

## Effect of Medium Composition on Xylanase Production by *Bacillus subtilis* using Various Agricultural Wastes

Muhammad Irfan, Muhammad Nadeem, Quratulain Syed and Shahjahan Baig

Food and Biotechnology Research Center, PCSIR Labs Complex, Ferozpure Road Lahore, Pakistan

**Abstract:** In the present study a strain of *Bacillus subtilis*-BS05 was grown in submerged fermentation of agricultural wastes for xylanolytic activity. Various agricultural wastes such as sugarcane bagasse, wheat straw, rice husk, soybean meal, corn cobs and wheat bran were evaluated for xylanolytic activity using different media (M-I, M-II, M-III & M-IV). Among all these tested substrates maximum xylanolytic activity was observed in sugarcane bagasse (439.5±2.84 IU) using medium constituents IV (M-IV) with agitation speed of 140rpm for 48h of fermentation period at 37°C. In static conditions maximum xylanase enzyme activity was recorded with rice husk (408.9±2.9 IU) at 37°C using media M-IV for 72h of fermentation period.

**Key words:** Xylanase • *Bacillus* Sp. Agricultural Wastes • Submerged Fermentation

### INTRODUCTION

Hemicellulose is the second most abundant polymer present on earth which is composed of xylan, arabinose, glucuronic acid and arabino glucuronic acid [1]. Xylan is hydrolysed by a mixture of glucyases such as endo-1,4- $\beta$ -xylanase,  $\beta$ -xylosidase,  $\alpha$ -glucuronidase,  $\alpha$ -arabinofuranosidase and esterase [2]. Xylanases have been produced from a variety of microorganisms such as bacteria and fungi [3-9]. Some species of genus *Bacillus* produce a large number of enzymes which are industrially very important [10]. Bacterial and fungal xylanases are of great interest due to its potential application in biobleaching of pulp and paper industry and recovery of fermentable sugars from hemicellulose [1, 11-13].

For industrial production of enzymes, production cost is very important for the successful industrial processes. 30-40% of the production cost of many industrial enzymes is affected by the cost of growth substrate [14]. Reduction in this cost is achieved by the utilization of low cost substrates in fermentation processes. For this, solid agricultural wastes can be used to make the process cost effective [15]. Sugarcane bagasse, wheat bran, rice bran and corn cob are agricultural wastes which are abundant in several countries and can be used as raw materials for developing biotechnological process of industrial interest [16]. The main objective of this study was to evaluate the various

agricultural waste and media optimization for maximum yield of xylanase enzyme from *Bacillus subtilis*-BS05 in submerged fermentation.

### MATERIALS AND METHODS

**Procurement of Substrates:** Various agricultural wastes such as sugarcane bagasse, wheat bran, rice husk, soybean meal, corn cobs and wheat straw were procured from local market of Lahore city and were used as substrates for xylanase enzyme production in submerged fermentation. All these wastes were washed and oven dried at 70°C and then ground to powder form (2mm) using hammer beater mill.

**Bacterial Strain:** Bacterial strain of *Bacillus subtilis*-BS05 was obtained from Fermentation Laboratory, Food and Biotechnology Research Center, PCSIR Laboratories complex, Ferozpure road Lahore, Pakistan. The strain was maintained on nutrient agar (Oxoid) slants and store at 4°C.

**Cultivation of Vegetative Cells:** Twenty five milliliter of nutrient broth (Oxoid) was inoculated with a loopful of 24h old *Bacillus subtilis*-BS05 and incubated at 37°C for 24h with agitation speed of 140 rpm. These vegetative cells were used as a source of inoculum throughout the study.

**Fermentation Media:** In the present study four different types of media were used to check the production of xylanase enzyme by *Bacillus subtilis*-BS05 in submerged fermentation. The composition of the media (g/L) used was as follows;

M-I: peptone 20, yeast extract 10, KH<sub>2</sub>PO<sub>4</sub> 1, Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O 2.5 and MgSO<sub>4</sub>.7H<sub>2</sub>O 0.32; M-II: yeast extract 2, KH<sub>2</sub>PO<sub>4</sub> 1.6 and MgSO<sub>4</sub>.7H<sub>2</sub>O 0.08, 5; M-III: KH<sub>2</sub>PO<sub>4</sub> 1, K<sub>2</sub>HPO<sub>4</sub> 1.76, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 5, CaCl<sub>2</sub>.H<sub>2</sub>O 0.08, and MgSO<sub>4</sub>.7H<sub>2</sub>O 0.16 and M-IV: sucrose 20, K<sub>2</sub>HPO<sub>4</sub> 0.5, NaCl 0.2, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.16 and yeast extract 0.5.

