Xylan Production from Corn Cobs for Isolation of Xylanase-Producing Bacteria

K S Sasmitaloka1,2*, A B Arif1, Juniawati1, C Winarti1, M Hayuningtyas1, Ratnaningsih1, and N Richana1

1Indonesian Center for Agricultural Postharvest Research and Development, Jln. Tentara Pelajar No. 12, Bogor 16112

*Email: kirana.sanggrami@gmail.com

Abstract. Xylan can be enzymatically degraded to establish environmentally tolerable and eco-friendly processes for the production of alcohol and others. It can be produced from agroindustrial wastes which is rich in lignocellulosic content, like corn cobs. However, there is still problem with the purity. This study investigated production of xylan from corn cobs for isolation of xylanase-producing bacteria. Raw material used in this study was corn cobs. The experiment was set up in complete randomized design with treatments of water soaking ratio (1:1, 1:2, and 1:3) and soaking repetitions (1, 2, and 3), in three replications. The results of analysis of variance (ANOVA) showed that between water soaking ratio and soaking repetitions have significantly different of their physicochemical characteristics (p<0.05). The best water soaking ratio and soaking repetitions was 1:2 for three soaking, with NaCl content of 0.17%, yield of 6.66%, and water content of 5.62%. Xylan produced from the best treatments used for isolation of xylanase-producing bacteria. Xylan with concentration of 1% can be used for isolation of xylanase-producing bacteria from specimens of mixture of decayed wood and sand, with potential index of 0.71, enzyme activity of 140.85 U/ml, specific enzyme activity of 319.788 U/ mg protein and namely Bacillus pumilus B21.

1. Introduction
Xylan is a branched, heterogeneous polymer with a backbone of β-1,4-linked D-xylopyranose units and different side chain residues such as arabinose, glucuronic acids, and other residues [1, 2]. It is considered as a second widely available polysaccharide in nature and can be enzymatically degraded to establish environmentally tolerable and eco-friendly processes for the production of alcohol, xylose, xylitol and xylooligosaccharides [3, 4, 5, 6, 7]. It can be produced from many agro industrial wastes which is rich in lignocelluloses content. Lignocelluloses materials are mainly composed by cellulose, hemicellulose and lignin, and smaller portion of pectin, waxes and mineral salts. About 25-40% of lignocellulose biomass consisted of xylan [8].

Corn cob has the highest xylan content than other lignocellulose waste. It consists up to 40% of xylan [9, 10, 11]. Various suitable methods for corn cob xylan extraction both chemically and mechanically have been developed by researchers. Alkaline treatment using NaOH is promising to be one of the best ways to extract xylan [8, 10, 12]. However, there is still problem with the purity, which still contains of high salt levels. The use of NaOH and HCl in xylan extraction can cause saturation by forming NaCl sediment.

Corn cob xylan can be used as a carbon source in the cultivation media of xylanase-producing bacteria. Therefore, the corn cob xylan that produced must have a high level of purity to support the
2nd International Conference on Agriculture Postharvest Handling and Processing  
IOP Conf. Series: Earth and Environmental Science 309 (2019) 012066  
doi:10.1088/1755-1315/309/1/012066

growth of bacteria. Salts and their concentration play very important role in microbial growth and enzyme production by providing ideal osmotic pressure. It was found that higher concentration of salts in production medium can make the water flows out from microbial cells through osmosis and causes the cells to shrink or in some cases die which ultimately decreases or stops the enzyme production [3, 13]. Moreover, salt also affects the water activity of substrate which can control microbial growth. So, it is necessary to modify the technology to produce xylan with minimal salt content.

The research result of Richana et al., [10] showed that xylan have solubility in hot water (90°C) but not in cold water (27°C), while salt is soluble in both hot and cold water [14]. The solubility properties can be used to produce xylan with low salt content. The salt content in xylan can be reduced by washing xylan using cold water.

