



Optimization of Xylanase Production from *Aspergillus spp.* under Solid-state Fermentation Using Lemon Peel as Substrate

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Xylanase is an enzyme that acts on xylan and degrades it into xylose. Xylanase may be produced by several different microbes such as bacteria and fungi. Xylanase has significant applications in food industries for improving dough handling and improving the quality of baked products in the extraction of starch, coffee, and plant oils. Xylanase, along with pectinase and cellulase, has enormous application in the clarification of fruit juices. In the present study, xylanase was produced by solid-state fermentation using lemon peel as substrates. *Aspergillus Niger* was induced to produce xylanase by frequent sub-culturing on a medium containing 2% xylan. OFAT was analyzed by using several fermentation parameters such as moisture content, particle size, incubation period, incubation temperature, peptone concentration, and pH of the extraction buffer. Under optimized conditions, the maximum xylanase activity was found for particle size of 1.7mm, moisture content of 80%, peptone concentration in nutrient solution at 0.3%, and extraction pH of 7.0. Hence, xylanase can be further subjected to down streaming processes and therefore for various applications.

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1. INTRODUCTION

Xylanase catalyze the hydrolysis of xylan. These enzymes actively take part in the breakdown of plant cell walls and other enzymes that hydrolyze polysaccharides and digest xylan during the germination of some seeds (e.g., in the malting of barley grain). The Xylanase can be found in the marine algae protozoans, crustaceans, insects, snails, and seeds of land plants. Among microbial sources, filamentous fungi are fascinating as they secrete these enzymes into the medium, and their levels are very much higher than those found in yeasts and bacteria. Xylanase genes have been isolated from microorganisms of various genera and expressed in *Escherichia coli*. The heterologous expression of the gene in A, encoding endo xylanase from *Bacillus*, in the yeast *Saccharomyces cerevisiae* has also been described [1]. The enzymes acting on the xylan backbone are classified in two groups: endo 1,4-xylanases (E.C. 3.2.1.8, xylan xylano hydrolase) and Exo 1,4-xylanases (E.C. 3.2.1.37, D- xylan hydrolase). Endo-1, 4- β -xylanase Endo-1, 4- β -xylanase (1,4- β -D-xylan hydrolase; EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate. Xylan is not attacked randomly, but the bonds selected for hydrolysis depend on the nature of the substrate molecule, i.e., on the chain length, the degree of branching, and the presence of substituent's. The utilization of fruit peels as fermentation raw material offers benefits for being cost-effective and can provide additional revenues to food-processing industries. Various literature reports; describe fermentative production of multienzyme using fruit peels such as those of Mango (*Mangifera indica*), Pomegranate (*Punica granatum*), Apple (*Malus pumila*), Mosambi (*Citrus limetta*), Banana (*Musa acuminata*), and Orange (*Citrus reticulata*) [2].

Xylanase can be produced both by either submerged or solid-state fermentation [3]. Most Xylanase manufacturers have these enzymes using submerged fermentation (SMF) techniques; in fact, SMF as a producing system accounts for nearly 90% of total Xylanase sales worldwide. There is, however, a significant interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes, including Xylanase from fungal origins.

The choice of the substrate is of great importance for the successful production of Xylanase. There are a series of fermentation conditions that also significantly affect the yield of Xylanase. The most critical conditions whose optimization has improved Xylanase production are pH value, temperature, oxygen saturation, and even the agitation of the culture broth [4]. Considerable progress has been made in identifying process parameters that lead to higher levels of xylanase production, influencing the economics of the xylanase production process [5]. Remarkable Fungal xylanases-mediated synthesis of silver nanoparticles for catalytic and biomedical applications [6,7].

