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STUDIES ON PRODUCTION OF CELLULASE FROM ANTIGONUM LEPTOPUS LEAVES USING *TRICHODERMA REESEI NCIM1186* UNDER SOLID- STATE FERMENTATION CONDITIONS

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ABSTRACT

Cellulase production was carried out in solid-state fermentation was investigated using isolated *Trichoderma reesei (NCIM1186*). Various agro –industrial waste materials and microorganisms are screened for obtaining maximum cellulase production.*Antigonum leptopus* leaves, as substrate and the *Trichoderma reesei* is taken as organism, since they showed maximum cellulosic activity, during solid state fermentation studies. The effect of incubation time, incubate temperature, particle size, weight of substrates, volume of inoculum and pH value in solid substrate on cellulose system were observed for optimal production in Erlenmeyer flasks. The physiological fermentation factors like temperature (30^{o}C), incubation time (72 h),volume of inoculum (2ml), weight of substrate (6gm), particle size (180µm) and pH (7.0) are optimized so that cellulase activity produced is maximum (4.36FPU/ml). The enzyme production was found to be associated with the growth of the fungal culture.

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Key Words
Cellulase, Solid state fermentation (SSF), *Trichoderma reesei*, cellulase activity, *Antigonum leptopus..*

INTRODUCTION

The interest in cellulase production is gaining significance because of its capacity to utilizing renewable biomass as a source of chemicals and liquid fuel (Mandels.M., 1981).Development of economical process for cellulase production is hindered because of high cost of substrate (pure cellulose).To overcome these bottle necks, cheap source which requires minimum treatment and purification with increased cellulase yields per unit of cellulose has to be used. The cheapest sources are lingo cellulosic materials such as corncobs, wheat straw, rice straw and bagasse.Hitherto the production of cellulase has been widely studying in submerged culture process. But the relatively high cost of enzyme has hindered the industrial application of the cellulose bioconversion solid state fermentation would be a better alternative for producing enzyme. A technique of solid state fermentation involves the growth metabolism of micro-organism on moist solids in the absence or near absence of any free flowing water. Such a system, being closer to natural habitat of microbes may prove more efficient in enzymes and metabolites (Narahara. H, Koyama. Y., 1982, Saloheino., 2002, Pelach.M.A., 2003).

Solid-state fermentation (SSF) involves the growth of microorganisms on moist solidSubstrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites. Agro-industrial residues are generally considered the best substrates for the process, including enzyme production, based on SSF (Ellaiah *et al.,* 2002). Compared with submerged fermentation, the use of SSF presents advantages such as lower power requirements, smaller reactor volume and high productivity (Bertolin *et al.,* 2001). Castilho *et al*., (2000) state that the conditions in solid-state fermentation were closer to those found in the natural habitat of filamentous fungi, which were, thus, able to grow better and excrete larger quantities of enzymes. This can be of special interest in those processes where the crude fermented product may be used directly, as the enzyme source. Low capital investment, low waste water output, higher concentration of metabolites obtained and low downstream processing cost (Kumaran., 1997). The cheapest sources are lingo cellulosic materials such as corncobs, wheat straw, rice straw and bagasse, etc. The agro industrial residues like Antigonum leptopus leaves, husk of maize corn, grape pomace and saw dust are used for enzyme production.

This study was taken up with the objective for the production of cellulase on various substrates (*Antigonum leptopus* leaves, husk of maize corn, grape pomace and saw dust) by different micro-organisms (*Aspergillus fumigatus, Trichoderma reesei, Trichoderma viride*)**.** Screening of substrates and micro-organisms, the conditions effecting the enzyme production such as incubation time, inoculums level, incubation temperature, pH, weight of solid substrate**,** particle size was studied and optimized.

MATERIALS AND METHODS

Microorganisms

All the fungal cultures were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, by order. The *Trichoderma reesei* (NCIM 1186) cultivated on potato dextrose agar medium containing 2.0% agar and incubated at 30^0C for 10 days until complete sporulation. The spores from slants were suspended in sterile water. The suspension is used as inoculum (10^7spores/ml).

