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ENZYMATIC HYDROLYSIS OF DELIGNIFIED CORNCOB USING COMBINED ENZYME

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Abstract: *Purpose:* Modification of corncob fiber by enzymatic hydrolysis will improve its function as functional fiber, which having many beneficial effects for health.
Methodology: In this research, the corncob was delignified using NaOCl 1% and its sugar composition was determined by GC-MS technique. Enzymatic hydrolysis was conducted using single and combined enzyme of cellulase and xylanase for 96 hours at pH 5 and temperature 50°C. The content of total and reducing sugar, the degree of polymerization of hydrolysis product and its microscopic structure were monitored.
Findings: The results showed that delignification removed about 60% lignin of undelignified corncob. Glucose and xylose were detected as major sugar in both undelignified and delignified corncob. Average total sugar, reducing sugar and degree of polymerization produced by enzymatic hydrolysis were fluctuating.
Value: The hydrolysis increased the soluble fractions of delignified corncob and its structure was converted to more amorphous states.

Keywords: *Cellulose; Corncob; Hydrolysis; Oligosaccharides; Xylanase*

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INTRODUCTION

Agricultural wastes are raw materials for industry because of their lignocellulose contents. Lignocellulose which consisted of lignin, hemicelluloses, and cellulose can be converted into many products such as organics solvent, oligosaccharides, xylitol, fertilizers and biofuel. Solomon *et al.* (1999) stated that lignocellulose can be converted into products that are of commercial interest such as ethanol, glucose, and single cell protein. But most of them were not utilized yet and caused negative effects for environment (Richana, 2002). Wu *et al.* (2011) stated that a large number of agricultural wastes have not been fully exploited. Most of the resources are constantly being wasted.

Corncob is one of potential agricultural waste because corn productivity is very high in Indonesia, it was about 16.48 tons in 2009 (BPS, 2009). Rangkuti and Djajanegara (1983) stated that corncob was about 30% of corn-agrowaste. Based on the two data, there were 7.06 million corncobs in 2009 and most of them is only used for feed or traditional fuel in households of rural farmers (Zakpaa *et al.*, 2009). The content of cellulose and hemicelluloses in corncob is very high. Meryandini *et al.* (2008) has reported that corncob from local variety contained 44.36% of cellulose, 30.38% of hemicelluloses and 19.21 % of lignin.

Modification of corncob fiber by enzymes will produce amorphous fiber and oligosaccharides. This process will improve the function of corncob fiber as a more functional fiber, cello-oligosaccharides (COs) and xylooligosaccharides (XOs), which having many beneficial effects for health. Oligosaccharides improve immunity, feeding efficiency and the quality of livestock products (Uma *et al.*, 1999). Many researchers are interested in

xylooligosaccharides production from corncob as prebiotic for food application. Xylooligosaccharides are used for food and pharmaceuticals applications, complementary in a variety of positive health effects (V´azquez *et al.*, 2001). Pangsri (2008) has compared the capacity in growth promotion of *Bifidobacterium* and lactic acid bacteria grown in xylooligosaccharides mixture from corncob autohydrolysis and commercial xylooligosaccharides. The result showed that xylooligosaccharides mixture from corncob auto hydrolysis exhibited a potential bifidogenic capability similar to commercial xylooligosaccharides.

At present, oligosaccharides is extracted from corn, soybean and yeast strain directly and it is also obtained by the reactions of artificial polysaccharide decomposition, monosaccharide binding and glycosyl transferase transfer (Crich and Vinogradova, 2007). This research explored the utilization of corncob to produce oligosaccharides by enzymatic hydrolysis using cellulase and hemicellulase. The objective of this research is to improve enzymatic hydrolysis of corncob to produce oligosaccharides as food ingredients.

EXPERIMENTAL

Materials

Corncob from local variety (Hawaii) was collected from farm around Bogor-West Java Indonesia. Commercial cellulase and xylanase (*Cellulast*) was obtained from Bioteknocare Company.

Methods

Delignification of corncob

The corncob was dried and milled using hammer mill (about

32 mesh). Delignification of corncob methods has been investigated previously (Widyani, 2002). It was prepared by immersing the sample in 1% of NaOCl solution for 5 hours at room temperature (28°C). This step removed lignin into soluble fraction, whereas cellulose and hemicelluloses were precipitated in the solid residue. The precipitated fraction was washed using distilled water until the pH become 8 and the hypochlorite adour did not exist anymore. The delignified corncob precipitant was then sun-dried. The fiber components (cellulose and hemicelluloses) were calculated as neutral detergent fiber and acid detergent fiber (Van Soest, 1963) and lignin (AOAC, 1984), whereas the structure of fibers were observed with polarized light microscope.

