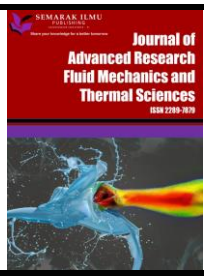
SEMARAK ILMU PUBLISHING

Journal of Advanced Research in Fluid Mechanics and Thermal Sciences

Journal homepage:
https://semarakilmu.com.my/journals/index.php/fluid_mechanics_thermal_sciences/index
ISSN: 2289-7879



Production of Hydrogen from Rice Straw through Enzymatic Hydrolysis by Optimally Prepared Crude Cellulase Mixture Followed by Fermentation and Its Hydrodynamic Study

Arief Widjaja[1,*], Nadiem Anwar[2], Tantular Nurtono[1], Sugeng Winardi[1]

[1] Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia
[2] Department of Chemical Engineering, Universitas Jenderal Achmad Yani, Cimahi, Indonesia

ARTICLE INFO

Article history:
Received 27 July 2023
Received in revised form 19 November 2023
Accepted 30 November 2023
Available online 15 December 2023

Keywords:
Hydrogen; rice straw; optimal crude cellulase mixture; hydrodynamic study

ABSTRACT

Production of hydrogen from rice straw was conducted through alkaline pretreatment, enzymatic hydrolysis, and fermentation. The hydrodynamic study was performed to investigate the effect of mixing to hydrogen gas produced during the heterogenous mixture in the fermentation tank. 2 % NaOH was found effective in reducing the lignin content in rice straw as evidenced from quantitative data and confirmed by the SEM image. Based on the optimum values of pH and temperature as well as the stability of enzyme, the hydrolysis was performed at pH 5.5 and 40 °C. The mixture of crude cellulases was able to give higher conversion than cellulase from one source of fungi and also higher than the conversion using pure commercial cellulase from *Aspergilus niger*. A unit activity ratio of 1 U *A. niger* cellulase to 2 U *Trichoderma reesei* cellulase was able to increase the reducing sugar concentration by 16%. Hydrogen fermentation was affected by the stirring speed in which increasing the stirring speed from 46 to 165 rpm increased the hydrogen recovery from 0.025 to 0.085 mol H_2/mol glucose. Simulations showed that the intensity of turbulence was greatest in the impeller area. By increasing rotational speed, the intensity of turbulence in all parts of the reactor will be more evenly distributed which can increase the diffusion of the substrate to the cell surface and increase the rate of biogas leaving out the solution. Furthermore, by increasing the stirring speed, the volume fraction of biogas at the bottom of the reactor is getting smaller which indicates more gas has risen to the surface and left the reactor. The study was the first report to study the hydrodynamic of hydrogen production from lignocellulose during the heterogenous fermentation mixture.

1. Introduction

The ever-increasing world population drives the demand of world energy, while at the same time, decreasing reserves of fossil fuels, the negative environmental impact as a result of combustion of fossil fuels, all these conditions, worsened by war and world politic condition, lead to an uncertainty

* *Corresponding author.*
E-mail address: arief_w@chem-eng.its.ac.id

https://doi.org/10.37934/arfmts.112.1.189203

of the supply and fulfilment of the conventional fuel. Researches dealing with new sustainable energy sources are therefore gaining more and more attention.

Indonesia is one of the largest producers of lignocellulose wastes which are not optimally utilized yet, including coconut husk, sugarcane bagasse and rice straw [1-3]. Rice straw contains 25–45% of cellulose that can be hydrolyzed chemically, fermentatively or enzymatically to glucose and biofuel such as bioethanol or biohydrogen [3-7]. Chemical hydrolysis takes place very fast but requires high temperatures, so it requires more energy and is less environmentally friendly. Xiang *et al.,* [4] reported that acid-catalyzed cellulose hydrolysis resulted in a 90% conversion of α-cellulose which was attained in five minutes at 245 ^{o}C and a 23% conversion at 185 ^{o}C in 40 minutes. Morya *et al.,* [3] reviewed the production of hydrogen from rice straw and discussed the policies and current situation of hydrogen production globally. Jin and Chen [5] showed that enzymatic hydrolysis of rice straw using *Penicillium decumbens* cellulase attained 95% cellulose conversion in 50 hours. Abedinifar *et al.,* [6] investigated enzymatic hydrolysis of rice straw using commercial cellulase from *T. Reesei*. Hydrolysis using dilute sulphuric acid on pretreated rice straw resulted in a 71.8% conversion of cellulose which was attained in 48 hours. Chang *et al.,* [8] investigated an enzymatic hydrolysis using commercial cellulase from *A. niger* on unpretreated and freeze pretreated rice straw with varying enzyme activity of 25–150 U/g rice straw at 37 °C and pH 5.5. The result showed that the freeze pretreatment increased digestibility of rice straw from 48% to 84%, attained after 48 hours of hydrolysis. Enzymatic hydrolysis can be conducted at low temperatures thereby offering a low energy consumption. However, long reaction time and high price of commercial enzyme are usually the drawbacks encountered using enzymatic process.

A cellulase enzyme has three component such as endo-1,4-β-D-glucanases (E.C.3.2.1.4) which cleave internal glycosidic bond, exo-1,4-β-D-glucanases or cellobiohydrolases (E.C.3.2.1.9) which cleave cellobiosyl unit from the ends of cellulose chain and 1,4-β-D-glucosidases (E.C.3.2.1.21) which cleaves glucose unit from cellooligosaccharides [9]. Although a large number of microorganisms are capable of degrading cellulose, only a few of them produce significant quantities of cellulase that are capable to completely hydrolyze crystalline cellulose. The genus *Trichoderma* is widely used in industrial applications and was selected as the best cellulase producing strain [9]. *T. Reesei* is able to produce cellulase with a composition of 60%–80% exo-1,4-β-D-glucanase, 20%–36% endo-1,4-β-D-glucanases and 1% 1,4-β-D-glucosidases [9]. The amount of 1,4-β-D-glucosidases is lower than that needed for an efficient cellulose to glucose hydrolysis. This will cause the main product of hydrolysis to be cellobiose. Cellobiose is a strong inhibitor to endo and exoglucanases, and the accumulation of cellobiose will slow down the hydrolysis rate significantly. The inhibitory effect of cellobiose can be ceased by addition of β-glucosidase from external sources or produce cellulase by cofermentation of *Trichoderma* and *Aspergillus* species. *Aspergillus* species are known for their ability to produce β-glucosidase with significantly higher yields than *Trichoderma* species [10].

