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STUDIES ON EFFECT OF NUTRIENT SOURCES ON XYLANASE PRODUCTION BY ENDOPHYTIC FUNGI *PHOMA EUROPHYRENA* SK 01 STRAIN

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ABSTRACT

Keywords:

Xylanase, *Phoma eupyrena*,
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The aim of the present work is to study the effect of various carbon and nitrogen sources on xylanase production by endophytic isolate supplemented in mineral salt media (MSM) with different concentration of carbon and nitrogen sources under submerged fermentation. The enzyme was purified by ammonium sulphate precipitation (60% saturation) followed by DEAE cellulose chromatography. Among the carbon sources, xylan at 1.0% concentration recorded maximum enzyme production followed by glucose at 0.8%. Carboxyl methyl cellulose and sucrose at all the tested concentration revealed least enzyme production. Among the nitrogen sources, peptone at 1.0 % recorded maximum enzyme production and urea at all the concentration showed least enzyme production. The enzyme was maximally active at pH 5.5 and temperature 50°C. The partially purified enzyme by ammonium sulfate precipitation at 60 % saturation followed by DEAE cellulose column chromatography yields pure xylanase with the molecular weight of 56 KDs.

INTRODUCTION

Xylan, after cellulose, is the most abundant polysaccharide present in wood, agricultural and several agro-industrial wastes. This complex heteropolysaccharide consists of a main chain of 1,4- β -D-xylose monomers containing different substituents or ramifications (Bailey et al, 1992). The substituents including arabinofuranosyl, glucuronyl and acetyl groups show a pronounced influence on its chemical and structural properties and also on the enzymatic degradability of xylan in lignocelluloses (Barbosa *et al*, 1992). In nature, xylan is completely hydrolyzed to monosaccharides by the synergistic action of different enzymes. The xylan-degrading system include endo-1,4-xylanases (1,4- β -xylan xylanohydrolase; EC 3.2.1.8), which release long and short xylooligosaccharides, or those that only attack longer chains and β -D-xylosidase (1,4- β -xylan xylohydrolase; EC 3.2.1.3.7), which remove D-xylose residues from short xylo-oligosaccharides. Enzymes such as β -arabinosidase, β -glucuronidase, ferulic acid esterase and acetyl xylan esterase are very important for the removal of side chain groups from xylan, especially when the fragments of the cleaved heteroxylans present high proportions of branched substituent. Xylanases belong to the group of hemicellulolytic enzymes which are required for the hydrolysis of β -1,4-xylans present in lignocellulosic materials. The use of xylanases in the utilization of lignocellulosic materials is under extensive study because of the production of xylose, which as a fermentation feedstock, is a raw material for single cell protein, or in the production of xylonic acid, xylitol, and ethanol (Beg *et al*, 2000). There is also much interest recently in the use of xylanases in the biobleaching of cellulose pulps, which decreases the demand for chlorines in conventional bleaching in paper making. Several microorganisms have been reported as xylanolytic (Viikari et al, 1994) and most of the bacteria, fungi and yeasts producing xylanases secrete the enzyme extracellularly (Shallom and Shoham, 2003, Collins et al, 2005). From an industrial point of view, filamentous fungi are interesting producers of these enzymes due to xylanases releasing and their easy cultivation. Endophytic fungi are the unique group of microorganism that inhabit in the interior live tissues of plants and to exhibit a wide range of biological activities (Saha, 2003). Pharmaceutical important products such as enzymes, anti microbials and anti tumour agents have extensively reported from

endophytic fungi residing in various plants. In the present study, production and characterization of xylanase produced from endophytic fungi *Phoma eupyrena* (SK1 strain) isolated from leaves of garden weed was discussed.

MATERIALS AND METHODS

Fungal stain *Phoma eupyrena*

Phoma eupyrena was isolated from leaves of *Cyperes* – garden weed adopted by modified method of Guo *et al* and the isolated fungi was identified based on cultural characteristics and microscopic examination of fungal spores with lactophenol cotton blue. The pure culture was maintained on potato dextrose agar slant.

Screening for xylanase-prductions

Purified isolates of *Phoma eupyrena* cultured on oat spelt xylan agar medium and incubated at 28°C for 4 days. The plates were then flooded with absolute ethanol and left for 16 h at room temperature to precipitate xylan. Colonies producing xylanase enzyme were surrounded clear zones against an opaque background of non-hydrolyzed media. Positive results were confirmed in a repeat test. The fungi which showed maximum zone of clearance are selected for further study.

