Optimization of physical and morphological regime for improved cellulase free xylanase production by fed batch fermentation using *Aspergillus niger* (KP874102.1) and its application in bio-bleaching

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**ABSTRACT**

The physiological and morphological changes were extensively studied during fed batch fermentation using newly isolated *Aspergillus niger* (KP874102.1). Significantly higher xylanase production was possible through optimization of environmental stresses by fed batch process. The fed batch fermentation was carried out for improved xylanase production (2524 U) where initial xylan was kept 1.5 g/L in the production medium. However, 3 g/L of xylan with 50 mM K$_2$HPO$_4$ having pH-7 was consecutively fed at 72 and 120 h of fermentation. K$_2$HPO$_4$ showed significant role both the morphology of the microorganism and produces enzymes in fed batch fermentation. During feeding phase, the pH was found in the range of 6.5 to 7 which was used as marker for the fed batch process. The crude enzyme was used for the bio-bleaching of banana pulp.

**Introduction**

Xylanase is an important enzyme to exploit xylan, the second most abundant polysaccharide in nature. In recent years, xylanases have been widely used in the animal feed, pulp and paper industries to create commercial value. Many forms of xylanases have been isolated and characterized from various microorganisms including bacteria and fungi. However, production of xylanases isolated from their original hosts is limited and the cost of enzymes is one of the main factors that determine the economics of a commercial process.

The submerged fermentation (SF) technique is widely used for production of enzymes due to its ease in operation. Given that the synthesis of a microbial enzyme is a complex process, therefore optimization of process parameters is an important task for bioprocess engineers for increasing xylanase production. In addition, morphology of filamentous fungi has a significant role on the rheological properties of cultivation medium that indirectly affects microbial xylanase production.

The most of the recent research focused on fungal growth, germination and control of morphology during fungal mediated xylanase fermentation. However, very little has been done for understanding the influence of metal traces during feeding process on both the morphology of the microorganism and production of enzymes in fed batch fermentation. In fermentation, identification of markers for the fed batch process is also utmost important for bioprocess engineers. Therefore, the potential contribution of these parameters for xylanase production was addressed by our present research work with *Aspergillus niger* (KP874102.1). Due to increase awareness on sustainable development and environmental pollution, cellulase free xylanases have received notable attention in the paper and pulp industry since it is especially helpful before Cl$_2$ or chlorine dioxide bleaching stages to the minimization the application of bleaching agent. Therefore, it is also eminent to find out an alternate technology that will replace the use of bleaching agents with microbial enzymes in the paper and pulp industry.

Therefore, the present work was focused on improving xylanase production by newly isolated *Aspergillus niger* (KP874102.1) through optimization of process parameters during fed batch fermentation. In addition,
marker identification for upholding the fed batch process and potential application of this xylanase as a bio-bleaching agent were also critically evaluated.

**Results and discussion**

**Variable volume fed-batch study**

The batch fermentation is normally preferred for secondary metabolite production. In our previous study batch fermentation was carried out in shake flask. The media was optimized through Taguchi methodology and xylanase production (1625.74 U) is found after 7 days of fermentation. The composition of medium was xylan from beechwood (3 g/L), potassium nitrate (10 g/L), magnesium sulfate (5 g/L), di-potassium hydrogen phosphate (50 mM), calcium carbonate (2 g/L), 1000X of trace element (1 ml), and sodium chloride (5 g/L). From optimization study, it was found that xylan from beechwood is an important medium component (carbon source) for xylanase production (Data not shown). It was also previously reported that xylose which is produced from xylan has repressive effect in its higher concentration on enzyme biosynthesis by *Aspergillus sp.* as it causes catabolic repression.\(^\text{15,16}\) However, there are several recent reports where fed-batch processes were used for noticeable increase of xylanase titer.\(^\text{17-19}\) Therefore, the potential contribution of fed batch fermentation process was brought to our attention for xylanase production by an experiment with *Aspergillus niger* (KP874102.1). So an attempt was made for fed batch fermentation where initial xylan concentration was optimized and kept 1.5 g/L. Initially, 0.2 litter fermentation medium was maintained and xylan was feed at concentration 3g/L with 50 mM K\(_2\)HPO\(_4\). The feeding of xylan was given at 72 and 120 h. The variable volume fed batch fermentation was carried out at initial pH of 7 with 5% inoculum for 7 d at 28°C and the effects of agitation speed, pH, residual xylan and reducing sugar were investigated on xylanase production. As it is difficult to understand the effect of these factors on bioreactor performance separately, therefore the interaction among these factors was considered on xylanase production. The ranges of these process parameters were chosen based on previous work.

