Enhancement of Indigenous Fungal Cellulase Production by Gamma Rays

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Abstract. The increasing demand for cellulas causes the need for a high cellulase-producing microbe. Mutagenesis is an efficient way to produce a high-titer cellulase-producing strain. Mutagenesis using gamma rays irradiation has the advantage that it can cause a double strand break of DNA. Repair of double-strand break tends to has an error-prone repair that leads to the alteration of DNA sequence. The aim of this study was to screen high cellulase-producing indigenous fungal mutants produced by mutagenesis. Trichoderma sp. PK1J2 was subjected to gamma irradiation at 300 Gy. The mutants produced were screened using a plate medium containing cellulose as a sole carbon source. After staining with congo red, colonies with wider clear zones were grown in a liquid medium for four days, and the cellulase activities were analyzed. Mutant M8 produces endoglucanase, FPase, and β-glucosidase at 0.46 U/ml, 0.18 U/ml, and 1.10 U/ml, respectively, which were 90%, 50%, and 30% higher than those of the parental strain.

1. Introduction
Cellulase is a biocatalyst indispensable in various commercial sectors, such as the pulp and paper industry, textile industry, detergent industry, pharmaceutical industry, animal feed industry, and food processing industry. Cellulase is also used to extract bioactive compounds from plants, remove bacterial biofilm, and biorefinery production. In the food industry, cellulases are utilized in various processes such as olive oil extraction, juice clarification, concentrating purees, and reducing the viscosity of nectars. Cellulase is also used to improve the quality of bakery products, pigment extraction from plants, and modification of fruit sensory properties, including flavor, aroma, and texture of fruits [1].

Many organisms can produce cellulases, including bacteria, actinobacteria, fungi, plants, and animals. Among these organisms, filamentous fungi are widely used for cellulase production, of which the genera Trichoderma, Penicillium, and Aspergillus are known as models for industrial-scale cellulase production [2]. One alternative that can increase cellulase production is the genetic modification of fungal strains. Fungal strains can be modified by mutagenesis into more robust ones in producing higher titers with better cellulase quality. There are two approaches to mutagenesis, namely physical...
by UV or gamma rays and chemical mutagenesis by N-methyl-N-nitro-N-nitrosoguanidine (MNNG), Ethidium bromide (EtBr), ethyl methyl sulphonate (EMS) or nitrous acid (HNO2) [3], [4].

Gamma-ray irradiation is a low energy transfer radiation (LET radiation) with a LET value of 1 keVµm-1 or lower produced by Co60 or Cs137 [5]. Gamma-ray radiation causes death or mutation in fungi due to DNA damage, either directly or indirectly. Gamma irradiation can directly break the single-strand and double-strand of DNA, remove nitrogen bases, and modify nitrogen bases. Indirectly, gamma-ray irradiation breaks the DNA by forming free radicals [6], [7]. The use of gamma-radiation has the advantage that it can cause double-strand breaks, besides single-strand break and deletion and modification of bases. In contrast to other types of DNA damage, which generally modify one strand of the double helix and can be repaired using the other strand as a template, double-strand breaks lead to the loss of integrity of both complementary strands. Error-prone repair of double-strand break causes more opportunities for inaccurate repair and leads to the transformation of the DNA sequence, resulting in alteration of genetic information [6], [8].

Gamma irradiation has been shown to cause changes in nitrogenous bases in genes in the form of transformations, transitions, or changes in the reading of the base sequence [9]. Gamma rays have been reported to enhance the production of endoxylanase, α, and β-galactosidase in Aspergillus niger [10], [11]. This study aimed to investigate the improvement of cellulase activity in indigenous fungi by mutation using gamma rays.

2. Material and Method
2.1 Microorganism
Five local isolates used in this were culture collections within Gadjah Mada University. Trichoderma sp. PK1J2, Trichoderma sp. MLT3J2, Aspergillus sp. FIG1, and Penicillium sp. G2J2 were obtained from the Laboratory of Biotechnology, Faculty of Agricultural Technology, Gadjah Mada University, while Aspergillus sp. FNCC 6151 was provided by Food and Nutrition Culture Collection, Gadjah Mada University. These microorganisms were maintained on Potato Dextrose Agar (PDA) and stored at 4 °C.

2.2 Screening indigenous fungi
Screening of the indigenous fungi was conducted by produced the cellulase of the five indigenous fungi and analyzed their cellulase activity. One milliliter of spore suspension (10⁷ spores/mL) of the fungi was transferred to 25 mL of medium contains Mandel’s mineral salt solution and 1% CMC as a substrate. The fermentation was carried out at 35 °C with a shaking speed of 100 rpm. After the fermentation, samples were withdrawn every day. The sample was filtered with filter paper and centrifuged at 12000 rpm and 4 °C for 10 min. The supernatant was analyzed for FPase activities using the Ghose method [12].

