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Beta-Glucosidase 1 (bgl1) Gene Analysis in Mutant and Wild-type of *Penicillium* sp. ID10-T065

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ABSTRACT

In the previous study, *Penicillium* sp. ID10-T065 has been mutated using ultraviolet (UV), ethyl methyl sulfonate (EMS) and the combination of UV-EMS to increase Beta-glucosidase (bgl) activity. There were three mutants selected, UV_{13} (UV mutant), EM_{31} (EMS mutant), and UM_{23} (UV-EMS mutant). This study examined the mutations in the bgl gene encoding (bgl1) as well as sequence differences between mutants and wild-type of *Penicillium* sp. ID10-T065. The gene analysis was performed by PCR amplification and sequencing of the bgl1 gene. The results of bgl1 gene sequences (600 bp) from mutants were aligned with the wild-type, it was discovered that there were base alterations from position 2025 to 2050. Mutant UV13 showed the highest base alterations (7 bases) which occurred at position 2027 (T→C); 2036 (T→G); 2040 (T→G); 2047 (G→C); and 2048-2050 (TTG→GGA). Mutant EM31 showed alterations in five bases at positions 2034 (G→A), 2036 (T→G), 2037 (G→C), 2044 (G→C), and 2047 (G→T). Mutant UM23 showed two base alterations at position 2025 (T→A) and 2037 (G→C). UV irradiation and EMS mutation resulted in transition and transversion DNA, whereas the combination of UV-EMS mutation resulted in transversion mutations. Base alterations in UV_{13} and EM_{31} mutants, causing missense and silent mutation, while in UM_{23} mutant only silent mutations occur. The bgl1 gene analysis showed that mutation using UV light was more effective than using EMS or a combination of UV-EMS in *Penicillium* sp. ID10-T065.

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INTRODUCTION

Beta-glucosidase (BGL) as one of the cellulase complexes has an essential role in cellulose hydrolysis. BGL is involved in the conversion of cellobiose to glucose at the end of the hydrolysis process (Keller et al., 2020; Srivastava et al., 2019). This action will reduce cellobiose accumulation in a cell which can inhibit the secretion of endoglucanase (EG) and cellobiohydrolase (CBH) (Singh et al., 2016; Sørensen et al., 2013).

BGL productivity in bacteria and fungi is considered low for industrial scale. Therefore the improvement of BGL activity is needed (Lynd et al., 2002; Srivastava et al., 2019). BGL activity could be improved through random mutation or genetic engineering (Singh et al., 2017). Strain

improvement through mutation can reduce industrial costs, elevate productivity, and emerge a special character (Afifi et al., 2014).

A mutation is an alteration made to the DNA sequence of an organism. In most cases, mutations are harmful, but they can occasionally lead to a better-adapted organism to its environment with improved biocatalytic performance. The potential of a microorganism to mutate is an important property conferred by DNA since it creates new variations in the gene pool (De Nicolás-Santiago et al., 2006; Habibi & Pezeshki, 2013).

It has been reported that random mutation followed by screening can improve enzyme activities in fungi. Random mutation can be done using a chemical, physic, or combine agent called mutagen (Adrio & Demain, 2006; A. Singh et al., 2017). These were several mutagens that have been reported that could improve BGL activity: proton beam, Ultraviolet (UV) radiation, Nitric Acid (HNO_2), Ethidium Bromide (EtBr), N-nitro-N-nitrosoguanidine (NTG), and Ethyl Methyl Sulfonate (EMS) (Jung et al., 2012).

UV light is one of the best physical mutagens for mutations in fungi. UV light can cause DNA damage and induce mutations. A chemical mutagen which is widely used in mutagenesis study is EMS. EMS is an alkylating agent that will alkylate guanine or thymine base, causing transition base pairs (Habibi & Pezeshki, 2013; Hogg 2005). The combined mutation of UV-EMS can induce a wide genetic variability (El-Bondkly & Keera, 2007). Several studies using a combination of UV light and EMS have proved the increase in the production of commercial enzymes, such as lipases (El-Bondkly & Keera, 2007); α-amylase (Shafique et al., 2009); phosphatase (Rajeshkumar & Ilyas, 2011); and ligninase (Ramzan et al., 2014).

