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Characterization and optimization of xylanase producing strain of *Bacillus subtilis* isolated from the cabbage looper (*Trichoplusia ni* (Hübner)) intestine

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ABSTRACT

Aims: To characterize xylanolytic enzyme producing strains of *Bacillus subtilis* from the intestinal tract of a cabbage looper (*Trichoplusia ni* (Hübner)) larvae.
Methodology and results: Approximately 5 g of intestinal content from the instar larvae were homogenated and serially diluted 10^{-4} -10^{-6} times with sterile normal saline before being spread onto duplicate tryptic soy agar plates. Every different colony was selected to test for xylanase production. Of six isolates, only one was found to be positive for xylanase production by screening agar. Biochemical characteristics and 16S rRNA gene sequencing indicated that the Bact-I was closely related to *Bacillus subtilis.* Optimization of xylanase enzyme production showed that *Bacillus subtilis* was able to produce xylanase enzymes when grown in a culture medium containing 2% (w/v) corn stover and 0.6% (w/v) yeast extract at pH 10 and 37 °C. The xylanase gene was cloned and characterized. The result revealed that the xylanase gene of *Bacillus subtilis* was homology to the β-1,4 endo-xylanase gene.
Conclusion, significance and impact of study: A xylanase producing *Bacillus subtilis* was isolated from the intestinal tract of a cabbage looper (*Trichoplusia ni* (Hübner)) larvae. Optimization and evaluation of the xylanase activity of *Bacillus subtilis* indicated that it could be useful for xylanase production or as a probiotic for improving animal feed stuff.

Keywords: Xylanase, *Bacillus subtilis, Trichoplusia ni*, intestinal tract

INTRODUCTION

Xylan compounds such as lignocellulosic biomass are the most abundant renewable organic materials found in agriculture (Kayserilioglu *et al.*, 2003; Faik, 2010). They have complex structures, which consist of substituent groups of acetyl l, 4-O-methyl-D-glucuronosyl and L-arabinofuranosyl residues, linked to the backbone of β-1,4-linked xylopyranosyl units (Bastawde, 1992). However, the utilization of agricultural waste requires improvement processes, such as the digestion of hemicellulosic enzymes prior to use. The enzyme used for xylan degradation is xylanase. This enzyme is widely used in many facets of the food and beverage industry, animal feed improvement and also for the bioethanol production industry (Beg *et al.*, 2001; Polizeli *et al.*, 2005).

Almost all of the xylanase used in industry is produced from microorganisms. Therefore, several xylanase producing microorganisms have been isolated from various sources, such as natural water, soil and the intestinal contents of various ruminant species, as well as assorted insect species (Cordeiro *et al.*, 2002; Ninawe *et al.*, 2006; Menon *et al.*, 2010; Subramaniyan, 2012). In contrast to mammals, insect larvae such as that of the cabbage looper, the instar larva of the *T. ni* (Hübner), are fascinating because they feed on plant leaves. The fact that they have a very short digestive tract, when compared to ruminants, which enables them to eat and digest plant matter with efficiency, is also of interest. Presumably, this may be due to the microbial flora within the gut of the butterfly larva producing the most powerful cellulolytic enzymes, including xylanase, which help in the digestion of fibrous compounds. Therefore, we conducted a study to isolate and identify the xylanase producing bacteria from the gut of *T. ni* (Hübner).

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MATERIALS AND METHODS

Samples and microorganism isolation

The fourth or fifth instar larvae of *T. ni* (Hübner) were collected from a cabbage. Five to six instar larvae (approximately 5 g of intestine) were used in this study. The surface area of the instar larva was sterilized twice with 70% (v/v) ethanol for 5 min and then treated once with 3% (v/v) sodium hypochlorite. Finally, the instar larva was washed twice in sterile normal saline and then allowed to air dry in a Class-II biosafety cabinet. The digestive tract was aseptically removed from the larva with caution. The digestive tracts were chopped and homogenized in a 15 mL centrifuge tube using a sterile stirring rod. The homogenate was serially diluted 10^{-4}-10^{-6} times with sterile normal saline before being spread onto duplicate tryptic soy agar plates (TSA: Himedia; India). The plates were then incubated separately at 37 °C for 18 to 24 h. Every different colony was selected to test for gram strain and xylanase production (Cordeiro *et al.*, 2002).

