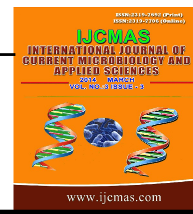

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Original Research Article

Production of Xylanase by Wild and Mutant strains of *Humicola insolens*

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A B S T R A C T

In the recent years lot of interest has been generated on the production of xylanase due to their potential application in paper, pulp and fiber processing industries. A thermophilic fungal strain of *H.insolens* was isolated from soil and the present work was carried out on the selection of hyper xylanase producing mutants of *H.insolens*. The U.V mutagenesis of *H.insolens* resulted in 74% killing rate. The six mutants of *H.insolens* designated as 8B, 12F, 13F, 8G, 2H, 13H selected were hyper producers of xylanase. It was observed that under optimized solid state condition, wild type *H.insolens* produced xylanase activity of 243.09 1U/g and CMCase activity of 11.05 IU/g, using rice straw as carbon source. The xylanase activity of mutant 8B was 517.99 IU/g under similar solid state conditions, which was 2.13 times higher than that of wild type *H.insolens*. The mutant 8B was further optimized for xylanase production using Box Behnken design. It was found that of the three independent variables tested, pH was the most significant parameter influencing xylanase and CMCase production. The maximum xylanase activity (1269 IU/g) could be achieved at pH 5.5 and using lowest level of inoculum (5.15 X 10^6/ml).

Keywords

Xylanase; *H.insolens;* Mutants; U.V mutagenesis; Box Behnken design.

Introduction

In recent years, growing interest has been generated on xylanase due to their potential applications in pulp and paper industry, in fibre industry, in food processing (cereals, starch, tea, vegetables) industry, in production of alcohol, isolation of protoplasts, degradation of lignocellulosic material and hydrolysis of baggase, etc. Recently interest has been increasing in applying xylanases to pulp bleaching process. Xylanases are the most studied hemicellulases because xylan is the principal constituent of hemicelluloses in plants (Biely, 1985). Xylanase levels from fugal cultures are generally much higher than those from yeasts or bacteria. The xylanases are able to remove hemicelluloses that forms bridge between liginin and cellulose. This action results in increased softness and brightness of paper

(Vikarii et. al, 1980). Some thermopilic fungi such as Thermomyces lanuginosus that produces cellulose free thermostable xylanase (Gomes et. al. 1994) Thermoscus aurantiacus, that produces high level of xylanase alongwith debraching enzymes and activity have been found to be useful in found to be useful in treatment of jute fibers (Hoq et al., 1992). Though, xylanase used in prebleaching process should be free of cellulases, however, xylanoytic enzmes alongwith cellulose, esterases can be useful in deinking of recycling paper. Thermostable fungi are particularly producers of xylanases from industrial point of view due to the fact that they excrete thermostable xylanases into the medium.Many thermophillic fungi and bacterial strains have been isolated, owing to increasing biotechnological importance of thermostable xylanases (Tan et.al., 1985, Venkitraman, 1994). These strains include *Thermomyces lanuginosus* RT9 (Hoq et. al., 1994), *Thermoasus aurantiacus (*Grajek,1987; Yu et. al., 1987; Tan et al.; 1987), *Humicola lanuginosa* (Anand and Vaithayathil, 1990), *Melanocarpus albomyces* IIS 68 (Saraswat and Bisaria,1987);*Talaromyces byssochlamyodoides* YH-50 (Yoshioka et. al., 1987), *H. grisea* (Monti et. al., 1991), *Talaromyces byssochlamyodoides* (Luthi et. al., 1990). *Thermotoga* sp.Strain Fjss3-B1 (Saul et. al.,1995), *Clostridum stercoraium* (Berenger et. al., 1985), *Pyrodictium abysii* (Andrade et. al, 1996), *Dictyoglomes thermophilum* Rt46 B.1 (Gibbs et.al, 1995), *Bacillus sp.* (Yang et.al. 1995) etc.An important factor for efficient xylanase production is the choice of an appropriate inducing substrate, ether insoluble or soluble, as well as optimization of the medium composition. In addition the substrate can influence the concomitant formation of enzyme in certain organisms. Wong et.al, (1998) suggested that the existence of a multiplicity of xylanases in microorganism may be strategy to achieve superior xylan hydrolysis. The xylanases from different sources differ in their molecular weight, isozyme profile, pH and temperature optimum characteristics. Application of xylanases is increasing in paper and pulp industry, jute, coffee, food and beverages, single cell protein etc. Xylanases from *Humicola sp.*(Silva et al., 1994, *Dictyoglomus sp.*(Ratto et. al., 1994), *Streptomycyes* (Elegir et. al., 1995), *Streptomyces thermoviolaceus* (Garg et.al., 1996), *Aureobasidium pullulans* (Youg and Eriksoon, 1992) are involved in bio bleaching of Kraft pulp. Xylanases from *Talaromyces emersonii* (Tuohy et. al., 1994) include their application in modification of cereal flours to enhance the volume and texture of bread through hydrolysis of arabinoxylans, saccharification of lignocelluleose together with cellulases and pectinases to produce the desired end products such as natural food flavours and additives. A xylanase from *Aspergillus flaviceps* (Mukohapadhyay et. al., 1997) is used in clarification of fruit juices, production of low viscosity beer, de-inking of waste newspaper, preparation of animal feed with increased digestibility, improving dough raising and quality of baked products in bakery and preparation of protoplast from the plant cells.

