Crude Xylanase Production in Bacteria using Corn Husk as Substrate in Submerged Fermentation

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ABSTRACT

Burning of agricultural waste leads to high levels of haze and negative impacts on human health. Management of crop waste is important for sustainable agriculture. We investigated xylanase producing strains of soil inhabiting bacteria using corn husk as a substrate in submerged fermentation. Soil samples were collected from corn silage waste areas in Mae Sai, Rong Kwang District, Phrae Province, Thailand. Primary screening of crude xylanase producing isolates was made with the enrichment method in Mineral Salt Medium (MSM) containing 1% (w/v) corn husk on a shaking incubator at 150 rpm at 37ºC for 7 days. In order to select potential isolates for further study, the serial dilution method was employed using MSM solid agar supplemented with xylan 0.1% (w/v). The enrichment method gave 236 bacterial isolates and these were used to determine their crude xylanase activity in spot diffusion and gel diffusion assays. Eleven isolates presented clear zones between 30 - 35 mm in diameter. The quantity of reducing sugars released in the supernatant in submerged fermentation was measured with the alkaline 3,5-dinitrosalicylic acid method. A Gram-negative isolate A023 had the highest crude xylanase activity with a value of 21.67 ± 0.38 Unit/mL in corn husk containing medium. Factors affecting xylanase expression and yield will be investigated further with this isolate.

Keywords: submerged fermentation, corn husk, crude xylanase production, xylan

INTRODUCTION

Corn (maize) is the third most important food crop after rice and wheat. The United States is the largest producer with 371 million metric tons (The Statistic Portal 2018). In Thailand, corn is mainly grown for the animal feed industry (Office of Agricultural Economics, 2016). In the highlands of northern Thailand, the area under corn-production has expanded due to the growing demand from the animal feed

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industry (Office of Agricultural Economics, 2014). After harvesting, large amounts of agricultural wastes such as corn husks, stems, leaves and cobs are burned in the field. The burning of agricultural wastes is causing serious air pollution in the region. Therefore, greater attention should be given to the utilization of agricultural biomass residues in place of burning.

Biological treatment of agricultural biomass can improve the nutritive value of animal feed (Abdel-Aziz et al. 2015). Many studies have reported the use of microorganisms to enhance the digestibility of animal feedstock by producing enzymes to break down plant cell walls (Kuhad et al. 1997; Burlacu et al. 2016). Corn husks contain 24% cellulose, 35% hemicellulose and 41% lignin (Yuan et al. 2004). Xylans contribute to the structural integrity of the cell wall; and they are a major component of the renewable heteropolymers found in hemicelluloses (Zhang and Sang 2015; Rohman et al. 2018). The heterogeneity and chemical complexity of xylans require several enzyme systems, with diverse specificity and modes of action, to be broken down into small oligomers (Collins et al. 2005; Motta et al. 2013; Uday et al. 2016).

On breaking down, xylan can provide biomolecules like low calory sugars, prebiotics and biofuel, thus many bacteria have been studied for xylanase production. Various sources of xylanase producing bacteria have been investigated such as soil from forests, gardens, termite-infested mounds and hotsprings (Rajaram and Varma 1990; An et al. 2005; Lee and Yun 2008; Hauli et al. 2013). In addition, novel xylanase-producing bacteria were isolated from soil in Nan Province, Thailand (Khianngam et al. 2009; Khianngam et al. 2011). To enhance production of xylanase, optimization of process parameters and the genetics of bacterial xylanase were investigated (Chakdar et al. 2016). Moreover, mixed microbial cultures (Murugan et al. 2015; Xu et al. 2018) and mono cultures of *Bacillus* and *Paenibacillus* were investigated for xylanase production using submerged fermentation (Kurrataa and Meryandini 2015; Bakri et al. 2016). Commercial products dependent on xylanase are expensive to produce and the yield of enzymes is often low. These are major problems for the animal feed industry. In this paper, we screened for xylanase producing strains of local bacteria using corn husk as substrate in submerged fermentation. The crude xylanase activity and break down of corn husk residue were observed.

