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การวิเคราะห์เชิงคุณภาพการผลิตเอนไซม์ย่อยสลายไฟเบอร์โดยบาซิลลัสที่แยกจากมูลสุกรพื้นเมือง

Qualitative Analysis of Fibre-Degrading Enzymes Production by *Bacillus* Isolated from Native Swine Manures

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บทคัดย่อ

เอนไซม์ย่อยสลายเส้นใยมีบทบาทสำคัญในการย่อยเส้นใยไฟเบอร์พอลิแซ็กคาไรด์ที่ไม่ใช่แป้ง และพอลิแซ็กคาไรด์ที่มีโครงสร้างอื่น ๆ โดยเฉพาะเส้นใยจากพืชที่เป็นส่วนประกอบในอาหารสัตว์ มีรายงานว่าจุลินทรีย์ในลำไส้ของสัตว์สามารถผลิตเอนไซม์เหล่านี้ได้ การศึกษานี้จึงทำการคัดแยกและคัดกรองเชื้อ *Bacillus* spp. ที่ผลิตเอนไซม์ย่อยสลายเส้นใย ได้แก่ เซลลูเลส ไซลาเนส และเพคติเนส จากมูลสุกรพื้นเมืองที่มีสุขภาพดี จำนวน 40 ตัวอย่าง พบแบคทีเรียที่คาดว่าเป็น *Bacillus* spp. จำนวน 103 ไอโซเลท และจากผลการคัดกรองความสามารถในการผลิตเอนไซม์เชิงคุณภาพโดยวิธี agar plate assay พบเชื้อ *Bacillus* spp. จำนวน 8 สายพันธุ์ ได้แก่ *Bacillus albus* NA11.3 *Bacillus cereus* NG3.6 *Bacillus cereus* NM1.1 *Bacillus amyloliquefaciens* NL1.2 *Bacillus amyloliquefaciens* NL1.3 *Bacillus subtilis* NM1.5 *Bacillus subtilis* NM1.7 และ *Bacillus subtilis* NM2.2 สามารถผลิตเอนไซม์เซลลูเลส ไซลาเนส และเพคติเนสได้ระดับสูงสุด ไอโซเลทที่แยกได้ใหม่เหล่านี้มีความเหมาะสมสำหรับการศึกษาเพิ่มเติมเพื่อประเมินระดับกิจกรรมของเอนไซม์ต่อไป

คำสำคัญ : บาซิลัส ; เซลลูเลส ; ไซลาเนส ; เพคติเนส ; การคัดกรองการสร้างเอนไซม์

Abstract

Fibre-degrading enzymes play an important role in fibre digestion, non-starch polysaccharides, and other structural polysaccharides in animal feed, especially in plant-based diets. It was reported that the microflora in an animal's gut could produce these enzymes. This study isolated and screened *Bacillus* spp. produces fibre-degrading enzymes (cellulase, xylanase, and pectinase) from native swine manures. One hundred and three isolates of the presumptive *Bacillus* spp. were isolated from 40 faecal samples of healthy native pigs, and their qualitative ability to produce fibre-degrading enzymes was screened by agar plate assay. Eight isolates of *Bacillus* spp., namely, *Bacillus albus* NA11.3, *Bacillus cereus* NG3.6, *Bacillus cereus* NM1. 1, *Bacillus amyloliquefaciens* NL1. 2, *Bacillus amyloliquefaciens* NL1. 3, *Bacillus subtilis* NM1. 5, *Bacillus subtilis* NM1.7, and *Bacillus subtilis* NM2.2, are the isolates that could produce cellulase, xylanase, and pectinase at the highest level. These newly isolated strains are the most suitable microorganisms for further studies to assess their quantitative enzyme production.