**Effect of agitation speed on xylanase production**

From the Fig. 1, it was found that fermentation was started with 120 rpm which continued up to 24 h. The significant fungal growth (branching of hyphal elements) was noticed after 12 h fermentation due to presence of maximum xylan (1.5 g/L). It is found that the concentration of xylan (1.14 g/L) decreases with increase in reducing sugar concentration (0.24 g/L) after 24 h of fermentation (Fig. 1). It is obvious that xylan cannot use directly by microorganism for their energy source. However, it can be utilized indirectly

![Figure 1. The effects of nutritional and environmental factors on xylanase production.](image-url)
after formation of reducing sugar (such as xylose) through the extracellular production of xylanolytic enzyme, required for cleaving the glycosidic bonds so the reducing sugars can be transported into the cell. In addition, pH of the fermentation decreases slowly from 7 to 6.8 (Fig. 1) which may be due to the production of organic acid.\textsuperscript{20} It is expected that the viscosity of fermentation media would be increased and DO would rapidly decrease due to formation of hyphae elements.\textsuperscript{12,13} Therefore, mass transfer within the shake flask will hampered which will indirectly inhibit the fungal growth. It was obvious that xylanase production depends on optimum fungal vegetative growth which will depend on optimum nutrients and physiochemical factors such as agitation, pH etc. However it was reported in several literature that better xylanase production was found from fungal pellet in the submerged cultivation of \textit{A. niger}.\textsuperscript{21} The quality and quantity of fungal pellet depends on optimum fungal vegetative growth and interaction of process parameters. Therefore, optimum fungal growth kept in shake flask will ascertain ideal pellet which eventually leads for maximum xylanase production.

To favor the optimum fungal growth, agitation speed was optimized and kept at 150 rpm up to 48 h which will help to maintain optimum DO demand in fermentation medium. However, the exponential growth was noticed in this phase and residual xylan concentration decreased to 0.84 g/L after 48 h fermentation (Fig. 1). The residual concentration of reducing sugar and pH were found to be 0.28 g/L and 6.8 respectively. To keep ideal fungal growth and DO demand in fermentation medium, the agitation speed was further optimized and kept at 180 rpm up to 72 h due to increase of mass transfer. However, the high viscosity of fermentation medium and low DO were expected in this phase due to exponential fungal growth. It was also observed that the residual xylan concentration was be low (0.04 g/L) and fungal growth was considered to be optimum after 72 h of fermentation. In addition the residual reducing sugar concentration and pH were found to be 0.36 g/L and 6.5 respectively.

\textbf{Effect of feed rate and feed concentration on xylanase production}

An extensive investigation was carried out to find out the optimum feed rate and feed concentration for maximum xylanase production. Therefore, experiment was carried out at different feed rate with various feed concentration and xylanase activity was measured. It has been found that the feed concentration containing 3 g/L of xylan with 50 mM of K\textsubscript{2}HPO\textsubscript{4} after 48 h interval was considered to be optimum in this study. Therefore, the feeding of xylan was given at 72 and 120 h. The residual reducing sugar concentration of 0.3 g/L was maintained at optimum feed rate (Fig. 1). As xylan was feed at 72 h therefore, there was a possibility for further fungal growth. To restrict fungal growth, the agitation speed was further increased to 210 rpm and continued up to 120 h that would inhibit the fungal hyphae formation due to high shearing force and helps in pellet formation. However, in this phase the transformation takes place continuously between hyphae and pellet. It was also found that xylanase production (1250 U) was also increased due to pellet transformation from hyphae. As any hyphae growth was not expected, the agitation speed was further increased to 240 rpm and continued up to 144 h which would enforce pellet transformation. Therefore, xylanase production increased to 2040 U after 144 h of fed batch fermentation. Optimal pH was found to be 6.5 after 72 and 120 h of fermentation. After 144 h of fermentation, the agitation speed was turned down to 150 rpm due to less oxygen demand of pellet. The xylanase production increased to 2524 U after 168 h of fed batch fermentation which was comparatively higher than the recent reports (Table 1).\textsuperscript{22-28} It was interesting to note that the productivity of xylanase production decreased after 168 h of fed batch fermentation. Therefore, fermentation process was turned down after 168 h of fed batch fermentation.