METHODOLOGY

Preparation of corn husk as substrate for enzyme production

Corn husk was collected from the local agricultural industries of Phrae Province, Thailand. Firstly, the corn husk was cleaned and dried in an oven at 70°C. Then, the clean and dry corn husk was chopped into smaller pieces of about 1 mm^2. Finally, the small pieces of corn husk were ground and sieved to obtain a fine corn husk powder. The powder was packed in zip lock plastic bags and kept at room temperature until use.

Isolation of bacteria

Soil samples were collected from corn silage waste areas in Mae Sai, Rong Kwang District, Phrae Province, Thailand. Ten grams of each sample were transferred

into 250 mL Erlenmeyer flasks contain 90 mL of modified Mineral Salt Medium (MSM) supplemented with 1% (w/v) of fine corn husk. The MSM in 1 L consisted of K_2HPO_4 1.6 g, KH_2PO_4 0.4 g, $(NH_4)_2SO_4$ 0.2 g, Yeast extract 5.0 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2$ 0.05 g and $FeSO_4 \cdot 7H_2O$ 0.01 g (Rousseaux et al., 2001). The flasks were incubated on a shaking incubator at 37ºC, at 150 rpm for 7 days. The culture fluid was serially diluted and spread on MSM agar plates with 0.1% (w/v) xylan as a carbon source. The plates were incubated at 37ºC and the emerging colonies were sub-cultured to obtain pure isolates. The selected isolates were examined for cell shape and colony appearance under a light microscope.

Primary screening method

Primary screening for the selection of crude xylanase producing bacteria was performed using a gel diffusion assay on MSM agar plate with xylan 0.1% (w/v) as the carbon substrate. Bacterial isolates were spotted on test plates and incubated at 37ºC for 2 days. Potential xylanase producing bacteria were revealed by staining the plates with 0.5% (w/v) Congo red solution for 15 min and destaining with 1 M NaCl (Downie et al. 1994). Xylan hydrolysis was detected by the appearance of clear zones on the test agar. The strains that produced clear zones greater than 10 mm in diameter were selected for secondary screening.

Secondary screening method

In the secondary screening step, two assay techniques including the gel diffusion assay (see primary screening method) and the xylanase enzyme activity assay were applied. The gel diffusion assay of the isolates obtained from the primary screening was performed by growing the selected isolates in test tubes containing 5 mL MSM with 1% (w/v) fine corn husk. The culture tubes were incubated at 37ºC in an incubator shaker at 200 rpm for 4 days. The culture broth was centrifuged at 10,000 rpm at 4ºC for 10 min and the supernatant was collected. Forty microliters of supernatant were applied onto the xylanase test agar medium that contained 0.1% (w/v) of xylan in 100 mM phosphate buffer pH 7.0. The plates were incubated at 37ºC for 16 hours and then the gel was stained. The presence of clear zones on the test agar were measured. For the xylanase enzyme activity assay, each sample was assayed by mixing 250 µl of an appropriately diluted culture supernatant with 250 µl of 1% (w/v) of xylan in a 100 mM phosphate buffer pH 7.0. The reaction mixture was incubated at 50ºC for 30 min. The xylanase activity was determined by the dinitrosalicylic acid (DNS) method (Miller 1959) using xylose as standard. One unit (U) of enzyme activity was defined as the amount of enzyme generating 1 µmol of xylose per minute under the assay conditions. All data represent the average of three replicate experiments.

Effect of time on crude xylanase production

The bacterial suspension was prepared to achieve 3.87 x 10^8 CFU/mL to use as pre-inoculum. Then, 0.5 mL of pre-inoculum was transferred to a 5 mL test tube contained MSM with 1% (w/v) fine corn husk. The cultures were incubated on a

shaking incubator at 37ºC for 10 days. The supernatants were collected daily for determination of crude xylanase production by the DNS method (Miller, 1959) using xylose as standard. The enzyme activity was calculated. Samples of corn husk residues, after incubation for 10 days, were dried at 60ºC and used to observe material degradation under a stereo microscope.