Xylan produced from the best treatments was used for isolation of indigenous xylanase-producing bacteria. The target microorganisms in this study were isolated from specimens of ex-mining land, mixture of decayed wood and sand, water of volcanic craters, and mixture of sea water and sand. The indigenous xylanase-producing bacteria were found to produce higher titres of xylanase. This enzyme has been used for preparation of xylooligosaccharides which are used as a prebiotics and also for the production of ethanol and xylitol [15]. This study investigated production of xylan from corn cobs for isolation of xylanase-producing bacteria.

2. Methods

2.1 Raw materials
Raw material used in the research was corn cob originated from Sukabumi, West Java. Chemicals used were NaClO, NaOH, HCl, ethanol, and Na₂CO₃. The nutrients used for isolation were yeast extract, polipeptone, K₂HPO₄, MgSO₄.7H₂O, and bacto agar. Equipment used were digital balance, soaking bucket, stirrer, filter cloth, hot plate, oven, aluminium tray, and other analytical equipment.

2.2 Production of corn cob xylan
The xylan extraction was prepared according to the method of Richana et al., [10] with modification, consists of size reduction, delignification, soaking, filtering, neutralizing, washing and drying. The corn cob was dried and milled to the size of 40 meshes. Corn cob powder (500 g) was used as grits by soaking it in 20% NaOCl solution at 28°C for 5 hours (delignification process). The grits were rinsed by distilled water and filtered to separate the lignin content from celluloses and hemicelluloses. Xylan extraction was carried out by soaking the delignified corn cob into 10% NaOH solution at 28°C for 24 h and was filtered. Filtrate which contained xylan was neutralized by 13 N HCl. Xylan was precipitated by addition of 95% ethanol into the filtrate with ratio 1:1 (v/v). The extracted corn cob xylan was dried by solar dryer (drying in the sun) until the moisture reach approximately 5%. Then xylan is washed using cold water (27°C) with treatments of water soaking ratio (1:1, 1:2, and 1:3) and soaking repetitions (1, 2, and 3), repeated three times. Observation carried out on the NaCl content using Mohr method [16], water content using oven method [16], yields [17] and xylan solubility [10].

2.3 Exploration
Exploration was carried out in several places, for mixture decayed wood and sand samples from West Java, ex-mining land samples from West Nusa Tenggara, water volcanic craters from Central Java, and mixture sea water and sand from DI Yogyakarta. In each location two or three exploration sites were established and each of them had 2-3 plots. Explored path length varies in the range of 1.3 to 2 km, depending on topography. Samples have been taken and put into plastic samples, then labeled (sample code, collection date, sample type and location). Samples were stored in the cooler-box.

2.4 Isolation of indigenous xylanase-producing bacteria
Isolation has been done by suspending one gram of soil or specimens that have been mashed into 10 ml of sterile distilled water, then shaken and made a series of 10¹ up to 10⁸ dilutions into separate test
tubes. Each sample dilution suspension, taken one ml then poured into a petri dish containing xylan and nutrient agar medium [18]. Composition in one liter of xylan medium consisted of 0.1 g of yeast extract, 0.5 g of polypepton, 0.1 g of K$_2$HPO$_4$, 0.02 g of MgSO$_4$.7H$_2$O, 1.5 g of bacto agar [19] and 1% xylan [20]. The culture was maintained on xylan medium adjusted to pH 9.5 by addition of 1% Na$_2$CO$_3$ [21].

Selection of bacteria was carried out in stages based on the clear zones produced around the colonies on solid media and potential index [22] that had been incubated for 48 hours. This stage was the first step to find out whether the isolate can degrade the substrate (xylan) in the growth medium. If it has been able to degrade the substrate with the formation of clear zones around the colony then the isolate has been declared to produce xylanase. At this stage, the selection for each isolate was carried out six times repetition.

Further testing for selected indigenous xylanase-producing isolates (potential index of more than 3 mm) was carried out by growing bacterial colonies from each isolate in liquid media. This is intended to find out how far the ability of bacterial isolates to produce xylanase. One ose of inoculants were placed in 100 ml xylan medium and incubated for 24 hours. Observation carried out on biomass expressed in dry cell weight [23], soluble protein using Bradford method [24], xylanase enzyme activity [25], and specific xylanase enzyme activity [25]. It was repeated four times.

