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Production and Optimization of Xylanase by *Penicillium chrysogenum* through Liquid State and Solid State Fermentation

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Abstract: **Xylanases are the hydrolytic enzymes that degrade natural polysaccharide, xylan into xylose. In natural ecosystem number of microorganisms such as bacteria, fungi and actinomycetes has been found to secrete xylanases. The present study has been undertaken with an objective to screen out xylanase producing fungi from degrading litter collected from Orchha Wildlife Sanctuary, M.P., India. Fungus *Penicillium chrysogenum* was isolated, identified and tested for its xylanase producing ability. The fungus have been found to produce a clear zone of 39 mm over malt extract agar (MEA) medium supplemented with 1% oat spelt xylan. In the present study substrates for xylanase production used were wheat bran and corn cobs. Xylanase production capability was optimized at different growth parameters under liquid as well as solid state fermentation conditions. Using wheat bran as substrate highest xylanase production was achieved on 6th & 5th day of incubation, temperature 30°C, pH 6.0 & 4.5, 180 mg/ml & 160 mg/ml wheat bran, 0.4 mg/ml & 0.2 mg/ml peptone and 0.85 mg/ml & 0.65 mg/ml yeast extract under LSF and SSF respectively. While with corn cobs as carbon source maximum xylanase produced in terms of activity was 7th & 5th day of incubation, 30°C temperature, pH 5.0 & 4.5, 160 mg/ml & 120 mg/ml corn cobs, 0.8 mg/ml & 0.2 mg/ml peptone, 0.75 mg/ml & 0.8 mg/ml yeast extract amount under LSF & SSF respectively.**

Index terms: **Litter, Liquid state fermentation, Solid state fermentation, xylanases.**

I. INTRODUCTION

Enzymes are biological molecules that catalyze the chemical reactions. In natural ecosystems a large number of complex polymers are converted into their simple units with the help of enzymes. Xylanases (3.2.1.8) are the one such enzyme that brings about complete hydrolysis of xylan into simple carbohydrates. Xylanase enzyme composed of endo and exo-xylanases, these two are the major consortium of enzymes, depolymerizing xylan from lignocellulosics in nature. Xylanases are extracellular enzymes produced by various microorganisms such as bacteria, actinomycetes, yeasts, fungi and many other microbial systems (Chen *et al*., 2018). In natural ecosystems, bio-decomposition of litter has been a key point in organic carbon recycling. This process is carried out by micro-organisms (decomposers). Micro-organisms especially saprophytic fungus plays a key role in degradation process by secreting extracellular enzymes. Plant litter (leaves, bark, twigs, flowers) consists of mainly three polymeric components – Cellulose (35-50%), hemicelluloses (20-35%) and lignin (10-20%) that are strongly bonded by non covalent as well as covalent cross linkages (Pèrez *et al*., 2002).

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Hemicelluloses being a heteropolysaccharide have xylan, mannan, galactan and arabinan as its major component (Harris *et al*., 2010). Among them, xylan is the major hemicellulosic component and is the second most abundant polysaccharide after cellulose in nature. This complex hetero-polysaccharide have β-1, 4-linked D-xylopyranose units with short chain branches of O-acetyl, α-L-arabinofuranosyl and α-D-glucurosyl residues (Nair *et al*., 2008). Xylanases are the important industrial enzymes used in depolymerization of xylan into simple sugars. The xylan hydrolyzing system include mainly two enzymes: endo-1,4-xylanase (1,4-β-D-xylan xylanohydrolase ; EC 3.2.1.8) and β-D-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). The endo-1,4-xylanase is the primary hydrolyzing enzyme that hydrolyzes the β-1,4-xylosidic bond of xylan backbone (Harris *et al*., 2010) and releases short and long xylo-oligosaccharides while β-D-xylosidase releases the D-xylose residues by hydrolyzing xylo-oligosaccharide and xylobiose (Saha, 2003). Considering its industrial applications, fungi are highly preferred over other microorganisms because of easy cultivation and production of extracellular enzymes in the medium during fermentation (Haltrich, 1996; Periasamy, 2017; Subramaniyam & Prema, 2002).

Microbial xylanases are important for their biotechnological applications in several industries such as food processing industry, textile industry, animal feed industry and paper making industry (Bajpai, 1994; Kumar and Singh, 2008). The most important use of xylanases is in pulp and paper industry to lower the use of toxic chlorine during bleaching process of pulp. They are also used in clarification of fruit juices and wines, in extraction of coffee and starch, plant oils and in production of valuable products such as xylitol (an artificial low calorie sweetner) and ethanol (Salles *et. al*., 2005).

