WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 7, 1146-1156. Research Article ISSN 2277– 7105

ISOLATION AND IDENTIFICATION OF XYLANASE PRODUCING MICROORGANISMS FROM SOIL

Rajakumar R.* and Rameshwara Reddy Naru

Post graduate and Research Department of Biotechnology, Marudupandiyar College, Thanjavur-613 403, Tamil Nadu, India.

Article Received on
10 Feb. 2018,

Revised on 02 Mar. 2018,
Accepted on 23 Mar. 2018,

DOI: 10.20959/wjpr20187-11655

***Corresponding Author**
Rajakumar R.
Post graduate and Research Department of Biotechnology, Marudupandiyar College, Thanjavur-613 403, Tamil Nadu, India.

ABSTRACT

The xylanase enzyme produced by microorganisms, can be used for food and non-food industries. Such as paper bleaching, waste processing, animal feed and area of food industry such as for juice purification, bread making, producing xylitol sugar and ethanol. In the present study investigated that the four bacterial and six fungal species were isolated from soil. Among the potent xylanse producers, *Bacillus amyloliquefaciens* and *Aspergillus niger* showed the highest xylanolytic activity (4.2 U/ml and 3.5 U/ml) at 48 hr after incubation. Temperature and pH are the important parameters that determine the enzyme activity. A high level of xylanase activity was obtained in the culture medium with optimum temperature at 37 °C at 48 hr (4.2 U/ml) in *B. amyloliquefaciens* and 45°C at 48 hr (3.5U/ml) in *A. niger*. The pH ranging from 5 to 10 was studied for the detection of optimum pH with high xylanase production by *A.niger*. The optimum pH found to be pH 8 (3.7 U/ml) and minimum was noted at pH 5 (2.2 U/ml).

KEYWORDS: Temperature, pH, Xylanase and *Aspergillus niger*.

INTRODUCTION

One of the major characteristics of all bacteria is their ability to produce and secrete a range of enzymes for the degradation of various organic compounds. Most of the screening procedures devised for the detection of xylanolytic microorganisms involve the enrichment technique. As we know that the required organism is accompanied by many unwanted species, so it is necessary to enrich such organisms. This is generally done by using enrichment technique. In this method, a specific environment is selected and a sample is

inoculated. The sample is incubated for one or more weeks and an inoculum is then transferred to a new container of the same selected environment. This is further incubated until the organism of choice appears. This may require few or many transfers. To isolate alkaliphiles alkaline medium must be used. Sodium carbonate or sodium hydroxide is generally used to adjust the pH, because alkaliphiles usually require at least some sodium ions.

Xylanase constitute up to 35% of the total dry weight of higher plant and is built from homopolymeric backbone chain of 1, 4-linked β-D-xylopyranose units, including short chains Oacetyl, α-L-arabinofuranosyl and D-glucuronyl or O-methyl- D-glucuronyl residues. The degradation of lignocellulosic materials by hydrolyzing xylan is an important step for nature. Chemical hydrolysis of xylan by the industries is accompanied with the formulation of toxic components which is hazardous to environment. The use of site specific microbial enzymes for xylan hydrolysis makes the process eco-friendly. Xylanases, catalyze the hydrolysis of 1, 4-β-D-xylosidic linkages in xylan. They are characterized as exo- and endo-xylanases and release xylo-oligosaccharides and xylose. Initially the main hydrolysis products are β-D-xylopyranosyl oligomer, but at later stage, small molecules such as mono-, di- and trisaccharides of β-D-xylopyranosyl may be produced (Kuhad *et al.*, 1997).

Xylanases occur widely in bacteria, yeasts and fungi. Many microorganisms are known to produce different types of xylanases, the nature of the enzymes varies between different organisms. Many reports on xylanases from *Clostridium sp* (Berenger *et al.*, 1985), *Streptomyces sp* (Sreenath and Joseph, 1982), and other microorganism are available. A *Bacillus sp* an alkaliphile isolated from an alkaline soda lake reported by Amare Gessesse (1998). An alkaline bacterium, also *Bacillus sp*, produces alkali stable xylanases without cellulose and one of them has a strong affinity for insoluble xylan. Xylanase have important applications in the pulp and paper industry (Viikari *et al.*, 1994). They can also be used to increase the digestibility of animal feed stock and in the baking and brewing industries (Linko *et al.*, 1989).