Isolation of crude enzyme

Phoma eupyrena isolate which shows maximum zone of clearance was inoculated into 100 ml of mineral salt medium supplemented with 0.5 % of soluble oat spelt xylan (Sigma) in 250 ml of conical flask at the spore concentration of 10^8 spores/ml. The seeded flasks were incubated at 30°C with 100 rpm for four days in orbital shaker (Scigenics). After the incubation the culture contents were filtered through a 0.45µm pore size filter (HA type; Millipore) and the collected filtrate was used as crude enzyme for further study.

Xylanase activity assay

Assays for crude xylanase were performed using 0.5% soluble oat spelt xylan (Sigma) in 50 mM sodium phosphate buffer, pH 7.0. The reaction mixture was composed of 1.8 ml substrate and 0.2 ml crude enzyme. The mixture was incubated in a water bath at 60°C for 15 min. The released reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNSA) method in which the reaction was stopped by adding 3 ml of DNSA acid

reagent. A reddish brown colour developed after placing the reaction tubes in a boiling water bath for 5 min. After cooling the reaction tubes to room temperature, the O.D. was measured at 575 nm with xylose as the standard, where one unit (U) of xylanase activity is defined as the amount of enzyme that releases 1 μ mol xylose/min/ml under the above mentioned conditions.

Effect of various carbon sources on xylanase production

To detect the effect of various carbon-sources on xylanase production 250 ml Erlenmeyer flasks were prepared containing 100ml of mineral salts medium supplemented with 0.1,0.2,0.4,0.6,0.8 and 1.0% concentration of one of the following carbon sources: glucose, xylose, arabinose, xylan, carboxymethyl cellulose (CMC). Xylanase assays were performed daily.

Effect of nitrogen source

To detect the effect of various nitrogen sources on xylanase production 250 ml Erlenmeyer flasks were prepared containing 100ml of mineral salts medium supplemented with 0.1,0.2,0.4,0.6,0.8 and 1.0% of concentration of one of the following nitrogen sources sodium nitrate, potassium nitrate and ammonium nitrate, peptone. Xylanase assay was performed as described earlier.

Purification

Culture filtrate was submitted to ammonium sulfate precipitation. The fraction from 60 % saturation, which contained 60 % of the total xylanase activity was dialysed for 24 hours at 5°C against three changes of 2 liters of distilled water. The dialysate was lyophilized and stored at -20°C. Twenty milligram of this material was applied to a Bio-gel P-70 DEAE cellulose column (Bio-Rad laboratories, California), eluted with 0.05 M Tris hydrochloric acid- 0.1M KCl (pH 7). Fractions showing xylanase activity were dialysed against 0.001 M Tris hydrochloride buffer (pH 7) for 24 hours at 5°C, lyophilized and stored at -20°C.

SDS- PAGE

SDS PAGE was carried to assess the molecular weight of the protein. The gel was polymerized from a mixture of 17.5 ml of 30% acrylamide -0.8% methylene bis acrylamide- 17.5ml of 1.5M Tris hydrochloride (pH 8.8) – 35 ml of distilled water –

35 ml of N,N,N',N' tetra methylene diamine -0.70ml of ammonium per sulfate (75mg/ml). Electrophoresis was performed at 10°C with 0.05M Tris-glycine buffer (pH 8.3). The protein band was visualized after staining with Commasive brilliant blue followed by destaining with methanol and acetic acid. The molecular weight of the band was identified using standard molecular markers.

Effects of pH and temperature on xylanase activity

The range of buffers were used at 50 mM in preparing 0.5% (w/v)xylan solution for detection of xylanase activity was aceto-acetate buffer (pH 4, 5 and 6), phosphate buffer (pH 7), Tris buffer(pH 8 and 9). The reaction conditions were as mentioned before using the culture filtrate as the enzyme source. The pH value giving the highest enzyme activity was used in further enzyme assays. The optimal temperature for enzyme activity was determined by performing the standard assay procedure at 40,50,60,70,80 and 90 °C was used to determine that is mentioned earlier. All further enzyme assays was performed at the optimum temperature.