### 2.5 Identification of indigenous xylanase-producing bacteria
Identification was carried out for potential xylanase-producing isolates. Identification of isolates was carried out based on 16S-ribosomal RNA sequences. A more diverse sequence of 16S-rRNA molecules is suitable for distinguishing an organism into lower taxa such as genera and species [26].

### 2.6 Statistical analysis
All data were subjected to the analysis of variance (ANOVA) using SAS 9.1.3 version. Differences between mean values were established using Duncan’s multiple range tests at a confidence level of 95%.

## 3 Results and Discussion

### 3.1 Corn cob xylan production

#### 3.1.1. Corn cob xylan production without soaking water
Corn cob xylan extraction by alkali method without water soaking had 9.90% NaCl content, 10.06% yields recovery and 7.64% water content (Table 1). Based on Table 1, corn cob xylan has high NaCl content. Gao et al., [27] reported that salt was a chemical component that was both bacteriostatic and bactericidal. Bacteria are able to be killed by salt because they are hygroscopic so they can absorb water (cytoplasm) bacteria in the end the bacterial cells shrink and die. NaCl will break down into sodium ions (Na$^+$) and chloride ions (Cl$^-$) which are toxic to some bacteria [3]. Therefore, efforts were needed to reduce the salt content.

| Parameters | Content (%) |
|------------|-------------|
| NaCl       | 9.90±0.16   |
| Yields     | 10.06±0.12  |
| Water      | 7.64±0.11   |

Xylan solubility test results showed that xylan had high solubility in hot water but low solubility in cold water. This condition is in line with the results of research by Richana et al., [10] which stated that xylan was less or rather difficult to soluble in cold water but easily soluble in water which was heated at a temperature of 100°C. Solubility of a biopolymer including carbohydrates will decrease with the higher molecular weight [28, 29, 30]. Xylan is a polysaccharide with a high molecular weight. The chemical structure of xylan in corn cob is mainly composed of D-glucuronic acid, L-arabinose and D-
xylose [31]. So the solubility of xylan in cold water is reduced. Based on data results (Table 2), xylan could be soaked in cold water to reduce the salt content.

### 3.1.2. Corn cob xylan production with soaking water
Soaking water could reduce NaCl salt content in xylan (Table 2). NaCl content was decreased if the soaking repetitions and water soaking ratio were increased. This condition is in line with the results of research by Cavanagh et al., [32] which stated that the more water used, the more soluble NaCl content would be. Based on the data in Table 2, treatments of soaking water can reduce NaCl content by 48.48-98.52%. Statistical analysis showed that the soaking repetitions, water soaking ratio, and interaction between soaking repetitions and water soaking ratio were significantly different for NaCl salt content of corn cob xylan.

| Water Soaking Ratio | NaCl Content (%) | Soaking Repetitions |
|---------------------|------------------|---------------------|
|                     | 1                | 2                   | 3                   | Averages |
| 1:1                 | 5.10±0.12         | 3.00±0.05           | 0.99±0.08           | 3.03^A   |
| 1:2                 | 4.22±0.04         | 2.09±0.04           | 0.17±0.02           | 2.16^B   |
| 1:3                 | 3.46±0.17         | 1.32±0.03           | 0.15±0.02           | 1.64^C   |

Remark: Different letters on the same lines indicate significantly different

### Table 3. Effect of water ration and soaking repetitions on the NaCl content of corn cob xylan

| Water Soaking Ratio | Yields (%) | Soaking Repetitions |
|---------------------|------------|---------------------|
|                     | 1          | 2                   | 3                   | Averages |
| 1:1                 | 9.07±0.09  | 7.55±0.09           | 6.59±0.28           | 7.74^A   |
| 1:2                 | 8.52±0.09  | 7.16±0.07           | 6.66±0.34           | 7.44^B   |
| 1:3                 | 8.23±0.15  | 6.81±0.02           | 5.43±0.06           | 6.82^C   |