Substrates and chemicals

Antigonum leptopus leaves were obtained from local market in vizag, India; husk of maize corn, grape pomace and saw dust were obtained from agricultural fields of aruku, India; Potato Dextrose Agar was obtained from Merck, India. All other chemicals and reagents used were obtained from sigma chemicals Co.Ltd, India and were of analytical grade.

Cultivation and cellulase production

The seed medium was that of Mendel's. (Mandels. M& Weber.J., 1969).The organism were grown on synthetic media containing (in 1liter of distilled water):NH_2CONH_2,0.3g;NH_4SO_4,1.4g;KH_2PO_4,2.0g;$CaCl_2 2H_20$,0.4g;$MgSO_4.7H_2O$,0.3g;$FeSO_4.7H_2O$,5mg;$MnSO_4.4H_2O$,1.6mg;$ZnSO_4.7H_20$,1.4mg;$CoCl_2.6H_20$,20mg;Peptone,0.75g;Yeast extract,0.25g with 2% Antigonum leptopus leaves as sole carbon source. The pH was adjusted 4.8

with 0.1N HCl. Erlenmeyer flask(250ml) containing 100 ml of the respective media were autoclaved at 121oc,15 lb pressure for 15 minutes, cooled and inoculated with 3 ml of 24h culture of organisms PDA plates. The cultures were incubated at 30ocin an orbital shaker (Remi, India) 220 rpm for 7 days. After incubation it was centrifuged (Remi cold centrifuge) at 10,000 rpm for 10 minutes at 4^{o}c.The supernatant were used as source of crude extracellular enzyme.

Cellulase Assay

Cellulase (EC 3.2.1.4) activity was analyzed by Filter paper assay method described by using Whatman No.1 filter paper strip as substrate (Ghose.T.K. 1987). The reaction mixture contained a piece of filter paper strip (1x6 cm) in 0.05M solution of sodium citrate buffer, pH 4.8, and 0.5 ml of the cell- free culture supernatant. The mixture was incubated at 50^{o}c with shaking for 60 minutes. The reducing sugar released by the enzyme was measured as glucose equivalent using dinitrosalicyclic acid reagent. A unit of activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions.

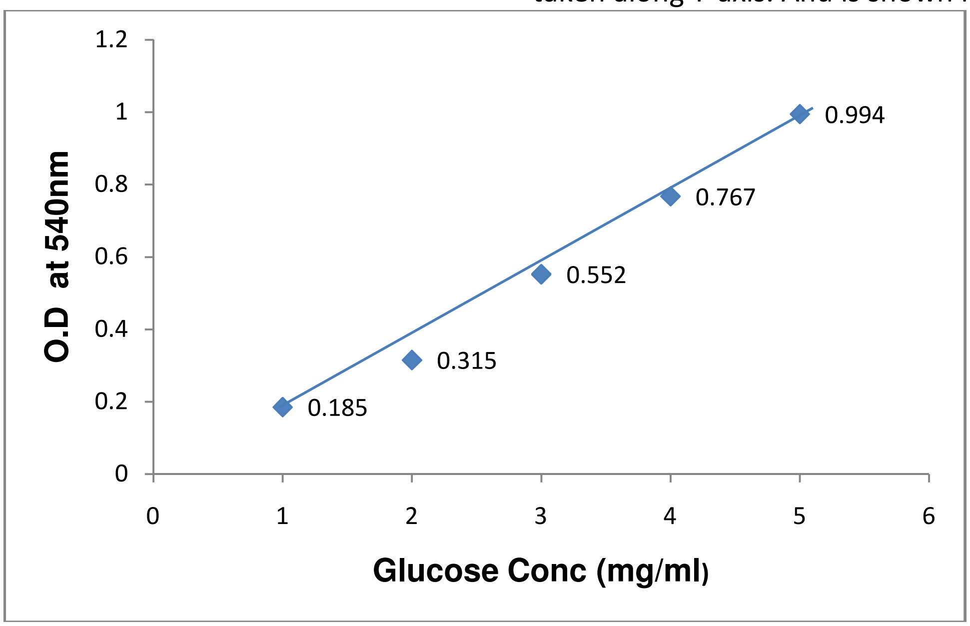
Construction of glucose standard graph

Stock solution (10mg/ml) was prepared by dissolving 1 gm of D-glucose in 100ml of distilled water in a volumetric flask.