RESULT AND DISCUSSION

Generic composition of endophytic fungi

A total of 76 fungi isolates belonging to six different genera such as *Phoma*, *Fusarium*, *Trichoderma*, *Alternaria* and *Aspergillus* were isolated from leaves of *Cyperes*. All the isolates were identified by cultural and morphological characteristics. As shown in the table: I *Phoma* was the predominant genera (45.5%). followed by *Fusarium* (20.5%), *Trichoderma* (17.0) *Pencillium* (10.0%) *Alternaria* (7.0%)

Xylanase producer- *Phoma eupyrena* (SK 01) strain

Among the endophytic fungi isolates, xylanase producers was observed in all the isolates belong to *Phoma eupyrena* and the *Phoma eupyrena* (SK 01) strain was further selected because of the maximum zone of clearance was observed in oat spelt xylan agar medium inoculated with *Phoma eupyrena* (SK 01)

Xylanase production by *Phoma eupyrena* (SK 01) strain

When *Phoma eupyrena* (SK 01) strain grown in mineral salt medium supplemented with 0.5 % of soluble oat spelt xylan, xylanolytic activity was appeared rapidly in the medium. Supernatants of *Phoma eupyrena* (SK 01) strain showed xylanolytic activity

during sixth hour of fermentation time and maximum enzyme production was recorded during 48 hour and the enzyme production was gradually decreased in successive time periods..(Table 2)

Effect of carbon source on xylanase production

Among the various carbon sources , maximum xylanase production with xylanase activity of 12.13 U/ml was recorded in 1.0 % of xylan supplemented MSM followed by 11.54,11.0, 8.99 and 7.86 U/ml of xylanase activity at 0.8,0.6,0.4 and 0.2% of xylan (Figure 1a).Glucose at the respective concentration and maltose at 0.2 % reveals 1.35,1.51,1.91,2.71, 2.34U/ml and 2,53 U/ml of enzyme activity respectively. ((Figure 1b and c)Carboxyl methyl cellulose (CMC) at the concentration of 0.2,0.4,0.6,0.8 and 1.0% reveals least enzyme activity of 0.7,0.3,0.08 ,0.04 and 0.03 U/ml (Figure 1d) 0.25, 0.98,1.44,1.04 and 0.13 U/ml of enzyme activity was recorded in respective concentration of sucrose (Figure 1e)

Effect of nitrogen source on xylanase production

Enzyme production was highly influenced by respective nitrogen source..Among the different nitrogen sources, MSM supplemented with peptone at 1.0% recorded maximum xylanase production with the xylanase activity of 1.01 U/ml (figure 2a) followed by 0.98U/ml at 1.0% of concentration of sodium nitrate (figure 2b).Least enzyme production with enzyme activity of 0.21,0.36,0.21,0.17 and 0.06 U/ml was recorded in respective concentration of urea (Figure 2c)

Purification of xylanase

Table 3 shows a summary of the purification steps.Ammonium sulfate precipitation of filtered culture supernatant showed that xylanase fractionated at 60% saturation.The fractions were submitted to Bio- gel P-70 DEAE cellulose column.Xylanase activity of all the fractions collected from DEAE cellulose column was obtained with a high purity since a single major band appeared after SDS- PAGE which revealed a monomer with a molecular weight of 56KD (Figure 3). Xylanase was lyophilized and stored at -20 ° C and no reduction in activity was observed after 25 days.

Effect of pH and temperature

Xylanase activity was found to be maximum at the pH of 6.0 (10.7U/ml) followed by 5.0 (7.6 U/ml) and the activity was significantly reduced at pH 8.0 and 9.0. (Table 4) The enzyme could retained the maximum activity at the temperature of 60⁰C (10.4U/ml) followed by 50⁰ C and the enzyme activity was significantly reduced in 70,80 and 90⁰ C.(Table 5)

Table 1. Generic composition (%) of endophytic fungi isolated from *Cyperes*

S.No	Fungal genera	Generic composition (%)
1	<i>Phoma</i>	45.5
2	<i>Fusarium</i>	20.5
3	<i>Trichoderma</i>	17.0
4	<i>Penicillium</i>	10.0
5	<i>Alternaria</i>	7.0

Table 2. Xylanase production by *Phoma eupyrena* (SK 01) strain in MSM media supplemented with 0.5 of xylan.

S.No	Fermentation time (Hour)	Enzyme activity (U/ml)
1	6.0	0.07
2	12.0	0.42
3	18.0	0.72
4	24.0	1.73
5	30.0	2.61
6	42.0	4.81
7	48.0	10.0
8	54.0	7.03
9	60.0	4.21
10	66.0	1.04
11	72.0	0.76
12	78.0	0.21
13	84.0	0.08
14	90.0	0.04
15	96.0	0.02

Table 3. Effect of pH on xylanase activity (U/ml)

S.No	pH	Xylanase activity (U/ml)
1	4	4.2
2	5	5.7
3	6	10.7
4	7	4.3
5	8	1.4*
6	9	0.6*