\begin{table}[h]
\centering
\caption{Present status of xylanase production of \textit{Aspergillus} sp.} \\
\begin{tabular}{|c|c|c|c|c|}
\hline
S. N & Fungal strain & Fermentation time (days) & Xylanase activity (U) & References \\
\hline
1. & \textit{Aspergillus} nidulans & 18 & 41 U & 22 \\
2. & \textit{Aspergillus} niger & 4 & 26 U & 23 \\
3. & \textit{Aspergillus} niger \textit{van Tieghem} & 15 & 9 U & 24 \\
4. & \textit{Aspergillus} fumigatus & 12 & 418 U & 25 \\
5. & \textit{Aspergillus} foetidus M RCC - 4898 & 3 & 210 U & 27 \\
6. & \textit{Aspergillus} carneus M34 & 6 & 3600 U & 28 \\
7. & \textit{Aspergillus} niger (KP874102.1) & 7 & 2524 U & Present study \\
\hline
\end{tabular}
\end{table}
Effects of pH on xylanase production

To study the effect of pH on fungal morphology, the variable volume fed batch studies were carried out at different initial pH viz., 7.5, 7 and 6.5 at constant temperature (28°C). The experiment was conducted in a shake flask (1L) containing 200 ml initial volume with optimum condition. As observed, the pH of the medium came down to 7, 6.6 and 6.2 from the initial pH of 7.5, 7 and 6.5 respectively after 48 h of fermentation (Fig. 1). To understand the morphological pattern of mycelia, the fermentation broth was observed under optical Inverted Microscope (Made –Olympus, Model No: CKX41). From the results (Fig. 2a, 2b and 2c), we observed a significant difference among mycelia with respect to morphology. The

Figure 2. The effects of pH on fungal morphology at fed batch fermentation. (Fig. 2a, 2b and 2c are fungal morphology after 48 h incubation at pH of 7.5, 7 and 6.5 respectively; Fig. 2d and 2e are pellet morphology after 72 h and 144 h incubation at different fed pH of 7.5, 7 and 6.5).
length of mycelia at different initial pH of 7.5, 7 and 6.5 were found to be 194, 205.2 and 205.6 μm respectively. Results revealed that fewer branches with shorter hyphae of mycelia at initial pH of 7.5 and 7 in comparison to pH-6.5. However, more mycelial network with higher number of cell aggregates was noticed at initial pH of 6.5. Therefore, final pH came down to 6 after 48 h of fermentation. We also observed a homogeneous dense mycelial network of Aspergillus niger (KP874102.1) at initial pH of 5.5 after 48 h of fermentation. It was interesting to note that at all initial pHs (7.5, 7 and 6.5), dispersed visible pellets were noticed after 72 h of fermentation. The radius of pellets at different initial pH of 7.5, 7 and 6.5 were found to be approx. 1, 1 and 2 mm respectively which may be due to effect of nutrient availability, environmental factors or changes in transport mechanisms.29 The morphology of pellets after 72 h of fermentation was illustrated at Fig. 2d. From the results, it was understood the exponential growth of pellets were noticed in this phases. Since small compact size pellets were expected for high xylanase titer therefore, initial pH of 7.5 or 7 was considered optimum for this study.