The low amount of free liquid in the substrate affects the whole process of SSF. Therefore it becomes the most critical feature of SSF. All the factors in SSF depend upon the amount of water present in the substrate. Due to the less availability of free water in the SSF process than most liquid fermentation, most SSF processes involve fungi. However, there are several reports involving bacteria and yeast. Different species of fungi used in the SSF process include many species of *Aspergillus niger*, *Rhizopus*, *Alternaria*, *Fusarium*, *Monilia*, *Mucor*, *Trichoderma*, and some species of *Penicillium*. Most of the species belong to filamentous fungi. These are best suited because of their ability to spread over and penetrate inside the solid substrate. The other advantage of using filamentous fungi is that the fungal mycelia synthesize and release significant quantities of extracellular hydrolytic enzymes [8].

Microbial growth in SSF generates a substantial amount of metabolic heat. It has been reported that 100-300 kJ of heat per kg of cell mass is developed in SSF process. The temperature increases rapidly because there is little water to absorb the heat or, in other words, mean specific heat capacity of the fermenting mass is much lower than that of water. Therefore, heat generated must be dissipated soon. Most of the microorganisms used in SSF are mesophilic, having optimal temperatures for growth between 20 and 40 °C and maximum growth below 50°C. There are also remarkable changes, which occur in the pH of the substrates. These are mainly for the production of acids due to incomplete oxidation of the substrate or uptake of ammonium ions. As we cannot monitor pH in the

SSF, it is difficult to control the pH. So, it is desirable to use microorganisms that can grow over a wide range of pH and have broad pH optima [6].

Due to the increase in environmental pollution, the demand for natural and eco-friendly methods has increased significantly. Especially in the traditional manufacturing industries such as the paper and pulp industry [9], Xylanase along with pectinase and cellulase are employed as food thickeners, [10] increasing the strength of dough and providing good tolerance during variations in processing parameters and quality of the flour [11]. In the bio-processing of the fabric where the hemicellulosic impurities can be removed without affecting the fiber's strength during the spinning process [12]. Of about 20 bleached kraft mills in the US, Finland, Canada, and other countries use Xylanase as bleaching agents in milling operations to decrease the expenses, decrease chlorine dioxide usage and increase the brightness of pulp [13].

The role of Xylanase in bread making has been studied intensively in recent years [12]. Also used in the bioconversion of lignocelluloses to ethanol, sugar, and wines, enhancing the nutritional aspects of green feed, silage, and deinking of waste paper [14]. The fruit juices are turbid due to the presence of pectic substances and other materials suspended in a stable colloidal system. Enzymes like Xylanase, pectinase, carboxymethyl cellulose, and amylase are commonly used to clarify fruit juices [15]. Xylanase are also used in enhancing the extraction of plant oils, coffee, and starch [16].

2. MATERIALS AND METHODS

2.1 Substrate Materials for Solid-state Fermentation

Fruits peels are reported to be good substrates for Xylanase production in Solid-state fermentation. In the present study, lemon peels were collected from different juice centers and houses in Hubballi city. The peels were sun-dried for 48 hours and then oven-dried at 70°C for 24 hrs. Once the substrates were dried, they were grounded to powder in an electric grinder. To these grounded substrates, a mineral solution was added whose composition is as follows (g/100 ml): yeast extract 0.3, peptone 0.5, NaCl 1.5, Na₂HPO₄·2H₂O 1.1, NaH₂PO₄ 0.61, KCl 0.3 and MgSO₄·7H₂O 0.01. These substrates were

moistened to 100% (W/V). The moistened substrates were autoclaved for 15 minutes at 121°C for proper cooking of the substrate and increased its amenability for microorganisms [17].

2.2 Microorganism Used in SSF

The bacterial and fungal strain *Aspergillums Niger* was employed for the production of Xylanase by SSF. Screening of the microbes was first performed using the xylan agar plate screening method. Screening of lipase-producing bacteria was done by TBA media [1]. A quantitative test for Xylanase production in solid-state fermentation was performed with the best xylanase-producing strains that were previously selected [18]. The organism was cultivated on potato dextrose agar at 37°C for two days and sub-cultured at regular intervals in the departmental laboratory [12].

