Abstract
Xylanase bioprocess by isolated Aspergillus terreus and Aspergillus fumigatus strains under solid-state fermentation using oil palm empty fruit bunch fiber as substrate was investigated. The productions of xylanase enzyme in these fungal strains were influenced by bioprocess parameters. Though enzyme production was noticed in the wide range of pH (3.0 – 8.0), effective xylanase production observed at pH 6.0 and 7.0 by A. terreus and A. fumigatus respectively. Similarly, enzyme titer values improved with increase in moisture content (70%) and inoculum concentration of 2.0 and 1.5 ml (1 x 10^6 spore solution per ml) for A. terreus and A. fumigatus respectively. Particle size mediated variation also noticed where 2.0 - 0.7 and 2.8- 2.0 mm (15,990 and 14,563 U/g) was effective for xylanase production by A. terreus and A. fumigatus, respectively. Whereas, supplementation of xylose and fructose to A. terreus and A. fumigatus were enhanced the xylanase production to 32,074 and 25,038 U/g, respectively. Over all xylanase enzyme productivity was improved to the tune of 3.0 and 2.8 folds with A. terreus and A. fumigatus after bioprocess optimization, respectively.

Keywords: Aspergillus, Bioprocess, Palm fiber, Solid state fermentation, Xylanase.

Introduction
Aspergillus sp are one of the most important group of filamentous fungi capable of degrading cellulosic and hemicellulosic part of the plant cell wall due to their capability to synthesize hydrolytic enzymes (cellulases and xylanases). These enzymes are responsible to degrade complex substrate molecules into low molecular weight compounds which are used by fungi for their nutrition (1). Among different hydrolytic enzymes, xylanases are mainly responsible for the hydrolysis of the main chain of xylan which has potential applications in many industries. Hence, many researchers focused their attention on production of xylanases using different fermentation strategies. Since, biotechnological applications require large amounts of low cost enzymes, which can be achieved by utilization of lignocellulosic waste or by products as substrates (2). From the literature reports it was revealed that, various lignocellulosic substrates and Aspergillus sp have
been used for the xylanase production under submerged and solid state fermentations. Though xylanase production is reported by different strategies, enzyme production by solid-state fermentation (SSF) is an attractive one because it presents many advantages, especially for fungal cultivation. Xylanase production from *Aspergillus niger* and *Aspergillus ochraceus* was improved by the combination of corn cobs and wheat bran as substrate (3). Whereas, similar strain *A. niger* has shown maximum xylanase production (3099 U/g) using 10 g of sugar cane bagasse and soybean meal in the ratio of 65 and 35%, respectively (4). Grape pomace is a suitable substrate for xylanase production (60U/g) by *A. awamori* (5). All the above reports have indicated that, each microbial strain has different profile in terms of enzyme productivity and utilization of different substrates as carbon sources. 

Hence, in the present study, an attempt has been made for xylanase production using isolated fungal strains, *A. terreus* and *A. fumigatus* under solid state fermentation. Here, oil palm empty fruit bunch fiber is selected as substrate based on the earlier studies performed under submerged fermentation (6). Furthermore, in this study, different physiological (pH, moisture content and particle size) and nutritional parameters (carbon and nitrogen sources) were optimized for effective xylanase production using these strains.

**Materials and Methods**

**Microorganisms and cultural conditions:** *A. terreus* and *A. fumigatus* were isolated in our laboratory from soil samples collected from Rajahmundry and in our laboratory premises. These isolates were identified and deposited as *A. terreus* and *A. fumigatus* at Institute of Microbial Technology (IMTECH), Chandigarh, India. The cultures were maintained regularly on potato dextrose agar slants and stored at 4° C.

