Production and Optimization of Xylanase by *Aspergillus niger* through Solid state fermentation using Lignocellulosic wastes as a substrate

[1]Mr. Jayaprakash S, [2]Dr.V.Swabna, [3]Ms. Hareeni selvam

[1, 3]Master's student, [2]Associate professor
Department of Biotechnology
St. Joseph's College (Autonomous), Tiruchirappalli.

***Abstract*: Xylanase is an enzyme which produced by various types of microorganisms such as bacteria and fungi. Studies shows that the fungal species particularly the *Aspergillus niger* play an important role in the production of the Xylanase. In this study, the solid state fermentation is used for the production of the xylanase which effective and less expensive. The aim of the study is to increase the production rate of the enzyme by optimize the parameters (Temperature, pH, Carbon source, Nitrogen source) of the Solid state fermentation. Raw materials used in this process are Sugarcane bagasse, Wheat straw, Rice straw and Rice husk. Among those substrates Wheat straw produce the high amount of Xylanase(1.21 U/ml) by optimizing the parameters such as pH at 6 and temperature 30° at the presence of Carbon source Xylose and Nitrogen source $NaNO_3$.**

***Keywords*: Xylanase, Xylanase production, Xylanase Activity, Fungal Xylanase, Xylanase Optimization, Solid state fermentation.**

1. INTRODUCTION

Xylanase is an enzyme which can degrade the xylan into xylose, by breaking down hemicelluloses [1]. It plays an important role in microorganisms for the degradation of plant matter into usable nutrients. Xylanases produced by some kinds of microbes (fungi, bacteria, yeast, marine algae and protozoa) snails, insects, etc. Due to xylans heterogeneity and complexity, the complete hydrolysis of this polysaccharide requires the action of an enzyme system with different specificities and ways of action [15].

Xylanase used in chlorine bleaching of wood pulp which important for the papermaking process, and the digestibility of silage [5]. Apart from this, xylanases from commercially relevant fungi such as *Myceliophthora thermophila* and *Trichoderma* are also used as food additives to poultry, in wheat flour for improving dough handling and improve the quality of bakery products, for the extraction of coffee from beans, plant oils from seeds, and starch, in the improvement of agricultural silage and grain feed, and in fruit juices and degumming of plant fiber sources such as flax, hemp, jute, and ramie [6].Filamentous fungi are produce rich amount of xylanases and other xylan-degrading enzymes because they produce the enzymes into the medium and their enzyme levels are higher than the yeast or bacteria. In addition to xylanases, fungi produce several auxiliary enzymes required for the degradation of substituted xylan [13]. The fungal families particularly *Trichoderma*, *Aspergillus*, *Fusarium*, and *Pichia* are viewed as incredible makers of xylanases.

Research facility information proposes that *Aspergillus niger* is a decent maker of multifunctional xylanolytic enzymes [2].Large-scale industrial studies demonstrated that a carbon-source-dependent response was conserved among *Aspergillus spp*. [1]. Some of the carbon sources, for example, glucose, xylose, and arabinose, have been used to consider the worldwide transcriptional reaction of *Aspergillus spp.*Many environmental factors affect microbial metabolic activity which can induce or repress enzyme biosynthesis such as, Temperature, pH, Substrate, Carbon Sources and Nitrogen Sources [3].

2. MATERIALS AND METHODS

2.1 ISOLATION OF MICROORGANISM

Isolation of microorganism carried out by serially diluted the 1g of agricultural soil sample in the distilled water and 0.5 ml of 10^4 dilution inoculated into plates containing PDA agar at 37ºC for 5 days.

2.2 SCREENING OF XYLANYTIC ACTIVITIES

The isolated colonies were screening for xylanolytic activities which was performed on Malt Extract Agar (MEA) containing 0.1% Wheat straw. Plates were incubated at 29ºC for 48 hours after, which they were stained with Iodine solution for 15minutes for clear zone formation.

2.3 PRODUCTION OF XYLANASES

2.3.1 COLLECTION OF RAW MATERIALS

Lignocellulosic wastes such as Rice straw, Sugarcane bagasse, Wheat straw and Rice husk are collected from the local area in and around Tiruchirappalli, Tiruchirappalli District, Tamil Nadu, India.

2.3.2 PRETREATMENT OF RAW MATERIALS
Raw materials are cut into small pieces at 35×45mm in size. The chopped substrates are dried overnight at 70°C and used for solid state fermentation.

2.3.3 CULTURE MEDIUM
The following ingredients were used for the preparation of Mandel and Stenberg's medium ($(NH_3)SO_4$ - 1.4g, $KH_2\ PO_4$ - 2.0g, $CaCl_2.2H_2O$ - 1.0g, $MgSO_4.7H_20$ - 0.3g, $FeSO_4.7H_2O$ - 5g, $ZnSO_4.7H_2O$ - 1.4g, $CoCl_2.6H_20$ - 2.0g, PEPTONE - 0.1g, TWEEN 20 - 0.1g in 1000ml of distilled water).

2.3.4 SPORE COUNTING
10µl of spore suspension was taken and was suspended in the well of hemocytomer and the cover slip was fixed on the wells. Then it was observed under microscope to count the number of spores.

