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Partial purification and characterization of anextracellular xylanase from Penicillium janthinellum and Neurospora crassa

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ABSTRACT

The aim of this experimental study was to isolate and partially purify extracellular xylanase from Penicillium janthinellum and Neurospora crassa.The species were inoculated in a xylanase fermentation medium .The supernatants were collected after 5 days. The partial purification was realized by applying respectively, ammonium sulfate precipitation, dialysis and DEAE-Cellulose ion exchange chromatography to the supernatant. Effect of pH and temperature on enzyme activity and stability were determined. In addition, the molecular mass of the obtained enzyme was investigated by Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE).The final enzyme preparation from Penicillium janthinellum and Neurospora crassa were 6.5 and 2.2 fold more pure than the crude homogenate respectively. The molecular mass of the partially purified enzyme from Penicillium janthinellum and Neurospora crassa were found to be 39kDa by using SDS-PAGE.

Key words: Xylanase, *Penicillium janthinellum, Neurospora crassa*, purification, characterization.

INTRODUCTION

Xylan is a major hemicellulose and considered with cellulose and chitin as being among the most abundant polysaccharides in nature.it is composed of a linear backbone of 1,4-β-linked –D-

xylopyranosyl units that often has side chains of O-acetyl,arabinosyl and methylglucuronosyl substituents(Maheshwari *et al*.,2000 and Blanco *et al*.,1997).endo-β-1,4-xylanase(1,4-β-D-xylan xylanohydrolase:E.C.3.2.1.8) is the main enzyme responsible for the cleavage of the linkages within the xylan backbone(Belfaquih *et al*.,2002).

Xylanase are commercially used in the pulp and Paper, food, beverage, textile and animal feed industries (Polizeli *et al., 2005).* A recent application of xylanase is in production of biofuels. It is estimated that the total energy content of global xylan and cellulose waste is equivalent to almost 640 billion tons of oil (Sa-Pereira *et al*., 2003).

Xylanases are used to convert the polymeric xylan into fermentable sugars for the production of ethanol and xylitol from plant biomass (Galbe and Zacchi, 2002 and Beg *et al*., 2001). Xylanases can be used for their tailor designing of drugs and modifying the properties of food. Xylanases have also been used in animal feed to improve the digestibility of animal feed for better feed utilization (Nortey *et al*., 2007; Uhlig., 1998).

The aim of the present work was to partially purify and investigate the characteristics of the extracellular enzyme xylanase from *Penicillium janthinellum* and *Neurospora crassa.*

MATERIALS AND METHODS

Isolation and Screening of xylanase producing fungi

Xylanase producing fungal strains were isolated from marine soil sample. xylanolytic fungi were screened on oat spelt xylan agar medium (Takashi *et al*.,1990) containing 0.1%Yeast extract,1% xylan from oat spelt,2% Agar, 0.4% KH_2PO_4,0.2% NaCl,0.1% $MgSO_4$ $7H_2O$,0.005%

$MnSO_4$, 0.005% $FeSO_4$. $7H_2O$, 0.2% $CaCl_2$ $2H_2O$, 0.2% NH_4Cl .The above contents were dissolved in 100ml of distilled water and the pH was adjusted to7.the medium was sterilized and it was poured into a sterilized petriplates. After solidification, the well was made by sterilized cork borer .The fungi were placed on the well. After 48 hours at 37^0C, the plates were stained with 1% Congo red and incubated for 15 minutes and then the plates were destained with 1M NaCl (Teather, 1982). Fungal isolates showing zone of clearance were picked up, purified by repeated streaking on the same medium and finally transferred to Potato dextrose agar slants and maintained at 4^oC. The best producing strains identified as *penicillium janthinellum* and *Neurospora crassa* were isolated from marine soil and were used for further studies.