Key Words : *Bacillus* ; cellulose ; xylanase ; pectinase ; enzyme production screening

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Introduction

In Thailand, native pigs have played an essential role in local communities for a long time. They are often raised in a backyard or in free-range farming. They adapt well to hot and humid climates, tolerate low-quality feed, and are probably resistant to infectious diseases and internal parasites (Charoensook *et al.*, 2013). Farmers often fed them plant-based diets, such as leftovers, fruit and vegetable scraps, rice bran, banana stalk, grass sapling, and other plant biomass. Although plant biomass is an inexpensive and highly available source of nutrients, most of it consists of fibre, such as cellulose, xylan, and pectin, which are non-digestible polysaccharides. In addition to indigestion, overfeeding fibre in the gastrointestinal tract may harm monogastric animals. The insoluble fibre traps proteins, amino acids and soluble sugars, causing inhibition of the nutrient absorption of the intestinal (Ojha *et al*., 2019). Digesting these substances requires fibre-degrading enzymes, the enzyme that breaks down fibre components of feedstuff, improving digestion. In the gastrointestinal tract of pigs, fibre is less digestible; most fibre constituents are digested by fibre-degrading enzymes produced by many bacteria in the gut (Ugwuanyi, 2016; Ojha *et al.*, 2019; Wang & McAllister, 2002). Fibre-digesting enzymes useful in pig feed include cellulase, xylanase and pectinase (Toushik *et al.*, 2017; Basit *et al.*, 2020). Supplementing these enzymes to pig feed, either with crude enzymes or enzyme-producing bacteria, can help promote pig growth (Kerr & Shurson, 2013; Taylor *et al*., 2018).

In commercial pig production, supplementing exogenous enzymes to feed has evolved to get around these challenges to feed utilization (Ravindran, 2013). Contrary to native pig, it is not supplemented with any exogenous enzymes. For this reason, we hypothesized that gut bacteria might contribute to digesting feed and improving feed utilization in native pigs by secreting various extracellular enzymes, especially fibre-digesting enzymes.

Bacillus is a Gram-positive, rod-shaped, spore-forming bacterium. It can be either obligate aerobes depending on oxygen or facultative anaerobes having the ability to continue living without oxygen (Logan & Vos, 2015). Although normally considered soil organisms, members of the genus *Bacillus* are also found in water (Yoon *et al.*, 2003), air (Dominguez-Moñino *et al.*, 2018), human (Hong *et al*., 2009), animal gut (Chaiyawan *et al*., 2010; Santos *et al*., 2021), as well as vegetables and food (Patel *et al.*, 2009; Yu *et al.*, 2019). *Bacillus* spp. secretes numerous enzymes to degrade various substrates, enabling the bacterium to survive in a continuously changing environment. Some species of *Bacillus*, such as *B. subtilis*, has a strong capacity for protein expression and secretion, which has led to its wide use in the production of industrial, and pharmaceutical proteins and enzyme preparations (van Dijl & Hecker, 2013). Moreover, it has excellent physiological characteristics and a highly adaptable metabolism, which makes it easy to cultivate on cheap substrates (Su *et al*., 2020).

One of the most efficient and successful means of finding new enzymes is to screen many microorganisms because of their characteristic diversity and versatility. Therefore, this study aimed to discover the new potential of *Bacillus* producing fibre-degrading enzymes, enzymes degrading fibres such as xylanase, cellulase, and pectinase from native swine manure.

Methods

Forty faecal samples were collected from healthy native pigs with no history of antimicrobial use and no diarrhoea raised in a backyard in Krabi, and Nakhon Si Thammarat province, southern Thailand. Fresh faecal samples were placed separately in a sterile tube. Due to the long distance, faecal samples were kept in iceboxes until transported to the laboratory and immediately analysed upon arrival. Bacterial isolation was performed as described in previous studies (Barbosa *et al*., 2005; Singh *et al.*, 2013) with minor modifications. The faecal sample (5 g) was suspended with 50 mL sterilised saline solution (0.85% w/v NaCl) in a conical flask, which was shaken at 180 rpm for one h at 37°C. Aerobic spore-forming isolates were then selected by heat treatment, and the suspension was further incubated at 65°C for 20 min. Subsequently, the heated suspension was diluted tenfold with sterilised saline solution (up to 10^{-5}). An aliquot of 100 µL of the appropriate dilution was spread plated on Difco nutrient agar or Luria-Bertani (LB) plates and incubated at 37°C for 24 h. After incubation, 5-10 colonies representing different morphologies were picked randomly and subcultured on the same media agar plates until pure cultures were obtained. Each isolate was preliminary identified based on morphology, Gram reactions, and endospore formation. The Gram-positive, rod-shaped, spore-forming bacilli were selected for further study. The isolates were preserved in LB broth supplemented with 20% glycerol and stored at -80°C.