2.3.5 SOLID STATE FERMENTATION (SSF)
15ml of Mandel's medium[8] was added to separate 250ml Erlenmeyer flasks containing 10g of each substrate such as Rice straw, Rice husk, wheat straw and Sugarcane bagasse. To that distilled water added to maintain the initial moisture content. Then it is autoclaved for 30 minutes in 121ºC. After autoclave it was allowed to cool. Then 13.5ml of spore suspension was inoculated into the flask. It was incubated at 28-30ºC for 5-7 days.

2.4 PROTEIN ASSAY
Protein contents of the culture supernatants were assayed by the Bradford method using Bovine Serum Albumin (BSA) as standard. Dilutions of bovine serum albumin was prepared and 30µl of culture supernatant taken as a test. Then 1.5ml of Bradford reagent added with that mixture. It incubated for 1 hour. After incubation period the readings were measured by using spectrophotometer at 595nm.

2.5 ENZYME EXTRACTION
After the fermentation period 70 ml of cold water was added to each Erlenmeyer flasks containing different solid state fermentation medium and was stirred. This mixture was centrifuged at 5000 rpm for 30 minutes. After centrifugation the solid biomass was separated from the mixture which is done by filtration using Whatman filter paper. The cell free supernatant is used as the source for the crude enzyme preparation.

2.6 XYLANYTIC ACTIVITY
Xylanase activity was assayed in 1.5 ml of a reaction mixture containing 0.5 ml of diluted enzyme solution and 1 ml of supernatant of 2% (w/v) wheat straw in 0.05 M citrate buffer (pH 4.8). It was incubated at 50°C for 20 min. After the incubation period, the reducing sugars were determined by the di-nitro salicylic acid method [9] with xylose as standard. The released xylose was measured by using spectrophotometer at 540 nm.

2.7 EFFECT OF TEMPERATURE
The effect of temperature on Xylanases production was examined at various temperature ranges up to 45°C to optimize the temperature.

2.8 EFFECT OF pH
The pH of medium was adjusted to variable pH range by HCl. The effect of Xylanase production by *Aspergillus niger* was examined at various pH ranges.

2.9 EFFECT OF CARBON SOURCES
The effect of carbon source in Xylanases production by *Aspergillus niger* was tested by flask containing production media with carbon sources, which was tested for the effect of carbon at 1% concentration.

2.10 EFFECT OF NITROGEN SOURCES
The effect of nitrogen sources in Xylanase production by *Aspergillus niger* was examined by flasks with nitrogen sources, which were tested for the influence of nitrogen sources at 0.075% concentration.

3. RESULTS

3.1 SELECTION AND SCREENING OF ISOLATES
Based on screening process, 4 isolates were identified. Those isolates were identified based on the structural morphologies. Among that *Aspergillus niger* identified and sub-cultured for the further process. By comparing others *Aspergillus niger* has capable of exhibiting xylanolytic activities on Malt Extract Agar (MEA) with the diameter of the clear zones ranging from 35-45mm.Growth on MEA showed that the initial white mycelia turned yellow and finally black on maturation stage. The formation of clear zone indicates the positive xylanolytic activity. So the isolates xylanolytic isolates were detected based on clear zones of hydrolysis.

3.2 SPORE COUNTING

After the spore counting the spore level adjusted at the range 1×10^4 to 1×10^6 spores/ml.

3.3 SOLID STATE FERMENTATION (SSF) AND ENZYME EXTRACTION

The result showed that *Aspergillus niger* produces highest xylanase enzyme (U/ml) when cultured in the media containing the different Lignocellulosic wastes (Rice husk, sugarcane bagasse, rice straw and wheat straw) as substrates. The result incubation at ambient temperature revealed that wheat straw remained the best substrate for xylanase production in Solid state fermentation with the enzyme production of 1.21 U/ml. (Table.1 and Figure.1)

Table 1 Xylanase enzyme activity by different types of microorganisms on various substrates

SUBSTRATE	ENZYME PRODUCTION (U/ml)			
	SPECIES			
	Aspergillus niger	***Aspergillus fumigatus***	***Aspergillus terreus***	***Penicillium citrinum***
Sugarcane Bagasse	0.92	0.48	0.75	0.32
Rice Straw	0.67	0.49	0.52	0.17
Rice Husk	0.42	0. 35	0.46	0.52
Wheat Straw	1.21	0.69	1.10	0.45

