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Standardization Of Bioprocess Parameters For Optimization Of Xylanase Production By Aspergillus Niger Soil Isolate

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ABSTRACT

In-vitro studies were carried out on *Aspergillus niger*, an indigenous strain, to determine the most optimize conditions for high xylanase production. The selected strain was boosted for xylanase enzyme production in czapek's dox broth medium using different carbon and nitrogen sources. Maximum enzyme activity was detected when xylan was used as carbon source and yeast extract was used as nitrogen source. Shake culture conditions, 1.0% NaCl, higher inoculums dose and 06 days of incubation were observed with high xylanase production. Submerged condition was found superior over solid-state condition; however, wheat bran substrate yielded more xylanase over others. Optimum pH and temperature for xylanase activity were found to be 7.0 and 28^{o}C. The enzyme was produced and extracted under optimized conditions and compared with values of before optimization. It was then purified and characterized for stability at different temperatures and pH. A high amount of xylanase was formed under optimized conditions and was found stable up to 45^{o}C temperature and at pH 8.0.

Key words: Bioprocess parameters, Optimization, *Aspergillus niger*, Xylanase

I. INTRODUCTION

Microorganisms are rich sources of xylanase enzymes, which are produced by diverse genera and species of bacteria, actinomycetes and fungi (Chandra *et al*., 2012 and Malhotra and Chapadgaonkar, 2018). Applications of xylanases can be found in the food and beverage industries (bakery goods, coffee, starch, plant oil and juice manufacture), feedstock improvement (increasing animal feed digestibility), and the quality improvement of lignocellulosic residues. The use of xylanases to allow such manufacturing processes to be run with less elements, further down less harsh conditions and also with less troubling side reactions. The cost of any enzyme production is one of the key factors, require optimization of conditions and parameters to achieve cost -benefit ratio.

Filamentous fungi are predominantly producers of xylanases. They excrete the enzymes into their surrounding and the enzyme levels are much higher than those of yeast and bacteria (Steiner *et al*., 1987). In addition to xylanase, fungi

typically produce several accessory xylanolytic enzymes, which are necessary for debranching substituted xylans (Jaafaru, 2013). An important factor for efficient xylanase production is the choice of an appropriate inducing substrate and bioprocess parameters that can affect activities and productivities of xylanase (Dietmar *et al*., 1996). Similarly, the activity of crude enzyme is also very important particularly when it is to be used in its crude form.

Considering these, a preliminary study had been done on 32 indigenous fungal isolates of social forest area of Bhilai Township to screen out the best xylanase producer among all and out of them *A.niger* was found the best xylanse producing strain among all (Kulkarni and Gupta, 2013). The main objective of present study is to standardize the parameters for xylanase production by *A. niger* under experimental conditions.

II. MATERIALS AND METHODS

Identification of isolate *Aspergillus niger* the potential producer of xylanase – Fungal identification was carried out based on the colony morphologies and structural characteristics as observed under the light microscope. The fungal characteristics were described and identified based on the description given in the manual of soil fungi (Gilman, 1959), Dematiaceous Hyphomycetes (Ellis, 1971) and illustrated genera of Imperfect fungi (Barnett *et al*., 1972). The ability to produce xylanase was primarily confirmed by Congo red test by rising on Oat spelt xylan agar plates (Kheng and Omar, 2005).

Xylanase assay – Xylanase activity was further assessed by using Oat spelt xylan (Sigma) as enzyme substrate. The enzyme substrate was prepared by 0.25 g of xylan wetted with 2ml 95% ethanol and 22.5ml of distilled water in a beaker and covered with aluminum foil and boiled to dissolve on hot plate at 100^{o}C for 10 min and made up the volume to 25ml with distilled water. The reaction mixture was prepared by adding 5.0ml of substrate, 1.0 ml of the culture filtered supernatant (crude enzyme) and 1.0 ml of sodium acetate buffer (pH 6.0). The mixture was incubated at 40^{o}C in water bath with shaking for 15 min. Released reducing sugar was measured using 3, 5-dinitrosalicylic acid and xylose as standard. Colour was developed by boiling in water bath for 5 min and read at 540 nm. One unit of enzyme activity was defined as amount of enzyme required to liberate 1µmol of xylose per minute under the assay conditions.

Optimization of cultural conditions for xylanase production – The optimization was carried out based on stepwise amendment of the main parameters for xylanase production. The growth was carried out keeping the temperature (28^{o}C) for 7 days.

A) **Effect of various carbon sources:** lactose, sucrose, maltose, dextrose, xylan, cellulose, and mannitol were examined using czapek's dox broth.
B) **Effect of various Nitrogen sources:** peptone, yeast extract, sodium nitrate, ammonium sulphate, ammonium nitrate, meat extract and beef extract were used.
C) **Optimization of salt concentration:** Using NaCl and KCl at different concentrations, varying from 1 to 6%.
D) **Effect of agitation:** The effect was examined by using regular incubator for non agitating flasks and shaker incubator for agitating flasks.
E) **Effect of incubation period:** Evaluated at 24 h interval over a period for 7days.
F) **Effect of pH:** Observed by changing the pH of growth medium by 05 to 10.

