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ISOLATION AND SCREENING OF CELLULOSE DEGRADING MICROBES FROM HARYANA REGION SOIL

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ABSTRACT

Saccharum munja biomass is a cheap lignocelluloses which has high carbohydrate content and, if properly pre-treated, could be converted to fermentable sugar. The aim of the study is to isolate cellulose degrading microbes from soil samples collected from different regions and to identify cellulose degrading microbes of fungi. A total of 20 soil samples were collected and processed for isolating the different cellulolytic fungi. Out of 20 soil samples, 82 fungal isolates were isolated by serial dilution (10^{-3}-10^{-7}) method and their pure cultures were maintained on potato dextrose agar (PDA) media. Clear zone around the colony was the indication of the cellulose degradation activity of the microorganisms. Then, all fungal isolates were screened primarily for cellulase production by plate assay method. Out of 82 fungal isolates, only 8 having maximum zone of hydrolysis were selected for carboxymethyl cellulase (CMCase) activity under submerged cultivation by making use of 1% carboxymethyl cellulose (CMC) acting as a carbon source. The CMCase and Xylanase activity was found to be maximum in Trichoderma sp. R-4 and Aspergillus sp. R-30 among all isolates of the genera Trichoderma and Aspergillus respectively.

Keywords: Aspergillus, Carboxymethylcellulase (Cmcase) Activity, Isolation, Screening, Fungi, Submerged Fermentation.

I. INTRODUCTION

The increasing concerns about the depletion, shortage of fossil fuels and air pollution caused by incomplete combustion of fossil fuels have led to specific focus on the production of cellulosic bioethanol from renewable lignocellulosic substrates such as wheat straw, rice straw, sugarcane bagasse, etc. (Sun and Cheng 2005). Hence, three major steps involved are: pretreatment process to release cellulose, hemicellulose and lignin from lignocellulose matrix, hydrolysis to produce reducing sugars (acid/enzyme) and fermentation to convert sugar mixtures to ethanol. One of these three steps is accomplished by many naturally occurring bacterial and fungal microorganisms that can saccharify/hydrolyse the major components of lignocellulose, cellulose, a linear

polysaccharide of glucose units linked by β-(1,4)-glucosidic bonds (Gielkens *et al.,* 1999; Han *et al.,* 1995) and hemicellulose with the help of extracellular hydrolytic enzymes, viz., cellulase and hemicellulase respectively. With the help of cellulolytic system, cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds, in a much cheaper and biologically favourable process (Chellapandi and Himansu, 2008). The cellulase enzyme system comprises three classes of soluble(hydrolytic) extracellular enzymes: endo-(1,4)-β-D glucanase [endoglucanase, endocellulase, CMCase (EC 3.2.1.4)] which cleaves β- linkage at random, exo-(1,4)-β-D glucanase [cellobiohydrolase, exocellulase (EC 3.2.1.91)] which releases cellobiose from non-reducing or reducing end, generally from the crystalline parts of cellulose and β-glucosidase [cellobiase (EC 3.2.1.21)] which releases glucose from cellobiose and short chain cello-oligosaccharides (Bhat and Bhat, 1997). Cellulose utilization is responsible for one of the largest material flow in the biosphere therefore the purpose of the study is to isolate cellulose degrading microbes from soil samples collected from different regions and to identify cellulose degrading microbes of fungi with better cellulases production.

II. MATERIALS AND METHODS

2.1 Collection of Lignocellulosic Biomass

Lignocellulosic biomass (LB) was collected from various places like *Saccharum munja* from Tilyar Lake on National Highway (NH) 10, Rohtak city which is located at latitude of 30°1' N and longitude of 75°17 E. The collected LB was washed with distilled water and then dried at 70°C till constant weight. The size of 0.1- 2 mm oven-dried feedstock was stored in sealed plastic bags at room temperature until use for pretreatment.

2.2 Isolation of Fungi for Better Production of Hydrolytic Enzymes

Isolation of Lignocellulose Decomposing Fungi-Soil is a high proliferation of microorganisms. Cellulases producing fungal strains were isolated from 20 soil samples collected from different areas of Rohtak (30°1'N and 75°17 E) and Jind (29°48' N and 78°26'E), Panipat in Haryana (INDIA) was screened for the isolation of decomposing fungi. One gram of soil from each sample was suspended in 100mL normal saline solution in a 250mL flask and incubated for 30 minutes at 180rpm. The serially diluted samples (10^{-3}-10^{-7}) were spread on the surface of potato-dextrose agar (PDA) media (prepared by adding potato peels 200g, dextrose 20g, agar agar 20g and streptomycin sulphate 70µg/mL to inhibit bacterial growth) and incubated for 3 days at 30ºC. The colonies were picked up and subcultured to obtain pure cultures. Stock cultures were maintained on PDA media at 4ºC. This final multistep enriched culture sample was used for further study.

