International Journal on Advanced Science Engineering Information Technology

Vol.6 (2016) No. 1
ISSN: 2088-5334

Saccharification Waste Biomass Rice Straw IR-64 by Using Xylanase from Indigenous Marine Bacteria *Bacillus safensis* LBF-002

Apridah Cameliawati Djohan[#1], Urip Perwitasari[#] and Yopi[#]

[#] *Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Bogor 16911, Indonesia,*
E-mail: a.camelia707@gmail.com or apri009@lipi.go.id

***Abstract*— Agricultural residues have an enormous potential as renewable carbon and energy sources. Saccharification of agricultural by microbial hydrolytic enzymes is the first step of bioconversion of organic material into reducing sugar. The main purpose of this research is converting rice straw IR-64 waste biomass into reducing sugar xylo-oligosaccharides by using xylanase from indigenous marine bacteria *Bacillus safensis* LBF-002. The optimation of xylanase in rice straw medium are using stability of pH and temperature were resulted higher activity in pH 7 and 50 °C which result 2.228 U/mL in 24 h incubation. The xylanase was concentrated with PEG 6000 in ratio (1:1) become 16.578 U/mL and was used for hydrolyze the rice straw for getting the sugar reduction product. The sugar reduction component from rice straw saccharification was analyzed using Thin Layer Chromatography and also High Performance Liquid Chromatography (HPLC). The present study was a first effort to explore marine bacteria *Bacillus safensis* LBF-002 to produce and obtain the optimum condition for producing xylooligosaccharide from rice straw waste. The best result for hydrolysis experiment showed in saccharafication with 2.5% rice straw and crude enzyme xylanase 4 U/mL for 1 h and 2 h incubation which is resulted xylose, xylobiose, and xylohexose.**

***Keywords*— Saccharification; Xylanase; Rice straw; Bacillus safensis LBF-002; Xylooligosaccharide.**

I. INTRODUCTION

Accumulation biomass from agroindustry in large quantity causes big problem. Biomass processing can help reducing agricultural waste also can added high value of this feedstock. Conversion this polymer into monomer or oligomer is important in biorefinery process [1]. Rice straw, corncob, empty fruit bunch, and tobacco stalk, are biomass from Indonesia agro-industrial production. This waste can be used to produce a potential product such as bioethanol, probiotic, or antioxidant [2];[3];[4]. In 2014, rice production in Indonesia within 70.83 million tons. One kilogram of grain harvested is accompanied by production of 1–1.5 kg of the straw. It means waste biomass from rice production above 70.83 – 106.245 million tons rice straw.

Several characteristics component from rice straw make it a potential feedstock for fuel production. Organic component in rice straw are hemicellulose 27%, cellulose 39%, xylan 20.6%, glucan 37.5%, lignin 12%, ash 11% [5]. Hydrolysis of rice straw can be carried out by chemical or enzymatic reaction. Chemical hydrolysis of rice straw to produce sugar has been reported [5]. Chemical hydrolysis of rice straw needs more energy than enzymatic reaction because it needs high pressure and temperature.

Xylanase is saccharification enzyme that able to hydrolysis polysaccharide from biomass into xylooligosaccharides. Xylanase can be produced by microorganism such as *Aspergillus foetidus*, *Aspergillus tubingensis*, *Bacillus aerophilus*, *Bacillus mojavensis*, *Promicromonospora* sp. [3];[6];[7];[8]. In addition xylanase from marine-bacteria, xylanase also can be produced from marine-derived endophytic fungi for produce xylanase [9]. The fungal xylanase has limited use in pulp bleaching, because they affect the viscosity and strength of the product. Bacterial xylanase has spesific activity and free from cellulose [10].

Some marine bacteria from Indonesia have been studied to produce mannanase, xylanase [11]; [12]. *Bacillus safensis* are known can produce xylanase, thus enzyme can be used to hydrolysis sugarcane baggase for xylooligosaccharide production [12]. In Indonesia, biodiversity of marine bacteria is still lack, whereas Indonesia has a wide ocean. Chemical hydrolysis biomass are not friendly for environmental, so we need hydrolysis biomass by enzymatic saccharification. Saccharification is conversion of lignocellulose in to fermentable sugar [13]. Conversion biomass from rice straw into sugar has been studied [5]. Ong et al [14] reported the optimum condition for hydrolysis rice straw using exoglucanase from *Aspergillus niger*.