**Fermentation Technique:** Twenty five milliliter of fermentation media with 2% substrates were sterilized in 250 ml Erlenmeyer flask at 121°C for 15 min. After sterilization, the media was inoculated with 2% solution containing vegetative cells of 24h old *Bacillus subtilis*-BS05 and incubated at 37°C for 24, 48 and 72h with agitation speed of 140 rpm.

**Production of Xylanase Enzyme:** After the completion of the fermentation period, the fermented broth was filtered through muslin cloth and centrifuged at 8000 xg, 4°C for 10 min to remove the bacterial cells and unwanted particles. The clear filtrate was used as a source of crude enzyme.

**Assay of Xylanase Enzyme:** Xylanase enzyme in the culture filtrate was estimated as reported earlier [17]. Reaction mixture containing 0.5 ml of appropriately diluted culture filtrate with 0.5 ml of 1% birchwood xylan (Sigma) solution prepared in citrate buffer (0.05M, pH5.0) for 15min at 50°C. After incubation the reaction was stopped by the addition of 1.75ml of 3, 5 dinitrosalicylic acid [18] and heated for 10 min in boiling water bath. After cooling the reducing sugars liberated were measured spectrophotometrically at 550nm and expressed as xylose equivalent. Xylose was taken as standard. One unit enzyme activity was defined as the amount of enzyme required to produce 1 µmole reducing sugar equivalent per minute under assay conditions. Units were calculated by using following formula [19].

$$\text{Xylanase activity (IU)} = \frac{\text{Reducing sugars (mg/ml)} \times 1000}{\text{Incubation time (15min)} \times 150}$$

**Statistical Analysis:** The data obtained after experimentation was statistically evaluated using ANOVA at significance level of p<0.05 by using Microsoft excel programme.

## RESULTS AND DISCUSSION

Present study described the production of enzyme xylanase from bacterial strain of *Bacillus subtilis*-BS05 in submerged fermentation using various agricultural wastes as a substrate. In this study fermentation media was optimized under two conditions i.e. agitated and static. Every microorganism requires proper nutrition for its growth and other processes. Media I (M-I) was examined for enzyme production under agitated (Fig. 1) and static conditions (Fig. 2) for 24,48 and 72h of fermentation period. Results of this experimentation revealed that highest level of enzyme production (36.8±1.21 IU) was observed using wheat bran as a substrate for 48h of fermentation period with agitation speed of 140 rpm and 24 h of fermentation period under static conditions. In this experiment both in static and agitation conditions wheat bran was found best inducer of xylanase enzyme production.

Figure 3 and 4 illustrate the xylanase production by *Bacillus subtilis* using medium constituent II (M-II) in submerged fermentation. Results of this experiment indicated that static conditions for 48h favored maximum enzyme (30.6±1.08 IU) production with wheat bran but in agitation condition Soybean meal was found best among various tested substrates. In this experimentation enzyme production was found better in static as compared to agitated. Archana and Satyanarayan [20] reported that xylanase enzyme production was found to be maximum by 72 h of fermentation period using wheat bran medium. Battan *et al.* [21] isolated a strain of *Bacillus pumilus* producing maximum xylanase activities on 2% wheat bran supplemented basal medium in submerged fermentation. Wheat bran and corn cob are thought to be the enhancer for xylanase production by *Streptomyces cyaneus* SN32 [22].

When medium constituents III were supplemented with various substrates, it was noted that sugarcane bagasse (44.3±2.11 IU) found to be best for xylanase production for 48h of fermentation period with agitation speed of 140rpm at 35°C (Fig. 5). When the same experiment was performed in static condition (Fig. 6), it was observed that wheat bran (30.6±1.36 IU) better induced the xylanase enzyme production for 48h of fermentation period.

