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Groundnut shell- A novel substrate for the production of xylanase from *Acremonium implicatum* GCKAP3SASTRA

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Abstract: Direct utilization of Groundnut shell for the production of xylanase by *Acremonium implicatum* GCKAP3SASTRA was performed under solid state fermentation (SSF). Maximum production of xylanase was obtained at 75% moisture level, initial pH 6.0, yeast extract and potassium nitrate as organic and inorganic nitrogen sources with groundnut shell as sole carbon source for 5 days. The optimum enzyme activity for crude was found to be at pH 6.0 and 40°C. Crude xylanase was stable at pH 6.0 and temperature 40°C for 2 hr and retains 95% of enzyme activity. The enzyme produced under optimized condition was 5 fold higher than unoptimized condition.
Keywords: Xylanase, *Acremonium implicatum*, Solid state fermentation.

Introduction

Agricultural residues are widely available and are usually left to rot or burned in the field after harvesting. They are lignocellulosic in nature composed of cellulose, hemicelluloses and lignin[1]. A large number of value added products are produced by bioprocess technique by using hemicelluloses rich agricultural leftovers as a alternative substrate and also reduces the risk of pollution[2]. Xylan is the primary hemicellulosic component and accounts for about 20-35% of dry weight of plant biomass[3]. It is a heteropolymer, which contains xylopyranosyl residues as building blocks linked together by β-1,4 linkages with different substituent as side chains. The complete degradation of xylan requires hydrolytic enzymes like xylanase, β-xylosidase, β-glucuronidase and esterase[4]. Because of wide variety of industrial applications, interests in xylanase have been increased tremendously. It is used in pretreatment process, biofuel production, poultry and bakery products, pulping and biobleaching in paper industry, clarification of fruit juices and wines, waste treatment, extraction of oils and pigments[5].

Xylanase (EC 3.2.1.8) are involved in depolymerization of xylan by cleaving β- 1, 4 glycosidic linkages of xylan[6]. Xylanase are produced by bacteria, filamentous fungi, yeasts and actinomycetes. Of this filamentous fungi are the most potential producer and cultured easily[7-8]. They can be produced by both solid state fermentation and submerged fermentation. Solid state fermentation is preferred over submerged process because of reduction in production cost by the use of ligocellulosic waste as substrate. SSF are advantageous because of low contamination risk, high productivity, high stability and low instrumentation costs[9]. Wheat bran, corncob and sugarcane baggasses are known lignocelluloses for the production of xylanase[10]. In this present work, the fungal was investigated for the production of xylanase from groundnut shell and its application in bakery and bleaching industry.

Methods

Isolation of xylanolytic fungi

The xylanase positive microorganism was isolated and screened from paper mill effluent collected from local paper and pulp industry. The isolate GCKAP3SASTRA shows promising xylanase activity in xylan plate assay as it produces large amount of xylanase. The organism was preserved in xylan agar slants (1% xylan, 0.5% yeast extract, 0.1% sodium nitrate, 0.1% KH_2PO_4, 0.03% $MgSO_4.7H_2O$) and preserved at 4°C and used for further studies.

Identification of fungal strain

The organism was identified based on morphology and 16S rRNA sequencing (Bhat Biotech, Bengaluru, India). Genomic DNA from fungal strain was isolated using fungus genomic DNA extraction kit (Bhat Biotech). The isolated genomic DNA was subjected to PCR amplification of ITS region using Universal primers (Primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3', Primer ITS4: 5'-TCCGTAGGTGAACCTGCGG-3'). The ampli fication was carried out for 40 cycles in Master cycler® Thermocycler (Eppendorf, Germany). Sequence was compared to the non-redundant NCBI database by using BLASTN, and aligned using CLUSTAL W2, and phylogram was generated (MEGA5 software).