The beneficial biomass saccharification using xylanase is thought to be the result of the improved cellulose accessibility as a result of xylan solubilisation [15]. Diep et al [16] showed techno-economic evaluation for ethanol production from rice straw is potential to be done for the future in Vietnam. Enzymatic saccharification can be improved to optimize the yield of oligosaccharide or the process. Simultaneous saccharification and acetone-ethanol-butanol (ABE) fermentation has been studied to reduce the number of steps involved in the conversion of lignocellulosic biomass into butanol [17]. Ethanol production can be enhancing by addition nutrient supplement in simultaneous saccharification fermentation [18].

Base on HPLC analysis in original rice straw concentration xylose is 16.1% of dry rice straw [19]. Chemical hydrolysis of rice straw by diluted acid achieved xylose 20.5 g/L [20]. Xylose concentration from hydrolysate rice straw after detoxified with activated charcoal is 18.2 g/L [21]. Hydrolysis rice straw with temperature treatment at 130, 160, 200 ^{o}C contain xylose 23 g/L, 25 g/L, and 17 g/L [22]. Saccharification are using enzymatic for rice straw using cocktail enzyme can produce xylose 74 mg/g rice straw [23].

The aims of this study are the utilization of rice straw to produce xylooligosaccharide using marine xylanase from *Bacillus safensis* LBF-002. Hopefully this technology can improve exploration marine microorganism biodiversity in Indonesia and also reduce waste biomass also can be alternative sugar material for biofuels production or functional food industry for the next studied.

II. Material and Method

A. Culture Collection

Local xylanolytic marine Bacteria from Pari Island *Bacillus safensis* LBF-002 used in these experiments were identified using direct sampling method, bacteria sample have been collected from sea water. The medium for Screening and purification bacteria was composed with Xylan substrate (0,5% Birchwood xylan, Sigma), 0.075% peptone, 0.05% yeast extract and some minerals compound (artificial sea water) and pH was adjusted to 6.0 [24].

B. Rice Straw Pre-treatment

The rice straw was obtained from commercial farm in Bogor, West Java, Indonesia and was harvested at the mature stage. A wet sample of approximately 5 kg was collected and immediately dried at 65 ^{o}C for 3 days (to the final moisture 3-5%). The rice straw was milled with a high-speed milling machine (Black & Decker, FX 350, England). Then, the milled samples gradually screened with a sieve shaker 80, 100 and 200 µm-mesh. The screened materials were stored in desiccators until their use.

C. Substrates and Enzyme

Commercial substrates such as xylan birchwood from Sigma, and raw material rice straw (*Oryza sativa*) powder were of 200 mesh analytical grade for lab scale experiment. Pre-treatment for rice straw by physical pre-treatment until 200 mesh availability using scientific sieves.

D. Xylanase Production

The most potential microbes were selected to produce spesific crude enzyme such as xylanase. Potential isolate Bacillus safensis LBF-002 was inoculated into pH 7 medium artificial sea water medium with spesific carbon sources (0.5% rice straw), extract and some minerals compound (Mandels & Sternberg, 1976). Incubation process in shaker incubator with optimum temperature 50˚C and agitation 200 rpm for 24 hours. Crude enzyme resulted after centifuge (5000 rpm, 4 °C, 10 min) the medium in after harvest time.

E. Enzyme Assays

Enzyme activity were assayed as follows: a reaction mixture containing 0.5 ml of 0.5% xylan birchwood in 20 mM acetate buffer as well as 0.5 mL of enzyme solution in the same buffer was incubated at room temperature for 30 min, the addition of 1.5 mL DNS reagent for deactivated the enzyme catalytic activity then measured the solution by spectrophotometer at 540 nm in wavelength. The resulting reducing power was determined by dinitrosalicylic acid (DNS) modification method [25], using xylose as a standard. One unit of the xylanase activity was defined as the amount of enzyme liberating 1 µmol of reducing sugar per minute under the above condition.

