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Research Paper

Production of Xylanase by *Aspergillus flavus* FPDN1 on *Pearl millet bran*: Optimization of Culture Conditions and Application in Bioethanol Production

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Abstract: *The production of extracellular xylanase by a locally isolated strain of* Aspergillus flavus FPDN1 *was studied for optimization of cultural conditions and saccharification followed by bioethanol production. Among the various agro residues used pearl millet (bajara) bran was found to be the best for high activity of xylanase with poor cellulase production under solid state fermentation with compared to submerged fermentation. During optimization, influence of different cultural conditions such as substrate concentration, incubation period, moisture level, inoculum size, pH, temperature, effect of carbon sources, orgenic and inorganic nitrogen sources were investigated. The production of the xylanase reached at maximum with substrate concentration (4g), incubation period (5 day), moisture level (45%), inoculum size (3 x 10^6 spores/mL), pH (6), temperature (25^0C), carbon source (xylose), organic nitrogen source (yeast extract) and inorganic nitrogen source [$(NH_4)_2SO_4$]. Under optimized conditions enhanced xylanase production achieved was 1530 IU/gds/min. The hydrolysis study of birch wood xylan by TLC was yielded xylose and other xylooligosaccharides as an end product. Crude xylanase was further utilized for enzymatic saccharification of pearl millet bran followed by bioethanol production from saccharified sugars. It was further confirmed by GLC analysis.*

Keywords: Xylanase, *Aspergillus flavus* FPDN1, Pearl millet bran, Optimization, Bioethanol.

Introduction

Xylan, the major hemicellulose component in plant cell walls, is found mainly in the secondary cell wall and is considered to be forming an interphase between lignin and other polysaccharides [9]. Xylan is typically comprised of a β-1, 4- linked xylopyranose backbone. Depending on its botanical origin, xylan can possess a number of side-linked groups, comprised of acetyl, arabinofuranosyl, and glucuronosyl residues [6]. The complete hydrolysis of xylan requires the combined action of various enzymes such as endo-β-1, 4-xylanase (EC 3.2.1.8), exoxylanase, β-D-xylosidase (EC 3.2.1.37) etc. xylanases randomly hydrolyze the β-1, 4-glycosidic bonds of xylan to produce several xylo-oligomers.

Xylanase has aroused great interest recently due to its biotechnological potential in many industrial processes, for example, in xylitol and ethanol production, in the cellulose and paper industry, in the production of oligosaccharides, to obtain cellular proteins, liquid fuels and other chemical substances, in the food industry and in poultry, pork, and caprine feeding [17]. A variety of microorganisms, including bacteria, yeast and filamentous fungi, have been reported to produce xylanase, of which the most potent producers are fungi. On an industrial scale, xylanases are produced mainly by *Aspergillus* and *Trichoderma* spp.

The cost of an enzyme is one of the main factors determining the economics of a process. Reducing the costs of enzyme production by optimizing the fermentation medium and process is the goal of basic research for industrial applications. In general, optimization by the traditional 'one-factor-at-a-time' technique was used. This method is determined by varying one factor while keeping the other factors at a constant level.

The aim of present study was to evaluate the xylanase production of *Aspergillus flavus* FPDN1grown on pearl millet bran through optimization of cultural conditions followed by its application in bioethanol production from saccharified sugars.

Material and Methods

Fungal strain and its growth conditions: The xylanase-producing fungal strain FPDN1 used in this study was isolated from soil collected locally from compost pit, Biogas Research Centre, Sadra, Gandhinagar, India., using potato dextrose xylan (Birch wood) agar medium (pH 7.0) at 35^{0}C temperature. For identification of fungal culture designated as a FPDN1 it was sent to The Agharkar Institute, Fungal Identification Service, Pune, India. It was maintained and stored at 4^{0}C on potato dextrose xylan agar medium.