Fungal xylanases of aspergillus and trichoderma spp, and bacterial xylanases of Bacillus sp, Streptomyces sp. and clostridium sp. have been intensively studied (Wong et.al., 1988; Erikisson et.al., 1990). The optimum pH for xylan hydrolysis is around 5 for most fungal xylanases and they are normally stable between pH values of 2 and 9. Xylanases which showed optimum enzymatic

activity at temperature of 70° to 80° C have been reported. The most thermostable xylanase described is that of an extremely thermophillic *Thermotoga sp*. with a half life of 20 min at 105°C (Brrager et. al., 1989). Under solid state fermentation high level of xylanase was produced at 50° C using wheat bran as carbon source by *T. lanuginosus* and *T. aurantiacus*, (Alam et. al., 1994). However *T. aurantiacus* produced small amount of cellulase in addition. The effect of initial moisture level was optimized for both fungi that performed best at 80% and 30% initial moisture, respectively. Xylanase from both was most active at respective, pH of 6.0 and 5.0 and temperature of 70°C.

True xylanase without cellulase activity (endo1,4-β- xylanase) has been isolated from *Trichoderma viridae* (UjiiE et. al., 1991). Many of mesophillic Aspergillus sp. are known to produce xylanase such as *A. niger* (Conard, 1981), A. awamori (kor melink et. al., 1993), *A. kwachii* (Ito et. al., 1992), *A. nidulans* (Fernandes et. al., 1983), *A. sojae* (Ishi et. al., 1964) and *A. terreus* (Ghareib et. al., 1992). Among these species, A. kawachii (Kinoshita et. al., 1995) and *A.sojae* (koji molds) were studied for xylanase production (Kimura et. al., 1995). Two isoenzymes of xylanase and arabino-furnosidase was purified by SDS page from solid state culture of *A. sojae*. The xylanase were stable at pH 5.0to 8.0 and up 50 ^{O}C and 35°C and its activity were inhibited by Mn^{2+} and EDTA. Many microorganism have been recognized as a Xylanase producers (Dekker and Richards, 1976; Woodword, 1984). Fourty Mesophilic and 13 thermophillic fungi were screened for their ability to produce extracellular xylanase (Endo 1,4 β-D- xylanase, EC 2.3.1.8) (smith et. al., 1991). Some of mesophillic fungi such as *Malbranchea pulchella* (Matsuo and yasui, 1985), *Melanocarpus albomyces* (Maheshwari and Kamalam,1985), *Sporotrichum thermophile* (Durand et. al., 1984), *Talaromyces byssochiamydoides* (Yoshioka, et.al., 1981), *T.emersonii* (Tuohy and coughlan, 1992), *Thermoascus aurantiacus* (yu et. al., 1987) and *Thielavia terrestris* (Durand etal.,1984) have been reported to produce xylanases. Of the 13 thermophillic strains studied *Malbranchea pulchella var. sulfurea (*Miehe), (Cooney and Emerso), *Sporotrichum thermophille* (Apinis), *Thielavia terrestris* (Apinis), *Humicola insolens* and *Acremonium alabamensis* (Morgan jones,1992) produced higher level of xylanses. *Thermomyces lanuginosus* (Gomes et. al., 1993) isolated from self heated jute stacks produced very high levels of xylanases using inexpensive lignocellulosic biomass . Maximum production of xylanases (4.2 units) was obtained by *T. viride* and *A.niger* on dried apple pomace (Bhalla et. al., 1993). Thermophillic *Humicola sp*. secreted thermostable xylanases when grown on wheat bran medium at 50 °C. Twenty one strains of thermophillic fungi in the Forintek culture collection were screened for their production of xylanolytic enzymes in both solid and media containing various hemicellulosic and cellulosic substrates (Yu et. al., 1987). *Thermoscas aurantiacus* was selected from 21 strains of thermophillic fungi as the best producer of extracellular xylanase. *Thermomyces lanuginosus* RT9 (Hoq et.al ., 1994), *Homicola grisea var. thermoidea* (Monti et. al., 1991), *Talaromyces byssochlamydoides* YH-50 (Yoshioka et. al., 1981) and thermophillic bacteria such as *Thermotoga sp*. strain Fjss3-B1 (Saul et. al., 1985), *Dictyoglomus themophilum* (Saiki et. al., 1985), *Coldocellum*