Analysis of sugar content by acid treatment

100 mg sample was suspended in 10 mL of sulfuric acid solution with various concentrations from 10-60% (v/v) and shaken at 120 rpm for 5 h at temperature 20°C. The samples treated with acid concentration higher than 45% (v/v) were mixed with 10 mL of distilled water to reduce their viscosity. All samples were centrifuged for 30 minute at 6000 rpm and at 0°C. One mL of the each supernatant was neutralized with 2 N sodium hydroxide. The amount of total sugar (TS) was estimated by phenol sulphuric acid method, while the content of reducing sugar (RS) was measured by modified Park-Johnson method. Average of degree of polymerization (DP) was determined by the ratio of TS and RS value (Thalagala *et al.*, 2009).

Analysis of sugar composition by GC-MS

Neutral sugars were prepared by acid treatment (Thalagala *et al.*, 2009). 100 mg sample was hydrolysis in 10 mL of sulfuric acid solution 50% (v/v) and shaken at 120 rpm for 5 h. The

solution was mixed with 7.5 mL of distilled water so that the concentration of the solution decrease to 30% (v/v) and then it was centrifuged at 6000 rpm at 0°C for 30 minute. The supernatant was shaken at 120 rpm for 5 h in a water bath at 60°C in order to get monosaccharide. The supernatant was then neutralized with 2 N sodium hydroxide and rotary evaporated to reach its total sugar content approximately 2 mg/mL. The sugars obtained were acetylated and analyzed by GC-Mass Chromatography (GCMS-QP2010, Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was performed with a 30 m x 0.25 mm SP™ 2380 fused SILICA capillary column operating with 1 mL/min helium as the carrier gas. The temperature was kept at 210°C for 3 min and then heated up to 240 °C within 2 min and this condition was continued for 7 min to complete the total separation time.

Assay of enzymes activity (Meryandini *et al.* 2008)

Cellulase activity was measured with CMC, Filter paper (FP), and Avicel as the substrates. Cellulase was added to CMC 0.5%, avicel 0.5%, FP (1 cm x 5 cm) with ratio 1:1 in citrate phosphate buffer pH 5. The enzyme mixture was incubated at 30-90°C in 10° interval for 60 minutes. Cellulase activity was measured using DNS (Dinitrosalisilic Acid) method by Miller (1959) with glucose as the standard. Xylanase activity was measured with oat-spelt xylan as a substrate. Xylanase enzyme (100 µL) was added to 1 mL substrate solution which contains 0.5% xylan and the mixture was incubated at temperature 30-90°C in interval 10°C for 60 minutes.

Xylanase activity was determined using DNS (Dinitrosalisilic Acid) method by Miller (1959) with xylose as the standard. The reducing sugar content of the references samples (substrate solution incubated without enzyme and

diluted enzyme solution in buffer) were deduced from the values of the test samples. The reducing-sugar was detected by spectrophotometer (=540 nm). One unit cellulase activity was defined as 1 µmol glucose released in one minute incubation under specified condition, whereas one unit xylanase activity was defined as 1 µmol xylose released in one minute incubation under specified condition.

Hydrolysis the delignified corncob using cellulase and xylanase

The delignified corncob flour was suspended in phosphate citrate buffer pH 5 with concentration 5% (w/v) in Erlenmeyer flask. Its optimum concentration had been investigated previously (Muttaqin 2010). The single cellulose and xylanase enzymes (20 unit/mL for each enzyme respectively) and combined cellulase-xylanase (1:1) enzyme (10 unit/mL for each enzymes) were used to hydrolyze the sample and incubated for 96 h in a shaking water bath at 120 rpm and at 50°C , then they shaken. The changes on the degree of polymerization were monitored every 12 h. The amount of TS was estimated by phenol sulphuric acid method and RS by DNS method. The values of average degree of polymerization (DP) were estimated by dividing the value of TS by that of RS. The changes of fiber structure were observed before and after hydrolysis with polarized light microscope.