Procedure

Different working standards were prepared from the stock solution (10mg/ml) by adding different volumes of buffer solution to 1ml of stock solution.0.5ml of each of the working Standards were taken in a series of test tubes and to each test tube 3ml of DNS reagent was added and heated in a water bath for 5 minutes. These test tubes were then cooled to room temperature and then 20ml of deionised water was added. The contents were mixed by completely inversing the test tube several times. The absorbance of the solution was measured at 540 nm in a U.V/Visible spectrophotometer (shimadzu 160A).

A standard graph is drawn between concentration of glucose taken along X-axis and absorbance at 540 nm taken along Y-axis. And is shown in Fig. 1.



Calculations

In according with the International Union of Biochemistry one enzyme unit equals 1 micromole of substrate hydrolyzed per minute.For cellulase ,it is “micromoles of glucose released per minute”.(one micromole of glucose equals 0.18mg).

The corresponding cellulase activity could be obtained from the following equation:

FPU/ml=mg glucose released x 0.185 x dilution factor

Optimization of fermentation process

Factors like selection of solid substrate,inoculum level, incubation temperature, initial pH, incubation time, particle size etc, are known to influence the production of metabolites during SSF.Experiments were conducted to improve the production of cellulase by *Trichoderma reesei*, by optimizing the following parameters. The optimum conditions obtained in each parameter were applied to the subsequent experiments. The following parameters were investigated on the production of cellulase.Effect of different lingo cellulosic substrates, Effect of incubation time, Effect of inoculum level, Effect of pH, Effect of Temperature, Effect of particle size, and Effect of weight of solid substrate Cellulase production with all the optimized parameters.

Effect of different substrates

The results in the present study may vary with the type of agro waste. This could be attributed to solid materials dual roles supply of nutrients to the microbial culture, different agro industrial lingo cellulosic substrates like Antigonum leptopus leaves, husk of maize corn, grape pomace and saw dust were used for the production of Cellulase. 5gm of solid substrate was transferred into 250ml Erlenmeyer flasks and 5ml of tap water was added to wet the solid substrate. The potato dextrose medium were inoculated with 3ml of inoculum as above and incubated at 30^0 C for 72 h. The general procedure mentioned earlier was followed for cellulase production and assay.

Effect of incubation time

To evaluate the effect of different incubation period on cellulase production, the incubation period of the medium range was varied from 24h to 168h. With a rise in incubation period. The duration of incubation plays an important role in the production of a microbial metabolite. To study the optimal incubation period for the production medium (pH 3.5) were inoculated and incubated at 30^0 C. Samples were withdrawn periodically at every 24 hours up to 168 hours and assayed for cellulase activity as described earlier.

Effect of temperature

To study the effect of different temperatures on cellulase production, the flasks containing medium kept at temperature range was varied from 25-30°C. With a rise in temperature, Incubation temperature was shown to effect cellulase production. To study the effect of incubation temperature for maximum cellulase production, the flasks with the production medium were inoculated and incubated at various temperatures such as 25, 28, 30, 32, 35, 37 and 40^o C for 72 h. The general procedure mentioned earlier was followed cellulase assay.

Effect of inoculum level

The effect of level of inoculum was studied for optimal cellulase production. Experiments was carried out using different volumes of inoculum such as 1, 2, 3, 4, 5ml. The flasks with the production medium were inoculated as above and incubated at 30^o C for 72 h. The general procedure mentioned earlier was followed for cellulase production and assay.

