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RESEARCH ARTICLE

Production of Endo-1,4-β-D-Glucanase and Exo-1,4-β-D-Glucanase on cellulosic substrates in solid state fermentation by *Hypocrea nigricans*.

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Abstract

Background:- This research article reports that the production of cellulases by *Hypocrea nigricans*; isolate of rotten wood barks. It was screened for their efficiency in cellulose degrading enzymes particularly endo and exo- β-1, 4-D-glucanases on different lignocellulosic solid substrates. The aim of the present study was to isolate a potent cellulolytic enzyme producing fungi from natural habitat; optimisation of enzyme production by one factor at a time (OFAT) strategy, then applied for purification and characterisation.

The purified cellulolytic enzymes was to be accessible for biofuel, textile, paper pulp industrial exploitation

Results:- Among the screened lignocellulosic substrates, paddy husk has been identified as the best source for maximum enzyme production and optimal cultural conditions were recorded as pH 6.0, incubation temperature 30°C, moisture content 40%, spore density 2 X 10⁶ spores/g of substrate. The production was increased (3 folds approximately) from 3.9 U/g to 11.84 U/g under conventional optimization studies. Each experiment was performed thrice, in each triplicate mean value of experimental results were taken into consideration. The two enzymes, endo and exo- β-1, 4-D-glucanases produced by *Hypocrea nigricans* were purified and characterised by estimating their molecular weights as 54 and 48kDa respectively through SDS-PAGE.

Conclusion:- The present study reveals the cellulolytic properties of *Hypocrea nigricans* with optimised production. Maximising cellulolytic enzymes production that will work under industrial conditions particularly with inexpensive, rapid and sensitive testing methods.

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Introduction:-

Cellulose is the earth's major biopolymer and assumes tremendous economic importance globally. It is the most occurring organic compound in the biosphere with an estimated synthesis at a rate of 10¹⁰ tonnes per year and is the most abundant constituent in plants while it generally constitutes 20-30% of the litter mass¹⁻⁷. Biological degradation of cellulose by cellulases is preferred for industrial purposes because it results in high yields of desired hydrolytic products with minimal by-products⁸. Cellulose degradation is achieved by a battery of enzymes out of which β (1→4) glucanases occupy highest degree of importance. The two most functional cellulolytic β(1→4) glucanases are endo-1,4-β-D-Glucanase (EC 3.2.1.4), exo-1,4-β-D-Glucanase (EC 3.2.1.91). Cellulose decomposition is an intensive issue that attracted a large input of research activity in the past few decades since total hydrolysis of cellulose into glucose could enable us to convert glucose into ethanol, isopropanol, and butanol through fermentation, where the usage of these alcohols can limit the emission of green house gases. Also, cellulose degradation is a prerequisite in many more industries and cellulases form a prime choice for the same and these industries include food, animal feed, textile, chemical etc⁹⁻¹¹. Most of the available data suggests that the major

degrading microorganisms are soil borne¹². It was first assumed that cellulolytic capabilities are limited to certain bacteria, but later updates proclaim the dominance of fungal species¹³⁻¹⁴. In the present study, the fungus chosen is *Hypocrea nigricans*, which is an anamorph of *Trichoderma sp.*, by nature a wood decaying fungus a common fungal species of moist forests. These fungi are easily recognized by their brightly coloured fructifications. Solid substrate fermentation is defined by those processes in which the substrate itself acts as carbon/energy source which occurs in the absence or near-absence of free water generally surviving on a natural substrate as above, or an inert substrate used as solid support. SSF is mostly the choice when the crude fermented product necessarily needs to be used directly as the enzyme source¹⁵. SSF has in its advantage list over submerged fermentation; high volumetric productivity, relatively high concentration of the products, less effluent generation, comparatively simple fermentation equipment etc¹⁶⁻¹⁸. However, solid state fermentation too is a cost-prohibitive one because of high cost of process engineering. The SSF supplies the nutrients to the culture and serves as anchorage to the microbial cells¹⁹. The current study is taken up with two essential objectives of isolation of highly efficient cellulase producing source organism, production of highly processive ($\beta(1\rightarrow4)$ -glucanases in higher quantities from the isolated source through solid state fermentation, and identification and optimization of the best substrate and other fermentation conditions.