saccharolyticum (Luthi et.al., 1990), *Thermomonospora fusca, T. Curvata* and *T. chromogena* (McCarthy et. al., 1985), Thermophilic *actinomycetes, Streptomyces thermoviolaceus* OPC-520 (Tsujibo et. al., 1991), *Thermomonospora sp*. (Ristroph and Humphrey, 1985) are known to produce thermostable xylanases. The existence of multiple forms of xylanase has been well documented for several microorganism (Wong et.al., 1988). Wong et.al. (1988) suggested the existence of a multiplicity of xylanases in microorganisms that may be the strategy to achieve superior xylan hydrolysis. The isozymes pattern of xylanase from diverse geographical strains of *T. lanuginosus* have shown two similar kind of xylanase; an endo xylanase of 25 kda and a 54 kda xylan hydrolyzing enzyme (Chadha et. al., 1999). Cellulase free xylanase was produced by the *Thermomyces lanuginosus* RT9 and *T. lanuginousus* strain MH_4 (Hoq and Deckwer, 1995). Two different xylanases CX-I and CX-II from an alkalophillic fungus *Cephalosporium* sp. strain RYM-202 have been purified. An enzyme with endo-1,4-β-D-xylanase (EC-3.2.1.8) activity was purified from *Aspergillus awamori* ATCC–11358 (CBS115.52) grown on wheat bran culture medium (Hessing et.al., 1994). One form of endoxylanase (25 kda) exists in *Thermomyces lanuginosus* (Cesar and Mrsa, 1986), whereas in Streptomyces thermoviolaceus OPC-m520, two types of xylanase (STX-I and STX-II) were isolated (Tsujibo et. al., 1991) in *Bacillus sp.* (VI-4) only one from of xylanase was found (Young et.al., 1995)and *Streptomyces sp.* TUB-12-2 produced five biochemically distinct xylanases (Elegir et. al., 1994).

The following study was taken up for xylanases and CMC'ase production by thermophillic fungus *H. insolens* with following objectives:

Mutagenesis of *H. insolens* for screening hyper xylanases producing mutants

Optimization of Xylanse production by xylanase hyper producing mutant by response surface method.

Materials and Methods

Collection of samples

A total of 150 soil samples (approximately 100 g) were collected by sterile forceps and tweezers, packed individually in sterile plastic bags. All samples analyzed for isolation of Xylanase producer *H.insolens.*

Isolation and identification of *H.insolens*.

The suspected strians of *H.insolens* were isolated by plating soil samples on to YPSs medium containing antibacterial antibiotic (ampicillin 50µg/ml). The plates were incubated at 50°C for 7 days, After 7 days whitish yellow colonies were picked and sub cultured to obtain a pure culture. All isolates were screened for xylanolytic ability. Prominant selected isolates were identified for their abilities to produce xylanase under solid state fermentation on the basis of morphological, cultural and biochemical properties. Xylanase activity of *H.insolens* was detected by congo red plate assay.10ml of congo red solution was poured on to each of xylan plates with suspected colonies. Plates were kept for 2 hours at 50^0c.Then 10ml of Nacl was poured on to plate and kept for 2 hours at 50^0c. Plates were flooded with HCI followed by incubation of plates for 2 days at 50^0c, for getting light blue clear Zone around the colonies.

Mutagenesis of *Humicola insolens*

Mutagenesiss of *H. insolens* strains was performed for screening of hyper xylanase producing mutants. Spore suspension of *H. insolens* culture was prepared in a 9 ml of sterilized water blank and spore count was taken 5.15 X10^5 spore/ml. Spore suspension was subjected to U.V irradiation for 30 minutes using (Phillips) germicidal U.V. lamp 30 w) from a distance of 20 cm in a laminar flow. Whole process was carried out under dark. After the U.V. exposure, spore suspension of mutated strains was prepared and diluted suitably (10^{-2} and 10^{-3} ratio). 0.1 ml (100 ul) spore suspension of each of 10^{-2} $_{and}$ 10^{-3} dilution prepared after mutagenesis of culture of *H. insolens* was spread on xylan medium plates with the help of spreader. All these plates were incubated for 5-7 days at 45° C in incubator. The U.V mutagenesis of *H.insolens* resulted in 74% killing rate The survivors colonies of *H. insolens* (Mutants) were randomly picked and transferred on to xylan medium containing sodium deoxycholate 0.01 (w/v),incubated for 4-5 days of incubation of at 45^{0}C in incubator.

Plate assay to screen potential xylanase hyper producer Mutants of *H. insolens*

Thirty selected hyper producing mutants of culture *H. insolens* showing larger clear zone were picked and transferred into xylan medium with wild type as control in each plate. These plates were incubated for 4-5- days at 45°C. The screening using clear zone method was adopted to select the mutant, hyper producer of xylanase. These mutants of *H. insolens* were finally selected for xylanase production under solid state fermentation. Six mutants of *H. insolens* designated as 8B, 12F, 13F, 8G, 2H, 13H to be hyper producers.The selected hyper producer mutants of culture *H. insolens* were picked and transferred into xylan medium. These plates were incubated for 4-5- days at 45°C .