Xylanase fermentation media

The Xylanase fermentation medium (Takashi *et al*.,1990)containing 2g Starch, 1g Yeast extract,0.05g Xylan,1g $KHPO_4$,1g Sodium Chloride, 6 mg Magnesium Sulphate ,4 mg Calcium chloride per 200ml at pH 7.0. The Xylanase positive cultures were inoculated at 37^oC for 5-7 days in a rotary shaker. The culture medium was centrifuged at 5000 rpm for 20 min in a refrigerated centrifuge at 4^oC to remove the fungal mycelia and medium debris the supernatant was used as crude enzyme solution.

Protein assay
Protein concentration was determined by the method of Lowry *et al.,* 1951 with Bovine serum albumin (BSA) as the standard.

Determination of xylanase activity
Xylanase activity was determined spectrophotometerically by Miller (1959) method. 0.5 ml of enzyme broth was taken and it was mixed with 0.5 ml of 2% Xylan suspension in 0.1 m acetate buffer, pH 6.0 and the mixtures were incubated at 55^0C for 30 minutes. After incubation, the mixtures were cooled rapidly on ice water. The insoluble Xylan was removed by centrifugation (10,000 rpm). To the resulting supernatant (0.5 ml), 1 ml of 3, 5 – dinitro salicylate (0.5%) solution was added and the mixture was cooked in boiling water. Then, the color intensity was measured spectrophotometer at 535 nm. For standard curve, xylose was used.

One unit of Xylanase is defined as the amount of enzyme that liberates 1μmol of Xylose equivalents per minute under the assay conditions.

Partial purification and characterization of xylanase
Fractionation with ammonium sulfate and dialysis
The crude enzyme was first saturated upto 20 % with solid ammonium sulfate and then centrifuged at 10000 rpm at 4^oC for 10 min. The supernatant obtained was further saturated upto 80 % with solid ammonium sulfate and again centrifuged. The pellets obtained were dissolved in minimum volume of 0.05 M citrate buffer; pH 6.2 these solutions were dialyzed against 500 ml of the same buffer at 4^OC to remove the excess salt. The xylanolytic activity was determined as described by Miller (1959) method.

Ion exchange chromatography
The dialyzed solution was applied to a DEAE – cellulose (DE - 52) column (11 x 1 cm) which had been previously balanced with citrate buffer (50 mM pH 6.2). The enzyme sample was stepwise eluted by using a discontinuous gradient of 150 – 200 mM of Nacl in citrate buffer (50 mM pH 6.2). The low rate was 0.1 ml min^{-1} and 20 fractions (2 ml each) were collected. Fractions containing the majority of the xylanase activity were pooled for activity assay. The activity of xylanase enzyme at the end of each step was measured by a spectrophotometric method.

Determination of molecular weight by SDS – PAGE
The molecular mass of enzyme was determined by Sodium Dodecyl Sulphate- Polyacrylamide gel electrophoresis (SDS - PAGE). SDS PAGE was carried out as descried by Laemmli, 1970.

Effect of pH on enzyme activity and stability
To assay optimum pH, xylanolytic activity was determined at 37^o C, at different pH values, using the 0.1M phosphate buffer solution (pH 4.5 – 8.5). For pH stability the enzyme was dispersed (1: 1) in the 0. 1 M phosphate buffer solution (pH 4.5 – 8.5) and maintained at 37^o C for 24 hours. Afterwards residual xylanolytic activity was determined under optimum conditions of pH and temperature (pH 4.5 and 37^o C respectively).

Effect of temperature on enzyme activity and stability

Optimum temperature was determined by incubating the reaction mixture at different temperatures ranging from 30 to 70^{o} C and assaying the activity at the pH determined as optimum.

Thermal stability was assayed by incubated the enzyme at different temperatures ranging from 30 to 70^{o}C for 1 hour at pH 6.5. Afterwards residual xylanolytic activity was determined under optimum conditions of pH and temperature (pH 4.5 and 37^{o}C respectively).

RESULTS AND DISCUSSION

The results obtained in this work revealed the ability of *Penicillium janthinellum* and *Neurospora crassa* to produce extracellular xylanase.