2.3 Inoculums Preparation

The spores of *Aspergillus niger* were transferred aseptically to 50 ml of sterilized inoculum medium (sterilized at 121°C for 15 minutes) containing (g/100ml): Glucose 2, yeast extract 0.3, peptone 0.5, NaCl 1.5, Na₂HPO₄·2H₂O 1.1, NaH₂PO₄ 0.61, KCl 0.3 and MgSO₄·7H₂O 0.01 in the laminar airflow. The flask was incubated at 37°C for 48 hrs. The homogenous mycelia were used as inoculums [17].

2.4 Solid-state Fermentation

The first trial of experiments was conducted in 250-ml flasks containing 10g (dry weight) powdered solid substrate [2]. The initial moisture content, determined gravimetrically, was adjusted to 100 % by nutrient solution containing(g/100ml) yeast extract 0.3, peptone 0.5, NaCl 1.5, Na₂HPO₄·2H₂O 1.1, NaH₂PO₄ 0.61, KCl 0.3 and MgSO₄·7H₂O 0.01. The flasks were sterilized at 121°C for 30 min and inoculated with 1 ml of spore suspension. After mixing, the flasks were incubated at 37°C for four days (due to the low production of enzymes). A previous kinetic study showed that the highest xylanase activity was achieved within four days of the culture inoculation. According to a preliminary investigation, the initial temperature for the improvement of SSF conditions was fixed at 37°C, which showed that the best xylanase production was obtained at this temperature [19].

2.5 Extraction of Enzyme

The extraction of Xylanase produced by *Aspergillus Niger* in SSF was performed by adding 100 ml of distilled water and keeping the flasks in the incubator shaker for 3 hours at 150 rpm at 37°C [20]. Fermented solids were separated by filtration using a muslin cloth. The Filtrate collected was centrifuged at 10000 rpm for 10 minutes at room temperature. The supernatant was carefully collected and used as crude enzyme extract for determining Xylanase activity [17].

Xylanase activity was measured by incubating 1mL of crude enzyme solution with 1mL of substrate solution at 37°C for 60 minutes. The substrate solution was made up of 1% birchwood xylan (Sigma) in 0.1 M sodium phosphate buffer (pH 7.0). For all the enzyme assays, the reducing sugars released were measured by the 3,5-dinitrosalicylic acid (DNS) method using D-xylose. A spectrophotometer determined xylanase activity at 540 nm [17]. In the current studies, enzyme activity is defined as Nano moles of product (xylose) produced per minute [13].

2.6 During the Initial Stage of Optimization

The effect of particle size, moisture content, incubation temperature, incubation period, peptone concentration, and the extraction pH on the Xylanase production during SSF was studied.

2.7 Statistical Optimization and Analysis

The statistical optimization of process parameters to get a better yield of Xylanase. The optimization is done at two stages screening of important variables by using PB design and RSM [8,21] and [19].

3. RESULTS AND DISCUSSION

3.1 The analysis of OFAT

3.1.1 Effect of particle size

Particle size has a significant effect on xylanase production. The result of varying particle size and moisture content on xylanase production was investigated [21]. The effect of particle size on enzyme activity was studied by taking lemon peel as a substrate. Particles of different sizes ranging

from 0.6mm to 2 mm were chosen. Sieves of varying mesh sizes arranged in decreasing order of mesh size as 2 mm, 1.7 mm, 1.18 mm, 0.6 mm were mounted on a vibrator. Substrates of each particle size were taken in a conical flask (250 ml), and solid-state fermentation was carried out for 48 hrs at 37°C. The crude enzyme was extracted; the absorbance reading of each particle size was recorded for enzyme activity [19]. Larger particles provide better respiration/aeration efficiency due to an increase of inter-particle space. In contrast, a small substrate particle may result in substrate accumulation, which may interfere with microbial respiration/aeration and therefore result in poor growth and enzyme production. In our study, particle size had a significant effect on xylanase production, with the optimum particle size of substrate required to achieve the highest specific activity being 1.5 mm [17].