Secondary screening of the mutants on rice straw containing medium

The spore suspension of six selected Mutants designated as 8B, 12F, 13F, 8G, 2H, 13H was prepared in sterilized water blank. The spore suspension was homogenized on cyclomixer and was inoculated into different flasks containing 25 ml of pre inoculum glucose medium (g100 ml); (Yeast extract, 0.4g; K_2HPO_4 01g; $MgSO_4$, 0.5g; glucose, 1.5 g) under aseptic conditions. Inoculum was developed by incubating culture flask at 45^{0}C for 24 h in shaker at 200 rpm. To identify the effect of carbon source on xylanase production, production medium containing 10gm rice straw grinded to 2mm particle size mixed with 18ml of basal nutrient medium was inoculated with 2ml of actively growing mycelium from culture flask. The inoculated flasks were incubated at 45°C under stationary conditions 5-6 day. After incubation fermentation spent; solid substrate was removed and transferred to 25 ml of 0.1 M sodium citrate buffer in flask and kept at 50^{0}C for 2 hours. The contents were thoroughly vortex to extract the xylanase. The sample was centrifuged at 5000 rpm at 4°C for 10 minutes .The supernatant was filtered through whatman No.1 filter paper. The enzymes extracts were stored in sterilized vials at 20^{0}C .The resultant clear filtrate was used for assay of xylanase and CMCase activates.

Xylanase activity

The reaction mixture containing suitably diluted enzyme(0.5ml) and(0.5ml) of 1%

solubilised birchwood xylan solution was incubated at 50^0C for 5 minute in water bath after that 4mlof DNS reagent was added and tubes were kept in boiling water for 10 minutes. The developed colour (absorbance) was measured at 575nm using spectrophotometer. From the xylanase standard curve concentration of the xylose released was calculated. The Xylanase activity was measured in international units/gm. One unit of xylanase activity was defined as the amount of enzyme required to release 1µmole of xylose per minute under assay conditions of incubation at 50^0C. Solubalised xylan was prepared by stirring birchwood xylan with 1M NaOH for 6 hours at room temperature followed by centrifugation and freeze drying the supernatant after neutralizing the alkali with 1m HCL.

$$\text{Xylanase activity} = \frac{1\mu\text{mole of xylose released x 2X x dilution factor}}{\text{Incubation time (minutes)}}$$

Carboxy methyl cellulase activity (CMCase activity)

The reaction mixture containing suitably diluted enzyme(0.5ml) and(0.5ml) of 1% carboxy ethyl cellulose was incubated at 50^0C for 5 minute in water bath after that 4mlof DNS reagent was added and tubes were kept in boiling water for 10 minutes. The developed colour (absorbance) was measured at 575nm using spectrophotometer. From the glucose standard curve concentration of the glucose released was calculated .The CMCase activity was measured in international units/gm. One unit of CMCase activity was defined as the amount of enzyme required to release 1µmole of glucose per minute under assay conditions of incubation at 50°C.

Results and Discussion

The results in Table.1 show the potency index of the mutants that show higher-clear zone as compared to wild type in *H. insolens*. The screening using clear zone method showed six mutants of *H. insolens* designated as 8B, 12F, 13F, 8G, 2H, 13H to be hyper producers. These mutants namely *H. insolens* were finally selected for xylanase production under solid state fermentation.

Production of xylanase and carboxymethyl cellulose by wild and mutant strains of *Humicola insolens*

The six mutans of *H. insolens* picked were hyper producer of xylanase. The xylanase and carboxymethyl cellulase activities were taken. The table.2 shows that maximum xylanase activity was shown by mutant 8B (517.99 units/g) which was 2.13 times higher than that of wild type strain of *H. insolens* . This was followed by xylanase activity of 8G (432.9 units/g), 12 F (399.6 units/g), 13 H (362.59 units/g), 13F(307.09 unit/g), 2H(256.41 units/g), respectively). The carboxymethyl cellulase activity of 12 F Mutant (13.71 units/g) was found to be maximum amongst the screened mutant.

The 8B mutant strain of *H. insolens* was selected as (in table-2) the maximum xylanase activity was observed for mutant 8B (517.99 units/g) which was 2.13 times higher than that of wild type strain of *H. insolens.* Box Behnken design with three variables like basal salt concentration ,pH and inoculum was used to test the effect of these variables on the xylanase, CMCase activity of selected mutant 8B.. As shown in the table-3, three different basal medium were selected at three concentrations(2.5x, 5.0x ,7.5x) and mixed

with different proportions of inoculums size(5. 15x10 ^{6}, 1. 30x10 ^{6}, 1. 95x10^{6}) at three different pH(4.5,5.5,6.5).

Optimization of Xylanase and carboxymethyl cellulase production by 8B mutant of *Humicola insolens* using response surface method

Optimization of the production of xylanase and carboxymethyl cellulase activities of mutant 8B was taken up. The xylanase and CMCase activity avd β Glcucosidase of 8 B mutant using three different variable in different combination was varied. The result in the table 5 showed that xylanase activity of 8B was much higher (1269.80 units/g) when medium concentration was 7.5 X, pH was 5.5 and inoculums 1 ml were used for it. The CMCase activity was much higher (25.10) when medium concentration was 7.5 X, pH was 5.5 and inoculum level was 3ml.