Statistical analysis

The experiments were performed in triplicate and the data were expressed as mean ± Standard Deviation (SD). All the experimental data were analyzed by SPSS program version 17.0. The data were subjected to analysis of variance (ANOVA) and using the Duncan's multiple range test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Isolation of bacteria

In this study, soil samples from under piles of corn waste were collected from Mae Sai, Rong Kwang District, Phrae Province, Thailand for isolation of xylanase producing bacteria by the enrichment culture method. Two hundred and thirty-six bacterial isolates were obtained and these were screened to detect high levels of crude xylanase production.

Primary screening method

The xylanase production ability of the 236 isolates was assessed using a Spot diffusion assay on MSM agar. After flooding with 0.5% (w/v) Congo red, 75 isolates were shown to be able to produce xylanase. However, only eleven isolates exhibited clear zone diameters (Figure 1) of between 30 - 35 mm (Table 1). The test plate showed an extensive clear zone on agar.

Figure 1 Clear zone formation by bacterial isolate A023 on an MSM agar plate using a spot diffusion assay. The plate was incubated at 37ºC for 2 days and stained with Congo red.

Table 1 Bacterial isolates that provided clear zones equal to or greater than 30 mm on agar plates using the spot diffusion assay. Plates were incubated at 37ºC for 2 days and stained with Congo red.

Bacterial isolate	Clear zone diameter (mm)*
A006	30.50 ± 0.70
A011	33.00 ± 4.20
A021	30.00 ± 0.00
A022	30.00 ± 1.40
A023	35.00 ± 2.80
A060	34.50 ± 2.10
A066	32.00 ± 0.00
A084	30.50 ± 2.10
A086	31.00 ± 1.40
B020	30.00 ± 0.00
B053	30.00 ± 2.80

* Each value is a mean of three replicates ± standard deviation.

Secondary screening method

Secondary screening entailed the use of the supernatant from 75 isolates collected from 4 day-MSM liquid cultures with 1% (w/v) corn husk as substrate. The evaluation of crude xylanase production was employed by using gel diffusion assay. In this assay, seven isolates gave a positive result for xylanase (Figure 2).

Figure 2 Clear zone formation by bacterial isolate A023 on an agar plate using gel diffusion assay. The plate was incubated at 37ºC for 16 hours and stained with Congo red.

Crude xylanase activity ranged between 15.31±9.08 - 21.67±0.38 (Unit/mL) (Table 2). The highest activity of crude xylanase was detected from bacterial isolate A023 (21.67±0.38 Unit/mL).

Table 2 Bacterial isolates that provided clear zones equal to or greater than 25 mm on agar plates using gel diffusion assay. Plates were incubated at 37ºC for 16 hours and stained with Congo red.

Isolate	Clear zone diameter* (mm)	Crude xylanase activity** (Unit/mL)
A023	26.50±0.50	21.67±0.38[a]
A051	28.50±0.55	17.45±2.78[abcd]
A053	30.50±0.25	19.67±2.97[abc]
A071	26.50±0.70	16.40±1.66[abcdefg]
A075	25.50±0.55	20.10±0.99[abc]
A089	26.00±0.25	16.75±13.04[abcde]
A124	25.50±0.75	15.31±9.08[abcdefg]

* Each value is a mean of three replicates; ± standard deviation.
**Means followed by different letters within each column differ significantly at $P \leq 0.05$.

Effect of time on crude xylanase production

Bacterial isolate A023 was cultivated and supernatant was collected daily to observe capacity for crude xylanase production. Crude xylanase activity was detected on day 1 and activity increased to a maximum on day 8 (12.92 ± 0.08 Unit/ml) as shown in Figure 3.