An enzyme screening assay was performed by adjusting the overnight bacterial cultures to turbidity equal to 0.5 MacFarland standard. After that, using a 10 µL disposable calibration loop, spot a suspension of bacteria onto each substrate agar and let the spotted bacteria dry before incubating at 30°C for 48 h.

Screening of cellulase-producing Bacillus spp.

A preliminary qualitative analysis of cellulolytic activity was performed as described previously (Kasana *et al.*, 2008), with minor modifications. Carboxymethylcellulose (CMC) sodium salt was used as a substrate for the cellulase enzyme. Ten microliters of 0.5 MacFarland standard of overnight bacterial cultures were spotted on carboxymethylcellulose agar plate (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC, 0.02% peptone, and 2% agar). Plates incubated at 30°C for 48 h were flooded with Gram's iodine (1% iodine, 2% potassium iodide in water) for 3 minutes. After incubation, the plates were observed for the zone of clearance around the colony.

Screening of xylanase-producing Bacillus spp.

Screening microbial isolates for xylanase activity by qualitative evaluations were evaluated according to the method described previously (Burlacu *et al.*, 2016), with minor modifications. Ten microliters of 0.5 MacFarland standard of overnight bacterial cultures were spotted on xylan agar plate (0.05 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L NaCl, 0.01g/L $CaCl_2$, 0.2 g/L yeast extract, 0.5 g/L peptone, 10 g/L xylan, 2 g/L agar). The plates were incubated at 30°C for 72 h. The xylanase activity of each isolate was observed by flooding the plates with Gram's iodine reagent for 3 min. If the isolates can produce xylanase, the zone of clearance around the colony was presented.

Screening of pectinase-producing Bacillus spp.

The bacterial isolates were screened for their pectinase activity using the plate screening method described previously (Mohandas *et al.*, 2018), with minor modifications. Ten microliters of 0.5 MacFarland standard of overnight bacterial cultures were spotted on the pectin agar plate (1.0 g/L $NaNO_3$, 1.0 g/L KCl, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.5 g/L yeast extract, 10 g/L pectin, and 20 g/L agar with pH adjusted to 7.0) and incubated at 37°C for 48 h. Then Gram's iodine solution was added to the sample plates and incubated for 5 min with intermittent gentle shaking. Subsequently, the plates were washed with distilled water, and the zones around the colony were observed.

Assessment of the qualitative enzyme production

In this study, qualitative enzyme activity was observed by the appearance of the hydrolyzed zone (diameter in mm) around the colony. The hydrolyzed zone was presented as score as follows, 0, no activity; 1, low (5-9 mm); 2, moderate (10-20 mm); 3, good (21-30 mm); 4, high (31-40 mm); and 5, excellent (>40 mm), respectively.

Identification of isolates by 16S rRNA gene sequence analysis

According to the manufacturer's instructions, total genomic DNA was extracted from pure bacterial cultures using the bacterial DNA extraction kit (GF-1 Bacterial DNA Extraction Kit, Vivantis Technologies, Malaysia). The amount and purity of the extracted DNA were determined using a spectrophotometer (NanoDrop Lite Spectrophotometer, Thermo Scientific, USA). PCR amplification followed a method described previously (Lan *et al.*, 2004), with some modifications. The universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. PCRs were performed in a total volume of 50 μL using a thermocycler (MultiGeneTM Mini Personal Cycler, Labnet, USA) under the following conditions: initial activation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The final cycle was 72°C for 5 min. Approximately 1,466 bp of the PCR products were then analysed by agarose gel electrophoresis and purified using the PCR clean-up kit (GF-1 AmbiClean Kit (Gel and PCR), Vivantis Technologies, Malaysia) before sending them for analyses of the nucleotide sequences (1st BASE DNA Sequencing Services: Base-Asia, Singapore). BLAST homology analysis was performed to compare and align with other 16S rRNA sequences retrieved from the EzBioClould database (https://www.ezbiocloud.net/). The phylogenetic tree relationship was constructed based on the maximum likelihood method using MEGA-11 software (Tamura *et al.,* 2021).