Xylanase production under submerged and solid state fermentation: For the production of extracellular xylanase, the modified Manedel and Sternburg (1976)[12] basal (MS) medium containing (g/L): Peptone 1.0, KH_2PO_4 3.0, $NaNO_3$ 2.6, $MgSO_4.7H_2O$ 0.5, $CaCl_2$ 0.5, (mg/L) $FeSO_4.7H_2O$ 0.75, $MnSO_4.H_2O$ 0.25, $ZnSO_4.7H_2O$ 0.36, $CoCl_2.6H_2O$ 0.37 and 10 mL Tween-80 was utilized. The initial pH of the fermentation medium was adjusted at pH 7.0 before autoclaving. Submerged fermentation (SmF) was carried out in 250 mL Erlenmeyer flasks, each containing 100 mL of MS medium and 5% agro residues. The flasks were inoculated with 2% inoculum (approximately 10^6 spores/ mL) prepared from one week old fungal culture and incubated at 30 ^{0}C in an incubator shaker at 200 rpm for 5 days. The contents of the flasks were centrifuged at 6000 x g for 10 min in refrigerated centrifuge at 4 ^{0}C, and the resultant clear supernatant was used for the xylanase, cellulase, FPase and total protein determination. In solid state fermentation (SSF) 5g moist substrates with above mentioned MS medium to maintain initial 50% moisture content. All the flasks were maintained at 30 ^{0}C under static condition for 5 days. Enzyme was extracted by adding 100 mL of sodium citrate buffer (0.1 M, pH 6.2) in flask and kept on shaker for 30 min followed by centrifugation at 6,000 x g for 10 min at 4 ^{0}C. The clear supernatant was used for enzyme assay.

Enzyme Assays: The xylanase activity was assayed according to the method of Biely *et al.*, (1992)[4] by measuring the amount of reducing sugars (xylose equivalent) liberated from birch wood xylan using 3, 5-dinitrisalicylic acid[13]. The reaction mixture contained 490 µL of 1% xylan as substrate and 10 µL of appropriately diluted enzyme extract was incubated for 5 min at 55^{0}C, and then reaction was terminated by adding 1.5 mL of 3, 5-dinitrisalicylic acid reagent. A control was also run simultaneously, contained all the reagents but the reaction was terminated prior to the addition of enzyme extract. The contents were placed in a boiling water bath for 10 min and then cooled to room temperature. The absorbance of the resulting red color was measured against the control at 540 nm in spectrophotometer.

Cellulase activity (CMCase and FPase) was determined according to the method of Ghose (1979)[8]. The reaction mixture for carboxymethyl cellulase (CMCase) activity containing 0.5 mL of 1% carboxymethyl cellulose prepared in 0.1 M sodium citrate buffer, pH 6.2 and 0.5 mL of enzyme was incubated at 50^{0}C for 30 min. The reaction mixture for Filter Paper (FPase) activity contained a Whattman No. 1 filter paper pieces (approximately 1x1 mm), 1.0 mL of 0.1 M sodium citrate buffer, pH 6.2 and 0.5 mL enzyme extract, followed by incubation at 50 ^{0}C for 60 min. The mixture was boiled for 5 min in a boiling water bath, cooled and added 15 mL of distilled water to it. The color intensity was measured at 540 nm. A control was run simultaneously, contained all the reagents but the reaction was terminated prior to the addition of enzyme extract.

One unit (IU) of xylanase or cellulase activity was defined as the amount of the enzyme that catalyze the release of 1µmol of reducing sugar as xylose or glucose equivalent per min under the specified assay conditions.

Protein estimation: Soluble protein was estimated by Folin Lowry's method using bovine serum albumin as standard [11].

Optimization of cultural conditions for xylanase production under SSF

Effect of various agro residues on xylanase production : The fungal strain was inoculated in 250 mL Erlenmeyer flasks each containing 5 g of various substrates (wheat straw, sugarcane baggase, sorghum straw and pearl millet bran) and moistened with MS medium. The flasks were incubated at 35 ^{0}C for 5 days. The enzyme was then extracted and assayed for activity as described above.

Effect of pearl millet bran concentration: Effect of the pearl millet bran concentration on xylanase synthesis was evaluated by varying the concentration from 2 to 6 g in 250 mL flask with initial 50% moisture content maintained with MS medium. The flasks were incubated at 35 ^{0}C for 5 days.

Effect of incubation period: A 4 g sample of pearl millet bran was moistened with MS medium to keep 50% initial moisture in 250 mL flasks which were then autoclaved, inoculated and incubated at 35 ^{0}C. The enzyme was extracted and assayed from 3rd day up to 7th day cultivated flask.

Effect of initial moisture level: The influence of initial moisture level on the enzyme production was evaluated by varying the initial moisture content 35, 40, 45, 50, 55 and 60%. The activity of xylanase was determined after an incubation of 5 days at 35 ^{0}C.