Xylanase production can be performed either by liquid state fermentation (LSF) or solid state fermentation (SSF) system. Most of the researchers have used SSF because it has several advantages over LSF i.e. low moisture content, low contamination probability, simplicity of equipments used and higher productivity per unit volume etc. (Archana, 1997; Prakash & Karmagam, 2014).

In the present study, ability of test strain *P. chrysogenum* to produce xylanases under LSF and SSF followed by different physical factors affecting enzyme production were analyzed and optimized.

II. MATERIALS AND METHODS

A. Sample Collection

Degrading litter samples were collected from 2-3 sites located at 25° 21' 6.91" N and 78° 38' 25.19" E of Orchha Wildlife Sanctuary, Madhya Pradesh (Fig.1). Litter samples were taken with the help of pre sterilized spatula and stored in polybags and brought to laboratory for further study.

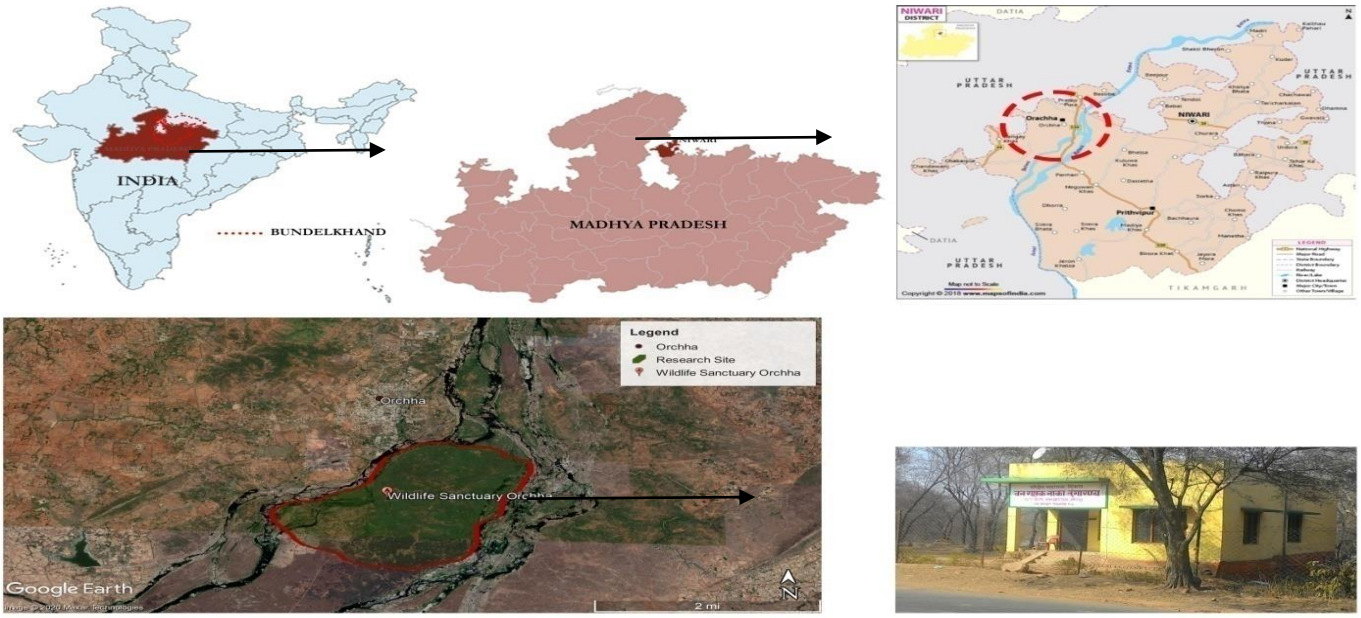

Fig. 1: Location of Orchha Wildlife Sanctuary

B. Isolation and Identification of Fungi

For fungal isolation, 1.0 g of collected litter sample was suspended in 10 ml of sterile distilled water and shaken on a shaker (REMI) at speed of 50 rpm for 3 minutes to get a homogenous suspension. The suspension was serially diluted upto 10^{-1} to 10^{-4}. 1.0 ml of each dilution was streaked over petriplates containing potato dextrose agar (PDA) medium (Potato – 200.0 g/L, dextrose – 20.0 g/L, agar – 15.0 g/L, pH - 5.6). All streaked agar plates were incubated at 30°C for growth. Fungal colonies appeared over the medium and they were examined morphologically as well as microscopically. In morphological characters, growth pattern of colony, colony color, absence and presence of aerial mycelium were considered. For the microscopic observation, hyphal growth pattern, conidiophores structure, spore structure and size were studied. Identification of fungi was done by using standard texts and photographs (Nagamani *et al*., 2006; Gilman, 1957).