F. Xylanase PEG 6000 concentrated

The crude enzyme containing xylanase activity was polled and concentrated using PEG 6000 and dialysis for 6 hours at 4 ˚C with 0.2 M phosphate buffer pH 7 before applied to hydrolysis process.

G. Hydrolysis Rice Straw Biomass

Enzymatic hydrolysis was carried on 1 mL reaction with pH 7 and temperature 50 ^{o}C. Treatment on 0.5%, 1%, 1,5%, 2.5% rice straw hydrolysis with enzyme concentration (2 U/mL; 4 U/mL; 8 U/mL), time incubation (0, 30 second, 1 h, 2 h, 3 h, and 4 h).

H. Reduction Sugar and Total Sugar analysis

Reduction sugar was analyzed using DNS modification method [21]. The hydrolysis sugars were centrifuge and take 0.5 mL the supernatant reacted with 0.5 mL DNS solution, heated at 95˚C and measure by spectrophotometry at 540 nm.

Total sugar analysis with phenol sulphuric modification method. The hydrolysis sugars were centrifuge and take 0.25 mL the supernatant reacted with 0.25 mL 2% phenol solution then add 0.5 mL concentrated H_2SO_4, heated at 95˚C and measure by spectrophotometry at 490 nm.

I. Thin layer chromatography analysis

Thin layer chromatography of the hydrolysis products was performed on a silica gel 60-plastic sheet (Merck & Co. Inc.) developed with a solvent of n-butanol–acetic acid–water (2:1:1), and the oligosaccharides were visualized by Reagent DAP contains of 0.2 g Diphenilamine, 0.2 mL Aniline, 10 mL Acetone, 1.5 mL Phosphoric acid. Then the plate TLC put into oven with temperature 120 ^{o}C for 15 minutes. The oligosaccharide standard such as xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose, glucose.

J. *High Performance Liquid Chromatography (HPLC) analysis*

Analysis of xylo-oligosaccharides the products resulting from the enzymic hydrolysis of xylan from pretreated rice straw were analyzed by HPLC Agilent 1263 Infinity. The supernatants were filtered through 0.45 µm syringeless filter device Mini-UniPrepTM (GE Healthcare Life Sciences). These filtrates were analysed by injecting 20 µl on to an HI-Plex Ca (Duo) Agilent Hi-Plex Ca (Duo), 6.5 × 300 mm, 8 µm (p/n PL1F70-6850) heated to 85 °C and using MilliQ water as the mobile phase at a flow rate of 0.6 mL/min. The xylo-oligosaccharides were detected with Refractive Index Detector (RID).

III. Result and Discussion

A. *Rice Straw Physical Pretreatment*

The cost of the substrate plays a crucial role in the economics of any industrial production process. Therefore, low-value crude agriculture-based raw materials may be employed as cost-effective. Substantial amounts of xylose and other reduced form of polysaccharides were found, whereas glucose was found in a relatively small amount among the sugar mixtures. Under the conditions used in this study, different enzymes could hydrolyze agricultural biomasses differently. This indicated the complexity of a solid material in each agricultural waste, which would allow for the access of particular enzyme to cellulose or hemicelluloses structure.

Fig. 1. Rice straw physical pretreatment (a). Rice straw (b). Drying process (Sun-dry) (c) rice straw powder 200 mesh.

The rice straw was used for optimize the xylanase activity has physical pretreatment to enhance the susceptibility of substrate for enzymatic hydrolysis. The utilization of agricultural residues for enzyme production may serve dual purpose, on one side it provides a valuable product (enzyme) and on the other side also help solving environmental waste disposal problem.

B. *Xylanase Production and PEG 6000 Concentrated*

Polyethylene glycol-6000 is a hydrophilic polymer and has effective effect for water deficit imposed. PEG 6000 can be easily synthesized by the anionic ring opening polymerization of ethylene oxide, into a range molecular weights and variety of end groups, When crosslinked into networks PEG can have high water content, forming "hydrogels".

Enzyme activity crude enzyme xylanase from marine bacteria *Bacillus safensis* LBF-002 in 0.5% rice straw medium was analyzed with DNS method and resulting 2.228 U/mL xylanase activity.

