Cellulolytic and xylanolytic faecal bacteria from tedong bonga, [Toraja buffalo, *Bubalus bubalis carabanensis*]

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Abstract. The gastrointestinal tract of ruminants contains a considerable number of microbes that can utilize lignocellulosic biomass from plants. In this study, faecal samples of a local white spotted swamp buffalo or tedong bonga from North Toraja, South Sulawesi [*Bubalus bubalis carabanensis*] were investigated to aim bacteria isolate that are capable of degrading both cellulose and xylan. Direct isolation and enrichment methods were performed by using selective medium containing 1% carboxymethyl cellulose [CMC] or xylan in the isolation process. Plate screening and enzyme quantification indicate that 11 out of 25 isolates have double actions of cellulase and xylanase with the range of 0.18-0.30 U/ml and 0.10-1.56 U/ml, respectively. Further, molecular identification using 16S rRNA gene for four selected isolates shows that three strains [KBX04, KBX07 and KBX08] were identical to *Bacillus altitudinis* 41KF2b [100%] while another strain [KBX03] were identical to *Cellulomonas flavigena* DSM 20109 [99%]. We demonstrated that faeces from ruminants are a promising source for lignocellulose degrading bacteria that could be used for biomass conversion.

Keywords: cellulase, faecal bacteria, lignocellulose, tedong bonga, xylanase

1. Introduction

Tedong bonga is a swamp buffalo [*Bubalus bubalis carabanensis*] with a white skin appearance and has social-cultural importance for Toraja peoples in South Sulawesi, Indonesia [1,2]. The body of tedong bonga is relatively bigger with a straight and massive back and weighing approximately 700-800 kg [3]. Unlike other buffalos which are widely used to plow paddy fields or produce meat and milk, tedong bonga is used by Toraja people for traditional rituals [i.e. funerals] and as a symbol of prosperity and social status [4]. Thus, tedong bonga has a very high economic value and usually special treatment including their special diet with high quality of grass or by adding milk or eggs.

Due to their unique diet in which may correlated with the gut microbial community, we hypothesised that tedong bonga also harbour an exceptional assembly of microorganisms that help the digestion of lignocellulosic biomass from the grass. Lignocellulose is composed of lignin, cellulose, hemicellulose and xylan, and their digestion requires the action of multiple carbohydrate-active enzymes [CAZymes] [5], which also include cellulase and xylanase [6]. Cellulolytic and xylanolytic bacteria have been successfully from various ruminants, including wild and domesticated [7] or local cattle [8], however, the potentials of these bacteria from tedong bonga remains unexplored. In this study we aimed to obtain
cellulolytic and xylanolytic bacteria from faecal samples of tedong bonga, a local swamp buffalo originated from Toraja region.

2. Materials and Methods

2.1. Bacterial isolation and plate screening

Fresh fecal samples from tedong bonga were collected in Rantepao, North Toraja, South Sulawesi. Two isolation approaches were applied, including direct isolation and enrichment methods with basic medium [9]. Carboxymethylcellulose [Sigma-Aldrich, Steinhein, Germany] or xylan from beechwood [Sigma-Aldrich, Steinhein, Germany] 1% were used as a carbon source. The presence of enzyme activity is determined by the presence of clear zones around the colonies after dropping 2.5% congo red reagents on the medium [10].

2.2. Enzyme activity measurement

Bacterial isolates with positive results on agar plate screening were further analysed for quantitative cellulase and xylanase activity [11]. Briefly, single colonies of bacteria were inoculated on an induction broth medium containing carboxymethylcellulose and xylan grown at 37°C for 20 hours. The crude extract of the enzyme was obtained by culture centrifugation at 2500xg for 5 min. The test of cellulase and xylanase enzyme activities were performed by measuring the formation of glucose and xylose reduction sugars. The glucose and xylose levels contained in each sample and control were determined based on the glucose and xylose standard curves for cellulase and xylanase, respectively, calculated by using linear regression.

2.3. Bacterial identification

Bacterial DNA was extracted using the Genomic DNA Purification Kit [Fermentas, Canada]. PCR was performed using Takara Gen Cycler's Thermal Cycle [Takara Bio Inc., Japan]. The PCR reagent used is GoTaq® Green Master Mix [Promega, USA]. Primer set of 27F [5'-AGAGTTTGATCCTGGCTCAG-3'] and 1492R [5'-GGTTACCTTGTAGACTT3'] was used for amplifying the conserved region of 16S rRNA gene [12]. Amplified 16S rRNA were subjected to Sanger dideoxy sequencing [1st BASE Pte Ltd, Singapore]. The sequences were analyzed in BioEdit program 7.0.5.3. Analysis of sequence was performed using the Basic Local Alignment Search Tool or BLAST and EzTaxon [13] to obtain high-homologous DNA sequences. Phylogenetic trees were made with alignment in the ClustalX program version 2.0.12. The alignment results are read using the NJ plot version 2.3 program.

3. Results and Discussions

3.1. Isolation and plate screening of cellulolytic and xylanolytic bacteria

A total 25 isolates were successfully purified from both cellulose and xylan based medium and then subjected to plate screening. The specific substrates used in the medium are carboxymethylcellulose [CMC] and xylan which play a role as the only carbon source in the media for rapid screening of microbial isolates with the ability to degrade cellulose using cellulase enzymes or to degrade xylan using xylanase enzyme. The microbial isolates that produced both cellulase and xylanase enzymes would show a clear zone around the colony after staining with 5% congo red solution [Figure 1].
Figure 1. The appearance of colonies and clear zones of bacterial isolates from gastrointestinal tract grown on agar medium containing CMC or xylan. The arrows indicate the presence of clear zones around the colony: KBX03 [A, B], KBX04 [C], KBX07 [D], and KBX08 [E].