Effect of inoculum size: Culture flasks containing pearl millet bran (4 g) kept at initial 45% moisture level were autoclaved and inoculated with 1 to 5 x 10^6 spores/mL (v/w). After 5 days incubation the crude enzyme was extracted and analyzed.

Effect of pH: Effect of medium pH on xylanase production

was estimated by culturing the fungal strain in media of different pH 5, 6, 7, 8 and 9. All flasks were kept at previously optimized condition.

Effect of temperature : Effect of temperature on xylanase synthesis was studied at different temperatures varying from 25 to 45 ^{0}C for 5 days. After incubation, xylanase was extracted and assayed.

Effect of additional carbon source: Various carbohydrates such as glucose, sucrose, fructose, lactose, maltose and xylose (0.5%) in MS medium were tested as an additional carbon source for enzyme production. After 5 days incubation at 25 ^{0}C enzymatic assays was performed.

Effect of organic nitrogen source: Various organic nitrogen sources (peptone, beef extract, yeast extract, meat extract, malt extract and tryptone) at 0.3% concentration in MS medium were studied to select best organic nitrogen source for xylanase production.

Effect of inorganic nitrogen source: Different inorganic nitrogen sources such as ammonium chloride, ammonium sulphate, ammonium hydrogen phosphate, ammonium nitrate, potassium nitrate and sodium nitrate was studied to check their effect on xylanase production.

Determination of hydrolytic products of xylan by TLC: The hydrolytic products of the soluble xylan by action of crude xylanase were identified by TLC. The hydrolyzed xylan broth along with xylan and xylose standards was applied on silica gel plate. The chromatogram was kept in acetone: isopropanol:water (6:3:1.5 v/v) solvent system. The chromatogram was developed by α-nepthol (3.5% in w/v in 83% ethanol and 10% H_2SO_4) and placed in oven at 100 ^{0}C for 10 min. The sugar spots appeared dark brown spots. Identification of sugar was studied by comparing it with the standards.

Bioethanol production from saccharified sugars: The physically grinded solid residue of pearl millet bran was hydrolyzed in SSF by crude xylanase enzyme produced by FPDN1 after 5 days incubation under all the optimized conditions. Due to enzymatic action simple sugars were produced which was extracted in 100 mL distilled water and sugar content was determined by Anthrone's method.

Fermented liquor containing sugars was directly used for ethanol production. The 100 mL liquor was sterilized, cooled and inoculated with 2% yeast suspension and incubated at 25 ^{0}C for 5 days. Ethanol content was determined by gas chromatography with an auto injector, split less injector system and flame ionization detector (FID). Separation was effected in a 30 m x 0.32 mm guard column. Samples of 0.1 µL were injected, and for quantitative analysis, ethanol used as an internal standard. An injector temperature of 200 ^{0}C, a detector temperature was maintained at 220 ^{0}C for 20 min. The column temperature was maintained at 220 ^{0}C. N_2 was used as a carrier gas.

Results and Discussion

Fungal strain and its growth conditions: In present study, total 5 morphologically different xylanase producing fungal cultures were isolated from different compost pit soil samples on potato dextrose xylan agar plates at 30 ^{0}C with 6 days incubation period. Based on the higher xylanase production under fermentation conditions FPDN1 was selected for further study. The fungal strain was identified as *Aspergillus flavus* on the basis of morphological and physiological characters.

Xylanase production under submerged and solid state fermentation: To select the best fermentation condition for xylanase production by *A. flavus* FPDN1 SmF and SSF was carried out (Figure 1), the higher xylanase production was achieved in SSF (710.4 IU/gds/min) with compared to SmF.

The technique of SSF involves the growth and metabolism of microorganisms on moist solid in the absence or near absence of any free-flowing water. These fermentation systems, which are closer to natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites [18]. Soliman *et al.*, 2012 [18] also reported the similar kind of the results for xylanase production under SSF with compared to SmF.

Effect of various agro residues on xylanase production: Various inexpensive agro-residues were tried as substrates for xylanase production by *A. flavus* FPDN1 in SSF. Among tested substrates, pearl millet (bajara) bran showed maximum activity of xylanase (906.3 IU/gds/min) as compared to other substrates viz. wheat straw, sugarcane baggase and sorghum straw after 5 days of incubation at 35 ^{0}C (Figure 2). This result may be attributed to the value of xylan or cellulose: xylan ratios in each substrate. In this connection, most literature proved that fungal xylanase could produce on different natural substrates during SSF[18]. Kaur *et al.*, (2011)[10] reported maximum xylanase activity with wheat bran.

