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Determination of optimum conditions for xylanase production by *Aspergillus niger* using solid state fermentation

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Abstract:
Aspergillus niger is one of the most important filamentous fungi that used in the fermentation industry. *Aspergillus niger* isolate was cultured on potato-dextrose agar (PDA) for activation, and the optimum conditions for xylanase production from this local isolate were studied by solid state fermentation, using a medium composed of wheat bran moisten with corn steep liquor at ratio 1:0.5 (v:w) at initial pH 5.5, inoculated with 1.6×10^6 spores/ml, and incubated at 30°C for 5 days.

Keywords: *Aspergillus niger* ; Xylanase **;**Solid state fermentation; Optimization

تحديد الظروف المثلى لإنتاج انزيم الزايلينيز من فطر *Aspergillus niger* باستخدام تخمرات الحالة الصلبة

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الخلاصة:
يعد فطر *Aspergillus niger* من اهم الفطريات الخيطية التي تستخدم في التخمرات الصناعية. نشطت عزله *Aspergillus niger* بزرعها على وسط مستخلص البطاطا (PDA)، ثم حددت الظروف المثلى لإنتاج انزيم الزايلينيز من العزلة المحلية *Aspergillus nige* باستخدام تخمرات الحالة الصلبة. حيث كانت باستخدام وسط نخاله الحنطة مرطب بمستخلص نقيع الذرة وبنسبه ترطيب 1:0.5 (حجم: وزن) وأس هيدروجيني 5.5، ولقاح 1.6×10^6 سبور/مل، وحضن المزروع بدرجه حراره 30 درجه مئويه ولمده 5ايام.

Introduction:

Xylanase (1,4-ß-d-xylan-xylanhydrolase, EC 3.2.1.8) is catalyzes the hydrolysis of xylan, the major component of hemicellulose in plant cell walls, to xylo-oligosaccharides and xylose. A variety of microorganisms, including bacteria, yeast and filamentous fungi, have been reported to produce xylanases. The potential applications of xylanase, with or without concomitant use of cellulase, include bioconversion of lignocellulose to sugar, ethanol and other useful substances, degradation of arabinoxylans in brewing, clarification of microflitration membrane, and nutritional value improvement of silage and green feed [1]. Xylan is an heterogeneous polysaccharide consisting of β-1,4-linked D-xylosyl residues on the back bone, but also contains arabinose, glucuronic acid and arabinoglucuronic acids linked to the D-xylose back bone. Considering the complexity of the molecular structure, xylan requires two different enzyme activities for hydrolysis. These are xylanase or endoxylanase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8) and β-xylosidase (1,4-D-β-xylan xylohydrolase, E.C. 3.2.1.37), which are responsible of the hydrolysis of main chain, the former attacking the internal main-chain linkages and the latter releasing xylosyl residues by endwise attack of xylooligosaccharides [2]. The genus *Aspergillus* is one of the most important filamentous fungal genera. *Aspergillus* species were used in the fermentation industry, but they are also responsible of various plant and food secondary

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rot, with the consequence of possible accumulation of mycotoxins [3]. *Aspergillus niger* is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose. Production of xylanase by fermentation which is a method of generating enzymes for industrial purposes. Fermentation involves the use of microorganisms, like bacteria and yeast to produce the enzymes. There are two methods of fermentation used to produce enzymes. These are submerged fermentation and solid-state fermentation (SSF) [4].The production of enzymes by SSF has gained much attention in biotechnology studies for production of lipases, inulinase, xylanase, proteases, etc. [5].

The aim of this study was to optimize the media components for xylanase production from *A. niger* by solid-state fermentation (SSF).

Materials and Methods:

Medium and chemicals:

Potato-dextrose agar (PDA) was obtained from Hi-media**,** Coomassie brilliant blue, bovine serum albumin (BSA) and other chemicals were supplied by BDH Chemicals.