The application of PEG 6000 is suitable material for biological applications because it does not trigger an immune response. The addition of the macromoleculear crowding agent of PEG 6000 haims to concentrated the xylanase enzyme for next hydrolysis processs. The crude enzyme containing xylanase activity was polled and concentrated using PEG 6000 with comparison (1:1), 200 mL enzyme in dialysis tubing was added by 200 g PEG 6000 incubated at 4 ˚C for 6 hours. The result of concentrated process is the volume of xylanase was decrease became 20 mL and the enzyme activity was increase became 16.578 U/mL.

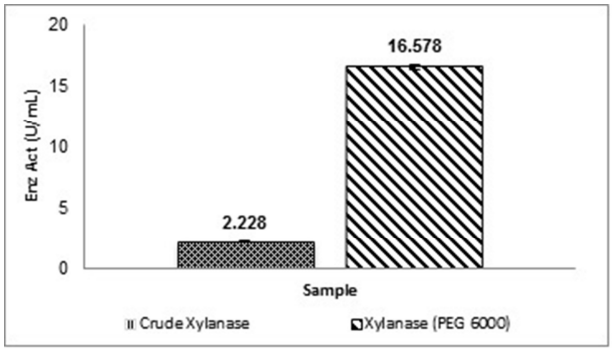


Fig. 2. Xylanase activity from *Bacillus safensis* LBF-002 in 0.5% rice straw medium concentrated with PEG 6000

C. *Thin Layer Chromatography Analysis*

Hydrolysis product from various rice straw concentrations with xylanase from marine was analyzed by silica gel thin layer chromatography. Spot sample hydrolysis rice straw with concentration 0.5%, 1%, and 1.5% were not detected (Fig. 3a).

The optimation of substrate concentration were not found in range 0.5%-1.5% because the substrate concentration is too low and it will produce small amount of reducing sugar.

The experiment showed that hydrolysis process by crude enzyme xylanase continuously produce various oligosaccharide product in difference reaction time and increase until completely degraded in 24 hours. The experiment of hydrolysis are using DAP as spray dye which is sensitive to oligosaccharide product. The XOSs production was characterized by rapid increase in the reducing sugar and also total sugar as a further experiment to know the production of sugar reduction from rice straw hydrolysis.

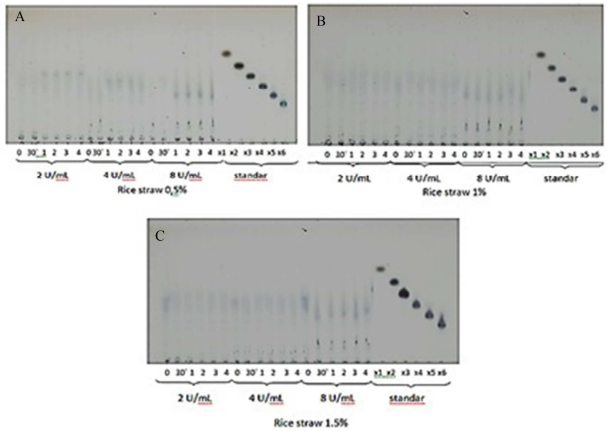
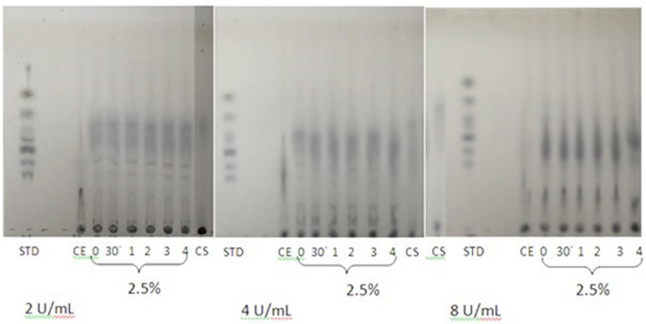


Fig. 3. Hydrolysis sugar 0.5%, 1%, and 1.5% rice straw biomass

STD: Standar xylo-oligosaccharide; CE: Control Enzyme; CS: Control substrate



STD: Standar xylo-oligosaccharide; CE: Control Enzyme; CS: Control substrate

Fig 4. Hydrolysis sugar 2.5% rice straw with concentrated xylanase 2 U/mL, 4 U/mL , and 8 U/mL in continuous sampling time (0, 30 s, 1 h, 2 h, 3 h, and 4h).