Screening for xylanase production

Screening for xylanase production was performed on xylan agar (composition in 1 L: birch wood xylan 2.5 g, yeast extract 5 g, peptone 5 g, $MgSO_4$ 0.2 g, K_2HPO_4 1 g and agar 15 g) (Sanghi *et al*., 2008). Approximately 5 µL of each overnight culture from Tryptic Soy Broth (TSB: Himedia; India) was dropped onto xylan agar and incubated at 37 °C for 18 to 24 h. In order to test for enzyme production, the plate was stained by overlaying with an iodine solution (2% w/v of iodine, 3% w/v potassium iodide mixed in distilled water) for 1-2 min. Xylanase production was observed in a clear zone around the colony. Only those isolates that produced a clear zone were selected for further observation (Cordeiro *et al.*, 2002).

Biochemical and molecular identification by 16S rDNA gene sequencing analysis

The biochemical properties of the isolate were characterized by HiBacillus^{Tm} identification kit (Himedia; India). The biochemical characteristics were identified according to standard protocols (Himedia, 2015). For molecular identification, bacterial isolates were grown on tryptic soy agar (TSA: Himedia; India) at 37 °C for 24 h. Then, 2-3 colonies of bacteria were collected in 200 µL of TE buffer. The bacterial cell was vortex mixed and boiled for 10 min. Extracted cells were stored at −20 °C until further analysis was conducted. Universal 16S rRNA primers Bact-0341 5'-CCTACGGGNGGCWGCAG-3' and Bact-0785 5'-GACTACHVGGGTATCTAATCC-3' were used in this study (Klindworth *et al.*, 2013). A polymerase chain reaction (PCR) was performed in a thermocycler (CC590 Bio-Rad, USA). Each reaction mixture (25 µL) contained 1× buffer, 4 mM $MgCl_2$, 50 pmole of each primer, 0.2 mM of each dNTP, 1.25 U of Taq DNA polymerase (*iTaq*; Bio-Rad) and 25 ng of template DNA. The amplifications were performed as follow: initial denaturation for 10 min at 94 °C, 40 cycles each of denaturation for 50 sec at 94 °C, annealing for 50 sec at 56 °C, and primer extension for 1 min at 72 °C. A final extension was performed for 10 min at 72 °C. The PCR products were analyzed by gel electrophoresis in 1.2% (w/v) agarose gels and stained with ethidium bromide (10 mg/mL). PCR products were purified using a gel extraction kit (Spinclean^{TM} PCR purification kit). Sequences were aligned against those found in the NCBI database by Bioedit and MEGA 6 program.

Cloning of the xylanase gene

Bacterial DNA was subjected to amplification of the xylanase gene using specific primers derived from previous report: xynF: 5'-ACGAATTCCATGTTTAAGTTTAAAAAGAATTTCTTAGTT-3', xynR: 5'-GAGGATCCTTACCACACTGTTACGTTAGACTTCCACT-3' (Huang *et al.*, 2006). The PCR reaction mixtures contained 1× of Taq reaction buffer, 0.2 mM dNTPs, 100 ng of genomic DNA, and 1 U of DNA Taq polymerase (*iTaq*; Bio-Rad) in a final volume of 50 µL. Amplification reactions were performed as described, with initial denaturation at 94 °C for 10 min, 40 cycles each of denaturation at 94 °C for 1 min followed by annealing at 55 °C for 1 min and primer extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The amplicons were analyzed by gel electrophoresis. The PCR product was purified before insertion into the plasmid pTZ57R/T (Isn TAclone Kit; Thermo Scientific: USA) and followed by transformation into *Escherichia coli* (DH5-α). The plasmid was extracted and purified before analyzing the nucleotide sequence. The amplified gene sequence was analyzed by Biobasic (Canada). Sequence alignment was employed using BLAST software from the GenBank. Alignment of the sequences determined was performed with a Bioedit program.