Xylanase production:

For activation of local *A. niger*, the isolate was cultured on potato-dextrose agar (PDA), pH 5.5, and incubation for 5 days at 30 ^{o}C. Xylanase production was performed by culturing *A. niger* on solid medium containing 10 gram from wheat bran with moisture ratio of Mandel's medium 1:0.5 (w:v) pH 5.5, (The Mandel's medium was prepared with the following composition (g/L) 10 urea, 0.3 peptone, 0.75 yeast extract, 0.25 $(NH_4)2SO_4$, 1.4 KH_2PO_4, 2.0 $CaCl_2$, 0.3 $MgSO_4.7H_2O$, and trace elements (mg/L): $FeSO_4.7H_2O$, 5; $MnSO_4.4H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4 and $CoCl_2.6H_2O$, 20) and inoculated with 1.6×10^6 spores/ml (the spores number were estimated by direct microscopic counting using haemocytometer), after 6 days of incubation at 30 ^{o}C, enzyme activity and protein concentration were estimated [1].

Estimation of xylanase activity and protein concentration:

Xylanase activity was estimated in solutions resulted after extraction of the enzyme by 50 ml distilled water, using the method described by [6], which depends on xylan as substrate (substrate concentration 1% in 0.2 M sodium acetate pH 5.0). One unit of enzyme activity (IU) is defined as the amount of enzyme which liberates 1 micro moles of xylose/ ml /minute under the assay condition. Protein concentration was estimated according to the method described by bradford depending on bovine serum albumin for standard curve preparation using Coomassie blue G-250, measured at 595 nm [7]. The specific activity was determined by using following equation:

$$\textbf{Specific activity U/mg protein} = \frac{\textbf{Enzyme activity U/ml}}{\textbf{Protein concentration mg/ml}}$$

Optimization for xylanase production:

Local isolate of *A. niger* had been taking from Biotechnology department / Science college-Baghdad University. These isolates were cultured on potato dextrose agar in slant tubes for 5 days at 28°C. Afterwards they were preserved in refrigerator at 4°C until use. Many factors that influence xylanase production from *A. niger*; these factors were; type of substrate, moisture ratio, moistening agent, pH, incubation temperature, Incubation period, carbon source and nitrogen source.

Type of substrate:

Six types of agricultural wastes were tested to determine the optimum substrate for xylanase production from *A. niger* isolate; these sources were wheat bran, corn, rice bran, sugarcane bagasse, corn cob and wood husk. All sources were washed with tap water then sliced to small pieces and dried. These dried parts were grinded until they became powder. 10 gm of each one was moistened with 5 ml Mandel's medium pH 5.5 in 250 ml flask, and inoculated with *A. niger* 1.6×10^6 spores/ml, then incubated at 30^{o}C for six days.

Moisturizing:

Ten gram of wheat bran was moistened with different volumes of Mandel's medium. Different moisture ratios were tested 1:0.25, 1:0.5, 1:0.75, 1:1, 1:1.25 and 1:1.5 (w:v) to select the optimum moisture ratio for xylanase production from *A. niger*.

Moistening agent:

Different moistening solutions were used for xylanase production from *A. niger*; these solutions were; tap water, distilled water, phosphate buffer, normal saline, Mandel's medium, Tris-HCl and mineral salts solution (consist of (g/l): $FeSO_4.7H_2O$, 5; $MnSO_4.4H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4 and $CoCl_2.6H_2O$, 20.0) [8].

Optimum pH:

Production media was distributed into flasks, the pH of tap water was then adjusted to 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, and inoculated with *A. niger*, and then incubated at 30 ^{O}C for 6 days. The xylanase activity and protein concentration was determined after incubation to determine the optimum pH for xylanase production.