Xylanase may also be used to prepare materials for scientific research, besides being research materials themselves. Well characterized xylanases may be useful for the characterization of polysaccharides and plant cell walls. Although many bacteria have been studied for xylanase production are alkaliphiles (Nakamura *et al*., 1995) only very few produced xylanases in neutral medium (Panbangred *et al*., 1983). Xylanases have considerable potential in several

biotechnological applications. Commercial applications suggested for xylanases involve the conversion of xylan, which is present in wastes from agricultural and food industry into xylose. Similarly, xylanases could be used for the clarification of juices, for the extraction of coffee, plant oils and starch and for the production of fuel and chemical feedstock (Linko *et al.,* 1989). Recently, the use of xylanolytic enzymes in pulp bleaching has considered as one of the most important new biotechnological applications of these enzymes. This study investigated that the isolation and identification of xylanase producing microorganisms from soil.

MATERIALS AND METHODS

Collection of soil sample

The soil sample was collected near farm land at Thanjavur, Tamil Nadu and India. The soil was collected in sterile container and stored at 4 °C until used.

Isolation of Microorganisms

One gram of soil sample was suspended in 10 mL sterile distilled water and serial dilutions were made. The 50 µL of the diluted sample (10^{-6}) was spread on nutrient agar plates and Czapek Dox agar plates. Pure cultures of individual isolates were identified by conventional methods (Gilman, 1959; Ellis, 1971 and Barnett *et al* 1972). The composition of the medium was (g.L-1): Xylan (Hi media), 0.1%; peptone, 5.0; yeast extract, 5.0; K2HPO4, 1.0; MgSO4.7H2O, 0.2 and agar, 20.0. The inoculated plates were incubated for 7 days at 30ºC. Plates were flooded with 0.1% (w/v) Congo red after 30 min, washed with 1 M NaCl and observed for zone of clearance around the bacterial and fungal growth. The clearing zones around their colonies were selected for further studies.

Identification of the bacterial isolate

The xylanase producing microorganisms were identified on the basis of their physiological, morphological and biochemical characteristics in our laboratory using *Bergey's* Manual of Systematic Bacteriology guidelines. The strain was stored and maintained on nutrient agar slants at 4°C. The various type of media used in the study were sterilized at 1.05 kg/cm2 for 20 min before utilized in the process.

Identification of the fungal isolate

Fungal identification was done based on structural characters and colony morphology observed under light microscope. Colour, texture and pigmentation of the isolates were

recorded. Mount prepared was stained with lacto phenol cotton blue. The fungal characteristics were described and identified based on the description given by Pitt and Samson (2000).

Xylanase production by Microorganisms

Bacteria

The selected bacterial isolates were incubated in medium for 18 h up to 24 h at 37 °C at 150 rpm. Then each strain with 0.1 OD was transferred to 250ml Erlenmeyer flasks containing 30ml liquid medium and incubated at 37°C for 4 days on rotary shaker (150 rpm). A 2 ml sample of culture media was removed every 24h and centrifuged at 10000 g for 10 min at 4 °C then, the supernatant were collected and used for extracellular enzyme assay. For obtaining the intercellular fraction of the xylanase, the cell pellet was washed three times with sodium phosphate buffer (pH7). After centrifugation at 10000 g for 10 min, the pellet was resuspended in the same buffer and sonicated using an ultrasonic cell disrupter in order to disperse bacterial aggregates. Then, the cell debris was removed by centrifugation at 10000×g for 10 min. The cell–free supernatant was used for intercellular enzyme assay.

Fungi

The fungal isolate which showed maximum zone consistently on the screening media was selected for the production of xylanase. The spore suspension was prepared by adding spore suspension of fungal growth from the 7 day incubated SDA plate in to 50 ml of sterile distilled water. The tube was shaken to make homogeneous mixture of spore suspension. The cultivation was carried out in a Czapek Dox liquid medium containing (1%) xylan as the sole carbon source. An Erlenmeyer flask (250 ml) containing 100 ml of the growth medium was inoculated with 5X108 spore suspension and cultured for 15 days at 28 °C on an orbital shaker set at 250 rev min-1.Broth was filtered with Whatsman filter paper. The Filtrate was centrifuged at 10,000 rpm for 15 min at 40° C. The culture supernatant obtained was used for the assay of xylanase activity.