* Significant at 0.5 % level by DMRT

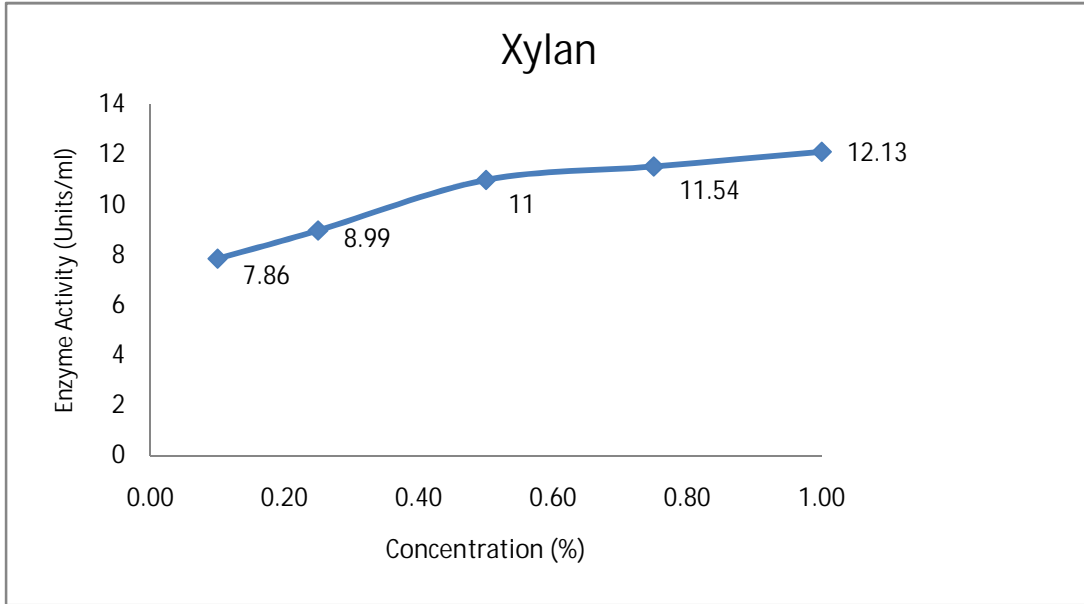
Table 4. Effect of temperature (° C) on xylanase activity (U/ml)

S.No (U/ml)	Temperature (° C)	Xylanase activity
1	40	7.4
2	50	8.7
3	60	10.4
4	70	2.0 *
5	80	0.91*
6	90	0.07*

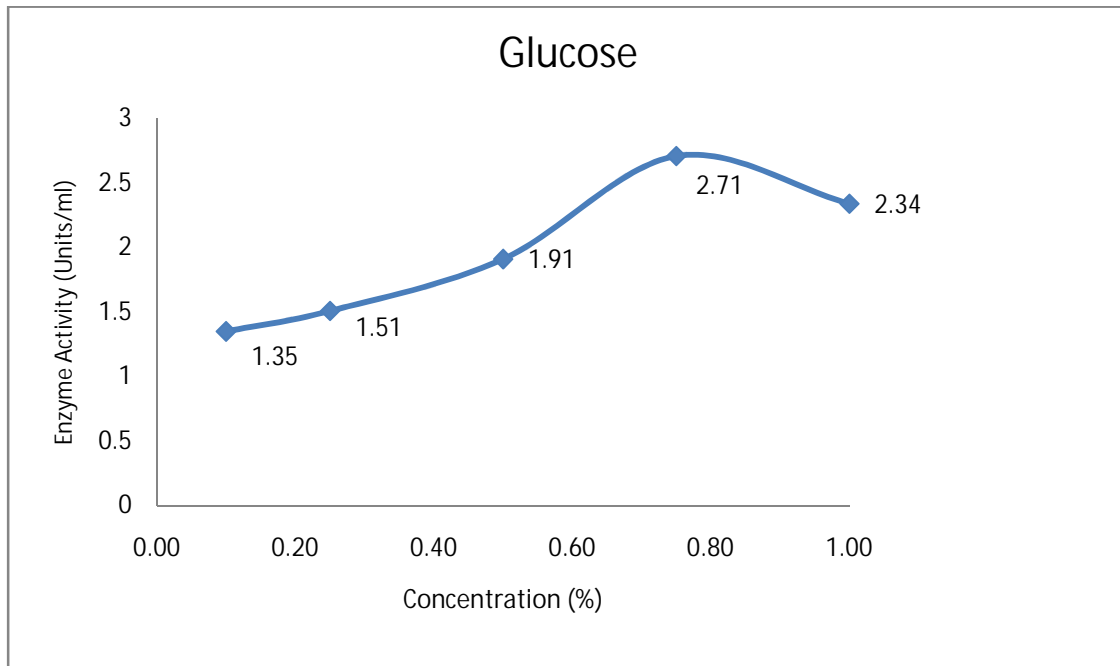
* Significant at 0.5 % level by DMRT

Figure 1. Effect of carbon sources a – xylan, b- glucose c- maltose d- CMC (carboxyl methyl cellulose) e- sucrose on xylanase activity (U.ml)