3.1.2 Effect of moisture content

The substrate was cooked by adding salt solution (w/v) to get different moisture content (60 to 120%). An increase in moisture content from 60% to 80% increases enzyme activity; further increase in moisture content of substrate from 90% to 120% decreases enzyme activity, as shown in Fig. 2. The moisture content of substrate was set using nutrient solution consisting of (g/100ml) yeast extract 0.3, peptone 0.5, NaCl 1.5, Na₂HPO₄.2H₂O 1.1, NaH₂PO₄ 0.61, KCl 0.3 and MgSO₄.7H₂O 0.01, to 60, 70, 80, 90, 100, 110 and 120 %. Xylanase activity was checked after four days of incubation [22]. The highest activity was seen for 60% moisture content [19] reported that the highest Xylanase activity was obtained from wheat bran when the moisture content had its highest value. Low moisture content is known to decrease the metabolic and enzymatic activity, probably due to reduced solubility of nutrients from the solid substrate, low substrate swelling, and higher water tension [21]. Therefore, to study the effect of moisture level, the substrate was moistened with different volumes of nutrient solution. It was taken into consideration that the concentration of medium ingredients was not changed [7].

3.1.3 Effect of incubation time

SSF was carried out at the temperature of 30°C, and activity was analyzed at the interval of 3 days, i.e., at 1st, 3rd, 5th, 7th, 9th, 12th, and 15th day. It was observed that Xylanase activity was high within a period of 48-72 hours, i.e., 4.25 to 6.81

U [23]. Then there was a decrease in activity, probably due to inactivation or hydrolysis by secreted proteases of fungi. Optimum activity of 7.81 U was observed on the 3rd day of incubation, as shown in Fig. 3. Gradual increase

in enzyme activity was observed during the incubation period from 6th day to 14th day due to the availability of desired moisture in the substrate [24].

