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Research Article

PRODUCTION AND EVALUATION OF EXTRACELLULAR XYLANASE FROM GUT MICROBES OF DRY-WOOD TERMITES: PAPER INDUSTRY

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ABSTRACT

Termites play a major role in tropical and subtropical ecosystems. They depend on plants debris and decaying wood for nutrition and helps in recycling the nutrition back to the ecosystem. Since the wood consist of various types of hemicelluloses compounds which highly varies in structure and properties, there is a need for a wide range of digestive enzymes and the termites cannot produce all the enzymes. They are dependent on the symbiotic microbes for the complete digestion of the consumed wood matter. These microbes found in the guts are able to synthesis enzymes, which are good both in quality and quantity. In this study the microbes capable of producing xylanase enzyme was screened and the most potential strain (zone of clearance 2.1cm) was isolated by Congo red dye staining. Such microbe was identified as actinomycetes by using 16s rRNA sequencing and this sequence was searched for similar matches using multiple sequence alignment in BLAST tool. The actinomycetes species was identified as *Streptomyces avidinii* and it was further used for the production of the enzyme. The optimum enzyme activity was observed in sixth day culture with a concentration of 0.5% Birchwood Xylan. The produced enzyme was then partially purified by using Ammonium sulfate precipitation followed by dialysis. The pulp bleaching ability of the enzyme was calculated from the treatment with the enzyme for a time period of 120 minutes there is a significant reduction in kappa number from 67.66 to 34.58. This passes to apply xylanase enzyme in the paper industry instead of chemicals for bleaching.

Keywords: *Streptomyces*, Actinomycetes, Xylanase, Termite gut microbe, Paper industry

INTRODUCTION

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the endohydrolysis of 1,4-b-D-xylosidic linkages in Xylan and produce xylose[1]. A number of Xylanases have been sublimated from a broad diversity of microorganisms such as *Bacillus* sp., *Clostridium* sp., *Streptomyces* sp., *Aspergillus* sp. *and Trichoderma sp*.[2]. There are many number of extremophilic xylanases have been isolated, especially thermophiles, alkaliphiles and acidiphiles, while little attention has been paid to cold-adapted xylanases for the industrial demand[3]. Xylan is present in the secondary cell wall of trees and it forms an interface between lignin and other hemicelluloses. It is one of the most abundant polysaccharide in nature[4]. It is present both in hardwoods and softwoods and account for 20-35% of the total dry weight in tropical plants and 8% in temperate soft woods[5].

Xylan hydrolysis is a key element in insect nutrition. The major diet of termites consists of wood matter and digestion of the structural polysaccharides like cellulose and other hemicelluloses is essential for energy metabolism[6]. These bio-organic compounds are resistant to degradation and the termites themselves cannot secrete all of the digestive enzymes to take down all the polysaccharides. They require the assistance of the enzymes produced by the microbial symbionts[7]. The termite gut is a differentiated organ which plays as a habitat for a complex microbiota comprising both resident and transient members from protozoa, bacteria and archea genera. Many of them are symbionts that contribute to carbon metabolism, they also aid in the nitrogen cycle and prevention of pathogenic infections[8]. The general aim of the termite gut and morphological variations within different taxa and working groups, especially those of the mid-gut and hid-gut regions with emphasis along the intestine as a director accommodating diverse organisms, many of which are as yet uncultured[9, 10].

Actinomycetes have been commercially used for the production of enormous quantities of pharmaceuticals, neutraceuticals, enzymes, antitumor agents, enzyme inhibitors, and assorted other useful compounds. Actinomycetes, a subfamily of bacteria make up a substantial portion of the microbial population in most territories and can produce extracellular enzymes which can decompose a wide range of materials. Their enzymes are more attractive than enzymes from other authors because of their high stability and unusual substrate specificity[11]. Actinomycetes found in extreme habitats produce potential enzymes with huge commercial value. Furthermore, xylanases from actinomycetes are stable on kraft pulps and can be applied in the crude form thereby making the process economical. High thermostability and specific activity, two desirable properties of enzymes to be employed in industrial processes, are reported in xylanases from *Actinomadura sp*. FC7 and *Nonomuraea flexuosa*[12]. Similarly, fused xylanases from fungi and actinomycetes have been used in the paper and pulp industries for significant bio-bleaching, due to high thermal and pH stability[13]. In the present study Actinomycete was isolated from the termite gut for Xylanase production.