Results

The preliminary identification by morphology, Gram reactions, and endospore formation showed 103 isolates were the presumptive *Bacillus* spp. Isolated for faecal samples. For qualitative estimation of enzyme production, all of the presumptive *Bacillus* spp. were screened for fibre-degrading enzyme production by agar plate assay. The CMC sodium salt, xylan, and pectin were used as a substrate to detect cellulase, xylanase, and pectinase, respectively. The enzyme production was observed by the appearance of the hydrolyzed zone around the bacterial colony. Details of the fibre degradation enzymes produced by *Bacillus* spp. isolated from native pig manures were shown in Table1. and Table S1.

Table 1 Production of fibre-degrading enzymes by *Bacillus* spp. isolated from native swine manures.

Enzyme production level [a]	Fibre-degrading enzyme producers					
	Cellulase		Xylanase		Pectinase	
	isolates	% Positive isolates	isolates	% Positive isolates	isolates	% Positive isolates
Low	0	0.00	0	0.00	6	5.82
Moderate	18	17.48	14	13.59	28	27.18
Good	41	39.81	24	23.30	12	11.65
High	8	7.76	25	24.27	15	14.56
Excellent	0	0.00	2	1.94	3	2.91
Total positive isolates	67	65.05	65	63.11	64	62.13
No activity	36	34.95	38	36.89	39	37.86

[a] The enzyme activity level was reported based on the hydrolyzed zone as follows, no activity; low (5-9 mm); moderate (10-20 mm); good (21-30 mm); high (31-40 mm); and excellent (>40 mm), respectively.

No significant differences were found among the number and typology of the enzyme activities expressed by the bacterial isolates obtained from the native swine manures. The cellulase enzyme-producing bacteria was the most isolated group in this study, followed by the xylanase and pectinase-producing bacteria. The enzyme production of some bacterial isolates screened by agar plate assay is shown in Figure 1.

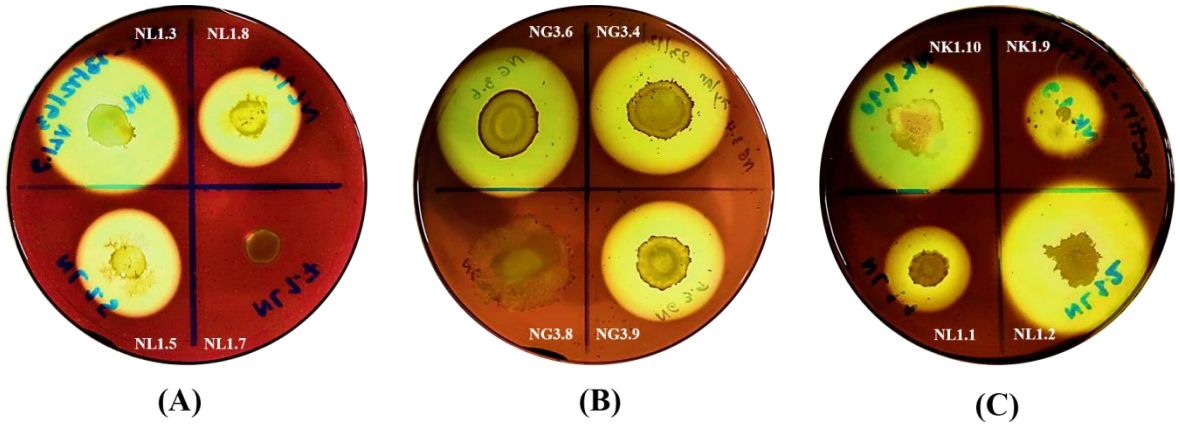

Figure 1 The enzyme activity of some bacterial isolates was estimated by plate assay, (A) Cellulase, (B) Xylanase, (C) Pectinase

For cellulase production, sixty-seven isolates (65.05%) could secrete cellulase to degrade CMC. The high level of cellulase production was detected by eight isolates, namely, NA11.3, NG3.6, NK1.4, NL1.2, NL1.3, NM1.5, NM1.7, and NM2.2. Most cellulase producers showed a high level, and the remaining isolates showed a moderate level. Xylanase is also an important fibre-degrading enzyme. In this study, 65 isolates (63.11%) produced xylanase. Xylanase was observed, ranging from a moderate to an excellent level. Among the xylanase producers, the isolates, namely NA11.3 and NM1.5, were the two excellent xylanase producers. The number of good and high xylanase producers was similar (24 and 25 isolates, respectively).