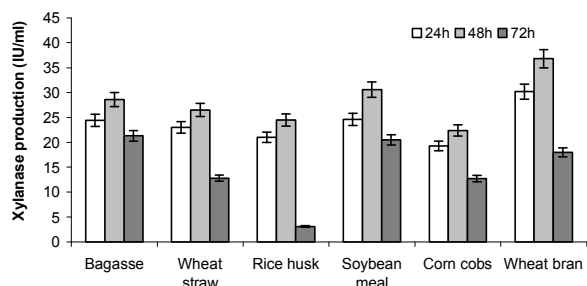


Fig. 1: Production of xylanase by *Bacillus subtilis*-BS05 using media-I at 37°C with agitation speed of 140rpm. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$

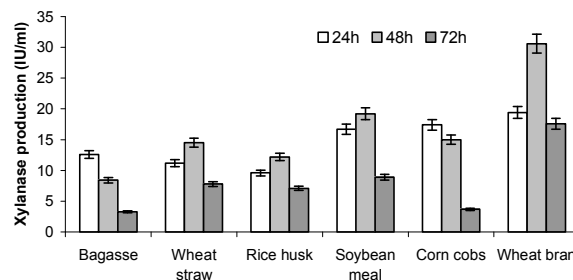


Fig. 4: Production of xylanase by *Bacillus subtilis*-BS05 using media-II under static conditions at 37°C. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$

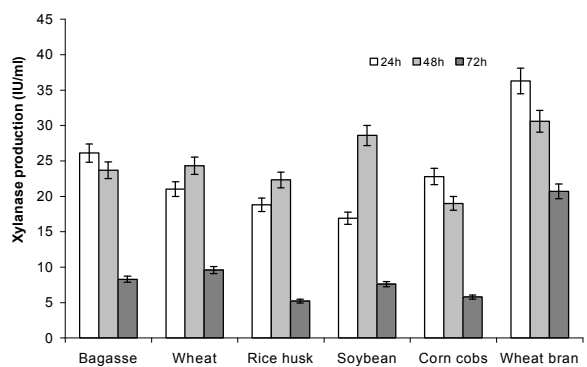


Fig. 2: Production of xylanase by *Bacillus subtilis*-BS05 using media-I under static conditions at 37°C. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$

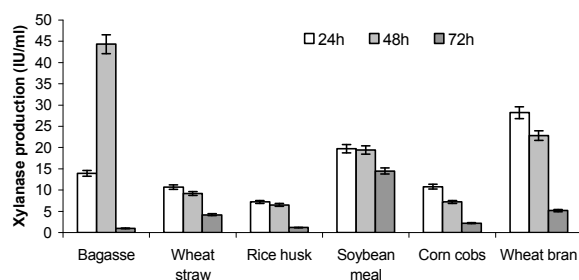


Fig. 5: Production of xylanase by *Bacillus subtilis*-BS05 using media-III at 37°C with agitation speed of 140rpm. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$

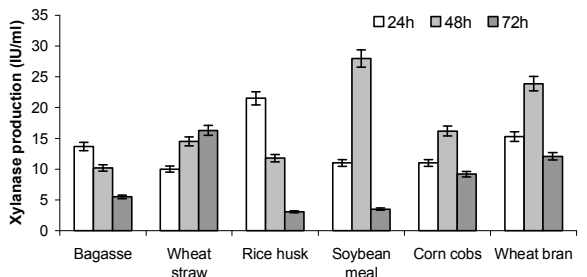


Fig. 3: Production of xylanase by *Bacillus subtilis*-BS05 using media-II at 37°C with agitation speed of 140rpm. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$

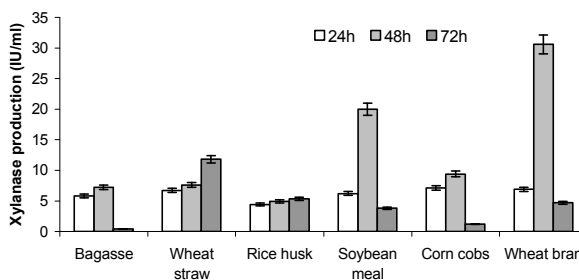


Fig. 6: Production of xylanase by *Bacillus subtilis*-BS05 using media-III under static conditions at 37°C. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$