MATERIALS AND METHODS

Sample collection

Matured termites (soldier and worker) have been picked up from bamboo wood infestations in the Garden of K. S. Rangasamy College of Technology, Tiruchengode, Tamil Nadu. The termites were trapped in ventilated plastic boxes and were preserved alive in a cool dark place until dissection. The collected termite's skin surface was then washed with 70% ethanol to remove all the dust. Under aseptic condition the abdomen part was then pierced openings to isolate the gut using sterile lancets with the aid of a magnifying glass.

Screening of Xylanolytic microbes

The colonies were screened in two different media each using peptone and ammonium sulfate as a sole nitrogen source and 1% of birch wood Xylan (Himedia) as a carbon source. The plates were then inoculated both by spread plate method and termite gut streaking and incubated at room temperature for 48 Hours[14].

Secondary screening

The pure culture of microbes was obtained by continuous and quadrant streaking techniques. The colonies observed in the primary screening plates were inoculated in nutrient agar media added with 5 g/l of birch wood Xylan (Himedia). The plates were then incubated for 48 Hours at room temperature without any disturbances[15].

Congo red and Gram staining

The plates were then stained with Congo red solution [0.5% (w/v) Congo red and 5% (v/v) ethanol in distilled water] for 15 minutes and destined with 1M NaCl. The enzyme producing ability of the microbes was qualitatively assessed by watching over the zone of clearance around the settlements. The zone of clearance was measured using a graded scale with the avail of the colony counter[16]. Gram's staining was performed to differentiate the Actinomycetes from bacteria.

Organism identification

The partial 16S rRNA gene was sequenced for the new isolate and aligned with sequences from NCBI server using BLAST tool. Multiple sequence alignment was performed for top 10 similar sequences using the clustalW2 program to place the organism[17].

Sequencing protocol

One-pass sequencing was done on each template using the 16S rRNA universal primers (Table 1). The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). Sequence data was aligned and analyzed for identifying the sample.

Enzyme production

The extracellular enzyme was synthesized by submerged fermentation in 500ml Erlenmeyer flasks incubated at 35°C in orbital shaker at 150 rpm for 144 hours. The culture was inoculated in nutrient broth media supplemented with 0.25% and 0.5% Xylan (Himedia) at pH 8.0 for xylanase production. The xylanase activity was measured at different time intervals[18] to ascertain the optimum enzyme production time[19].

Xylanase assay

Xylanase activity was evaluated[18] using 1% (w/v) of Xylan as a substratum. Three ml of reaction mixture having one ml of crude enzyme, 1% Xylan substrate and McIlvaine buffer. The mix was incubated at 50 °C for 30 minutes and the reaction was stopped by adding three ml of Dinitrosalysilic acid (DNS reagent). After heating for 5 min in a boiling water bath and the absorbance was noted at 550 nm. Xylanase activity was shown in terms of international units (IU). One IU was the amount of enzyme required to release the 1µl xylose equivalent in one ml of the enzyme in one minute.

Standard curve construction

A different concentration of 0.1% xylose was taken and diluted to 1 ml with distilled water. One ml of McIlvaine buffer at pH 5 was mixed and finally 1ml of Distilled water was added. Three ml of DNS Reagent was added and placed in boiling water bath for 15minutes. A blank was also run in separate flask. After boiling the flasks were cooled to room temperature and the absorbance was measured at 550 nm[18].

RESULTS AND DISCUSSION

Screeing of xylanolytic microbes

Xylanase from the bamboo manifesting termite (soldier and worker) was illustrated in Figure 1. Finely crushed termite gut was cultured in the media as mentioned in the section of screening of enzyme producing microbe. After incubation the microbial colonies were discovered where the only nitrogen source for the microbial growth as peptone added media had the number of colonies of 3 fungi, 3 bacteria and 2 actinomycetes and ammonium sulfate as a sole nitrogen source added media had the sum of 3 fungi, 2 bacteria and 1 actinomycete colonies.

Congo red staining of pure cultures

For the isolated pure cultures of bacteria, fungi and actinomycetes, congo red dye staining was performed to determine its xylanase producing ability. The size of the zone of clearance formed around the colonies was used as a criterion to determine its enzyme producing ability. Among the different isolates the Actinomycetes colony was found to possess good Xylan degrading ability and was applied for further studies (Figure 2). *Streptomyces cyaneus*, *Streptomyces tendae* and *Streptomyces caelestis* have been identified as the xylanase producing microbes using congo red stain[16].
As mentioned in table 2 the zone of clearence for the isolated fungi, bacteria and actinomycetes have been measured.