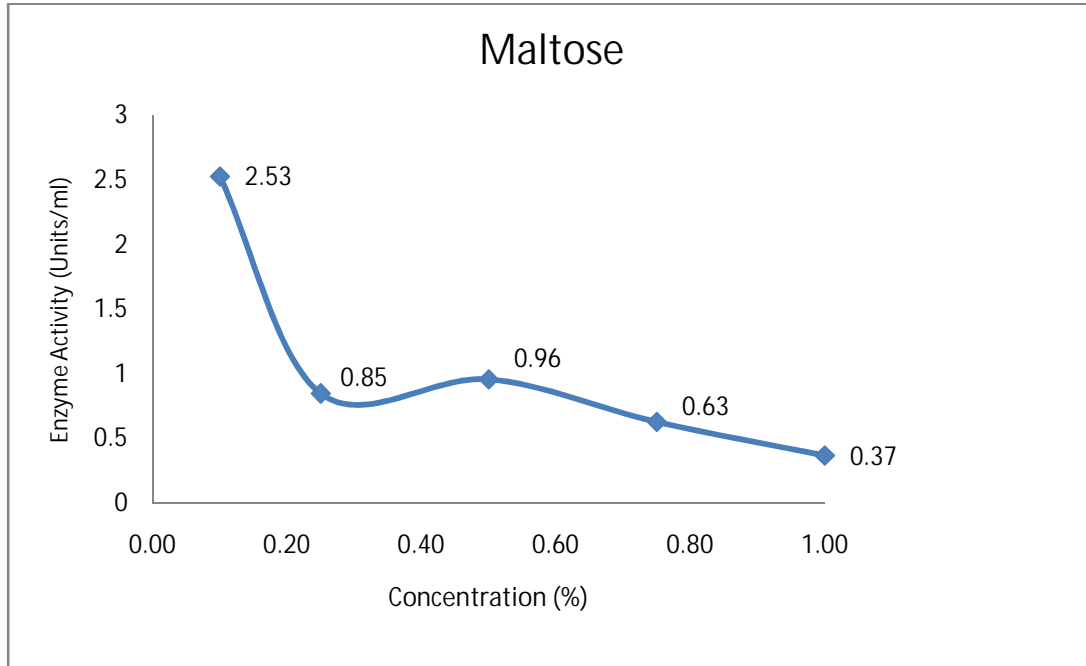
a.



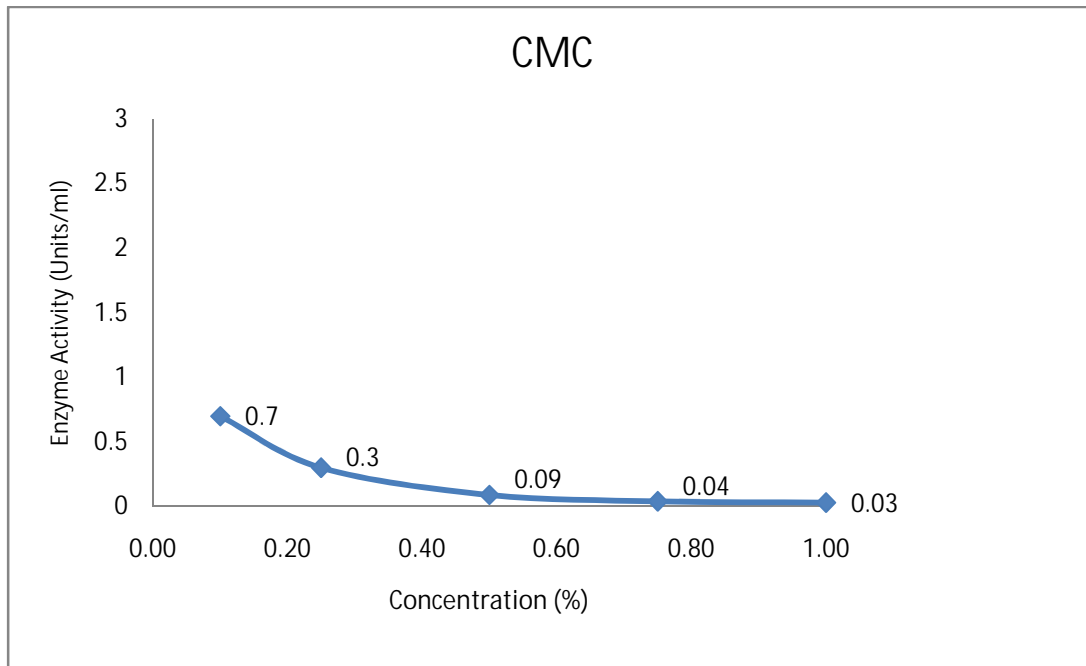
b.