G) Effect of cultivation temperature: The enzyme production was examined by incubating the growing culture at different temperatures starting from 28ºC, 32ºC, 37ºC, 40ºC.

H) Optimization of inoculum size: Approximately 100 to 150 spores of *A.niger* were transferred to 9 ml of sterile water blank. This suspension was serially diluted to 10^{-1} to 10^{-6} and each dilution was transferred to sterile media and incubated at 28ºC for 7 days.

I) Xylanase production under solid state fermentation: The fungi were cultured in Erlenmeyer flasks (250 ml) containing 10.0 g of paddy straw, paddy husk, rice bran and wheat straw (particle size 300 - 500 mm) moistened with 10 ml of mineral salts solution. The composition of the mineral salts solution was (g./L): NaCl, 1.0; $MgSO_4.7H_2O$, 0.5; $(NH_4)_2HPO_4$, 2.5; NaH_2PO_4, 0.5; $CaCl_2.2H_2O$, 0.01; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.002. The pH was adjusted and the medium was then autoclaved. The flasks were inoculated with 1 ml of spore suspension containing $1x10^6$ spores/ ml obtained from 7-day-old pure cultures. The flasks were incubated at 28ºC under shaking conditions (100 rpm) for 7 days. The enzyme was harvested in sodium acetate buffer (50 mM, pH 7.0). The fermented slurry was filtered through cheese cloth and centrifuged at 10000 x g for 20 min at 4°C. The supernatant was used for further enzyme study.

J) Xylanase production under submerged fermentation: It was carried out with Birch wood xylan as the carbon source along with mineral salts medium. The pH of the medium was adjusted to 7.0 and 50 ml of the medium was transferred into a 250 ml Erlenmeyer flask and inoculated with 1 ml of spore suspension containing $1x10^6$ spores/ ml. The flasks were incubated at 28ºC on a shaking incubator (100 rpm) for 7 days. After incubation, the medium was filtered through Whatman No.1 filter paper and the filtrate was centrifuged at 10000 x g for 15 min at 4ºC. The supernatant was used as enzyme for further evaluation.

Enzyme characterization –

A) Partial purification of xylanase: It was done by adding chilled Ethanol drop wise to the culture filtrate (100 ml) at 4 °C with continuous stirring and left at –20°C for 24 h. The precipitate was collected by centrifugation and was then dissolved in 30 ml of a sodium acetate buffer (50 mM, pH 6.0).

B) Stability of xylanase activity on different temperature and pH range – The temperature stability/ tolerance of purified enzyme was determined by the incubation of the reaction mixture from 15 to 70ºC and at pH 7.0. Enzyme activity was measured at 45ºC in different pH values by the use of sodium acetate buffer from 3.0 to 8.0.

SDS-PAGE and xylanases detection by zymography – Sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS-PAGE) was carried out using a 10% gel and low molecular weight standards from 14 to 66 kDa. After the electrophoresis, the xylanase polypeptide was detected using oat spelt xylan (Sigma) as substrate.

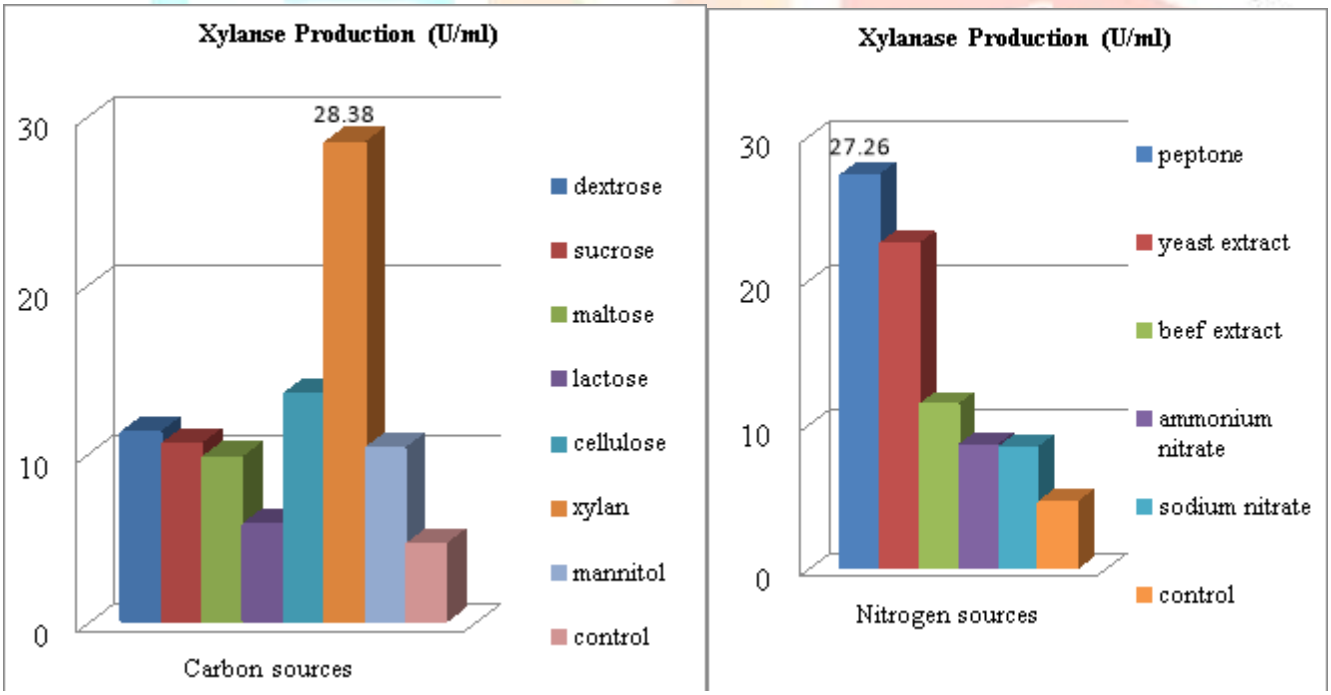
Statistical Analysis – All the above experiments were carried out maintaining 05 replicates and obtained values were subjected to find out standard error of mean.