Partial purification and Characterization of extracellular xylanase

xylanase enzymes from *Penicillium janthinellum* and *Neurospora crassa* were partially purified with 80 % ammonium sulfate precipitation followed by dialysis.The dialyzed enzyme from *Penicillium janthinellum* and *Neurospora crassa* were loaded onto a DEAE -cellulose ion -exchange column .Their specific activities and degrees of purification are given is tables 1 and 2.from the results ,*Penicillium janthinellum* is the best xylanase producer than of *Neurospora crassa.*

TABLE-1: Purification of extracellular Xylanase from *Penicillium janthinellum*

Purification step	Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme extract	40.0	65.0	0.61	100	1.0
After Ammonium Sulfate fractionation	35.0	40.0	0.90	87	1.5
DEAE Cellulose Ion -exchange chromatography	20.0	5.0	4.0	50	6.5

TABLE-2: Purification of extracellular Xylanase from *Neurospora crassa*

Purification step	Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme extract	34.0	75.0	0.45	100	1.0
After Ammonium Sulfate fractionation	20.0	40.0	0.50	59	1.1
DEAE Cellulose Ion -exchange chromatography	18.5	19.0	1.0	54	2.2

Effect of pH on enzyme activity and Stability

The study revealed that the best pH for xylanase activity from *Penicillium janthinellum* and *Neurospora crassa* were 4.5 and 5.5 respectively (Fig-I).Studies carried out with *Penicillium funiculosum*(Furniss *et al*.,2002) as well with other fungal species (Saha,2002) also conducted that the most suitable pH value for xylanase activity was within the acid region.

Fig-I: Effect of pH on activity of Xylanase from *Penicillium janthinellum*(♦) and *Neurospora crassa*( )

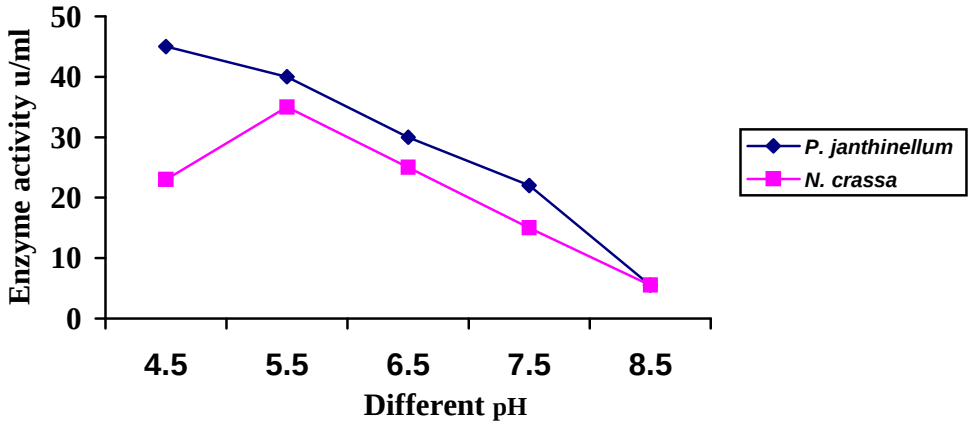
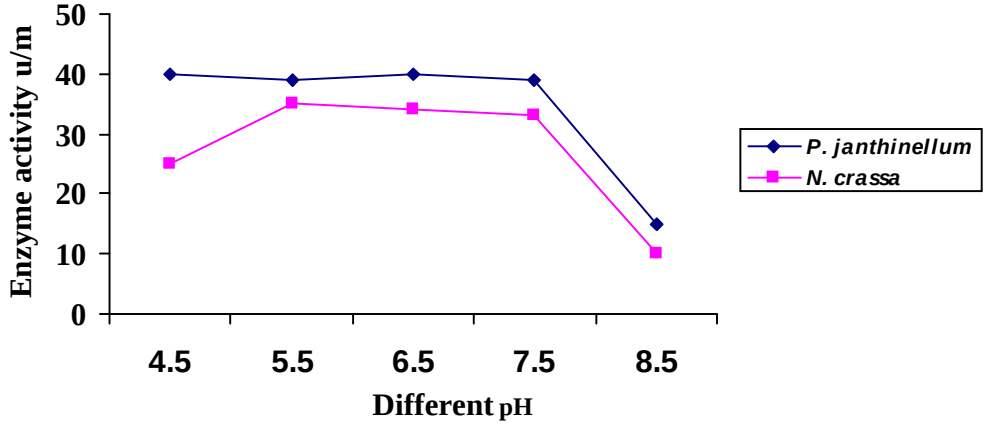