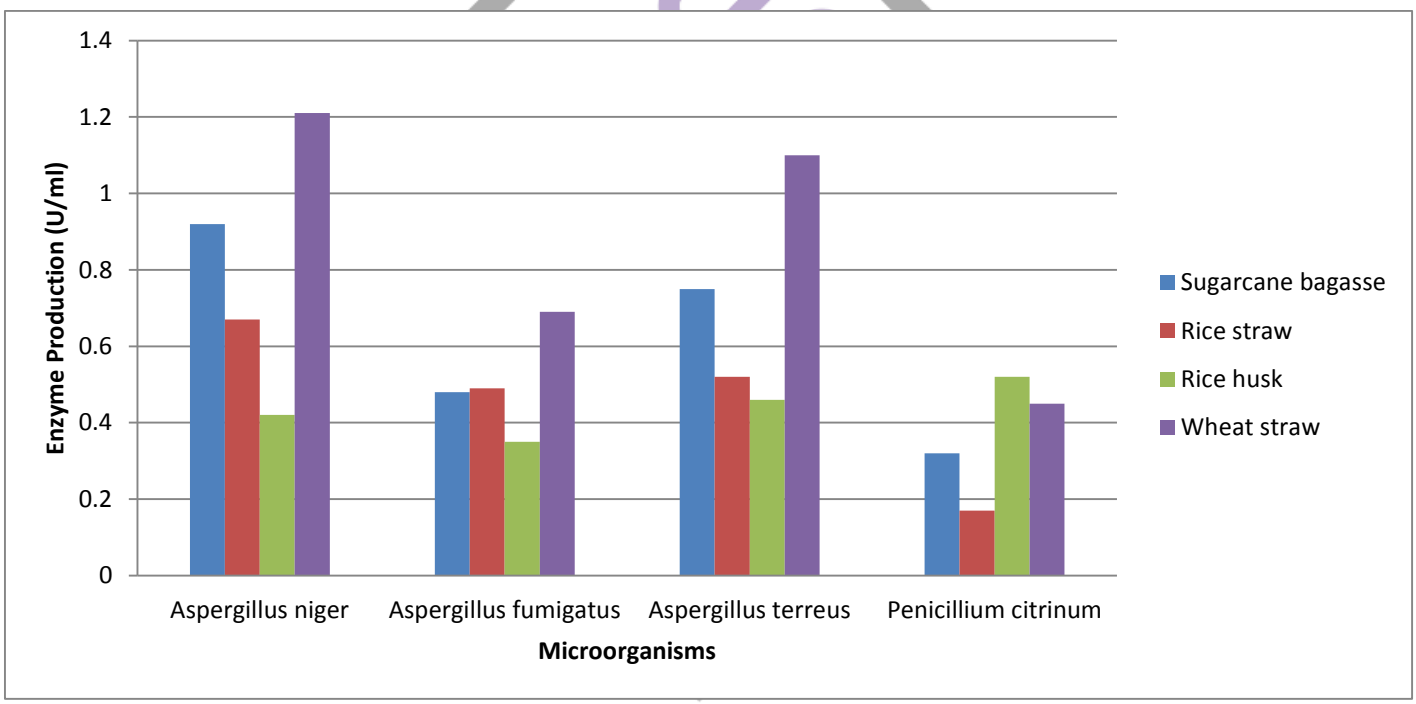

Fig 1: Graphical representation of SSF cultivation system by different microorganisms on various Lignocellulosic wastes as substrate

3.4 PROTEIN ASSAY

The released protein levels of the *Aspergillus niger* were represented in Table.2 and Figure.2. The highest value was obtained with wheat straw cultures which gave maximum protein concentration of 1.17 mg/ml at 120 h. protein peaks of 0.59 and 0.46 mg/ ml were obtained respectively from cultures containing rice straw and sugarcane bagasse at 120 h. The least protein peak value of 0.16 mg/ml was obtained from cultures containing rice husk at 120 h.

Table 2 Protein content of culture supernatant of *Aspergillus niger* using substrate

SUBSTRATES	PROTEIN CONTENT (U/ml)						
	INCUBATION PERIOD (hours)						
	24	**48**	**72**	**96**	**120**	**144**	**168**
Sugarcane Bagasse	0.05	0.21	0.22	0.25	0.46	0.48	0.52
Rice Straw	0.04	0.23	0.34	0.37	0.59	0.65	0.71
Rice Husk	0.02	0.06	0.08	0.12	0.16	0.21	0.30
Wheat Straw	0.23	0.24	0.36	0.66	1.17	1.69	1.73

