Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

SUPPLEMENTATION OF NITROGEN SOURCE IN WHEAT STRAW FOR IMPROVING CELLULOLYTIC POTENTIAL OF G*ANODERMA LUCIDUM.*

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ABSTRACT

Ganoderma lucidum is recognized as a powerful medicinal fungus because of its pharmacological effects and it is cultivated worldwide, using hardwood sawdust as a substrate in synthetic formulation. However, relative abundance of cereal straws such as wheat straw in an agricultural country like India could be exploited for commercial production of this mushroom. Therefore, solid state fermentation of *G. lucidum* was carried out to study the effect of supplementation of wheat straw as carbon source with different nitrogen sources, viz, Peptone, Ammonium sulphate, Wheat bran and Rice bran on mycelial growth and cellulolytic potential. The comparison of cellulase and hemicellulase activities revealed that supplementation of nitrogen resulted in increased mycelial growth and cellulolytic potential with a highest ß-glucosidase activity of 1200µg/min/ml, Xylanase activity of 600 µg/min/ml and Endo-1,4-ß-D-glucanase activity of 213 µg/min/ml at 20% rice bran concentration. So, it can be inferred that Wheat straw can be optimized to be a good substrate for cellulase production and its utilization by *Ganoderma lucidum* can be improved considerably with the supplementation of rice bran.

KEYWORDS: ***Ganoderm lucidum*, ß-glucosidase, Xylanase, Endo-1,4-ß-D-glucanase, SSF**

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INTRODUCTION

Ganoderma lucidum is a basidiomycota white rot macrofungus known by its many names like "Reishi," "Ling Zhi," and "Mannentake," and is recognized as a powerful medicinal fungus because it has properties often associated with health, healing, long life, knowledge and happiness. The basidiocarp, mycelia and spores of *Ganoderma lucidum* contain approximately 400 different bioactive compounds, which mainly include triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins/peptides and trace elements having a number of pharmacological effects.[1-3] This macrofungus is very rare in nature therefore cultivation either on solid substrates or by submerged cultivation has become essential to meet the increasing demands in the international market.[4-6] Cultivation of this mushroom uses a vast amount of cellulosic substrates. Worldwide, the most popular basal ingredient used in synthetic formulation of substrate for the commercial production of *G. lucidum* is hardwood sawdust which is not a substrate of choice in regions where sawdust is not easily available,and therefore many alternate substrates have been investigated by by various workers for the cultivation of this muchroom.[7-9] The relative abundance of cereal straws like wheat straw could be exploited for commercial production of this mushroom especially in India where wheat is one of the major agricultural crops. The growth on lignocellulosic substrate is dependent upon the ability to synthesize lignolytic, three extracellular cellulolytic enzymes: Endo-1,4-ß-D-glucanase (EC 3.2.1.4), ß-glucosidase (EC 3.2.1.21), Exocellobiohydrolase (EC 3.2.1.91) and hemicellulolytic enzyme : Xylanase (EC 3.2.1.8).[10] Moreover, its lignocellulolytic potential has been reported to be directly correlated with its efficient substrate utilization and biological efficiency. So improving enzyme activity on a particular substrate can be considered as a suitable method for increasing its production on a variety of substrates to replace the use of sawdust. Physiological demands for cellulolytic and hemicellulolytic enzymes production varies among white-rot species, and even among strains of a species. The factors that can affect enzymes activity and hence can affect the ability of this mushroom to grow on a particular substrate are: cultivation type (submerged or solid-state), carbon and nitrogen sources and their concentrations, presence or absence of different inducers, medium pH, temperature, agitation, cultivation period etc.[11-12] Results of numerous studies have demonstrated that various agricultural and industrial residues can be better utilized in the presence of suitable type and concentration of nitrogen source .[13-16] Keeping this in mind the present study was undertaken to improve the ability of *G. lucidum* to degrade wheat straw by supplementing with organic and inorganic nitrogen sources and evaluating their impact on cellulolytic and hemicellulolytic enzymes.

MATERIALS AND METHODS

Ganoderma lucidum strain used in this study was purchased from "National Research Centre for Mushroom", Solan. All the constituents used for preparation of media were procured from Hi Media Pvt. Limited (India). The composition per litre of the growth media used is given in Table 1. The media was sterilised by autoclaving at 121^0C (15psi) for 15 minutes.

Table 1
Composition of Growth Media

Media	Components	Amount(g/l)
Potato Dextrose Agar	Potato	200
	Dextrose	20
	Agar	20
Synthetic Media	Glucose	10
	NH_4NO_3	2
	K_2HPO_4	1
	$NaH_2PO_4.H_2O$	1
	$Na_2HPO_4.H_2O$	0.4
	$MgSO_4.7H_2O$	0.5
	Yeast Extract	2
Modified Synthetic Media	K_2HPO_4	1
	$NaH_2PO_4.H_2O$	1
	$Na_2HPO_4.H_2O$	0.4
	$MgSO_4.7H_2O$	0.5
	Yeast Extract	2
Basal Media	Yeast Extract	5
	K_2HPO_4	1
	Magnesium sulphate	0.2
	Agar	20
Media	**Components**	**Amount(g/l)**
Potato Dextrose Agar	Potato	200
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Synthetic Media	Glucose	10
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	$Na_2HPO_4.H_2O$	0.4
	$MgSO_4.7H_2O$	0.5
	Yeast Extract	2
Basal Media	Yeast Extract	5
	K_2HPO_4	1
	Magnesium sulphate	0.2
	Agar	20

(i) Enzyme Screening

The fungus was screened for the production of cellulolytic and hemicellulolytic enzymes on basal media as suggested by Jo et al.[10] The inoculum for the solid state fermentation was prepared by inoculating 50 ml of synthetic medium (Table 1) with 0.5 cm mycelial discs taken from a 7 day-old *G. lucidum* culture grown on Potato Dextrose Agar. Each flask was inoculated with 15 mycelial discs and incubated at 28ºC on shaking incubator (160 rpm), for 7 days.