Regarding pectinase production, 64 isolates (62.13%) are pectinase-producing bacteria. Fifteen isolates produced a high level, whereas most showed a moderate level. Only six isolates could produce pectinase at a low level. Among pectinase producers, three isolates, NL1.2, NL1.3, and NM1.1, were excellent.

Considering the nutritional versatility, we also noticed the ability to produce multi-enzymes of the bacterial isolates in our study, and the results are shown in Figure 2. Most isolates (57 isolates) could produce all the enzymes investigated. Seven isolates produced two enzymes. Among these, five isolates were cellulose and xylanase producers; one isolate was a cellulose and pectinase producer, and another was a xylanase and pectinase producer. Eleven isolates produced only one enzyme. Out of these, five isolates were pectinase producers, four isolates were cellulase, and only two were xylanase producers.

In comparison, twenty-eight isolates showed no activity in any enzymes screened. The isolates showing a multi-enzyme production (2–3 activities) revealed a wide nutritional versatility that may assist in the synergistic degradation of complex substrates. In contrast, those having only one enzyme could be considered to have high specificity and limited versatility.

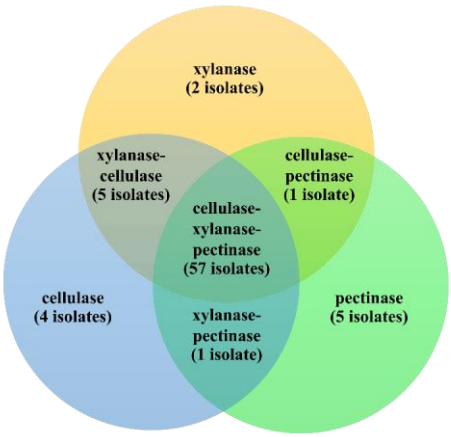

Figure 2 The nutritional versatility of *Bacillus* spp. isolated from native swine manures.

This study selected eight isolates with the highest presumptive enzyme activity for molecular identification of 16S rRNA gene (approximately 900-1,100 bp) sequencing analysis. BLAST homology analysis was carried out to compare with other 16S rRNA sequences in the EzBioClould database. The results revealed that the sequence of the PCR product of 3 isolates (NM1.5, NM1.7, and NM2.2) showed a high similarity to closely related *B. subtilis*. Two isolates showed similarity to be closely related to *B. amyloliquefaciens* (NL1.2 and NL1.3), two isolates showed similarity to be closely related to *B. cereus* (NG3.6 and NM 1.1), and one isolate showed similarity to be closely related to *B. albus* (NA11.3). (Table 2 and Figure 3).

Table 2 Qualitative fibre-degrading enzyme production of potential *Bacillus* isolated from native swine manures.

Strains	Species identification (Accession No.)	% similarity (Accession No.)	Qualitative enzyme activity (score)[a]			Total scores
			Cellulase	Xylanase	Pectinase	
NG 3.6	*Bacillus cereus* (OP599545)	99.58 (AE016877)	4	4	3	11
NA 11.3	*Bacillus albus* (OP599546)	99.67 (MAOE01000087)	4	5	3	12
NL 1.2	*Bacillus amyloliquefaciens* (OP599547)	100.00 (FN597644)	4	4	5	13
NL 1.3	*Bacillus amyloliquefaciens* (OP599548)	100.00 (FN597644)	4	3	5	12
NM 1.1	*Bacillus cereus* (OP599549)	99.19 (AE016877)	3	4	5	12
NM 1.5	*Bacillus subtilis* (OP599550)	99.04 (ABQL01000001)	4	5	4	13
NM 1.7	*Bacillus subtilis* (OP599551)	99.75 (ABQL01000001)	4	4	4	12
NM 2.2	*Bacillus subtilis* (OP599552)	98.96 (ABQL01000001)	4	4	4	12

[a]Score of enzyme activity was reported based on hydrolyzed zone as follows, 0, no activity; 1, (5-9 mm); 2, (10-20 mm); 3, (21-30 mm); 4, (31-40 mm); and 5, (>40 mm), respectively. All sequences were blasted and aligned with the type strain sequence retrieved from EzBioCould.

