Optimal Conditions For Production Acidic D-Xylanase By *Aspergillus niger* in Submerged Fermentation

الظروف المثلى لانتاج انزيم الزايلييينيز الحامضي من عفن *Aspergillus niger* بتخمرات المزارع السائلة

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المستخلص

استخدمت عزلة *Aspergillus niger* المعزولة من التربة والمنماة على وسط dextrose agar potato والحاوي على الزايلان كمادة اوليه لانتاج انزيم الزايلييينيز ، وعلى وسط التخمر السائل المضاف اليه الزايلان (قش الرز) كمادة اساس للعزل . وجد ان اضافة الزايلان الى وسط النمو رفع من انتاجية انزيم الزايلييينيز ووجد ايضا كمدعم أولي لانتاج الانزيم . قدرت الظروف المثلى لانتاج الانزيم كالاتي: سجلت أمثل فعالية لانتاج الانزيم عند الرقم الهيدروجيني الامثل 5.0 (61.221 U/ml) ، كان أفضل مصدر نيتروجيني لانتاج الانزيم مستخلص خميرة سجل عنده اعلى فعالية (89.71 U/ml) ، سجلت اعلى فعالية للانزيم عند افضل مصدر كاربوني سكر الزيلوز (88.69 U/ml) درجة الحرارة المثلى 40 م سجلت عندها امثل فعالية كانت (35.15 U/ml) ، مدة الحضن 7.0 أيام وسجلت أمثل فعالية عندها (52.33 U/ml) ، وامثل تركيز مادة اساس 0.1 % سجلت عنده الفعالية (45.95 U/ml) ، وامثل حجم القاح كان 10^6 1X سبور / مل كانت أمثل فعالية عنده (57.19 U/ml) .

Abstract

The Xylanase producing strain *Aspergillus niger* was isolated from soil on potato dextrose agar in the presence of xylan as its first substrate for primary isolation, and then grown under liquid medium fermentation in the presence of crude xylan (rice husk) to produce D-Xylanase. the optimum conditions were determined as follows: the Optimum pH for xylanase production was found pH 5.0, xylanase was induced by xylan (rice husk) 0.1% and the production was (61.221 U/ml) and nitrogen source Yeast extract recorded highest enzyme production(89.71 U/ml), and repressed by carbon source xylose the highest enzyme production (88.69 U/ml). The optimum temperature was 40°c for xylanase production was (35.15 U/ml), the optimum period after 7 days of incubation was (52.33 U/ml), the optimum substrate concentration 0.1% was (45.95 U/ml), and the optimum inoculum size was 1 x 10^6 (spore /ml) recorded (57.19 U/ml).

Introduction

Xylan is the major hemicellulose constituent of hard wood and soft wood, and is the next most abundant renewable polysaccharide after cellulose. Xylan is a heterogeneous carbohydrate, consisting of a homopolymeric backbone of β-1, 4 linked D-xylopyranose units and short chain branches consisting of O-acetyl, α -L-arabinofuranosyl and α-D-glucuronyl residues. Endo-xylanase (β-1, 4-Dxylan-xylanohydrolase, E.C 3.2.1.8) is the key enzyme for xylan depolymerization. A large number of bacteria and fungi are known to produce xylanases [1; 2]. Filamentous fungi are industrially important producers of this enzyme due to extracellular release

of xylanases, higher yield com-pared to yeast and bacteria and production of several auxiliary enzymes that are necessary for debranching of the substituted xylans [3]. However, fungal xylanases are generally associated with concurrent production of cellulases. Xylanases are produced by either solid state or submerged fermentation [3]. Microbial xylanases are used in the animal feed, textile and food processing industries, and in the production of several valuable products like xylitol and ethanol [4]. Biobleaching of pulps using xylanase is one of the most suitable applications in the pulp and paper industry to reduce and/or eliminate the use of chlorine and chlorine dioxide. Main objectives of the study included isolation of potential xylanase-producing fungi from soil and to determine the optimum conditions.

