Improvement of bioethanol production in cornstalk fermentation through hydrolysis by fungi *Trichoderma reesei* exposed to gamma rays

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**ABSTRACT.** The utilization of cornstalk as a raw material for bioethanol production is an alternative solution to increase the added value of lignocellulose from agricultural wastes. The glucose content of lignocellulose material can be increased through the hydrolysis process by cellulolytic microorganisms. This research aims to obtain fungi *Trichoderma reesei* which capable of optimizing glucose content on lignocellulose materials and influence to increase in bioethanol production by *Saccharomyces cerevisiae*. The experiments were conducted with 2 factorials consist of substrates pre-treatments and hydrolysis. Pre-treatment of substrates by aqua dest (S1), 1% H$_2$SO$_4$ (S2) and 1% NaOH (S3) solution. Treatments of hydrolysis consist of control (K) and fungi *T.reesei* 750 Gray (H). The fungi *T.reesei* 750 Gray was obtained from optimizing by gamma rays at a dose of 250, 500, 750 and 1000 Gray. The fungi *T. reesei* 750 Gy have the specific cellulase activity about 0.24 U/mg higher than the fungi *T. reesei* 0 Gy about 0.16 U/mg. Pre-treatment of substrates with 1% NaOH (1: 5 w/v) solution and hydrolysis by fungi *T.reesei* 750 Gray (S3H) is suitable for solid fermentation with *S.cerevisiae*. After 2 days of solid fermentation by *Saccharomyces cerevisiae*, the bioethanol contents in S3H medium about 387 ppm while S1K (control) about 196 ppm or increase about 98% (ppm/ppm). This result is expected to be an alternative solution for the utilization of cornstalk for bioethanol production.

1. **Introduction**

Cornstalk is one of the largest agricultural waste lignocellulosic materials in Indonesia. Unwise management of lignocellulosic materials such as burning on agricultural land can have an impact on environmental pollution [1]. Efforts to use lignocellulosic materials as raw material for bioethanol production can be an alternative to increase the added value of agricultural waste without threatening the balances of food and feed availability [2].

The use of lignocellulosic as a raw material for bioethanol is required preliminary treatment to break lignin bonds and damage the crystalline structure of cellulose so that it can easily be converted into glucose [3]. The delignification process will reduce the lignin content and hydrolysis will increase glucose levels in the substrate of lignocellulosic materials. The delignification and hydrolysis process of the substrates can be physical,
chemical and biological. Chemical hydrolysis with acids and bases and enzymatic by Aspergillus niger, Penicillium funicul or Trichoderma reesei able to increase glucose content in the substrates of lignocellulosic materials [4,5].

Cellulase enzymes play an important role in converting cellulose to glucose. This cellulase enzyme is produced by cellulolytic microorganisms such as Trichoderma sp. and Aspergillus sp. Gamma rays treatments are able to increase the specific cellulase activity of cellulolytic fungi [4,6]. Gamma irradiation is able to stimulate the growth and activity of fungi. The fungi Aspergillus niger exposed to gamma rays at 500 Gray have 3.9 times higher than the wild types. The cellulase activity of Aspergillus niger 500 Gray about 31.01 U/g while the wild types about 7.85 U/g [4]. The fungi Aspergillus niger 500 Gray can produce higher glucose (2.6 times) is about 125.79 mg/g while the control (wild-types) is about 48.00 mg/g.

The increasing glucose content of the lignocellulosic substrate will affect the efficiency of bioethanol production by Saccharomyces cerevisiae. This unicellular yeast is nonpathogenic and nontoxic, so it has long been used in various fermentation processes such as in making bread, lactic acid, and alcohol [7]. Yeast S.cerevisiae is an aerobic facultative microbial that can use both aerobic and anaerobic systems to obtain energy from the process of breaking down glucose, resistant to high sugar and ethanol content, remains active in its activities at temperatures of 28-32°C [8].