III. SCREENING OF FUNGAL STRAINS FOR CELLULASE AND XYLANASE USING PLATE ASSAY

3.1 Qualitative Plate Assay: The fungal discs (= 4mm diameter) of different fungal strains were inoculated on Mandels and Reese medium (Table-2) plates containing 0.5% (w/v) carboxymethyl cellulose, 0.5% (w/v) Birchwood xylan, 0.1%(w/v) for the screening of various fungal strains for the production of cellulose and xylanase respectively. Then, the agar plates, in triplicates, were incubated at 30°C for 72 hours. The agar plates were then stained with 0.1% Congo red dye (w/v) (mixed with 0.1g in 100mL disilled water) for 15 minutes and then destained with 1M NaCl solution (mixed with 58.5g NaCl in 1000mL distilled water) for 15 minutes

(Ninawe *et al.*, 2005). The strains that showed a clearing zone around the colony were isolated as potential cellulose producing fungi. The zone of hydrolysis was observed and its diameter was measured.

3.2 Quantitative Screening: Screening for Cellulase Production

The cellulase producing fungi selected by qualitative screening were screened for cellulase production in liquid medium. They was inoculated into 250mL Erlenmeyer flasks containing 50mL of Mandels and Reese medium along with 1% carbon source carboxymethyl cellulose (CMC) and birchwood xylan. The inoculation of the medium was done with 1.0mL spore suspension and incubated at 30^{o}C and 180 rpm for 3 days. The contents of the flasks were filtered through Whatmann No. 1 filter paper and cell-free culture filtrates were used in enzyme assays.

Carboxymethyl Cellulase (CMCase) Assay: The endoglucanase/carboxymethyl cellulase (CMCase) activity was measured according to IUPAC (Ghose, 1987). In the test tube, 0.5mL carboxymethyl cellulose (1%, 4.8pH, and 0.05M citrate buffer) was added with 0.5mL appropriately diluted enzyme and incubated at 50ºC for 30 minutes. The reducing sugar concentration was estimated by DNSA method (Miller, 1959). At the end of incubation period, the reaction was stopped by adding 3.0mL DNSA reagent. The tubes were incubated for 5 minutes in boiling water bath for colour development and the optical densities (OD) were taken at 540nm. The CMCase activity were calculated following the concept that 0.185 units of enzymes will liberate 0.5mg of glucose under the assay conditions and was expressed as U/mL.

Xylanase Assay: Xylanase activity in the culture filtrate was measured by adding 0.5mL of 1% birchwood xylan prepared in 0.05 M sodium citrate buffer of pH 4.8 and 0.5mL of appropriately diluted enzyme were incubated at 50°C for 30 minutes. At the end of the incubation period, 2.0mL of DNSA reagent was added and sugar concentration was estimated as per the method of Miller (1959). One unit of enzyme activity was defined one micromole of xylose released per mL per minute.

IV. OPTIMIZATION OF FUNGAL GROWTH USING SUBMERGED FERMENTATION METHOD

Microbial growth and cellulase production was optimized by varying different physical (temperature, pH and agitation etc.) as well as chemical parameters (C and N source) in submerged as well as solid state fermentations.

4.1 Effect of Incubation, Temperature and pH - The effect of **incubation (**incubation period at regular intervals upto seven days)**, temperature** (pH 5.0 at 20°C, 25°C, 30°C, 35°C and 40°C) and **pH** on cellulase production was studied by cultivating the fungi in a set of Mandels and Reese medium prepared by adjusting the pH from 3.0 to 8 at 30°C and 180 rpm for 120 hours. The cellulose enzyme in the culture filtrates was assayed.