Remark: Different letters on the same lines indicate significantly different
Table 4. Effect of water ration and soaking repetitions on the water content of corn cob xylan

| Water Soaking Ratio | Water Content (%) | Soaking Repetitions |
|---------------------|-------------------|---------------------|
|                     | 1     | 2     | 3     | Averages |
| 1:1                 | 7.40±0.07<sup>A(a)</sup> | 6.65±0.16<sup>A(b)</sup> | 5.97±0.04<sup>A(c)</sup> | 7.74<sup>A</sup> |
| 1:2                 | 7.27±0.07<sup>B(a)</sup> | 6.37±0.09<sup>B(b)</sup> | 5.62±0.11<sup>B(c)</sup> | 7.44<sup>B</sup> |
| 1:3                 | 6.96±0.14<sup>C(a)</sup> | 6.08±0.06<sup>C(b)</sup> | 5.39±0.03<sup>C(c)</sup> | 6.82<sup>C</sup> |

Averages: 7.21<sup>a</sup> 6.37<sup>b</sup> 5.66<sup>c</sup>

Remark: Different letters on the same lines indicate significantly different

3.1.3. Determination of the selected soaking treatment

Selected soaking treatment carried out based on data of NaCl content and yield. Selected soaking treatment is a treatment that produces high of yields with low NaCl content. Treatment with water soaking ratio 1:2 for three soaks and 1:3 for three soaks have low NaCl content (Table 2). However, yields of treatment with water soaking ratio 1:2 for three soaks higher than yields of treatment with water soaking ratio 1:3 for three soaks. Results showed that ANOVA between the two treatments have not significantly different of their NaCl content (p<0.05). So, the selected soaking treatment is water soaking ratio 1:2 for three soaks.

3.2 Isolation of xylanase-producing bacteria

Isolation of bacteria has produced 5 colonies from specimens of mixture decayed wood and sand (3 isolate), water volcano crater (1 isolate) and sea sand (1 isolate) which could grow on xylan media and produce a clear zone with a diameter of more than 3 millimetres (Table 5). Colonies less than 3 mm in size, even though clear zones were not used. The clear zone is formed due to the xylan hydrolysis activity by the xylanase enzyme. The potential index of the five isolates ranges from 0.11 to 0.71. This showed that the five isolates have the ability to secrete xylanase enzymes.

Figure 1. Clear zone of indigenous xylanase-producing isolates

The five isolates could grow well on the agar medium slant. Therefore, the five isolates were selected to test the ability to produce xylanase. Analysis was carried out on liquid media. Observations include biomass, soluble protein, enzyme activity, and specific enzyme activity are presented in Table 6.

Observation of biomass at the end of cultivation showed that each isolate had a different ability to grow (Table 6). Isolates that could grow well provide an indication that it was able to utilize the only carbon source in the growth medium (xylan). Thus the enzyme production will be better if using isolates
which can grow well on the substrate induced by the xylan. Presence of substrate in xylan-containing medium is known to induce xylanase productivity [34]. High protein can also show high enzymes [35]. However, the enzyme that is produced cannot be ascertained is xylanase. Therefore, it is necessary to know xylanase activity.

### Table 5. Potential index of xylanase-producing bacteria

| Isolate | Potential Index |
|---------|----------------|
| F61     | 0.66±0.06a     |
| B21     | 0.71±0.06a     |
| B72     | 0.47±0.07b     |
| B82     | 0.30±0.07c     |
| C53     | 0.11±0.04d     |

Remark: Different letters on the same lines indicate significantly different

Protein content of cultivation results from bacterial isolate between 0.208-0.440 ml / mg protein (Table 6). Based on Table 6, highest soluble protein found in isolates B21, although the isolate produces low biomass. High biomass does not always produce high xylanase secretion as well [10]. This is supported by potential index data (Table 5) that has been obtained, where isolate B21 had the highest potential index value (0.71) so that the isolate had a high ability to secrete xylanase enzymes.