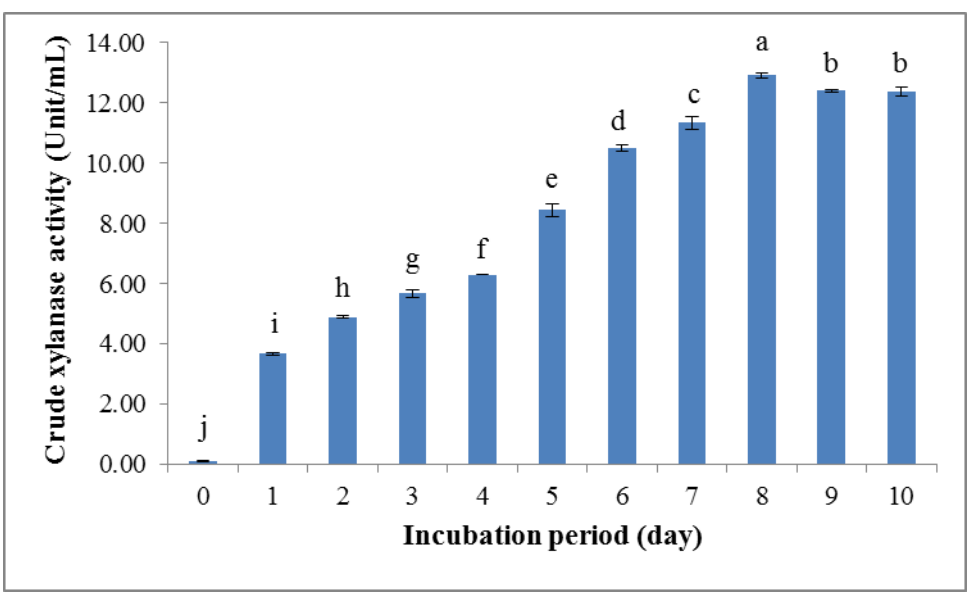

Figure 3 Crude xylanase activity of bacterial isolate A023 cultivated in MSM liquid medium using corn husk as substrate. Different letters indicate significant differences according to the Duncan's multiple range test at $P \leq 0.05$ between treatments.

The histological structure of corn husks during decomposition under submerged fermentation for 10 days is shown in Figure 4. The samples were randomly picked and illustrate the occurrence of cell wall decomposition due to enzyme activity from bacteria. Other researchers have employed scanning electron microscopy to observe decomposition of cell wall constituents (Khattab et al., 2013).

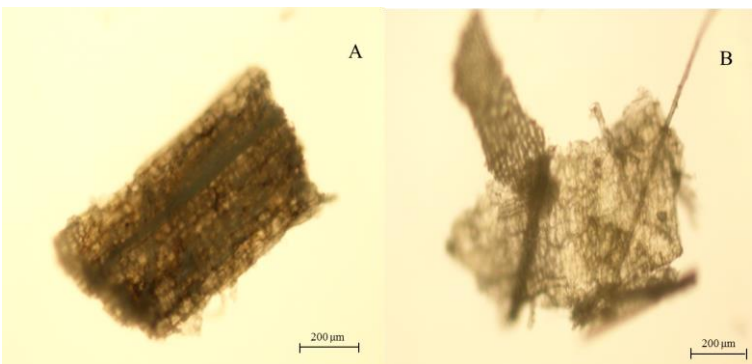

Figure 4 Comparison of corn husk residues observed under a stereo microscope at the commencement of the trial (A) and after 8 days (B). Notice that light passed easily through the husk residue on day 8 compared to the residue from day 0.

Isolate A023 colony morphology and Gram staining

Isolate A023 formed yellow, raised, circular, smooth, glistening and weakly cohesive colonies on Nutrient agar (Figure 5A). It was a rod shaped bacterium, and individual cell arrangement was identified by Gram-negative staining (Figure 5B).

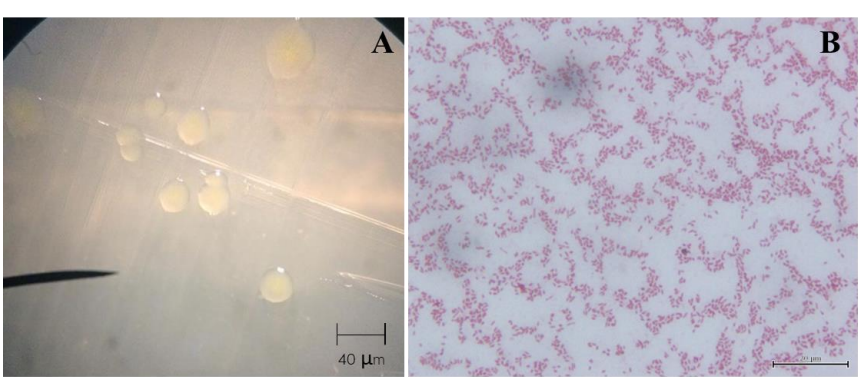

Figure 5 Bacterial isolate A023: **A** colony form on agar (bar = 40 µm); **B** Gram-negative staining observed under a compound light microscope (bar = 20 µm).