V. RESULTS AND DISCUSSION

5.1 Composition of Lignocellulosic Biomass: Percent composition of cellulose, hemicelluloses and lignin of lignocellulosic biomass, *Saccharum munja*, was studied. The maximum cellulose content in *S. munja* was found to be 45.68 but maximum hemicelluloses content was 22.4%. The lignin content was found to be quite

high in *S. munja*, i.e., 24.1%. *S. munja* may be used for bioethanol production because it contained high percentage of halo (Cellulose+hemicellulose) cellulose content.

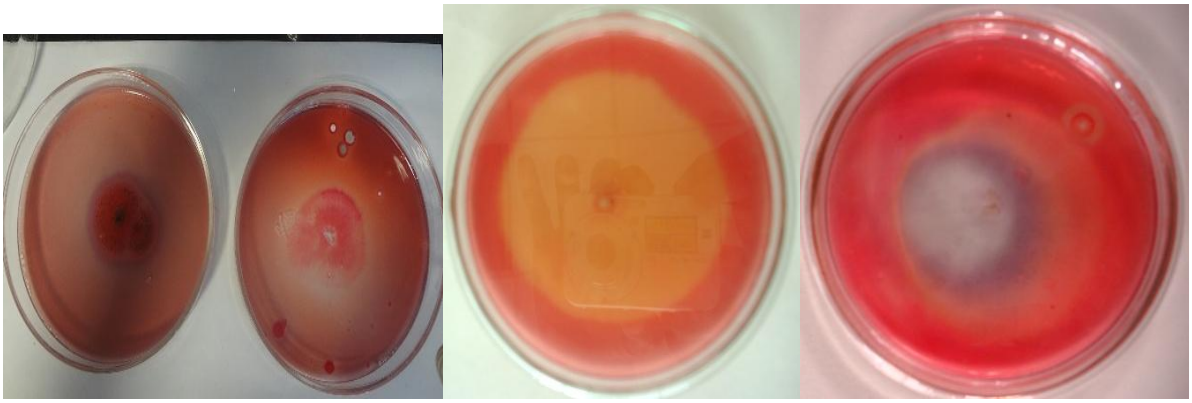
VI. ISOLATION AND SCREENING OF FUNGAL STRAINS FOR CELLULASE AND XYLANASE PRODUCTION

Primary Screening: In the present study, a total of 82 fungal isolates were isolated from 20 soil samples collected from various regions of Rohtak from the sites rich in decomposing organic matter (Table 1). The main fungi *spp*. isolated from different soil samples was *Aspergillus sp., Rhizobium sp., Fusarium oxysporium, Alternaria sp. , Penicillium sp., Mucor sp., Trichoderma sp.*. The fungal isolates with larger zones of decolorization (Figure 1) belonged to genera *Trichoderma* and *Aspergillus* which were identified by microscopic examination of conidia, spore, mycelium structure and colony morphology characteristics.

Table 1: Fungal Isolates from Different Geographical Regions

S. No.	Geographical region	No. of samples	No of fungal isolates
1.	Jind	7	26
2.	Rohtak	6	32
3.	Panipat	7	24

On the basis of zone of decolorisation (Table 2), the fungal isolates 2 (8.3cm), 4 (9.0cm), 12 (8.5cm) and 15 (8.6cm) belonging to genera *Trichoderma* and 30 (5.9cm), 38 (5.7cm), 47 (5.7cm) and 80 (5.6cm) belonging to genera *Aspergillus* were selected for CMCase production in submerged cultivation.



Trichoderma sp. R- 30 & 97 **Trichoderma sp. R- 4** **Aspergillus sp. R- 30**

Figure 1: Zone of decolorisation of different fungal isolates of Aspergillus and Trichoderma spp..

6.1 Qualitative Screening : The CMCase activities of all the selected fungal isolates were analysed on third day. Among all the selected *Trichoderma* and *Aspergillus* isolates, the CMCase activities of isolates 4 and 30 named *Trichoderma* sp. R-4 (0.193IU/mL) and *Aspergillus* sp. R-30 (0.0971IU/mL) respectively were found to be maximum (Figure 2).