Effect of weight of solid substrate

The effect of various weights of Antigonun leptopus leaves powder (3, 4, 5, 6, 7, 8gm) on cellulase production was studied under batch process in 250ml flasks. The flasks with the production medium were inoculated as above and incubated at 30^o C for 72 h. The general procedure mentioned earlier was followed for cellulase production and assay.

Effect of particle size

The effect of particle size was tested by grinding the substrate into powder and passing through different mesh sizes, 44, 52, 72, 85 and 100 to obtain particles of 355,300,212,180 and 150µm respectively. The flasks with the production medium were inoculated as above and incubated at 30^o C for 72 h. The general procedure mentioned earlier was followed for cellulase production and assay.

Effect of pH

Cellulase production by microbial strains strongly depends on the extra cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes which in turn support the cell growth and product production (Ellaiah *et al*., 2002). The effect of initial pH on cellulase production was studied. The production medium was adjusted at various levels of pH by 1N HCL and 1N NaOH solution (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0and 10.0.). General procedure mentioned earlier was followed for cellulase production and samples were assayed as described earlier.

RESULTS

Cellulase was analyzed by using three different organisms namely *Aspergillus fumigates, Trichoderma reesei, T.viride*. The procedure was carried out on a variety of substrates like Antigonum leptopus leaves, husk of maize corn, grape pomace and saw dust. Different parameters were standardized by using a range of values for optimum production of cellulose. The process parameters like incubation time, temperature, particle size, inoculum size, substrate weight, and pH were studied optimized and the results obtained were compared with those in literature.

Screening of Microorganisms and substrate

Effect of mode of fermentation

Traditionally enzymes were produced by using submerged fermentation. The production of enzymes by solid state fermentation has gained momentum recently, in order to check which mode of fermentation offers better activity of cellulose enzyme, experiments were conducted for cellulase production in both submerged and solid state fermentation for a period of seven days by three fungi (*Aspergillus fumigates, Trichoderma reesei, Trichoderma viride)*.Samples were drawn and analyzed for cellulase activity which is shown in (Fig .2A).As shown in figure, cellulase production was found to be maximum in solid state fermentation carried out with *Trichoderma reesei* (2.6018 FPU/ml).similar observations were reported by various authors(Jecu.L.,2000).

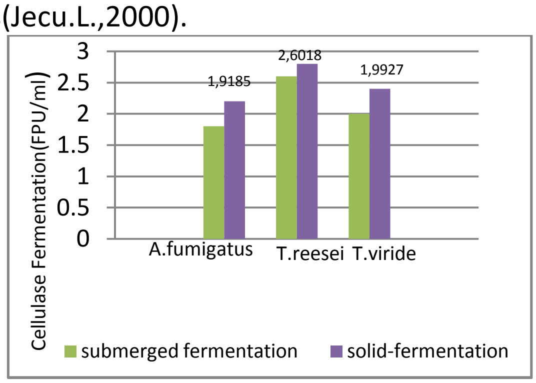


Fig 2 (A) Effect of mode of fermentation on cellulase production

Effect of type of inoculums

Cellulase can be produced either by spore suspension or by seed culture of the fungus. Experiments were conducted to check further inoculum type, which gives maximum cellulose activity by inoculating solid substrate with the 3ml of spore suspension and seed culture, and inoculated for seven days. At the end of incubation period the samples were withdrawn and assayed for cellulase activity. The results are shown in (Fig.2B). Results indicate that the seed culture of *Trichoderma reesei* (2.6542FPU/ml) was exhibited maximum cellulase activity.Further it can be observed that seed culture, as inoculum, gave higher yields when compare to spore suspension for all fungi applied.