III. RESULT AND DISCUSSION

Soil fungi are gaining considerable importance as good producers of xylanase over others from the industrial point of view. Their non-pathogenic nature, easy cultivation and capability of producing high levels of extracellular enzymes attract researchers to work on parameter optimization procedures. and to study the factors responsible for increasing enzyme activity in its crude form (Simoes *et al*., 2009; Sridevi and Charya, 2011 and Parihar and Rai, 2015). In the present study, different bioprocess parameters were investigated for indigenous soil fungal isolate *Aspergillus niger*.

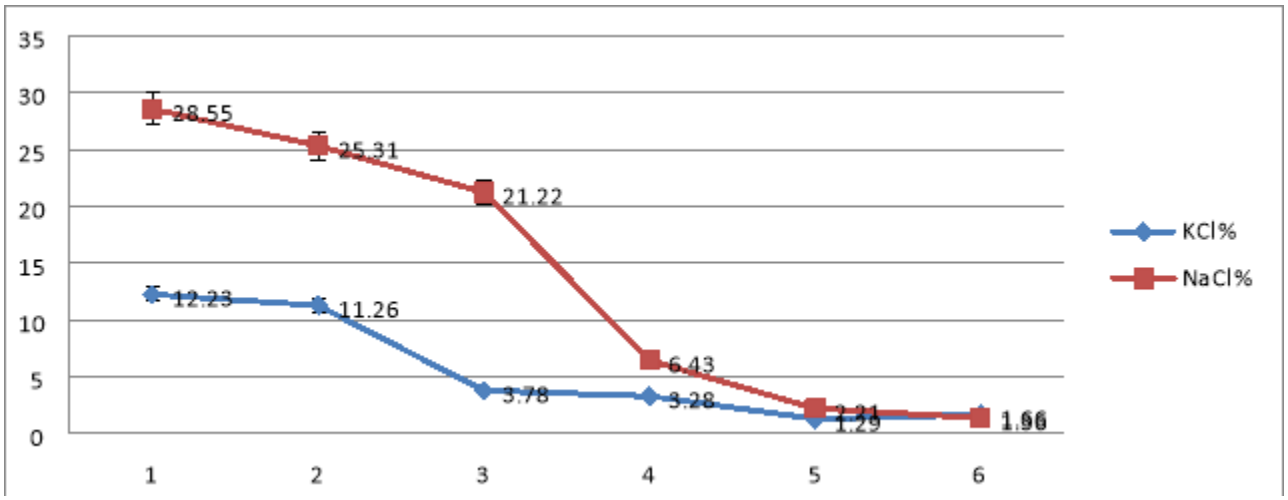
The effect of additional carbon and nitrogen sources in the growth medium affected positively. Kheng and Omar (2005) also reported that supplementation of additional carbon and nitrogen sources in the growth medium could enhance enzyme productivity. The enzyme production was found significantly higher when supplemented with xylan (28.38U/ml) as carbon source (Fig.-1). The results showed that xylan served as a superior carbon source and seems to be efficient inducers of xylanase activity by *A. niger*. Okafor *et al.* (2005) suggested the initiation of xylanase production through low molecular weight degradation products of xylan. Peptone as nitrogen source was found best over others (27.26U/ml). This result however, was in contrast to that reported by Naby *et al.* (2005). On the other hand, Tallapragada *et al*. (2011) reported organic nitrogen sources (meat extract, peptone and yeast extract) relatively suitable for xylanase production.