The aim of this work is to investigate the hydrogen production from rice straw through enzymatic hydrolysis using mixed crude cellulase produced from *T. reesei* and *A. niger*. Several important parameters such as combination of strains in the production of cellulase, pretreatment condition, effective pH and temperature of the hydrolysis as well as enzyme concentration were investigated in order to perform an efficient hydrolysis of rice straw. Hydrodynamic study of the fermentation of the obtained glucose was conducted to investigate the effect of the mixing in the heterogenous mixture to the production of hydrogen gas. The study was the first to report the hydrodynamic study of hydrogen production from sugar derived from lignocellulose during the heterogenous fermentation mixture.

2. Methodology

2.1 Raw Material and Microorganism

Rice straw was collected from a local farmer near the campus of Institut Teknologi Sepuluh Nopember Surabaya and corn stem was collected from farmer in Jombang, a small city not far from Surabaya. Rice straw and corn stem were both used for enzyme production while for enzymatic hydrolysis, only rice straw was utilized. Commercial cellulase was purchased from Fluka Biochemica and has an activity of 0.93 U/mg. All chemicals used were of analytical grade and purchased from commercial sources.

A. niger ITBCC F74 was obtained from the Culture Collection of Department of Chemical Engineering, Bandung Institute of Technology and *T. reesei* was obtained from Department of Biology, Airlangga University Surabaya. *Enterobacter aerogenes* NBRC 13534 was given by Prof. Hiroyasu Ogino from Osaka Prefecture University, Japan. Stock cultures were maintained on potato dextrose agar slants and regeneration was conducted regularly every 4 weeks.

2.2 Preparation and Pretreatment of Raw Material

Rice straw and corn stem were sun dried for about four days and cut to approximately 5 mm length and grinded using cereal grinder. For enzyme production, rice straw and corn stem were sieved to 80–100 mesh. For hydrolysis, rice straw was sieved to 120–140 mesh. Prior to enzymatic hydrolysis, the rice straw particle was subjected to alkaline pretreatment. It was boiled at 80 °C with 2% sodium hydroxide solution for six hours and then neutralized with tap water and dried at 105 ^{o}C for six hours. The morphological changes of rice straw after pretreatment were obtained with a scanning electron microscope (SEM) (Zeiss EVO MA 10, British).

2.3 Preparation of Enzyme

Production of crude enzyme was performed using single and mixed culture of *A. niger* ITBCC F74 and *T. reesei* in a solid-state fermentation using undelignified rice straw and corn stem as the substrate. Into 5 grams of substrate in 250 mL Erlenmeyer flask, 25 mL nutrition solution were added. The nutrition contained (per liter): 2.5 g yeast extract (Oxoid-England), 1.5 g bacterial peptone (Oxoid-England), 0.35 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.1 g $CaCl_2.2H_2O$, 0.00125 g $FeSO_4.7H_2O$, 0.0004 g $MnSO_4.H_2O$ and 0.00035 g $ZnSO_4.7H_2O$ in a 0.1 M acetic acid buffer pH 5.5. The medium was sterilized at 121 ^{o}C for 15 minutes and then cooled to room temperature. Spore of *T. reesei* and *A. niger* was suspended in 0.85% saline solution containing 0.1% Tween 80. Six milliliters spore suspension of *T. reesei* and *A. niger* with the ratio 0/1, 1/1, 1/2 and 1/0 which contained a total of approximately 1.0 x 10^8 spores per mL was inoculated aseptically into medium in an Erlenmeyer flask and then incubated at 30 ^{o}C for 4, 6 and 8 days. Crude enzyme was harvested using 100 mL of 0.1% Tween 80 solution in 0.1 M acetate buffer pH 5.5, shaken at 175 rpm for 135 minutes and then centrifuged at 3000 rpm for 60 minutes. The supernatant of crude enzyme was separated from solid by using cloth filter equipped by vacuum pump.

The commercial enzyme solution was prepared by dissolving powder of 100 mg of the commercial enzyme in 100 mL of 0.1 M acetic acid buffer pH 5.5 to obtain an enzyme solution with an activity of 0.93 U/mL.

2.4 Stability of Enzyme

Experiments to investigate enzyme stability were conducted by incubation of 25 mL enzyme solution for 10 h at various pH and temperature. Activity of enzyme was determined at the initial time and at the end of the incubation. Enzyme assay was conducted at pH 5.5 and 35 ^{o}C using the same procedure for enzyme assay as described in Analytical Methods and Enzyme Assay.

2.5 Enzymatic Hydrolysis

Hydrolysis was conducted in a 300 mL flask equipped with a mechanical stirrer. The optimum ratio of crude enzyme concentration from *T. Reesei* to that from *A. niger* as well as the optimum ratio of mixed crude enzyme concentration to substrate concentration were investigated to obtain the best hydrolysis performance. Five grams of pretreated rice straw of 120–140 mesh and 150 mL of mixed crude enzyme, both at 40 °C and pH 5.5 were mixed and stirred at 160 rpm. Samples were taken every hour and the reducing sugar produced during hydrolysis was analyzed by DNS method [11]. All analyses were performed in duplo or in triplicate.

2.6 Production of Hydrogen

Hydrogen production was conducted in 4 parallel batch reactors, each containing 500 mL fermentation medium, which is equipped with stirrer and heater. Fermentation media contains 2% glucose and / or xylose 1.5%, 0.5% yeast extract and 350 mg / L $FeSO_4.7H_2O$. This media was sterilized at 121 ^{o}C for 15 minutes. *E. aerogenes* was two-step grown at 50 and 200 mL using the same medium as the fermentation. 50 mL of 200 mL preculture was inoculated into each reactor containing 450 mL fermentation medium. pH medium was adjusted by using 4 N NaOH. Reactor, 1, 2, 3 and 4 have been shut down after 6, 12, 18 and 24 hours operations respectively. The concentration of reducing sugar, bacterial cells and hydrogen was determined as described in the analytical method. The composition of hydrogen was determined by using Panterra hydrogen sensors from Neodym.

2.7 Analytical Methods and Enzyme Assay

The content of cellulose, hemicellulose and lignin in rice straw was determined using Chesson method [12]. The enzyme activity was determined as CMCase. Two hundred µL of crude enzyme was incubated with 1.8 mL of 1% CMC for 10 minutes at pH 5.5 and 35 ^{o}C. One unit activity was defined as the amount of enzyme that produces 1 µmol glucose per minute. The glucose concentration was determined by dinitro salicylic acid (DNS) method [11]. The optimum pH and temperature of enzyme were determined by using the same method for activity by varying pH and temperature.