RESULT AND DISCUSSION

Delignification of corncob

Cellulose and hemicelluloses are major components of corncob. Cellulose is homopolymer of 8,000-12,000 units of glucose joined by -1,4 linkages and hemicelluloses is branched-chain polymers consists of xylose, mannose,

galactose, rhamnose, and arabinose. The two components can be hydrolyzed into their oligomers and monomers, but the presence of lignin makes the access of enzymes to them difficult, thus reducing the efficiency of the hydrolysis. Pretreatment (delignification) is required to remove lignin and hemicelluloses, reduce cellulose crystallinity, and increase the porosity of the materials (Sun and Cheng, 2002). **Figure 1** presents that the structure of corncob fiber is change after delignification. The structure has broken and the fiber looks more rigid and amorphous after delignification by NaOCl 1%.

The delignification also influenced the corncob fiber content (Table 1). Based on the Table 1 it can be seen that delignification could remove the lignin about 60%, and increase the holocellulose content. Hypochlorite ion of NaOCl could cleave the carbon linkage on lignin structure and cause the opening of the linkages between lignin and the other polysaccharides. Delignification could not eliminate the lignin content completely, because the lignin linked with holocelluloses by covalent linkages and the cellulose microfibril was integrated in hydrophobic matrix covered by lignin.

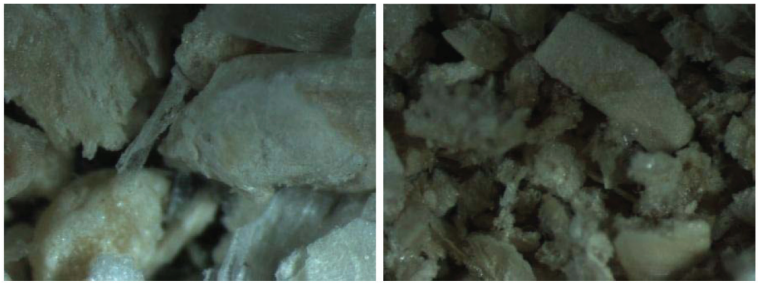

Figure 1:
Structure of Corncob Fiber Before Delignification (a) and After Delignification (b) (Light Polarized Microscope in Magnification 20)

Constituent	Before delignification (%db)	After delignification (%db)
Cellulose	65.53	71.00
Hemicelluloses	9.48	11.32
Lignin	11.18	4.51

Table 1: Composition of Corncob Fiber Before and After Delignification

Characteristics of sugar components in undelignified and delignified corncob

Table 2 showed that acid treatment with various concentration of sulphuric acid 10-60% (v/v) at 20°C for 5 h produced oligomers and monomers with average degree of polymerization (DP) value 1.0-3.3 from corncob and 2.8-4.0 from delignified one. Total sugar (TS) amount of hydrolyzed delignified corncob was higher than hydrolyzed undelignified corncob.

It could be found (Figure 2) a unique rising patterns with

Concentration of sulphuric acid (%)	Corncob		Delignified corncob	
	TS (mg)	DP	TS (mg)	DP
10.0	4.8	1.0	16.0	4.0
20.0	9.4	2.0	30.6	4.0
30.0	12.3	2.6	44.4	3.6
35.0	34.5	2.3	138.5	4.0
37.5	67.0	2.5	203.0	3.9
40.0	67.5	2.7	206.0	2.8
42.5	70.0	2.3	212.5	2.8
45.0	90.4	3.3	303.0	3.5
50.0	93.0	2.7	321.0	3.0
55.0	88.0	1.3	315.0	2.8
60.0	83.0	1.2	303.7	2.5

Table 2: Content of Sugar in Undelignified and Delignified Corncob by acid Treatment with Various Concentration of Sulphuric Acid at 20°C

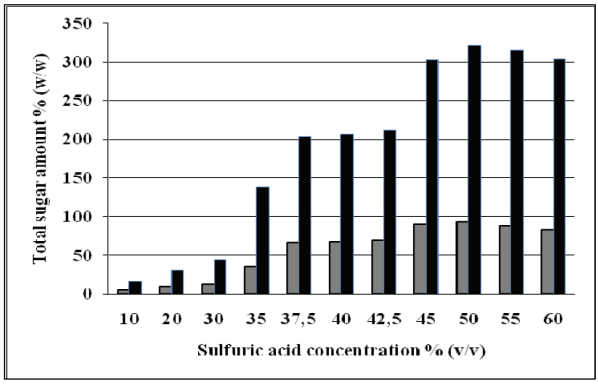

Figure 2:
Total Sugar Amount % (w/w) of Saccharides Extract from 100 mg Each of Undelignified Corncob ( ) and Delignified Corncob ( ) Treated with Various Concentration of Sulphuric Acid at 20°C.