Fig. 1 Effect of different carbon and nitrogen sources on xylanase production



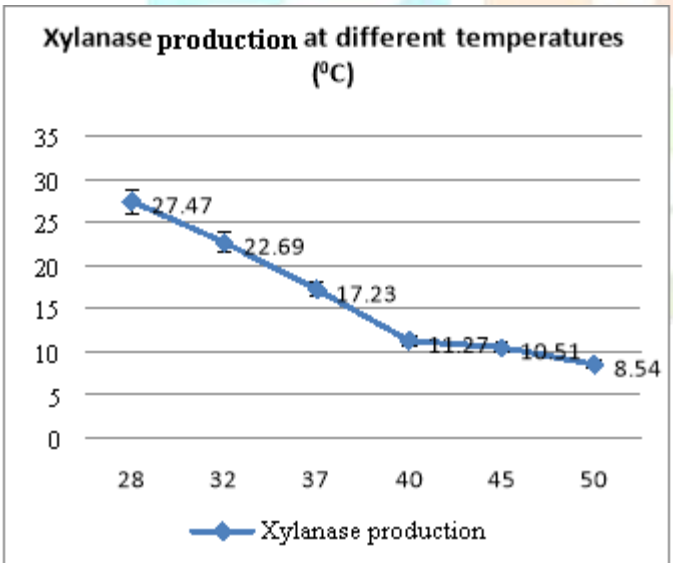
Lower concentrations of salts were affected positively. However, NaCl (1%) was found superior over the KCl (1%) and produced significantly high (28.55U/ml) xylanase (Fig.-2).

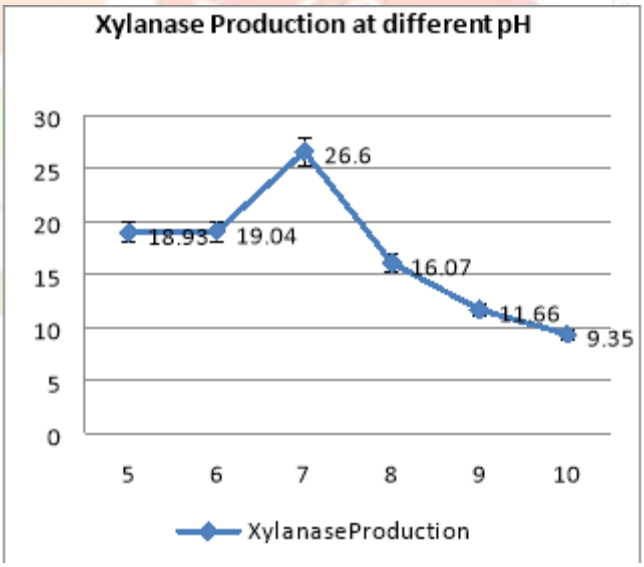
Fig. – 2 Effect of NaCl and KCl at different concentrations on xylanase production



Aspergillus niger grew well at 28ºC, pH 7.0 and produced significantly more enzyme (27.47U/ml; 26.6U/ml respectively (Fig. -3). The net temperature and pH influenced not only by the environment, but also the changes generated from the metabolic activities of the growing fungi. The results revealed that the enzyme production linked closely to the growth of the fungus. The xylanase production correlated to the growth and availability of fungal biomass. This observation was in agreement with those reported by Kheng and Omar (2005) and Simoes *et al*. (2009).

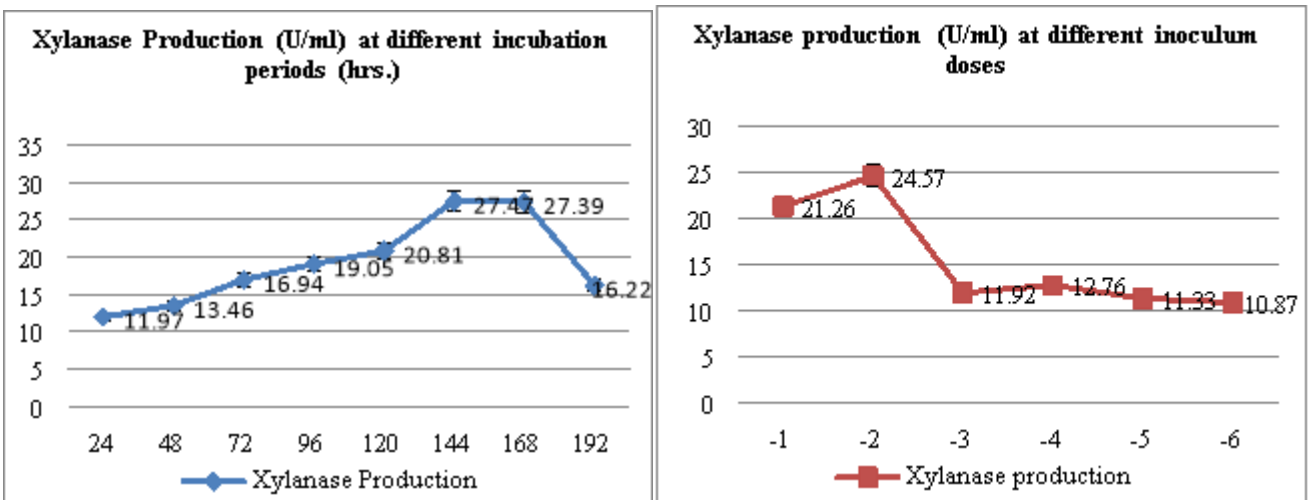
Fig. - 3 Effect of incubation temperature and pH on xylanase production