2.3 Mutagenesis with gamma rays treatment
The fungi were inoculated in a PDA medium and incubated at 30°C for 7 days. After the incubation, the spores were harvested using 0.05% tween 80. Ten milliliters of spore suspension (1×10⁶ spores mL⁻¹) were exposed by Cobalt-60 gamma rays (at 100, 200, 300, 400, and 500 Gy). The mutagenesis by gamma rays was held at Polytechnic Institute of Nuclear Technology, National Nuclear Energy Agency of Indonesia, Yogyakarta, Indonesia.

2.4 Analysis of fungal viability
Serial dilutions were made from each treated spore suspensions, then 0.1 ml of each dilution was poured onto DRBC (Dichloran Rose Bengal Chloramphenicol) medium using the spread plate method. After 3 days of incubation at 30°C, the fungal viability percentage was calculated by the plate count method.
2.5 Clear zone assay
Plate screening medium contains 10 g/L Carboxymethyl Cellulose (CMC) as a carbon source, 17.5 g/L agar, 4 g/L sorboses, 0.1% Triton-X, Mandels mineral salt solution, and 1% glucose as a repressor. The fungi were inoculated in each plate screening and incubated at 28 °C for 3 days, then incubated at 50 °C for 18 h [13]. The plates were flooded with 0.5% congo red for 15 minutes and destained with 1 M NaCl solution twice [14]. The clear zone ratio was calculated by dividing the clear zone diameter by the colony diameter.

2.6 Enzyme production
One milliliter of spore suspension (10⁷ spores/mL) transferred to 25 mL of medium contains Mandel’s mineral salt solution and 1% CMC as a substrate. The fermentation was carried out at 35 °C with a shaking speed of 100 rpm. After the fermentation, samples were withdrawn, filtered with filter paper, centrifuged at 12000 rpm and 4 °C for 10 min, and the supernatant was analyzed for endoglucanase, β-glucosidase, and FPase activities.

2.7 Enzyme assay
Endoglucanase activity assay was carried out using an endo-cellulase CellG5 assay kit from Megazyme according to manufacture instruction. The β-glucosidase activity was analyzed by adding 0.2 mL of enzyme sample to 1.0 mL p-nitrophenyl-β-D-glucopyranoside substrate and 1.8 ml of citrate buffer (0.1 M, pH 4.8). The solution was incubated at 50 °C for 30 min, then the solutions were added with 4.0 mL of glycine buffer (0.4 M, pH 10.8), and read the absorbance of the solutions at 430 nm [15]. The FPase activity was analyzed according to Ghose [12] by adding 0.5 mL of enzyme sample in 1.0 ml citrate buffer (0.05 M, pH 4.8) with one Whatman No.1 filter paper strip 1x6 cm (50 mg). The reaction mixture was incubated at 50 °C for 60 min, then added the solutions with 3.0 ml DNS. The reaction was stopped by boiled the solution for 5 min. After boiling, the solution was transferred to a cold water bath and added 20 mL distilled water. Wait for 20 min to settle the pulp. The color formed of the solutions was measured at 540 nm.

3. Result and Discussion
3.1 Screening indigenous fungi
Penicillium, Trichoderma, and Aspergillus are high titer cellulase-producing fungi. The results of cellulases activity assay produced by Penicillium sp. G2J2, Trichoderma sp. PK1J2, Aspergillus sp. FIG1, Trichoderma sp. MLT3J2, and Aspergillus sp. FNCC 6151 are presented in Figure 1.

![Figure 1. FPase activity of indigenous fungi](image-url)
The result showed that Trichoderma sp. PK1J2 produces the highest FPase activity on the third-day incubation of 0.101 U/mL. Aspergillus sp. FIG1, Aspergillus sp. FNCC 6012, and Penicillium sp. G2J2 have the highest FPase activity on the fourth, fifth, and six-day incubation of 0.083 U/mL, 0.048 U/mL, and 0.045 U/mL, respectively, while Trichoderma sp. MLT3J2 produces the same amount of FPase on days four, five, and six of incubation with 0.078 U/mL. In this study, fungi with the highest cellulase activity would be selected for mutagenesis. Trichoderma sp. PK1J2 has the highest FPase activity from the 3rd until the 7th day compared with the four fungi, so Trichoderma sp. PK1J2 was selected for mutagenesis using gamma rays irradiation.

3.2 Analysis of fungal viability in different doses of gamma rays
Trichoderma sp PK1J2 was exposed to different doses of gamma rays. Each spore suspension was exposed to 100, 200, 300, 400, and 500 Gy of gamma rays. In this study, an irradiation rate of 3885 Gy / hour was used, which requires 1.54 minutes of exposure time to produce an irradiation dose of 100 Gy. The effect of various gamma radiation doses on fungal viability is presented in Figure 2.