**Xylanase production by solid state fermentation and enzyme extraction:** Solid state fermentation experiments were performed using OPEFB fiber as solid material. This material was collected from palm oil industry and processed using USA standard sieve set Nos. 7, 10, 25, 50 and 70 to obtain mean particle sizes of 2.8 - 2.0; 2.0 - 0.7; 0.7 - 0.3 and 0.3 - 0.2 mm and used for bioprocess experiments. Five grams of substrate was used in 250 ml Erlenmeyer flasks and moistened to 70% with pH 7.0 supplemented with (for 5.0 g solid material) yeast extract - 0.2 g; peptone - 0.2 g; KH₂PO₄ - 0.05 g; K₂HPO₄ - 0.05 g; NaCl- 0.05 g and MgSO₄ - 0.05 g unless otherwise stated. The contents of the flasks were mixed thoroughly and autoclaved at 121° C for 15 min. After cooling to room temperature, the flasks were inoculated with 1 ml of *A. terreus* and *A. fumigatus* spore suspensions (1.0 x 10⁶ spores per ml) and incubated at 30°C for 30 min for three times. Then the pooled contents were centrifuged and the supernatant was collected and used as source of xylanase enzyme.

**Measurement of xylanase activity:** Xylanase activity was determined using DNS method (7). Enzyme assay was performed at 50° C for 30 min using 1.0% (w/v) oat spelt xylan as substrate. One unit (U) of xylanase activity was defined as the amount of enzyme that releases 1μmol of xylose per min under the standard assay conditions. Xylanase production was expressed as U/g dry substrate. Enzyme assays were performed in triplicate with analytical grade reagents and average values were represented.

**Evaluation of different fermentation factors for xylanase production using one variable at a time approach:** To investigate the optimum
physical and nutritional parameters for xylanase production the following conditions were optimized. The moisture content was optimized by moistening the solid substrate to 60, 65, 70 and 75% moisture level. The particle size of the substrate was optimized by using 2.8 to 2.0, 2.0 to 0.7, 0.7 to 0.3 and 0.3 to 0.1 mm particle size. The effect of medium pH and inoculum level were optimized in the pH range from 3.0 to 8.0 and 0.25 to 2.5 ml inoculum (1x10^6 spores per ml) respectively. Moreover, effect of metabolizable sugars and different nitrogen sources on xylanase production was investigated by using 0.2 g per 5 g of OPEFB fiber.

Results and Discussion

Time course of xylanase production by A. terreus and A. fumigatus: To investigate the suitable fermentation time for xylanase production using A. terreus and A. fumigatus, bioprocess was carried out over a period of 96 h. The data revealed that xylanase titer values of A. terreus and A. fumigatus were improved with increase in incubation time up to 60 h and 72 h, respectively. Further increase in incubation time resulted in decreased xylanase production in both strains. At 60 h of incubation time A. terreus produced 13,370 U/g whereas A. fumigatus produced 12,898 U/g xylanase (Fig.1). Xylanase production values noticed at 24, 36 and 48 h of incubation time with A. terreus were 59%, 74% and 91%, respectively when compared to xylanase production at 60 h of incubation time. After 72, 84 and 96 h of incubation time, xylanase production was gradually decreased to 17, 25 and 27% respectively. In case of A. fumigatus after 24, 36, 48 and 60 h of incubation time 48, 50, 58 and 78% of xylanase production was measured. After 72 h of incubation time xylanase production was decreased to 11 and 13% at 84 and 96 h respectively. These variations in xylanase enzyme production values over incubation time may be attributed to insufficient growth of the microorganism, loss of moisture, inhibition of the enzyme by end products, depletion of macro-and micronutrients in the fermentation medium, alteration in the medium pH, etc (8). This data indicated that biocatalyst production by these fungal strains is growth associated and produced xylanase helps in increasing the availability of carbon source for their growth by hydrolyzing the xylan portion of the OPEFB. Similar trend was reported in other extracellular enzyme production by other microbial strains: Penicillium canescens and Paecilomyces thermophila J18 produced maximum xylanase after 7 and 8 days of incubation time (9, 10). Ghanem et al., (11) and Nogueira et al., (12) reported that A. terreus produced maximum xylanase (922 U/ml) after 4 days of incubation whereas A. fumigatus and A. niveus showed maximum activity (370 and 180 U/g, respectively) after 96 h and 120 h of incubation time. From all these literature reports, it is evident that among fungal xylanase producers the isolated Aspergillus species of this study produced maximum xylanase in shorter incubation periods. However, isolated A. terreus produced maximum xylanase titers in lesser incubation period (60 h) when compared to A. fumigatus (72 h) which is advantageous for industrial application. In addition, the presented

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Fig 1. Effect of incubation time on xylanase production by A. terreus and fumigatus
data denoted that both these strains differ in their metabolic pathways involved in xylanase production and utilization of OPEFB as substrate material.