Pure isolates were maintained at 4°C on Czapek's Yeast Autolysate Agar (CYA) medium (K_2HPO_4 – 1.0 g, Sucrose – 30 g, Yeast extract – 5 g, Agar – 15 g, Czapek's concentrate – 10 mL, distilled water – 1L) suggested by Pitt, 1973.

C. Primary Screening of Fungus for Xylanase Production

Xylanase production capability of the isolated strain was studied by well diffusion technique suggested by Nakamura *et al.* (1993). Agar wells were prepared on solidified malt extract agar (MEA) medium (K_2HPO_4 – 0.2, peptone - 5.0, yeast extract – 5.0, agar – 20.0 and Oat spelt xylan – 10.0 g/L, pH – 5.5) then the fungus was inoculated in the wells and petriplates were incubated at 30°C for 5 days. Appearance of clear zone indicates that fungus has ability to produce xylanase.

D. Secondary Screening

The petriplates of above experiment (primary screening) were stained with 0.1% congo red solution for 15 minutes. Excess of congo red solution was removed and plates were washed with 1M NaCl solution (Teather & Wood, 1982). Using the isolated fungal strain, xylanase production, xylanase assay and

optimization of physical parameters for production of xylanase under LSF and SSF were performed.

E. Substrate Preparation

Xylan being expensive, cheaper substrates such as wheat bran and corn cobs was used as carbon sources for xylanase production. Substrates were collected from locals then pulverized, washed with running tap water, dried and then treated with 4% NaOH. After drying, treated wheat bran and corn cobs were stored in air tight containers.

F. Liquid State Fermentation

The selected strain, *Penicillium chrysogenum* was tested for its ability to produce extracellular xylanase enzyme through liquid state fermentation. Fermentation process was performed in 100 ml Erlenmeyer flask containing 20.0 g/L of wheat bran was used as substrate with the addition mandels medium (Modified) (g/L): KH_2PO_4 – 2.0, $MgSO_4.7H_2O$– 0.3; $CaCl_2$ – 2.0; $FeSO_4.7H_2O$ – 0.5; $ZnSO_4.7H_2O$ – 1.4; $MnSO_4.H_2O$ – 1.6; peptone – 0.3; yeast extract – 0.75; distilled water – 1L and pH – 5.5 (Mandels & Sternburg, 1976). After sterilization fungal spores were inoculated.

G. Solid State Fermentation

In SSF, 160 g/L of substrate (wheat bran and corn cobs) was taken. The medium composition was same as that of liquid state fermentation. The medium was autoclaved and inoculated with 1.0 ml of spore suspension and incubated at 30°C for fermentation. Enzyme was harvested by adding 50 ml of sodium citrate buffer (pH 5.3) into each flask and left it for 1 hr. The mixture was shaken for 15 min on magnetic stirrer. The fermented slurry was squeezed through muslin cloth.

H. Enzyme Harvesting

The biomass of each flask was filtered through Whatman no.1 filter paper and filterate was centrifuged with cooling centrifuge (REMI) at 10,000 rpm for 10 min. The clarified supernatant was used as crude enzyme source.

I. Optimization of Growth Parameters for Xylanase Production

In present study, factors such as incubation period, temperature, pH, concentration of carbon and nitrogen sources affecting enzyme production has been studied. Incubation period has been one of the important criteria in fermentation studies The effect of incubation period was determined by harvesting the flasks at various incubation days such as 3rd day, 4th day, 5th day, 6th day, 7th day, 8th day, 9th day, 10th day, 11th day and 12th day. The effect of incubation temperature on the xylanase production was examined on different temperature ranges from 20°C to 40°C. The pH effect was also determined by growing the fungal strain in Mandel's medium having different pH range from 3.0 to 8.0. To observe the substrate concentration optimization, wheat bran and corn cobs were taken in different amount. Similarly, the effects of nitrogen sources such as peptone and yeast extract were investigated by varying their quantities in culture medium. After getting optimized xylanase enzyme on above mentioned parameters, some parameters such as incubation period, temperature, pH and substrate i.e. oat spelt xylan were also optimized for maximum xylanase assay.

J. Xylanase Assay

Xylanase enzyme activity was assayed by using crude enzyme (1.0 ml) with oat spelt xylan (1.0% in sodium citrate buffer at pH 5.3). The reaction mixture was incubated at 55°C for 10 min (Biely, 1992). The reaction was stopped by adding 3.0 ml of 3,5-dinitrosalicyclic acid (DNS). Released reducing sugar

was measured spectrophotometrically at 540 nm. The xylanase activity was expressed in terms of International Units (IU).