Materials and methods:-

Isolation and screening:-

The endophytic Fungi were isolated from samples collected from Tirumala forest areas of Andhra Pradesh, India, and cultured on sterilised Potato Dextrose Agar. The morphological characteristics were observed for better identification. Exo-1, 4- β -D-Glucanase activity was tested by growing the fungal isolates on Mandel's medium. Endo-1, 4- β -D-Glucanase activity of isolated fungi was tested by culturing them on Czapek Dox medium amended with carboxy methyl cellulose - 5.0 g/L followed by 1% congo red staining and 1M NaCl destaining²⁰.

Molecular Identification of a fungal culture using 18S region:-

Total genomic DNA was isolated from fungal mycelia using C-TAB method²¹, followed by qualitative assessments at 260/280 nm confirmed in a Nanodrop spectrophotometer (ND-1000, USA)²². 18S rRNA gene fragments were subjected to PCR mixture of universal primers - forward (51-CTGGTGCCAGCAGCCGCGGYAA-31) & reverse (51-CKRAGGGC ATYACWG ACCTGTTAT-31) 10 pmole/ μ l - 1 μ l each, DNA (30ng/ μ l) 3.0 μ l, DNase-RNase free water 7.50 μ l, 2X PCR master mix (MBI Fermentas) 12.50 μ l. Initial denaturation 95°C/5 min - 1 cycle followed by 30 cycles of 94°C/30 sec, 50°C/30 sec, 72°C/90 sec and final extension at 72°C for 10 min. The PCR products were analysed on 1% agarose gel in 1X TBE buffer. Amplified PCR products were gel purified and subjected to automated DNA sequencer (ABI 3730xl Genetic Analyzer - Applied Biosystems, USA). The sequence was deposited in NCBI GenBank with an accession number KJ914660.

Collection of Substrates:-

Substrates used in the present study were Paddy husk, Wheat husk, Groundnut haulms, Coir dust and Wood shavings collected from local markets of Tirupati. The substrates were individually grounded and sieved through a 2 mm screen, for uniform particle size.

Analysis of Substrates:-

Knowledge of chemical composition of lignocellulose is a key feature in determining potential uses. Therefore, the lignocelluloses were analyzed for their chemical constituents²³. Composition of lignocellulose in terms of lignin, hemicelluloses, and cellulose were determined by sequential chemical extraction according to the method of gravimetric analysis²⁴⁻²⁵. The amounts of nitrogen present in lignocelluloses were estimated by Kjeldahl method,²⁶⁻²⁷. Distillate sample recovered from Kjeldahl digestion was used for further analysis of chemical constituents present in solid substrates.

Production of endo, exo -1, 4- β -D-Glucanases:-

Production of enzymes in laboratory scale was carried out in 250 ml Erlenmeyer flasks, containing 10 g of each solid substrate initially moistened to 50% (w/v) with Czapek Dox liquid medium supplemented with 0.5% cellulose which was autoclaved. Flasks were inoculated with the spores of *Hypocrea nigricans* at a density of 2×10^6 spores/gm of substrate and incubated at ambient temperature ($30 \pm 2^\circ\text{C}$). During the 10 days course of incubation, water loss by evaporation from the flask was aseptically replaced with addition of sterile distilled water to maintain 40% or 50% moisture content. Entire fermented substrate in the flask was mixed with acetate buffer (0.2 M; pH 5.0)

or distilled water, the slurry was filtered through nylon cloth and the filtrate was centrifuged at 10,000 rpm for 20 min at 4°C. The clear filtrate obtained was used for enzyme assays.

Enzyme Assays:-

Activities of individual enzyme components of cellulase system secreted into the culture medium were estimated²⁸. The leachate, recovered after centrifugation in solid state fermentation was used as a source of enzyme. Exo-1,4-β-D-Glucanase activity²⁹ and Endo-1,4-β-D-Glucanase activity³⁰ were measured by established protocols. Exo-1, 4-β-D-Glucanase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from filter paper per min. Endo-1, 4-β-D-Glucanase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 μmole of reducing sugar from carboxymethyl cellulose per min. Secreted protein content was estimated³¹.