Response surface regression analysis for Xylanase production by 8B mutant of *H. insolens*

A xylanase hyper mutant of *H. insolens* (8B) was used. The box Behnken design used in the present study was intended to determine the optimization level. Response surface methodology (RSM) using thebox Behnken design of experiments was used to develop a mathematical correlation between the effect of basal medium concentration pH and inoculum level on the xylanase, CMCase by the mutant 8B of *H. insolens*. A class of three level of complete factorial design for the determination of parameters in a quadratic model was developed by Box Behnken design (Box and behnken 1960). The behavior of the system is explained by quadratic model equation: $G= \beta_0+\sum\beta_i X_i +\sum\beta_{ii} X_i^2+\sum\beta_{ijh} X_i X_j X_h$

Where G = predicted response, β_0 = offset term, β_i = linear effect, β_{ii} = squared effect

The three chosen critical variables were designated as X1, X2 , X3 , respectively. The low middle and high concentration levels of each variables was designated as -1 , 0 and +1 , respectively and listed in the Table 3 . The actual design of experiment is listed in Table 4. The three significant independent variables i.e. X1 ,X2 , X3,and the mathematical relationship of the response G to these variables was approximated by the quadratic model equations (Box 1962 ; Box and Wilson 1957 ; Box and Behnken 1960) . $G = \beta_0+ \beta_1X_1+ \beta_2+X_2+\beta_3X_3+ \beta_{11}X_1^2+ \beta_{22}X_2^2+ \beta_{33}X_3^2+ \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{23}X_2X_3$

Where G = Predicted response, β_0 = Constant, X_1= Basal medium, X_2= pH, X_3= Inoculum,

β_{12} , β_{13} , β_{23} = Cross product coefficients

Using multiple linear regression analysis a set of coefficients namely xylanse, CMCase activity were determined .

Coefficients of regression for production of xylanase and CMCase by *H. insolens*

The coefficients of regression model for xylanase and CMCase as function of basal medium concentration, pH and inoculums were reported in Table 6. To predict the relationship between independent variable X1, X2, X3,regression analysis was performed and the mathematical relationship of the response G to other variable was approximated by the following quadratic model equation:

For Xylanase G=12807+379

$X_1+4216X_2+849_X3-4X1^2-28^3X_2^2+316X_3^2-51X_1X_2-44X_1X_3-373X_2X_3$

For CMCase $G=122.5+8.7X_1+49.1X_2-22.8X_3-0X_1^2-3.4X_2+7.8X_2^3-1.5X_1X_2-0.1X_1X_3-1.4X_2X_3$

All models correlated well with measured data and were significant. The results in the Table 6.showed that the R2 Table for xylanase and CMCase activities as a function of basal medium concentration, pH and in inoculums was 90.7% and 93.9%, respectively.

As in the figures (2-7) among the independent variables, pH showed the most prominent effect followed by basal medium concentration and inoculums level. The pH and inoculums level showed significant effect both in linear and squared terms, whereas basal medium concentration showed significant effect in linear terms only. The pH in interaction with inoculums level showed highly significant effect on xylanase activity (p <0.002). .

The effect of pH, inoculum level and basal medium concentration on xylanase and CMCase activities is illustrated with increasing the pH from 4.0 to 5.5 and further increase resulted in decrease in the enzyme activities. The xylanase activity progressively increased with the increase in basal medium Concentration, whereas, reverse was true in xylanase. Lower Inoculum level showed higher xylanase as well as CMCase activities.

Interest has been growing in xylanase free of the cellulase to remove xylans selectively from ligno cellulosic material without effecting the cellulose, fiber strength. Xylanases from Aspergillus flavipes are also used in bio bleaching of high grade cellulosic pulps.

The xylanases from Theromphilic fungi are being studies extensively, because of their potential use in paper and pulp, textile and cereal breakfast industry. Humicola insolens has an efficient celluloytic system which finds application in deinking of recycling paper as well as dyed cellulosic fabreics for stone wash effect (Franks et. Al., 1996 Onishi et. al, 1997.) T. lanuginouses, is an excellent source of enzyme that produces up to 2880 units/ml of xylanase when grown in corn cobs containing liquid medium (Bernmet et. Al, 1997. T. aurantiacus and T. lanuginosus also produce very high amounts of xylanase in solid state fermentation. Humicola insolens, was found to produce comparatively low amounts of xylanase (243.09 units/g DW substrate) during solid substrate fermentation on rice straw, and therefore, this industrially important fungus was subjected to u.v. mutagenesis and screening. Mutagenesis for improvement of xylanase activity has been reported in T. lanugisnosus (Chadha et al., 1999) A niger (kim et al, 1996), Penicillium Pinophilum (Wood et al, 1992), Coprinus filanatus (Stephens et al, 1991). Potentially important from industrial viewpoint.