Morphology and staining of actinomycetes

The morphological characteristics of the actinomycetes colony were observed as spongy and gray in color (Figure 3a) and suggested as *Streptomyces*, the biochemical characteristic as Gram's staining and Catalase test was performed. It was found to be filamentous and Gram positive (Figure 3b) in nature and also showed negative for catalase test. This confirmed that it was actinomycetes species as the majority of actinomycetes are Gram positive and catalase test negative in nature[20].

Table 1 Details of forward and reverse primer

Primer	Sequence	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

Table 2: Zone of clearance observed by Congo red dye screening

Organism	Zone of Clearance (cm)
Actinomycetes (grey)	2.1
Fungi (Black)	1.2
Fungi (Green)	0.9
Fungi (White)	0.7
Bacteria (Transparent)	0.5
Bacteria (yellow)	0.2

Table 3 Calculation of kappa number in xylanase treated wood pulp

Treatment time (min)	Kappa number
0	67.66
30	50.98
60	49.51
90	39.4659
120	34.5853

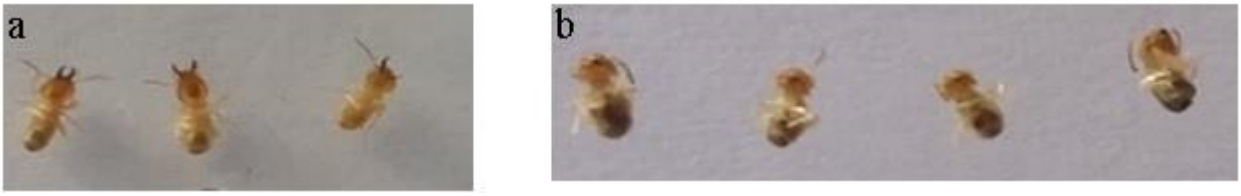

Figure. 1. Bamboo manifesting Termites for the isolation of xylanase producing microbes. a) Soldier termites b) Worker termites

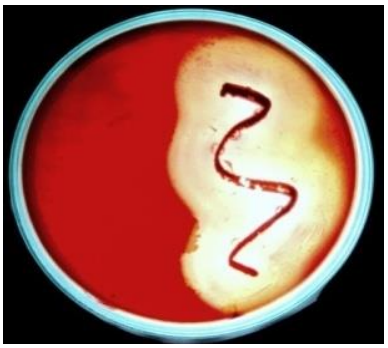

Figure. 2. Zone of clearance around the actinomycetes colony

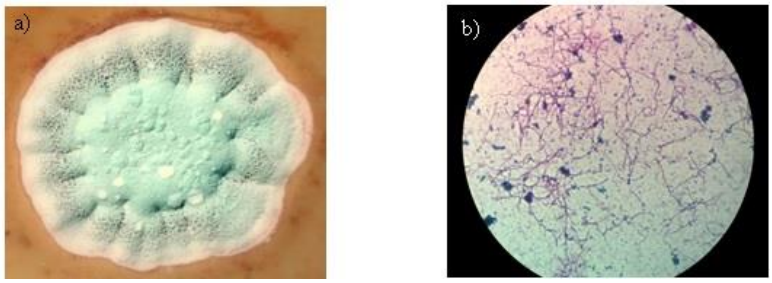

Figure. 3 Characterization of the actinomycete. a) Naked eye view and b) Microscopy view (Gram stained Actinomycetes -100x).

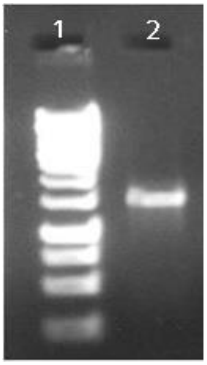

Figure 4 Electrophoresis of Amplified PCR Product. Lane 1: Ladder DNA; Lane 2: PCR Amplified Gene fragment.