Production and extraction of xylanase

Xylanase production was carried out in solid state fermentation containing groundnut shell as a sole carbon source. The strain GCKAP3SASTRA was cultured in 250 ml conical flask encompassing 5% of groundnut shell, yeast extract 0.5%, KH_2PO_4 1%, NaCl 1%, $MgSO_4.7H_2O$ 0.03% and moistened using deionized water. The initial pH was maintained at 5.8 and sterilization was performed at 121°C for 20 min. The fungal isolate was cultivated in PDB medium for 3 days under shaking (150 rpm) and was inoculated into the production medium and allowed to grow for 5 days at a temperature of 28±2°C. The enzyme was extracted using distilled water at solid to liquid ratio of 1 g initial dry weight of substrate per 4ml of distilled water. The mixture was kept in shaker at 150 rpm for 1 h and then subjected to centrifugation at 8000 rpm for 15 min at 4°C to separate solid from extract. The crude enzyme (supernatant) was analyzed for enzyme activity.

Xylanase assay

Xylanase enzyme was analyzed spectrophotometrically using 1% beech wood xylan as substrate at room temperature. Dinitrosalicylic acid method[11] was employed to quantify the amount of reducing sugar liberated with standard xylose. One unit xylanase was the quantity of enzyme that were required to liberate 1µmol of xylose per minute.

Effect of nitrogen source and moisture level

For studying the effect of different nitrogen sources on xylanase production, the production media was supplimented with different organic (yeast extract, peptone, beef extract, urea) and inorganic (ammonium sulphate, potassium nitrite, sodium nitrate) nitrogen sources and fermentation was carried out for 5 days. Xylanase activity was determined for crude enzyme.

The influence of moisture content on xylanase enzyme production was explored by inoculating the fungal strain in production media containing different moisture level (30-90%) and the enzyme activity was determined. Moisture content was calculated by considering all liquids added to the medium.

Effect of fermentation time and initial medium pH

The optimum fermentation period for xylanase production was determined by incubating microorganism in production medium and sampled at various time intervals (1-14 days). The initial pH of the medium was varied from 3.0-10.0 using various buffer systems and adjusted using 0.1N HCl and 2% Na_2CO_3. The organism was grown in medium for 5 days at room temperature.

Effect of pH and temperature on xylanase activity

The enzyme activity was influenced by pH of the solution and that was investigated by incubating xylanase at room temperature in different pH ranges from 4.0 to 10.0. The following buffer solution was

employed: 10mM Citrate buffer pH 3.0 – 5.0, 10mM phosphate buffer pH 6.0-7.0, 10mM Tris HCl pH 8.0, 10mM carbonate-Bicarbonate buffer pH 9.0-10.0. The stability was investigated after incubating the enzymes at a pH of 4-10 at room temperature for 2 h and the residual activity at different time interval was determined.

To study the effect of temperature on xylanase enzyme activity, the enzyme was incubated at different temperature (30-70°C) at pH 6.0 in 1% beech wood xylan solution. The activity of enzyme was measured at a time intervalof 30 minute for 2 h at various temperature from 30 - 70°C to determine the thermal stability.

Results

Isolation and Identification of Fungal Strain

Diverse fungal species were isolated from the paper mill effluent and screened for xylanase production using xylan plate assay. The isolate GCKAP3SASTRA shows most prominent zone of clearance on congo red staining was selected. The organism was analyzed based on morphologic al characteristics and 16S rRNA sequencing. The morphological features were slow growing white colonies with thin and long hyphae. Analysis by 16S rRNA confirmed the isolate GCKAP3SASTRA as *Acremonium implicatum* (GenBank Accession number KC832475). The phylogenetic analysis was depicted in Fig.1.