Chemically pre-treatments of cornstalk substrate and hydrolyzed by fungi Trichoderma reesei exposed to gamma rays. The Gamma-ray treatment at the dose of 250, 500, 750 and 1000 Gray were intended to obtain fungi T. reesei with high specific cellulase activity. The selected fungi T. reesei were used in hydrolysis of cornstalk substrate with pre-treatment using 1% H2SO4 and 1% NaOH solution. This treatment aims to increase the glucose content of cornstalk substrates. Furthermore, a solid fermentation of cornstalk substrates was carried out with S.cerevisiae so that the conversion of glucose into bioethanol.

This study aims to obtain fungi T.reesei with high specific cellulase activity and increase bioethanol production by S. cerevisiae. The results are expected to be an alternative solution to increase the added value of cornstalk and contribute to agricultural waste management and environmental quality improvement.

2. Material and Method
2.1. Material and Equipment
Cornstalks (Zea mays L.) were used from experimental fields at the Centre for Application of Isotope and Radiation, National Nuclear Energy Agency (CAIR-BATAN), T.reesei dan S.cerevisiae strains from culture collection in the Environmental Group (CAIR-BATAN), potatoes dextrose broth (PDB), potatoes dextrose agar (PDA), yeast extract, bacterial peptone, NaCl, HCl, H2SO4, NaOH, dinitrosalisilic acids (DNS), carboxymethyl cellulose (CMC), Na2CO3, K-Na tartrate, CuSO4.5H2O, bovin serum albumin (BSA), folin, Na-citrat, citric acids, K2HPO4, KH2PO4, MgSO4.7H2O and aqua dest.

The equipment used consisted of Gamma Chamber 4000A (Co-60), cutting machine, analytical balance (Acculab), incubator (Heraeus), autoclave (Wisclave), pH meter (Pestesr 35), centrifuge (Hitachi), Spectrophotometer (Shimadzu), laminar airflow, GC-FID (Shimadzu), ovens, furnaces, mechanical shakers, Whatman filter paper, hot plates, micropipette, microtube, porcelain plates and glasses equipment.

2.2. Gamma Rays Treatment
The culture 4-7 days old of fungi T. reesei in PDA media were exposed to gamma rays at the dose of 250, 500, 750 and 1000 Gray. Gamma irradiation treatment at the Gamma Chamber 4000A facility uses a Co-60 source with an activity of about 479.54 Ci and the dose rate of 356.18 Gray/hour. The test of gamma irradiation impact on the enhancement
of the specific cellulase activity of fungi \textit{T. reesei} was carried out in the PDB medium containing 1% CMC (pH 5.5). About 0.5 x 0.5 cm of fungi \textit{T. reesei} was transferred into a 25 ml test medium then incubated in a mechanical shaker at 100 rpm and 28-32 °C for 48 hours. The observation parameters consist of cellulase activity and dissolved protein in medium.

2.3. \textit{Preparation of Cornstalk Substrates}

Cornstalk (\textit{Zea mays} L.) were chopped washed with tap water and then dried in the oven at 60-70 °C for 48 hours. Furthermore, it is ground with a cutting mill and sifted to obtain a substrate with particle size about <200 µm. The S1, S2 and S3 substrates are obtained by adding distilled water, 1% H\textsubscript{2}SO\textsubscript{4} and 1% NaOH solution. Into the substrates were added distilled water or acid or base solution in a ratio of 1: 5 then stirred evenly and allowed to stand for 2 hours. All of the substrates were dried in an oven at 60-70 °C for 48 hours. The observation parameters consist of pH, water content, glucose, lignin and cellulose.