c.



d.



e.

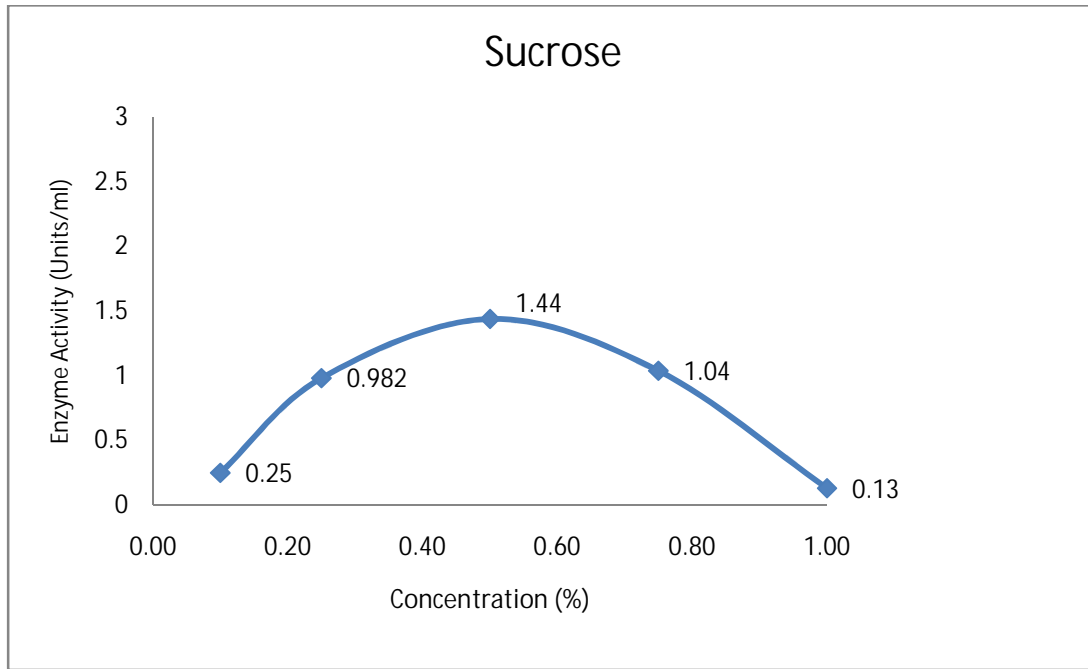