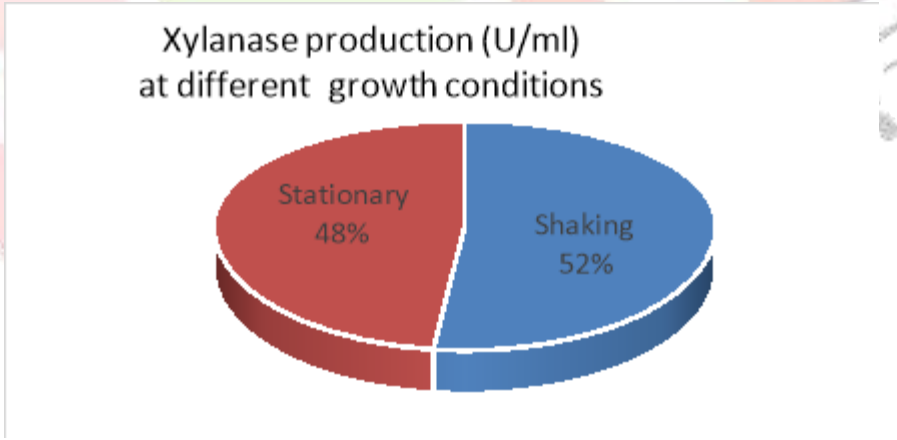
The effect of incubation period and inoculam dose on xylanase production is depicted in Fig.-4. It is clear from the figure that the fungus attains its maturity after 6-7 days and produced maximum enzyme (27.4U/ml). This is due to exhaustion of growth the substrate from the medium after 6-7 days and thus not available to trigger the extracellular enzyme production. High spore contents during inoculation caused faster growth and prompt enzyme production (24.57U/ml), supporting the fact that the time taken to achieve maximum growth or enzyme productivity was much longer at lower inoculum sizes. Tallapragada and Venkatesh (2011) also reported higher degradation of the substrates and increase availability of the nutrients for higher enzyme production during their studies.

Fig. – 4 Effect of incubation period and inoculums dose on xylanase production



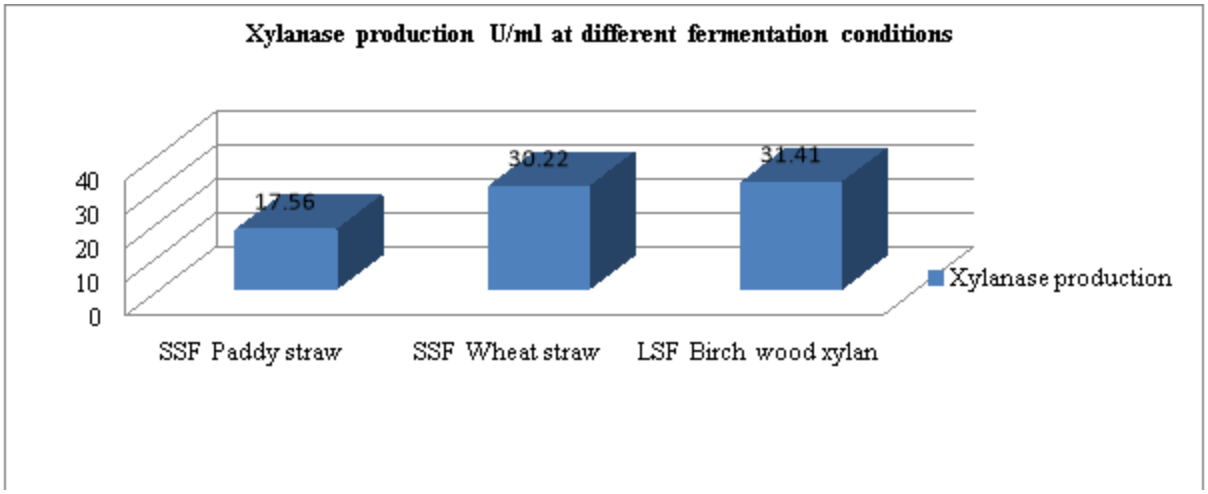
The effect of agitation was found important to ensures aeration, supply of oxygen for aerobic metabolism, and also for removal of CO_2, heat, water vapor, and volatile components produced during the growth. It also has very important effects on hydration properties. The effect of shaking on xylanase production was studied under above optimized growth conditions. It was observed that shaking conditions supported the enzyme production over the stationary condition (Fig.–5). However, the difference was not significant. Several studies have reported different results regarding agitation during fermentation and stated that some organisms require more air for growth as compared to others.