2.4. \textit{Hydrolysis of Cornstalk Substrates}

This experiment uses a completely randomized design (CRD) with 2 (two) factorial consisting of f1 = substrates S1 (aqua dest), S2 (1% H\textsubscript{2}SO\textsubscript{4}) and S3 (1% NaOH) and f2 = K (without fungi) and H (fungi \textit{T. reesei} 750 Gy). The fungi \textit{T. reesei} 750 Gy from cellulase test medium was transferred into PDB medium then incubated in a mechanical shaker at 100 rpm and 28-32 °C for 3-4 days to obtain the fungi cultures about of 10\textsuperscript{7} cfu/ml. The S1, S2, S3 substrates each about 3 g put into a plastic bag (polyethylene) and added 6 ml of mineral salt nutrient solution containing 1.65 g/l PDB, 0.1 g/l peptone, 0.1 g/l yeast extract, 0.1 g/l KH\textsubscript{2}PO\textsubscript{4}, 0.1 g/l K\textsubscript{2}HPO\textsubscript{4}, 0.01 g/l MgSO\textsubscript{4}.7H\textsubscript{2}O and 0.1 g/l NaCl. All of the substrates were sterilized by autoclave at 121 °C for 15 minutes and cooled. About 500 µl of fungi \textit{T. reesei} 750 Gy were inoculated into sterile substrates in a plastic bag and sealed then incubated at 28-32 °C for 12 days. The observation at 4, 8 and 12 days after inoculation consists of fungi viability, cellulase activity and glucose content. The optimum time is used in the solids fermentation of cornstalk substrates by \textit{S. cerevisiae} for bioethanol production.

2.5. \textit{Solids Fermentation of Cornstalk Substrates}

The substrates after hydrolysis (S1H, S2H, S3H) and substrates without hydrolysis (S1K, S2K, S3K) were used as raw material for bioethanol production through solid fermentation by \textit{S. cerevisiae}. Into 2 g of substrates were added 6 ml of a nutrient solution containing 10 g/l yeast extract and 20 g/l peptone and then pH was adjusted to about 5 with the solution of 1 N NaOH and 1N HCl. All of the substrates were sterilized by autoclave at 121 °C for 15 minutes and cooled. About 500 µl of \textit{S. cerevisiae} culture were inoculated into 2 g of the sterile substrate and incubated at 28-32 °C for 2 and 4 days. The observation parameters consist of pH, water content, cellulase activity, glucose and bioethanol contents.

2.6. \textit{Sample Analysis}

The sample observation parameters consist of pH, fungi viability, water content, lignin and cellulose content, cellulase activity, dissolved protein, glucose and bioethanol content. The pH value of the sample is determined by a digital pH meter. The viability of fungi was determined by total plate counts [9]. Into 1 g of sample 10 ml was added to the 0.85% NaCl solution shaken in a mechanical shaker at 100 rpm for 60 minutes. After diluting up to 10\textsuperscript{7} times with a solution of 0.85% NaCl, it is poured and spread on the surface of the Potatoes Dextrose Agar (PDA) medium and incubated at 30 °C for 2-4 days.

Glucose content and cellulase enzyme activity were determined by the DNS method [10]. Into 1 g of sample was added 10 ml of citrate buffer solution (0.1 M; pH 5.5) and shaken at 100 rpm for 60 minutes. About 1 ml of supernatant or crude cellulase enzyme
was transferred into the microtube and centrifuged at 12000 rpm for 10 minutes. The crude cellulase enzyme about 500 µl was added into a solution of 1% CMC and incubated at 50 °C for 30 minutes. The sample about of 500 µ and 500 µl DNS solution was heated at 100 °C for 3-5 minutes, cooled and added 3 ml of distilled water then measured of glucose content by spectrophotometer at λ = 540 nm. One unit of cellulase enzyme is equivalent to the formation of 1 µmol glucose/minute.

The dissolved protein content was determined by the Lowry method [11,12]. Standard curves are made with a solution with standard bovine serum albumin (BSA) solution with concentrations of 0, 50, 100, 200, 400, 600 and 800 µg/ml. Reagent I was made from 48 ml of reagent A (2% Na2CO3 in 0.1 N NaOH), 1 ml of reagent B (1% K-Na tartrate in distilled water) and 1 ml of reagent C (0.5% CuSO4.5H2O in distilled water). Reagent II is folin and distilled water with a volume ratio of 1: 1. About 500 µl of the sample was added to 4 ml of reagent I and incubated for 5 minutes then 500 ml of reagent II was added and incubated for 30 minutes. Determination of dissolved protein content with a spectrophotometer at λ = 600.

Lignin and cellulose content was determined by the modification Chesson method [13]. About 2.5 g sample was extracted with 150 ml of acetone and dried at 105 °C for 24 hours. Extractive free samples about 0.5 g were hydrolyzed with 5 ml of 72% H2SO4 solution for 2 hours then 140 ml of distilled water were added and heated in an autoclave at 121 °C for 4x15 minutes. The hydrolyzate is separated with a vacuum filter and the precipitate is dried in an oven at 105 °C for 24 hours. The dry precipitate is heated in the furnace at 575-650 °C for 5 hours.