General discussion

Agricultural wastes are available natural carbon sources for the production of industrially important enzymes. In this paper, we investigated xylanase producing strains of local bacteria using corn husk as a substrate in submerged fermentation. To obtain xylanase producing bacteria, an enrichment culture method was employed to obtain bacterial isolates from soils in corn silage waste areas. Enrichment culture is a reliable technique for the isolation of microbes with specific metabolic properties (Steele and Stowers 1991). In a xylano-cellulolytic enzyme screening program, thirty bacterial isolates were obtained from termite guts and termitaria using enrichment culture. The authors claimed the procedure to be simple and cost-effective (Prajapati et al. 2018).

In this study, we used the spot diffusion assay for primary screening rather than serial dilution because we were targeting bacteria with the capacity to produce xylanase. About 30% of the total number of isolates obtained from the enrichment culture method were found to have potential for xylanase production in the spot diffusion assay. From enriching the group of bacteria of interest in the favored medium, our objective was to multiply the xylanase producing bacteria in submerged fermentation. Ghasemi et al. (2014) also used enrichment culture before isolating xylanase producing bacteria from farmland soils. Their high xylanase producing isolate was identified as *Sphingobacterium* sp. SaH-05. To detect crude xylanase production, spot diffusion and gel diffusion assays were used as they are time and cost effective. However, these methods can not be used to confirm enzyme yields and activities. Thus, further studies are needed to evaluate xylanase production in both primary and secondary screening (Shanthi and Roymon 2014; Burlacu et al. 2016). As xylanases are inducible enzymes, fermentation and optimization factors influencing induction have been reported (Gupta and Kar 2009; Irfan et al. 2016).In 2015, Kurrataa and Meryandini showed that *Paenibacillus* sp. XJ18 had the highest activity of xylanase production at 36 h at 27ºC in submerged fermentation. Submerged fermentation increased enzyme activity about 5 times after being concentrated in 70% acetone. Furthermore, *Bacillus subtilis* XP10 produced thermostable xylanase with maximum yield after 4 days at 40ºC in submerged fermentation (Tork et al. 2013). In terms of efficiency and effectiveness, potential isolates should be selected by optimizing factors that promote the highest yield in a suitable time. Modern techniques can help to simplify the screening and identification of target organisms for application in the industrial utilization of agricultural wastes. In order to speed up the research on bacterial xylanase and to understand the biology and biochemistry, a multidisciplinary approach is required to satisfy the industrial purposes.

Xylanase producing bacteria can be used to enhance the digestibility of animal feed. Before they are used, extensive screening through qualitative and quantitative assays are essential before mass production and commercialization. Previously, bacteria such as *Burkhoderia* sp. DMAX (Mohana et al. 2008), *Bacillus* sp. GRE7 (Kiddinamoorthy et al. 2008) and *Arthrobacter* sp. MTCC6915 (Murugan et al. 2011) were isolated from mesophilic, thermophilic and marine environments, then purified and identified for xylanases. Interestingly, *Bacillus* spp. were proven to

be a good source of commercial xylanases for feed additives and biotechnological industries (Chakdar et al. 2016). However, the search for new sources of xylanase production continues. For example, some novel bacterial strains in the genus *Paenibacillus* have been tested for xylanolytic enzyme production on agar (Park et al. 2007; Lee and Yoon 2008; Khianngam et al. 2009). It is challenging to improve xylanase yield and activity during fermentation. In our study, simplified screening methods were undertaken in order to isolate bacteria with potential to turn agricultural waste into commercial products. Strategies for increasing xylanase yield and activity require further study.

CONCLUSIONS

The results of the present study revealed that isolate A023 expressed high xylanase activity of up to 21.67±0.38 Unit/mL after 4 days fermentation in submerged culture using finely ground corn husks as a substrate. Enzyme activity reached a maximum on day 8. Isolate A023 was a Gram-negative and rod shaped bacterium. The isolate will be identified and xylanase production will be characterized in further studies.

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