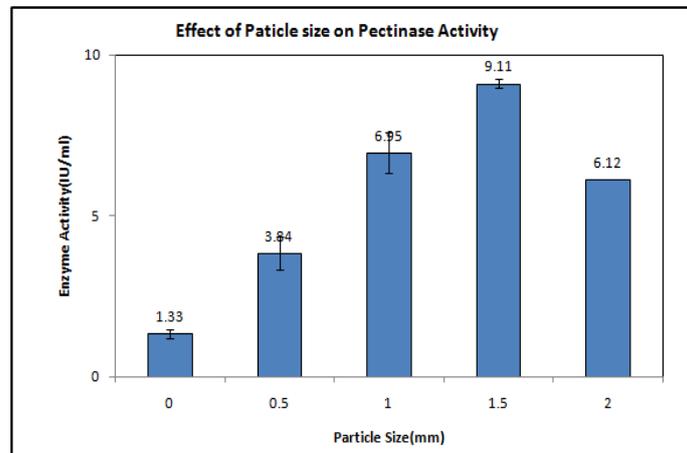


Fig. 1. Graph representing the effect of particle size on Xylanase activity

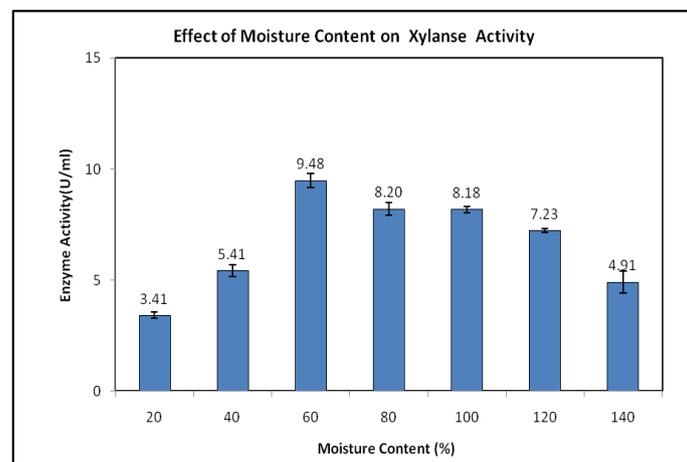


Fig. 2. Graph representing the effect of Moisture content on Xylanase production

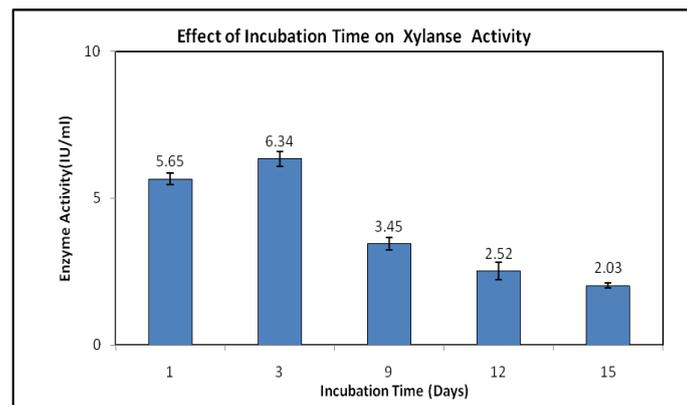


Fig. 3. Graph representing the effect of Incubation time on Xylanase activity

3.1.4 Effect of temperature on enzyme activity

Solid-state fermentation was performed to study the effect of temperature on enzyme activity. Different temperatures considered were 15°C, 30°C, 35°C, and 40°C. After extraction of the enzyme, activity was measured. Enzyme activity increases with an increase in incubation temperature to a maximum and then declines. An increase in temperature increases metabolism and the organisms can consume the substrate, and the enzyme production is higher. An increase in temperature from 30°C to 40°C decreases enzyme activity, whereas an increase in temperature from 25° C to 30° C increases enzyme activity as shown in Fig. 4 The optimum enzyme activity obtained was 9 U at 30°C. A drastic decrease in enzyme activity was observed when the temperature was increased from 30°C to 40°C during SSF [9].

3.1.5 Effect of pH on enzyme activity

SSF was performed to check the effect of extraction buffer pH on enzyme activity. The

crude enzyme was extracted by using buffers of different pH from 3 to 8. The acetate buffer of 0.2M was used for pH 5. A phosphate buffer of 0.1M was used for pH 6, 7, and 8. The enzyme activity was recorded to study the effect of pH of the extraction buffer and also to optimize the condition for pH. An increase in the hydrogen ion concentration considerably influences the enzyme activity. Each enzyme has an optimum pH at which the enzyme activity was maximum. Hydrogen ions affect the enzyme activity by altering the ionic charges on the amino acids, particularly at the active site, substrate, etc. When an enzyme assay for the enzyme was carried out, it was observed that the pH 7 buffer gave the highest enzyme activity of 7.56U [25]. The enzyme activity increased with an increase in pH, with maximum activity at pH 7 and then decreased in activity as shown in Fig. 5. However, in 2014 reported, the production of xylanase was found to be the best at pH 6 of 1.48 U/ml on Wheat straw by *Aspergillus niger* [13,26].