Fig. – 5 Effect of agitation on xylanase production



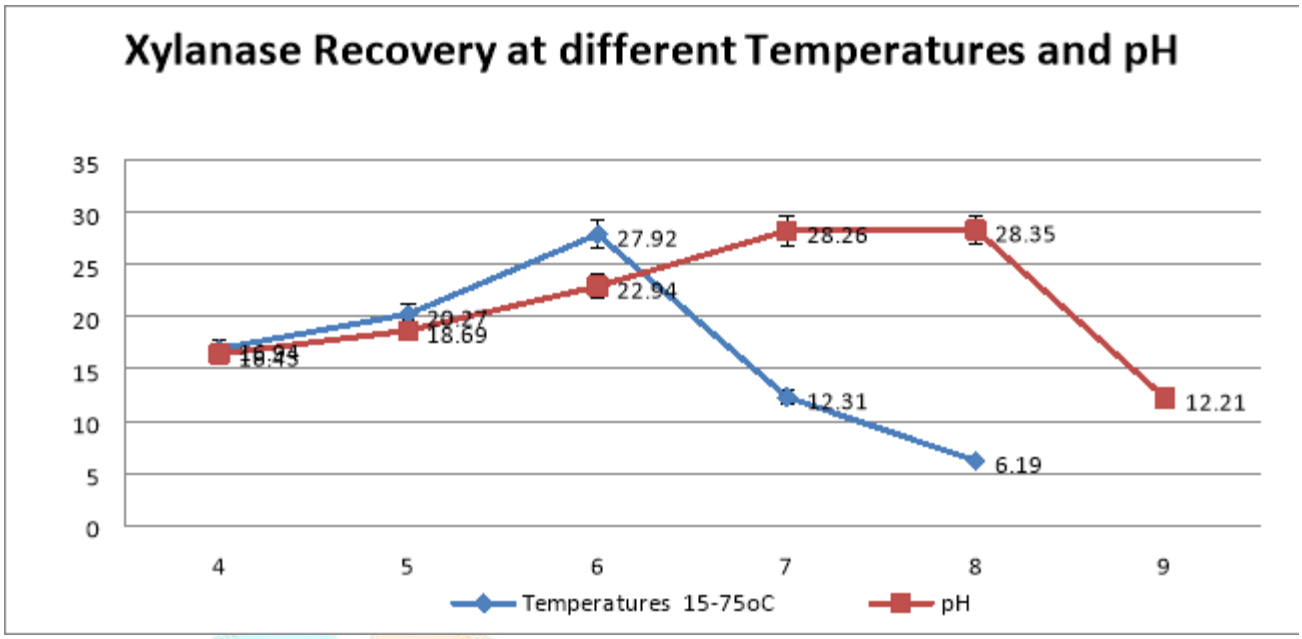
The media types are one of the most responsible factors for the higher value of crude enzyme activity obtained from fungal strains. Solid-state fermentation (SSF) has significant economical in producing the food, feed, pharmaceutical and agricultural industrial products. Due to the rough nature of the substrates, strong agitation is not required during the fermentation. The products thus obtained are more concentrated and need less purification procedures. Additionally, the countries with abundance of biomass and agro-industrial residues, these can be used as cheap raw materials. Xylanase initiation by lignocelluloses substrates have been shown by several reports. The major substrates used in their studies were wheat bran, rice straw, corncobs, saw dust, sugarcane bagasse, palm kernel cake and soybean meal (Reis *et al*., 2003; Okafor *et al.*, 2007 and Simoes *et al*., 2009). In the present study, wheat straw under SSF condition and birch wood xylan under LSF condition yielded better xylanase (30.22U/ml and 31.41U/ml respectively).

Fig. – 6 Effect of fermentation condition on xylanase production



Temperature during growth plays a critical role in enzyme productivity. The enzyme activity was investigated at different reaction temperatures ranging from 25-60°C (Fig. -7). The *Aspergillus niger* contributed promising results, with maximum activity of xylanase, when incubated at 45°C (16.00 Unit/ml) and minimum was observed at 25°C (7.33 Unit/ml). The enzyme activity was found to increased or decreased below and above from 45°C temperature. This was attributed to denaturation and conformation changes of enzymatic proteins. Optimum pH not only provides suitable condition for growth and enzyme production, but will also determine the enzymatic action on the substrates and enhance the enzyme stability. In the present study, the enzyme exhibited activity up to pH 7.0 and stability up to pH 8.0. Reis *et al*. (2003) reported high stability of fungal xylanase under alkaline conditions and temperature up to 55°C. Shah and Madamwar (2005) and Jin *et al.* (2012) also reported maximum recovery of partial purified xylanase at 50 -55°C and pH 5.0.