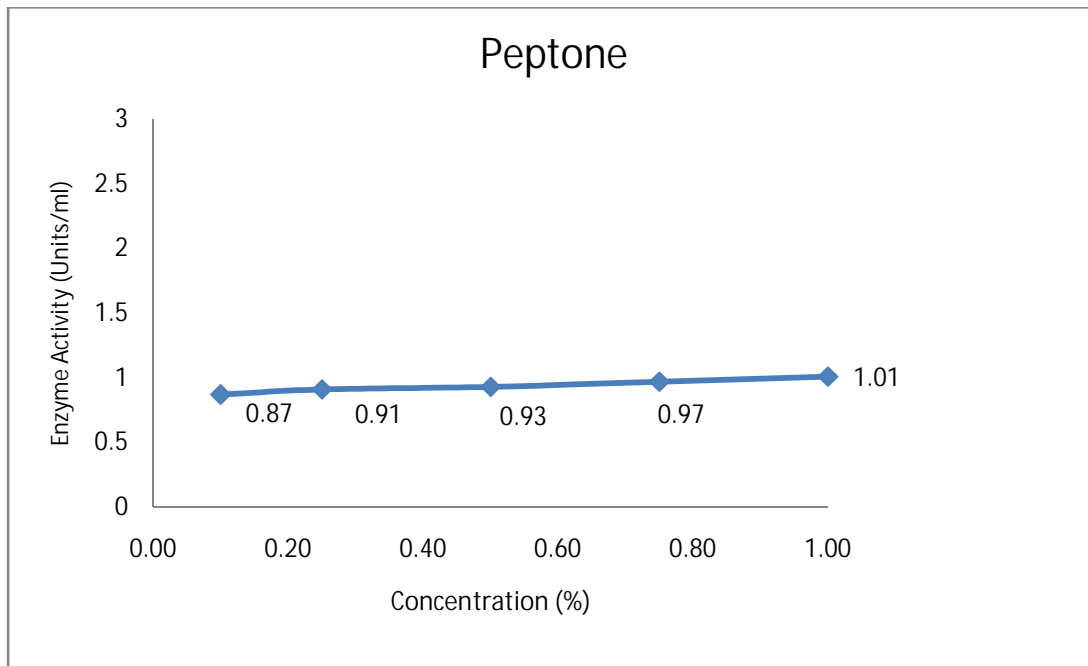
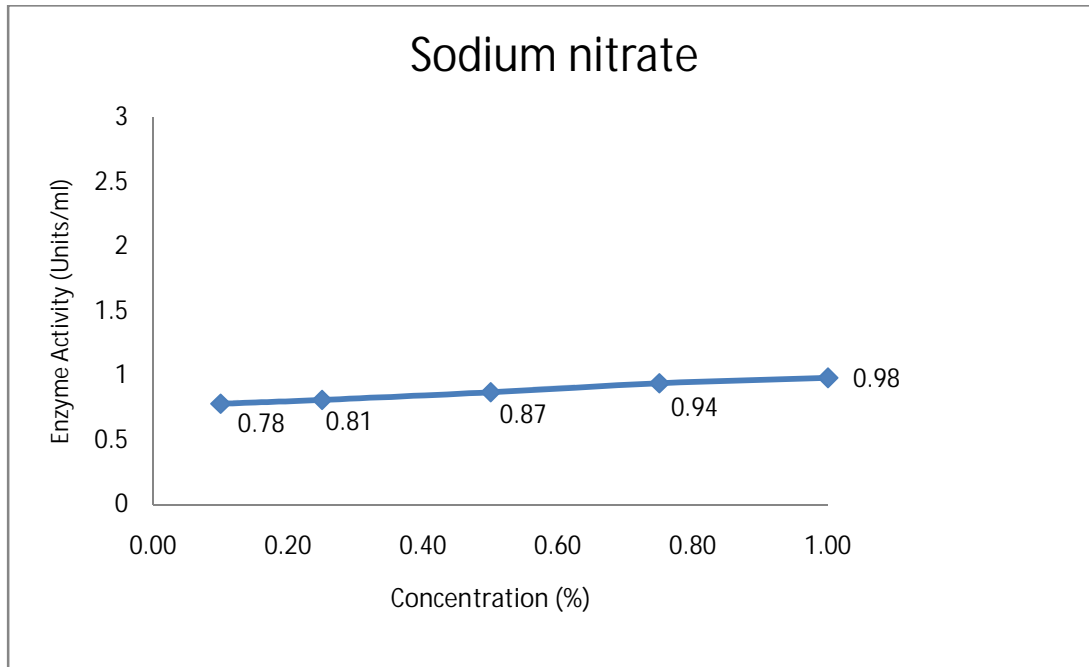