Enzymatic activity assay

Total xylanase activity in the culture media was determined with reducing sugars by dinitro salicylic acid (DNS) method (Miller, 1959). In brief, bacteria was grown in 50 mL of nutrient broth (NB: Himedia; India) at 37 °C for 48 h. Then, 10 mL of inoculum was added to the xylan solution (0.2% w/v birch wood xylan in sterile distilled water) and incubated at 37 °C for 48 h. The supernatant was collected by centrifugation at 10,000 rpm at 4 °C for 10 min. After that, 0.5 mL of 1% (w/v) xylan solution, 0.5 mL of crude enzyme and 0.5 mL of 0.1 M sodium phosphate buffer pH 7 were mixed together and incubated for 10 min at 50 °C. Subsequently, 1 mL of DNS solution was added to the tube. After mixing thoroughly, samples were boiled for 15 min and immediately cooled in ice water for stabilization of color. The reactions were added to 2 mL of distilled water prior to measurement with an absorbance at 540 nm using a spectrophotometer (ThermoSpectronic, USA) (Cordeiro *et*

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al., 2002). The standard curve of xylose was used to determine xylanase activity. One unit of xylanase activity is defined as the amount of enzyme releasing 1 µmol of reducing sugar equivalent to xylose per minute.

Optimization of xylanase production

The optimization of xylanase production was carried out based on stepwise modification of the various parameters for the growth of bacteria. For carbon source utilization, various concentrations (0.5, 1, 1.5 and 2%) of rice straw, corn stover and corn cob were used as carbon sources in the production medium (composition in 1 L: yeast extract 5 g, peptone 5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, K_2HPO_4 0.4 g pH 7) (Sanghi *et al*., 2008). The optimization of nitrogen source was carried out with various concentrations (0.2, 0.3, 0.4, 0.5 and 0.6%) of yeast extract, peptone, ammonium sulphate and urea in the production medium containing the optimal carbon source. The effect of pH was performed by varying the pH from 6 to 10 in the production medium containing the optimal carbon and nitrogen sources. The effect of cultivation temperature on the growth and enzyme production was examined at 30, 37 and 40 °C.

Statistical analysis

The results of enzyme production in each optimized condition were analyzed by GraphPad Prism program (V 5.0). All statements of significance were calculated using one-way ANOVA method, based on a confidence level of 95%.

RESULTS AND DISCUSSION

Isolation of xylanase producing bacteria

In this study, bacterial samples were isolated from the intestinal tract of *T. ni* (Hübner) larvae. Only different bacterial colonies were selected to screen for xylanase production in a xylan medium. Altogether, 6 different bacterial colonies were isolated (Bact-I, Bact-II, Bact-III, Bact-IV, Bact-V and Bact-VI). However, only 1 isolate (Bact-I) was found to be positive for xylanase production by screening agar (Figure 1).

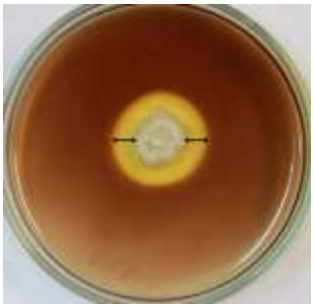

Figure 1: Hydrolysis zone produced by *Bacillus subtilis* (Bact-I) on xylan agar.

Identification of xylanase producing bacteria

Morphology and Gram strain indicated that the Bact-I isolate was Gram positive for bacilli. The preliminary results of morphological and biochemical characteristics suggested that this isolate closely related to *Bacillus subtilis.* The Bact-I isolate was further confirmed by 16S rRNA gene sequencing. The nucleotides blast against the GenBank database demonstrated a consistent result, which indicated that this strain had 98% similarity to *B. subtilis* (Table 1 and Figure 2).

Table 1: Biochemical characteristic tested for the isolate Bact-I (HiBacillus^{Tm} identification kit).