Clear zone measurements on the medium were performed to estimate the potential bacterial isolates producing cellulase and xylanase rapidly. Of the 25 isolates isolated, 11 isolates showed the activities of cellulytic and xylanolytic with the range of 0.1-1.7 cm clear zone in diameter. Interestingly, 11 isolates had double enzyme activity that demonstrating both cellulase and xylanase activity.

Table 1. Plate screening measurement of clear zone surrounding colonies.

| ID   | Methods   | Clear zone diameter in CMC medium [cm] | Clear zone diameter in xylan medium [cm] |
|------|-----------|----------------------------------------|----------------------------------------|
| KB1008 | direct    | 0.1                                    | 0.1                                    |
| KB1009 | direct    | 0.3                                    | 0.7                                    |
| KB1013 | direct    | 0.1                                    | 0.1                                    |
| KB1015 | direct    | 0.1                                    | 0.1                                    |
| KBX03  | direct    | 1.7                                    | 0.5                                    |
| KBX04  | direct    | 0.8                                    | 0.8                                    |
| KBX06  | enrichment| 0.6                                    | 0.9                                    |
| KBX07  | enrichment| 0.9                                    | 1.5                                    |
| KBX08  | enrichment| 0.5                                    | 0.7                                    |
| KBX09  | enrichment| 0.5                                    | 0.4                                    |
| KBX10  | enrichment| 1.6                                    | 1.0                                    |

3.2. Enzyme activity

The cellulose and xylanase activity of the eleven selected bacterial isolates were varied. Overall, the average cellulase activity was 0.26 U/mL with the highest activity in the KBX03 [0.3 U/mL] isolate while the xylanase activity was 0.62 U/mL with the highest activity on the KBX07 isolate [1.3 U/mL]. However, xylanase activity in some selected bacterial isolates was known to be more dominant than cellulase, including KBX03, KBX04, KBX06, and KBX07 with the ratio of xylanase/cellulase activity.
in each isolate were 3.7; 5.6; 3.4 and 4.6; respectively. In addition, the xylanase enzyme activity of the isolates obtained through the enrichment step tends to be greater when compared with isolates obtained by direct isolation. Enzyme activity of cellulase and xylanase were presented in Figure 2.

Figure 2. The activity of cellulase and xylanase of bacterial isolates from the gastrointestinal tract of Toraja’s buffalo.

Several studies on the activity of cellulase and xylanase from bacteria that originated from ruminants have been reported, including double enzyme activity of Ruminococcus flavefaciens, Rumininococcus albus and Bacteroides succinogenes [14]. In the previous studies reported that B. succinogenes were isolated from cow rumen when induced using CMC substrate, the enzyme secreted not only cellulase, but also the xylanase and aryl-13-xylosidase. The magnitude of xylanase activity tends to be higher than that of cellulase activity, similar to this study with four isolates [KBX03, KBX04, KBX06 and KBX07] showing three to seven times higher or in xylanase activity [0.99-1.56 U/mL] compared to cellulase activity [0.27-0.30 U/mL]. Whereas in other samples of cellulolytic bacteria, Clostridium papyrosolvens C7, was reported to have high-molecular-weight system enzymes that can hydrolyze cellulose and xylan. This enzyme molecule consisted of seven different protein complexes with each other [15].

3.3. Bacterial identification using 16S rRNA

A total of four selected isolates with high cellulase and xylanase enzyme activity was performed on four isolates namely KBX03, KBX04, and KBX07 and KBX08. The result of both BLAST and EzTaxon showed the relationship to the closest species of two genera, Cellulomonas and Bacillus. Based on EzTaxon, KBX03 isolate had homology of up to 99.87% with Cellulomonas flavigena DSM 20109T and Cellulomonas iranensis NBRC 101100T [Table 2]. While the other three bacterial isolates of KBX04, KBX07 and KBX08 had homology up to 100% with Bacillus altitudinis 41KF2bT and Bacillus xiamenensis HYC-10T. The results of phylogenetic analysis showed that isolate KBX03 was in the same clade of Cellulomonas flavigena and Cellulomonas iranensis. Other three isolates, KBX04, KBX07, and KBX08 were in the clade of Bacillus altitudinis and Bacillus xiamenensis [Figure 2]. All the reference strains have been reported to have cellulase or xylanase activity in several studies [16-18]. The ability to degrade cellulose, xylan and starch is the characteristic of C. flavigena and the genomic data has also been well identified and characterized [17].
Table 2. The closest relationship of bacteria isolated from Toraja’s buffalo analyzed by EzTaxon

| Strains | Species [Accession number] | Homology [%] |
|---------|-----------------------------|--------------|
| KBX03   | *Cellulomonas flavigena* DSM 20109\T [CP001964] | 99.87        |
| KBX04   | *Bacillus altitudinis* 41KF2b\T [ASJC01000029] | 100.00       |
| KBX07   | *Bacillus altitudinis* 41KF2b\T [ASJC01000029] | 100.00       |
| KBX08   | *Bacillus altitudinis* 41KF2b\T [ASJC01000029] | 100.00       |

Figure 2. Phylogenetic tree of faecal cellulolytic and xylanolytic bacteria. The code inside the brackets shows the GenBank\® access code.

4. Conclusions

In total, we obtained 11 isolates with both cellulase and xylanase activity and four of them were belong to genus of *Bacillus* and *Cellulomonas*. Xylanase activity of four isolates were up to seven times higher compared to cellulase activity.

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Conflict of interest

The authors declare that there is no conflict of interest.
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