The reducing sugar content in the hydrolysates produced from the enzymatic hydrolysis was analyzed by DNS method. For data confirmation, some samples were also analyzed by HPLC (Shimadzu, Kyoto, Japan). A 300 mm x 7.8 mm HPLC column containing Aminex HPX-87P Heavy Metal, 9 mm particle size (Bio-Rad Laboratories, Richmond, CA) was used for the analyses and water was used as the eluent. The detailed analytical procedure followed the method reported by Sharma *et al.,* [13].

2.8 Study of Hydrodynamic

The study used ANSYS FLUENT 13.0 software in which for geometric modelling the ANSYS Design Modeler application was used with grid determination using Meshing and calculation of CFD simulation iterations using ANSYS FLUENT. The procedure for simulating includes pre-processor, solver and post-processor. The simulation involves a multiphase system with a two-phase Eulerian approach. The liquid phase as the primary phase initially only consisted of 0.02 mass fraction of $C_6H_{12}O_6$ and 0.98 mass fraction of H_2O, while the gas phase as the secondary phase consisted of CO_2 and H_2 with a mass fraction of 0.5 each. The gas bubble is assumed to have a constant diameter of 0.1 mm. To model turbulent flow in agitating tank, the standard k-ε model is used. Transport species are used to define model species in which heterogeneous reactions using a modified Arrhenius equation, are used to solve the rate equations of the reaction. To model the impeller, the Multiple Reference Frame (MRF) approach is used.

3. Results and Discussion

3.1 Production of Crude Enzyme

Figure 1 shows the effect of incubation time on the activity of enzyme produced by single or mix culture of *T. reesei* and/or *A. niger* using rice straw or corn stem as the substrate. Both Figure 1(a) and Figure 1(b) show that cellulase activity produced by mixed culture were lower than that produced by a single culture. This is not in accordance with the results obtained by Ikram-ul-Haq *et al.,* [14]. This might indicate the existence of metabolic disorders if both strains are mixed, so that the strains cannot produce enzymes optimally as they do in a single culture. The figures showed that the maximum enzyme activity produced by single culture of *T. reesei* (Tr/An = 1/0) and *A. niger* (Tr/An = 0/1) were attained at six- and eight-days incubation, respectively. When rice straw was used as the substrate, the highest activity produced by *A. niger* was 1.69 U/mL nearly equal to that obtain by *T. reesei* i.e., 1.66 U/mL. When the substrate was corn stem, the highest activity produced by *A. niger* was 1.15 U/mL that was slightly lower than that produced by *T. reesei* of 1.30 U/mL. Cellulase activity produced using rice straw as the substrate was around 23%–47% higher than that produced using corn stem as the substrate. It was reported that cellulose of rice straw is one of the most difficult to degrade by fungi [15]. If such compound was used as the substrate for enzyme production, the fungi will produce enzyme with higher activity [16]. Ikram-ul-Haq *et al.,* [14] also stated that cellulase production depends on crystallinity of cellulose used as the substrate, in which the higher the crystallinity the better the cellulase produced.

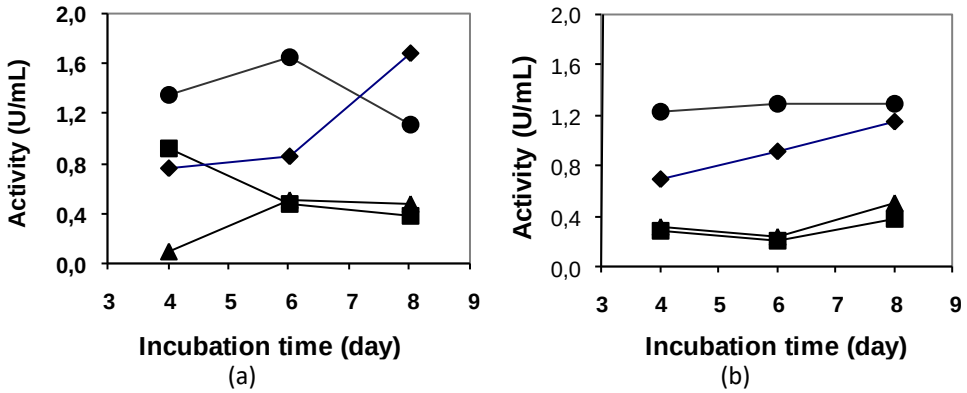


Fig. 1. Effect of incubation time on the enzyme activity produced using single or mixed culture of *T. reesei* (Tr) and/or *A. niger* (An) using (a) rice straw and (b) corn stem as the substrate at a spore ratio of (●) Tr/An = 1/0 (pure *T. reesei*), (■) Tr/An = 1/1, (▲) Tr/An = 2/1, and (◆) Tr/An = 0/1 (pure *A. niger*)

3.2 Optimum Temperature and pH of Crude Cellulase

The optimum pH and temperature of crude cellulase produced by a single culture of the fungi were investigated and the results are shown in Figure 2 and Figure 3 for cellulase from *T. reesei* and *A. niger,* respectively. In Figure 2, it can be seen that at pH values of 4 and 5.5, the enzyme has relatively similar values of activities and the values were higher compared to those obtained at pH 7. Figure 2 shows that the activities of cellulase from *T. reesei* increased when the temperature increased. Activity at 50 °C was relatively the same as that at 60 °C. The results of pH change on cellulase from *A. niger* was nearly the same, i.e., lower pH of 4 or 5.5 gave higher activities (Figure 3). In Figure 3, it can be seen that the cellulase from *A. niger* works optimally in the temperature range of 40 – 60 °C.

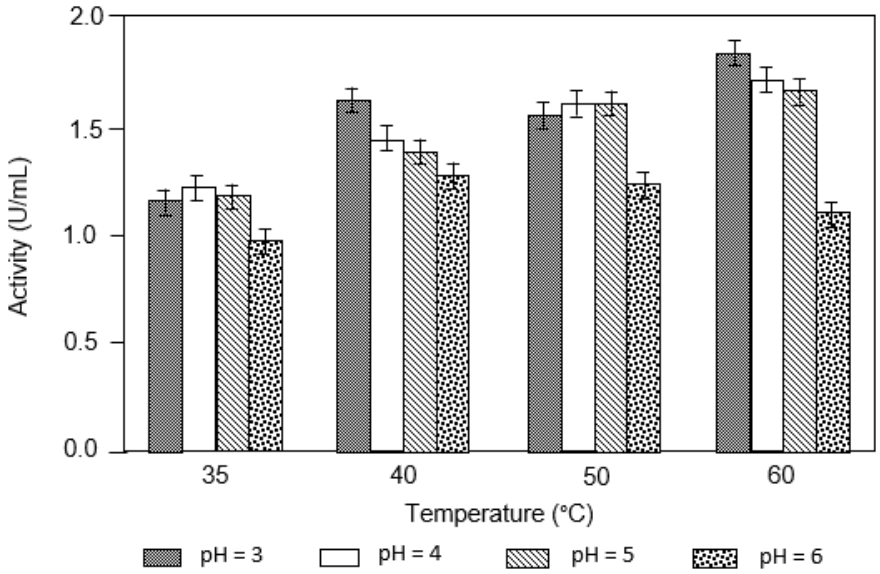


Fig. 2. Effect of pH and temperature on the activity of crude cellulase from *T. reesei*. Effect of pH was conducted at 50 °C, while effect of temperature at pH 5.5. Error bar indicates the standard deviation of the data