Optimization of xylanase producing microbes

Effect of temperature

To determine the temperature optimum xylanase activity was measured at different temperatures (30°C to 45°C) under standard assay conditions. The temperature stability of xylanase was determined by pre -incubating an aliquot of enzyme at 30°C to 45°C for 12 to 120 h and the residual activity (%) was calculated.

Effect of pH

The effect of pH on xylanase activity was studied by using the substrate prepared in buffer (0.05 M) of different pH ranging from 5.0 to 10.0 viz, citrate buffer (pH 5-6); phosphate buffer (pH 6-8) and Tris-HCl buffer (pH 8-10), under standard assay conditions. The pH stability of the enzyme was determined by pre -incubating an aliquot of enzyme with buffers of different pH for 24 h at 37°C followed by measurement of xylanase activity using sodium phosphate buffer (0.05 M, pH 7) after varying time interval for 5 h. The residual activity (%) at each pH was calculated.

RESULTS AND DISCUSSION

Isolation and Identification of microbial strains

For a primary isolation, several morphological and biochemical tests were performed to characterize the isolated bacterial and fungal species. In the present study four bacterial and six fungal species were isolated from soil samples as a source of xylanolytic organisms. The both bacterial and fungal strains were collected by spreading sample dilution on different xylane agar plates (Table.1). Among this *Bacillus amyloliquefaciens* and *Aspergillus niger* were showed the xylanolytic activity in the samples.

Xylanase production

Four bacterial and six fungal isolates which produced large and clear zones in shorter time were transferred in to liquid medium. The xylanase activity was measured by DNS method. Among the potent xylanse producers, *Bacillus amyloliquefaciens* and *Aspergillus niger* showed the highest xylanolytic activity (4.2 U/ml and 3.5 U/ml) at 48 hr after incubation. Regarding the specific activity, the best results were obtained with the strains *A.niger* and *Bacillus amyloliquefaciens.* For this reason, in the further studies the combination of the best xylanolytic strains will be evaluated. The data obtained with the strains tested in these experiments are comparable with those communicated by other authors for *Aspergillus niger, A. flavus, A brasiliensis, Trichoderma viride, T. harzianum* (Jafaru, 2013). For *P.digitatum* the synthesis of xylanases is less documented in literature (Cole and Wood, 1970) and the results obtained suggest that the strain used in this experiment is promising for further studies related the xylan degradation. The selected fungal isolates are dominant heterologous enzyme producing microorganism (Sathiyavathi and Parvatham, 2011), which are very much attracted by industries for a varying reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete extracellular proteins. The biochemistry,

physiology and generics of some of these isolates are well studied, facilitating further development and greater exploitation for industrial purposes (Alexopoulus and Mims, 1952).

Optimization of xylanase producing microbes

Effect of temperature on Bacteria

Temperature is one of the important parameters that determine the enzyme activity. The effect of temperature on xylanolytic enzymes production by *B.amyloliquefaciens* was investigated by growing culture at different temperature at 30°C, 37°C and 45°C. A high level of xylanase activity was obtained in the culture medium with optimum temperature at 37 °C at 48 hr (4.2 U/ml) (Fig.1). Higher xylanase production was also reported from alkalophilic *Bacillus* sp. using SSF at the optimum growth conditions (Gessesse and Mamo, 1999). Similar findings were also reported by some species i.e. *Bacillus* sp and *B.subtilis* YJ1 which have optimum temperature of 50ºC (Kim *et al*. 2011).

Effect of temperature on Fungai

The high level of xylanase activity was obtained in the optimum temperature at 48 hr (3.5U/ml) by *A. niger*. The different temperature at 35°C, 45°C and 55°C were examined for the detection of optimum temperature required for the production of enzyme and the results showed optimum to be 45°C at 48 hr (3.5U/ml) and minimum was observed at 35°C at 12 hr (1.2U/ml) (Fig.2). At 50°C, a significant decline in xylanase activity was evident. The optimum temperature for xylanase production by *P. oxalicum* was similar to some thermophilic fungi, such as *Thermomyces lanuginosus* (Purkarthofer *et al*., 1993), *Thermoascus aurantiacus* (Kalogeris *et al.*, 1998), and *Sporotrichum thermophile* (Topakas *et al.*, 2003) which were grown in SSF. The results clearly indicated the thermophilic nature of the fungus. The optimum temperature for xylanase production is similar to the optimum temperature for the growth of the fungus. This observation was in agreement with those reported by Sudgen *et al.*, (1994), who showed that the highest xylanase activities were obtained at temperatures that were optimum for the growth of the fungi in solid-state fermentation.