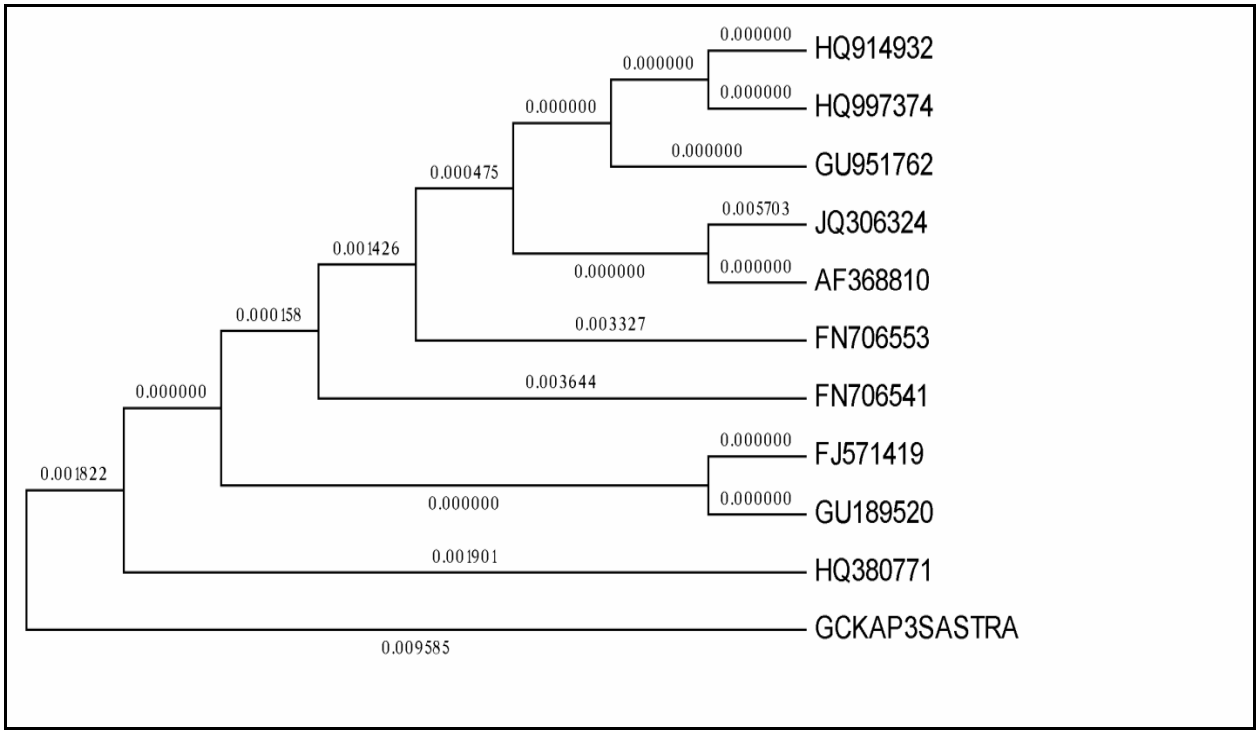

Fig 1: Phylogenetic analysis of isolated strain GCKAP3SASTRA

Optimization of SSF

The production of xylanase by *Acremonium implicatum* GCKAP3SASTRA was studied in shake flask cultures by SSF using lignocellulosic residue groundnut shell as a sole carbon source. The chemical composition of groundnut shell contains cellulose 38%, hemicelluloses 36%, ash 5%, moisture 5% and lignin 16%[12]. Xylanase activity was examined after 5 days. Optimization was carried out to increase the xylanase production. Moisture content, initial pH, fermentation time, nitrogen source were investigated for maximum xylanase production.

Effect of Nitrogen Source

The nitrogen source influences the mycelia growth and xylanase production. Different organic and inorganic nitrogen sources were supplied with groundnut shell as a carbon source. Among various nitrogen sources examined xylanase production was high in yeast extract followed by peptone and beef extract. Potassium nitrate shows enhanced enzyme production among inorganic nitrogen sources as shown in Fig 2.

Yeast extract facilitated the growth and maximum enzyme production in *Melanocarpus albomyces*[13], *Paecilomyces themophila* J18[14]. Inorganic nitrogen source nitrate accumulates more xylanase compared to organic nitrogen source yeast extract. This result was in accordance with xylanase production by *Aspergillus awamori*[15].

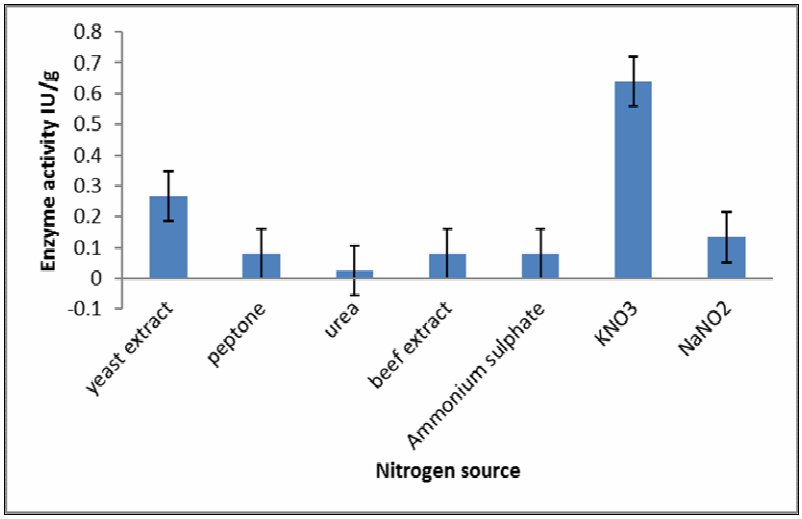

Fig 2: Influence of different organic and inorganic nitrogen sources on enzyme production