the first increasing at 35.0-42.5% (v/v) sulphuric acid concentrations and the second increasing at 45-50% (v/v) sulphuric acid concentrations. It could be assumed that there were two kinds of extracted materials. Thalagala et al. (2009) suggested that lots of chemical linkages of hemicelluloses were broken preferentially and those of cellulose were substantially kept up to around 42.5% (v/v) sulphuric acid. At the second increase of TS amount, crystalline lattices of cellulose were disrupted and converted into a completely amorphous state forming and gelatinous substances. The cellulose was extremely susceptible to be hydrolyzed by sulphuric acid at concentration higher than 60% (v/v).

Sugar composition in undelignified and delignified corncob

Since the highest value of TS amount (Table 2) was resulted by treatment of 50% (v/v) sulphuric acid concentration for the two substrates (93 mg for undelignified corncob and 321 mg for delignified one), then the concentration was used to

produce the oligosaccharides and monosaccharide from the two substrates. The oligosaccharides and monosaccharide produced by delignified corncob were higher than undelignified corncob after acid treatment (Table 3).

The monosaccharide composition was analyzed by GC/MS method. **Table 4** showed that both substrates contained glucose and xylose as the major sugar, which were 36.27 and 50.22 (undelignified corncob) and 43.00 and 49.00 (delignified corncob). Glucose was the result of cellulose hydrolysis, whereas xylose, arabinose, galactose and mannose was the result of hemicelluloses hydrolysis.

Based on the characterization of the sugar in undelignified corncob and delignified one, it can be concluded that corncob is potential sources to produces oligosaccharides by enzymatic hydrolysis because it contained a lot of cellulose and hemicelluloses. Cellulose or ß -1-4-glucan is a polymer of glucose made of cellobiose units with about 2,000 to 27,000 glucose residues (Delmer and Amor 1995; Morohoshi 1991). These chains are packed by hydrogen bonds in socalled 'elementary fibrils' originally considered to be 3 to 4 nm wide and contain about 36 chains, although larger crystalline fibrils

Sugar components % (w/w)	Undelignified Corncob	Delignified corncob
Oligosaccharides		
Total sugar amount (mg)	93.0	321.0
Average DP value	3.0	2.3
Monosaccharide		
Total sugar amount (mg)	87.0	285.0
Average DP value	1.0	1.0

Table 3:
Content of Sugar in Undelignified Corncob and Delignified Corncob by Acid Hydrolysis (Sulphuric Acid 50%)

Sugar components % (w/w)	Raw materials (100 mg)	
	Undelignified Corncob	Delignified corncob
Xylose	50.22	49.00
Glucose	36.27	43.00
Arabinose	4.93	7.00
Galactose	0.74	1.00
Mannose	0.19	ND*

*ND (not detected)

Table 4: Composition of Monosaccharide Sugar Analyzed by GC/MS in Undelignified and Delignified Corncob After Hydrolysis at 60°C for 5 h

up to 16 nm were also discovered (Ha *et al.*, 1998). Xylan is the major hemicellulose component in nature. The sugars in xylan are primarily pentoses (D-xylose and L-arabinose) and harbor as minor constituents' hexoses (D-galactose, D-glucose and D-mannose) as well as uronic, acetic and cinnamic acids (Collins *et al.*, 2005; Squina *et al.*, 2009).

Hydrolysis of delignified corncob using cellulase and xylanase

The assay of enzymes activity was done to get informations about the optimum condition of hydrolysis. The characteristics of two enzymes were shown at **Table 5**.

Characteristics	Cellulase	Xylanase
Optimum pH	5.0	6.0
Optimum temperature	60°C	50°C
Enzyme activity	1.83×10^5 unit/mL	2.45×10^7 unit/mL

Table 5: Characteristics of Cellulase and Xylanase

Based on the data in Table 5, enzymatic hydrolysis was done at pH 5 and temperature 50°C. Sugar content was monitored every 12 h for 96 h of hydrolysis (by analyzing TS and RS, then calculating the DP value). The result of sugar composition after enzymatic hydrolysis is shown in Table 6.