Materials and methods

Isolation of fungi

Fungal isolate was isolated from soil samples collected from different places. The dilution plate-method was employed for the isolation of fungal strains [5]. Potato dextrose agar (PDA) medium containing 0.1% (w/v) xylan was used as isolation medium. The plates were incubated at 25ºC for 7 days. The fungal strains were transferred to fresh PDA plates containing 0.1% (w/v) to determine quantity activity.

Xylanase production by liquid medium fermentation:

The composition of mineral salts medium was (g.L-1): KCl, 0.5; MgSO4.7H2O, 0.5; (NH4)2HPO4, 2.5; NaH2PO4, 0.5; CaCl2.2H2O, 0.01; FeSO4.7H2O, 0.01; ZnSO4.7H2O, 0.002 and rice husk xylan, 1.0 as a substrate. The pH of the medium was adjusted to 5.0. Fifty ml of the medium was transferred into a 250 ml erlenmeyer flask, and after autoclaving was inoculated with 1 ml of spore suspension containing 1 x 10^6 spores/ ml. The flasks were incubated at 25ºC on a rotary shaker (100) rpm for 7 days. After incubation, the medium was filtered through gauze cheese cloth and the filtrate was centrifuged at 6 000 x g for 15 min at 4ºC. The clear supernatant was used as crude xylanase [6].

Enzyme assays

Xylanase activity was determined by mixing 0.9 ml of 1% (w/v) xylan (prepared in 50 mM Na-citrate buffer, pH 5.3) with 0.1 ml of suitably diluted enzyme and the mixture was incubated at 50°C for 5 min [7]. The reaction was stopped by addition of 1.5 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 5 min [8]. After cooling, the color developed was read at 540nm. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 mmol of xylose equivalents per minute under the assay conditions.

Optimization of the xylanase production

1. **pH optimum**

   The optimum pH of the acidic D- xylanase enzyme production was determined by using different pH (4.0, 5.0, 6.0, 7.0, 8.0) after fixation the rest factors.

2. **Nitrogen source**

   The nitrogen source optimum of D- xylanase enzyme production was determined by using different nitrogen sources (2.5 g.L-1) (peptone, ammonium nitrate, yeast extract, ammonium sulphate and urea) after fixation the rest factors.

3. **Carbon source**

The carbon source optimum of D- xylanase enzyme production was determined by using different carbon sources (1.0 gL-1) (maltose, fructose, sucrose, lactose, xylose) after fixation the rest factors.

Temperature optimum

The influence of temperature of the production of D- xylanase was studied by incubating media at different temperatures (25, 30, 35, 40, 45) °C after fixation the rest factors.

4. **Incubation period**

The incubation period of D- xylanase enzyme production was determined by using different (5, 7, 9, 11, 13) days after fixation the rest factors.

5. **Substrare concentration**

Different substrate concentrations of rice husk (0.05, 0.1, 0.3, 0.5, 0.7)% were used in the submerged fermentation medium after fixation the rest factors.

6. **Inoculum size**

The influence of inoculation represented 1 x 10^6 of the production D- xylanase was studied by inoculating media at different volumes (1 x 10^6, 2 x 10^6, 3 x 10^6, 4 x 10^6 , 5 x 10^6) spore /ml after fixation the rest factors.

Results and Discussion

Effect of pH on xylanase production

The results showed that xylanase production by *A. niger* was very much dependent on pH, and the optimum initial pH was between pH (5, 6) Figure (1). However, when the pH was increased or decreased to values other than 4.5, the production of xylanase activity gradually decreased. This might be due to the fact that increasing of pH has inhibitory effect on the growth of *A. niger* and enzyme production. The initial pH influences the transport of several species of enzyme across the cell membrane. In addition, cultivation of fungi at an unfavorable pH value may limit growth rate and xylanase production by reducing accessibility of the hemicellulosic substrate [9, 10].