Optimisation of cellulase production:-

The cultural conditions were optimized for higher yield of cellulase enzyme. For the initial optimization of the medium, the traditional method of “one variable at a time approach” was used by changing one component at a time while keeping the others at their original level. The cultural conditions like pH, temperature, spore density, moisture levels, supplementation of carbon and nitrogen sources. pH ranging from 3.0 to 7.0, temperature levels at 25°C, 30°C, 35°C and 40°C, moisture levels within a range of 20 – 80%, spore densities of 2×10^4 , 2×10^5 , 2×10^6 and 2×10^7 spores/gm of substrate (where the spore densities were quantified by using neubauer chamber), Supplementation of different carbon sources to Czapek Dox medium i.e., Sucrose 0%, 2.5%, 5.0%; Glucose 0%, 0.5%, 1.0%, 2.5%, 5.0%; and Cellulose 0%, 0.5%, 1.0%, 1.5%, 2.0% individually. Also different nitrogen sources such as urea, ammonium sulphate, peptone, and yeast extract were added individually at 1% (w/v) to Czapek Dox medium. The optimization of the conditions for maximum efficacy is a measure derived of observations made over a 10 days period.

Partial purification of cellulose:-

The enzyme production was performed in optimised cultural conditions to amplify the yield of cellulolytic enzymes. The culture filtrate was centrifuged at 8,000 X g for 20 min followed supernatant by filtration with 0.45 μm membrane²⁸. The filtrate was precipitated by 80% saturation of ammonium sulphate. The collected precipitate was dialysed against 20mM Tris-HCl buffer (pH- 8.0) overnight. Dialysed contents were passed through sephadex G 100 (Sigma, USA) column gel filtration chromatography³². Column fractions (5ml each) were collected with regular time intervals and enzyme activity was determined by well diffusion method where carboxymethyl cellulose (0.5%) and Canged (0.1%) incorporated agarose (1%) gel was used as solid matrix. Final confirmation purified enzymes was done by by SDS- PAGE by determining their molecular weight³³.

Results and Discussion:-

Several Fungal species with appropriate cellulolytic activity were isolated from collected samples. According to the characters – morphological; colour of the colony, vegetative and reproductive structures; certain growth pattern studies; and 18S rRNA sequence (Genbank ACC No. KJ914660) – the organism has been identified as *Hypocrea nigricans*. Most anamorphs in *Hypocrea* are readily recognizable as being *Trichoderma*. The *Trichoderma* anamorph is unique in the ascomycetes while verticillium- and gliocladium-like morphologies are commonly found in moist tropical or subtropical forests although they also occur in arid, temperate or boreal forests, even in the most extreme north and south latitudes. The *Hypocrea* teleomorph can be found on wood, on other members of the Ascomycota, on resupinate basidiomycetes, or on perennial bracket fungi in varying stages of decay, and less commonly on herbaceous substrata. All investigated *Trichoderma* species are intimately related to *Hypocrea*, and, increasingly, named *Trichoderma* species are being shown to be the anamorphs of *Hypocrea* species³⁴⁻³⁶. In addition to this use of *Hypocrea* species in the biological control of plant pathogenic fungi, many species have the ability to break down cellulosic materials through the production of cellulases like *Trichoderma*. This ability has led to the commercial exploitation of some *Hypocrea* and *Trichoderma* species in production of cellulolytic enzymes used in manufacture of denims, animal feed and bio fuels³⁷. Several studies have shown that *Trichoderma* sp. are preferred for cellulose degradation using SSF, exhibits the same wood decaying property and genetically too is similar in terms of cellulolytic activity. On screening for the enzymes, exo-1, 4-β-D-Glucanase has shown characteristic cellulose clearance (Fig.-1) and endo -1,4-β-D-Glucanase has displayed a clear zone around the colony due to CMC depletion (Fig.-2). Molecular identification and characterisation was done by sequencing of 18S rRNA where it has yielded a consensus sequence of PCR amplified 829 bp. Based on results of homology search, Bootstrapped Neighbor joining tree was constructed using Clustal X v. 2.1 with 1000 replicates and 111 random odd