Bioethanol content was determined by gas chromatography (GC) with a flame ionization detector (FID) and integrated software [14]. The temperature of the FID detector is about 270 °C and the injection port temperature is about 195 °C. Nitrogen is used as a carrier gas and is used at a flow rate of 3 °C/minute. The capillary column used was SGE ID-BP20, 30 m, and a diameter of 0.25 mm. The oven temperature is set at 60 °C at the start, increased to 80 °C and programmed 175 °C/min and held for 5 minutes. Standard curves are made with various variations, namely 2000 ppm, 4000 ppm, 6000 ppm and 8000 ppm. The sample about 2 ml was put into a microtube and centrifuged at 12000 rpm for 2x15 minutes then the supernatant was filtered with a syringe filter and inserted into the GC-FID auto sample vial. Data from this study were analyzed using analysis of variance (ANOVA) on SPSS version 20.0 with a confidence limit of 95% (α = 0.05).

3. Results and discussion
3.1. Capability Improvement of Fungi T.reesei

The culture of fungi T.reesei in PDA medium was exposed to gamma rays at the dose of 250, 500, 750 and 1000 Gray. The interaction of gamma rays with cells and the medium in order to produce free radicals or reactive oxygen species [15,16]. Free radicals will disrupt the structure and function of random cell components, after exposure to gamma rays ends there will be a rearrangement with various cell structures. The diversity of cell structures influences the specific cellulase activity of fungi T.reesei as shown in Figure 1.
Figure 1. Specific cellulase activity of fungi \textit{T.reesei} fungi exposed to gamma rays.

Fungi \textit{T.reesei} with the highest specific cellulase activity was obtained at a dose of 750 Gray which is about 0.24 U/mg. Fungi \textit{T.reesei} without gamma-ray treatment (wild types) have specific cellulase activity around 0.16 U/mg. Thus, the treatment of gamma rays at a dose of 750 Gray produces fungi \textit{T.reesei} with specific cellulase activity higher than the wild types. These results indicate that gamma-ray exposure in 750 Gray can induce and stimulate the ability of fungi \textit{T.reesei} to produce cellulase enzymes. The exposure gamma rays on 750 Gray, oxidative stress causes cells to develop protective mechanisms to counteract the effects of reactive oxygen by producing more enzymes.

3.2. Pre-treatment and Hydrolysis of Cornstalk Substrates

In the utilization of lignocellulose cornstalk as raw material for bioethanol production, a pretreatment process is needed to open the lignin structure and facilitate the substrate's hydrolysis process. Lignin is one of the phenylpropanoid polymers composed of three compounds namely \textit{p}-cumaryl alcohol, coniferyl alcohol and sinapyl alcohol as shown in Figure 2. Lignin protects cellulose, hydrophobic and resistant to degradation by most microorganisms.

The pre-treatment of cornstalk was carried out with 1\% H\textsubscript{2}SO\textsubscript{4} (S2) and 1\% NaOH (S3) solution, the substrates and solution ratio about 1: 5 w/v for 2 hours. The addition of distilled water (S1) is used as a control. The use of these two types of delignification agents has a different effect on changes in the lignin and cellulose content in the cornstalk substrates as shown in Table 1. The solution of 1\% H\textsubscript{2}SO\textsubscript{4} has no effect on lignin degradation but decreases the cellulose content in the cornstalk substrates. Lignin
degradation and increased cellulose content were obtained in the initial treatment with a 1% NaOH solution.