Usually for isolation of xylanase hyper producing strains, clear zone formed around colonies on xylan containing medium, flooded with congo red dye was in use . Therefore, a plate assay methods has been found to be objective and useful in selecting the potential hyper producing strains.

The plate assay method has been quantitatively used for screening amylase hyper producer as well as picking catebolite repression resistant and constitutive mutants/recombinants strains (Rubinder et al. 2000). The best selected hyper producing *H. insolens* mutant 8B

Fig.1 (A-H) Xylanense Activity shown y the different mutants of *Humicola. Insolens*

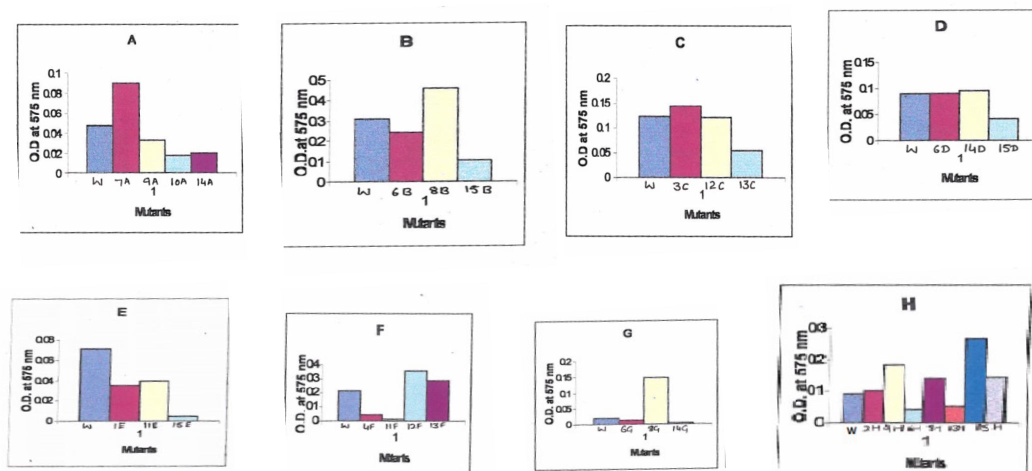


Table.1 Screening of Mutants of *H. insolens* on xylan containing medium

Strain	Colony diameter	Clear zone diameter	Potency Index
Wild	0.6	0.8	1.33
7A	1.2	1.7	1.44
9A	1.2	1.7	1.41
10A	1.2	1.7	1.41
14 A	1.2	1.8	1.5
6B	1.4	1.9	1.35
8B	1.2	1.7	1.41
15B	0.8	1.6	2.0
3C	0.7	1.8	2.57
12C	1.1	1.7	1.54
13C	1.1	1.7	1.54
6D	1.1	1.7	1.54
14D	1.0	1.5	1.5
15D	0.9	1.5	1.66
IE	0.8	1.5	1.66
IE	0.8	1.5	1.87
11 E	0.8	1.5	1.87
15 E	0.3	0.8	2.66
4F	0.4	1.0	2.5
II F	0.4	0.9	2.25
12F	0.3	0.8	2.66
13F	0.4	0.8	0.2
6G	0.4	1.1	2.75
8 G	0.5	0.1	2.0
14G	0.9	1.5	1.66
I H	0.6	1.3	2.16
2 H	0.8	1.5	1.87
4 H	0.6	1.4	2.33
6 H	0.5	1.3	2.6
8 H	0.6	1.3	2.16
13 H	0.8	1.3	1.62
15 H	0.7	1.3	1.85

Table.2 Production of solid substrate fermentation Enzyme activity (International units/g)

Mutant /Strain	Xylanase Activity	CMcase activity
Wild Type	243.09	11.09
12F	399.6	13.71
13F	307.09	11.034
8 G	432.9	13.65
2 H	256.41	11.40
8 B	517.99	12.43
13 H	362.59	13.53

Table.3 The three level of variables chosen for trails

Basal Medium Concentration (X1)	pH (X2)	Inoculum levels (X3)
2. 5X	4.5	5. 15x10 ^{6}
5. 0X	5.5	1. 30x10 ^{6}
7. 5X	6.5	1. 95x10^{6}

Table.4 The Box Behnken design with three independent variables

Trial No.	Basal Medium (X1)	pH (X2)	Inoculum level in ml (X3)
1	2.5X	4.5	2
2	7.5X	4.5	2
3	2.5X	6.5	2
4	7.5X	6.5	2
5	2.5X	5.5	1
6	7.5X	5.5	1
7	2.5X	5.5	3
8	7.5X	5.5	3
9	5X	4.5	1
10	5X	6.5	1
11	5X	4.5	3
12	5X	6.5	3
13	5X	5.5	2
14	5X	5.5	2
15	5X	5.5	2
16	5X	5.5	2
17	5X	5.5	2

Table.5 Production of xylanase and CMCase by selected mutant 8B using three different variable (Medium concentration, pH, Inoculum).