III. RESULTS AND DISCUSSION

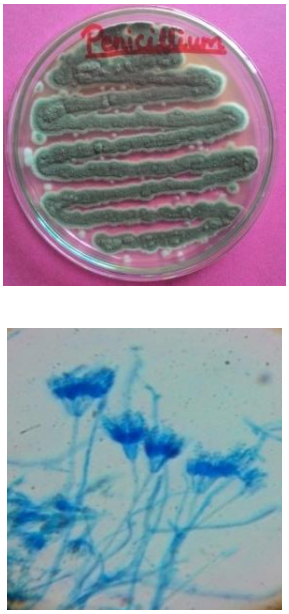

Fig. 2: *Penicillium chrysogenum* (A) Culture (B) Microscopic view

Isolated fungal strain from degraded litter was identified as *Penicillium chrysogenum* with the help of standard texts and photographs. *P. chrysogenum* was characterized to have yellow green or near glaucous blue green color, mycelium white to buff, phialides 4-6 per verticle with ellipsoidal, smooth conidia (Fig.2).

When fungus was subjected to test its xylanase producing ability it was allowed to grow on MEA medium supplemented with 1% oat spelt xylan as sole carbon. After 72 hrs of incubation, clear halo region has been observed around its colony on xylan agar plates. Appearance of clear zone around the fungal growth confirms that fungus secretes hydrolyzing enzymes that can hydrolyze the xylan which was present in the medium. The same have been confirmed with congo red test (Fig.3).

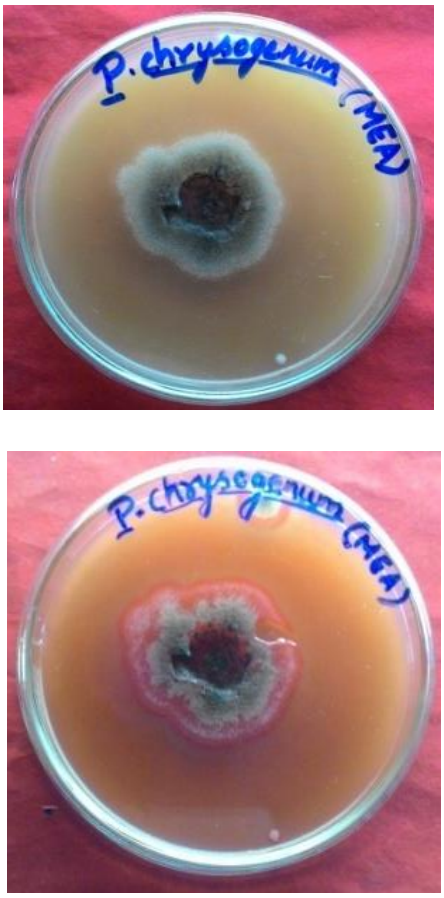

Fig. 3: Showing zone of hydrolysis (A) Before congo red test (B) After congo red test

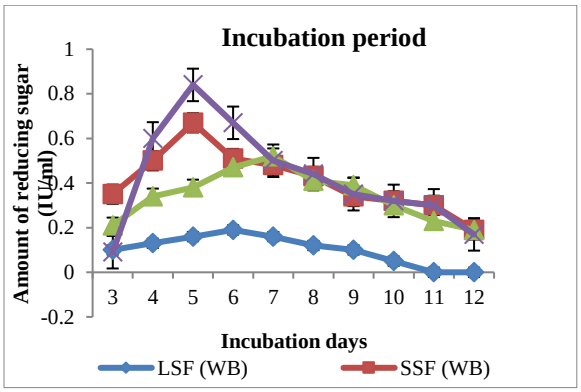

Fig.4: Effect of incubation period on xylanase production

After confirming xylanolytic activity of fungal strain, xylanase production has been performed by fermentation processes: Liquid state fermentation (LSF) and Solid state fermentation (SSF). Agro-wastes i.e. wheat bran (WB) and corn cobs (CC) were used as substrate i.e. carbon source or as raw material for enzyme production. Enzyme production was further optimized at several parameters such as incubation time, temperature, pH, carbon and nitrogen sources.