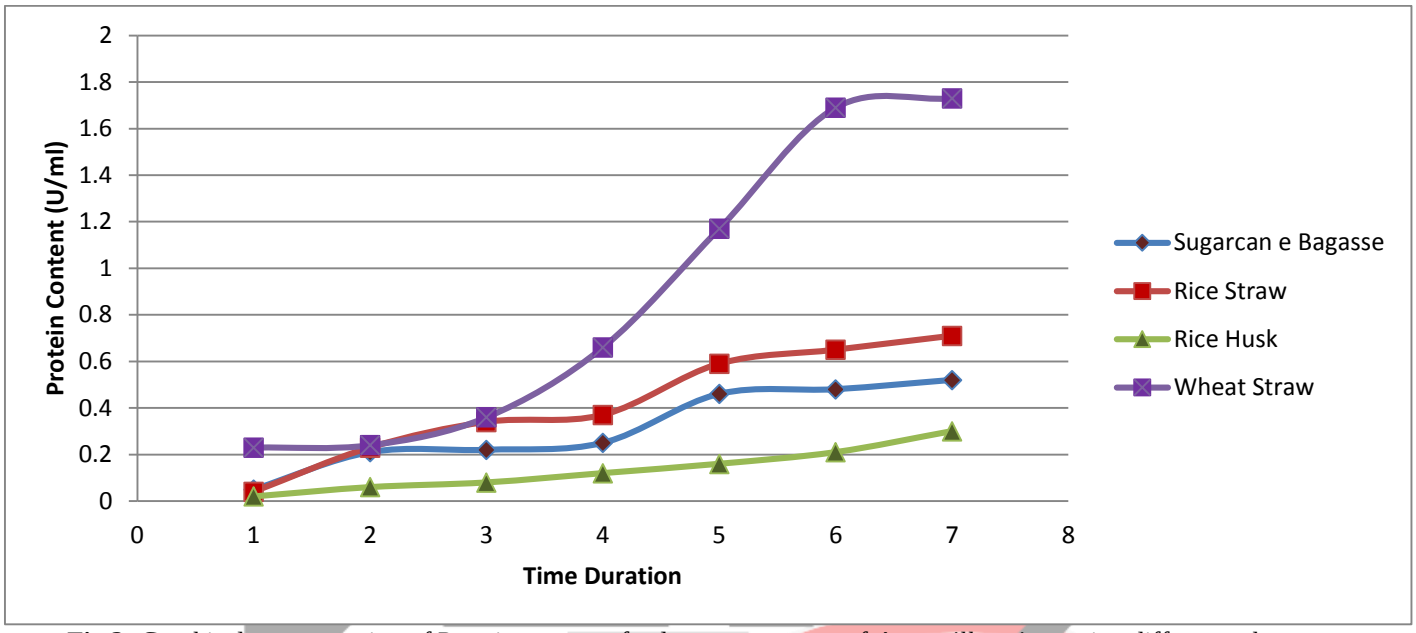

Fig 2: Graphical representation of Protein content of culture supernatant of *Aspergillus niger* using different substrates

3.5 EFFECT OF SUBSTRATE

Various types of substrates such as wheat straw, sugarcane bagasse, rice straw and rice husk were examined for the growth and xylanase production by *Aspergillus niger*. After incubation, the results showed that wheat straw remained the good substrate for xylanase production by *Aspergillus niger* (1.21U/ml). (Table.3 and Figure.3)

Table 3 Effect of *Aspergillus niger* cultivated on various substrates

SUBSTRATE	ENZYME PRODUCTION(U/ml)
Sugarcane bagasse	0.92
Rice straw	0.67
Rice husk	0.42
Wheat straw	1.21

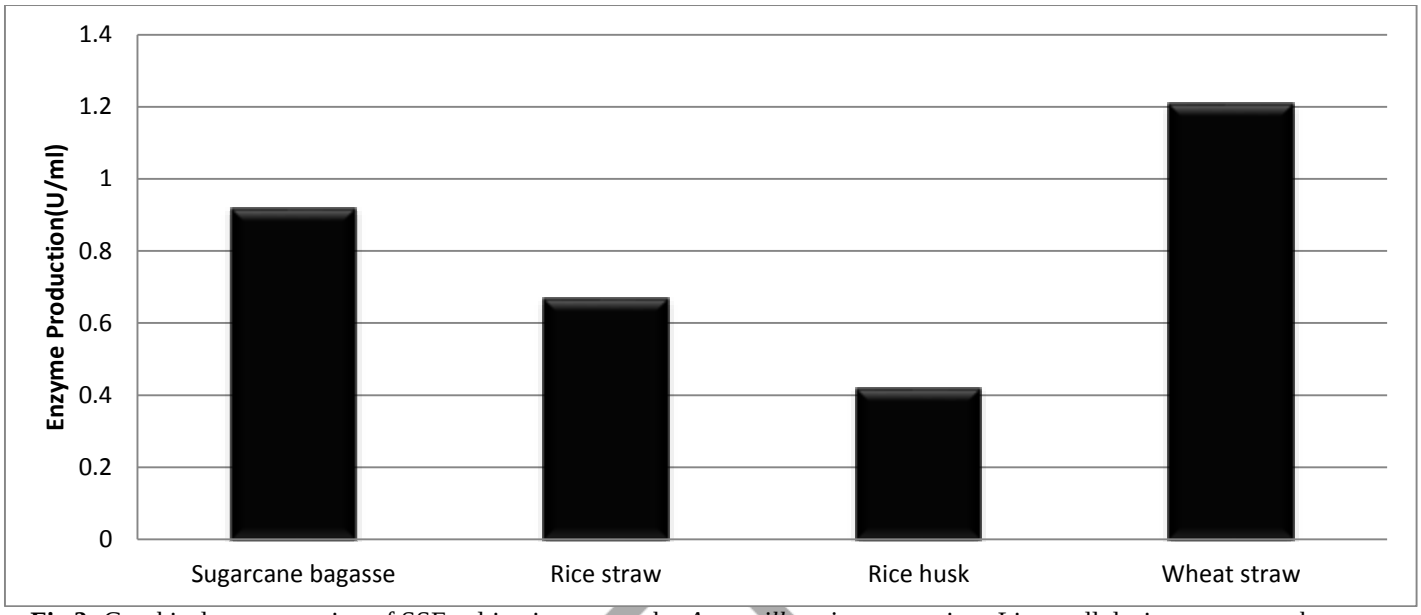

Fig 3: Graphical representation of SSF cultivation system by *Aspergillus niger* on various Lignocellulosic wastes as substrate

3.6 EFFECT OF TEMPERATURE

Several temperatures are examined to determine the optimum temperature level. The maximum production of xylanase was observed at the temperature 30°C with an activity of 1.63 U/ml on wheat straw as substrate by *Aspergillus niger*. (Table.4 and Figure.4)

Table 4 Xylanase enzyme activity by *Aspergillus niger* on various substrates at different temperature

SUBSTRATE	ENZYME PRODUCTION(U/ml) TEMPERATURE				
	25°	**30°**	**35°**	**40°**	**45°**
Sugarcane Bagasse	0.73	0.92	0.86	0.79	0.68
Rice Straw	0.34	0.42	0.38	0.30	0.27
Rice Husk	0.61	0.67	0.54	0.50	0.31
Wheat straw	1.05	1.63	1.19	1.0	0.92