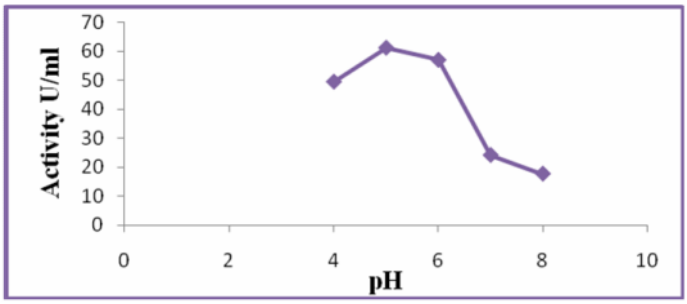

Fig (1): Effect of pH on the production of xylanase by *Aspergillus Niger*, at pH5, Temp.40◦C in liquid medium at 7 days

Effect of nitrogen source on xylanase production

Varying of nitrogen sources include peptone, ammonium sulphate, yeast extract, ammonium nitrate, urea were used. We studied, in which yeast extract gave the maximum enzyme activity (89.71)U/ml Among the various inorganic and organic nitrogen sources tested, 0.25% of yeast extract was the best in stimulating xylanase production by *Aspergillus niger* and increase in enzyme activity was obtained

compared to others Figure (2). Nitrogen sources have a dramatic effect on the production of xylanolytic enzyme by fungi [11]. Our results are in good agreement with those of [12]. Peptone, ammonium sulphate, ammonium nitrate and urea were also effective in inducing the enzyme. The other nitrogen compounds tested were less efficient. These results are in agreement with [12, 13].

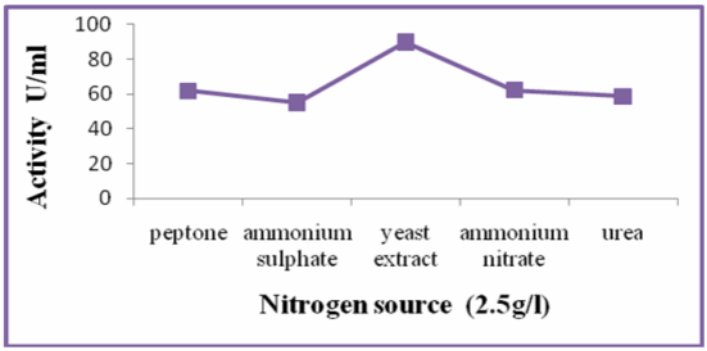

Fig (2): Effect of nitrogen source on the production of xylanase by *Aspergillus Niger*, at pH5, Temp.40◦ C in liquid medium at 7 days

Effect of carbon sources on xylanase production

The results in Figure (3) showed the highest activity of enzyme production in carbon source D- xylose recored (88.69) U/ml, While low xylanase production of sucrose, maltose, lactose (14.62, 10.19, 14.17) and fructose (83. 92) U/ml was detected in the medium containing sucrose, fructose, maltose and lactose respectively, in the medium containing xylan indicate the inducible nature of enzyme production by *A. niger*. The results are in agreement with the results of [14] on *Aspergillus nidulans.* [1] suggested that low molecular mass degradation products of xylan and cellulose hydrolysis penetrate into the cells and induce the production of hydrolytic enzymes [15] reported that xylose, the ultimate breakdown product of xylan, serves as a good inducer of this enzyme.

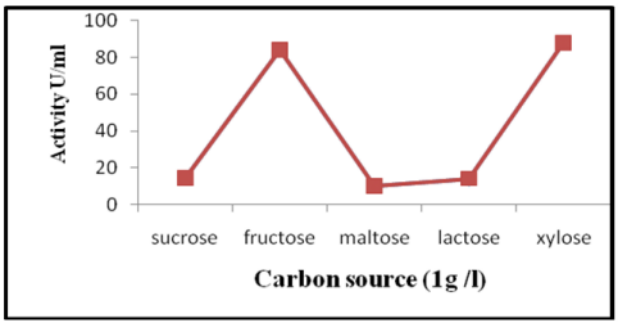

Fig (3): Effect of carbon source on the production of xylanase by *Aspergillus niger*, at pH5, Temp.40◦ C in liquid medium at 7 days