FIG-II: Effect of pH on stability of Xylanase from *Penicillium janthinellum*(♦) and *Neurospora crassa*( )



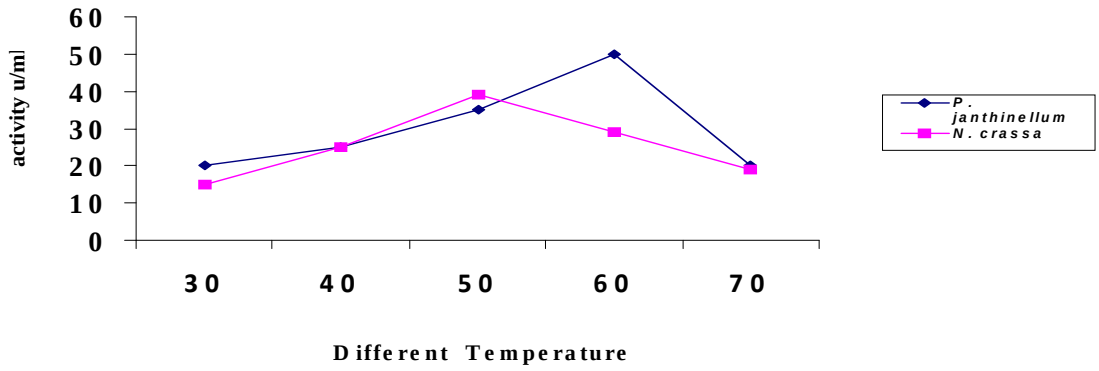
The pH stability of xylanase from *Penicillium janthinellum* and *Neurospora crassa* were observed from 4.5-7.5 and 5.5-7.5, respectively (Fig - II). After a pH of approx 7.5, a less of activity was followed by a slight decrease, as shown in Fig -II. Xylanase from *Penicillium sclerotiorum(*Adriana knob *et al.*,2008) also exhibited a broad range of stability from pH 3.5 - 7.5.

Effect of Temperature on Enzyme Activity & Stability

The optimum temperature for xylanase activity from *Penicillium janthinellum* and *Neurospora crassa* were 60 ^{o} C and 50 ^{o} C respectively(Fig-III).similarly ,in other studies with *Penicillium* spp,it was concluded that the optimum temperature varied between 40 & 50 ^{o} C(Krogh *et al.*,2004 & Ryan *et al.,2003).*besides other fungal xylanases show optimum temperature at 50^{o} C(Bakir *et al.*,2001 & Carmona *et al.*,2005*).*

Thermal stability of Xylanase from *Penicillium janthinellum* and *Neurospora crassa* was determined to be 30 - 50 ^{o} C and 30-40^{o}C respectively (Fig -IV).thermal stability is an interesting enzymes property due to the great industrial importance(Eijsink *et al.*,2005)

Fig-III: Effect of Temperature on activity of xylanase from *Penicillium janthinellum*(♦) and *Neurospora crassa*( )



Determination of Molecular Weight by SDS- PAGE

The molecular mass of the partially purified Xylanase from *Penicillium janthinellum* and *Neurospora crassa* were estimated to be approximately 39 kDa as measured on SDS - PAGE (Fig V) . *Penicillium chrysogenum* and *Penicillium purpurogenum* have the molecular weight of 35 kDa and 33 kDa respectively.(Haas *et al.*,1992 and Belanic *et al*.,1995)