The concentration of rice straw was increased become 2.5% for increasing the production of reduction sugar. Hydrolysis of rice straw with marine xylanase from Bacillus safiensis LBF-002 resulted xylotriose as the main products and the other component are xylopentose and xylohexose with little amount (Fig. 4). The results indicate that xylanase from Bacillus safiensis P20 degraded rice straw become xylooligosaccharide and from the spot pattern can be indicated that Bacillus safiensis P20 is produce endoxylanase [26].

Variation enzyme concentration to hydrolyze rice straw are shown on (Fig. 4). Xylanase 2 U/mL are able to hydrolysis xylan to form xylotriose, xylopentose and xylohexose after 1 hour incubation.Hydrolysis rice straw with xylanase 4 U/mL showed spot xylotriose, spot for xylopentose and xylohexose almost not detected. It means the optimum enzyme concentration for hydrolysis rice straw is 4 U/mL.

D. Total Sugar and Sugar Reduction

The use of phenol–sulfuric acid with a phenol concentration of 2% provided a relatively simple and reliable colorimetric method to quantify the total soluble-sugar concentration. Total sugar from hydrolysis process 2.5% rice straw by 4 U/mL xylanase has showed positive degradation and relatively fast for enzymatic reaction.

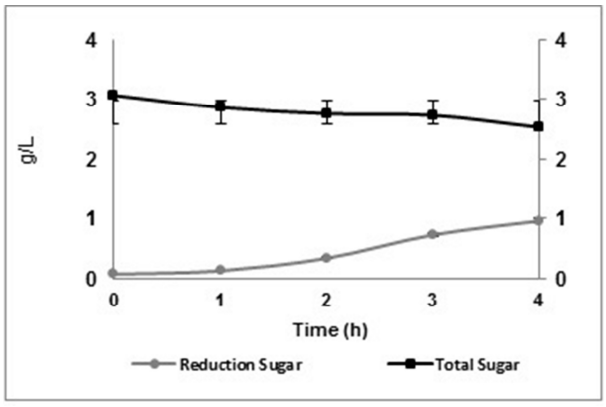


Fig. 5. Total sugar and reduction sugar from hydrolysis 2.5% rice straw with concentrated xylanase 4 U/mL in continuous sampling time (0, 1, 2, 3, and 4 h).

The total sugar was gradually decreased after being added with xylanase enzyme, it showed at Fig 5. At initial reaction from 0 hour is 3.0748 g/L and for 4 hours reaction the total sugar was degrease became 2.5353 g/L. It was concluded that in 4 hours hydrolysis the sugar in rice straw are decrease 0.5 g/L degraded become mono or oligosaccharide product.

The analysis of sugar reduction is showing the compatible result conduct to total sugar experiment which is in Fig. 5. The sugar reduction analysis with DNS method was showed there is increasing reducing sugar amount significantly from 0 hour (0.071 g/L) become 0.959 g/L in 4 hours enzymatic reaction at optimum temperature 50˚C.

E. High Performance Liquid Chromatography (HPLC) Analysis

The result of HPLC analyses of the samples on hydrolysis sugar from rice straw with final concentration 2.5% by 4U/mL crude xylanase from marine bacteri Bacillus safensis LBF-002, which is include optimize condition of hydrolysis at temperature 50˚C and agitation 150 rpm.

The hydrolysis of rice straw is shown in Fig. 6a. from the chromatogram of hydrolyze sugar at 1 hour hydrolysis was showed the oligosaccharide product such as xylohexaose as dominant sugar from degradation product and a little of xylobiose sugar has found in the sample.