Mutation using UV light and EMS in fungi was reported can increase the activity of BGL (Chandra et al., 2009; Ega et al., 2020; Ike et al., 2010; Liu et al., 2013; Syafriana et al., 2014). However, information on the base changes that occur after a random mutation in the coding gene of the BGL mutant with wild-type was underreported. It was reported only by Ega et al. (2020) which found that there was a base substitution in *bgl*H gene of *Bacillus subtilis* after shuffling mutation using the EMS, NTG, and UV. Larue et al., (2016) reported another base alteration in the *bgl* gene, demonstrating that after directed mutagenesis in *Aspergillus niger* resulted in several substitution mutations. The substitution causes changes in several amino acids, which probably affected the substrate's hydrolysis. However, analysis of alteration in *bgl* gene after random mutations in *Penicillium* has not been reported. The research that has been published was only in *eg1* gene by Caniago et al. (2015). It was reported that sequence analysis of *eg1* gene on wild-type strains and mutants of *Penicillium oxalicum* showed that some changes in nucleotide bases cause amino acid mutation. Bases mutation which was occurred in *eg1* gene could influence the enhancement of endoglucanase activity in the mutant (Caniago et al., 2015).

We have been mutated *Penicillium* sp. ID10-T065 using UV irradiation, EMS, and the combination of UV-EMS (Syafriana et al., 2014). The results showed that BGL activity in selected mutants was increased. This study examined the mutations in the bgl gene encoding (bgl1) as well as sequence differences between mutants and wild-type of *Penicillium* sp. ID10-T065.

MATERIALS AND METHODS

Sample

DNA of mutants UV_{13}, EM_{31}, and UM_{23}, also wild-type of *Penicillium* sp. ID10-T065 were obtained from Laboratory of Applied Microbiology, Research Center for Biotechnology, Indonesian Institute of Sciences. Mutant UV_{13} was mutated by UV irradiation; EM_{31} was mutated using EMS; while UM_{23} was mutated by a combination of UV irradiation and EMS (Syafriana et al., 2014).

Primers

Primers were designed based on *bgl*1 gene from reference strain *Penicillium brasilianum* with accession number EF527403 (Krogh et al., 2010). We designed five pairs of primers (Table 1). Among those five primers, we chose BG_2 to amplify our mutants and wild-type gene. Primers were edited using BIOEDIT Sequence Alignment Editor and DNA Star Programme. Optimization was done by varying the annealing temperature ± 5. optimization results showed that only BG2 primers meet the requirements based on the band formed in electrophoresis. BG1, BG3, and BG5 bands were not detected, while BG4 bands were too thin.

Data analysis

The DNA sequences of a *bgl*1 gene from mutants and wild-type were aligned to be compared with each other using BIOEDIT Sequence Alignment Editor. The base alterations and the amino changes among wild-type and mutants were analyzed to see the pattern of mutation that occurred (Luo et al., 2016; Saitou, 2013).

Table 1. Five pairs' primers of *bgl*1

Name of Primer	Tm (Melting Point) (°C)	Sequences *5'-3'*	Number of Bases	G-C Content (%)	Base Pair (bp)
BG_1 F1	61.4	5'-gccattgcgcagcccataca-3'	20	60.0	430
BG_1 R1	59.3	5'-aagcggaggaataatctgcgaacc-3'	24	50.0	
BG_2 F2	59.2	5'-tggccgccgcaacattt-3'	17	58.8	389
BG_2 R2	63.3	5'-accagcgcgaacggcatcag-3'	20	65.0	
BG_3 F3	60.2	5'-tcggcgctagctggacttgata-3'	22	54.5	947
BG_3 R3	61.0	5'-cgacttttggcccaggtgaacg-3'	22	59.1	
BG_4 F4	61.0	5'-cgttcacctgggccaaaagtcg-3'	22	59.1	425
BG_4 R4	61.4	5'-cgcgtggggtgggataaagtct-3'	22	59.1	
BG_5 F5	57.8	5'-tgacctccgtgttgtgaagaagta-3'	24	45.8	453
BG_5 R5	56.8	5'-tgaccgggcagcagataaaa-3'	20	50.0	

RESULTS AND DISCUSSION

DNA sequences alignment

Results of DNA sequences alignment of *bgl*1 from wild-type and three mutants were shown in Figure 1. Sequence alignment showed base alterations from base 2025 - 2050 (Figure 1). The results showed that the mutant UV_{13} had the most alteration (7 bases) compared to EM_{31} (5 bases) and UM_{23} (2 bases). Base alterations from mutant UV_{13} were showed at base position 2027 (T→C); base 2036 (T→G); base 2040 (T→G); base 2047 (G→C); base 2048 (T→G); base 2049; (T→G); base 2050 (G→A). Meanwhile, mutant EM_{31} showed five alterations from base 2034 (G→A), base 2036 (T→G), base 2037 (G→C), base 2044 (G→C), and base 2047 (G→T). Whereas the mutant UM_{23} has two alterations which were at base position 2025 (T→A) and 2037 (G→C). These alterations were in line with the results of the enzyme BGL activity which showed that the highest activity value was mutant UV_{13}, followed by EM_{31}, and UM_{23} (Syafriana et al., 2014). These findings support the hypothesis that the number of mutations has an impact on BGL activity.