Effect of pearl millet bran concentration: The effect of available substrate concentration plays an important role on the enzyme production and their activity. In present investigation, we employed the pearl millet bran at various concentrations ranged from 2 to 6 g under SSF. Improvement in xylanase production was observed with increased substrate concentration from 2 to 4 g and decreases thereafter at 4 to 6 g substrate concentration. Maximum xylanase activity was obtained (1171.06 IU/gds/min) at 4 g concentration (Figure 3), this might be due to the fact that high concentration of substrate influenced the maximum medium component and oxygen transfer rate. Our observed results are in tuning with the work reported by Ahmed *et al.*, (2012)[2]. They got

optimum xylanase production at 3.5% substrate concentration.

Effect of incubation period: Time course plays a very critical role in fungal metabolic activity and growth. In present study xylanase production was determined starting from 3rd day up to 7th day of inoculation at regular interval of 24 h (Figure 4). The obtained results indicated that, the highest yield of xylanase was 1438.7 IU/gds/min at 5th day of incubation. Further increase in incubation period resulted decrease in subsequent xylanase production, this might be due to the fact that higher number of spores may utilize available nutrients rapidly to achieve log phase of growth, the subsequent decrease in xylanase synthesis could be due to exhaustion of nutrients in medium which stressed the fungal physiology resulting in the inactivation of secretary mechanism of fungi. In the connection of our findings, Neves *et al.,* (2011)[14] carried out higher xylanase production at 5th day of incubation by *Lichthemia blakesleeana.*

Effect of initial moisture level: The moisture content in SSF is a crucial factor, which determines the success of the overall process. In present work, SSF was carried out by potent fungi *A. flavus* FPDN1 with varied different moisture level ranged from 35 to 60% (v/w). As shown in Figure: 5, our results indicated that the optimum moisture level for xylanase production was 45% (1012.5 IU/gds/min). Increase in moisture content influenced the enzyme biosynthesis negatively, it reduced surface area of the substrates and made the water film thicker which affected the accessibility of the air to the substrate. The obtained results are similar with the reported work of [7]. They observed maximum activity of xylanase at 48% moisture content.

Effect of inoculum size: The culture used to inoculate the fermentation medium must be in healthy, active state and of optimum size, and possibly minimizing the length of growth phase. Thus in its high rate of substrate conversion we have varied the size of inoculum between 1 to 5 x 10^6 spores (1 O.D = 1 x 10^6 spores/mL at 660 nm). The maximum xylanase activity (1051.7 IU/gds/min) obtained at 3 x 10^6 spore density. A further increase or decrease from 3 x 10^6 spore density showed gradual decrease in xylanase production (Figure 6). The results suggest that lower inoculum size resulted in less number of spores and requires a longer time to grow to optimum number to utilize substrate, while higher inoculum results in greater number of spores which might cause nutrient limitation condition, resulted in decrease in metabolic activity of *A. flavus* FPDN1. Soliman *et al.,* (2012)[18] reported maximum xylanase synthesis at higher inoculum size at 1- 4 x 10^7 spores/mL.

Effect of pH: Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both substrate and in particular enzyme molecules. Thus, pH is known to affects the synthesis and secretion of xylanase. During optimization study pH ranged from 5 to 9. Among which, maximum xylanase production (1274.5 IU/gds/min) was achieved at pH 6 (Figure 7). In the harmony of our findings, Abdelrahim *et al.,* (2011), reported maximum xylanase production at pH 6 by *S. thermophile* and *C. thermophile.*

Effect of temperature: Temperature is of much influencing parameter in SSF system, because during fermentation there is a general increase in the temperature of the fermenting mass due to respiration. As shown in: Figure 8, the maximum xylanase activity (966.8 IU/gds/min) was observed at 25 ^{0}C. At above 25 ^{0}C, rapid decline in xylanase biosynthesis was observed. This result might be due to the fact that high temperature can change membrane composition and caused the protein catabolism and inhibition of fungal growth or it may be due to drying of substrate at higher temperature. The observed results clearly indicate the mesophilic nature of the fungus. In the line of observed results, Neves *et al.,* (2011)[14] showed maximum xylanase production at 26 ^{0}C by *Lichthemia blakesleeana*.