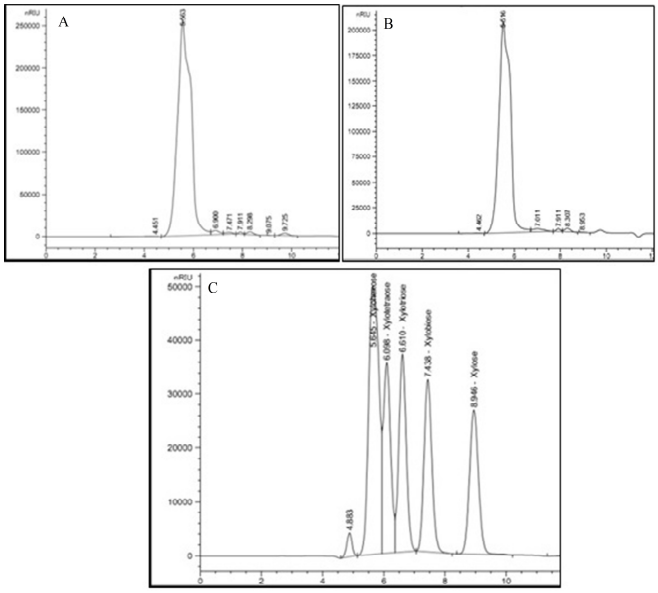


Fig. 6. Chromatogram obtained from hydrolysis 2.5% rice straw with 4 U/mL crude xylanase from Bacillus safensis LBF-002. Product reduction sugar from 1 h hydrolysis in temperature 50˚C (b), reduction sugar from 2 h hydrolysis in temperature 50˚C (c), and Xylo-oligosaccharide standard (a).

The xylanase enzyme from *Bacillus safensis* can cleaves sugar bond randomly, it means the enzyme was active to produce oligosaccharide sugar.

Fig. 6b. showed chromatogram of hydrolyze sugar at 2 hours reaction in temperature 50˚C and agitation 150 rpm and produce mono- and xylo-oligosaccharide sugar such as xylose and xylohexaose if it compare with chromatogram standard at fig. 6c.

IV. CONCLUSIONS

Extensive studies have been done concerning hydrolysis of various lignocellulosic materials including pretreatment strategies and enzymatic hydrolysis by commercial enzymes or enzymes from marine microbial. The use of microbial enzymes for the hydrolysis of lignocellulosic materials is therefore widely researched because the hydrolysis products do not harm microorganisms used in fermentation processes.

This study has resulted that marine bacteria *Bacillus safensis* LBF-002 can produce xylanase type endoenzyme which can degrade polimer rice straw randomly and can produce several type of xylo sugar mono- and oligosaccharide such as xylose, xylobiose and xylohexaose in optimal condition at temperature 50˚C and agitation 150 rpm. This study showed a potential use of marine xylanase from *Bacillus safensis* LBF-002 to hydrolyze specific kinds of agricultural wastes such as rice straw.

ACKNOWLEDGMENT

The authors acknowledge the financial support from Science and Research Grant from Indonesia Toray Science Foundation (ITSF) years 2014.

REFERENCES

[1] Hoang TMC., ERH. van Eck, WP Bula, JGE. Gardeniers, L. Leffertsa and K. Seshan. 2015. Humin based by-products from biomass processing as a potential carbonaceous source for synthesis gas production. Green Chem., 17:959-972.

[2] Chapla D, J. Divecha, D. Madamwara, and A. Shah. 2010. Utilization of agro-industrial waste for xylanase production by Aspergillus foetidus MTCC 4898 under solid state fermentation and its application in saccharificationBiochemical Engineering Journal 49: 361–369.

[3] Chapla D, P. Pandit, and A. Shah. 2012. Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. Bioresource Technology 115: 215–221.

[4] Gowdhaman D, V. Ponnusami. 2015. Production and optimization of xylooligosaccharides from corncob by Bacillus aerophilus KGJ2 xylanase and its antioxidant potential. International Journal of Biological Macromolecules 79: 595–600.

[5] Karimi K, S. Kheradmandinia, and MJ Taherzadeh. 2006. Conversion of rice straw to sugars by dilute-acid hydrolysis. Biomass and Bioenergy 30: 247–253.

[6] Adhyaru DN., NS Bhatt, HA Modi. 2015. Optimization of upstream and downstream process parameters for cellulose-poor-thermosolvent-stable xylanase production and extraction by Aspergillus tubingensis FDHN1. Bioresources and bioprocessing 2(3): 1-14.