Figure 2. Effect of nitrogen sources a – peptone b- sodium nitrate c- urea On xylanase activity (U/ml)

a.



b.



c.

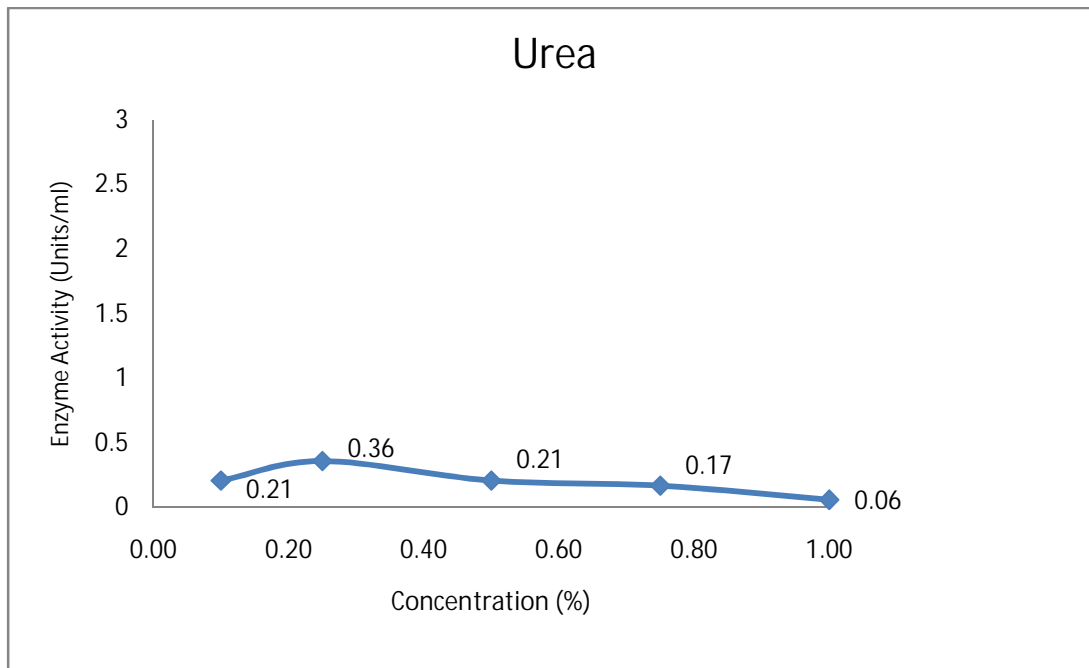
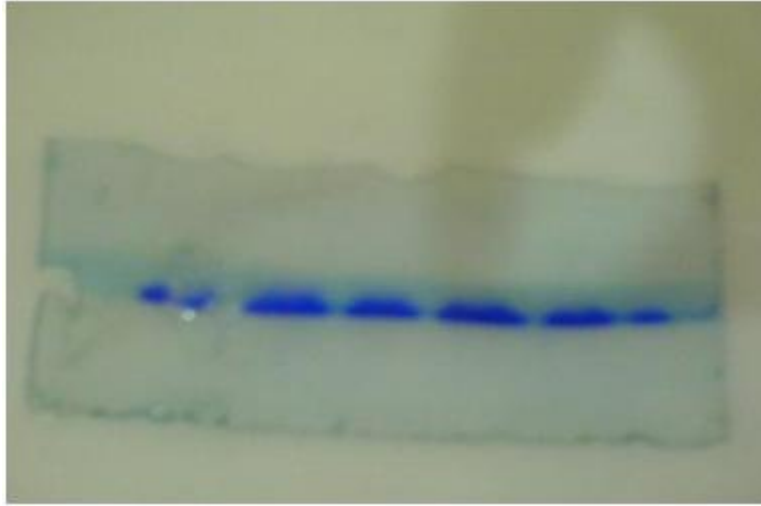


Figure 3. SDS- PAGE analysis of xylanase with the molecular weight of 56 KD

DISCUSSION

Fungi produce a large variety of enzymes of which xylanases are particularly significant industrial importance. Endophytic fungi are the unique group of microorganism that inhabit in the interior live tissues of plants and to exhibit a wide range of biological activities. Pharmaceutical important products such as enzymes, anti microbials and anti tumour agents have extensively reported from endophytic fungi residing in various plants. In the present study, *Phoma eupyrena* (SK 01) strain isolated from leaves of *Cyperes* produced extracellular xylanase in MSM media supplemented with various carbon and nitrogen sources. There have been many reports of xylan binding xylanases that remove side chain from xylan back bones. The culture conditions mainly nutrient factors were found to have profound influence on xylanase production. In the present study, xylanase production by *Phoma eupyrena* (SK 01) strain was maximum in MSM media supplemented with all the concentration of xylan as the carbon source. Similarly, *Streptomyces albus* soil isolate produced maximum production of xylanase in xylan supplemented media. In the case of nitrogen sources, a maximum increase was observed with peptone which is in accordance with finding of Balakrishnan *et al* , Purification of the enzyme revealed that 60 % of the ammonium sulfate fractions further purified by DEAE cellulose column showed xylanase activity was more than 99% of pure as a single

protein band was obtained in SDS- PAGE. A similar purification step was reported for xylanases from *Bacillus stearothermophilus* T-6 and *B.subtilis*.(Khasim ,1993, Bernier,1983) Effect of pH and temperature on xylanase activity revealed the enzyme could retained maximum activity at pH 6 and 60 ° C.. This finding was supported by xylanase from *Bacillus subtilis* and *Penicillium sclerotiorum* In the present study, xylanase from *Phoma eupyrena* (SK 01) strain produced xylanase in a low cost synthetic media and being active at acidic pH and high temperature. These are desirable properties used in large scale production of xylanase and application in paper,pulp and food industries.

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