Initial Moisture Content

SSF is the process of growing microorganism in solid substrate with lack of free water and it particularly stimulates the growth and activity of filamentous fungi by creating natural habitat[16]. Moisture level is the crucial factor for enzyme production in SSF because free water and oxygen is required for the metabolism of microorganism[17]. The relation between various moisture content (30-90% initial moisture level) and enzyme production was depicted in Fig 3. The growth and enzyme production was poor at low water level and it steadily increased as the water content increases. Maximum xylanase production was achieved at 75% of moisture level. Enzyme production decreases with further increase in moisture level. This is due to the fact that increase in moisture level above its optimum level will cause reduction in porosity that affects oxygen and heat transfer[18]. In contrast, lower moisture level leads to poor dispersion of nutrients and also causes water tension[19]. Moisture content with similar effect was observed by many researchers[20-22]. Thus, in SSF when agro residues were used as carbon source with high moisture content facilitates enhanced fungal growth and enzyme production.

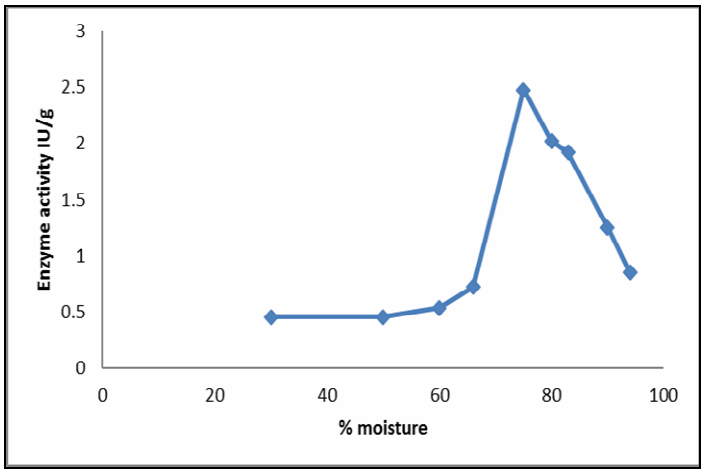

Fig 3: Impact of moisture level on xylanase production

Effect of initial pH and fermentation time

Microbial growth and activities were effected greatly by pH of the medium[23]. Xylanase production was evaluated for all pH ranges (pH 4.0-10.0). Highest production was obtained at pH 6.0 over pH 7.0 and 5.0 (Fig 4). This indicates the acidophilic character of the isolated fungal species. Certain fungal xylanase from *Aspergillus flavus* FPDN1[24], *Aspergillus terreus*[23] exhibited optimum production at initial pH 6.0.

To evaluate the relation between fermentation time and the growth of fungal isolate, the production medium was inoculated with fungus and xylanase activity was measured at different time intervals (1-14 days). The growth and enzyme production were determined by standard xylanase assay with 1% beech wood xylan as substrate. Fig 5 shows low level of xylanase production was observed initially and the maximum xylanase activity was reached at 5th day. The enzyme production was declined after 10th day of incubation. The reduction in enzyme production during SSF after prolonged incubation period was due to fungal autolysis or decline in medium pH[19,25]. *Streptomyces galbus* NR[26], *Aspergillus flavus* FPDN1[24], *Penicillium citrinum* MTCC 2553[27] have maximum xylanase production during 5th day.