Table 2. Plate Assay Screening of Fungal Isolates

Fungal isolates	Zone of Decolourization (cm)		Fungal isolates	Zone of Decolourization (cm)	
	A	B		A	B
1	6.5	7.1	28	3.3	4.1
2	**8.3**	**8.6**	29	3.5	4.3
3	4	4.7	**30**	**5.9**	**11.4**
4	**9**	**9.5**	31	3.6	4.5
5	3	3.3	32	3.3	3.9
6	3.8	4.4	33	3.6	4.2
7	6.1	7.6	34	3.5	4.3
8	5.5	6.8	35	3	3.8
9	8	8.7	36	3.4	4.1
10	6.2	7.2	37	3.2	4.0
11	3.2	3.9	**38**	**5.7**	**10.7**
12	**8.5**	**10.1**	39	3	3.2
13	3.4	4.0	40	3.2	3.3
14	5.3	6.8	41	3.1	3.4
15	**8.6**	**9.1**	42	4.2	5.1
16	3.5	4.2	43	3.4	4.0
17	3	3.9	44	7.2	7.9
18	3.2	3.9	45	3.1	4.0
19	7.9	8.5	46	4	4.8
20	3.8	4.6	**47**	**5.7**	**10.9**
21	6.4	7.3	48	3.3	3.7
22	7.0	7.3	49	4.7	5.4
23	3.6	4.1	50	8	8.4
24	3.3	3.7	**51**	**5.6**	**11.1**
25	3.4	3.9	52	4.6	5.3
26	3.8	4.5	53	3	3.6
27	4	4.7	54	7	7.4

The xylanase activity of the selected fungal isolates was also analyzed on third day (Figure 2.1). Ahmed *et al.* (2009) could achieve maximum production of FPase, CMCase and β-glucosidase from *T. harzianum* at 120 hours. Among all the selected *Trichoderma* and *Aspergillus* isolates, the xylanase activity of isolates 12 and 30 named *Trichoderma* sp. R-12 (25.01 IU/mL) and *Aspergillus* sp. R-30 (48.25 IU/mL) respectively were found to be maximum (Figure 2.1). Due to highest cellulase activity required for hydrolysis of cellulose of the lignocellulosic biomass, *Trichoderma* R-4 was selected among all the fungal isolates for further experiments.

Likewise, maximum cellulase production from *A. niger* KK2 and *A. phoenix* was reported at 120 hours incubation by Kang *et al*. (2004) and Dedavid *et al*. (2008) respectively.

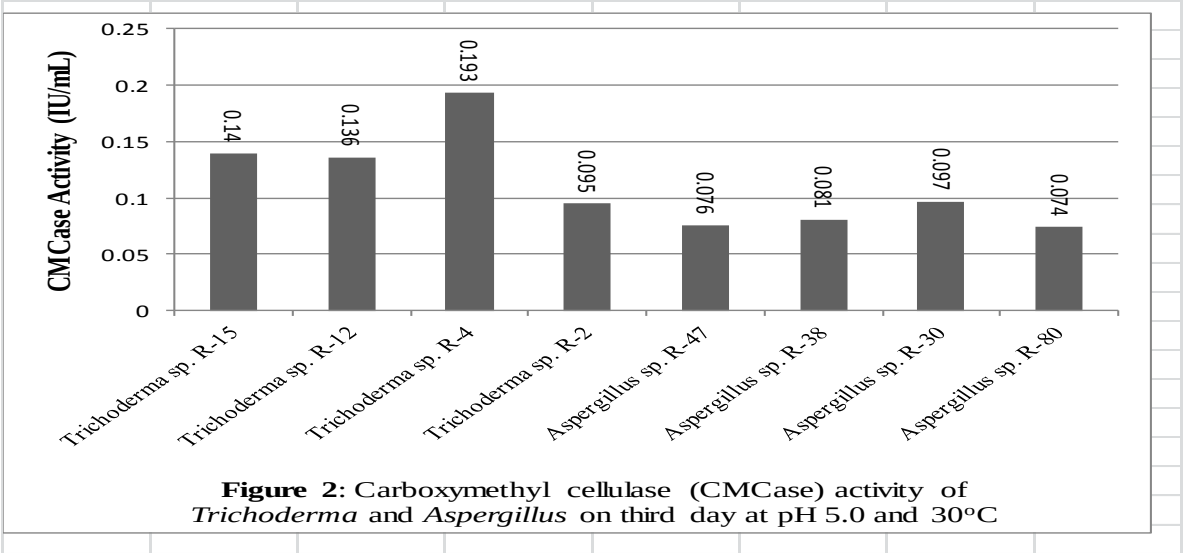


Figure 2: Carboxymethyl cellulase (CMCase) activity of *Trichoderma* and *Aspergillus* on third day at pH 5.0 and 30°C