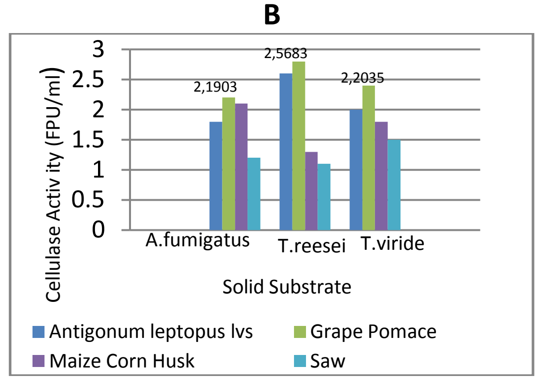


Fig.2 (B) Effect of various substrates on cellulase production

Effect of various solid substrates

Cellulase production by various strains of microorganism using purified substrates suchas Avicel and solka floc, and the use of soluble inducers such as lactose, sophorose were reported (Mandels.M. and Reese.E.T., 1964).In present study various substrates are employed in the production of cellulase, such as Antigonum leptopus leaves, husk of maize corn, grape pomace and saw. The results are shown in (Fig.2C).It is observed that Antigonum leptopus leaves produced maximum cellulase activity when the inoculum employed is *Trichoderma reesei* (2.5863FPU/ml).

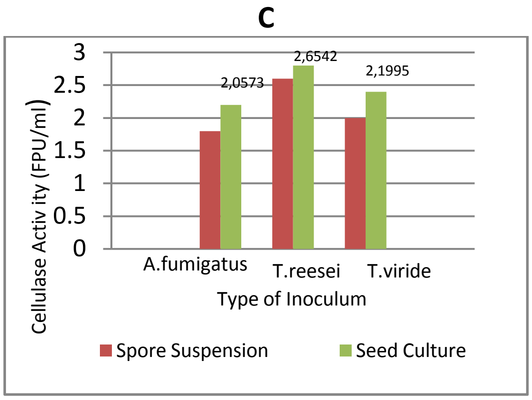


Fig.2 (C) Effect of type of inoculum on cellulase production

Optimization of parameters

Effect of Incubation Period

Incubation time has profound effect on enzyme production. To determine optimal incubation time for production of enzyme, inoculated flasks were incubated for 24, 48, 72, 96, 120, 144 and 168hrs duration. After inoculation period the enzyme was extracted and assayed .The maximum activity was found to be 2.3835FPU/ml at 72h. The decrease in activity after 72h may be due to denaturation of enzyme, resulting from variation in pH and cellular metabolism during fermentation (Table 1). Similar trends were observed by various authors for the production of cellulases (Allen ad Roche, 1989; Krishna, 1999; Chahal, 1985).

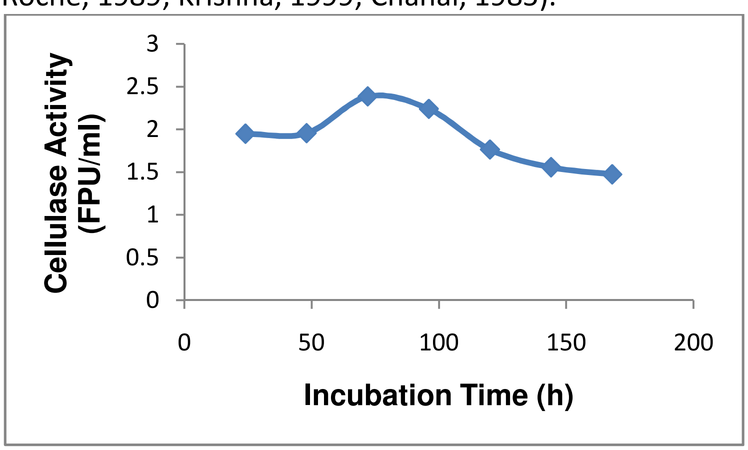


Figure 3: Effect of Incubation Time on Cellulase Production

Effect of incubation temperature

The incubation temperature is a factor regulating the enzyme synthesis. The enzyme activity was increased with the increase of temperature from 25-30°C.the maximum cellulose activity was found to be 2.396FPU/ml at 30^{o}c as shown in (Table 2).Further increase in temperature caused a slowed in activity probably due to deactivation of enzyme. So 30^{o}C was considered as optimum temperature for cellulase production.