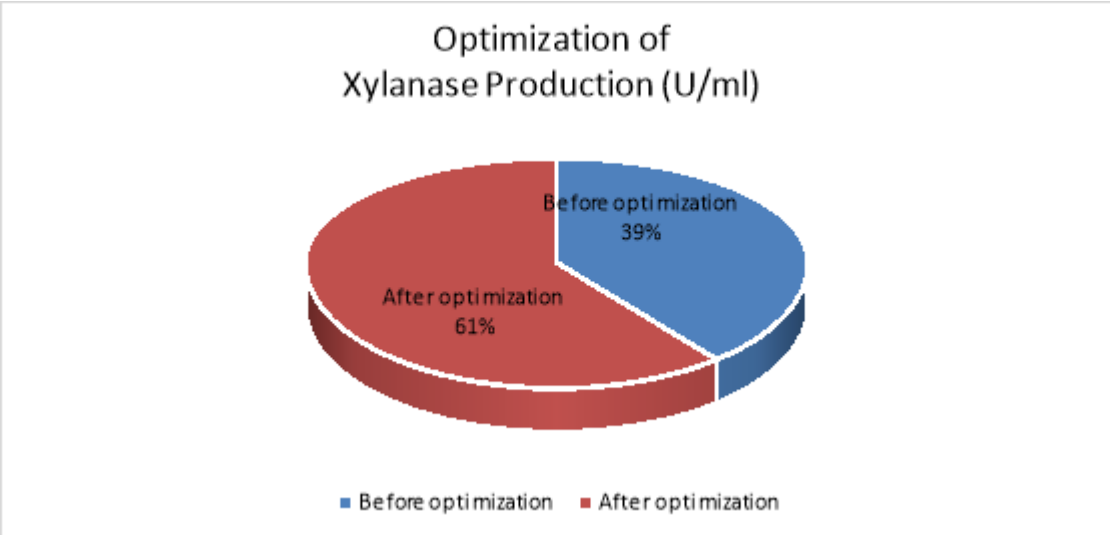
Fig. -7 Effect of reaction temperature and pH on Xylanase recovery



Important factor for effective enzyme production is the choice of an appropriate inducing substrate, either insoluble or soluble, as well as optimization of the medium composition. Bioprocess parameters, that can affect activities and productivities of xylanase attained in a fermentation process, include the pH, temperature, or agitation (Haltrich *et al.*, 1996 and Venkatesh and Tallapragada, 2009). Garapati *et al*. (2009) reported that moisture content, Incubation time and inculation concentration were major influential parameters for xylanase production by *Aspergillus terreus*.

It was noticed that all factors and interactions considered in the experimental design were statistically significant at 95% confidence limit (Fig. – 8) indicating that nearly all the variability of experimental data can be explained in terms of significant effects. Statistical analysis of the xylanase production data in above experimental designs revealed that among all selected factors type of carbon and nitrogen source, salt concentration, growth temperature and pH, incubation time and inoculums dose contributed the maximum impact on the overall enzyme production followed by temperature and pH of reaction mixture. Effect of agitation and fermentation under SSF or LSF condition did not affected significantly in this study. Naby *et al.* (1992) reported 5.7fold increased xylanase activity after optimization of culture medium.

Fig. – 8 Comparison of Xylanase production before and after Optimization



Zymogram analysis through SDS PAGE gel showed two different polypeptides at 32 and 38 kDA position. Coral *et al.* (2002) reported one xylanase band to be around 36 k DA in their isolate *A.niger* Z1 wild strain. Presence of multiple xylanases has been reported in a number of fungal and bacterial isolates from terrestrial sources (Ghatora *et al.*, 2006). Raghukumar *et al.* (2003) identified Endo-β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase; EC3.2.1.8) belong to the class of glycosyl hydrolases, with a molecular mass over 30 k Da and an acidic pH in their studies.

Sakthiselvan *et al.* (2014) isolated xylanase from *Hypocrea lixii* SS1 by utilizing sunflower oil sludge (waste from the oil industry) as the sole carbon source for xylanase production and the enzyme was further affirmed by SDS-PAGE with their standard molecular weight (29 k Da).

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REFERENCES

1. Barnett H.L., Hunter S. and Barry B. (1972) Illustrated genera of Imperfect fungi. 3rd ed., Burgess Pub.
2. Chandra, S., Sharma, R., Jasuja, N. D., Saxena R. and Rana S. (2012) Xylanase assay of fungal isolates from semi – arid soil of india. Indian Journal of Fundamental and Applied Life Sciences. 2 (4):7-12.
3. Coral, G., Arikan, B., Unaldi, M.N. and Guvenmez, H. Korkmaz (2002) Some properties of thermostable xylanase from an *Aspergillus niger* strain. Ann. Microbiol. 52, 299-306.
4. Dietmar H., Bernd N., Klaus D. K., Walter S. and Silvia Z. (1996) Production of fungal xylanases. Bioresource Technology, 58, (2): 137-161
5. Ellis M.B. (1971) Dematiaceous hyphomycetes. Commonwealth mycological institute, Kew, England.
6. Garapati Suvarna Lakshmi, Chaganti Subba Rao and Ravella Sreenivas Rao (2009) Enhanced production of xylanase by a newly isolated *Aspergillus terreus* undersolid state fermentation using palm industrial waste: A statistical optimization. Biochemical Engineering Journal. 48 :51–57.
7. Ghatora, S., Chadha, K., Bhupinder, S., Badhan, A.K., Saini, H.S. and Bhat, M.K. (2006) Xylanases from fungi. Bioresourses. 1(1): 18-33.