Incubation temperature:

The culture which consist of the medium contained on wheat bran (10 gm), moisture by tap water (5 ml), pH 5.5 and inoculated with 1.6×10^6 spores/ml of *A. niger*, was incubated in different temperature degrees (25, 30, 35, 40 , 45 , 50) ^{O}C to estimate the optimum incubation temperature for enzyme production.

Incubation period:

After inoculation the medium wheat bran (10 gm), moisture by tap water (5 ml) at the pH adjusted 5.5 and inoculated with 1.6×10^6 spores/ml of *A. niger*, the culture was incubated at 30 ^{o}C and checked every day for 7 days to estimate enzyme activity, protein concentration.

Carbon source:

Eleven different carbon sources examined to determine the best carbon source for xylanase production from *A. niger*; these sources include; sucrose, glucose, starch, maltose, fructose, xylan, raffinose, mannitol, galactose, cellulose, and xylose at 5% (w/v) [9].

Nitrogen source:

Nitrogen sources were examined to determine the best source for xylanase production from *A.niger*, these sources were; peptone, urea, $NaNO_3$, yeast extract, $(NH_4)2SO_4$,corn step liquor, NH_4Cl, $Ca(NO_3)$, gelatin and KNO_3 at 0.075% (w/v) [9].

Results and Discussions:

Type of substrate:

Six types of agricultural wastes were tested to determine the optimum substrate for xylanase production from *A. niger* isolate. These sources were wheat bran, corn, rice bran, sugarcane bagasse, corn cob and wood husk Figure-1. The results shown that best substrate for enzyme production was wheat bran with high specific activity 46.7 U/mg, while corn cob, rice bran, corn, sugarcane bagasse, and wood husk showed specific activities as follows 36.2, 33.5, 25.14, 23.7 and 11.8 U/mg, respectively. This indicates that wheat bran is the most efficient source for xylanase production from *A. niger*, because wheat bran has been mentioned as a good carbon source owing to its nutritional characteristics, such as 14% proteins, 27% carbohydrates, 5% minerals, 6% fatty acids, vitamin B and 64% nitrogen [9].

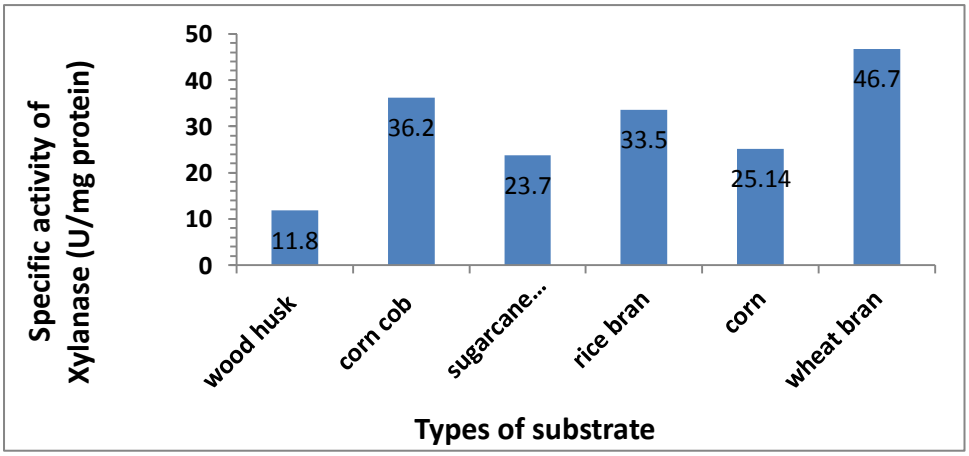

Figure 1- Effect of different agricultural wastes as substrate on xylanase production from *A. niger*, moistened with (1:0.5 w:v) Mandel's medium pH 5.5, incubation for 6 days at 30 ^{o}C.

Kanimozhi and Nagalakshmi [9], proved that production of xylanase from *A. niger* depends on changing the media components, and found that wheat straw was efficient medium for xylanase production from same isolate. While Kheng and Omar [8], found that the best medium for xylanase production from *A. niger* was wheat bran with maltose as carbon source.