Fig. 7 and 8 indicated the xylanase enzyme production using medium constituent IV (M-IV) with various agricultural wastes. In this experiment three substrates i.e. sugarcane bagasse, wheat straw and rice husk produced better levels of enzyme production in both

agitation and static conditions. Soybean meal, corn cobs and wheat bran showed no remarkable production with this medium supplementation. In agitation, maximum enzyme synthesis was observed with sugarcane bagasse ( $439.5 \pm 2.84$  IU) for 48h of fermentation period but in static condition, rice husk ( $408.9 \pm 2.92$  IU) favored maximum xylanase enzyme production for 72h of fermentation period. Nutrient supplementation to the medium greatly affects the enzyme production. Virupakshi *et al.* [23]

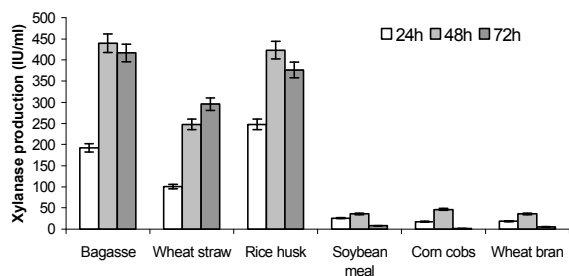


Fig. 7: Production of xylanase by *Bacillus subtilis*-BS05 using media-IV at 37°C with agitation speed of 140rpm. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$



Fig. 8: Production of xylanase by *Bacillus subtilis*-BS05 using media-IV under static conditions at 37°C. Bars represent the standard deviation among replicates which differ significantly at  $p \leq 0.05$

observed maximum enzyme production by *Bacillus* sp. in rice bran medium with 72 h of fermentation period. Gupta and Kar [24] isolated a strain of *Bacillus lichniformis* reporting highest yield of xylanase enzyme using corn cob as substrate for 72 h of fermentation period in solid state fermentation. Kumar *et al.* [25] isolated two strains of *Bacillus* sp from flour mill waste. Among the two isolated strains, *Bacillus* sp. FME 2 has highest production of CMCase (100 U/ml), FPase (45U/ml) and  $\beta$ -glucosidase (3.5U/ml) using rice husk as substrate for eight days of submerged fermentation. Johnvesly *et al.* [26] isolated a strain of *Bacillus* sp. JB99 from sugarcane molasses which is a good producer of cellulose free xylanase using rice bran as a carbon source in submerged fermentation.

In conclusion the results of the present study showed that maximum xylanase activity ( $408.9 \pm 2.92$  IU) was obtained by rice husk after 72 h of fermentation period under static conditions. However, under shaking condition bagasse resulted in maximum activity ( $439.5 \pm 2.84$  IU) after 48h of incubation period with agitation speed of 140rpm. All these findings indicated that

selection of suitable medium and fermentation conditions plays a vital role in xylanase production and is a prerequisite to make the process cost effective.

## REFERENCES

1. Wong, K.K.Y., T. Larry and J.N. Saddler, 1988. Multiplicity of  $\beta$ -1, 4- xylanase in microorganisms: functions and applications. *Microbiology Rev.*, 52: 305-317.
2. Bakir, U., S. Yavascaoglu and G.F. Ersayin, 2001. An endo  $\beta$ -1, 4-xylanase from *Rhizopus oryzae*: Production, partial purification and biochemical characterization. *Enzyme. Microbiol. Technol.*, 29: 328-334.
3. Angelo, R., C. Aguirre, E. Curotto, E. Esposito, J.D. Fontana, M. Baron, A.M.F. Milagres and N. Durán, 1997. Stability and chemical modification of xylanase from *Aspergillus* sp (2MI strain). *Biotechnology Applied Biochem.*, 25: 19-27.
4. Anuradha, P., K. Vijayalakshmi, N.D. Prasanna and K. Sridevi, 2007. Production and properties of alkaline xylanases from *Bacillus* sp. Isolated from sugarcane fields. *Current sci.*, 92: 1283-1286.
5. Bailey, M.J., J. Buchert and L. Viikari, 1993. Effect of pH on production of xylanase by *Trichoderma reesei* on xylan and cellulose based media. *Applied Microbiol. Biotechnol.*, 40: 224-229.
6. Shoham, Y., Z. Schwartz, A. Khashin, O. Gat, Z. Zosim and E. Rosenberg, 1992. Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* T-6. *Biodegradation*, 3: 207-218.
7. Yang, S.Q., Q.J. Yan, Z.Q. Jiang, T. Lil, H.M. Tian and Y.Z. Wang, 2006. High level of xylanase production by the thermophilic *Paecilomyces thermophila* J18 on wheat straw in solid state fermentation. *Bioresource Technol.*, 97: 1794-1800.
8. Morales, P., A. Madarro, A. Flors, J.M. Sendra and J.A. Pérez-González, 1995. Purification and characterization of a xylanase and an arabinofuranosidase from *Bacillus polymyxa*. *Enzyme Microbiol Technol.*, 17: 424-429.
9. Kar, S., A. Mandal, P.D. Mohapatra, K. Mondal and K. Pati K. 2006. Production of cellulose-free xylanase by *Trichoderma reesei* SAF3. *Brazilian J. Microbiol.*, 37: 462-464.
10. Bajpai, P., 1998. Microbial xylanolytic enzyme system: properties and applications. *Advance Applied Microbiol.*, 43: 141-9.