Biochemical reactions	Bact-I
Malonate	-
VogesProskauer's	+
Citrate	+
ONPG	+
Nitrate reduction	+
Catalase	+
Arginine	-
Sucrose	+
Manital	+
Glucose	+
Arabinose	+
Trehalose	+

Bacillus spp. is a spore-forming Gram positive bacteria. These bacteria are normally found in environments including the intestinal tract of humans and various animals. It is not a pathogenic strain in animals. This result is according to previous reports that have described the isolation of bacteria from the digestive tract of different insects indicating that *Bacillus* spp. was the main genus found in insect intestines (Gusmão *et al.*, 2007; Anand *et al.*, 2010). Since these results were from single-sampling experiments, we were unable to determine whether *B. subtilis* is a normal flora bacteria or just a temporary colonization in the insect larva. However, the ability to produce xylanase enzyme probably implies that this strain plays an important role in the digestion of hemicellulose compounds in the intestines of the *T. ni* (Hübner) larvae.

Optimization of xylanase production

To optimize the conditions for xylanase production from *B. subtilis* (Bact-I), various types of hemicellullose (rice straw, corn stover and corn cob), nitrogen sources (yeast extract, peptone, ammonium sulphate and urea), pH and temperature were used to create the optimal conditions for xylanase production. It was found that *B. subtilis* (Bact-I) produced the highest level of xylanase with statistical significance (p<0.001) when cultured in a medium composed of 2% (w/v) corn stover, which produced enzyme activity equal to 1.5 U/mL at 24 h. For optimization of the nitrogen sources, 2% of corn stover was used as a carbon source in the culture medium

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containing various types of nitrogen sources. The results found that yeast extract at 0.6% was able to induce the highest level of xylanase production, which produced enzyme activity equal to 1.2 U/mL at 12 h (p<0.001). The best suitable pH to produce xylanase was 10, with the optimal temperature for growth of bacteria at 37 °C (Figure 3).

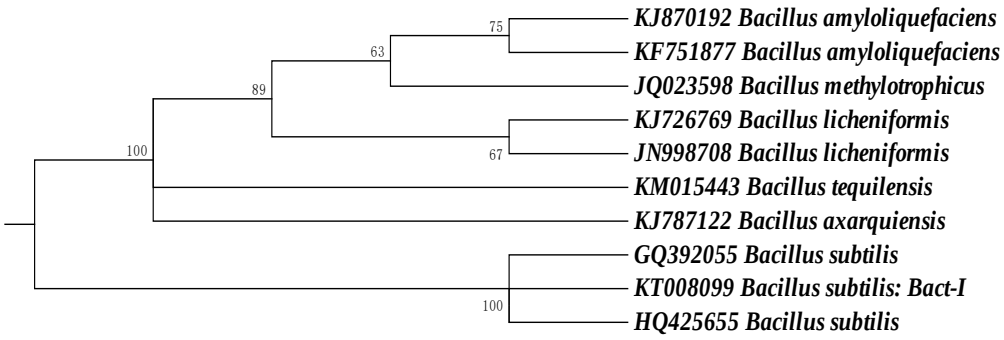

Figure 2: A. Nucleotides sequence analysis of 16S rRNA gene (accession number: KT008099) of *Bacillus subtilis* (Bact-I) against sequence retrieved from the GenBank database. The phylogenetic tree was performed with a Bioedit and MEGA 6 program (Neighbor Joining: 1,000 bootstrap).