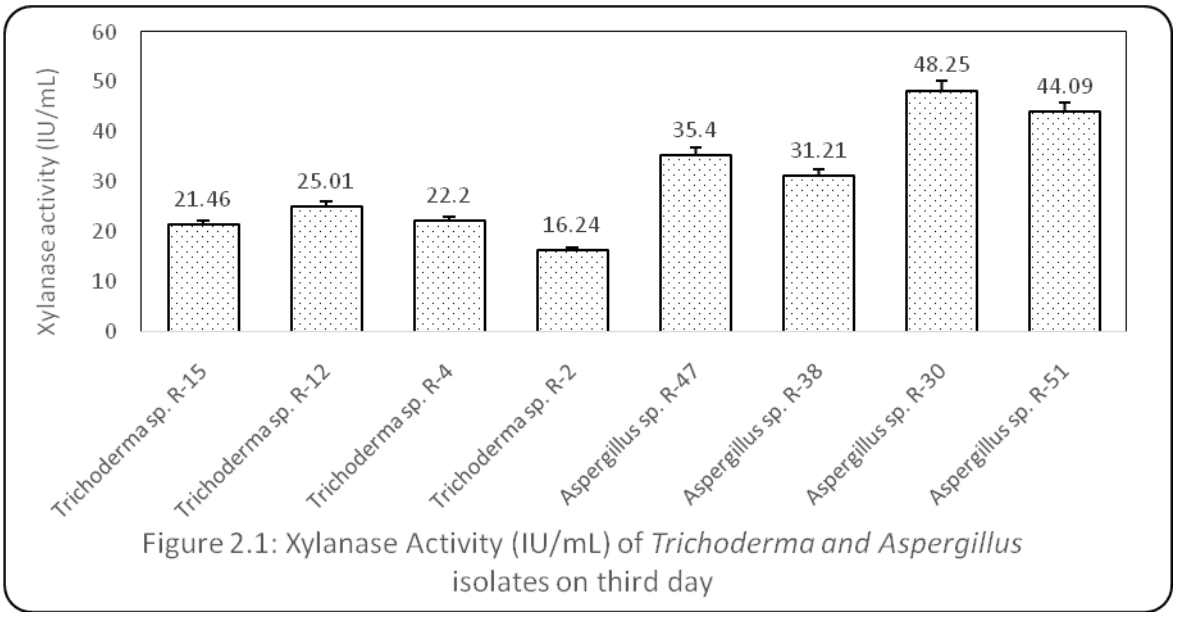


Figure 2.1: Xylanase Activity (IU/mL) of *Trichoderma and Aspergillus* isolates on third day

VII. OPTIMIZATION OF ENZYME PRODUCTION UNDER SUBMERGED CULTIVATION

7.1 Effect of Incubation Period on Enzyme Production of Trichoderma sp. R-4: In fig. 3.1, the effect of incubation period at regular intervals upto seven days on FPase (U/mL) production by *Trichoderma* sp. R-4 on lignocellulosic biomass of *S. munja* under submerged cultivation has been shown. In case of *S. munja*, FPase activity (U/mL) ranged from 0.4 to 1.01 respectively and found to be maximum on fifth day.