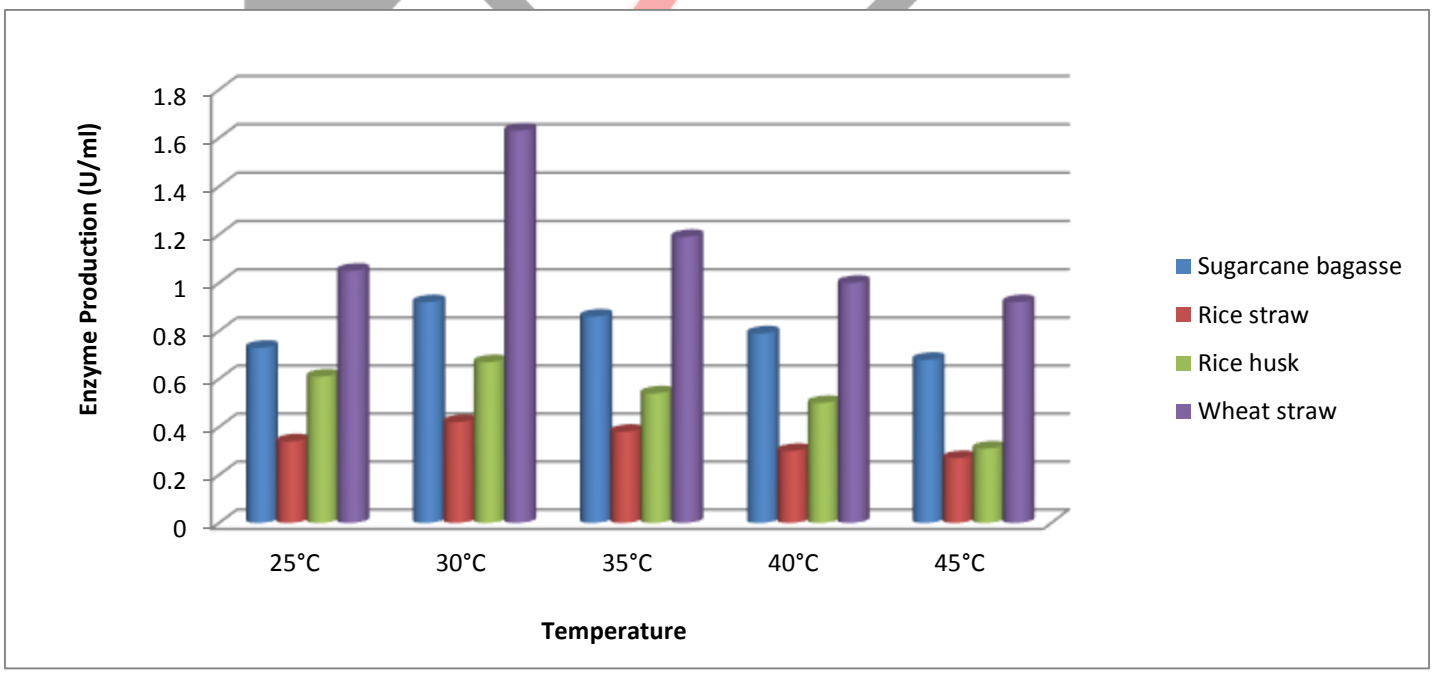

Fig 4: Graphical representation of Xylanase enzyme activity by *Aspergillus niger* on Lignocellulosic substrates at different temperature by Solid State Fermentation system

3.7 EFFECT OF pH

To estimate the optimum pH of the enzyme, the activity was determined under various levels of pH range between 5-9.The pH range of the medium was adjusted by adding 0.1N HCl and NaOH. Compared with other pH ranges the high production rate of the xylanase enzyme was determined at pH 6 of 1.65 U/ml on Wheat straw by *Aspergilllus niger* (Table.5) (Figure.5).

Table 5 Xylanase enzyme activity by *Aspergillus niger* on various substrates at different pH

SUBSTRATE	ENZYME PRODUCTION(U/ml)				
	pH				
	5	6	7	8	9
Sugarcane Bagasse	0.82	1.32	1.19	1.12	1.06
Rice Straw	0.71	1.20	1.04	1.01	0.77
Rice Husk	0.87	1.05	1.01	0.98	0.90
Wheat Straw	1.35	1.65	1.47	1.39	1.25