number; the isolate has the maximum homology (99%) and closest phylogenetic relationship with *Hypocrea nigricans* (Fig.-3). Agro-industrial residues rich in cellulosic materials and thus are generally considered the best substrates for solid state fermentation processes³⁸. The solid substrates such as Paddy husk, Wheat husk, Groundnut haulms, Coir dust and Wood shavings were screened to conclude on the ideal substrate for maximum cellulolytic activity by *Hypocrea nigricans* in solid state fermentation. The chemical compositions of all the substrates were determined and the results were shown in Fig.-4 and 5. The production of exo and endo-1,4- β -D-Glucanase in terms of their activities were estimated on various lignocellulosic substrates for a period of 10 days. The highest activities; for exo-1,4- β -D-Glucanase of 2.98 FPU/g, and for endo-1,4- β -D-Glucanase of 3.9 CMC U/g are obtained with paddy husk. Highest amounts of secreted proteins by *Hypocrea nigricans* viz 5.48 mg/g is obtained with paddy husk as substrate in solid state fermentation. (Fig.-6 to 8). So, in the wake of the results obtained, paddy husk has been selected as best substrate for the optimisation of enzyme production.

The effective utilization of the substrate depends on many factors than simply on carbon content and cellulose content. This is the theme clearly visible through the whole set of experiments. The wood shavings have clearly had highest carbon content, and cellulose levels; however the paddy husk was found to be a better substrate according to the study. The wheat husk has very similar carbon content like paddy husk, but the lesser levels of cellulose in wheat husk compared to paddy husk have shifted in favour of paddy husk. The growth of *Hypocrea nigricans* and the enzyme activities are also dependent on physical properties like pH, temperature, etc. However, porosity of the substrate is one factor that has not been considered during the study plan and looks to have played a role in determining the growth favourable conditions. The optimised cultural conditions where the maximum enzyme activity were recorded are as follows; pH 6.0, temperature 30°C, moisture levels of 40%, spore density 2×10^6 spores/g of substrate, carbon sources i.e., sucrose 5%, glucose 2.5%, cellulose 1.5% and among the nitrogen sources ammonium sulphate resulted good yield of enzyme. The optimised cultural conditions results were shown in Fig.-9 to 11 and Table.- 1. The enzyme production was increased from 2.98 U/g to 11.89 U/g for exo-1, 4- β -D-Glucanase, 3.9 U/g to 11.89 U/g for endo -1,4- β -D-Glucanase respectively under conventional optimization studies. Among them temperature and spore density majorly affected the enzyme production where 3 folds increased enzyme activity was recorded at optimum conditions. Temperatures higher than optimal temperatures result in enzyme denaturation and inhibition, excess moisture losses and growth arrest while lower temperatures lead to lower metabolic activity³⁹. Although most filamentous fungi are mesophilic requiring optimal temperatures between 25 and 35°C, some species thrive at 50°C⁴⁰. The importance of the inoculum in solid state fermentation is well recognized⁴¹. In SSF involving fungi, spores are generally used. Spore inoculum allows a greater flexibility in coordination of inoculum preparation with the cultivation process. The spores are more viable than fungal mycelia and are less susceptible to any variation that may occur between harvesting and inoculation. The positive activity of fractions was determined by using gel diffusion method, (Fig.-12) and further characterisation was done by determining their molecular weight. Zymogram of partially purified exo, endo-1,4- β -D-Glucanase was obtained by SDS- PAGE. Comparing with the molecular mass of protein marker (Fermentas) bands by staining with Coomassie brilliant blue R-250 (Sigma) the molecular weight of lignocellulase bands were compared and determined⁷. As per the zymogram the molecular weight of endo, exo -1,4- β -D-Glucanase is of 54 kDa, 48kDa respectively showing the similarity with endoglucanase I, Cellobiohydrolase I of Genencor (Palo Alto, CA) commercial Cellulase a purified from *Trichoderma reesei* (Fig.-13).

Statistical analysis:-

Each experiment was performed thrice, in each triplicate the mean value was taken into consideration as experimental result; bar diagrams were constructed using Origin 7.0.

Figures:-

Fig.-1 Positive strain of *Hypocrea nigricans* showing cellulose clearance in the Mandel's medium.



Fig.-2 Positive strain of *Hypocrea nigricans* showing clearing zone in CMC agar.



Fig.-3 Phylogenetic analysis by Bootstrapped Neighbor joining tree with Genbank ACC No. *KJ914660*.