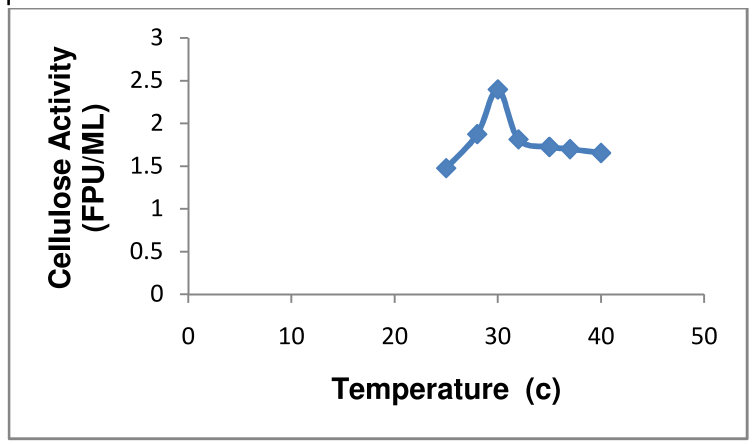


Figure 4: Effect of Incubation Temperature On cellulose Production

Effect of particle size

In the presence study, the solid substrate of various particle sizes weighed in to flasks and SSF was carried out under same conditions. Results indicated that as the particle size decreases from 355µm to180µm, the enzyme activity was found to increase and enzyme activity was maximum for a particle size of 180µm(3.15FPU/ml) as shown in (Table 3).Further decrease in the particle size, caused a decrease in the enzyme activity. This was probably due to overlapping of the particles which masks some of the active sites.

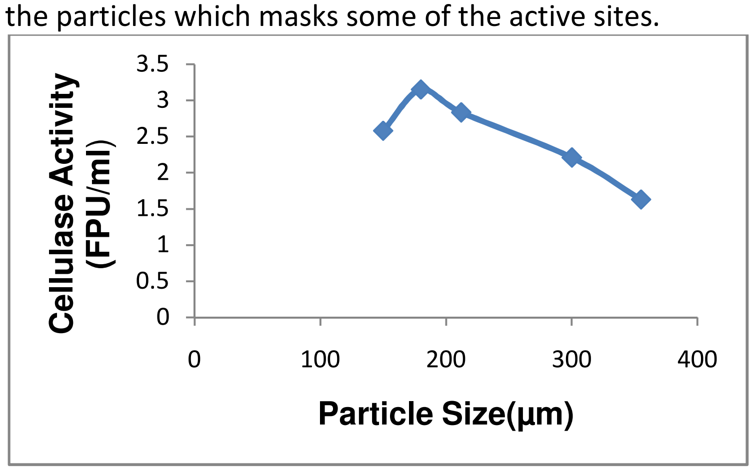


Figure 5: Effect of Particle Size on Cellulase Production

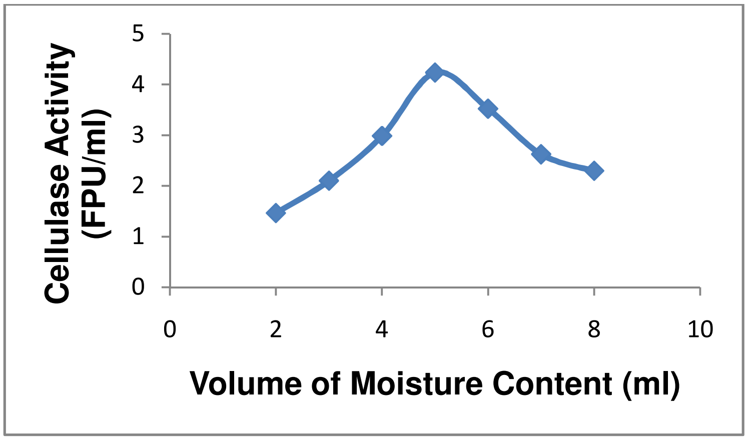


Figure 6: Effect of Volume of moisture content on cellulase Production

Effect of weight of solid substrate

The influence of various weights of Antigonun leptopus leaves powder (3, 4, 5, 6, 7, 8gm) on cellulase production was studied under batch process in 250ml flasks. The results indicated that the cellulose activity was increased when *T. reesei* was cultivated at increasing concentrations of leaves powder (Table 5). The optimum weight was found to be 6gm where enzyme activity was maximum at 4.149FPU/ml. Further increase in the weight caused a decrease in the activity because of resistance of mass transfer was increased, a longer fermentation period was needed and productivity decreased (Romero., 1999 and Ketal., 1996).