The range of average DP value for all treatment was 3.442 - 16.908. The least average of DP value (3.442) was obtained in enzymatic hydrolysis using single cellulase for 12 h. The less average of DP value explained that polysaccharides in delignified corncob were depolymerized into short-chain compounds during enzymatic hydrolysis. It indicates that enzymatic hydrolysis of delignified corncob produced oligosaccharides. Pangsri (2008) stated that oligosaccharides are carbohydrate containing 310 units of monosaccharide. Oligosaccharides have linear or side chain structure and low molecular weight with a degree of polymerization (DP)>10. Oligosaccharides are also small polymer with 2 to 10 monosaccharide joint to form straight or branched-chain by means of the glucosidal bonds (Liab *et al.*, 2000; Teramoto *et al.*, 2008).

Enzyme	Hydrolysis period (h)	Total sugar (mg/ml)	Reducing sugar (mg/ml)	Degree of polymerization (DP)
Cellulase (20 unit/g)	0	1,138	0,144	7,903
	12	0,592	0,172	3,442
	24	1,322	0,133	9,940
	36	1,801	0,159	11,327
	48	1,504	0,139	10,820
	60	1,588	0,162	9,802
	72	1,874	0,133	14,090
	84	2,925	0,173	16,908
	96	1,357	0,141	9,624
Xylanase (20 unit/g)	0	1,138	0,144	7,903
	12	0,923	0,171	5,398
	24	0,982	0,153	6,418
	36	1,275	0,172	7,413
	48	1,426	0,153	9,320
	60	1,360	0,167	8,144
	72	1,617	0,155	10,432
	84	1,058	0,170	6,224
	96	1,872	0,157	11,924
Cellulase-Xylanase (20 unit/g)	0	1,138	0,144	7,903
	12	0,876	0,157	5,580
	24	1,096	0,136	8,059
	36	1,672	0,155	10,787
	48	1,324	0,144	9,194
	60	2,140	0,150	14,267
	72	1,776	0,145	12,248
	84	2,661	0,168	15,839
	96	1,932	0,145	13,324

Table 6:
The Change of Total Sugar, Reducing Sugar, and DP Value in Enzymatic Hydrolysis of Delignified Corncob for 96 h

The patterns of TS amount produced by single and combined enzymatic hydrolysis were various, but the patterns of RS were the same (**Figure 3**). The TS patterns were fluctuating for each enzyme treatment because the sugars released could inhibit the hydrolysis reaction (Taherzadeh and Karimi, 2007). High substrate concentration can cause substrate inhibition, which substantially lowers the hydrolysis rate. The extent of the inhibition depends on the ratio of total enzyme to total substrate (Sun and Cheng 2002).

The various patterns of TS also indicated that cellulose and xylanase were complex enzymes. Cellulase is a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-b-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades

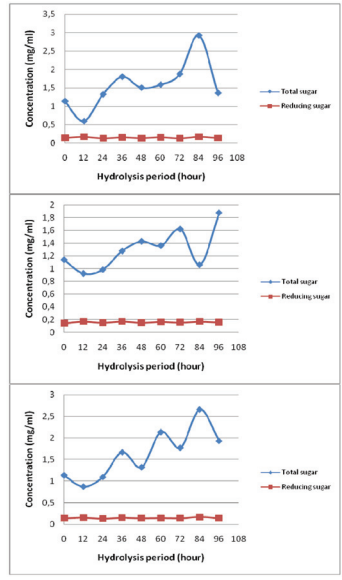

Figure 3:
The Changes of Total Sugar and Reducing Sugar Amount % (w/w) in Hydrolysis of Delignified Corncob 5% (w/v) use 20 Unit/ml Single Cellulase (a), Single Xylanase (b) and Combined Cellu-Lase-Xylanase 1:1 (c) for 96 h

the molecule further by removing cellobiose units from the free chain-ends; (3) -glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988).

There are three major groups of xylanase and also a number of ancillary enzymes that attack hemicelluloses, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). Complete depolymerization of xylan is accomplished by the synergistic action of endo-xylanases and xylosidases along with arabinofuranosidases, ferulic acid esterases, uronidases and other enzymes, which respectively act on the xylan backbone, side chains and decorating units, producing fermentable xylo-oligomers and monomers (Remond *et al.*, 2010).

Based on **Figure 3**, it can be also presented that the pattern of RS obtained by enzymatic hydrolysis of delignified corncob was flat for both single and combined enzymes. It indicates that the change of RS during enzymatic hydrolysis was not significant. Some factors could influence the RS content, they are porosity (accessible surface area) of the waste materials, cellulose fiber crystallinity, and the content of lignin and hemicelluloses. The presence of lignin and hemicelluloses makes the access of cellulose enzymes to cellulose diffcult, thus reducing the effciency of the hydrolysis (McMillan, 1994).