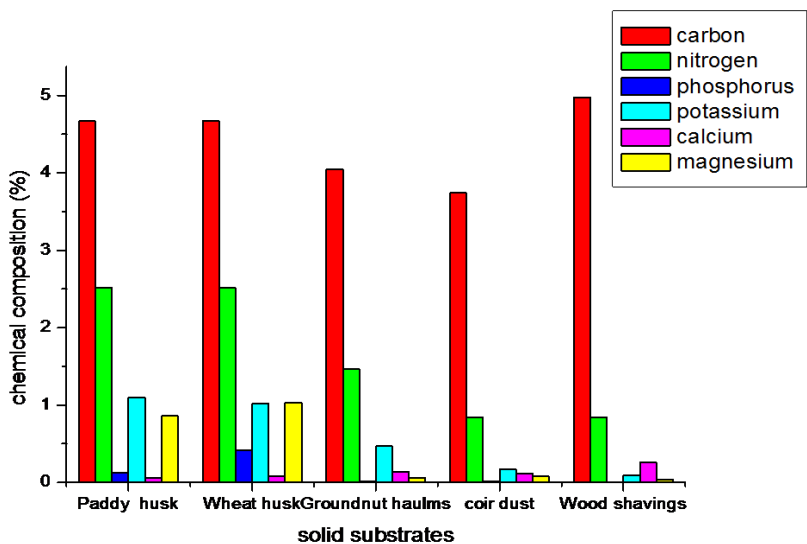


Fig.-4 Chemical compositions of solid substrates

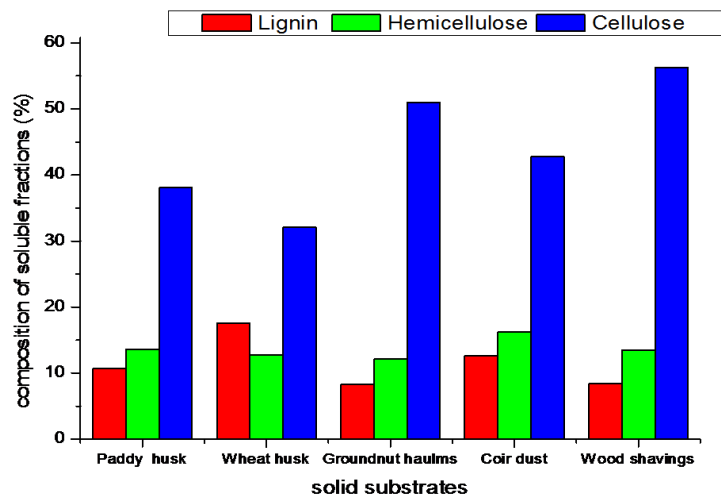


Fig.-5 Chemical components such as soluble fractions, lignin, hemicellulose and cellulose present in native solid substrates.