3.3 pH and Temperature Stability of Cellulase

In order to determine the pH and temperature to be used in the hydrolysis of rice straw, an investigation of the stability of enzyme was further conducted. This is because the hydrolysis will be conducted for a relatively long time. The optimum pH and temperature were obtained under a very short reaction time (10 min) and it therefore should be compromised with the data of the stability of the enzyme.

Table 1 shows the results of experiments to investigate the remaining cellulase activity after exposure to certain pH and temperature for 10 h. The table shows that in general, the activity of cellulase declined when the pH was decreased or when the temperature was increased, revealing that 40 ^{o}C and pH 7.0 was the condition by which both the enzymes were most stable. The table also shows that cellulase from *T. reesei* has same stabilities under a temperature range of 40 – 60 ^{o}C. However, since the activity of cellulase from *A. niger* decreased significantly when exposed for 10 h at higher temperature, 40 ^{o}C was determined as the temperature of the hydrolysis. On the other hand, the table shows that both the enzymes were most stable under pH 7.0. However, since the enzyme activity under this pH was not so high, as shown in Figure 2 and Figure 3, this pH was not applied in the hydrolysis process. Instead, pH 5.5 was chosen since stability under this pH was still good (≥ 80%) and much better than stability under pH 4.0. As has been shown in Figure 2 and Figure 3, the activity under pH 5.5 was relatively the same as the activity under pH 4.0.

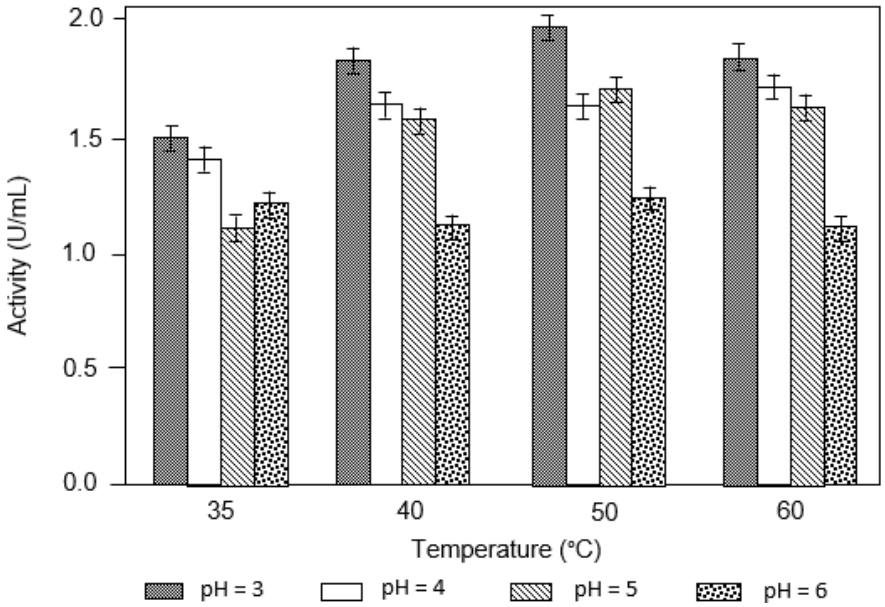


Fig. 3. Effect of pH and temperature on the activity of crude cellulase from A*. niger*. Effect of pH was conducted at 50 ^{o}C, while effect of temperature at pH 5.5. Error bar indicates the standard deviation of the data

Table 1
Enzyme stability when exposed under certain pHs and temperatures

pH of incubation	Temperature of incubation (°C)	Remaining cellulase activity after 10 h of incubation*)	
		T. reesei	*A. niger*
5.5	40	82 %	86 %
	50	80 %	85 %
	60	82 %	78 %
4.0	40	76 %	79 %
5.5		80 %	86 %
7.0		100 %	91 %

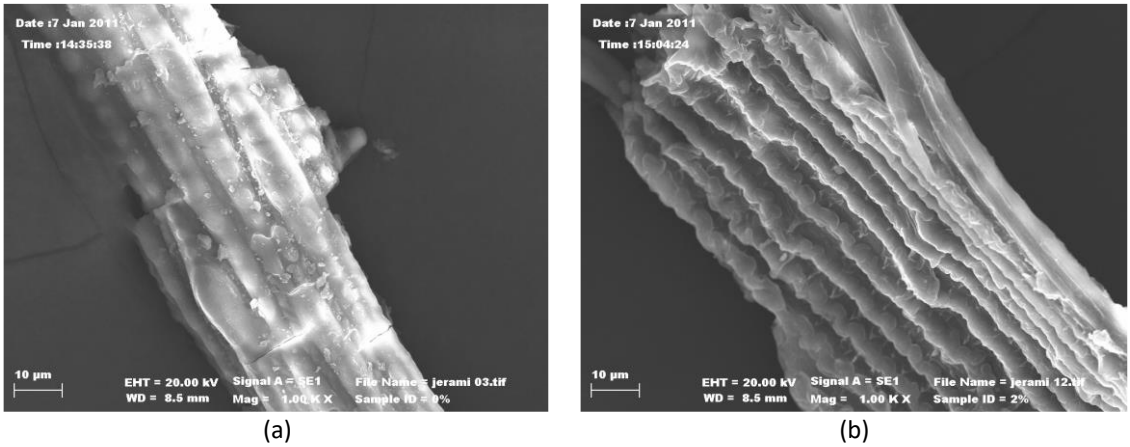
*) determined as the ratio of the activity of the enzyme after incubated at certain pH and temperature for 10 h to the initial activity of the enzyme at the corresponding pH and temperature.