![Figure 2. Fungal viability in different gamma irradiation doses](image)

After exposure to gamma-ray irradiation on Trichoderma sp. PK1J2, it was found that there was decreasing fungal vitality along with the increasing dose of gamma-ray irradiation. Higher radiation doses cause more DNA damage and increase the potential for fungal death. The spores viability was decreased 65% after exposure to 100 Gy gamma rays, and only 0.5% of spores survived in 500 Gy. The decreasing of spore viability after gamma-ray irradiation was reported elsewhere. About a half of T. reesei’s spore died under the 500 Gy gamma rays [16], and 90% lethality of A. niger’s spore on 1400 Gy [11]. Fungal viability was used to estimate the D10 value. D10 value represented the gamma irradiation dose that reduced the fungal viability of 1 log cycle or 90%. Gamma rays tended to generate multiple mutations in one gene at a dose higher than the D10 value [9], [17]. In this study, the dose of 300 Gy was selected for further analysis.

3.3 Screening mutant
Mutations of Trichoderma sp. PK1J2 was performed using gamma-ray irradiation at a dose of 300 Gy. After the mutation, the mutants were grown on plate screening media, and the clear zone formation was assayed using congo red. Clear zone considers as a qualitatively total cellulase. The wider clear zone formation represented the higher cellulase activity.
Based on the clear zone assay, some mutants formed a wider clear zone than the parental strain. Figure 3 shows the top ten mutants with the highest clear zone ratio, and they were M1, M2, M4, M5, M6, M8, M13, M20, M22, and M28. The mutants formed a clear zone with a ratio ranged from 1.64 to 2.11, while the parental strain produced a clear zone ratio of 1.44. Mutant M8 has the widest clear zone compared to other mutants. For further analysis, mutants with wide clear zone formation inoculated in submerge fermentation media to produce cellulase and analyzed the enzyme activity.

3.4 Enzyme analysis of mutant

Enzyme activity assay was carried out on mutants that produce a clear zone that wider than parental strain. Ten mutants were analyzed for endoglucanase activity. The results of the endoglucanase activity of the mutants examined are presented in Figure 4.

As seen in Figure 4, the ten mutants produced endoglucanase higher than the parental strain. Endoglucanase activity of the ten mutants after 4 days ranged from 0.24 U/mL to 0.46 U/mL, whereas the parental strain had endoglucanase activity of 0.24 U/mL. Mutant M4, M5, M13, M22, and M8 produced endoglucanase slightly higher than the parental strain. However, mutant M1, M2, M6, M8, and M20 produced endoglucanase considerably higher than the parental strain. Mutant M8 has the
highest endoglucanase activity among the other mutants. This result is similar to the clear zone assay. Mutant M8 forms the widest clear zone compare with the other mutants.

Mutant M8 produces endoglucanase activity of 0.46 U/mL, which was 1.90 times higher than the parental strain. The increasing of cellulase production by gamma-ray irradiation was reported on *Chaetomium cellulyticum*, *Penicillium cyclopium*, and *Trichoderma reesei* 2414, which increased the CMCase activity by 1.60, 1.40, and 1.23 time, respectively [16], [18], [19]. The other study also reported that *T. reesei* produced CMCase activity by 1.50 times higher than its parent after exposed to gamma rays at 750 Gy [20].

![Figure 5. FPase and β-glucosidase activity of mutant](image)

FPase and β-glucosidase activity of mutant M8 were analyzed. As seen in Figure 5, mutant M8 produces FPase and β-glucosidase of 0.18 U/mL and 1.1 U/mL, respectively, which was higher than the parental strain. FPase activity of mutant M8 was 50% higher than the parental strain. The other study was reported that mutant *T. reesei* CCT 2414 and *T. viride* had about 30% and 38% FPase activity higher than its parent after exposure by gamma irradiation [16], [21].

Gamma rays irradiation could increase the cellulase activity produced by fungi, probably due to the gamma rays alter the DNA sequence of fungi at protein secretion gene, transcriptional regulation gene, or gene of sugar transport and metabolism, either directly or indirectly. The research mentioned that the mutant with higher cellulase than the parental strain derived from random mutagenesis has a mutation in RNA metabolism, protein secretion/vacuolar targeting, and transcriptional regulation gene. The other mutant reported has a mutation in the gene of sugar transport and general metabolism [22]. Mutagenesis *Trichoderma sp.* PK1J2 using gamma rays irradiation produces mutant with higher endoglucanase, β-glucosidase, and FPase activity than the parental strain.

### 4. Conclusion

Mutagenesis of *Trichoderma sp.* PK1J2 using gamma rays irradiation at 300 Gy produced a mutant with cellulase activity higher than the parental strain. Mutant M8 had endoglucanase, FPase, and β-glucosidase activity at 0.46 U/ml, 0.18 U/ml, and 1.10 U/ml, respectively, which were 90%, 50%, and 30% higher than the parental strain.
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6. Reference

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