Mutation Analysis

The mechanisms of mutation that occurred in mutants UV_{13}, EM_{31}, and UM_{23} were summarized in Table 2. The data showed that all isolates had substitution mutation, which is a mutation that occurs as a result of the replacement of one base by another base. If a substitution is between chemically similar bases, such as between purines (adenine and guanine) or pyrimidines (cytosine and thymine), it is called transition. If a substitution is between a purine and a pyrimidine, or vice versa, it is called transversion (Saitou, 2013). The data showed that transversion substitution occurred about 11 events (5 events at UV_{13}, 4 events at EM_{31}, and 2 events at UM_{23}); while the transition was about three events (2 events at UV_{13} and 1 event at EM_{31}) (Table 2). These events contradicted the literature, which stated that transitions happen more frequently than transversions (Luo et al., 2016; Porceddu & Camiolo, 2017; Saitou, 2013). The process of a transversion is known more complicated than transition due to different structures between purine (bicyclic structure) and pyrimidine (single ring structure) (Luo et al., 2016).

Single base alteration showed that transversion from T→G was the most frequent event (5 events), followed by G→C (4 events). These findings were also inversely proportional to the claim that the most common transversion alteration is the C→A alteration (Saitou, 2013). In transition, the data showed that G→A (2 events) was higher than T→C (1 event). Even though this result showed no significance, but it suited the literature which said Guanine and Cytosine had the highest frequencies of transition (Porceddu & Camiolo, 2017). G:C → A:T more frequent than A:T → G:C (Luo et al., 2016; Saitou, 2013).

Besides single base, we also analyzed the changes that happen in a codon (triplet) and in amino acid translated. The analysis revealed that the amino acid sequence had changed, but that some had remained the same despite the base changes (Table 2).

UV-irradiation Mutation

Mutations that occurred in mutant UV_{13} at base positions 2027 and 2050 showed a transition substitution. Substitution at base-2027 was a pyrimidine base transition in which thymine was replaced by cytosine. While substitution on the base 2050 revealed purine base transition event in which guanine was replaced by adenine (Table 2) (Hogg, 2005; Saitou, 2013).

Figure 1. Sequence alignment of the reference strain, mutants, and wild-type of *Penicillium* sp. ID10-T065

Table 2. Mutation analysis of mutants *Penicillium* sp. ID10-T065

Mutant Code	Base number	Base Alteration	Mechanisms of Mutation	Triplet Changes	Amino acid Changes	Results of Mutation
UV_{13}	2027	T → C	Transition	TTT → CTT	Phe → Leu	Missense mutation
	2036	T → G	Transversion	TGC → GGC	Cys → Gly	Missense mutation
	2040	T → G	Transversion	CTT → CGT	Leu → Arg	Missense mutation
	2047	G → C	Transversion	CCG → CCC	Pro → Pro	Silent Mutation
	2048	T → G	Transversion			
	2049	T → G	Transversion	TTG → GGA	Leu → Lys	Missense mutation
	2050	G → A	Transition			
EM_{31}	2034	G → A	Transition	GGA → GAA	Gly → Glu	Missense mutation
	2036	T → G	Transversion	TGC → GCC	Cys → Ala	Missense mutation
	2037	G → C	Transversion			
	2044	G → C	Transversion	TCG → TCC	Ser → Ser	Silent Mutation
	2047	G → T	Transversion	CCG → CCT	Pro → Pro	Silent Mutation
UM_{23}	2025	T → A	Transversion	ATA → AAA	Ile → Lys	Missense mutation
	2037	G → C	Transversion	TGC → TCC	Cys → Ser	Missense mutation

Note:
→ : shows alteration in base/amino acid sequence

A : adenine	Ala : alanine	Gly: glycine	Phe : phenylalanine
G : guanine	Arg : arginine	Ile : isoleucine	Pro : proline
C : cytosine	Cys : cysteine	Leu : leucine	Ser : serine
T : thymine	Glu : glutamate	Lys : lysine	

Base alterations at base positions 2036, 2040, 2048, and 2050 showed similar changes, which were thymine replaced by guanine; whereas at base position 2047, guanine became cytosine (Table 2). This kind of substitution is referred to as a transversion mutation, in which a purine is replaced with a pyrimidine or vice versa (Hogg, 2005; Saitou, 2013).