3.4 Alkaline Pretreatment of Rice Straw

Significant reduction of the lignin contained in lignocellulosic materials is one of the most important conditions required for effective access of cellulase enzyme into the cellulose structure so that degradation of cellulose can be better performed. For that reason, effective pretreatment to conduct the delignification process is highly required prior to enzymatic hydrolysis. The results of the alkaline pretreatment using 2% NaOH were given in Table 2. The table shows that 2% NaOH was effective in reducing the lignin content in rice straw. Figure 4 shows the SEM images of rice straw particle before and after alkaline pretreatment at 1000x magnification. It can be seen from Figure 4(a) that before alkaline pretreatment, a layer which looked like a blanket was covering the surface of the particle. The blanket may represent mostly lignin component that protects cellulose against enzymes attack. After pretreated with 2% NaOH, this blanket was removed, leaving a more opened cellulose part of the particle (Figure 4(b)).

Table 2
Comparison of the lignocellulosic composition in Rice Straw before and after the alkaline pretreatment

Component	Before pretreatment	After pretreatment using 2% NaOH
Cellulose	31.8	69.8
Hemicellulose	29.4	27.5
Lignin	38.7	2.6



(a) (b)

Fig. 4. SEM image of rice straw (a) before alkaline pretreatment and (b) after pretreated with 2% NaOH

3.5 Hydrolysis of Rice Straw

Figure 5 shows the time course of glucose concentration produced in the hydrolysis of rice straw using cellulase of different source and composition. Dashed line of Figure 5 shows that crude cellulase from *T. reesei* gave higher glucose yield of 5.5 g/L in comparison to the yield by using crude cellulase from *A. niger* (3.2 g/L) or by using pure commercial cellulase from *A. niger* (4.2 g/L) after 7 hours of hydrolysis. This indicates that crude cellulase from *T. reesei* may have more suitable composition of endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and 1,4-β-D-glucosidase so that degradation of cellulose results in higher yield of glucose. Figure 5 also shows that mixing 1 U activity of crude cellulase from *T. reesei A.* with 2 U activity of crude cellulase from *A. niger* (data represented by ◆) produced glucose with a concentration of 3.75 g/L after 7 hours of incubation. The data lay between the concentration of glucose produced by crude cellulase from *T. reesei* (□) and crude cellulase from *A. niger* (△). Reducing the amount of cellulase from *A. niger* added to the crude cellulase from *T. reesei*, i.e., under an enzyme ratio of 1/1 (●) resulted in a hydrolysis performance which nearly coincide with the performance by using crude cellulase from *T. reesei* (□). Further reducing the amount of cellulase from *A. niger* added until the ratio of crude cellulase from *T. reesei* to the crude cellulase from *A. niger* was 2 : 1 (▲) resulted in further improvement of the performance of the enzymatic hydrolysis of cellulose. After 7 hours of hydrolysis, the concentration of glucose attained 6.4 g/L, around 16 % higher than that generated by crude cellulase from *T. reesei* alone.

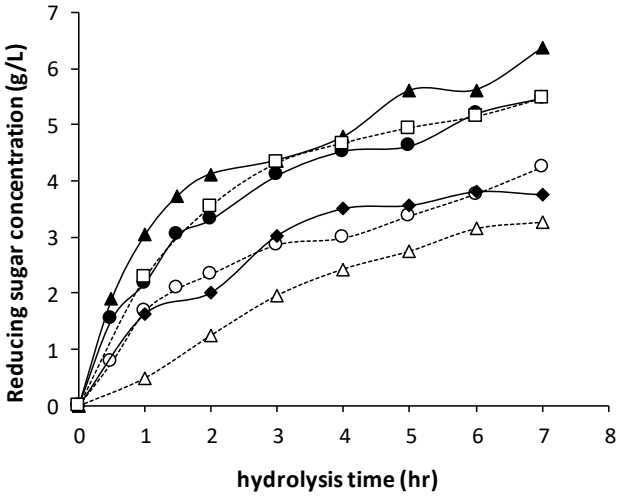


Fig. 5. Time course of glucose concentration produced in the hydrolysis of rice straw at 40 ºC, pH 5.5, enzyme concentration was 0.47 U/mL, straw weight 5.0 g, under stirring speed of 160 rpm, and particle size of straw of 120–140 mesh using cellulase of different source and composition. Legend --△--, --○--, --□--, represent single crude enzyme from *A. niger*, single commercial enzyme from *A. niger* and single crude enzyme from *T. Reesei*, respectively. Legend —◆—, —●—, —▲— represent mixed crude enzyme under a Unit/Unit ratio of crude cellulase from *T. reesei* to crude cellulase from *A. niger* of 1/2, 1/1 and 2/ 1, respectively

Figure 6 shows the time course of enzymatic hydrolysis of rice straw under the enzyme ratio of 2 Units crude cellulase from *T. reesei* to 1 Unit crude cellulase from *A. niger* using various total enzyme concentration. The figure shows that after 7 hours of incubation, the greater the total enzyme activity used, the higher the concentration of glucose produced. This is possible because the greater the level of activity of enzymes used, the more the active sites of enzyme available so that cellulose can be degraded into glucose in a higher speed.

Chang *et al.,* [8] obtained a reducing sugar of 4 g/L after hydrolyzing the "freeze pretreated" rice straw using a cellulase concentration of 150 U/g rice straw conducted at 37 °C and pH 5.5 for 48 h incubation. The results obtained in the present experiment seem to be superior with 8.7 g/L sugar obtained using 28 U cellulase per gram rice straw after 7 h incubation.

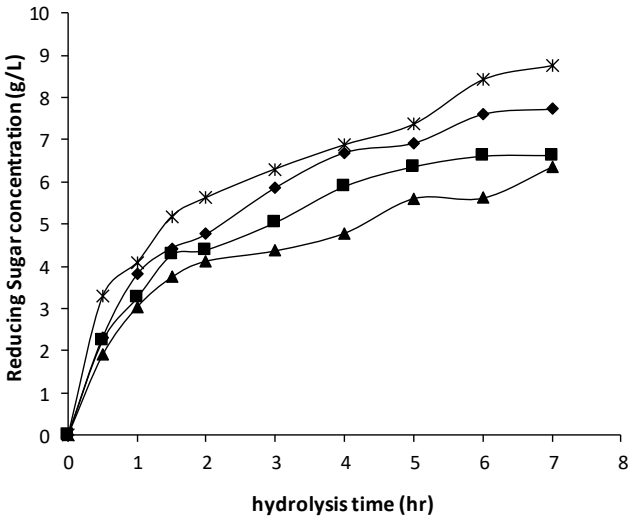


Fig. 6. Time course of glucose produced using an crude enzyme ratio Tr : An = 2 : 1 at different total enzyme concentration. Liquid volume 150 mL, straw weight 5 g, stirring speed 160 rpm, straw particle size 120-140 mesh, pH 5.5. Legend —▲—, —■—, —◆— and —✳— represent enzyme concentration of 0.47, 0.62, 0.78 and 0.93 U/mL, respectively

However, the increase of glucose concentration is not proportional to the increase of enzyme used for the hydrolysis. For example, a nearly double increase of enzyme used from 0,47 U/mL to 0,93 U/mL only increases the level of glucose concentration from 6.4 to 8.7 g/L. A detailed economic calculation should therefore be made if the process is to be commercialized or to be taken to bigger production scale.

