containing PDA and incubated for 7 days at 30^{o}C.

UV and EMS mutagenesis (combined mutation). The combined mutation was done by combination treatment from UV and EMS exposure above which showed generated mutant. Spore suspension (10^4 CFU/ml) was first exposed to UV irradiation for 3 min (0.1 J/cm^2, 13 cm apart from the lamp). The treated spore was then inoculated into Eppendorf tubes which contained 1%, 2% and 3% of EMS solution (v/v). Treated spore was incubated for 1 hour in a rotary shaker (60 stroke/min) at 26-28^{o}C. Exposed spore was washed with 500 µl distilled water and centrifuged for 5 min at 13,000 rpm. Spore sedimentation as much as 100 µl was then transferred into a Petri dish containing PDA and incubated for 7 days at 30^{o}C.

Screening of *Penicillium* sp. mutants. Mutants were screened using Teather and Wood (1982) method. Surviving spores which developed in PDA plates were inoculated onto the plate-screening medium contain 1% CMC and incubated at 30^{o}C for 4-7 days (until sporulated). Sporulated mutants were stained with 0.5% Congo Red for 30 min and washed with 2% NaCl solution. Mutants were selected based on Enzymatic Index (=EI): the ratio of clearing zone (halozone) diameter that developed and colony diameter (Florencio *et al.*, 2012).

Enzyme production and bioassay. BGL activity was determined by using Haggett *et al.* (1979) methods with modification according to Zhang *et al.* (2009). Selected isolates were inoculated into a liquid medium which contained 0.1% and 1% substrate (cellobiose). The cultures were incubated at 26-28^{o}C for 5 days in a rotary shaker (60 strokes/minutes). The cultures were afterwards centrifuged at 8,000 rpm for 20 minutes. Supernatant was subsequently filtered as crude enzymes.

Supernatants were assessed for BGL activities using cellobiose as their substrates (in phosphate buffer 20 mM, pH 7.0). The mixture reactions were incubated at water bath shaker for 30 min at 30^{o}C. Enzymatic reactions were stopped by boiling at 100^{o}C using DNS method (Miller, 1959).

The absorbance was measured by spectrophotometer at 540 nm. The enzyme activity was calculated as U/ml. Units of BGL were defined 1 µmol of glucose released per minute under assay conditions.

Results and Discussion

Mutagenesis

Random mutagenesis is a simple method to attain genetic variation and functional modifications of an organism (Chandra *et al.*, 2009). Here, we report mutagenesis of *Penicillium* sp. ID10-T065 using UV, EMS and combination of those two mutagens.

The result using mutagenesis UV irradiation showed that spore killing rates achieved 100% at 6 minutes exposure. *Penicillium* sp. reached 96% mortality when exposed at the minutes 3 (7 colonies) (Table 1). Meanwhile the killing rate of 100% using EMS mutagenesis was achieved when spore exposed to solution which contain 4% of EMS. Spore exposures to EMS solution with 2 and 3% concentrations generated 2 and 1 colonies respectively (97-99% killing rate) (Table 2). Combination of UV-EMS mutagenesis displayed that on the treatment 2 (3 minutes UV, 2% EMS concentration) and treatment 3 (3 minutes UV, 3% EMS concentration) spore resulted in killing rates only reached 72-78% (10 and 8 colonies, respectively) (Table 3).

Screening of mutants

Total mutants that generated from the UV, EMS, and UV-EMS treatment were 57 isolates. We selected several mutants randomly to screen their cellulolytic activity. Fifteen isolates (14 mutants + 1 wild-type) were analyzed on the ability of generating halozone using Congo Red method (Teather & Wood, 1982). The halozone formation indicated that the isolates can hydrolyze the polysaccharides into their monomers unit by producing hydrolysis enzymes (Castro *et al.*, 1995). The halozone was then converted onto

EI value. The potential isolate showed EI value bigger than 1.5 cm (>1.5 cm) (Florencio, *et al.*, 2012). Isolates which have the highest EI from each treatment were chosen for BGL activity analysis.

Table 1. Spore survival upon UV treatment

Treatment (minutes)	Colony (CFU/ml)	Killing Rate (%)
0	175	0
3	7	96
6	0	100
9	0	100
12	0	100
15	0	100

Table 2. Spore survival upon EMS treatment

Treatment (concentration) (%)	Colony (CFU/ml)	Killing rate (%)
0	76	0
1	6	92
2	2	97
3	1	99
4	0	100
5	0	100

Table 3. Spore survival upon UV-EMS treatment

Treatment (minutes — concentration (%))	Colony (CFU/ml)	Killing rate (%)
0 — 0	37	0
3 — 1	31	16
3 — 2	10	73
3 — 3	8	78

We screened 5 mutants from the UV treatment for Congo Red Method. The mutants are UV11, UV12, UV13, UV14 and UV15. Among the 5 mutants, UV13 has the highest EI (2.40), while others only 2.21 (UV11); 2.32 (UV12); 2.30 (UV14); and 2.27 (UV15) (Table 4).

We selected 3 mutants from EMS exposure mutants. We investigated two mutants (EM21 and EM22) from treatment 2 (2 µl EMS) and one mutant (EM31) from treatment 3 (3 µl EMS). Among those three mutants, EM31 showed the highest EI ratio (2.73), while EM21 only 2.38 and EM22 2.45 (Table 4).

There are six mutants from combination treatment of UV and EMS that we screened. Mutant UM23 showed the highest EI ratio (2.66) among 5 other mutants 2.52 (UM21), 1.39 (UM22), 2.62 (UM31), 2.52 (UM32), and 0.89 (UM33) (Table 4).