A. Incubation period optimization

To observe the effect of incubation period on the enzyme production by *P. chrysogenum*, flasks were incubated at 30°C and harvested at regular time intervals from 3rd day to 12th day. It has been found that by using wheat bran as substrate, 6th day (0.19 IU/ml) and 5th day (0.67 IU/ml) was optimized for LSF and SSF respectively while on 7th (0.52 IU/ml) and 5th day (0.84 IU/ml) maximum enzyme production was observed in LSF and SSF respectively when corn cobs was used as substrate. Results presented in figure 4 showed that in the beginning of fermentation process, *P. chrysogenum* produced enzyme in small concentration but as time passes enzyme production was also increased upto maxima then after the production was decreased. This was due to scarcity of nutrients in the medium and accumulation of toxic waste materials in the flask. *Penicillium sps.* produced maximum xylanases after 2 and 3 days of incubation (Prakash & Karmagam, 2014), 6th day of incubation (Bajaj *et al*., 2011) and 5th day of incubation (Knob & Carmona, 2008) by using different agro industrial based carbon sources such as wheat bran, saw dust or rice bran.

A. Temperature optimization

Temperature is an important factor for growth of fungi as well as production of enzyme. During present investigation it has been found that 30°C is the optimum temperature for LSF and SSF. In LSF, maximum enzyme production was found on wheat bran – 0.67 IU/ml & corn cobs – 0.76 IU/ml and in SSF, enzyme production on wheat bran – 0.8 IU/ml & on corn cobs – 1.029 (Fig. 5). It has been found that enzyme production was declined below and above than the optimum temperature because enzyme production is directly proportional to fungal growth and temperature. Fungi are generally mesophilic, they require temperature between 25°C - 35°C for their maximal growth. Nutrients of raw material which are used in the medium were destroyed at higher temperature and this affects the fungal growth thus decreasing enzyme production in the fermentation process (Bharti *et al.*, 2018). Earlier optimum xylanase production was reported at 26.5°C from *Penicillium brasilianum* (Panagiotou *et al.*, 2006).

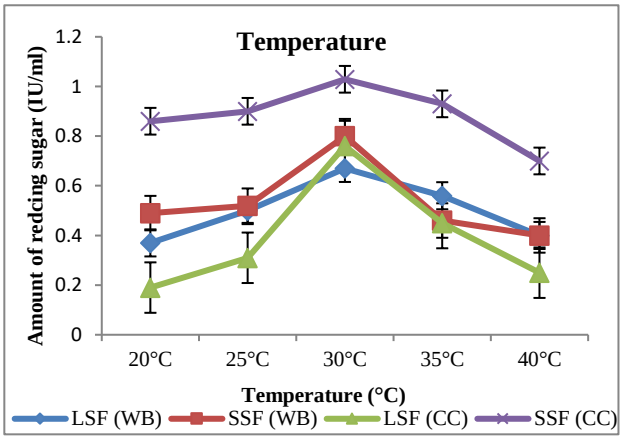

Fig.5: Effect of temperature on xylanase production

B. pH optimization

pH is one of the important cultural parameter that determine growth rate of fungi and remarkably affect the enzyme production level. Previously it was found that different pH ranges of medium were used to study maximum enzyme production in fermentation process. In our study, xylanase from *P. chrysogenum* showed 0.94 IU/ml activity at pH 6.0 under LSF while 1.2 IU/ml activity at pH 4.5 in SSF by using

wheat bran as substrate. When corn cobs were used as raw material in enzyme production, pH 5 (1.0 IU/ml) and pH 4.5 (1.2 IU/ml) were recorded as optimized pH for optimum xylanase production under LSF and SSF respectively (Fig 6). Based on our results, lower xylanase production was observed when pH medium was shifted from acidic to alkaline condition at 7.0, 7.5 and 8.0. This indicates that *P. chrysogenum* was not able to grow at alkaline condition so the enzyme production was also decreased. Similarly, highest xylanase production at pH range of 4 – 7 was found for *Aspergillus sps*. (Ghosh *et al*., 1993; Raj & Chandra, 1995; Sharma *et al*., 2020). Another research work carried out with *P. funiculosum* found that pH 6.0 was the optimized pH for xylanase production (Burugu *et al*., 2020).