S.No	C1 Medium (X)	C2 pH	C3 Inoculum	C4 Xylanase	C5 CM Case
1	2.5	4.5	2	163.75	6.55
2	7.5	4.5	2	149.65	13.00
3	2.5	6.5	2	723.70	19.00
4	7.5	6.5	2	196.10	10.95
5	2.5	5.5	1	1118.10	21.45
6	7.5	5.5	1	1269.80	24.50
7	2.5	5.5	3	763.65	23.50
8	7.5	5.5	3	479.50	25.10
9	5.0	4.5	1	275.50	19.00
10	5.0	6.5	1	1100.00	22.40
11	5.0	4.5	3	950.00	21.20
12	5.0	6.5	3	299.00	19.00
13	5.0	5.5	2	588.00	17.00
14	5.0	5.5	2	625.00	11.00
15	5.0	5.5	2	635.00	16.20
16	5.5	5.5	2	621.50	16.55
17	5.0	5.5	2	621.50	13.70

Fig.2 Effect of Basal Medium concentration on xylanase activity .

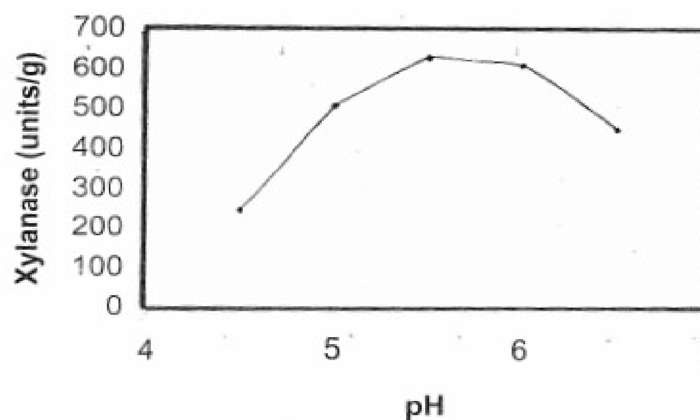


Fig.3 Effect of pH on xylanase activity.