Table 1. Lignin and cellulose content in cornstalk substrates after pre-treatment.

| Treatment   | Lignin | Cellulose |
|-------------|--------|-----------|
| S1 (akuadest) | 4,382$^b$ | 46,278$^b$ |
| S2 (1% H$_2$SO$_4$) | 4,375$^b$ | 45,416$^a$ |
| S3 (1% NaOH) | 4,048$^a$ | 48,247$^c$ |

NaOH is the best delegating agent compared to other chemicals such as ammonia, NaClO$_2$ and H$_2$O$_2$[18]. The particles NaOH will enter the material and break down the structure of lignin so that lignin is more soluble which results in the decrease of lignin content. The OH$^-$ ion from NaOH will break bonds from the basic structure of lignin while the Na$^+$ ion will bind to lignin to form sodium phenolate. This phenolic salt is formed after bonding between cellulose and lignin so that cellulose is obtained in a lignin-free state. Reducing lignin content and increasing cellulose are expected to increase the ability of T.reesei 750 Gray mold on the hydrolysis of corn stalk substrates.

Figure 3. shows that the optimum cellulase activity of the fungi T.reesei 750 Gray was obtained on the hydrolysis medium with a pre-treatment of 1% H$_2$SO$_4$ solution (S2). In this medium the cellulase activity of fungi T.reesei 750 Gray after 8 and 12 days of hydrolysis was about 8,877 and 9,496 U/g. The highest cellulase activity of T.reesei 750 Gray in the medium with pretreatment of 1% NaOH (S3) solution was obtained on the 12th day which was about 4.145 U/g. In the control medium (S1), the highest cellulase activity of T.reesei 750 Gray was obtained on the 8th day which was around 7,391 U/g then decreased to 4,535 U/g on the 12th day.

Figure 3. Cellulase activity in hydrolysis medium of cornstalk substrates by fungi T.reesei 750 Gray.

The difference in cellulase activity of fungi T.reesei 750 Gray and hydrolysis time can affect the glucose content in the hydrolysis medium of cornstalk as shown in Figure 4. The highest increase in glucose content about 4.7 times from 10.444 to 60.031 mg/g was obtained at the pretreatment of the substrate with a 1% H2SO4 solution (S2) after 12 days of hydrolysis. In this medium, cellulase activity of fungi T.reesei 750 Gray was also the highest around 9,496 U/g. The low cellulase activity of fungi T.reesei 750 Gray in the substrates medium with 1% NaOH (S3) solution and control (S1) affects the lower glucose content which is lower than the S2 treatment. The increase in glucose content in the S3
medium was about 1.6 times from 3.889 to 10.227 mg/g and in the S1 medium about 0.5 times from 7.470 to 11.276 mg/g.

Figure 4. Glucose content in the hydrolysis medium of cornstalk substrates.

After the substrate pretreatment and hydrolysis with fungi T. reesei 750 Gray has obtained the cornstalk substrates with different characteristics. The S1 and S3 substrates have higher lignin content than S2 substrates. After hydrolysis, HS1 and HS3 substrates contain lower glucose than HS2 substrates. Thus, pretreatment with 1% NaOH solution can reduce the lignin content but less or less support the performance of fungi T. reesei 750 Gray in the process of cellulose hydrolysis. The pre-treatment of a 1% H2SO4 solution can be selected before the hydrolysis process with fungi T. reesei 750 Gray to increase glucose content in the cornstalk substrate.

3.3. Solid Fermentation of Cornstalk Substrates

Solid fermentation with S. cerevisiae in the medium of substrates without hydrolysis (S1K, S2K, S3K) and the results of hydrolysis (S1H, S2H, S3H) at pH about of 5 and room temperature of 28-32 °C. After 2 and 4 days of solid fermentation, there was a change in the pH of the medium but the viability with S. cerevisiae was relatively the same as shown in Table 2. These results indicate that S. cerevisiae is able to well growth in all treatments of solid fermentation medium but the difference in pH of the medium can affect the activity and process of converting glucose into bioethanol.

| Fermentation time, day | Medium | pH    | Viability, cfu/g |
|------------------------|--------|-------|------------------|
| 2                      | S1K    | 4.92  | 7.38x10^{11}    |
|                        | S1H    | 5.60  | 1.77x10^{11}    |
|                        | S2K    | 6.29  | 2.04x10^{11}    |
|                        | S2H    | 6.71  | 1.43x10^{11}    |
|                        | S3K    | 6.08  | 2.29x10^{11}    |
|                        | S3H    | 6.69  | 1.70x10^{11}    |
| 4                      | S1K    | 4.24  | 1.08x10^{11}    |
|                        | S1H    | 4.24  | 1.79x10^{11}    |
|                        | S2K    | 4.88  | 1.68x10^{11}    |
|                        | S2H    | 5.02  | 1.47x10^{11}    |
Note: S1 = control (distilled water), S2 = 1% H2SO4, S3 = 1% NaOH, K = control (without fungi), H = hydrolysis by fungi T.reesei 750 Gray, cfu = colony forming units.