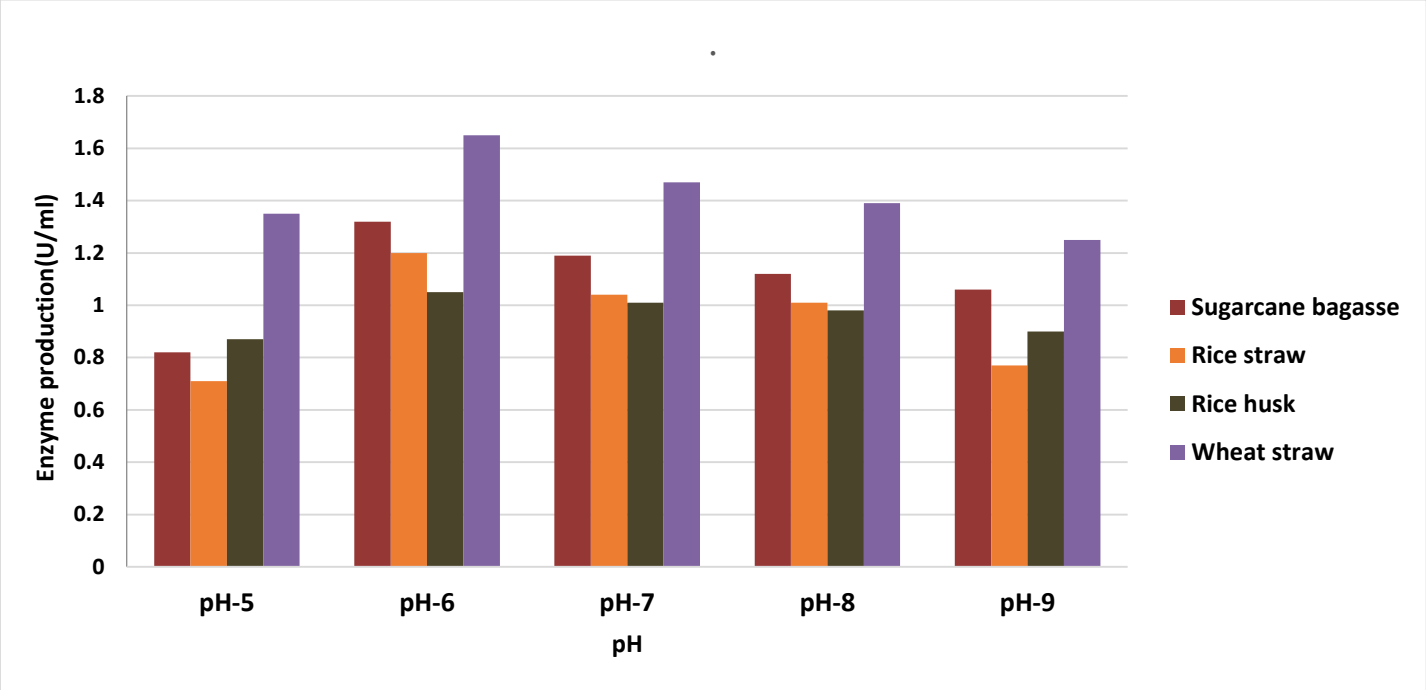

Fig 5: Graphical representation of Xylanase enzyme activity by *Aspergillus niger* on Lignocellulosic substrates at different pH by Solid State Fermentation system

3.8 EFFECT OF CARBON SOURCES

Here the carbon sources are the supplemented sugars such as Glucose, Xylose, Lactose and Maltose. Among those sugars the addition of xylose resulted as a high production of Xylanase (1.69 U/ml) when compared with the other carbon sources. (Table.6)(Figure.6

Table 6 Xylanase enzyme activity by *Aspergillus niger* on various substrates using different carbon sources

SUBSTRATE	ENZYME PRODUCTION			
	CARBON SOURCES			
	GLUCOSE	XYLOSE	LACTOSE	MALTOSE
Sugarcane Bagasse	1.21	1.52	0.95	1.32
Rice Straw	1.13	1.49	0.92	1.17
Rice Husk	1.02	1. 35	0.76	1.02
Wheat Straw	1.37	1.69	1.10	1.21