8. Gilman J.C. (1959) A manual of soil fungi. Oxford & IBH Pub. New Delhi.
9. Haltrich, Dietmar , Nidetzky, Bernd, Kulbe, Klaus D., Steiner, Walter and Zupancic, Silvia (1996) Poduction of fungal xylanases. Bioresource Technology.58 (2): 137-161.
10. Jaafaru, M. I. (2013) Screening of Fungi Isolated from Environmental Samples for Xylanase and Cellulase Production. Hindawi Publishing Corporation ISRN Microbiology. 2013 (7), Article ID 283423. http://dx.doi.org/10.1155/2013/283423
11. Jin, Nan, Ma, Shuhan, Liu, Yan, Yi, Xiuli, He, Rong, Xu, Hui, Qiao, Dai Rong and Cao, Yi (2012) Thermophilic xylanase production by *Aspergillus niger* in solid state fermentation using wheat straw and corn cob. African Journal of Microbiology Research. 6(2): 2387-2394.
12. Kheng, P. P. and Omar, I. C. (2005) Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate. Songklanakarin J. Sci. Technol.,27 (2): 325-336.
13. Kulkarni, P. and Gupta, N. (2013) Screening and evaluation of soil fungal isolates for xylanase production. Recent Research in Science and Technology, 5(2): 33-36.
14. Malhotra G. and Chapadgaonkar, S.S. (2018) Production and applications of xylanases – an overview. BioTechnologia. 99(1)C: 59–72. http://doi.org/10.5114/bta.2018.7356.
15. Naby A., Mohamed A. and Dae Young Kwon (1992) Production of Xylanase and Xylosidase by *Aspergillus niger* NRC 107 Kor. Appl. Microbial. Biotechnol. 20(5): 543-550.
16. Okafor, U. A., Okochi, V. I., Onyegeme-okerenta, B. M. and Nwodo-Chinedu, S. (2007) Xylanase production by *Aspergillus niger* ANL 301 using agro – wastes. African Journal of Biotechnology. 6 (14): 1710-1714.
17. Parihar, Preeti Singh and Rai, Vibhuti (2015) Isolation, Screening and selection of xylanase producing thermophilic fungi from Raipur. Research in Biotechnology, 6(4): 27-37.
18. Raghukumar, Chandralata , Muraleedharan, Usha, V. R. Gaud and Mishra, R. (2009) Xylanases of marine fungi of potential use for biobleaching of paper pulp. J. Ind. Micribiol. Biotechnol. 31:433-440.
19. Reis, Simone dos, Costa, Maria Aparecida Ferreira and Peralta, Rosane Marina (2003) Xylanase production by a wild strain of *Aspergillus nidulans* . Acta Scientiarum: Biological Sciences, Maringá. 25, (1,): 221-225.
20. Sakthiselvan P., Balakrishnan N. and Nagarajan P (2014)Molecular characterization of a Xylanase-producing fungus isolated from fouled soil. Braz J Microbiol, 45(4): 1293–1302
21. Shah, Amita, R., and Madamwar, D. (2005) Xylanase production by a newly isolated *Aspergillus foetidus*strain and its characterization. Process biochemistry. 40(5): 1763-1771.
22. Simoes, Maria Lúcia Garcia, Samia Maria Tauk-Tornisielo and Tapia, Daniel Mario (2009) Screening of culture condition for xylanase production by filamentous fungi. Tapia1African Journal of Biotechnology. 8 (22): 6317-6326.
23. Sridevi, B. R. and Charya, M. A. S. (2011) Isolation, identification and screening of potential cellulase-free xylanase producing fungi. African Journal of Biotechnology. 10(22): 4624-4630.

24. Steiner W., Lafferty R.M., Gomes I., Esterbauer H. (1987) Studies on a wild type strain of *Schizophyllum commune*: Cellulase and xylanase production and formation of the extracellular polysaccharide schizophyllan. Biotechnol. Bioeng. 30: 169-170.
25. Tallapragada P. and Venkatesh K. (2011) Isolation, identification and optimization of xylanase enzyme produced by aspergillus niger under submerged fermentation. J. Microbiol. Biotech. Res. 1 (4):137-147.
26. Venkatesh, K. and Tallapragada, P. (2009) Optimization of growth condition for xylanase production by Aspergillus niger in Solid state fermentation. Polish Journal of Microbiology. 58(2): 125-130.