Effect of pH on Bacteria

The effect of pH on xylanolytic enzyme production by *B. amyloliquefaciens* was investigated at different pH between 5 and 10. A high level of xylanase activity was obtained in the culture medium with pH 8 (4U/ml). Three different buffers (0.05 M) were used. Citrate buffer was used for pH 5 to 6. Phosphate buffer was used for pH from 6 to 8 and glycine-

NaOH buffer was used for pH from 8 to 10 (Fig.3). To test the optimum pH, the purified enzyme recorded (4U/ml) at pH=8.0 and the decline of the enzyme activity noted in the pH 9 and 10 (3.8 and 3.4 U/ml). In the previous study, Among the xylanases from *Bacillus* species, highest pH optima had been reported for *Bacillus* Tar-1, C-125 and *Bacillus* sp. NCL-86-6-10 (Techapun *et al.*, 2003). However, several alkaline-tolerant fungal xylanases have also been characterized recently (Tseng *et al*., 2002).

Effect of pH on Fungai

The pH ranging from 5 to 10 was studied for the detection of optimum pH with high xylanase production by *A.niger*. The optimum pH found to be pH 8 (3.7 U/ml) and minimum was noted at pH 5 (2.2 U/ml) (Fig.4). The xylanase has pH optima around 8 but the activity at pH value of 7 – 9 makes it suitable for enzyme production. The residual enzyme activity was estimated at 1 h intervals during the 2 h period of incubation. Xylanase from a fungal source *A. terreus* UL 4209 showed maximum pH activity at 6.0 (Nakamura *et al*.,1993) Thermostable alkaline xylanase from a *Bacillus sp.* showed three optimum peaks for pH 6.5, 8.5 and 10.5. A wide range of pH activity from 5.0 - 8.0 was observed in *B. circulans* BL53 upon solid state cultivation (Júlio *et al*., 2006)The persistence of activity in a large range of pH is a desirable quality of an industrial enzyme.

CONCLUSION

In present study, 10 microorganisms were subjected to a screening for their ability of xylan degradation. Among this, the highest xylanase activity was obtained with *B. amyloliquefaciens* and *A. niger*. This enzyme was found to be cellulase free and exhibits alkalophilic and thermophilic properties that suggest its potential role in industrial application. The exploration of the biodiversity and exploitation of microbial enzyme is good for the human welfare and for the environment.

Table 1: Preliminary screening of isolated microorganisms.

S.No	Microorganisms	Xylenase activity (U/ml)
1	*Bacillus amyloliquefaciens*	4.2
2	*B. subtilis*	2.2
3	*B.pumilus*	1.8
4	*Pseudomonas sp.*,	1.5
5	*Fusarium culmorum*	2.5
6	*Aspergillus flavus*	2.8
7	*A.niger*	3.5
8	*A.brasiliensis*	2.5

9	*Trichoderma viride*	2.2
10	*Penicillium digitatum*	1.9

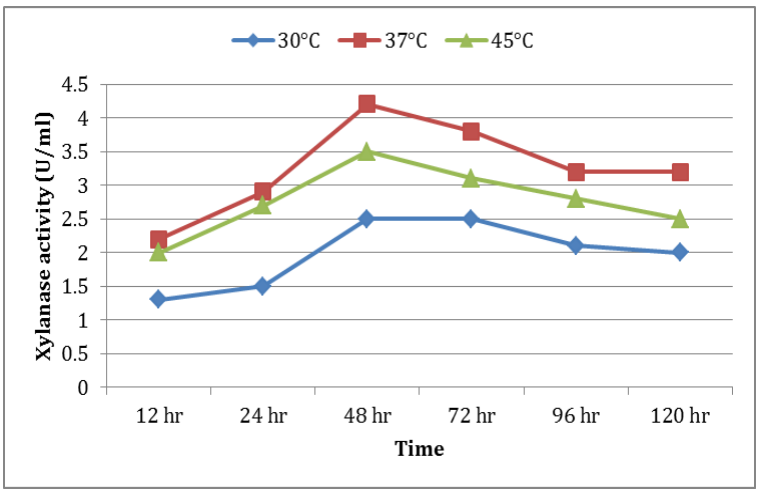

Fig. 1: Effect of Temperature on xylanase production by *Bacillus amyloliquefaciens.*