Effect of tempertuer on xylanase production

To study the effect of different temperature, fungal was grown at various temperatures (25, 30, 35, 40, 45) °C using production medium containing 0.1% xylan as the main substrate. Maximum xylanase production was observed between temperatures (35-40)°C Figure (4). An optimum temperature of 40°C for maximum biomass and xylanase production has also been reported by [16]. Xylanase production by this fungus was tested at four different temperatures (25, 30, 35, 45) °C, with 40°C being the best temperature for xylanase production (35.15) U/ml. The resu

similar to those obtained by other authors who established that the best temperature range for xylanase activity is between 20 °C and 30 °C [17, 18, 19, 20].

The best growth temperature for the fungus is not always the best for enzyme activity. However, [21], using *Aspergillus niger*, verified that optimal temperatures for enzyme activity and fungal growth were similar, which is also in agreement with the results are obtained by [22]. According to [23], the large decreases at very low or high temperatures are because the fungal growth is inhibited at these temperatures, leading to a decrease in enzyme synthesis.

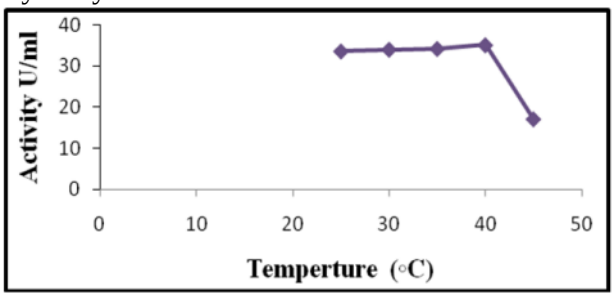

Fig (4): Effect of temperature on the production of xylanase by *Aspergillus niger.* at pH5, Temp 40◦ C in liquid medium at 7 days.

Effect of Incubation period on xylanase production

The xylanase production was investigated and maximum production was observed after 7 days (52.33 U/ml) while minimum was noted at 13 days (14.56) U/ml Figure (5). Further incubation after this did not show any increment in the level of enzyme production. [13] calculated highest activities of xylanase during the present study, *A. niger* produced maximum enzyme at 7 days of fermentation. Xylanase yield increased gradually with time however, after 9 days the depletion of nutrients from the culture medium caused negative impact on fungal growth thus resulted in reduced enzyme synthesis. The findings of the present study are supported by the results reported by [24].

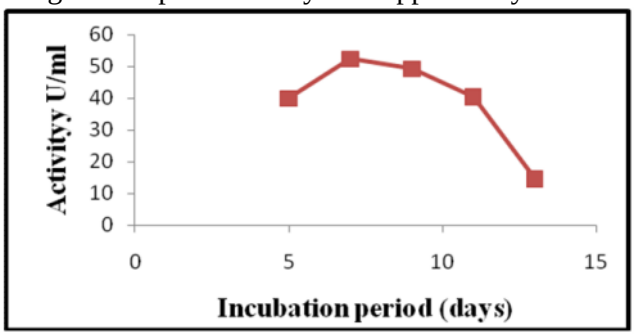

Fig (5): Effect of incubation period on the production of xylanase by *Aspergillus Niger,* at pH5, Temp.40◦ C in liquid medium at 7 days.

Effect substrate concentration on xylanase production

When different substrate concentrations rice husk (0.05, 0.1, 0.3, 0.5, 0.7)% were used in the submerged fermentation medium, the highest enzyme activity was obtained in 0.1% concentration (45.95) U/ml and minimum at 0.7% (22.54)U/ml Figure (6), rice husk is an inexpensive byproduct, which contains a lot of xylan. Therefore, it is one of the most popular components of complex media for xylanase production [25, 15]. However, the particles suspended in the cultivation medium have to be decomposed to form soluble compounds to be used by the fungus and also protects the fungal mycelium from the shear force. Xylanase is an in[illegible]

and the xylan present in rice husk (xylan) acted as a good inducers for enzyme production [3], reported that addition of small amounts of purified xylan to complex lignocellulosic substrates like wheat bran resulted in considerable enhancement of xylanase production.