Moisturizing ratio:

To determine the best moisturizing ratio for xylanase production from *A. niger*, six proportions were used. These results prove that the highest specific activity of xylanase produced from *A. niger*, was obtained from the moisture ratio 1:0.5 (w:v), with specific activity 48.3 U/mg Figure-2. While the ratio 1:0.25, 1:0.75, 1:1, 1:1.25 and 1:1.5 (w:v), gave 37.8, 22.6, 21.8, 19.9 and 15.2 U/mg respectively. Shahi *et.al.*[10], found that the optimum moisture ratio for xylanase produced from *A. niger* was 83 %. While Ghoshal *et al.*, [11], found that best method for xylanase produced from *Penicillium citrinum* was submerged fermentation.

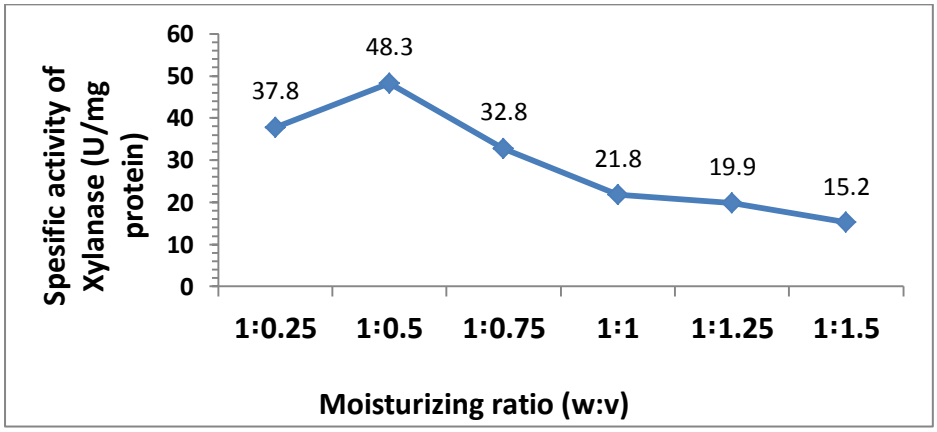

Figure 2- Effect of moisturizing ratio on xylanase production from *A. niger*, using wheat bran moistened with Mandel's medium pH 5.5, incubation for 6 days at 30 °C.

Most of solid substrates used in solid state fermentation are insoluble in water; therefore water will have to be absorbed onto the substrate particles, which can be used by the microorganisms for growth and metabolic activity [12]. Thus, it is concluded that the degree of hydration of the substrate plays an important role in the growth of the fungi and subsequently the enzyme production. Water causes the swelling of the substrate and facilitates good utilization of substrates by the microorganisms. Increasing moisture level is believed to have reduced the porosity of substrate, thus limiting the oxygen transfer into the substrate [13, 14]. Likewise, a lower moisture ratio leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling and a higher water tension [15].

Moistening solutions:

The results showed that tap water was efficient moistening agent for xylanase production from *A. niger* with specific activity 54.9 U/mg Figure-3, while distilled water, phosphate buffer, normal saline, Tris-HCl and mineral salts solution were 51, 8, 48.3, 37.8, 49.7 and 50.3 U/mg respectively. Kheng, and Omar [8], found that best moistening agent for xylanase production from *A. niger* was mineral salts solution.