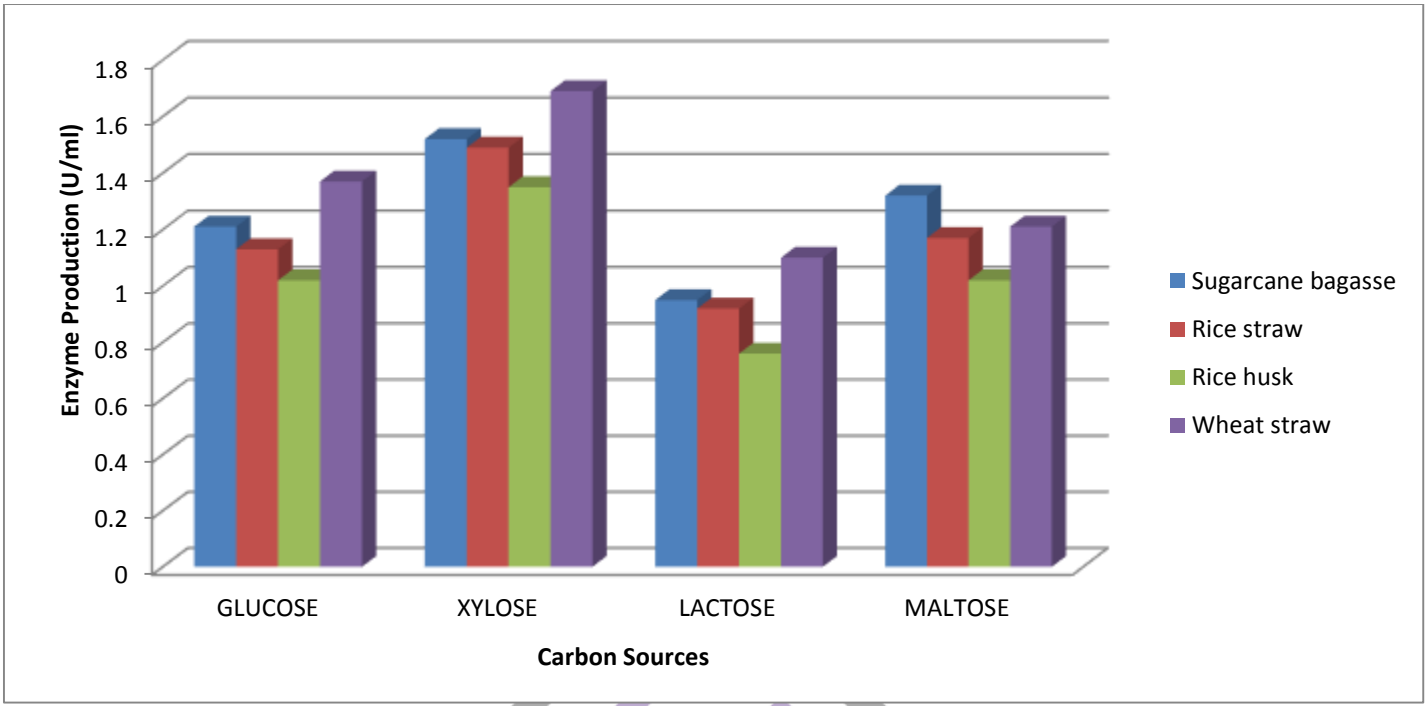

Fig 6: Graphical representation of Effect of Carbon Sources supplemented to the Solid State Fermentation by *Aspergillus niger* for Xylanase Production

3.9 EFFECT OF NITROGEN SOURCES

The effect of nitrogen source supplementation on the production of xylanase was examined by using various nitrogen sources. The results show that NaNO3 was found to increase the production of xylanase by *Aspergillus niger* (1.67U/ml). (Table.7)(Figure.7)

TABLE 7 Xylanase enzyme activity by *Aspergillus niger* on various substrates using different nitrogen sources

SUBSTRATE	ENZYME PRODUCTION NITROGEN SOURCE			
	PEPTONE	UREA	YEAST EXTRACT	$NaNO_3$
Sugarcane Bagasse	1.24	0.83	1.37	1.62
Rice Straw	1.17	0.76	1.41	1.61
Rice Husk	1.12	0.87	1.32	1.59
Wheat Straw	1.37	1.26	1.44	1.67

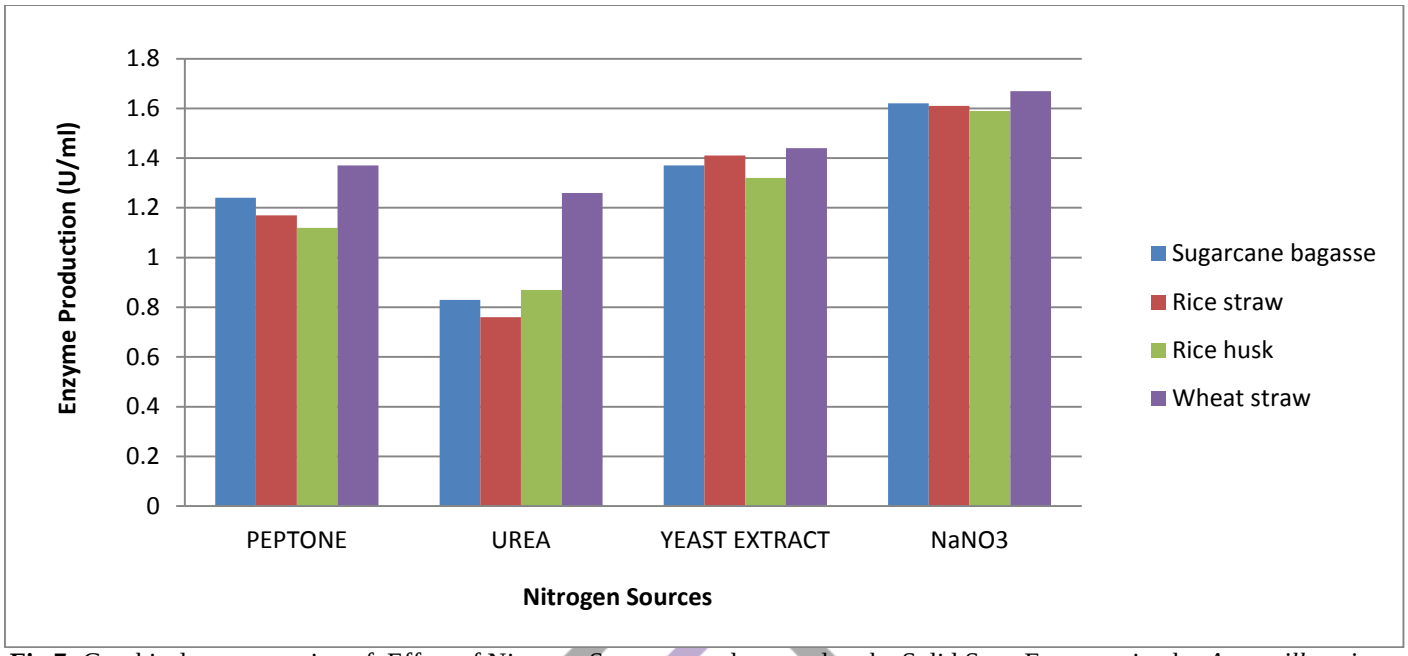

Fig 7: Graphical representation of Effect of Nitrogen Sources supplemented to the Solid State Fermentation by *Aspergillus niger* for Xylanase Production

4. DISCUSSION

4.1 SELECTION AND SCREENING OF ISOLATES

After the incubation period the individual colonies were observed and the specific species were identified based on the characteristic features described by Gautam A K and Bhadauria R (2018) [4]. The fungal isolates which formed were sub cultured to purify and examined for further enzymatic activities.