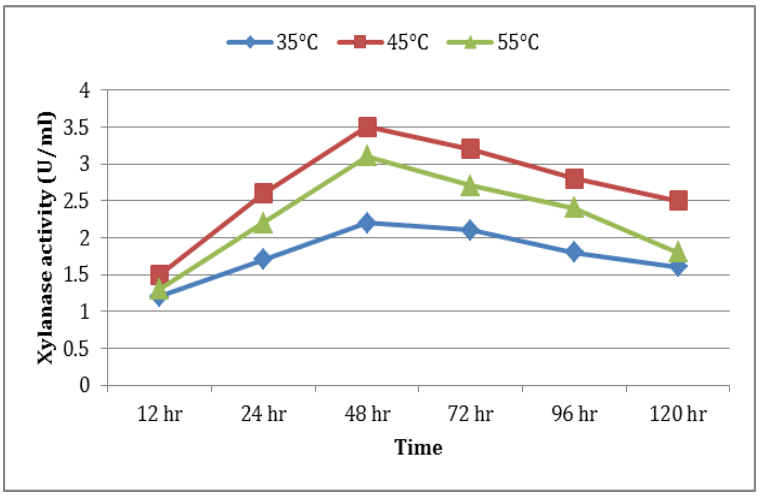

Fig. 2: Effect of Temperature on xylanase production by Aspergillus niger.

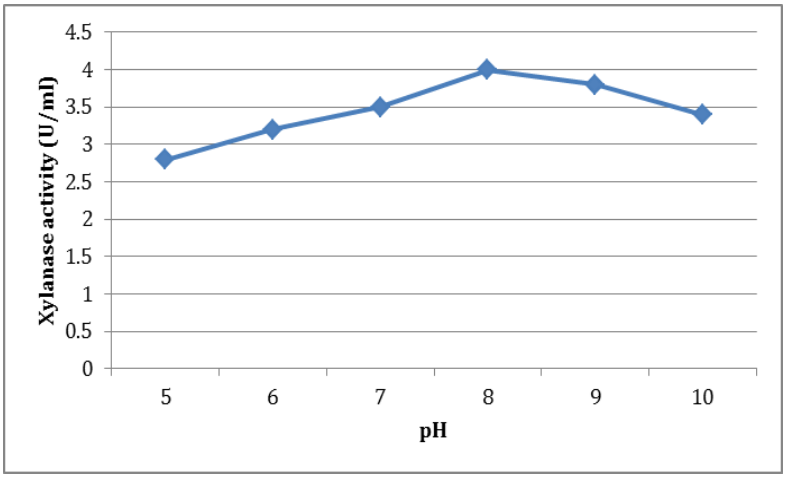

Fig. 3: Effect of pH on xylanase production by *Bacillus amyloliquefaciens.*

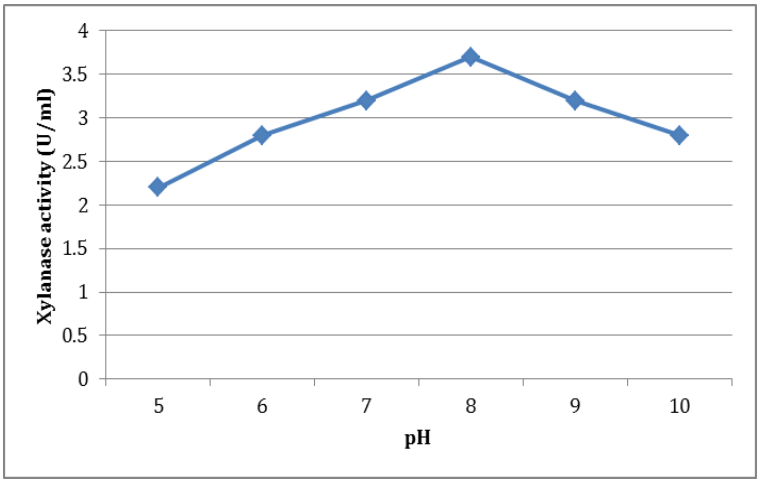

Fig. 4: Effect of pH on xylanase production by *Aspergillus niger*.

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