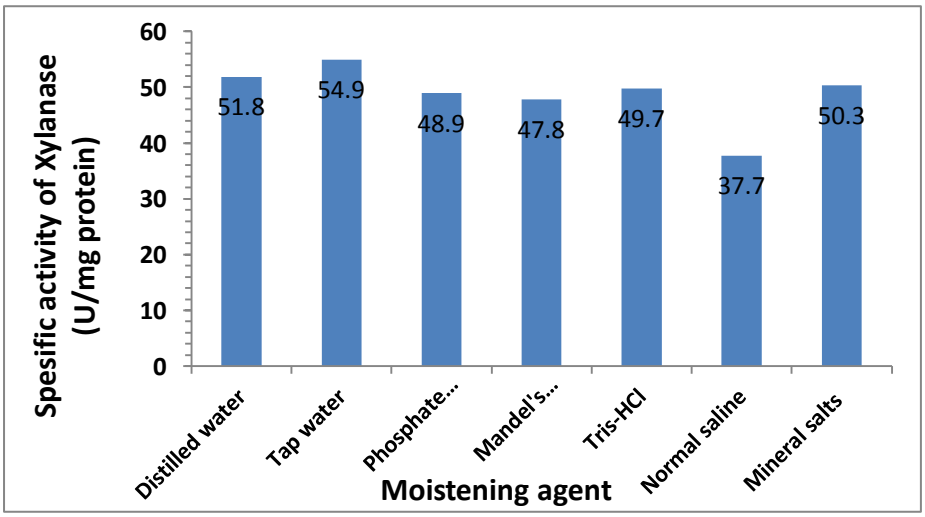

Figure 3- Effect of Moistening agent on xylanase production from *A. niger*, using wheat bran pH 5.5, incubation for 6 days at 30 °C.

Initial pH:

The specific activity of xylanase was estimated after incubation to determine the optimum pH and the results were illustrated in Figure-4, the optimum pH for enzyme activity was 5.5 because gave high specific activity 54.8 U/mg. Umsza-Guez et. al. [16], found that optimum pH for xylanase production from *Aspergillus awamori* was 4 pH, while Kanimozhi and Nagalakshmi [9], found that best pH for xylanase production from *A. niger* was 6 pH.

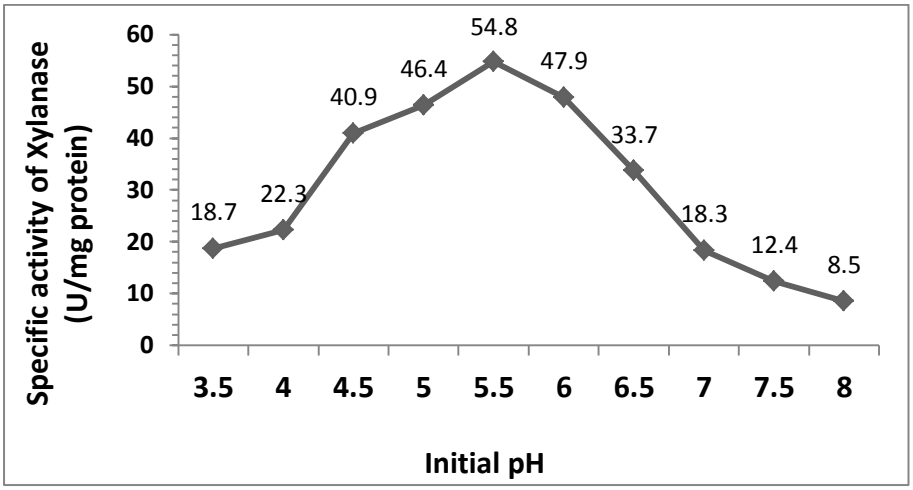

Figure 4- Effect of pH on xylanase production from *A. niger*, using wheat bran moistened with tap water and moisture ratio 1:0.5 (w:v), pH 5.5, incubation for 6 days at 30 °C.

Fungi generally prefer slightly acid conditions and therefore tend to dominate bacteria when these prevail. The reason for the growth rate falling away either side of the optimum value is again due to alterations in three-dimensional protein structure. The pH affects in enzyme production because of its role in the solubility of medium substrates and its effect on the ionization of the substrate and it's availability for the fungal growth. Moreover the pH affects the productivity and enzyme stability [14].