This extensive study was carried out to explore the role of fermentation pH on morphology of pellet. It was obvious that the uniform small size of pellet and high number was expected for high xylanase titer. Therefore, fed concentration with feed rate and pH of fed were optimized to achieve high numbers of uniform small sized pellets. In this study, the optimum fed concentration, pH of fed and fed rate were found to be 3g/L of xylan, pH of 7 and 48 h respectively. Therefore, xylan (3 g/L) having a pH of 7 was given at 72 and 120 hr to the feed. It was obvious that the pH of fermentation medium would remain in the range of 6.5 to 7 under optimum condition (Fig. 1). Therefore, the high xylanase titer would be expected if pH of the fermentation medium varies from 6.5 to 7 that could be used as marker for continuation of fed batch process.

In addition, significant number of pellets (approx. 20 pellets per ml) formation was found after 144 h of fermentation at initial pH of 7 which was the maximum in comparison with the other initial pHs. However, diameter of the pellets was found minimum at initial pH of 7.5 (Fig. 2e). It was expected that complete pellet transformation had been taken place after 144 h of fermentation. As the production of xylanase depends on quality and quantity of pellets, optimum pH was determined based on xylanase production in this phase. Maximum xylanase production (2000 U) was found at initial pH of 7 after 144 h of fermentation. Therefore, initial pH of 7 was considered optimum for this study. The decreased pellet formation at higher pH (7.5) could be explained due to inactivation of enzyme which were directly or indirectly responsible for fungal growth.30 The catalytic activity of these enzymes depends on pH of the medium. The enzymes have ionic groups on their active sites and these ionic groups must be in stable form. Variations of the pH of the medium change the ionic form of the active site that affects the reaction rate. Therefore, the number of pellets had decreased with increase of initial pH of the medium after 144 h of fermentation.

**Effects of K2HPO4 on xylanase production**

Earlier studies have suggested a direct influence of various metal ions such as Mn2+, Fe2+, Cu2+, Zn2+ and Mn2+ on the morphology and cell wall composition of A. niger and also in controlling the enzyme production by Aspergillus sp.31-33 However, the influence of these metal ions has been widely reported for medium design purpose. There are few studies reported in fed batch culture about the influence of metal ions on the morphology of the microorganism and the production of enzymes. Therefore, to evaluate the effect of K2HPO4 on fungal morphology, the variable volume fed batch studies were carried out in a 1 l shake flask containing 0.2 litter fermentation medium at pH (7) and temperature (28°C). 50 mM K2HPO4 was used to maintain pH-7 of fed concentration which contained 3 g/L of xylan. The feed was given at 72 and 120 h. Samples were taken after 144 h and analyzed for fungal morphology. Result showed that the presence of K2HPO4 significantly affected both the diameter and perimeter of the pellet (Fig. 2e). In addition, significant number of homogeneous pellets (approx. 20 pellets per ml) was found after 144 h of fermentation which was higher in absence of 50 mM K2HPO4. Obviously high xylanase titer depends on quality and quantity of pellets, therefore, 50 mM K2HPO4 was fed and 3 g/L of xylan based on xylanase production in the feeding phase. Maximum xylanase production (2524 U) was found after 168 h of fermentation when xylan was fed with 50 mM K2HPO4 which was probably due to formation of diffused pellets. The diffused pellets favors nutrient flux and indirectly helps in enzyme synthesis.34 The
experiment was also carried out in presence of 50 mM of Fe$^{2+}$, Zn$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, and Mg$^{2+}$. However, no significant improvement in xylanase production was observed in presence of these metal ions.