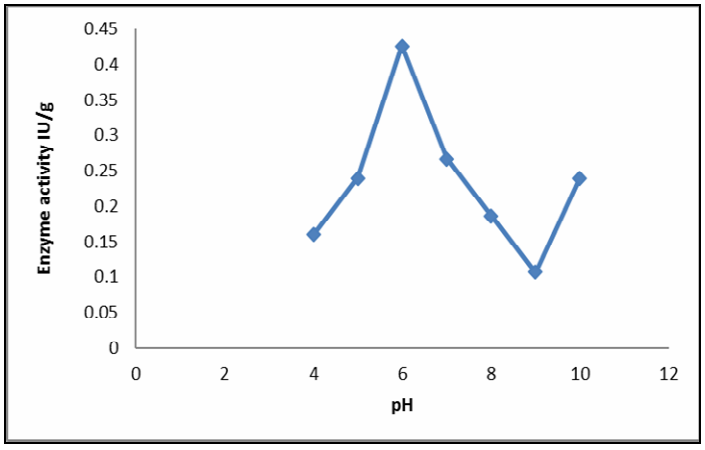

Fig 4: Effect of initial pH on enzyme production

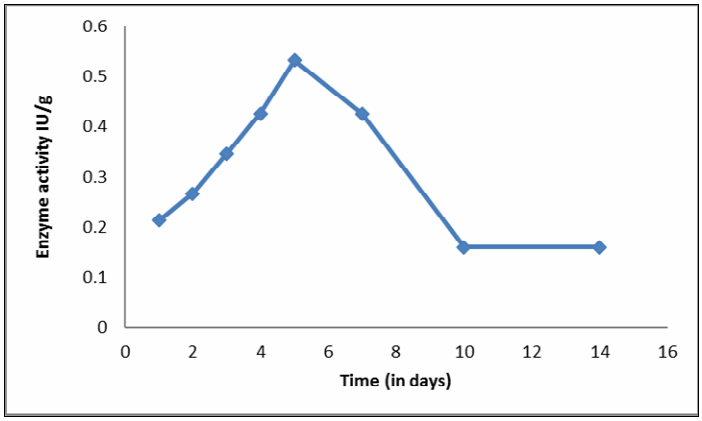

Fig 5: Fermenation time profile of xylanase production

Effect of pH and temperature on xylanase activity

The xylanase activity pH profile was illustrated in Fig 6A. The xylanase activity was studied over different pH range (3-10). The xylanase activity was detected in wide range of pH of 5.0-9.0. The optimum enzyme activity was observed in pH 6.0. The acidic pH of the enzyme favors the fruit juice clarification process[8]. *Penicillium oxalicum* ZH-30[28], *Aspergillus carneus* M34[2], *Streptomyces sp.* 7b[29], *Chaetomium thermophilum*[30] have optimum activity at pH 6.0. The relative activity of xylanase at pH 7.0 is 83% after 2 h of incubation. The pH stability of xylanase was depicted in fig 6B. and it shows activity of 95% at pH 6.0 after incubation of 2 h and it maintains 52% activity at pH 3.0.

The temperature activity pattern was depicted in Fig 7A. The enzyme activity was studied over temperature range of 30 - 70°C. Xylanase activity was found over broad range of temperature. The maximum activity was found at 40°C and enzyme activity declines after 70°C. Enzyme from *Aspergillus niger* DFR-5[8] showed maximum activity at same temperature. Thermal stability of different fungal xylanase varies between 40 - 70°C[31]. The temperature stability profile was depicted in fig 7B. The enzyme incubated for 2 h shows 76%, 97%, 86.6%, 75% at temperature of 30, 40, 50 and 60°C respectively.