**Effect of moisture content on xylanase production by A. terreus and A. fumigatus:** Solid-state fermentation unlike submerged one occurs at near absence or free flow of water in the fermentation vessel. Hence, supplementation of appropriate quantity of moisture plays critical role for microbial growth and xylanase production in solid-state fermentation (13, 14).

So in the present study to evaluate the xylanase production by *A. terreus* and *A. fumigatus*, OPEFB fiber was moistened with different moisture levels. The data revealed that the maximum xylanase production by *A. terreus* and *A. fumigatus* (13,565 and 13,248 U/g) was noticed at 70% moisture content (Fig. 2). At 60 and 65% of moisture content, xylanase production by *A. terreus* and *A. fumigatus* were 9855, 11458 and 9129, 10892 U/g, respectively. Further varying the moisture content to 70%, little variation in xylanase production was noticed. This may be due to the fact that, low moisture content decreases the metabolic and enzymatic activities owing to the reduction of the solubility of nutrients. Whereas, a level of moisture content higher than the optimum causes a decrease in porosity, an alteration in substrate particle structure, a gummy texture, and a lower oxygen transfer is influencing the mass transfer, thus, limiting the nutrients utilization level (15, 16).

Authors working with *Penicillium canescens* and *Paecilomyces thermophila* J18 reported that 80% and 83% of initial moisture level was the most suitable environment for optimum xylanase production using soya oil cake and wheat straw as substrates (9, 10). In another study, 75% moisture level was noticed effective for maximum xylanase production by *A. terreus* with wheat straw as carbon source (11). These reports supported that moisture content in solid-state fermentation plays an important role on xylanase production. In the present study both *Aspergillus* strains showed maximum xylanase activity at 70% moisture content. This similarity may be due to the same substrate (OPEFB) fiber was used for the production of xylanase enzyme by these strains. So level of moisture content is mainly dependent on the type of substrate used for the enzyme production (15).

**Influence of particle size on xylanase production by A. terreus and A. fumigatus:**

Particle sizes of the substrate during solid-state fermentation influence the microbial growth and allowed heat and mass transfer (13, 15, and 17). From literature, it was reported that *Trichoderma reesei* showed highest xylanase and cellulase activities using small particle size of horticultural waste (15). But *A. terreus* and *A. fumigatus* showed high xylanase titers when used large particle size of palm fiber. In view of the above, different particle sizes of palm fiber tested in order to determine their effects on xylanase production by these two *Aspergillus* sp. It was apparent from the experimental data that particle size affected the enzyme production (Fig. 3). The highest titers of xylanase (15,990 and 14,563 U/g) produced by *A. terreus* and *A. fumigatus* using 2.0 - 0.7 and 2.8 - 2.0 mm size of the OPEFB
fiber respectively. After screening of the particle size of the OPEFB fiber, xylanase production by *A. terreus* and *A. fumigatus* was improved to 17% and 9%, respectively and all other tested particle sizes revealed lower productivity values especially with smaller particle sizes. The observed variation in enzyme production values with particle size could be due to its impact on available surface area for microbial growth. This is because, use of smaller particles provide larger surface area for microbial adhesion thus making the environment advantageous for heat transfer and exchange of oxygen and carbon dioxide between the air and the solid surface. However, too small particles may result in substrate agglomeration, which may interfere with microbial respiration and thus result in the poor cell growth (15). However, even though large particle size provides better aeration to microbe may result in poor accessibility of nutrients which limits the microbial growth leads to decreased enzyme production. The literature reports revealed that influence of the particle size varied to the type of the substrate and microorganism used for the enzyme production. *Penicillium canescens* and *Paecilomyces thermophila* J18 produced maximum xylanase using 5.0 mm size of soya oil cake and 0.45 - 0.3 mm size of wheat straw as substrates (9, 10).