[7] Kallel F, et al. 2015. Production of xylooligosaccharides from garlicstraw xylan by purified xylanase from Bacillus mojavensis UEB-FK and their in vitro evaluation as prebiotics. Ffood and Bioproducts Processing 94: 536–546.

[8] Kumar M, A. Joshi, R. Kashyapa, S. Khanna. 2015. Production of xylanase by Promicromonospora sp MARS with rice straw under non sterile conditions. Process Biochemistry 46: 1614–1618.

[9] Thirunavukkarasu N. et al. 2015. Screening marine-derived endophytic fungi for xylan-degrading enzymes. Current Science, 109 (1): 112-120.

[10] Mathur N., GK Goswami, and AN Pathak. 2015. In silico study of Bacillus brevis xylanase - structure prediction and comparative analysis with other bacterial and fungal xylanase. Biomedical Data Mining, 4:1-5.

[11] Yopi, A. Purnawan, A. Thontowi, H. Hermansyah, and A. Wijanarko. 2006. Preparasi Mannan dan Mannanase Kasar dari Bungkil Kelapa Sawit. Jurnal Teknologi, 4: 312-319.

[12] Rahmani N, NU J. Robbani, IH Suparto, Yopi. 2014. Optimization of Production Xylanase from Marine Bacterium Bacillus safensis P20 on Sugarcane Baggase by Submerged Fermentation. Advanced Science Engineering Information Technology 4(6):31-34

[13] Zhang Y, et al. 2012. High solid and low enzyme loading based saccharification of agricultural biomass. BioResources, 7(1): 345-353.

[14] Ong LGA, CH Chan, and AL Chew. 2012. Enzymatic hydrolysis of rice straw: process optimization. Journal of Medical and Bioengineering (JOMB), 1 (1):14-16.

[15] Kumar R, Wyman CE. 2009. Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. Bioresour Techno, 100 (18):4203-4213.

[16] Diep NQ, et al. 2012. Comparison of the Potential for Ethanol Production from Rice Straw in Vietnam and Japan via Techno-economic Evaluation. International Energy Journal 13: 113-112.

[17] Ibrahim MF, S. Abd-Aziz, M. Ezreeza, M. Yusoff, LY Phang, MA Hassan. 2015. Simultaneous enzymatic saccharification and ABE fermentation using pretreated oil palm empty fruit bunch as substrate to produce butanol and hydrogen as biofuel. Renewable Energy, 77:447-455.

[18] Castro RC. d A. and I. C. Roberto. 2015. Effect of nutrient supplementation on ethanol production in different strategies of saccharification and fermentation from acid pretreated rice straw. Biomass and Bioenergy, 78:156-163.

[19] Hsu TC., GL. Guo, WH Chen, and WS Hwang. 2010. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresource Technology, 101:4907–4913.

[20] Roberto IC, SI Mussatto, RCLB Rodrigues. 2003. Dilute-acid hydrolysis for optimization of xylose recovery from rice straw in a semi-pilot reactor. Industrial Crops and Products 17:171-176.

[21] Silva JPA, SI Mussatto, and IC Roberto. 2010. The Influence of Initial Xylose Concentration, Agitation, and Aeration on Ethanol Production by Pichia stipites from Rice Straw Hemicellulosic Hydrolysate.. Appl Biochem Biotechnol, 162:1306–1315.

[22] Lin TH., CF Huang, GL Guo, WS Hwang, and SL Huang. 2012. Pilot-scale ethanol production from rice straw hydrolysates using xylose-fermenting Pichia stipitis. Bioresource Technology, 116:314–31.

[23] Hideno A. et al. 2009. Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. Bioresource Technology, 100:2706–2711.

[24] Mandels, M, Sternberg, D. 1976. Recent advances in cellulose technology.Journal of Fermentation and Technology. Vol-54, page 267-286.

[25] Miller GL. 1959. Use of Dinitrosalysilic Acid Reagent for Determining Reducing Sugar. Analytical Chemistry: 426-428.

[26] Collins T, C. Gerday, G. Feller. 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiology Reviews 29:3-23.