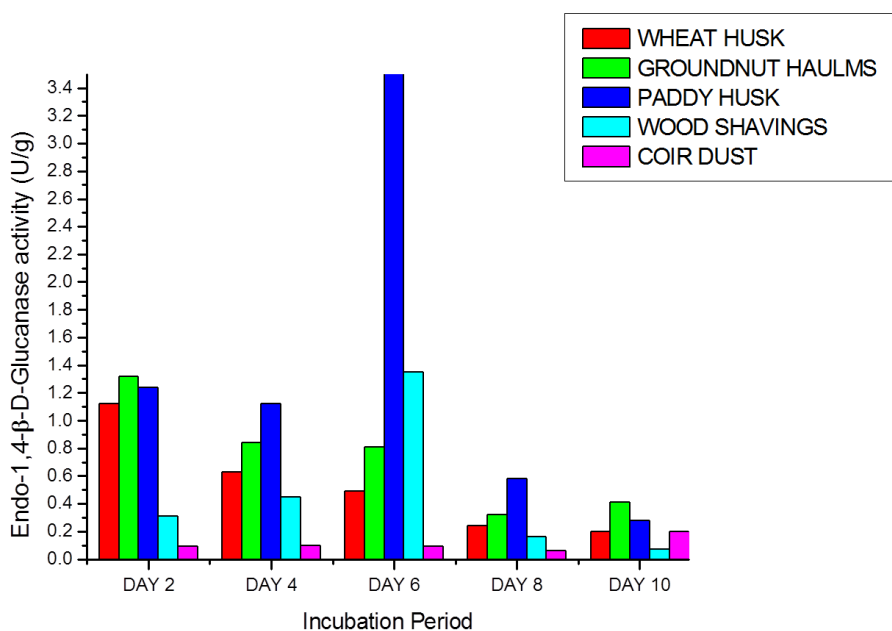


Fig.-6 Production of Endo -1,4-β-D-Glucanase on SSF by *Hypocrea nigricans*

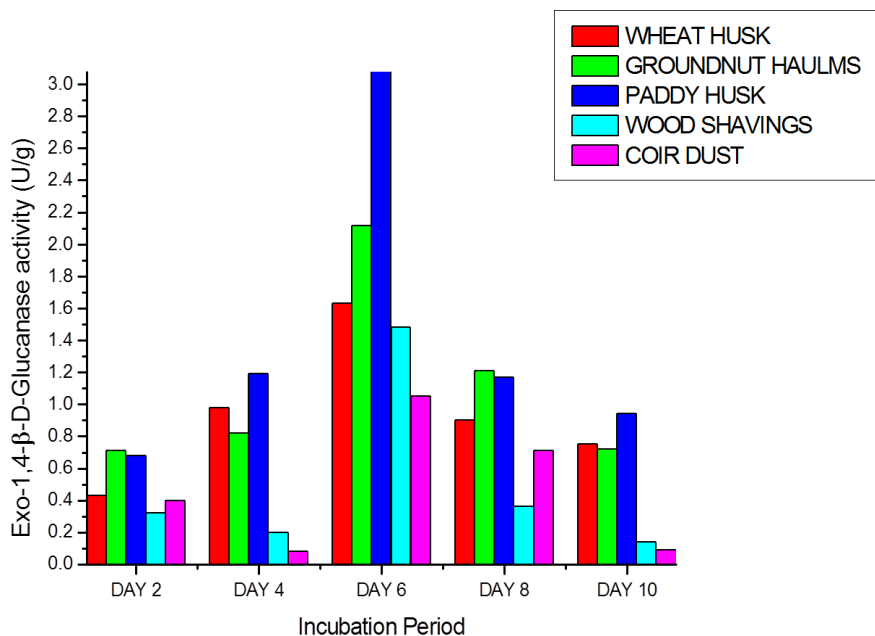


Fig.- 7 Production of Exo -1,4-β-D-Glucanase on SSF by *Hypocrea nigricans*

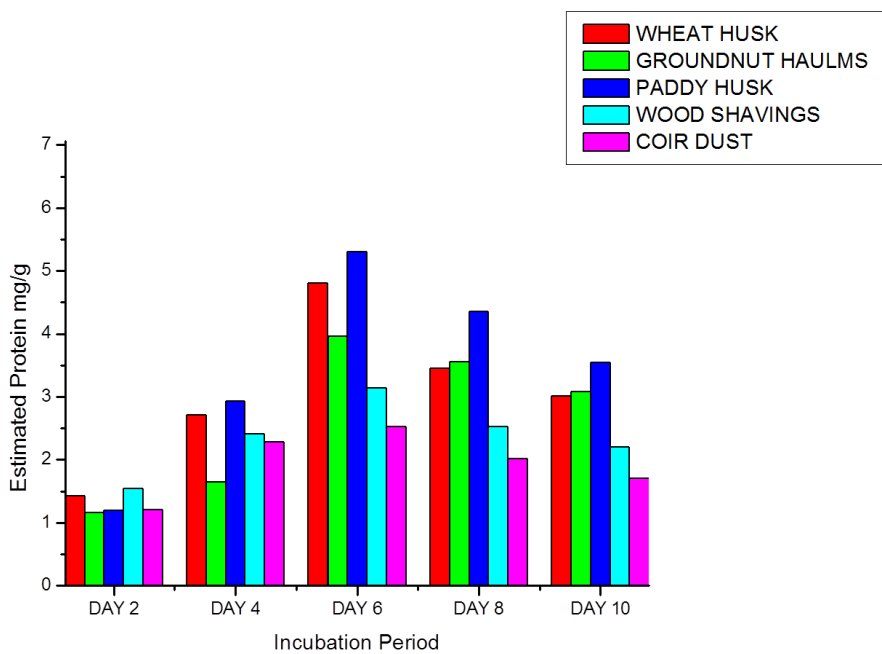


Fig.-8 Estimation of Secretion of protein by by *Hypocrea nigricans* on SSF