Here, 2.0 to 0.7 mm and 2.8- 2.0 mm size of palm fiber were found to be suitable for higher titers of xylanase production by *A. terreus* and *A. fumigatus*.

**Effect of medium pH on xylanase production by *A. terreus* and *A. fumigatus***: pH is an important environmental factor, which significantly affects the production of microbial enzymes and microbial growth (6,18). Analysis on xylanase production by *A. terreus* and *A. fumigatus* at different pH environments ranging from pH 3.0 to 8.0 suggested that the xylanase production is regulated by medium pH. Maximum enzyme production by *A. terreus* and *A. fumigatus* (18,684 and 15,258 U/g, respectively) was noticed with pH 6.0 and 7.0 (Fig. 4). Altering of the pH on either sides of the optimum pH caused the decreased xylanase production. The data further confirmed that these two microbial strains has potential to grow in pH range of 3.0 to 8.0 and produces appreciable quantities of xylanase enzyme. When compared to pH 6.0, at pH 3.0, 4.0 and 5.0, *A. terreus* produced 77, 78 and 85% of xylanase respectively. But when compared to pH 6.0, at pH 7.0 and 8.0 xylanase production was decreased to 14 and 32%, respectively. While in case of *A. fumigatus*, the respective xylanase titers at pH 3.0, 4.0, 5.0, 6.0 and 8.0 were noticed.
as 64, 80, 92, 96 and 97% when compared to xylanase activity at pH 7.0. From these results it was concluded that, pH 3.0 to 6.0 was favorable for xylanase production by *A. terreus*. While in the case of *A. fumigatus*, neutral and alkaline pH were favorable for xylanase production. Such pH dependent xylanase production in solid state fermentation was observed in other microbial strains. *Paecilomyces thermophila* J18 and *Fusarium oxysporum* produced maximum xylanase at pH 7.0 and *A. awamori* showed optimum xylanase production at pH 4.0 (10, 19, and 20). *A. fumigatus* and *A. niveus* produced maximum xylanase in the pH range of 5.0 to 5.5 and 4.5 to 5.0, respectively (12). From these reports it can be showed that fungal xylanases were produced in the pH range of 4.0 to 7.0.

**Effect of inoculum concentration on xylanase production by *A. terreus* and *A. fumigatus***: All microbial growth associated enzymes production is directly proportional to microbial biomass (13). To optimize the initial inoculum concentration for xylanase production by *A. terreus* and *A. fumigatus*, different concentration of spore suspension (1 x 10⁶ spores per ml) ranging from 0.25 to 2.5 ml (v / 5.0 g of substrate) were evaluated and xylanase production at 60 and 72 h of growth were measured. The data clearly indicated that enzyme productions by both these strains were influenced by inoculum concentration. Xylanase production by *A. terreus* and *A. fumigatus* was increased by using 2.0 ml and 1.5 ml of spore solutions respectively and further increase in inoculum level, decreased the enzyme production (Fig. 5). Similar effects of inoculum level on xylanase production are reported in the literature. Gaffney *et al.*, (21) reported that *Thermomyces lanuginosus* produced higher titers of xylanase using higher inoculum level. Ghanem *et al.*, (11) reported that maximum xylanase production was obtained by *Aspergillus terreus* using 1.2 x10⁴ spores per 2.0 ml. These reports suggested that lower inoculum volumes in SSF might not accommodate mycelial expansion and subsequent product formation. Increased levels of inoculum typically improve growth-related activities, but after a certain point, they serve to restrict gaseous exchange, reduce heat removal, and increase the demand for nutrients from the substrate. Furthermore, a higher inoculum volume in SSF can increase the incidence of bacterial contamination (22). From this data it was concluded that inoculum level plays an important role on xylanase production. Lower or higher levels of inoculum level inhibit the xylanase production.