Effect of volume of inoculum

The importance of inoculum size was emphasized by Raimbault and Alazard (1980) and Pandey (1999). Different volumes of inoculum such as 1, 2, 3, 4, 5ml were added to the medium during solid state fermentation. The results indicated that maximum activity was obtained when inoculum volume was 2ml, 3.96FPU/ml. Further increase in the volume led to decrease in the activity as shown in (Table 4).At higher inoculum sizes, the moisture content was increased significantly and thus was detrimental to growth and production. The free unabsorbed liquid will give rise to a diffusional barrier imposed by the solid nature of the substrate. This will lead to a decrease in enzyme production and growth.

Effect of pH

There exists a strong influence of pH of the medium on enzyme production. To evaluate the effects 0f initial pH value in solid substrate on cellulase synthesis ,the initial pH values were adjusted by the addition of HCl or NaOH to 3.0,4.0,5.0, 6.0,7.0,8.0 ,9.0and 10.0.Different pH values were used in the medium and incubated at 30°C for 3 days. It was observed that the enzyme activity increased from 3-7 and maximum was found at neutral pH with a maximum enzyme activity of 4.36FPU/ml as shown in (Table 6). Further increase in the pH, decreased the activity. This indicates that alkaline conditions are more favorable for production of cellulase by *T. reesei*.

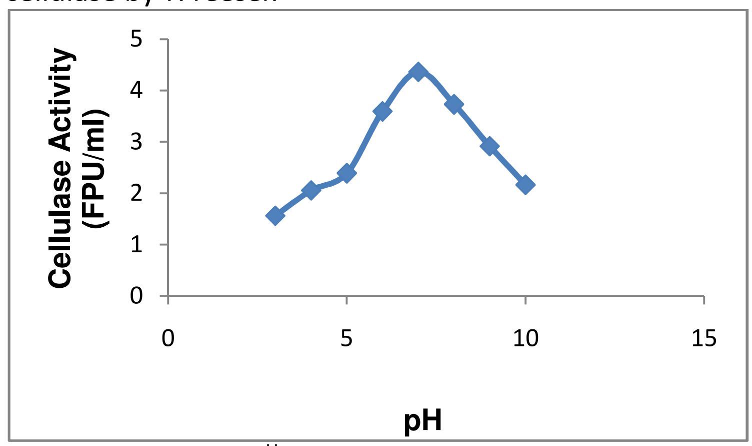


Figure 7: Effect of P^H on Cellulase Activity

CONCLUSION

The present work has been taken up with a view of exploring the possibilities of using Antigonum leptopus as a substrate and *Trichoderma reesei* as a microbial source for the production of cellulase which can hydrolysis of 1, 4-beta-D-glycosidic linkages in cellulose, lichenin and cereal beta-D-glucans. Cellulase is an industrially important enzyme has several applications in food and textile industries. In accordance with the results, taking all the influence factors and the results into Consideration, the optimal cultural process was considered as follows: Incubation time 72hrs, Incubation temperature 30^{o}c, Particle size 180µm, Volume of inoculum 2ml and pH 7.0. In the present study, cellulase enzyme was produced from *Trichoderma reesei* using Antigonum leptopus leaves as a substrate in solid state fermentation. When compared with submerged conditions, solid state conditions were found to be more favorable. The results suggest the suitability of using cheap and abundantly available Antigonum leptopus leaves as solid substrate for large scale production of cellulase in an SSF system in order to reduce the high costs. Maximum utilization of this plant waste can also contribute to efficient solid waste management. This process therefore has a high potential for comprehensive utilization of renewable lignocellulosic resources.

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