The mutations that occurred also affected the translation of the amino acids. Substitution at base 2027 caused an alteration from phenylalanine into leucine; the substitution at base 2036 altered cysteine into glycine; whereas substitution at base-2040 altered leucine into arginine. The next base alteration was on the three bases sequence at positions 2048-2050. The three bases were a triplet codon that will be translated into mRNA. The triplet changes caused an alteration in the amino acid leucine into lysine (Table 2). This type of mutation referred to as a missense mutation, caused a change in amino acid translation (Habibi & Pezeshki, 2013; Saitou, 2013).

Base alteration at position 2047 didn't affect the translation of amino acids. The amino acid at that position was persistently proline (Table 2). That type of mutation does not alter an amino acid translation is known as silent mutation (Habibi & Pezeshki, 2013). This happened because one amino acid can be coded by multiple codons, for example, amino acid proline can be coded by CCU, CCA, CCG, and CCC (Saitou, 2013; Voet & Voet, 2011).

UV irradiation is one of the inducers which can affect a mutation. UV irradiation affects the structure of pyrimidine (Cytosine = C and Thymine = T). Pyrimidine is sensitive to UV light, so it is easily modified when UV light is absorbed into the cell. UV absorption can lead to the formation of pyrimidine dimers in two adjacent pyrimidine bases that can damage the structure of the DNA chain or hinder the passage of the replication process (De Nicolás-Santiago et al., 2006; Irfan et al., 2011; Shafique et al., 2009). The damage is caused by photochemical reactions triggered by UV energy absorption on DNA bases. The double bonds in the pyrimidine bases (either thymine or cytosine) absorb UV light, causing the adjacent pyrimidine bases to react (Figure 2) (Goodsell, 2004; Ikehata & Ono, 2011).

Sequence analysis showed that the nucleotide changes that occurred in the mutant UV_{13} were a position adjacent pyrimidine bases, namely TT (at base position 2027, 2048-2050); CT (base position 2040); and CC (base position 2047), except at the base 2036 (T adjacent to purine) (Figure 1). A base substitution mutation of cytosine into thymine is the most common mutation due to UV irradiation, even though there may be transversion, frameshift mutations, duplications and deletions (Ikehata & Ono, 2011; Livneh et al., 1993).

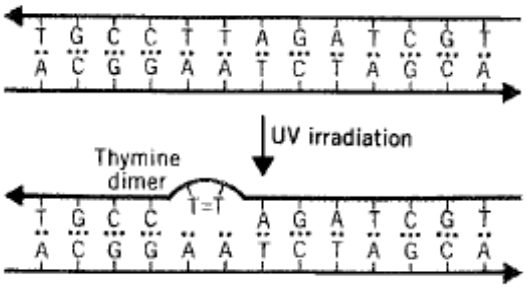

Figure 2. Formation of pyrimidine dimers induced by UV light (Source: Hogg, 2005)

EMS Mutations

Mutations in the mutant EM_{31} indicated substitution mutations (transitions and transversions). Transition mutation was found at base position 2034 which altered guanine into adenine, whereas transversion mutations occurred at the base 2036 (thymine into guanine), 2037 and 2044 (guanine into cytosine), as well as to the 2047 base (guanine into thymine) (Table 2). This substitution caused an alteration in amino acid translation (missense mutation), there was glycine into glutamate (at base position 2034), and cysteine into alanine (at base position 2036--2037). Besides that, it was also found a silent mutation event in mutant EM_{31}. It was found at base position 2044 (constantly serine) and base position 2047 (constantly proline) (Table 2).

Alkylating agents such as EMS works on the base guanine or thymine by adding an alkyl group to the oxygen atoms bonded to hydrogen bonding (position 6). The addition of alkyl groups to form a new molecule, namely alkylguanine or alkylthymine (Figure 3). Alkylguanine pairs with thymine (can be not paired with cytosine), while alkylthymine pairs with guanine (can be not paired with adenine) (Hogg, 2005; Hooley et al., 1988; Sega, 1984). This resulted in a change of GC base pairs into the AT or of TA into CG (Figure 3).