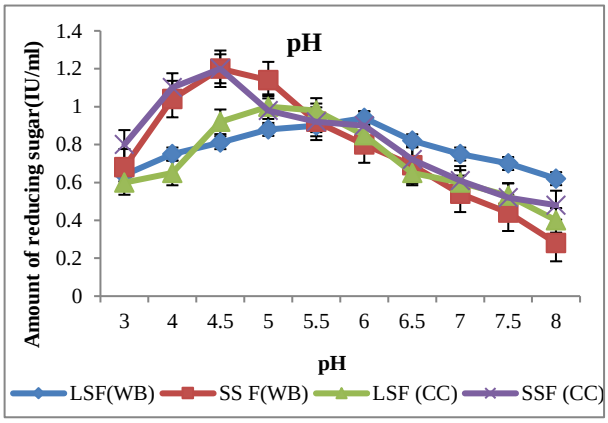

Fig.6: Effect of pH on xylanase production

C. Substrate optimization

Results presented in figure 7 and 8 showed that substrate (wheat bran and corn cobs) concentration affects xylanase production with LSF and SSF conditions. These substrates not only serve as carbon sources for growth of fungi but also serve as precursor for enzyme production (Bhavsar *et al.*, 2016). The results showed that 16 mg/ml (1.26 IU/ml) and 18 mg/ml (1.23 IU/ml) of wheat bran and corn cobs respectively supported maximal xylanase production in LSF (Fig 7) while in SSF optimum enzyme production was found at 160 mg/ml (1.32 IU/ml) of wheat bran and 200 mg/ml (1.3 IU/ml) for corn cobs. Among both carbon sources wheat was found to be best preferred substrate for xylanase production (Fig 8). The result was similar to that reported by many workers (Bakri *et al*., 2008; Nair *et al.*, 2008).The highest enzyme activity was obtained in wheat as compared to others such as rice bran, corn cobs, soya bran, saw dust, sugarcane husk and ragi bran because wheat bran contains lot of xylan as compared to others (Venkatesh & Tallapradaga, 2009; Richhariya *et al.*, 2020).

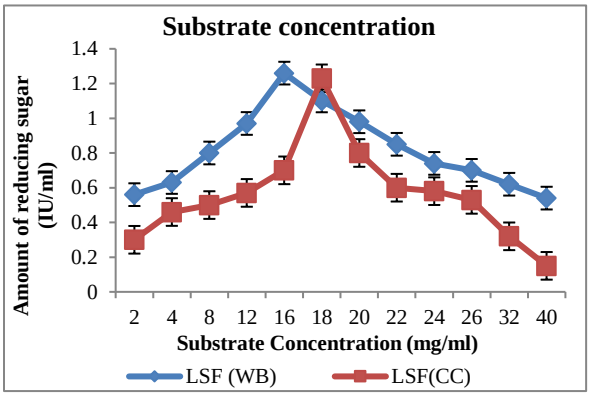

Fig.7: Effect of substrate concentration on xylanase production under LSF

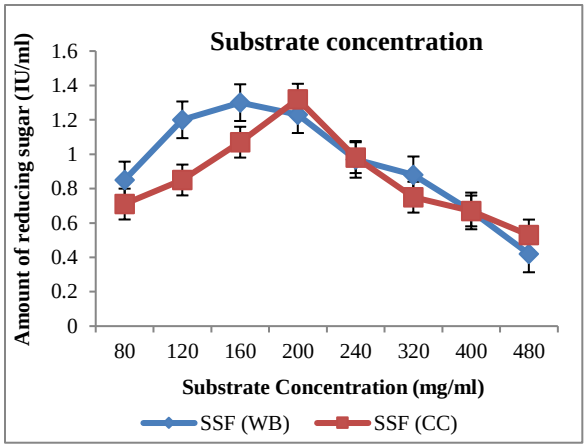

Fig.8: Effect of substrate concentration on xylanase production under SSF

D. Nitrogen sources concentration optimization

The optimal microbial growth is very much dependent on nitrogen source provided in the medium. After optimizing physical parameters and carbon sources for maximum enzyme production nitrogen sources i.e. peptone and yeast extract were also optimized for different fermentation conditions. In case of LSF, 0.4 mg/ml (1.29 IU/ml) and 0.8 mg/ml (1.27 IU/ml) peptone concentration was required for maximum xylanase production on wheat bran and corn cobs respectively. On the other hand, it has been observed that when peptone was present in the medium at concentration 0.2 mg/ml (1.34 IU/ml for wheat bran & 1.38 IU/ml for corn cobs), maximum xylanase was obtained on both substrates (Fig.9). Extracellular enzyme synthesis is affected in presence of nitrogen sources as it is the ultimate precursor for protein synthesis (Bajaj *et al.*, 2011).