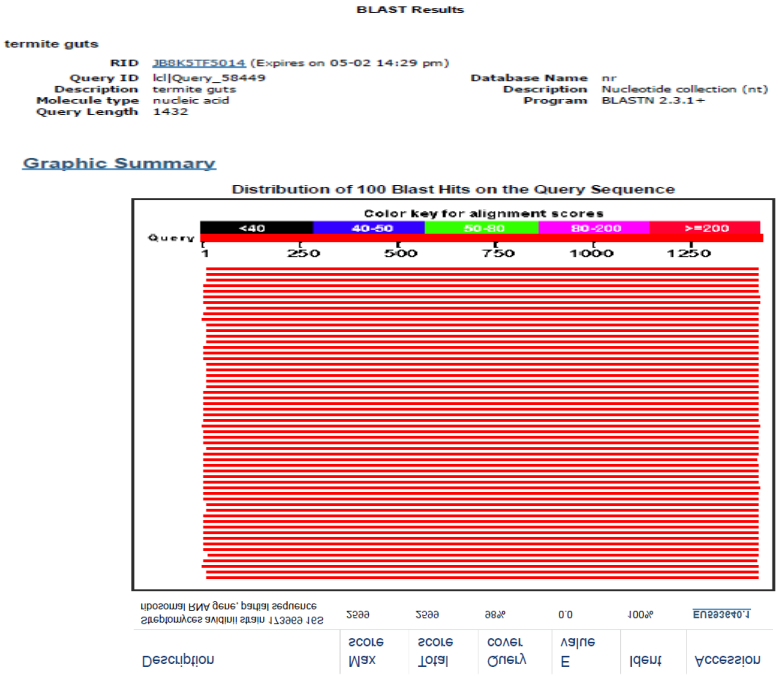

Figure. 5 Sequence alignment of new isolate

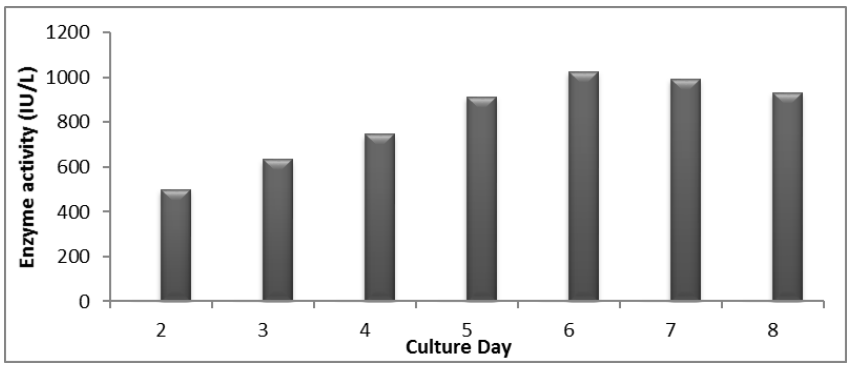

Figure. 6 Effect of incubation time (as day) on enzyme production

Molecular identification of Actinomycetes

The genomic DNA of the actinomycetes was isolated using InstaGene™ Matrix Genomic DNA isolation kit. 16S rRNA gene fragment was amplified using Universal primers (MJ Research Peltier Thermal Cycler). The reaction was included with a positive control and a negative control. The PCR Product was purified to remove unincorporated primers and dNTPs by using the Montage PCR Cleanup Kit (Millipore). The purified DNA fragment ranging about 1,400bp (Figure 4) was sequenced using the same 27F/1492R primers in ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The fluorescent-labeled fragments were subjected to electrophoresis in an ABI 3730XL Sequencer (Applied Biosystems). The obtained sequence was aligned using BLAST (Figure 5) with the sequence available at NCBI data base. From the aligned sequences, the top 10 sequences were downloaded and run with the Clustal W2 program. It discloses that the unknown isolate have 100% sequence identity with *Streptomyces avidini* strain 173969.

Production and concentration of Xylanae

The culture broth was measured for xylanase activity using Bailey *et al.* method[18] in regular time intervals to find out the peak production of the enzyme. The maximum activity of the enzyme was observed at sixth day culture with xylan concentration of 0.5% (3317 IU/L). The culture broth was centrifuged and xylanase was precipitated with ammonium sulfate. The precipitate was resuspended in phosphate buffer (pH 7.0) and dialyzed against the same buffer for overnight at 4°C. The activity of the enzyme was found to be higher in 0-40% ammonium sulfate saturation (4517 IU/L) than in 40-80% ammonium sulfate saturation (3365 IU/L). The enzyme activity was significantly higher when compared to earlier studies[19].

Pulp bleaching ability of the enzyme

The kappa number is the volume (in milliliters) of 0.1N potassium permanganate solution consumed by one gram of moisture-free pulp. The kappa number of the pulp was measured by using TAPPI industrial Standards. It is practiced to set the comparative hardness, bleaching ability and degree of

delignification of pulp. The undamaged fibers have been delivered from the Bamboo wood pulp and its kappa number was calculated as 67.66. The dried pulp (0.1gram) has treated with the xylanase enzyme at 55°C for different time intervals (Table 3).

It has been a significant reduction in Kappa number (67.66 to 34.58) of enzyme treated fibers. This confirms the ability of the enzyme to bleach and delignify the wood pulp and increase its whiteness. With further optimization of the pretreatment process, the Xylanase enzyme can be utilized as an alternative to toxic chemical agents used as a bleaching agent.

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