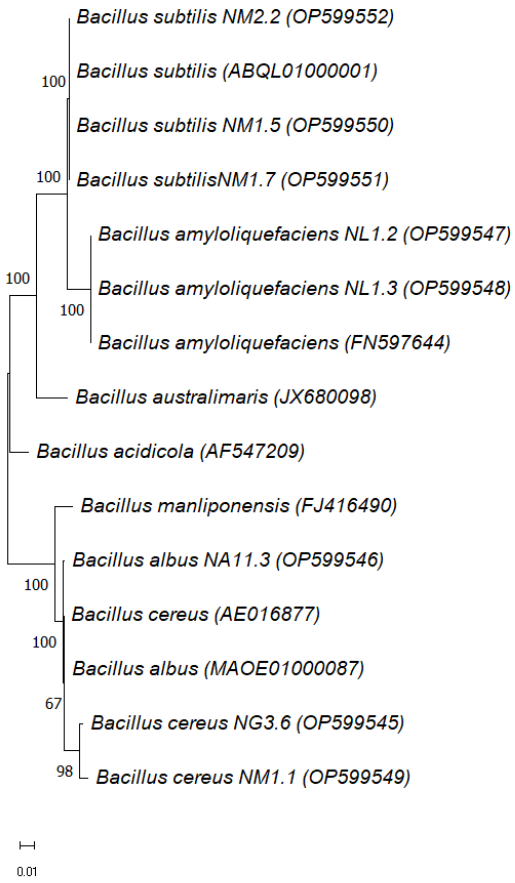

Figure 3 The relationship of *Bacillus* spp. analyses results and constructs a phylogenetic tree with the MEGA-11 program by the maximum likelihood method (500 bootstraps). All sequences were aligned with the type strains sequence retrieved from EzBioCould. All nucleotide sequences were submitted to GenBank; the access number is OP599545-OP599552.

Discussion

In this study, we isolated and screened *Bacillus* sp. producing fibre-degrading enzymes (cellulase, xylanase, and pectinase) from native swine manures. The results showed that the native swine manures as a good source represented by several *Bacillus* species produced cellulase, xylanase, and pectinase, similar to the previous studies showing that *Bacillus* sp. can produce various necessary enzymes and found in a wide variety of sources. For example, *B. amyloliquefaciens* from sago pith waste produces cellulase and amylase (Apun *et al*., 2000). *B. subtilis* from the empty fruit bunch's compost produce xylanase-pectinase enzymes, *B. pumilus* from oil palm empty fruit bunch produce cellulose (Ariffin *et al*., 2006), *B. licheniformis* from compost (Archana & Satyanarayana, 1997). *B. arseniciselenatis* from soil produced xylanase, *B. subtilis* and *B. pumilus* from sanitary landfills produce pectinase and xylanase (Ahlawat *et al*., 2007). However, the isolation and screening of *Bacillus*-producing fibre-degrading enzymes from native pig manures have

not previously been reported. Therefore, our report is one of the important pieces of information on microbial enzyme production in monogastric animals, such as native pigs, and also contributes to the knowledge of the nutritional versatility of the bacterial isolates. Concerning biodiversity, in this study, native pigs seem to be a reservoir of beneficial gut bacteria that could be an asset for future use, such as microbial enzyme production.

Our results indicated that the feed habit might be correlated to the occurrence of enzyme-producing bacteria within the native pig's gut. Our finding is consistent with a previous study showing that feed habits correlated to the discovery of extracellular enzyme-producing microorganisms in the animal gut (Ahmad *et al*., 2019). Production of cellulase, xylanase and pectinase by the gut bacteria noticed in this study might indicate their ability to degrade plant cell wall polymers owing to the diet of native pigs primarily consisting of vast amounts of cellulose, xylan, and pectin.