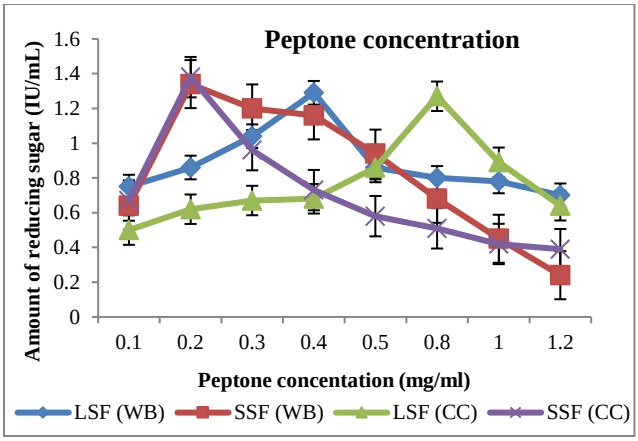

Fig.9: Effect of peptone concentration on xylanase production

Yeast extract have also been used as nitrogen source in the present investigation. In LSF, 0.85 mg/ml (1.35 IU/ml) and 0.75 mg/ml (1.29 IU/ml) concentration of yeast extract was optimized for wheat bran and corn cobs respectively while for SSF, 0.65 mg/ml (1.37 IU/ml) for wheat bran and 0.8 mg/ml (1.4 IU/ml) was optimized for corn cobs (Fig.10). Similar to above results, organic nitrogen sources such as peptone and yeast extract usually enhanced the enzyme production (Aqeel & Umar, 2010). Among different nitrogen sources i.e. yeast extract, tryptone, ammonium sulphate, urea and peptone, Yeast extract was proved as good nitrogen source to enhance the xylanase production (Akinyele *et al.*, 2020).

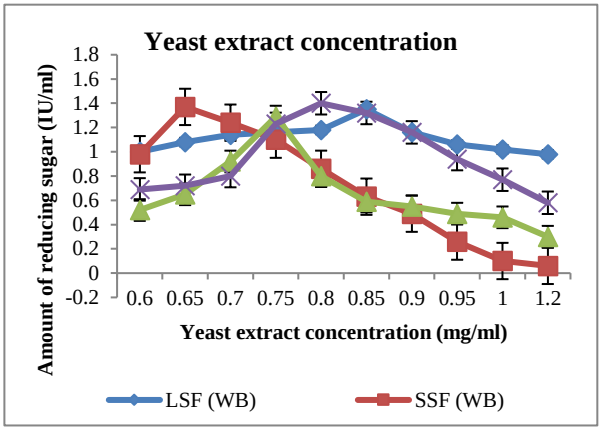

Fig.10: Effect of yeast extract on xylanase production

After different physical and chemical parameters optimization for maximum xylanase production, xylanase enzyme activity has been assayed at few conditions such as incubation period of the reaction mixture, temperature, pH and xylan concentration. Based on our results it was observed that when xylanase assay was performed for 15 min incubation, maximum xylanase activity was found in LSF on both substrates i.e. wheat bran (1.39 IU/ml) and corn cobs (1.36 IU/ml) while in SSF, 10 min and 30 min of incubation period was optimized for wheat bran (1.4 IU/ml) and corn cobs (1.46 IU/ml) respectively (Fig 11). In figure 12, to find the optimum temperature at which maximum xylanase assay was observed. Xylanase activity assay was carried out at different temperatures such as 40°C, 45°C, 50°C, 55°C, 60°C and 65°C. In this work it was found that enzyme used in assay obtained from fungal strain with wheat bran, 45°C was optimized for LSF (1.4 IU/ml) as well as SSF (1.47 IU/ml). when corn cobs xylanases were used for enzyme assay it was observed that at

55°C temperature maximum enzyme activity was found on LSF (1.39 IU/ml) and SSF (1.51 IU/ml) both. When pH of buffer solution was varied from 4.0 to 6.0 (Fig. 13), it was found at in LSF, 5.5 was optimum pH for wheat bran (1.45 IU/ml) and 5.0 pH was for corn cobs (1.4 IU/ml) while in SSF maximum enzyme activity was found at pH 5.0 for both wheat bran (1.52 IU/ml) and corn cobs (1.53 IU/ml). When oat spelt xylan was used as substrate for xylanase activity assay optimization (Fig. 14), it was observed that on wheat bran, 10 mg/ml of xylan concentration optimum enzyme activity was found under both fermentation processes (1.53 IU/ml & 1.61 IU/ml for wheat bran). From the corn cobs, 10 mg/ml (1.46 IU/ml) concentration of xylan was optimized for LSF while 5.0 mg/ml (1.59 IU/ml) concentration is the maximum amount for SSF.