The enzymatic hydrolysis of delignified corncob also affected the structures of delignified corncob fiber. **Figure 4** showed the changes of crystalline state to amorphous state.

The fiber structure of the delignified corncob before hydrolysis was in a complex and solid form **(Figure 4a)**. After hydrolysis for 96 h, the fiber structure of the delignified corncob

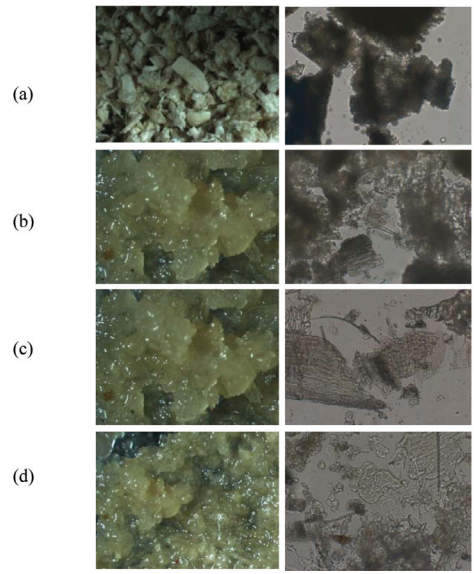

Figure 4:
Microscopic Structure of Delignified Corncob Fiber Before Hydrolysis (a) and After Hydrolysis for 96 h Using 20 Unit/g Cellulase (b), Xylanase (c) and Combination of Cellulose-Xylanase 1:1 (d) ((Light Polarized Microscope in Magnification 100x)

was more rigid and amorphous **(Figure 4b-4d).** The changes of crystalline state to amorphous state affected by complex cellulase and xylanase. Endoglucanase hydrolyzed regions of low crystallinity (amorphous state) in the cellulose fiber, creating free chain-ends (oligosaccharides) with different chain-length. Exoglucanase hydrolyzed regions of high crystallinity (crystalline state) efficiently (Perez *et al.*, 2002).

CONCLUSION

The delignified corncob contained 71.00 % of cellulose and 11.32% of hemicelluloses. The delignification removed about 60% lignin. Glucose and xylose were detected as major sugars in undelignified and delignified corncob. Average total sugar, reducing sugar and degree of polymerization produced by enzymatic hydrolysis were fluctuating. The hydrolysis increased the soluble fractions of delignified corncob fiber and its structure was converted to more amorphous states.

BIOGRAPHY

Eka Ruriani, is a lecturer and scientist with master degree (M.Si. Agroindustrial Technology), at Department of Agroindustrial Technology, Bogor Agricultural University. Since the year 2006, she has worked at Department of Technology of Agricultural Products, Faculty of Agricultural Technology, Jember University, Jember, East Java, Indonesia. Her experience and special training are *Alternative Energy Production and Management* in Japan (2010) and *Marketing Strategy and Research in Netherland (2011)*. Some topics of her research are Process Development of Bioethanol Production from Corncob by Simultaneous Saccharification and Fermentation, Production of Cassava Sour Starch by Fermentation Using Acid Lactic Bacteria, and The Synthesize of Galactooligosaccharide Enzimatically Using β-Galactosidace Enzyme from *Aspergillus oryzae*. Since 2011 she is the secretary of Indonesian Food Technologist Association for Jember Region. She was also a manager of executive editor of *J Agroteknologi* (Indonesian Journal was published by Jember University) in 2011.

Anja Meryandini, is a scientist with PhD degree (PhD Microbiologist), at Biology Faculty, Albert Ludw=if Universitaet, Freiburg Germany. Since the year 1988, she has worked at the Biology Department, Bogor Agriculture University at Bogor Indonesia. She works with microbes and enzymes.

Titi Candra Sunarti is a scientist with doctor degree (PhD. Applied Bioscience and Biotechnology), at Graduate School of Bioresources, Mie University, Japan. Since the year 1991, she has worked at Division of Bioindustry, Department of Agroindustrial Technology, Bogor Agrocultural University, Indonesia. Her experiences in research are bioprocess technology and carbohydrate technology. She was also interesting in exploring new local indigenous carbohydrate resources in Indonesia.

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