Mutations in the mutants EM_{31} showed a pattern that EMS could cause a change in the guanine bases (at base 2034, 2037, 2044 and 2047), and thymine (at base 2036) (Table 2). This indicated that there was the addition of an ethyl group to the two bases, resulting in errors of base pairs. EMS in small amounts can also induce transversion of G/C

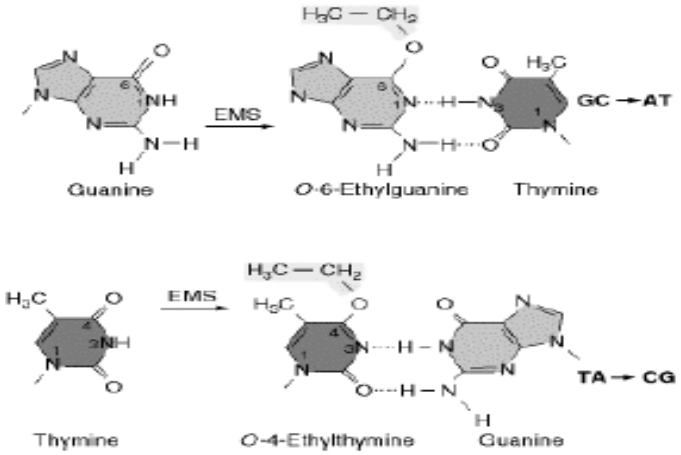

Figure 3. Mechanism of an alkylating agent in DNA bases (Source: Griffiths et al., 2000)

to C/G as can be seen at the base 2037; or transition A/T to G/C as can be seen at the base 2036 (Kim et al., 2006; Sega, 1984).

Combination Mutation of UV and EMS

Transversion mutations were observed in a mutant combination of UV-EMS (UM_{23}), with thymine being replaced by adenine (at base 2025) and guanine being replaced by cytosine (at base 2037). Change at base 2025 led to the reading of the amino acid isoleucine altered into lysine and change at base 2037 altered serine into cysteine (missense mutation) (Table 2).

UV and EMS interactions are not associated with an increase in the mutation or gene conversion, but a combination of both mutagens affects the possibility of DNA repair processes inhibition. Combined treatment with UV and certain alkylating agents (MMS or MNNG) in human and Chinese hamster ovary cells has revealed inhibition of excision repair of UV-induced lesions. These results indicated that the inhibition of repair might be due to the alkylation of repair enzymes, thus leading to reduced enzyme activity (Ager & Haynes, 1990). Based on the results of the activity of the mutant enzyme UM_{23} (Syafriana et al., 2014), the enzyme activity decreased when UV and EMS mutations were combined. This indicates that there was inhibition of the mutant enzyme's pathway at UM_{23}.

The results of gene analysis revealed that the base alterations caused by mutations had an impact on BGL's increased activity. Mutant UV_{13} is a mutant with the highest number of mutated bases compared to EM_{31} and UM_{23}. The results of BGL activity in the previous study showed that the mutant UV_{13} (5.53 U/ml) has BGL activity higher than EM_{31}(4.26 U/ml) and UM_{23}(1.75 U/ml) (Syafriana et al., 2014). This indicated that the mutation using UV light was more effective than EMS or a combination of UV and EMS to increase the yield of BGL activity.

The effectiveness of UV as mutagen was also reported by De Nicolás-Santiago et al. (2006) who got increased activity of mannanase, cellulase and xylanase in *Aspergillus niger* mutant UAM-GS1 after exposure to UV light for 3 minutes. Increased activity of the enzyme was thought to be related to alterations in promoter zones of the genes coding of the enzyme due to UV exposure. This radiation might have deregulated the transcription of the mRNA corresponding to the enzyme, leading to an increased secretion production. Since UV radiation affects mainly the hydrogen bonds of pyrimidine bases (C + T), the most vulnerable regulatory sequences must have been those containing the highest concentration of C + T. It also suggested that the promoter zone was strongly affected by the UV radiation and it might have affected the mechanism of cellulolytic enzymes expression.

CONCLUSION

It was concluded that mutant UV_{13} showed the highest base alterations (7 bases) compared to the other two mutants, EM_{31} (5 bases) and UM_{23} (2 bases). UV irradiation and EMS mutation resulted in transition and transversion DNA, whereas the combination of UV-EMS mutation resulted in transversion mutations. Base alterations in UV_{13} and EM_{31} mutants, causing missense and silent mutation, while in UM_{23} mutant only silent mutations occur. The bgl1 gene analysis showed that mutation using UV light was more effective

than using EMS or a combination of UV-EMS in *Penicillium* sp. ID10-T065.

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