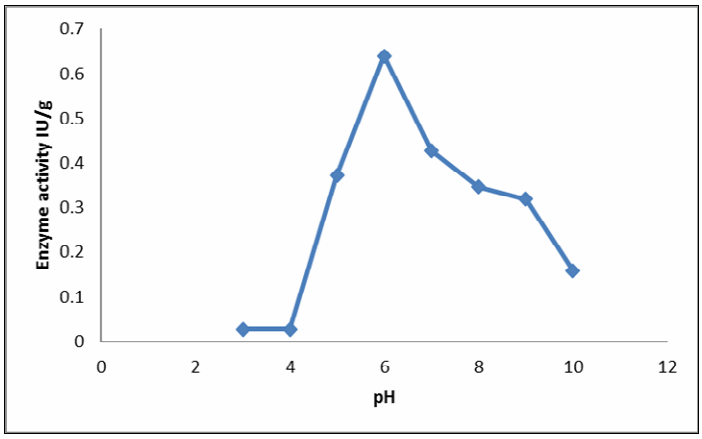

Fig 6a: pH profile of xylanase activity

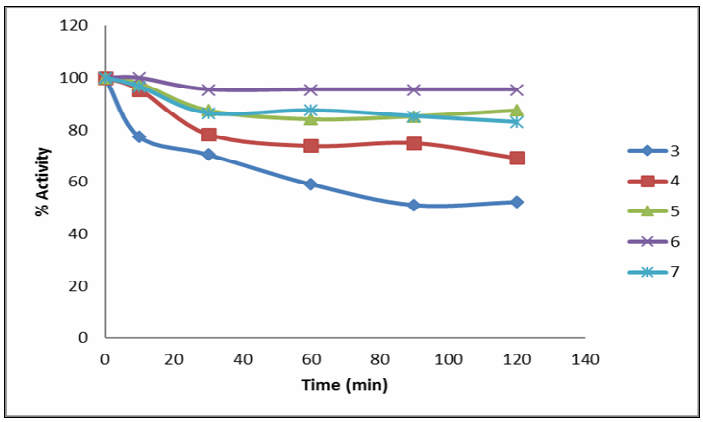

Fig 6b: pH stability of xylanase from *A.implicatum* GCKAP3SASTRA

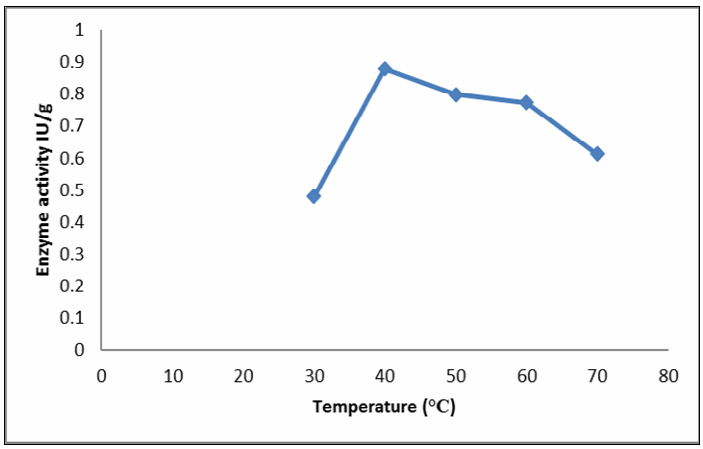

Fig 7a: Temperature profile of xylanase activity

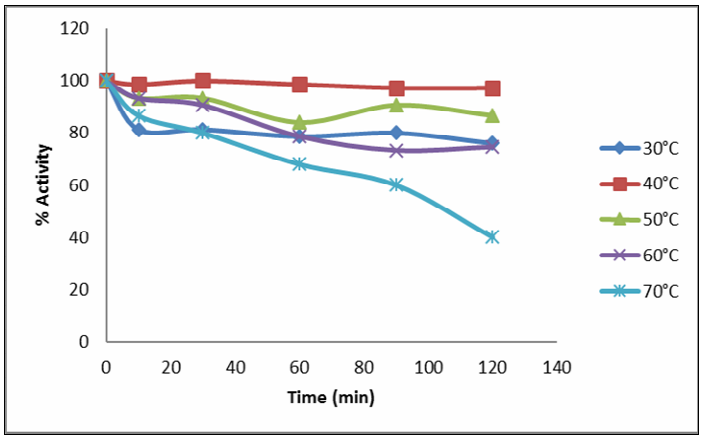

Fig 7b: Temperature stability of xylanase

Conclusion

Feasibility of groundnut shell to produce maximum level xylanase by solid-state fermentation was studied. It also highlighted a newly isolated fungal strain, *Acremonium implicatum* GCKAP3SASTRA showed ability to hydrolyse complex groundnut shell. Maximum level of xylanase was obtained with 75% moisture content, initial pH 6.0, potassium nitrate and yeast extract as inorganic and organic nitrogen sources with fermentation time of 5 days. Xylanase secreted by *Acremonium implicatum* GCKAP3SASTRA showed maximum activity at 40°C and pH 6.0.

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