3.6 Production of Hydrogen

The effect of stirring intensity on the rate of glucose consumption is shown in Figure 7. Stirring can increase the rate of glucose diffusion in the substrate to the cell surface. It can be seen that at the beginning of the fermentation the glucose degradation at a stirring speed of 81 rpm was slower than at 46 rpm, but in general the increase in the stirring speed accelerated the consumption of glucose by microorganisms and produced products.

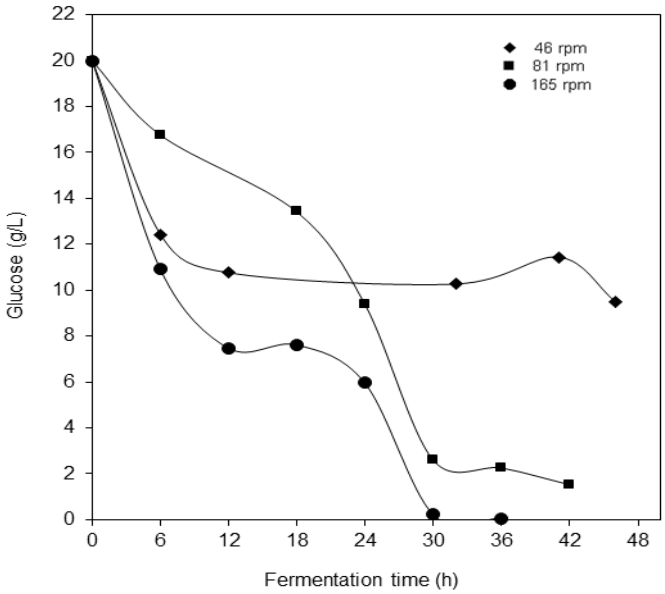


Fig. 7. Effect of stirring intensity on glucose consumption in hydrogen fermentation by *E. aerogenes* NBRC 13534

Figure 8 shows that cell growth corresponds to glucose consumption (Figure 7), where the lowest substrate conversion and cell concentration are obtained at the lowest speed, whereas the highest substrate conversion and cell concentration are obtained at the highest stirring speed. Deviations occurred at a stirring speed of 81 rpm, where the yield of cells to the substrate was smaller than the yield of cells to the substrate at 46 and 165 rpm. This is possible because the substrate is not only converted into cells, but also converted into various products such as CO_2 and H_2 [17].

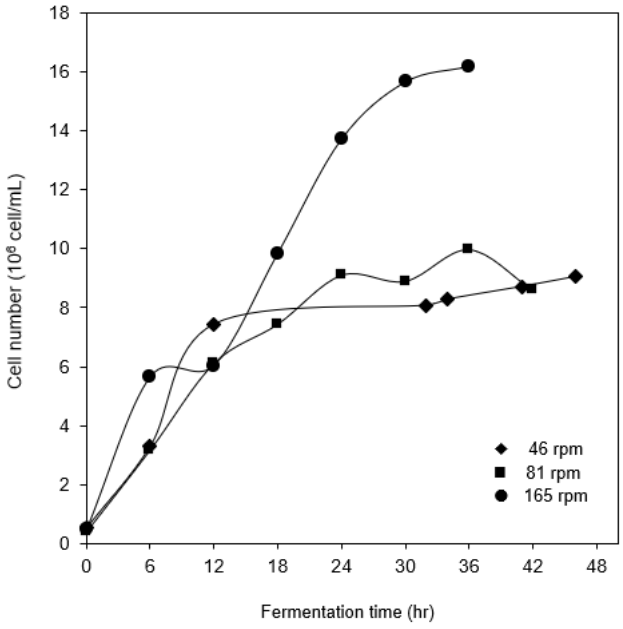


Fig. 8. Effect of stirring intensity on cell concentration in hydrogen fermentation by *E. aerogenes* NBRC 13534

Figure 9 is the number of moles of H_2 produced during fermentation, at different stirring intensities. In general, at higher stirring intensity, the rate of hydrogen production at the start of fermentation was higher and gas was detected more quickly. Increasing the stirring intensity results in a wider fluid circulation area. The highest circulation speed flowing from the bottom to the top of the reactor becomes higher and reaches the liquid surface. This flow pattern can break the foam layer so that the gaseous products can escape from the liquid phase more easily, as a result the concentration of CO_2 in the solution is smaller and the reoxidation of NADH by H+ to H_2 is faster. The lowest number of moles of hydrogen was produced at a stirring intensity of 64 rpm and the highest was achieved at a stirring speed of 165 rpm according to the number of cells produced (Figure 8). This can be understood because the stirring speed is related to the rate of substrate diffusion through the cell wall of microorganisms [18].

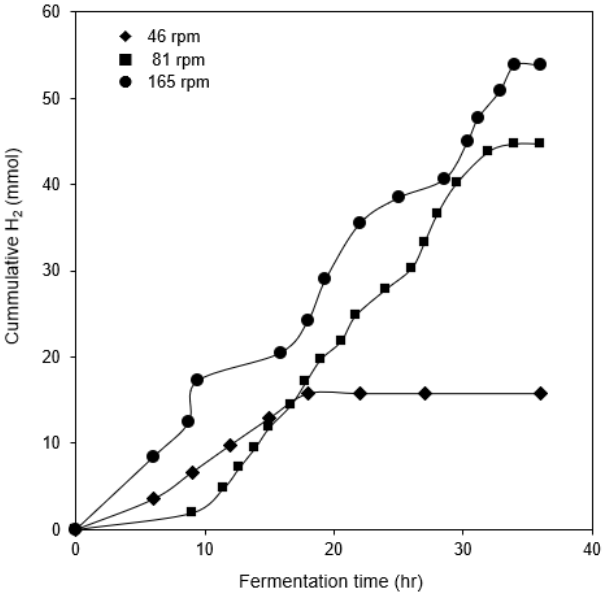


Fig. 9. Effect of stirring intensity on hydrogen produced in hydrogen fermentation by *E. aerogenes* NBRC 13534

At a stirring speed of 81 rpm, the increase in the amount of hydrogen was not proportional to the increase in the number of cells, whereas the production of hydrogen was related to the production of cells. This is possible because the stirring speed of 81 rpm produces a better stirring effect so that hydrogen can exit the liquid phase very quickly.