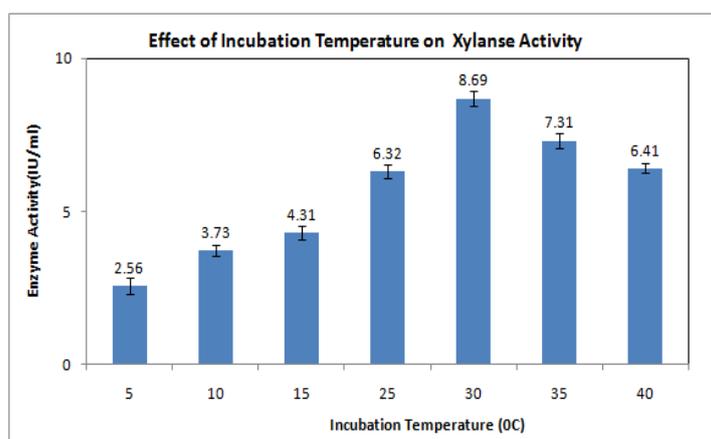


Fig. 4. Graph representing the effect of temperature on xylanase activity

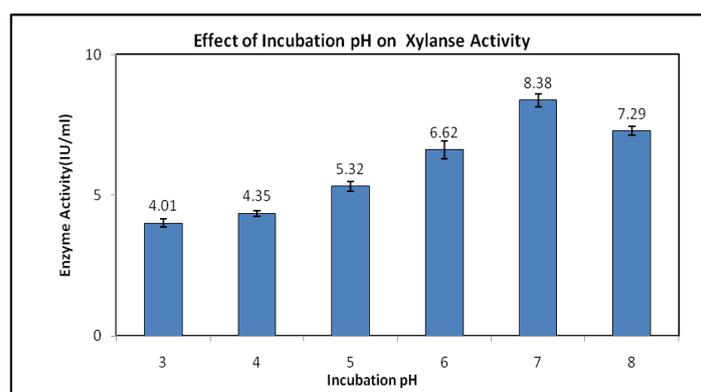


Fig. 5. Graph representing the effect of pH on Xylanase activity

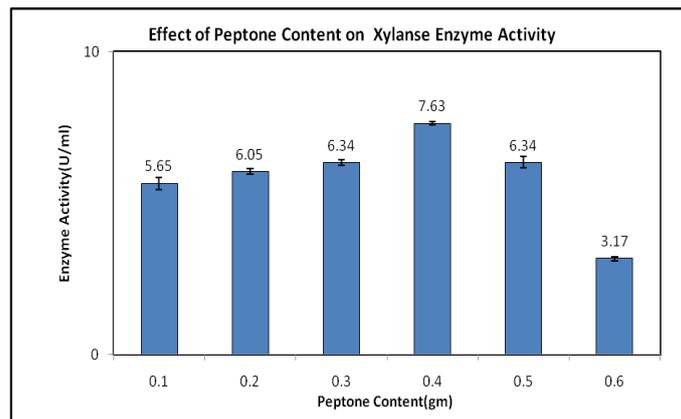


Fig. 6. Effect of peptone concentration on xylanase activity

3.1.6 Effect of peptone on enzyme activity

The additional nitrogen source like peptone enhances the production of hydrolytic enzymes. In this work effect of different concentrations of peptone, the extract was checked on enzyme activity. An increase in peptone content from 0.1 g to 0.3 g increases enzyme activity; further, an increase from 0.4 to 0.6gm should decrease enzyme activity. A typical bell-shaped graph was obtained, as shown in Fig. 6, with maximum enzyme activity of 7.52U at 0.4g [2,11].

4. CONCLUSION

This study reveals that the production of extracellular Xylanase under solid-state fermentation using lemon peels as one of the critical substrate material. This study found that production of extracellular Xylanase by isolated *A. niger* under SSF using lemon peels as substrate/support material has been investigated. SSF is a well-adapted process for the fermentation of filamentous fungi on solid biomass, which is broken down by excreted hydrolytic enzymes. Enzymes can be produced by SSF using solid agro wastes, which is one of the ways by which value addition can be achieved. *Aspergillus Niger* can be induced to make xylanase by growing on a medium containing xylan and minute amounts of glucose as a carbon source. Solid-state fermentation can be carried out for the production of xylanase on lemon peels and parameters like particle size, moisture content, incubation period and temperature, pH of extraction buffer have a significant effect. The optimum conditions for xylanase production obtained in this work were the following: initial moisture content of 60 %, pH of the extraction buffer 7.0, incubation period of 5

days, the particle size of 1.5 mm, peptone content of 0.4g, and temperature at 30°C. The condition of temperature and pH for maximum xylanase activity was similar to other *Aspergillus sp.*

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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