Effect of additional carbon sources: The production of primary metabolites by microorganisms is highly influenced by their growth which is determined by the availability of the nutrient in the substrates. In present study, maximum xylanase production obtained in case of xylose as carbon source (1315.5 IU/gds/min) (Figure 9). Many researchers have been reported xylose as an inducer for the xylanase production. Noted results were confirmed with the work reported by Murthy and Naidu (2012)[15], showed maximum activity of xylanase using xylose as carbon source from *Penicilluim* sp.

Effect of organic nitrogen source: Nitrogen have influencing effect on xylanase production thus in present investigation different organic nitrogen sources such as peptone, yeast extract, beef extract, meat extract, malt extract and tryptone at 0.3% was evaluated (Figure 10). The optimum xylanase production 1417.6 IU/gds/min was obtained in yeast extract as most effective organic nitrogen source. This finding is in contradiction with the reported work by Murthy and Naidu, (2012) [15]. They showed optimum xylanase production by using peptone as organic nitrogen source.

Effect of inorganic nitrogen source: Present study was designed to evaluate the effect of inorganic nitrogen sources such as NH_4Cl, $(NH_4)_2SO_4$, NH_4HPO_4, $NaNO_3$, KNO_3 and NH_4NO_3 at 0.3% concentration. Among evaluated inorganic sources maximum xylanase activity was observed 1395.1 IU/gds/min. with $(NH_4)_2SO_4$ (Figure 11). Our results are supported with work reported by Dai *et al.* (2011) [5], they also showed maximum xylanase activity

at 3.1% $(NH_4)_2SO_4$ during optimization of SSF from *Aspergillus niger* JL-15.

Determination of hydrolytic products of xylan by TLC: The hydrolysis study of the xylan with crude xylanase was carried out by TLC. The study revealed the end products of xylan hydrolysis as xylose, xylobiose and other xylooligosaccharides (results not shown), the end products xylose and xylobiose indicates the nature of the enzyme as an endoxylanase. It might be due to the presence of the xylanase accessory enzymes.

Bioethanol production from saccharified sugars: The present study showed successful production of ethanol from the saccharified sugars of agricultural waste pearl millet bran through solid state fermentation by *A. flavus* FPDN1. The obtained results by gas chromatography clearly shows that the distilled samples of produced bioethanol chromatograms were compared with used standard and it indicates the presence of ethanol. However with compare to ethanol standard, test samples gave the concentration of ethanol content 88% with 3.31 min retention time (Figure 12). Our GC analysis reports are in accordance with Banerjee *et al.*, (2011)[3] they carried out experiments on bioethanol by GLC during alkaline peroxide pretreatment on agriculture residue.

Conclusion

The present investigation successfully shows production and application of *Aspergillus flavus* FPDN1 xylanase under optimized cultural conditions. The potent fungal culture *A. flavus* FPDN1 shows very good xylanase production on pearl millet bran with compared to other agricultural residues. Optimized conditions gave maximum xylanase activity 1530 IU/gds/min.

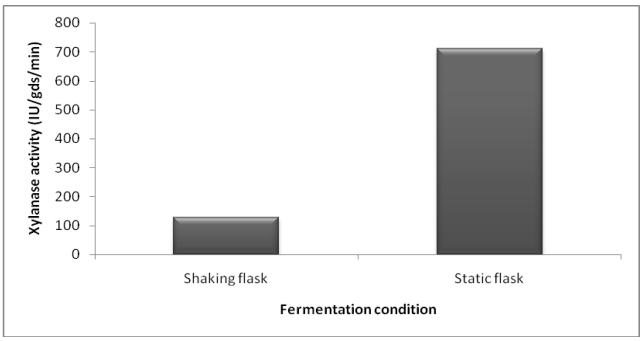

Figure 1: Effect of different fermentation condition

Furthermore, the hydrolytic products of xylan were identified as xylose and other xylooligosaccharide by TLC which has great industrial importance. The saccharified sugars were produced by the hydrolysis of pearl millet bran has been successfully used for the bioethanol production. It has good potency for scale up and large scale ethanol production.