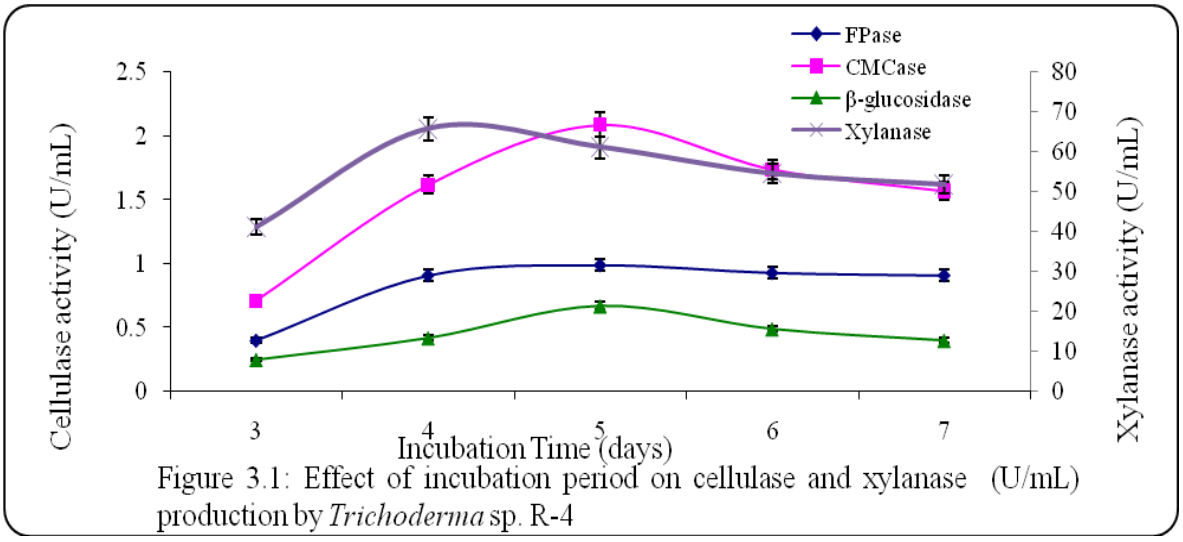


Figure 3.1: Effect of incubation period on cellulase and xylanase (U/mL) production by *Trichoderma* sp. R-4

Different researchers found different incubation times for maximum cellulase production as the time-course required to reach maximum level of cellulase activity may be affected by several factors, including the presence of different ratios of amorphous to crystalline cellulose (Ogel *et al*., 2001). It was found just like that of FPase activity as the fungal isolate *Trichoderma* sp. R-4 showed maximum CMCase activity (2.69 U/mL) on *S. munja* under submerged cultivation on fifth day. The CMCase activity of *Trichoderma* sp. R-4 on *S. munja* was found to be ranging from 0.71 to 2.51 from 3rd to 7th day. Similarly, maximum production of CMCase was achieved on 3rd day by Gomathi *et al.* (2012) by the cultivation of *A. flavus* under SmF using wheat bran as substrate. Anthony *et al.* (2003) reported the maximum production of xylanase by *Aspergillus fumigates* AR1 at 60 hours of incubation period.

Under submerged cultivation, the xylanase activity (U/mL) of *Trichoderma* sp. R-4 on *S. munja* and Municipal Solid Waste (MSW) varied from 23.29 to 82.99 U/mL respectively. However, highest xylanase activity (99.56 U/mL) was found to be present on 6th day of submerged cultivation of *S. munja* (82.99 U/mL).). Bakri *et al*., (2008) reported highest xylanase production by *Aspergillus niger* SS7 at 120 hours. *Aspergillus fumigates* NITDGPKA3 also exhibited maximum cellulase and xylanase activity at 5 days (Sarkar and Aikat, 2014).

Thus, it can be predicted from the effect of incubation period on the production of various enzymes, viz., CMCase, and xylanase released by *Trichoderma* sp. R-4 under submerged cultivation of lignocellulosic biomass of *S. munja* that the fungus expressed its maximum enzymatic activity of all the enzymes on fifth day except that of xylanase which was found to be maximum on 6th day.

7.2 Effect of pH on Enzyme Production of Trichoderma sp. R-4: The production FPase enzyme (U/mL) by *Trichoderma* sp. R-4 grown under submerged cultivation using various lignocellulosic biomass of *S. munja* has been found to be varying from 0.81 to 1.01 U/mL as also shown in Fig. 3.2. With the increase in pH, the production of FPase was found to increase upto pH 6.0. Sharp decrease in FPase enzyme production was observed beyond pH 6.0.