Table 4. Enzymatic Index (EI) of wild-type and mutants

Treatment	Code of mutant	EI (cm)
Wild-type	Wt	2.13
UV	UV11	2.21
	UV12	2.32
	UV13	2.40
	UV14	2.30
	UV15	2.27
EMS	EM21	2.38
	EM22	2.45
	EM31	2.73
UV-EMS	UM21	2.52
	UM22	1.39
	UM23	2.66
	UM31	2.62
	UM32	2.52
	UM33	0.89

BGL activity

We selected one potential mutant from each treatment (UV, EMS, UV-EMS) according to screening analysis. The three mutants were UV13, EM31 and UM23. We examined the BGL activities from two different concentrations of substrate.

According to Breuil *et al.* (1986), there are several substrates which commonly used for BGL activity assay. One of well-known substrate is cellobiose (Breuil *et al.*, 1986; Dasthban *et al.*, 2010). Cellobiose is commonly used in BGL assay because it also known as one of the natural inducer of cellulase (Mandels & Reese, 1960). Breuil *et al.* (1986) also stated that when cellobiose concentration is being elevated then the BGL activity will increase too.

Our research also proved similar outline. We inoculated the selected mutants and wild-type isolate into two different substrate concentrations. One production medium contains 0.1% cellobiose and the other medium contains 1% cellobiose. The results from this experiment showed that the activities of the mutants grown in 1% cellobiose medium were higher than those in 0.1% cellobiose, except for mutant UM23 (Figure 1). Roy *et al.* (1998) reported that BGL activity in fungi was increased linearly with increasing concentration of the inducer (substrate).

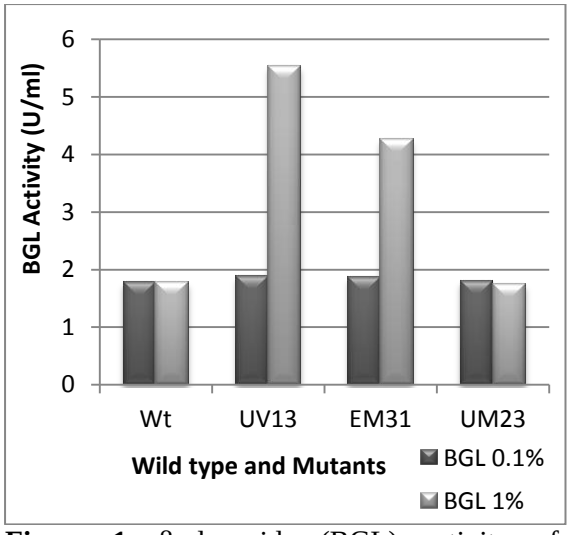

Figure 1. β-glucosidase(BGL) activity of wild-type and mutants in 0.1 and 1% cellobiose

The differences in activities between mutants and wild-type were also analysed. In the first substrate containing 0.1% cellobiose, the results showed that the three mutants (UV13, EM31 and UM23) had higher activities compared to the wild-type strain. Different enzyme activity pattern was shown when the isolates were grown in a medium production containing 1% cellobiose. In this condition, two mutants (UV13 and EM31) showed higher activities compared to the wild-type, while mutant UM23 showed lower activity than that found in wild-type. The most significant improvement was shown by the mutant UV13 while the lowest one was shown by mutant UM23.

Figure 1 shows that UV mutation could improve BGL activities better than EMS and combination of UV-EMS, whereas the combination of UV-EMS mutation had minor effect in increasing the activity of the enzyme. Ager and Haynes (1990) stated that UV is more efficient mutagen than EMS. Ultraviolet (UV) light can cause DNA damage and induce mutation (Ikehata & Ono, 2011). Mutation through UV irradiation can alter the pyrimidine structure (Cytosine = C or Timine = T) which mostly cause pyrimidine dimer. Pyrimidine dimer can damage double helix of DNA and inhibit the next replication process (Pfeifer *et al.*, 2005; Irfan *et al.*, 2011). According to Pfeifer *et al.* (2005) and Agrawal *et al.* (2013), UV light is one of potential and effective mutagen to enhance commercial enzymes activities.

Ethyl Methyl Sulfonate (EMS) known as an alkylating agent that has been recognized to be mutagenic in any genetic assessment from viruses to mammals (Sega, 1984; Hogg, 2005). Mutant EMS (EM31) showed less activity enhancement compared to UV13. The enhancement is about 0.08 U/ml in 0.1% cellobiose. Significant improvement emerged when the mutants were cultivated in an elevated substrate (1% cellobiose). The enhancement of the activity is about 2.48 U/ml.

The *Penicillium* sp. was also mutated by combination of UV and EMS exposure. The result showed that the mutation by the combination of UV-EMS in *Penicillium* sp. was ineffective. The BGL activities of mutant UM23 had the lowest activity among other mutants and also demonstrated unstable BGL activities. This characteristic was shown by its activity which sometimes higher than that in wild-type but lower the other time. This phenomenon possibly occurs because mutant UM23 did not reach 90-99% of killing rate when it was first mutagenized. In the opposite, mutants which generate from UV and EMS reached the sublethal concentration until 96-99%. Chand *et al.* (2005) reported that mutation approach using sublethal concentration can generate stable mutants and increase enzyme production.

Conclusion

UV mutagenesis was found to be the method of choice of producing b-glucosidase mutants from *Penicillium sp*. The ease of mutagen application with high spore killing rate resulting in the mutant selection with higher b-glucosidase activity compared with the EMS treatment and its combination with UV.

In view of the DNA repair mechanisms that would occur in every transient mutation, this result however, needs further studies especially on the elaboration of the genetic stability studies to ascertain a stable mutant. Subsequent molecular analysis should also be done to pinpoint the position of base changes.

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