**Effect of different easily metabolizable sugars on xylanase production by *A. terreus* and *A. fumigatus***: To evaluate the effect of easily metabolizable sugars, different sugars such as glucose, fructose, xylose, galactose, maltose, arabinose, mannose and lactose were taken at 0.2% level and sterilized separately from fermentation medium and used for the xylanase production. The data indicated that enzyme production differed with the type of carbon source used (Fig. 6). Maximum xylanase production by *A. terreus* and *A. fumigatus* (32,074 and 25,038 U/g) occurred in xylose and fructose supplemented conditions indicating an increase of 45% and 16%, respectively over

Fig 5. Effect of inoculum concentration on xylanase production
control (without any addition of carbon source). Not only xylose, other simple sugars glucose, maltose and mannose also increased (17, 23 and 31%, respectively) the xylanase production by A. terreus when compared to control. But xylanase production by A. fumigatus was only enhanced by fructose supplementation and all other sugars inhibited the xylanase production. This data suggested that utilization pattern of externally supplemented carbon sources by these two strains differ and only fructose is effective for xylanase production by A. fumigatus and not for A. terreus. Effect of simple sugars on xylanase production was studied by many other researchers. Shah and Madamwar (23) reported that fructose and lactose inhibited the xylanase production which was similar to xylanase production by A. terreus of this study.

Xylanase production by Thermomyces lanuginosus was enhanced by the addition of glucose and cellulose whereas xylose and sucrose reduced the xylanase activity by 9 and 8% (21). Christakopoulos et al., (24) reported that xylanase production was repressed by the addition of simple sugars such as glucose, xylose and lactose. Smith and Wood (20) also reported the similar result about the effect of simple sugars on xylanase production. In this case, except xylose, all the sugars repressed the xylanase production. This data revealed that maximum number of tested simple sugars repressed the xylanase production. In addition, xylanase production by A. terreus and A. fumigatus was enhanced by the addition of low concentrations of xylose and fructose. Further increase in carbon source did not improve the enzyme production and showed catabolic repression (data not shown).

**Effect of different nitrogen sources on xylanase production by A. terreus and A. fumigatus:** Nitrogen sources are known to regulate the production of extracellular enzymes in different microbial strains by altering the availability of precursors for protein synthesis (25). In the present study, to evaluate the effect of different nitrogen sources on xylanase production by A. terreus and A. fumigatus, various organic nitrogen sources such as yeast extract, peptone, beef extract, tryptone, soya bean meal, corn steep liquor, urea and casein (0.2 g per 5.0 g of substrate) and inorganic nitrogen sources such as (NH₄)₂SO₄, NH₄Cl, NH₄NO₃, NaNO₃ and KNO₃ (0.2%) were added in the production media and checked for the xylanase production. As shown in Fig. 7, among all the organic nitrogen sources tested, urea and peptone were the best for achieving optimal xylanase production (37,799 and 28,520 U/g, respectively) by A. terreus and A. fumigatus, and least xylanase production (13,462 and 15,180 U/g dry substrate, respectively) was obtained with corn steep liquor as nitrogen source by both strains. Other nitrogen sources have shown nearly similar effect on xylanase production by both strains. While, in the case of A. terreus, when compared to the xylanase production by using urea, addition of yeast extract, peptone, beef extract, tryptone, soybean meal and casein decreased the xylanase production to 35, 38, 30, 35, 33 and 38% respectively. While, in the case of xylanase production by A. fumigatus, the differences in
xylanase production by using other nitrogen sources was less, when compared to the xylanase production by using peptone. Here, addition of casein and yeast extract have shown similar impact on xylanase production. When compared to the xylanase production by peptone, both of these nitrogen sources have shown 25% decrement in xylanase production followed by tryptone (22%). Whereas, supplementation of beef extract was caused 11% reduction in xylanase production and soybean meal has shown similar effect on xylanase production like as peptone. However, addition of corn steep liquor to the production media led to the 64% and 46% of reduction in xylanase production by A. terreus and A. fumigatus respectively. Even though, corn steep liquor is a rich nitrogen source, both of these fungal strains were unable to utilize this source properly due to its non-synthetic and complex nature which led to the lower xylanase production. In this study, urea supplementation has given higher titers of xylanase production by A. terreus compared to other studied organic nitrogen sources. The results are in agreement with Chaetomium cellulolyticum, Phanerocheate chrysosporium and Rhizopus stolonifer (26, 27). In contrast to the above result, urea inhibited the xylanase production by using A. niveus, A. ochraceus and A.niger (3). A.niger showed maximum xylanase activity using peptone as nitrogen source which was similar to the xylanase production by A. fumigatus (3). The above data revealed that supplementation of the organic nitrogen sources greatly enhanced the xylanase titers in the present study by isolated fungal strains.