Fig-IV: Thermostability of xylanase from *Penicillium janthinellum*(♦) and *Neurosporacrassa*( )

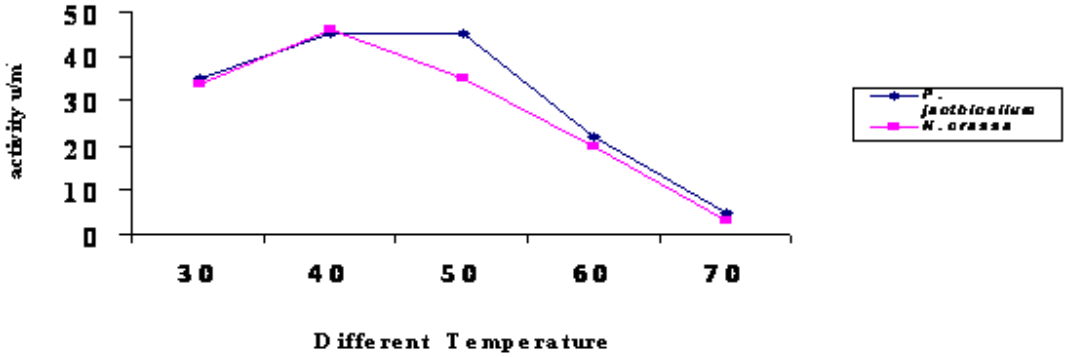
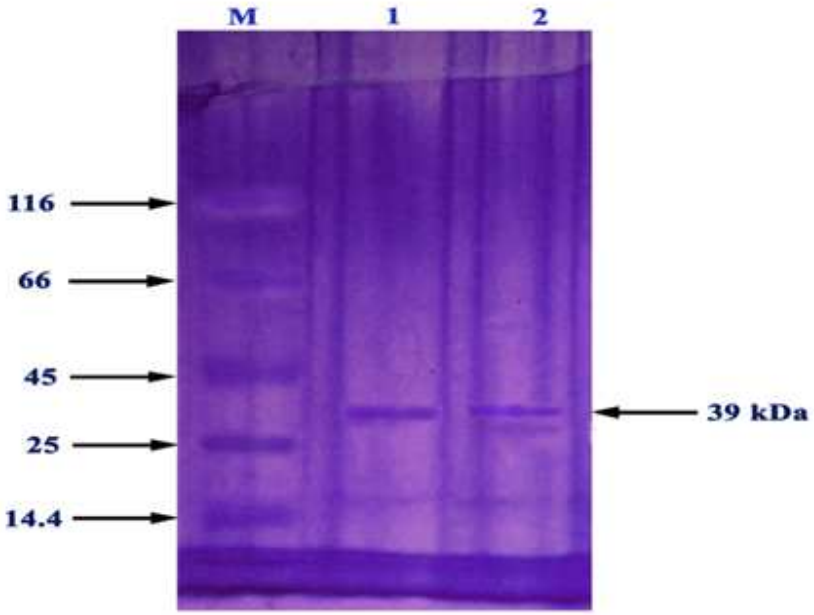


Fig V

Molecular Weight Determination of Xylanase by SDS PAGE



M - Molecular Weight Markers :

14.4 kDa - Lysosyme, 25 kDa - Endonuclease Bsp 981
45 kDa - Ovalbumin, 66 kDa Bovine serum albumin,
116 kDa - β - Galactosidase

Lane1 - Purified fraction of Xylanase from *Penicillium janthinellum*
Lane2 - Purified fraction of Xylanase from *Neurospora crassa*

CONCLUSION

In the present study, an extracellular xylanase were partially purified and characterized from *Penicillium janthinellum* and *Neurospora crassa.*The isolated strain of *Penicillium janthinellum* was found to be a potential producer of extracellular xylanase than *Neurospora crassa*.The enzyme yield were more in *Penicillium janthinellum* than that of *Neurospora crassa*.These are desirable properties for application in the pulp and paper, as well as in food industries.

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