Conclusions

These results indicate that indigenous pig manure is a good source of a wide variety of beneficial microbes that could be isolated and classified for exploitation. The study focused on isolating bacteria that produce fibre-degrading enzymes. As a result, we could isolate and identify eight isolates of fibre-degrading enzymes producing *Bacillus* spp., which were *B. albus* NA11.3, *B. cereus* NG3.6, *B. cereus* NM1.1, *B. amyloliquefaciens* NL1.2, *B. amyloliquefaciens* NL1.3, *B. subtilis* NM1.5, *B. subtilis* NM1.7, and *B. subtilis* NM2.2. However, the results of this study were a preliminary screening assay for their enzyme activity. Enzyme activity levels and optimization need to be studied to obtain the information necessary for developing bacteria for further supplementation in pigs.

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References

Ahlawat, S., Battan, B., Dhiman, S. S., Sharma, J., & Mandhan, R. P. (2007). Production of thermostable pectinase and xylanase for their potential application in bleaching of kraft pulp. *Journal of Industrial Microbiology and Biotechnology, 34*(12), 763-770.

Ahmad, R., Bashir, S., & Tabassum, R. (2019). Evaluation of cellulases and xylanases production from Bacillus spp. isolated from buffalo digestive system. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi, 25*(1), 39-46.

Apun, K., Jong, B. C., & Salleh, M. A. (2000). Screening and isolation of a cellulolytic and amylolytic *Bacillus* from sago pith waste. *The Journal of general and applied microbiology, 46*(5), 263-267.

Archana, A., & Satyanarayana, T. (1997). Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state fermentation. *Enzyme and Microbial Technology, 21*(1), 12-17.

Ariffin, H., Abdullah, N., Umi Kalsom, M., Shirai, Y., & Hassan, M. (2006). Production and characterization of cellulase by *Bacillus pumilus* EB3. *International Journal of Engineering and Technology, 3*(1), 47-53.

Barbosa, T. M., Serra, C. R., La Ragione, R. M., Woodward, M. J., & Henriques, A. O. (2005). Screening for *Bacillus* isolates in the broiler gastrointestinal tract. *Applied and Environmental Microbiology*, 71(2), 968-978.

Basit, A., Jiang, W., & Rahim, K. (2020). Xylanase and Its Industrial Applications. In T. P. Basso, T. O. Basso, & L. C. Basso (Eds.), *Biotechnological Applications of Biomass*. IntechOpen. https://doi.org/10.5772/intechopen.92156.

Burlacu, A., Cornea, C., & Israel-Roming, F. (2016). Screening of xylanase-producing microorganisms. *Research Journal of Agricultural Science*, *48*(2), 8-15.

Chaiyawan, N., Taveeteptaikul, P., Wannissorn, B., Ruengsomwong, S., Klungsupya, P., Buaban, W., & Itsaranuwat, P. (2010). Characterization and probiotic properties of *Bacillus* strains isolated from broiler. *The Thai Journal of Veterinary Medicine*, *40*(2), 207-214.

Charoensook, R., Knorr, C., Brenig, B., & Gatphayak, K. (2013). Thai pigs and cattle production, genetic diversity of livestock and strategies for preserving animal genetic resources. *Maejo International Journal of Science and Technology*, *7*(1), 113-132.

Dominguez-Moñino, I., Jurado, V., Gonzalez-Pimentel, J. L., Miller, A. Z., Hermosin, B., & Saiz-Jimenez, C. (2018). *Bacillus onubensis* sp. nov., isolated from the air of two Andalusian caves. *Systematic and Applied Microbiology*, *41*(3), 167-172.

Hong, H. A., Khaneja, R., Tam, N. M., Cazzato, A., Tan, S., Urdaci, M., & Cutting, S. M. (2009). *Bacillus subtilis* isolated from the human gastrointestinal tract. *Research in Microbiology*, *160*(2), 134-143.

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology*, *57*(5), 503-507.

Kerr, B. J., & Shurson, G. C. (2013). Strategies to improve fiber utilization in swine. *Journal of Animal Science and Biotechnology*, *4*(1), 11. https://doi:10.1186/2049-1891-4-11.