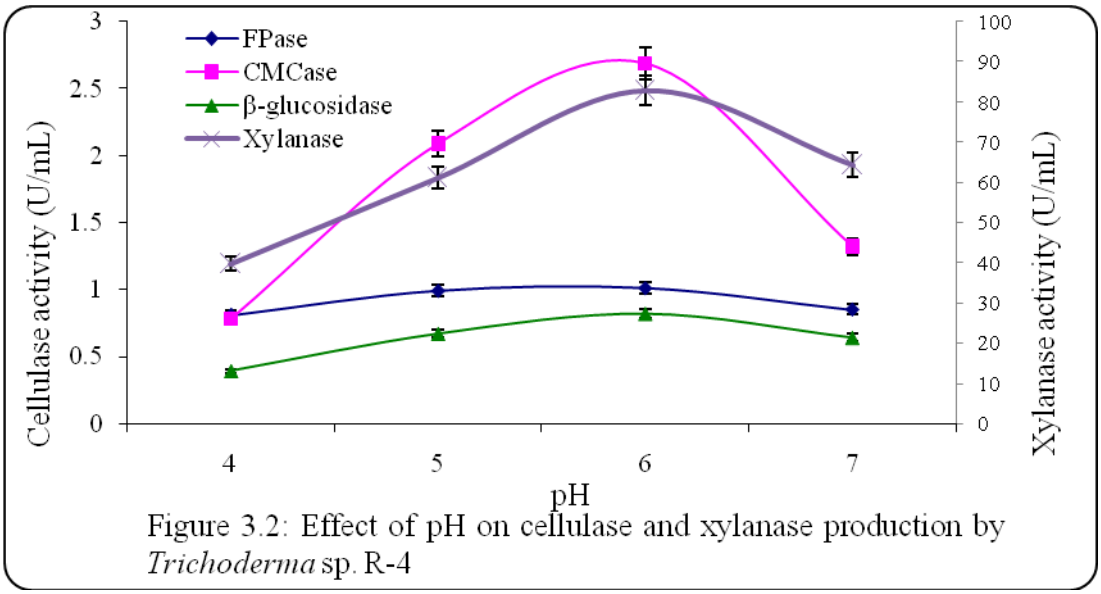


Figure 3.2: Effect of pH on cellulase and xylanase production by *Trichoderma* sp. R-4

Kirchner e*t al.* (2005) produced maximum β-glucosidase activity from *A. niger* C-6 after 96 to 120 hours. Matkar *et al.* (2013) reported maximum production of cellulases under SmF by *A. sydowii* on 6th day. The production of this enzyme by the fungus was also found to be maximum at pH 6.0 (2.69 U/mL) just like that of FPase. The CMCase activity of *S. munja* was also found to increase along with the increase in pH upto 6.0. Very sharp decrease in the CMCase enzyme production was observed beyond pH 6.0 which also indicated that the optimum pH for the production of CMCase by *Trichoderma* sp. R-4 should be 6.0 only.

The production of enzyme xylanase (U/mL) by the fungus *Trichoderma* sp. R-4 grown under submerged cultivation using biomass *S. munja* has been found to be varying from 39.75 to 82.99 U/mL. Deshpande *et al.* (2009) found maximum cellulase production by *T. reesei* on 15th day of incubation period.Here, it is worth mentioning that pH 6.0 was found to be most suitable for the production of xylanase enzyme by the fungus *Trichoderma* sp. R-4 grown under submerged cultivation. Ahmed *et al.* (2009) reported maximum production of FPase, CMCase and β-glucosidase from *T. harzianum* at pH 5.5. Sohail *et al.* (2009) studied *A. niger* MS82 with different initial pH values (4.0 to 7.0) and found that a higher CMCase and β-glucosidase activity was obtained at pH 4.0. Ibrahim *et al.* (2013) found maximum production of cellulases at pH 7.0 by *T. asperellum* UPM1 and at pH 6.0 by *A. fumigatus* UPM2. However, further studies are required to be carried out to determine different medium, the optimum catalytic activity of the cellulases produced by each of the cellulases producing fungal isolates so that the tested highly efficient fungal strains could be used optimally in a H effective manner for the protection of environment through solid waste management by the environmental agencies as the solid waste is mostly in the form of cellulose, the world's most common organic substance (Ruttloff, 1987), which can be decomposed easily by the investigated potential cellulolytic fungal strains, *i.e.*, *Trichoderma* sp. R-4 and *Aspergillus* sp. R-30.

VIII. CONCLUSION

Out of 84 fungal isolates, isolated from 20 soil samples, the fungal isolate-4 was found to be most potent cellulase producer and identified as *Trichoderma* sp. R-4 on the basis of macro and micro-morphological

characteristics. Optimization of cellulase and xylanase production by *Trichoderma* sp. R-4 was carried out under SmF using *S. munja* as substrate. *Trichoderma* sp. R-4 gave maximum production of cellulase and xylanase on 5th and 4th day respectively. The gradual increase in production of cellulase and xylanase by *Trichoderma* sp. R-4 was observed along with the increase in pH upto 6.0. Decrease in production of cellulose and xylanase was observed beyond pH 6.0. *Trichoderma* sp. R-4 gave maximum production of cellulase and xylanase at 30°C. Decrease in cellulase and xylanase production was observed at temperature above 30°C.

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