3.7 Hydrodynamic Study

Figure 10 shows the change in the intensity of the flow turbulence in the reactor with an increase in the impeller rotational speed. The color change from blue to red indicates higher turbulence intensity. The highest intensity of turbulence is found in the impeller area, where with increasing rotational speed of stirring, the intensity of turbulence near the impeller is higher and the turbulence is more evenly distributed. This, in addition to increasing the volume of biogas that can come out of the liquid body, will also increase the rate of substrate diffusion to the bacterial cells and ensure a more even distribution of bacterial cells in the reactor so that sedimentation of bacterial cells in the reactor can be avoided.

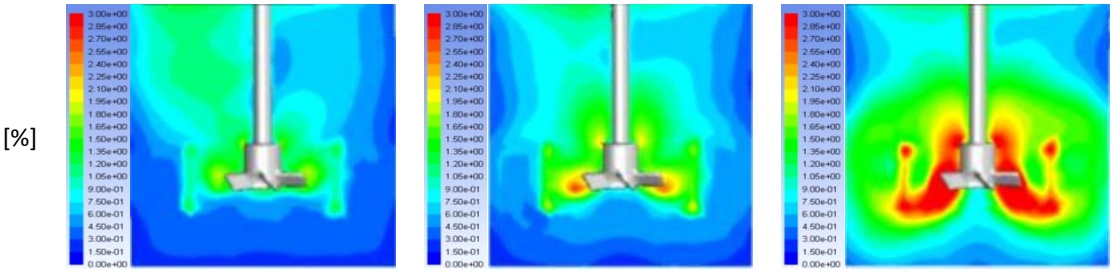


Fig. 10. Contour of turbulence intensity (%) at t = 20 s (a) 46 rpm, (b) 81 rpm and (c) 165 rpm

Figure 11 describes the contours of the volume fraction of biogas in a stirred tank at different impeller rotational speeds for each given time interval. The color change from blue to red indicates that the volume fraction of biogas is getting bigger. It can be seen that for each time interval, with increasing impeller rotation speed, the volume fraction of biogas in the bottom area of the tank decreases. This indicates that in the PBT impeller with pumping down flow, the increase in the rotational speed of stirring causes the fluid to have greater energy to push the biogas in the media to flow axially. Therefore, the biogas in the reactor can be easily pushed to the liquid surface and eventually escape from the bulk liquid to the bulk gas. Yusefi *et al.,* [19] studied the hydrodynamic size of magnetic nanoparticles using fruit peel extract but almost no study concerning the study of hydrodynamic of hydrogen produced in a heterogenous mixture in a fermenter.

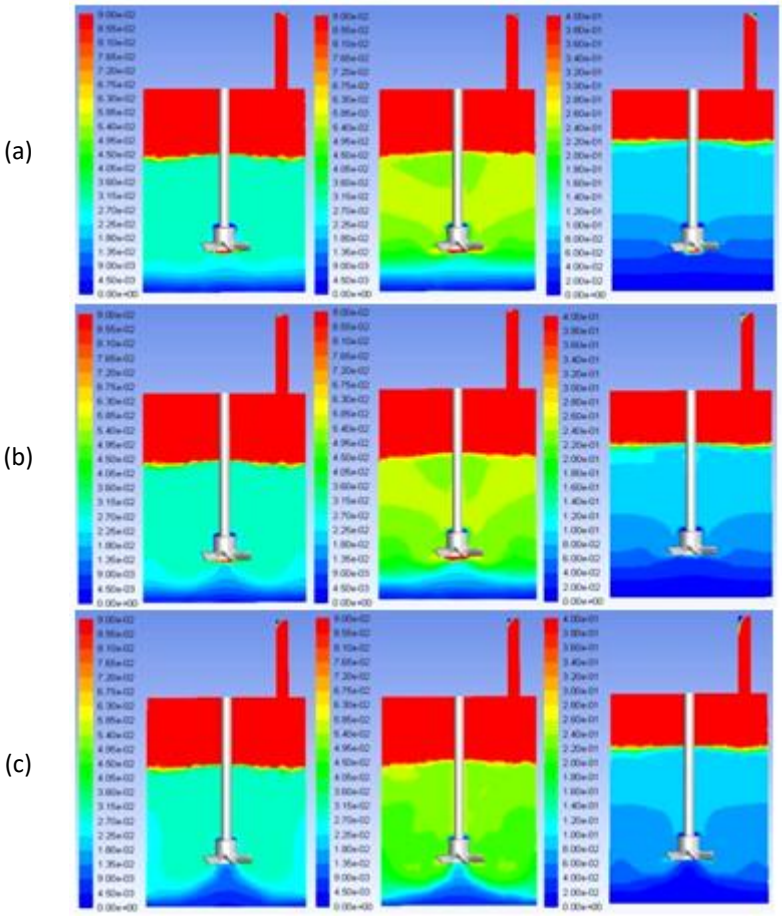


Fig. 11. Contour of biogas volume fraction at t = 20s. (a) 46 rpm, (b) 81 rpm and (c) 165 rpm

4. Conclusion

Crude cellulose degrading enzymes cellulases were produced from fungi strains *T. reesei* and *A. niger*. Cellulase activity produced by mixed cultures was lower than that produced by a single culture. Rice straw was a better substrate for cellulase production than corn stem by which cellulase activity produced by this substrate was more than 20% higher. A compromise between the optimum pH and temperature and the stability of enzyme after incubation for 10 h was used to determine the pH and temperature to be used in the hydrolysis experiment. 2% of NaOH was effective in reducing the lignin content of rice straw prior to enzymatic degradation. This was confirmed by the SEM image which showed that the layer covering the rice straw particle has been removed after this alkaline pretreatment. Although the value of cellulase activity obtained in the present experiment was somewhat below the activity obtained by some researchers such as cellulase from *T. Reesei* RUT-C30 which has an activity of 4.2 U/mL, however, the performance of the crude cellulase utilized in the present experiment was quite good, and even better than the performance by using pure commercial cellulase from *A. niger* [9]. A combination of cellulase from *T. reesei* and cellulase from *A. niger* used in the present experiment obviously showed excellent performance in the enzymatic hydrolysis of rice straw. Hydrogen fermentation is affected by the speed of stirring. Increasing the stirring speed from 46 to 165 rpm increased the hydrogen yield from 0.025 to 0.085 mol H_2/mol glucose. Hydrodynamic simulation shows that the highest turbulence intensity occurred at the impeller region, by which by increasing the stirring speed, the turbulence intensity in all part of the reactor will be more uniformly distributed. This will result in the diffusion of substrate to the cell surface and increasing the rate of biogas flowing out from liquid bulk. The study also shows that the volume fraction of biogas in the bottom of the tank decreased by increasing the stirring speed. This shows that more gas goes to surface and leaves the reactor.