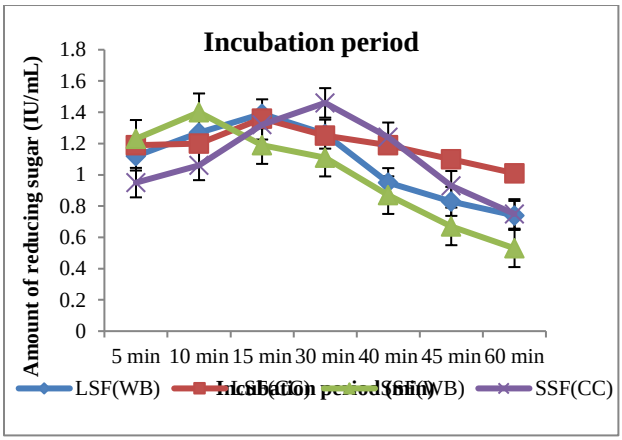

Fig.11: Effect of incubation time on xylanase activity

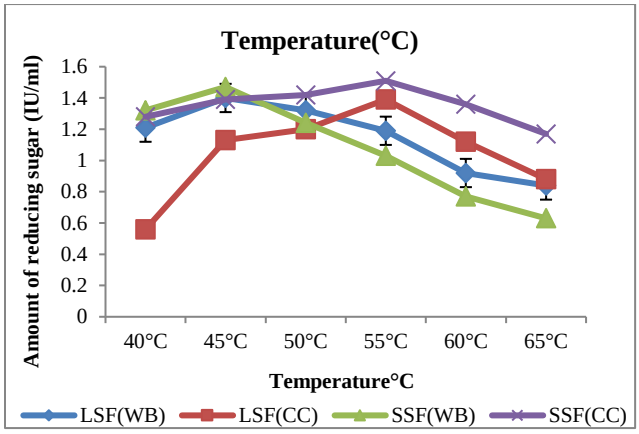

Fig.12: Effect of temperature on xylanase activity

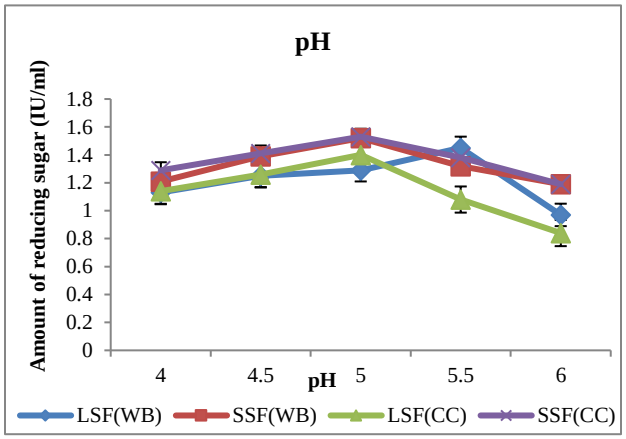

Fig.13: Effect of pH on xylanase activity

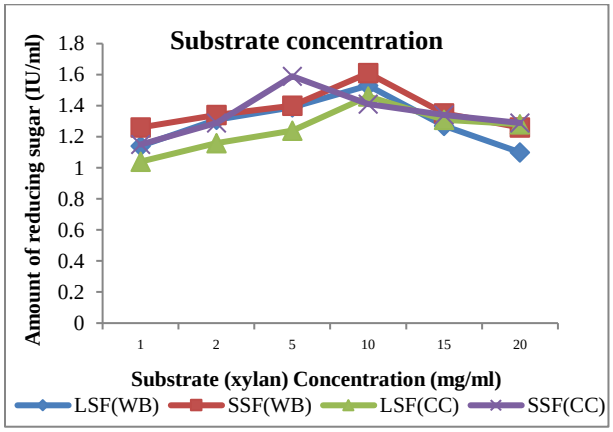

Fig.14: Effect of xylan (oat spelt) used as Substrate on xylanase production

CONCLUSION

Penicillium chrysogenum has good potential for xylanase production utilizing lignocellulosics like wheat bran and corn cobs as carbon source. So the replacement of expensive xylan with the agro-residual wastes makes it cost effective and environment friendly. Different parameters such as incubation period, temperature, pH, substrate concentration, peptone and yeast extract concentration have been optimized to enhance the yield of xylanase production. Based on the above results wheat bran is proved a better carbon source for enzyme production in comparison to corn cobs. Among both fermentation processes, SSF have been found to produce higher enzyme concentration when compared with LSF. It has been found that there was more than three times enhancement in the xylanase enzyme production in terms of enzyme activity when optimized with wheat bran as a substrate. When powdered corn cobs were used as substrate then also two times increment in the enzyme production has been found under solid state fermentation condition. The enzyme xylanase having high stability and activity will be used in various industrial and biotechnological uses.

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