Incubation temperature:

The culture which consist of the medium (wheat bran) with pH 5.5, inoculated with 1.6×10^6 spores/ml of *A. niger* isolate was incubated in different temperature degrees (25, 30, 35, 40, 45, 50) °C to find the optimum incubation temperature for enzyme productivity. The result in Figure-5 shown that the optimum incubation temperature is 30 °C which gave the specific activity of 54.3 U/mg. Lower and higher temperatures decreases the specific activities because of the thermal effects of these temperatures on the microorganism growth and on the enzymatic reaction rate inside the cells which reflects on the vital creation of the enzyme**.** These results agree with the result by Ghoshal, et.al [11]. While Pang et.al. [17], found that optimum temperature for xylanase production from *Trichoderma spp***.** was 30 °C.

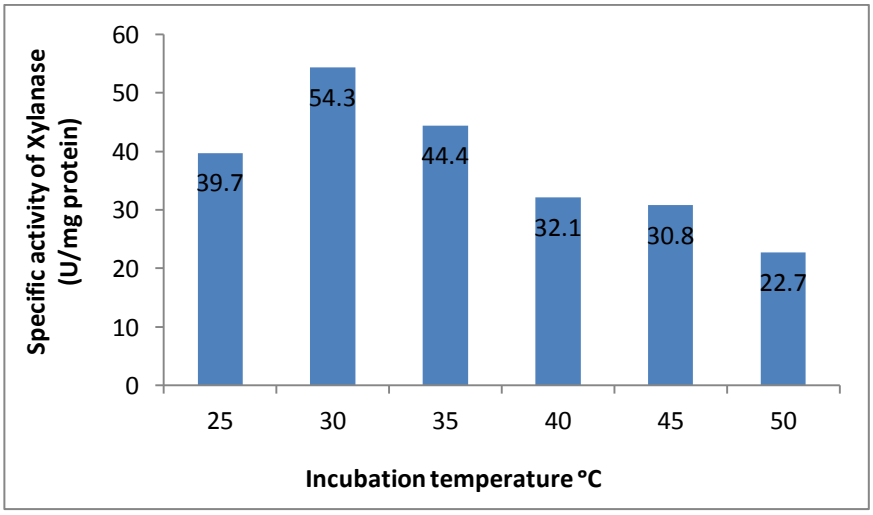

Figure 5- Effect of Incubation temperature on xylanase production from *A. niger*, using wheat bran moistened with tap water and moisture ratio 1:0.5 (w:v), incubation for 6 days at 5.5 pH.

Incubation period:

The results in Figure-6 show the effect of incubation period 1–7 days on xylanase production from *A. niger*. The highest specific activity 57.4 U/mg, was at 5 days of incubation. This result agrees with the result by Umsza-Guez, et.al. [16], while Bhosale, et.al. [18], found that best Incubation period for xylanase production from *Streptomyces rameus* grown on agricultural wastes was 7 days.