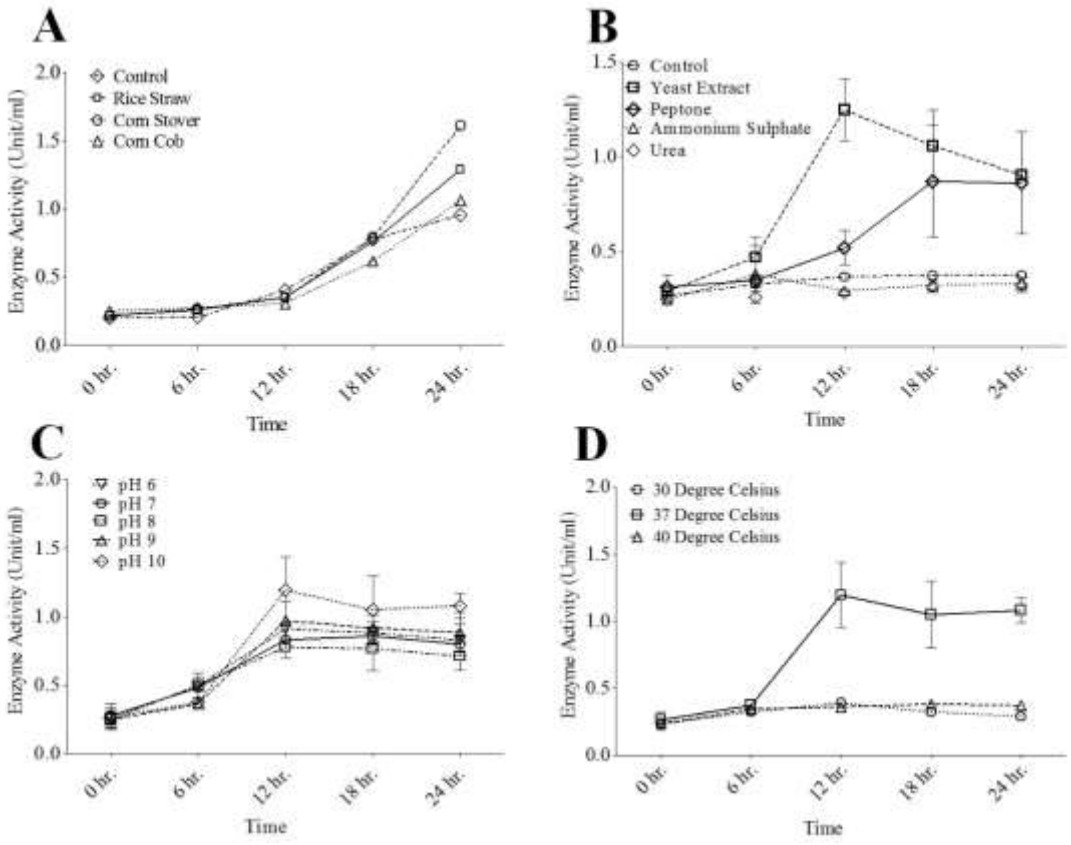

Figure 3: Enzyme activity of *B. subtilis* (Bact-I) cultured in various condition. A, the enzyme activity observed from various carbon sources; B, the enzyme activity observed from 2% of corn stover and various nitrogen sources; C, the enzyme activity observed from 2% of corn stover, 0.6% of yeast extract and various pH conditions; D, the enzyme activity observed from 2% of corn stover, 0.6% of yeast extract at pH 10 and various growth temperature. All experiments were done in duplicate and showed as mean and SD. ** Statistical analysis significantly (p< 0.001).

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Xylanase activity indicated that the *B. subtilis* (Bact-I) is a potential strain when compared to other xylanase-producing *Bacillus* spp. from previous reports in Thailand, which displayed circa 1.6 U/mL (Leartslarus *et al.*, 2002). Interestingly, our results indicated that the xylanase from *B. subtilis* (Bact-I) is an alkaline and probably a thermotolerant xylanase. This is because the optimal pH and temperature of xylanase function were 10 and 50 °C, respectively. Optimization by varying the carbon sources indicated that corn stover was the best carbon source to induce xylanase production. This was consistent with previous studies, which showed that corn stover was the most suitable substrate for the production of xylanase from microorganisms (Leartslarus *et al.*, 2002; Selig *et al.*, 2008; Ghori *et al.*, 2011). This may be due to the fact that corn stover contains sufficient xylan and more than the other hemicelluloses used in this study.