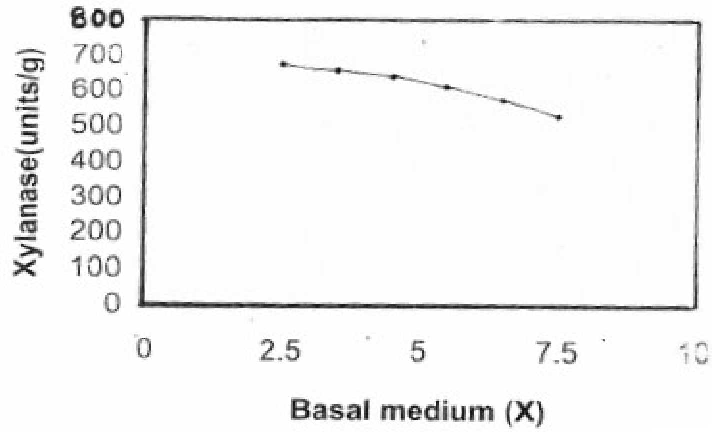


Fig.4 Effect of Inoculum level on xylanase activity

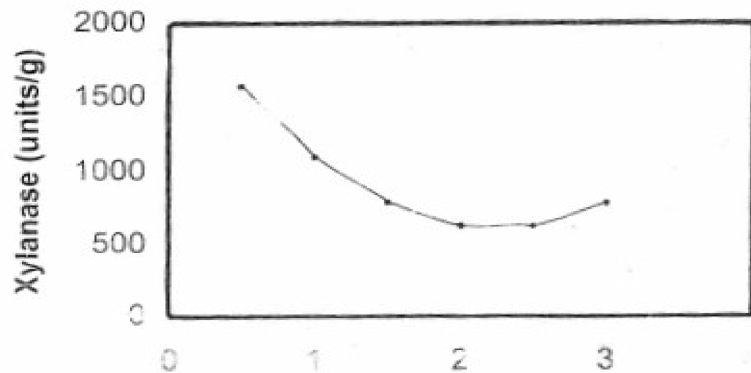


Fig.5 Effect oBasal Medium Concentration on CMCase activity

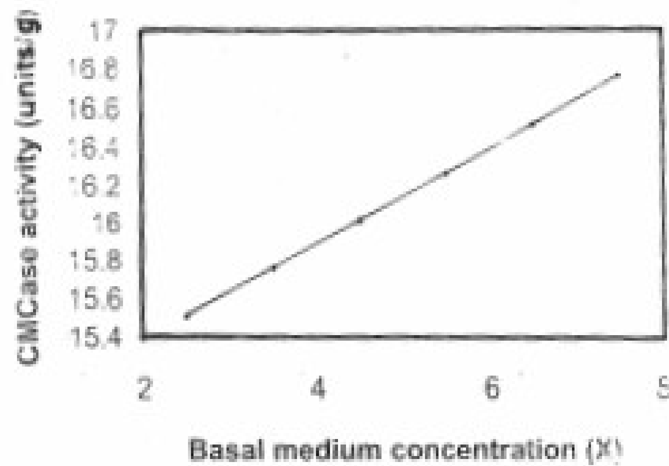


Fig.6 Effect of pH on CMCase activity

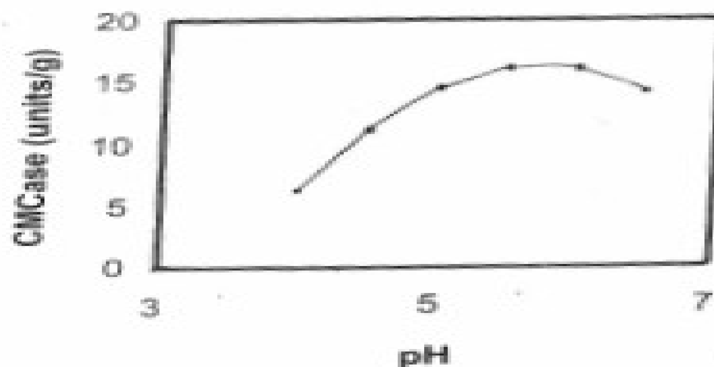
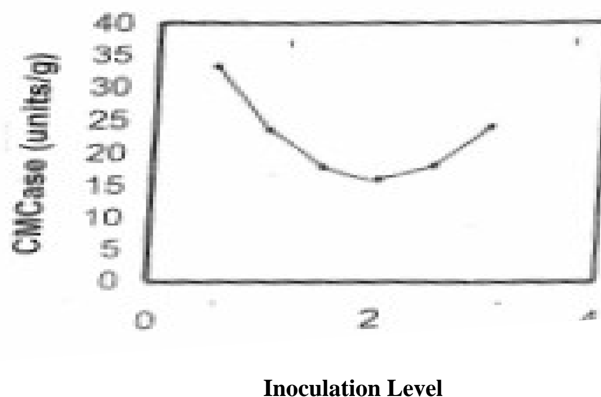


Fig.7 Effect on Inoculum level on CMCase activity



Inoculation Level

Table.6 Coefficients of regression for production of xylanase and CMCase by *H. insolens*

Term	**Xylanase**	**CMCase**
Constant	-12807	-122.5
Basal Medium Concentration	397*	8.7**
pH	4216***	49.1***
Inoculum	849*	-22.8**
Medium concentration X medium concentration	-4	-0.0
pH X pH	-283**	-3.4**
Inoculum X Inoculum	316**	7.8***
Medium concentration X Inoculum	-44	-0.1
pH X Inoculum	-373***	-1.4
R2	90.7%	93.9%

was optimized for xylanase production using response surface methodology (Box GBehnken) employing three independent variables i.e. pH, inoculums level and basal medium concentration during solid state fermentation of rice straw as carbon source. Rice straw was the most suitable carbon source for production of xylanase by *H. insolens* (Aujla, 1998). The Mutant 8B screened on the rice straw produced two folds higher xylanase activity as compared to wild strain. Further optimization of xylanase production was carried out using Box Behnken design and interaction of three variables pH, basal medium concentration and inoculums content on the xylanase production and inoculums content on the xylanase production was analysed. The regression analysis showed pH to be the most important parameter influencing xylanase production. A ph of 5.5 was optimum for xylanse production by *H. insolens*, whereas, a pH of 6.5 and 8.5 were found to be optimum for production of xylanase by *T. lanuginosus* and *Fusarium oxysporum*, respectively (Hoq et al, 1994; christakopoulos et al., 1996). It was found that inoculums raised from an initial spore count 5.15 x 10^5 / ml was most suitable for xylanase production, increased spore concentration had a negative impact on the xylanase production. It was observed that inoculum grown using 5.15 x $10^{5,}$ spore formed very small pellets after 24 hrs.of growth in glucose medium and that with high spore count i.e. 1.3x 10^6 and 1.95x10^6 / ml ,the growth of pellet was more of mycelial branching type. Our study showed that it may be the morphology of inoculums that played an important role in the xylanase production. We found that an interaction between pH and inoculums was significant and at pH of 5.5 and lowest level of inoculum was best for achieving very high activity to the extent of (1269.89 units/g DW.) The increasing basal medium concentration, component comprising of yeast extract , KH_2PO_4, K_2HPO_4, $MgSo_4$ had a negative effect. Lower requirement for such medium component favoured the xylanase production and process economics . Since production of microbial enzymes has a large impact on overall process economy, it may be desirable to use thermophillic microorganisms which produce appreciable amount of thermostable enzyme while growing on cheap lignocellulosic substrate. The diverse application of xylanases and their sources suggests that novel strains must be isolated and screened from nature to meet the ever-growing demand of this important enzyme. Present study can be proved beneficial for stain development using approach of mutagenesis for improved quality and quantity of xylanase

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