11. Bastawde, K.B., 1992. Xylan structure, microbial xylanases and their mode of action. *World J. Microbiol. Biotechnol.*, 8: 353-368.
12. Kang, M.K., P.J. Maeng and Y.H. Rhee, 1996. Purification and characterization of two xylanases from alkalophilic *Cephalosporium* sp. strain RYM-202. *Applied Environmental Microbiol.*, 62: 3480-3482.
13. Kuhad, R.C. and A. Singh, 1993. Lignocellulose biotechnology: current and future prospects. *Critical Reviews Biotechnol.*, 13: 151-172.
14. Hinnman, R.L., 1994. The changing face of the fermentation industry. *Chemical Technol.*, 24: 45-8.
15. Wizani, W., H. Esterbauer, W. Steiner, J. Gomes and H. Verfauser, 1990. Exo and Endo-cellulase xylanase. A Patent 1030/ 90 1990.
16. Kewalrami, N., D.N. Kamra, D. Lall and N.N. Pathak, 1988. Bioconversion of sugarcane bagasse with white rot fungi. *Biotechnol. Lett.*, 10: 369-72.
17. Irfan, M., M. Nadeem, Q. Syed and S. Baig, 2010. Submerged cultivation of *Aspergillus niger* on pretreated sugarcane bagasse. *World J. Agric. Sci.*, 6: 466-472.
18. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chem.*, 31: 426-428.
19. Ray, A.L. and T. Rahim, 2010. Cellulase and xylanase activities of *Neurospora crassa* induced with Leaf extract of *Aroma augusta* L. *Bangladesh J. Scientific and Industrial Res.*, 45: 151-154.
20. Archana, A. and T. Satyanarayan, 1997. Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid state fermentation. *Enzyme. Microbial. Technol.*, 21: 12-17.
21. Battan, B., J. Sharma, S.S. Dhiman and R.C. Kuhad, 2007. Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. *Enzyme Microbial Technol.*, 41: 733-739.
22. Ninawe, S. and R.C. Kuhad, 2005. Use of xylan rich cost effective agro residues in the production of xylanase by *Streptomyces Cyaneus* SN32, *J. Appl. Microbiol.*, 99: 96631141-1148.
23. Virupakshi, K., B. Gireesh, R. Satish and G.R. Gaikwad, 2005. Production of a xylanolytic enzyme by a thermoalkaliphilic *Bacillus* sp. JB-99 in solid state fermentation. *Process Biochem.*, 40: 431-435.
24. Gupta, U. and R. Kar, 2008. Optimization and Scale up of Cellulase free Endo xylanase Production by Solid State Fermentation on Corn cob and by Immobilized Cells of a Thermotolerant Bacterial Isolate. *Jordan J. Biological. Sci.*, 1: 129-134.
25. Kumar, G.S., M.S. Chandra, M. Sumanth, A. Vishnupriya, B.R. Reddy and Y.L. Choi, 2009. Cellulolytic enzymes production from submerged fermentation of different substrates by newly isolated *Bacillus* spp. *FME. J Korean Society of Applied Biological. Chem.*, 52: 17-21.
26. Johnvesly, B., S. Virupakshi, G.N. Patil and G.R. Naik, 2002. Cellulase-free thermostable alkaline xylanase form thermophilic and alkalophilic *Bacillus* sp. JB-99. *J. Microbiol Biotechnol.*, 12: 153-6.