Acknowledgement
This study was financially supported by The Directorate of Research and Community Development, Ministry of Education and Culture, Republic of Indonesia.

References

[1] Muharja, Maktum, Nur Fadhilah, Tantular Nurtono, and Arief Widjaja. "Enhancing enzymatic digestibility of coconut husk using nitrogen-assisted subcritical water for sugar production." *Bulletin of Chemical Reaction Engineering & Catalysis* 15, no. 1 (2020): 84-95. https://doi.org/10.9767/bcrec.15.1.5337.84-95

[2] Widjaja, Arief, Silvya Yusnica Agnesty, Hanny F. Sangian, and Setiyo Gunawan. "Application of ionic liquid [DMIM] DMP pretreatment in the hydrolysis of sugarcane bagasse for biofuel production." *Bulletin of Chemical Reaction Engineering & Catalysis* 10, no. 1 (2015): 70-77. https://doi.org/10.9767/bcrec.10.1.7143.70-77

[3] Morya, Raj, Fanirintsoa Herivonona Andrianantenaina, Saurabh Singh, Ashutosh Kumar Pandey, Gi-Beom Kim, Jay Prakash Verma, Gopalakrishnan Kumar, Tirath Raj, and Sang-Hyoun Kim. "Exploring rice straw as substrate for hydrogen production: Critical challenges and opportunities." *Environmental Technology & Innovation* (2023): 103153. https://doi.org/10.1016/j.eti.2023.103153

[4] Xiang, Qian, Y. Y. Lee, Pär O. Pettersson, and Robert W. Torget. "Heterogeneous aspects of acid hydrolysis of α-cellulose." In *Biotechnology for Fuels and Chemicals: The Twenty-Fourth Symposium*, pp. 505-514. Humana press, 2003. https://doi.org/10.1007/978-1-4612-0057-4_42

[5] Jin, Shengying, and Hongzhang Chen. "Structural properties and enzymatic hydrolysis of rice straw." *Process Biochemistry* 41, no. 6 (2006): 1261-1264. https://doi.org/10.1016/j.procbio.2005.12.022

[6] Abedinifar, Sorahi, Keikhosro Karimi, Morteza Khanahmadi, and Mohammad J. Taherzadeh. "Ethanol production by Mucor indicus and Rhizopus oryzae from rice straw by separate hydrolysis and fermentation." *Biomass and Bioenergy* 33, no. 5 (2009): 828-833. https://doi.org/10.1016/j.biombioe.2009.01.003

[7] Ogino, Hiroyasu, Takashi Miura, Kosaku Ishimi, Minoru Seki, and Hiroyuki Yoshida. "Hydrogen production from glucose by anaerobes." *Biotechnology Progress* 21, no. 6 (2005): 1786-1788. https://doi.org/10.1021/bp050224r

[8] Chang, Ken-Lin, Jitladda Thitikorn-amorn, Jung-Feng Hsieh, Bay-Ming Ou, Shan-He Chen, Khanok Ratanakhanokchai, Po-Jung Huang, and Shui-Tein Chen. "Enhanced enzymatic conversion with freeze pretreatment of rice straw." *Biomass and Bioenergy* 35, no. 1 (2011): 90-95. https://doi.org/10.1016/j.biombioe.2010.08.027
[9] Muthuvelayudham, R., and T. Viruthagiri. "Fermentative production and kinetics of cellulase protein on Trichoderma reesei using sugarcane bagasse and rice straw." *African Journal of Biotechnology* 5, no. 20 (2006).
[10] Juhász, Tamás, Krisztina Kozma, Zsolt Szengyel, and Kati Réczey. "Production of b-glucosidase in mixed culture of Aspergillus niger BKMF 1305 and Trichoderma reesei RUT C30." *Food Technology and Biotechnology* 41, no. 1 (2003): 49-53.
[11] Miller, Gail Lorenz. "Use of dinitrosalicylic acid reagent for determination of reducing sugar." *Analytical Chemistry* 31, no. 3 (1959): 426-428. https://doi.org/10.1021/ac60147a030
[12] Datta, Rathin. "Acidogenic fermentation of lignocellulose-acid yield and conversion of components." *Biotechnology and Bioengineering* 23, no. 9 (1981): 2167-2170. https://doi.org/10.1002/bit.260230921
[13] Sharma, D. D., V. F. D'Souza, M. B. McConnell, and G. Mazza. "Identification and quantification of sugars in winter-hardy apples by high performance liquid chromatography." *Canadian Institute of Food Science and Technology Journal* 21, no. 4 (1988): 435-437. https://doi.org/10.1016/S0315-5463(88)70982-7
[14] Ikram-ul-Haq, Muhammad Mohsin Javed, Saleem Khan, and Zafar Siddiq. "Cotton saccharifying activity of cellulases produced by co-culture of Aspergillus niger and Trichoderma viride." *Research Journal of Agriculture and Biological Sciences* 1, no. 3 (2005): 241-245.
[15] Havannavar, Raghavendra B., and G. S. Geeta. "Pre-treatment of agroresidues for release of maximum reducing sugar." *Karnataka Journal of Agricultural Sciences* 20, no. 4 (2010).
[16] Chen, Shu, and Morris Wayman. "Novel inducers derived from starch for cellulase production by Trichoderma reesei." *Process Biochemistry* 27, no. 6 (1992): 327-334. https://doi.org/10.1016/0032-9592(92)87010-E
[17] Colin, Verónica Leticia, Analía Rodríguez, and Héctor Antonio Cristóbal. "The Role of Synthetic Biology in the Design of Microbial Cell Factories for Biofuel Production." *Journal of Biomedicine and Biotechnology* 2011 (2011): 1-9. https://doi.org/10.1155/2011/601834
[18] Chou, Chia-Hung, Cheng-Wei Wang, Chieh-Chen Huang, and Jiunn-Jyi Lay. "Pilot study of the influence of stirring and pH on anaerobes converting high-solid organic wastes to hydrogen." *International Journal of Hydrogen Energy* 33, no. 5 (2008): 1550-1558. https://doi.org/10.1016/j.ijhydene.2007.09.031
[19] Yusefi, Mostafa, Ong Su Yee, and Kamyar Shameli. "Bio-mediated production and characterisation of magnetic nanoparticles using fruit peel extract." *Journal of Research in Nanoscience and Nanotechnology* 1, no. 1 (2021): 53-61. https://doi.org/10.37934/jrnn.1.1.5361