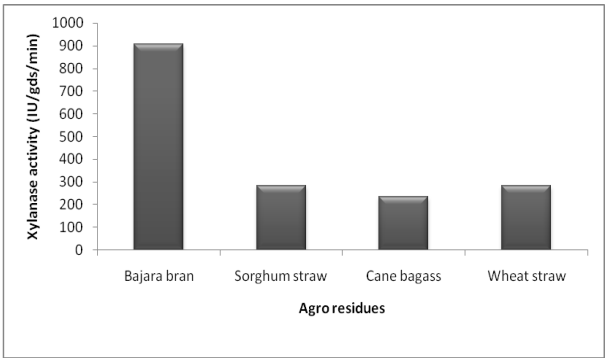

Figure 2: Effect of different agro residues on xylanase production

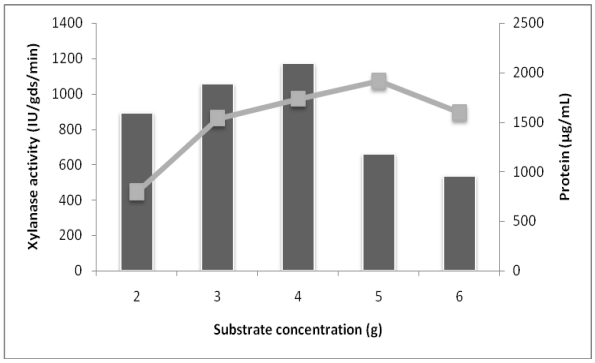

Figure 3: Effect of substrate concentration on xylanase production

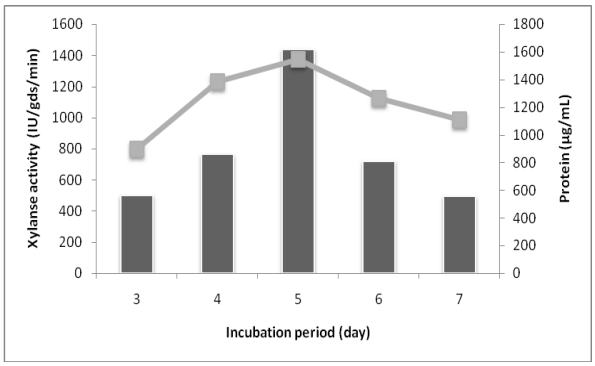

Figure 4: Effect of incubation period on xylanase production

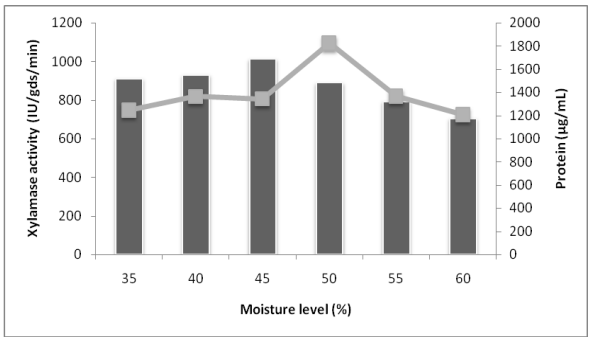

Figure 5: Effect of moisture level on xylanase production

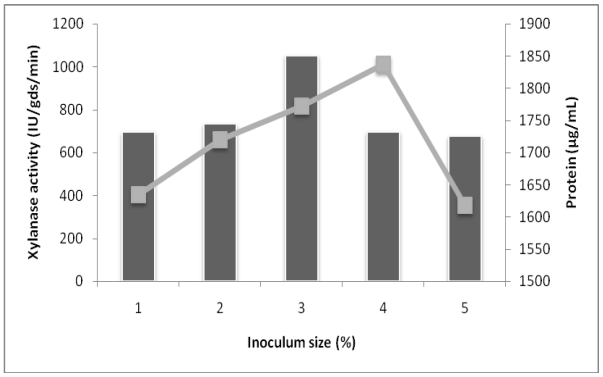

Figure 6: Effect of inoculum size on xylanase production

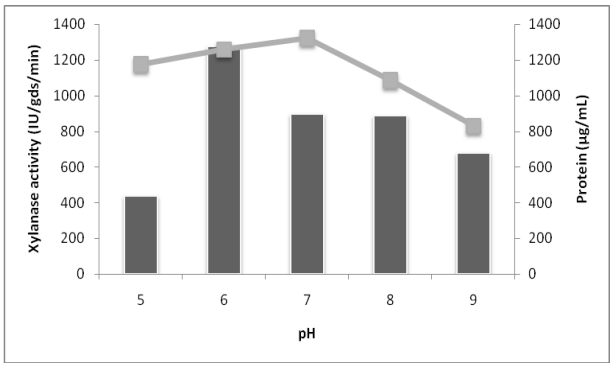

Figure 7: Effect of pH on xylanase production