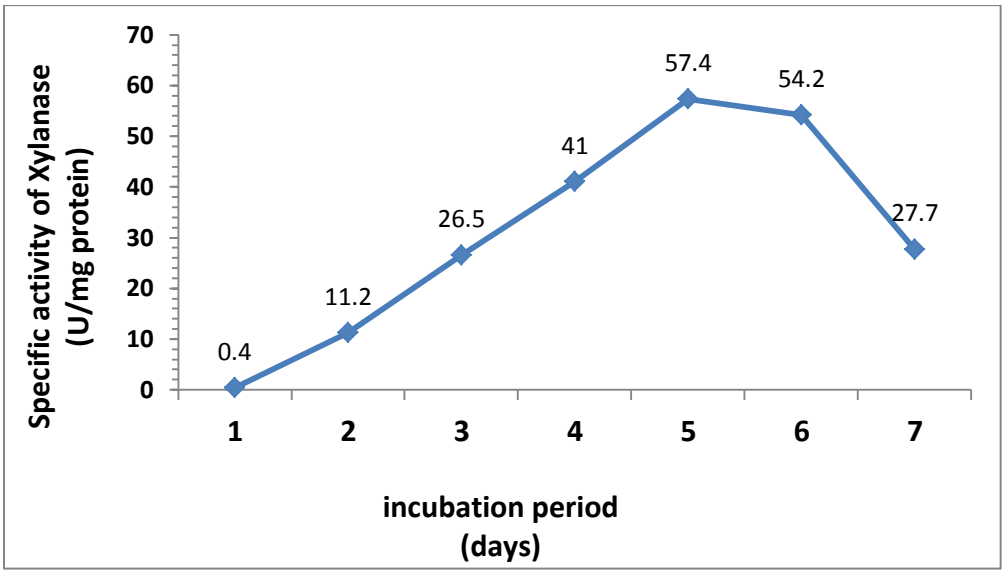

Figure 6- Effect of Incubation period on xylanase production from *A. niger*, using wheat bran moistened with tap water and moisture ratio 1:0.5 (w:v) pH 5.5, incubation at 30 °C.

The enzyme production decreases after 5 days of incubation is due to the production of reducing sugar such as xylose, glucose and fructose in culture medium which may lead to repression of xylanase production because these sugars are more readily carbon source than sucrose. This decrease in enzyme production occurred as a result of the reduction in nutrients of the medium and as a result of accumulation the catabolic repression of enzyme [19].

Optimum carbon source:

Elven carbon sources were tested for their efficiency in xylanase production from *A. niger*. Figure - 7, showed that xylan was efficient carbon source for enzyme production, with specific activity 75.1 U/mg. Knob and Carmona [20], found that Oat spelts xylan was best carbon source for xylanase production by *Penicillium sclerotiorum* with specific activity 24.51 U/mg. while Kanimozhi and Nagalakshmi [9], found that efficient carbon source for xylanase production from *A. niger* was xylose.

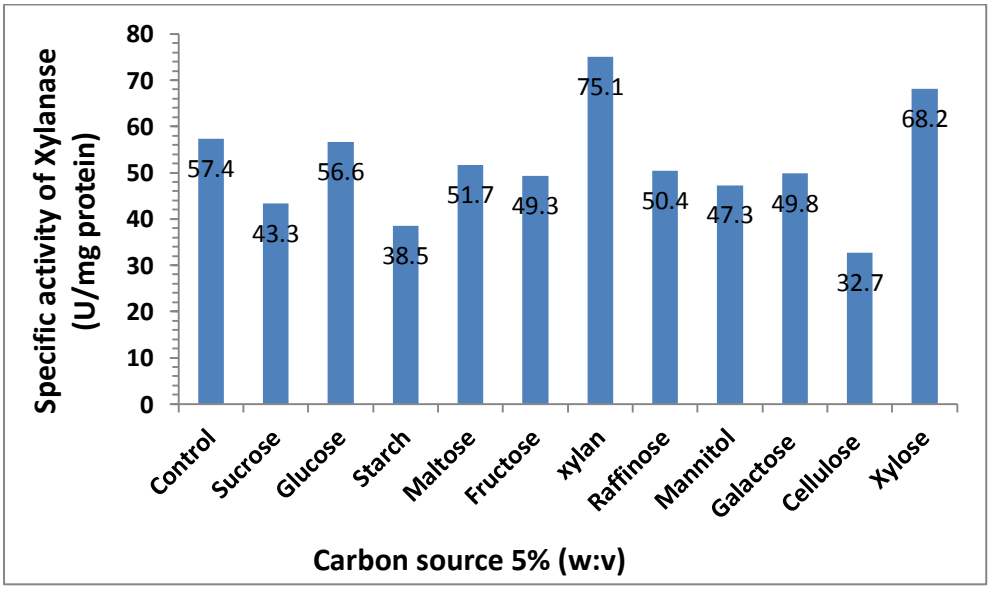

Figure 7- Effect of carbon source on xylanase production from *A. niger*, using wheat bran moistened with tap water and moisture ratio 1:0.5 (w:v) pH 5.5, incubation at 30 °C for 5 days.