During solid fermentation the use of glucose by S.cerevisiae is indicated from the decrease in glucose content in the medium as shown in Figure 5. After 2 and 4 days of fermentation by S.cerevisiae, the optimum utilization of glucose occurs in the S2H medium were about 81.245% and 87.334% or about 48.772 mg/g and 52.428 mg/g. The utilization of glucose in the S3H medium lower than the S2H medium. In S3H medium after 2 and 4 days of fermentation, the decrease of glucose content was about 15.645% and 37.092% or about 1.600 mg/g and 3.793 mg/g. The utilization of glucose by S.cerevisiae will produce ethanol as the main products and by-products in the form of glycerol, 2,3-butanediol, and acetate which are smaller than ethanol as shown in Figure 6. Ethanol or bioethanol is obtained through the glucose glycolysis pathway to glyceraldehyde-3-phosphate to produce 1 NAD$^+$ [19].

![Figure 5. Decrease of glucose content in the fermentation medium of cornstalk substrates.](image)

![Figure 6. Pathways of glucose fermentation S.cerevisiae [19].](image)
Pre-treatment, hydrolysis and solid fermentation time of substrates by *S.cerevisiae* were affected to the content of bioethanol in the medium as shown in Figure 7. After 2 days of solid fermentation, the highest bioethanol content was obtained in S2H medium about 267.57 ppm and S3H about 387.32 ppm then after 4 days of solid fermentation decrease to 191.19 and 183.59 ppm. The longer the time of solid fermentation will increase the amount of CO$_2$ gas in the medium so that it affects the pH decrease of the S2H medium from 6.71 to 5.02 and the pH of the S3H medium from 6.69 to 5.98. The decrease in pH is also possible because of the increased formation of acetic acid in the fermentation medium.

The glucose content in the S2H medium is higher than S3H but bioethanol production by *S.cerevisiae* in the S2H medium is lower than S3H. This condition is thought to be related to the lignin content in S2 substrates which is higher than S3. Lignin can directly or indirectly be an inhibiting factor for enzyme catalysis [20]. Lignin degradation is an important step to release amorphous sugar and cellulose polymers needed to be hydrolyzed by cellulase enzymes [21]. The substrate which has a low lignin content (S3) and is hydrolyzed by fungi *T.reesei* 750 Gray (S3H) suitable for *S.cerevisiae* so that it can efficiently converting glucose into bioethanol. The utilization of glucose in S1K (control) and S3H medium was not significantly different but the production of bioethanol in the S3H medium was about 1.98 times higher than control. The increase of bioethanol production in the S3H medium was about 98% from 195.59 to 387.28 ppm.

4. Conclusion
Gamma irradiation at a dose of 750 Gray able to obtain the fungi *T.reesei* with specific cellulase activity higher than the wild type. The specific cellulase activity of fungi *T.reesei* 750 Gray was about 0.24 U/mg while fungi *T.reesei* 0 Gray was about 0.16 U/mg. Pre-treatment of substrates using 1% H$_2$SO$_4$ solution (1: 5 w/v) is suitable for increasing glucose content in the hydrolysis of cornstalk by fungi *T.reesei* 750 Gray (S2H). In this treatment was obtained the increase in glucose content about 4.75 times from 10.44 to 60.03 mg/g. Pre-treatment of substrates with a solution of 1% NaOH (1: 5 w/v) and hydrolysis by fungi *T.reesei* 750 Gray (S3H) is suitable for solid fermentation with *S.cerevisiae*. The improvement of bioethanol production in the fermentation of the S3H medium by *S.cerevisiae* was about 98% or increased from 196 ppm to 387 ppm.

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