### Table 6. Biomass, soluble protein, xylanase enzyme activity and specific enzyme activity of xylanase-producing bacteria

| Isolate | Biomassa (g/l) | Soluble Protein (ml/mg protein) | Xylanase Enzyme Activity (U/ml) | Specific Enzyme Activity (U/mg protein) |
|---------|----------------|---------------------------------|---------------------------------|----------------------------------------|
| F61     | 0.033 ± 0.005a | 0.248±0.02a                     | 17.505±2.36a                    | 70.968±11.35a                          |
| B21     | 0.025 ± 0.002b | 0.440±0.01b                     | 140.85±5.79b                   | 319.788±12.72b                         |
| B72     | 0.031 ± 0.001a | 0.296±0.01c                     | 87.58±3.12c                    | 296.341±20.84c                         |
| B82     | 0.019 ± 0.007c | 0.270±0.01c                     | 76.53±3.41d                    | 269.813±20.99d                         |
| C53     | 0.023 ± 0.004c | 0.208±0.02d                     | 38.63±2.85c                    | 186.61±18.97d                          |

Remark: Different letters on the same lines indicate significantly different

The rate of xylanase production was usually found to be highest towards the late exponential or stationary phase [36,37,38]. It is probably happened because other carbon source in medium was existed and played as repressor in xylanase production. The xylanase enzyme activity is a reflection of the ability of the bacteria to produce xylanase. The higher enzyme activity, the higher xylanase produced. Observation of xylanase activity ranged from 38.63 to 140.85 U/ml, the highest was achieved by B21 (140.85 U / ml).

Specific enzyme activity is calculation results of xylanase activity divided by soluble protein [8]. This data shows the ability of xylanase per unit of proteins in hydrolyzing xylan (Table 6). The highest specific enzyme activity was obtained by isolate B21. Based on observations of it, isolate B21 was the most potential isolate of the five isolates.

#### 3.3. Identification of xylanase producing-bacteria

The colony of B21 was circular, raised, translucent and smooth. It was white and butyrous. The edge was undulate. This indicated that the isolate J12 belonged to a group of rod, spore-forming bacteria based on Bergey’s Manual of Systematic Bacteriology [39]. The genomic DNA of B21 isolate was successfully extracted and served as a template for PCR-amplification. The chromatogram of 16S rRNA gene indicated that bacterial isolate B21 belonged to the genus Bacillus was highly similar to Bacillus pumilus (99% sequence similarity). Therefore, in this study, the bacterial isolate B21 was identified as Bacillus pumilus.
Figure 2. Chromatogram sequence of isolate B21

TCAGTATTTCGATGGACGAAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTC
TGTTGTTAGGGAAGAACAAGTGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
ACTACGTGCCACAGCCCGGTGTAATAGCTAGGTTGGCAAGCTGTTCCCGAAATTTATGCGGCTTCAGGGC
AGCGGTTTTCTAATTGCTAGTGAAAACCCGGCGCTCAACCGGAGGGTGACTTGAAACCTGGAAACCTTGA
GTGCAAGAGAGAGAGTGGCTTCCACGTGAGCGGAAATGCTAGTGGAGATGTGGAGGAACACCAGTGGCC
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCGGCT
CAACCGGGGAGGGTCATTGGAAACTGGGAAACTTG
GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGC
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAGCTTACCAGGTCTTGACATCCTCTGACTACCTTAGAC
AGGGCTTTCCCTTCGGTGAC

Figure 3. Base sequences of isolate B21 base sequences

4 Conclusions

The results of analysis of variance (ANOVA) showed that between water soaking ratio and soaking repetitions have significantly different of their physicochemical characteristics (p<0.05). The best water soaking ratio and soaking times for xylan production is 1:2 for three soaks, with NaCl content of 0.17%, yield of 6.66%, and water content of 5.62%. Xylan with concentration of 1% can used for isolation of xylanase-producing bacteria from specimens of mixture decayed wood and sand, with potential index of 0.71, biomass of 0.025 g/l, soluble protein of 0.440 ml/mp protein, enzyme activity of 140.85 U/ml, specific enzyme activity of 319.788 U/mg protein and namely Bacillus pumilus B21, based on identification of 16S-ribosomal RNA sequence.

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