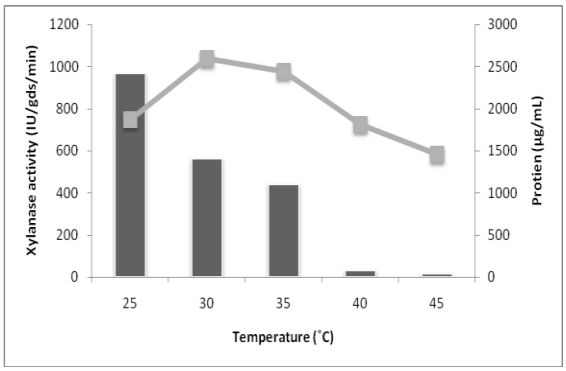

Figure 8: Effect of temperature on xylanase production

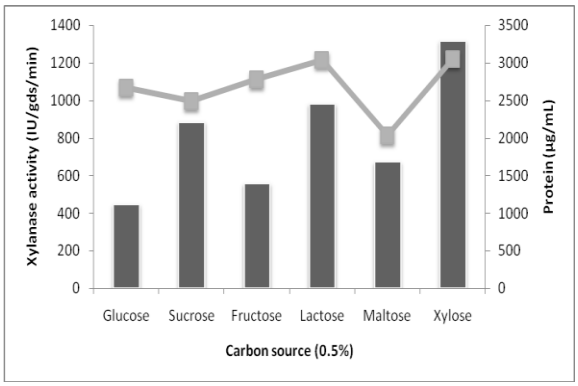

Figure 9: Effect of carbon source on xylanase production

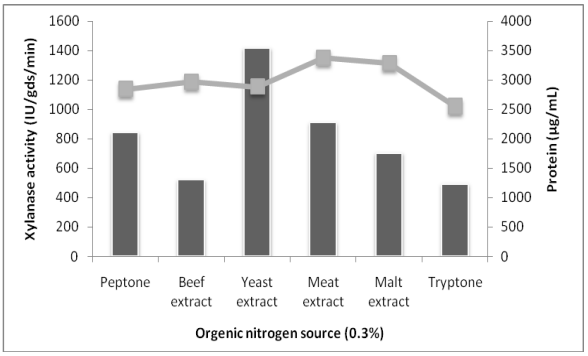

Figure 10: Effect of organic nitrogen source on xylanase production

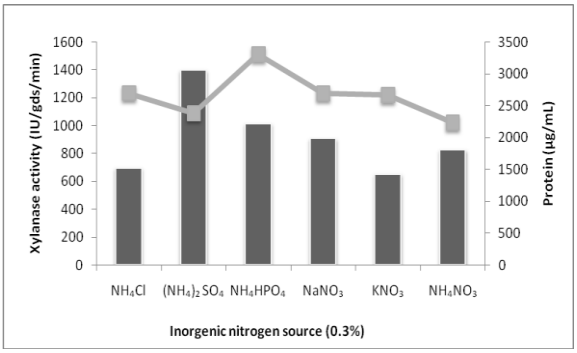

Figure 11: Effect of inorganic nitrogen source on xylanase production

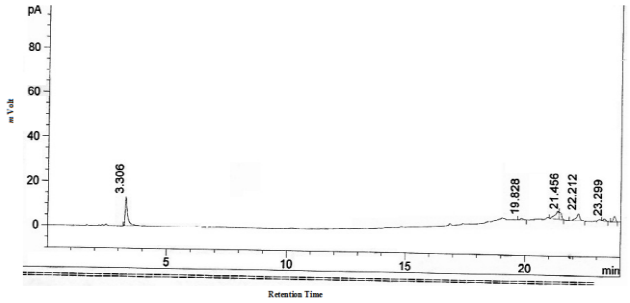

Figure 12: Chromatogram of saccharified sample (X axis: Retention Time and Y axis: *m* Volt)

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