**Application of acid-stable xylanase for bio-bleaching of pulp**

Swelling of fiber wall is an important factor for disruption of xylan since xylan is intimately linked to cellulose and lignin within pulp fibers. Increased fiber swelling enhanced the speed of diffusion of bleaching agent. Therefore, xylanase treatment eventually improved the extraction of lignin from pulp as xylanases hydrolyse the xylan polymer. Morphological changes of the banana pulp before and after xylanase treatment were studied by Scanning electron microscopy (SEM) and showed in Fig. 3. From the Fig. 3, it revealed significant changes on the surface of xylanase treated pulp which was clearly visible in scanning electron micrograph. Cracked and flaked off surfaces of xylanase treated banana leaves was due to the action of xylanase which opened the micro fibrils present on the surface. Since modern bleaching does not include chlorine gas and the first stage is nowadays usually carried out with chlorine dioxide only. Conditions normally used in such a stage are: pH 2–3, 40–70°C, 9–13 % pulp consistency, 0.5–1.0 h retention time. It has been found that in this new bleaching process the pulp is treated at acidic pH (2–3), showing better performance than the previous kraft pulp bleaching. Recently it was found that US8426181 B2 xylanase was active and stable at pH 3, therefore, it was restricted only for use at pH 3. In the present study, we observed no significant cellulase activity from fed batch fermentation by *Aspergillus niger* (KP874102.1). However, xylanase from *Aspergillus niger* (KP874102.1) was active and stable over a this pH range of pH 2–5 (Filing for Indian patent; application no. 201631005766) therefore, it could be used widely over pH of 2–3 for modern bleaching process.

The present investigation reports that improved xylanase production could be achieved through optimization of environmental factors during fed batch fermentation of *Aspergillus niger* (KP874102.1). The process parameters viz. initial substrate concentration, feed rate with feed concentration, agitation and pH during feeding phase play a critical role on cell morphology and xylanase production. In the present investigation, pH was considered as a marker for optimizing fed batch process. In submerged cultivation the morphology of filamentous fungi varied from suspension of mycelia to compact pellets. Thus it became obvious that optimal xylanase production could be expected from fungal pellet in submerged cultivation of *A. niger*. We also observed that the quality and quantity of fungal pellet depended the on optimum fungal vegetative growth along with the interaction of process parameters. In addition, the nature of the pellets reduced the viscosity of cultivation medium and facilitated the separation of broth in downstream process. The xylanase from *Aspergillus niger* (KP874102.1) could be used as a bleaching agent.

**Materials and methods**

**Chemicals and analysis**

Beech wood xylan was purchased from Sigma (Sigma Aldrich Co. Ltd., USA). DNS reagent (Merck India), bovine serum albumin (Himedia, India), xylose
(Sigma, USA), bradford reagent (Himedia, India) were used in this study. All other chemicals were of analytical grade commercially available in India. Optical Inverted Microscope (Made –Olympus, Model No: CKX41) with M shot Digital Imaging System 9.0 (Microshot Technology Co. LTD) was used for microscopic analysis.

**Microorganism**

The microorganism used in the present study was a strain of *Aspergillus niger* (KP874102.1) isolated from soil sample (Baramura forest, Tripura west, India) and characterized. The newly isolated strain was submitted at MTCC, Chandigarh, India having accession number MTCC 25055. The microorganism was grown on PDA medium for 5 d at 28°C. It was maintained by regular sub-culturing on PDA medium for 5 d at 28°C. After incubation the plates were stored for 28 d at 4°C for further experiment.

**Seed culture**

A $5 \times 10^8$ fresh culture was inoculated into a 50 ml complex media (pH-7), contained in a 250 ml Erlenmeyer flask containing (g/L); xylan, 1; KNO$_3$, 10; MgSO$_4$, 7H$_2$O, 5; NaCl, 5 and a 1000X trace element solution, 1 ml/L (pH 7). Trace element solution contains (g/L, w/v) ZnSO$_4$, 7H$_2$O, 20; H$_3$BO$_3$, 10; MnCl$_2$, 7H$_2$O, 5; FeSO$_4$, 7H$_2$O, 5; CoCl$_2$, 5H$_2$O, 1.5; CuSO$_4$, 5H$_2$O, 1.5; Na$_2$MoO$_4$, 4H$_2$O, 1) and incubated at 28°C with shaking at 120 rpm.