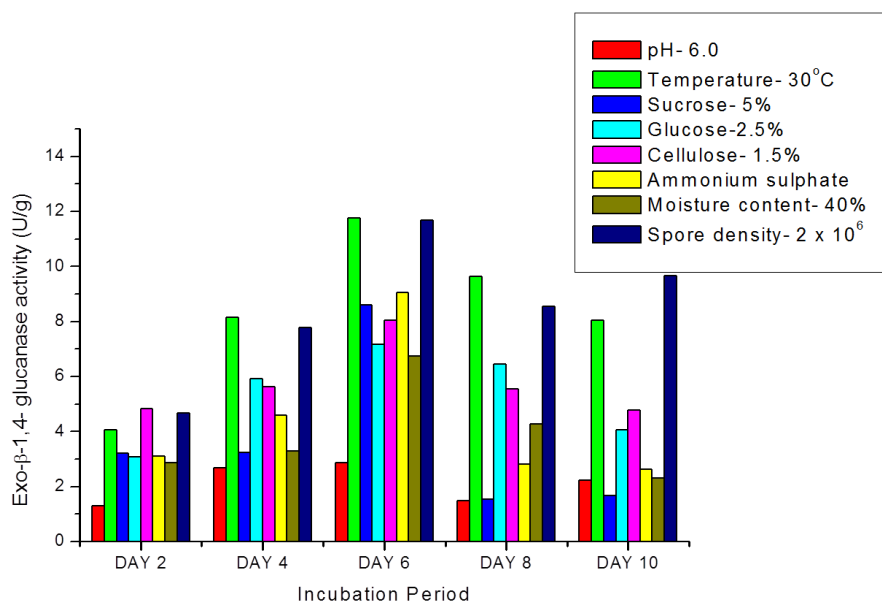


Fig.-9 Optimized Endo -1,4-β-D-Glucanase(U/g) activity with Paddy Husk on SSF by *Hypocrea nigricans*

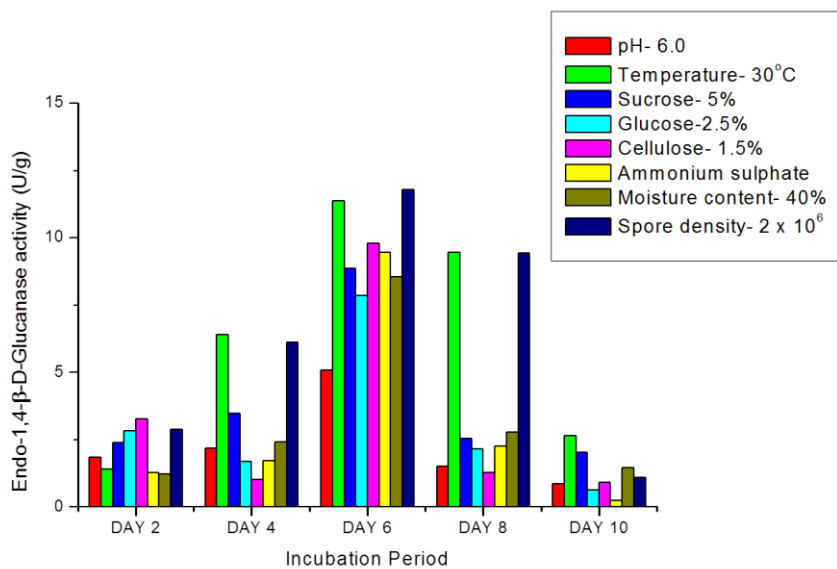


Fig.-10 Optimized Exo -1,4-β-D-Glucanase(U/g) activity with Paddy Husk on SSF by *Hypocrea nigricans*