4.2 PROTEIN ASSAY

Cultures containing the Lignocellulosic wastes gave higher protein levels. Among those substrates the highest protein value was obtained from the cultures containing wheat straw which gave a high protein concentration of 1.17 mg/ml at 120 hours (Table.2) (Figure.2). It shows that of the all Lignocellulosic wastes, wheat straw is the best prospective carbon source for the production of the enzyme [11].

4.3 EFFECT OF TEMPERATURE

The rice husk, rice straw and sugarcane bagasse are although supporting the growth it produces enzyme activity as 0.67, 0.42 and 0.92 (U/ml) after the incubation (Table.4) (Figure.4). Higher xylanase production was also reported from *Aspergillus niger* using SSF at optimum temperature (30°C). It shows that among all temperatures the ambient temperature for the xylanolytic activity of the *Aspergillus niger* is 30℃. [7]

4.4 EFFECT OF pH

It was found that the xylanase from *Aspergillus niger* exhibited the activity at increased pH range. The optimum pH of the enzyme was found to be 6.0 with an activity of 1.65 U/ml in the wheat straw (Table.5) (Figure.5). The *Aspergillus niger* holds a range of pH at 6 for its growth and activity with an optimum values, between these ranges the initial pH influences enzymatic system and the transport of enzyme across cell membrane [10].

4.5 EFFECT OF CARBON SOURCE

In addition of xylose, maltose, lactose and glucose, the xylose resulted in an increment in xylanase production (1.69 U/ml) by SSF (Table.6) (Figure.6). The production of essential metabolites by microorganisms, which profoundly affected by their development which is controlled by the accessibility of the supplements in the substrates. Therefore, it is expected that nutritional value of substrates by the supplementation of carbon sources will also improve the growth of *Aspergillus niger* and subsequently the enzyme production. [14]

4.6 EFFECT OF NITROGEN SOURCE

In addition of Peptone, Urea, $NaNO_3$ and Yeast extract, the $NaNO_3$ produces high yield of xylanase production (1.67 U/ml) by Solid State Fermentation (Table.7) (Figure.7). Therefore, it is expected that nutritional value of substrates by the supplementation of nitrogen sources will also improve the growth of *Aspergillus niger* and subsequently the enzyme production. [12]

5. CONCLUSION

In this study, xylanase activity and its biomass concentration from *Aspergillus niger* was evaluated by DNS method and optical density via spectrophotometer, respectively. Various carbon sources, nitrogen sources, pH, Temperature and different

Lignocellulosic wastes on xylanase production were also investigated. In solid state fermentation wheat straw was found to be the optimum carbon source for xylanase activity, producing 1.21 U/mL of xylanase by *Aspergillus niger* was obtained by optimizing the pH at 6 and Temperature at 30°C, Xylose and $NaNO_3$ as Carbon source and Nitrogen source, followed by Sugarcane bagasse, Rice straw and Rice husk are produced respectively 0.92, 0.67 and 0.42 U/ml of xylanase. Therefore, Wheat straw was concluded as a best substrate to produce the microbial Xylanase enzyme because of its high productivity range.

REFERENCES

[1]. **Andrews S R, Taylor E J and Pell G** (2004) The use of forced protein evolution to investigate and improve stability of family 10 xylanases the production of $Ca2^{+}$-independent stable xylanases.
[2]. **Beg Q K, Kapoor M, Mahajan L and Hoondal G S** (2001) Microbial xylanases and their industrial applications: a review
[3]. **Buswell J A and Chang S T** (1994) Biomass and extracellular hydrolytic enzyme production by six mushroom species grown on soybean waste. Biotechnology Letters; 16: 1317– 1322.
[4]. **Gautam A, Kumar A, Bharti AK and Dutt D** (2018) Rice straw fermentation by *Schizophyllum commune* ARC-11 to produce high level of xylanase for its application in pre-bleaching. J Genetic Engineering Biotechnology; 16:693–701
[5]. **Gulzar** (2004) Production and partial purification of Xylanase from *Trichodermalongibrachiatum*.
[6]. **Kulkarni N, Shendye A and Rao M** (1999) Molecular and biotechnological aspects of xylanases.
[7]. **Lu W, Li D and Wu Y** (2003) Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solid-state fermentation by *Aspergillus sulphurous*. Enzyme Microbial. Technol., 32: 305-311.
[8]. **Mandels M and Stenberg D** (1976) Recent advances in cellulase technology. Journal of Fermentation Technology; 54: 267–286.
[9]. **Miller G L** (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry; 31: 426-428.
[10]. **Mohana S, Shah A, Divecha J and Madamwar D** (2008) Xylanase production by Burkholderia sp. DMAX strain under solid state fermentation using distillery spent wash. Bioresour Technol 99: 7553-7564.
[11]. **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. Afr J Biotechnology; 6(14):1710–1714.
[12]. **Pang Pei Kheng and Ibrahim C O** (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., 2005, 27(2) : 325-336
[13]. **Polizeli M L, Rizzatti A C, Monti R, Terenzi H F, Jorge J A and Amorim D S** (2005) Xylanases from fungi: properties and industrial applications.
[14]. **Priem B, Dobberstein J and Emeis C C** (1991) Production of β-1,4-xylanase in continuous culture by Aureobasidium pullulans CBS 58475. Biotechnol. Lett. 13: 149-154.
[15]. **Wong K K, Tan L U and Saddler J N** (1988) Multiplicity of beta-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev; 52:305–317
[16]. Appl. Microbiol. Biotechnol. 62: 374–