**Xylanase assay and protein measurement**

Xylanase activity was determined according to Uday et al. (2016). Briefly, 200 μl of fermentation supernatant was added in the reaction mixture containing 1.8 ml of 1% (w/v) beechwood xylan dissolved in 0.2M potassium chloride buffer (pH 2.0) and kept at 50°C for 10 min. The amount of reducing sugar liberated was determined by the 3,5-dinitrosalicylic acid (DNS) method using xylose as a standard. One unit of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1 μ mol of xylose equivalent in one minute under the standard assay conditions.

**Carboxymethyl cellulase activity**

Carboxymethyl cellulase (CMCase) activity was measured according to modified Ghose (1987) and reducing sugar was measured using the 3,5-dinitrosalicylic acid. Briefly, 200 μl of fermentation supernatant was added in the reaction mixture containing 1.8 ml of 1% (w/v) CMC dissolved in 0.2M potassium chloride buffer (pH 2.0) and kept at 50°C for 10 min. The amount of reducing sugar liberated was determined by the 3,5-dinitrosalicylic acid (DNS) method using xylose as a standard. One unit of CMCase activity was defined as the amount of enzyme that catalyzes the release of 1 μ mol of glucose equivalent in one minute under the standard assay conditions.

**Estimation of residual xylan and reducing sugar**

Estimation of residual xylan was carried out by Phenol-Sulfuric Acid method using xylose as a standard. Residual sugar concentration was determined by 3,5-Dinitrosalicylic acid (DNS) method.

**Variable volume fed-batch study**

The experiments were carried out in a 1 L shake flask where 0.2 L fermentation medium was maintained initially. A 5% (v/v) fresh culture was inoculated in shake flask, containing medium at pH-7. The agitation speed was kept at 120 rpm for 3 d at 28°C. The cultivation medium contained the following components viz. (g/L) of xylan (1.5); KNO$_3$ (10); NaCl (5); magnesium sulfate (5); K$_2$HPO$_4$ (5) and CaCO$_3$ (2) and 1000X of trace element (1ml). The xylan at concentration 3 g/L was used as feeding substrate and given at 72 and 120 hr. Samples were taken periodically at an interval of 12 h and analyzed for xylanase production, residual xylan and residual sugar concentration. All experiments were done in triplicate.

To study the effect of initial pH on fungal morphology, the variable volume fed batch study was carried out at different initial pH of 7.5, 7 and 6.5 at constant temperature (28°C). The fed batch fermentation was conducted in a shake flask (1L) contacting 200 ml initial volume with optimum condition. To investigate the effect of feed pH on pellet morphology, the feed (3 g/l of xylan) at different pH (viz., 7.5, 7 and 6.5) was given at 72 and 120 h. Samples were taken after 12 h interval and analyzed for fungal morphology. All experiments were done in triplicate.
To study the effect of K$_2$HPO$_4$ on fungal morphology, the variable volume fed batch studies were carried out in a 1 L shake flask containing 0.2 L fermentation medium at pH (7) and temperature (28°C). The xylan at concentration 3 g/L along with 50 mM K$_2$HPO$_4$ were used as feeding substrates (pH-7) which was given at 72 and 120 h. Samples were taken after 144 h and analyzed for fungal morphology. All experiments were done in triplicate.

**Application of acid-stable xylanase for bio-bleaching of pulp**

To evaluate the potential use of xylanase produced by *Aspergillus niger* (KP874102.1) as bio-bleaching agent, crude xylanase was used for pre-bleaching of banana leaves. The surface of xylanase treated and untreated banana leaves sample was observed with scanning electron microscope (SEM). Banana stem waste after harvesting of the fruits procured as raw material for bio-bleaching studies. It was grinded using mixer grinder, dried in an oven for 2 h. One gram each of dried banana leaves were soaked in 1 mL of 0.2M Potassium chloride buffer (pH 2.0) for a period of 1 h at 40°C ± 1°C. Partly purified xylanase (30 U/mg) from *Aspergillus niger* (KP874102.1) was added and incubated at 40°C for 24 h. Xylanase treated and untreated pulp samples were mounted on stubs and observed under scanning electron microscope.41

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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