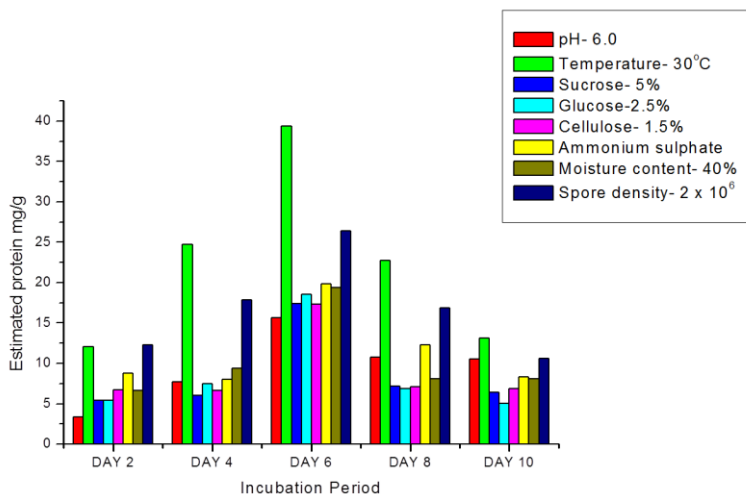


Fig.-11 Secretion of protein (mg/g) with optimized conditions on SSF by *Hypocrea nigricans*



Fig.-12 Detection of enzyme activity of column fractions by diffusion method

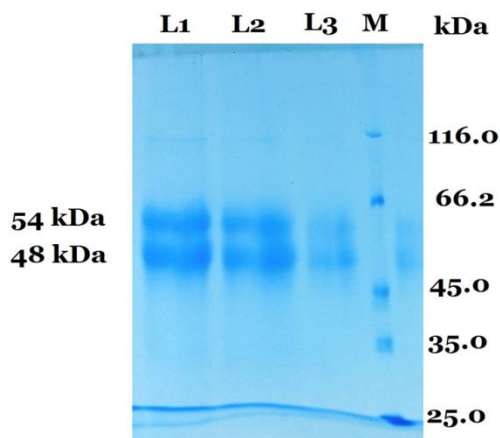


Fig.-13 SDS- PAGE of partially purified exo, endo-1,4-β-D-Glucanase by SDS-PAGE.

M- Protein marker –unstained- middle range- life technologies.

Lane 1,2- purified exo, endo-1,4-β-D-Glucanase with molecular weight of 54 kDa, 48 kDa respectively (produced under Optimised conditions)

Lane 3- purified enzymes under non optimized conditions.

Tables:-**Table. 1: Optimisation of cultural conditions of, endo-1,4-β-D-Glucanase by *Hypocrea nigricans*.**

Factor	Trail Range	Optimized Cultural Conditions
Lignocellulosic substrates	Paddy husk, Wheat husk, Groundnut haulms, Coir dust and Wood shavings	Paddy husk
Incubation period	2 days, 4 days, 6 days, 8 days, 10 days,	6 days
pH	3.0, 4.0, 5.0, 6.0, 7.0.	6.0
Incubation temperature	25°C, 30°C, 35°C, 40°C.	30°C
Spore density	2X10 ⁴ /gram of substrate 2X10 ⁵ /gram of substrate 2X10 ⁶ /gram of substrate 2X10 ⁷ /gram of substrate	2X10 ⁶ /gram of substrate
Moisture content	20%, 40%, 60%, 80%	40%
Carbon sources	Sucrose, Lactose, Glucose	Glucose (2.5%)
Nitrogen sources	Urea, Peptone, Ammonium Sulfate, Yeast extract	Ammonium sulfate

Conclusion:-

The main objective of this paper was to identify fungal strain and their production of cellulolytic enzymes that will work under industrial conditions particularly with inexpensive, rapid and sensitive testing methods. In the present study, a fungal isolate of litter, *Hypocrea nigricans* was screened for their efficiency in cellulose degrading enzymes particularly endo and exo- β-1, 4-D-glucanases on different lignocellulosic solid substrates. Among the screened lignocellulosic substrates, paddy husk has been identified as the best source for maximum enzyme production and optimal cultural conditions were recorded as pH 6.0, incubation temperature 30°C, moisture 40%, spore density 2 X 10⁶ spores/g. The enzyme production was increased from 2.98 U/g to 11.89 U/g for exo-1, 4-β-D-Glucanase, 3.9 U/g to 11.89 U/g for endo -1,4-β-D-Glucanase respectively. The two enzymes, endo and exo- β-1, 4-D-glucanases produced by *Hypocrea nigricans* were established after estimating their molecular weights as 54 and 48kDa respectively through SDS-PAGE.

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