Among all the tested inorganic nitrogen sources, supplementation of NaNO₃ and NH₄Cl caused maximum and minimum xylanase production by A. terreus (39,136 and 23,545 U/g dry substrate) respectively (Fig.7). When compared to the organic nitrogen source (urea), addition of inorganic nitrogen source (NaNO₃) showed maximum influence on enzyme production (Fig 7). In contrast to the above result, A. fumigatus produced higher titers of xylanase using NH₄Cl (35,380 U/g) as nitrogen source which caused lower xylanase production using A. terreus. As per literature report, Trichoderma harzianum showed maximum xylanase activity by the addition of NaNO₃ which was similar to the xylanase production by A. terreus (28). Seyis and Aksoz (29) reported that addition of ammonium sulphate enhanced the xylanase production by Trichoderma harzianum. NH₄NO₃ and (NH₄)₂HPO₄ supplementation in Schizophyllum commune increased the xylanase production (30). By observing these literature reports, it was found that not only organic nitrogen sources but also inorganic nitrogen sources positively affected the xylanase production. In the present study among all the organic and inorganic nitrogen sources, addition of NaNO₃ and NH₄Cl enhanced the xylanase production by A. terreus and A. fumigatus. Hence, further optimization of xylanase production by A. terreus and A. fumigatus was done by supplementing different concentrations of NaNO₃ and NH₄Cl (0.2, 0.4, 0.6, 0.8 and 1g). The results revealed that 0.6 g of NaNO₃ showed maximum xylanase production by A. terreus,

![Fig. 7. Effect of different nitrogen sources on xylanase production](image-url)
whereas 0.4 g of NH$_4$Cl has shown maximum xylanase production in the case of _A. fumigatus_. Overall, after optimization of the nitrogen source, xylanase production by _A. terreus_ and _A. fumigatus_ was improved by 5 and 4.5%, respectively.

**Conclusion**

Overall xylanase production pattern was investigated under solid state fermentation by isolated _A. terreus_ and _A. fumigatus_ strains using oil palm empty fruit bunch fiber as solid support material. Different fermentation parameters such as incubation period, moisture content, particle size, medium pH, inoculum concentration, carbon and nitrogen sources were optimized. These isolated strains showed effective enzyme production ( _A. terreus_ : 41,000 and _A. fumigatus_ : 36,985 U/g) upon supplementation of sodium nitrate and ammonium chloride as nitrogen sources, xylose and fructose as inducers at the optimum pH of 6.0 and 7.0 with 70% moisture content in 60 and 72 h of incubation with an initial inoculum concentration of 2.0 ml and 1.5 ml (1 x 10$^6$ spore solution) respectively. The xylanase production by both these strains and was increased by 3.0 ( _A. terreus_ ) and 2.8 ( _A. fumigatus_ ) folds under optimized conditions. When compared to _A. fumigatus_ , _A. terreus_ showed higher xylanase production.

**Acknowledgments**

Authors are thankful to Council of Scientific and Industrial Research, New Delhi for financial support and Director, Indian Institute of Chemical Technology, Hyderabad for his encouragement.

**References**