Xylanase gene cloning and nucleotides analysis

The PCR used specific primers for the xylanase gene, which resulted in amplicons of approximately 600 bp. The amplicon was inserted into a plasmid pTZ57R/T TA-cloning vector and transformed into *E. coli* DH5-α. The partial nucleotide sequence of the insert was determined in one direction and one open reading frame of 600 bp encoding protein comprised of 181 amino acid residues. The nucleotide blast against the sequence from the GenBank database indicated that this xylanase gene is 100% identical to the β-1,4 endo-xylanase gene (EU233656) (Figure 4). According to previous reports, this indicated that most of the bacteria produced endo-xylanase enzymes other than exo-xylanase enzymes (Anand *et al.*, 2013; Bhalla *et al.*, 2014; Sheng *et al.*, 2014).

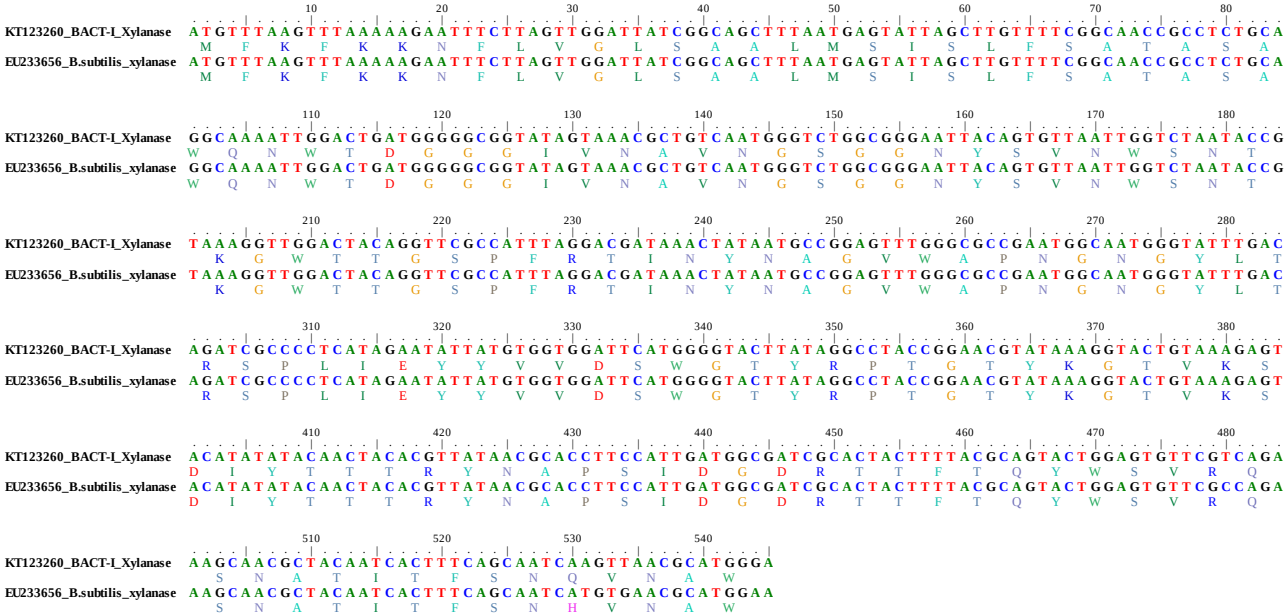

Figure 4: Nucleotide and deduced amino acid sequences of the xylanase gene (accession number: KT123260) from *Bacillus subtilis* (Bact-I) against with the nucleotide sequence of β-1,4 endo-xylanase gene retrieved form GenBank (EU233656).

For the preliminary experiment in this study, bacterial samples were isolated from the gut of *T. ni* (Hübner) larvae. Only different bacterial colonies were selected to screen for xylanase production in a xylan medium. Although we selected different colonies, only one isolate was found to be positive for xylanase production in our observations. For reasons of application, anaerobic bacteria are more difficult to cultivate and manipulate than aerobic bacteria. Thus, we focused only on aerobic bacteria which are able to grow at 37 °C. This temperature may not be suitable for anaerobic bacteria or certain species of insects, which usually grow at between 27-28 °C (Zhao *et al.*, 2012). This is based on a recent study that showed the majority of microflora in insect intestines are anaerobic bacteria (Yun *et al.*, 2014).

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