Nitrogen source:

Figure-8 showed that corn step liquor was efficient nitrogen sources for xylanase production from *A.niger*, with specific activity 81.3 U/mg. While other nitrogen sources peptone, urea, $NaNO_3$, yeast extract, $(NH_4)2SO_4$, NH_4Cl, $Ca(NO_3)$, gelatin and KNO_3, with specific activity 75.6, 64.8, 79.3, 76.5, 66.5, 73.7, 56.6, 68.8 and 79.1 U/mg, respectively. The presences of nitrogen sources have improved xylanase production by about 12-46%.

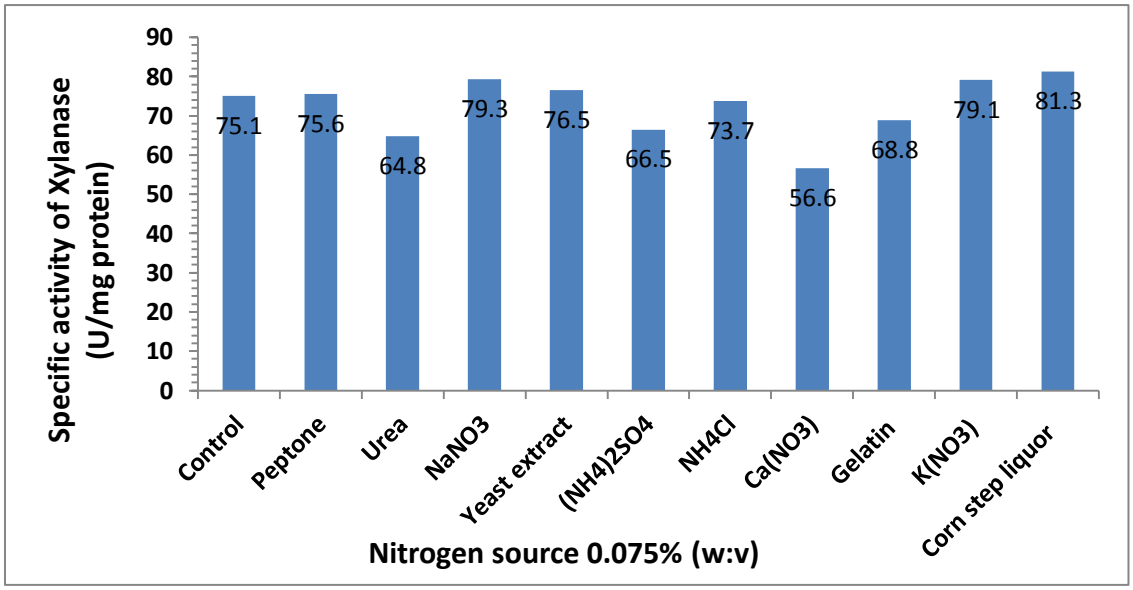

Figure 8- Effect of nitrogen source on xylanase production from *A.niger*, using wheat bran moistened with tap water and moisture ratio 1:0.5 (w:v) pH 5.5, incubation at 30 °C for 5 days.

Bhosale et.al. [18], found that best nitrogen sources for xylanase production from *Streptomyces rameus* was peptone with concentration 1.5%, while Kanimozhi and Nagalakshmi [9], found that NaNO3 was best nitrogen sources for xylanase production from *A.niger*. Kheng and Omar [8], also found that efficient nitrogen sources for xylanase production from *A.niger* was NaNO3. Bedan et.al. [21], found that ammonium nitrate was best nitrogen source for amylase production from mutant isolate of *A. niger* N11.

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