Lan, P. T. N., Sakamoto, M., & Benno, Y. (2004). Effects of two probiotic *Lactobacillus* strains on jejunal and cecal microbiota of broiler chicken under acute heat stress condition as revealed by molecular analysis of 16S rRNA genes. *Microbiology and Immunology*, *48*(12), 917-929.

Logan, N. A., & Vos, P. D. (2015). *Bacillus*. In *Bergey's Manual of Systematics of Archaea and Bacteria*, 1-163.

Mohandas, A., Raveendran, S., Parameswaran, B., Abraham, A., Athira, R. S., Kuruvilla Mathew, A., & Pandey, A. (2018). Production of pectinase from *Bacillus sonorensis* MPTD1. *Food Technology and Biotechnology*, *56*(1), 110-116.

Ojha, B. K., Singh, P. K., & Shrivastava, N. (2019). Chapter 7 - Enzymes in the Animal Feed Industry. In M. Kuddus (Ed.), *Enzymes in Food Biotechnology* (pp. 93-109): Academic Press.

Patel, A. K., Ahire, J. J., Pawar, S. P., Chaudhari, B. L., & Chincholkar, S. B. (2009). Comparative accounts of probiotic characteristics of *Bacillus* spp. isolated from food wastes. *Food Research International*, *42*(4), 505-510.

Ravindran, V. (2013). Feed enzymes: The science, practice, and metabolic realities. *Journal of Applied Poultry Research*, *22*(3), 628-636.

Santos, R. A., Oliva-Teles, A., Pousão-Ferreira, P., Jerusik, R., Saavedra, M. J., Enes, P., & Serra, C. R. (2021). Isolation and Characterization of Fish-Gut Bacillus spp. as Source of Natural Antimicrobial Compounds to Fight Aquaculture Bacterial Diseases. *Marine Biotechnology*, *23*(2), 276-293.

Singh, S., Moholkar, V. S., & Goyal, A. (2013).Isolation, identification, and characterization of a cellulolytic *Bacillus amyloliquefaciens* strain SS35 from rhinoceros dung. *International Scholarly Research Notices Microbiology*, 728134. https://doi:10.1155/2013/728134.

Su, Y., Liu, C., Fang, H., & Zhang, D. (2020). *Bacillus subtilis*: a universal cell factory for industry, agriculture, biomaterials and medicine. *Microbial Cell Factories*, *19*(1), 1-12.

Tamura K., Stecher G., and Kumar S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, https://doi.org/10.1093/molbev/msab120.

Taylor, A. E., Bedford, M. R., & Miller, H. M. (2018). The effects of xylanase on grower pig performance, concentrations of volatile fatty acids and peptide YY in portal and peripheral blood. *Animal*, *12*(12, 2499-2504. https://doi:10.1017/s1751731118000277.

Toushik, S. H., Lee, K.-T., Lee, J.-S., & Kim, K.-S. (2017). Functional applications of lignocellulolytic enzymes in the Fruit and Vegetable Processing Industries. *Journal of Food Science*, *82*(3), 585-593. doi: https://doi.org/10.1111/1750-3841.13636.

Ugwuanyi, J. (2016). Enzymes for nutritional enrichment of agro-residues as livestock feed. In *Agro-Industrial Wastes as Feedstock for Enzyme Production* (pp. 233-260): Elsevier.

van Dijl, J., & Hecker, M. (2013). *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microbial Cell Factories*. *12*, 3. https://doi.org/10.1186/1475-2859-12-3.

Wang, Y., & McAllister, T. (2002). Rumen microbes, enzymes and feed digestion-a review. *Asian-Australasian Journal of Animal Sciences*, *15*(11), 1659-1676.

Yoon, J.-H., Kim, I.-G., Kang, K. H., Oh, T.-K., & Park, Y.-H. (2003). *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from seawater of a tidal flat of the Yellow Sea in Korea. *Journal of Medical Microbiology*, *53*(5), 1297-1303.

Yu, P., Yu, S., Wang, J., Guo, H., Zhang, Y., Liao, X., & Xue, L. (2019). *Bacillus cereus* isolated from vegetables in China: incidence, genetic diversity, virulence genes, and antimicrobial resistance. *Frontiers in Microbiology*, *10,* 948.