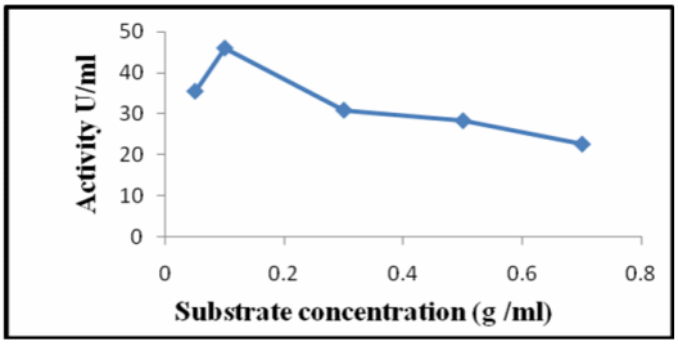

Fig (6): Effect of substarte concentrations on the production of xylanase by *Aspergillus Niger*, at pH5, Temp40◦ C in liquid medium at 7 days

Effect of inoculum size on xylanase production

To study the effect of inoculum size, fungi was grown at various inculum size ($1x10^6$, $2x10^6$, $3x10^6$, $4x10^6$, $5x10^6$) spore /ml using production medium containing 0.1% xylan as the main substrate. Maximum xylanase production was observed in $1x10^6$ of spore/ml suspension the inoculum concentration of 1 $x10^6$ spores/ml contributed to the maximum xylanase activity (57.19 U/ml) relative to the other concentrations. The lowest activity was observed when using a concentration of 5 x 10^6 spores/ml (20.69) U/ml Figure (7). In order to verify the enzyme activity, the spore concentration in fungi cultivation must be high enough to colonize the substrate particles [26] ; many studies, however, have indicated that there can be a decline in this activity over a determined spore concentration. [11] obtained maximum xylanase activity by *Fusarium oxysporium* using $1x10^7$ spores/ml; on the other hand, using 2 x 10^7 spores/ml, they achieved the same level of activity, and the one containing higher concentrations of spores led to a decrease in activity. [19] observed that, during the cultivation of *Aspergillus foetidus*, maximum xylanase activity (210.0) U/ml occurred when the inoculums used had a concentration of 1.5 x 10^8 spores/ml, two times higher than that obtained using 1.5 x $1x10^4$ spores/ml. However, the increase in the inoculum concentration was not beneficial for xylanase activity, verifying that over 10^8, a drastic decrease occurred in activity. As a general mean, the optimal spore concentration is between (1 x 10^6 , 4 x 10^7) spores/ml: outside this range, a decrease in xylanase activity occurred [27].

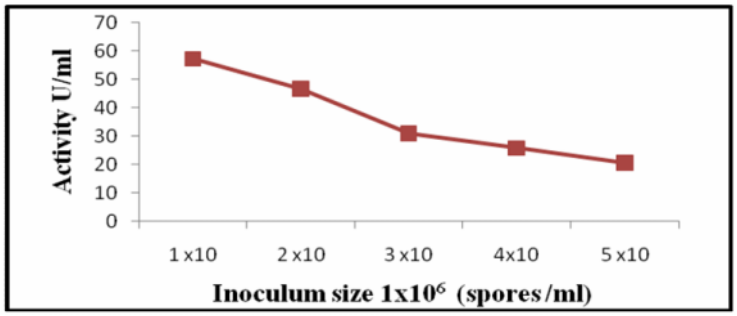

Fig (7): Effect of inculoum size on the production of xylanase by *Aspergillus Niger*, at pH5, Temp.40◦ C in liquid medium at 7 days

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