(ii) Solid State Fermentation

Wheat straw was used as carbon source for the solid state fermentation. Wheat straw procured from the market was dried in oven at 60^0C for two hours prior to use.

Four different inorganic and organic nitrogen source viz, Ammonium sulphate (1-5Mm), Peptone (0.25-4.0%), Rice bran (0.25-20.0%) and Wheat bran (0.25-20.0%) were used as nitrogen supplements. Solid-state fermentation was carried out at 28ºC in 100 ml flasks containing 2 g of wheat straw as the carbon source, and 10 ml of the modified synthetic medium (without glucose and supplemented with one of the selected nitrogen source at different concentration. The medium composed of wheat straw and modified synthetic media (without any nitrogen source supplementation) was used as the control. Each flask was inoculated with 3 ml of the prepared inoculum under sterile conditions and incubated at 28^0C under static conditions. Four sets were prepared for each nitrogen source and they were subjected to an incubation of 4, 8, 12 and 16 days respectively.

Samples from flasks were harvested after the required days of cultivation, and the

extracellular enzymes were extracted by stirring of samples with 25 ml sterile distilled water for 30 min at 28ºC in a orbital incubator. The obtained extracts were separated by centrifugation (4ºC, 5000 rpm, 15 min), and supernatants were used for measurements of activities of cellulolytic enzymes.

(iii) Enzyme Activity Assays

Endoglucanase (CMCase) activity*:* was measured by estimation of the reducing sugars released during incubation of carboxymethyl cellulose with enzyme extract according to the method of Mandels et al.[17]

ß-glucosidase (CBase) activity: was measured according to the method described by Toyama and Ogawa.[18]

Exocellobiohydrolase (FPase) activity*:* was measured using Whatman filter paper as substrate. Test tubes containing a mixture of 0.25ml acetate buffer, 0.25 ml of the enzyme extract and a filter paper strip (Whatman No. 1, 1x6 cm) were incubated at 50^0C for 1 hour in water bath.[19] The released glucose concentration was measured at 575 nm.[20]

Xylanase activity*:* was determined using xylan as substrate by the procedure described by Bucht and Eriksson. [21] The released reducing sugars were measured as xylose equivalents at 575 nm. Enzyme activity was expressed as µg/ml/min.

(iv) Electrophoresis

The SDS-PAGE was performed by using 5% stacking and 10% separating gel according to the method of Laemmli. [22] The protein samples were prepared by heating at 100°C for 5 min in SDS-protein loading dye to denature the proteins. The gel slab was inserted in the electrophoresis apparatus and sample was then loaded into the respective wells. The gel was run on 50–100 volts electric supply.

(v) Partial Purification by Ammonium sulphate precipitation

Extracellular enzyme extract obtained was subjected to protein fractionation by ammonium sulphate precipitation. Fractionation of protein was made by addition, at 2-5°C with stirring, of increments of solid ammonium sulphate. The amount of ammonium sulphate to be added was calculated from percent saturation of ammonium sulphate from the table given in the protocols of the protein purification by Deutscher .[23] Each fraction was checked for the enzyme activity on Native PAGE containing enzyme substrate.

(vi) Zymogram

The modified method of Teunissen et al [24] was followed. Purified enzyme fraction produced in rice bran was resolved in 10% polyacrylamide native gels containing 1% cellobiose. The characteristic CBase profile for the samples was recorded.

RESULTS AND DISCUSSION

1. Primary Screening

The *G. lucidum* strain was subjected to screening for the production of CMCase, CBase and Xylanase by the method of Jo et al 2011. The diameters of the zone of clearance formed are given in Table 1. Maximum zone of clearance was observed for CBase followed by xylanase. Comparatively a smaller zone was formed for CMCase.

Table 2
Primary screening for enzymes

Enzyme	Zone of Clearance (diameter in mm)
CMCase	28
CBase	32
Xylanase	30

2. Solid State Fermentation

Solid state fermentation was carried out at 28^0C in 100 ml flasks containing 2g wheat straw as carbon source and 10 ml of modified synthetic medium. Four different nitrogen sources, viz, Peptone, Ammonium sulphate, Rice bran and Wheat bran were supplemented for the growth of the fungus. Out of the four

sources, maximum growth was observed in flasks supplemented with higher concentrations of rice bran followed by wheat bran supplementation while comparatively lesser growth was observed in flasks supplemented with ammonium sulphate. However compared to control, mycelial growth increased on supplementation with nitrogen except in case of ammonium sulphate supplementation. A comparison of mycelial impregnation in wheat straw revealed maximum mycelial growth with rice bran (20%), wheat bran (2%), followed by peptone (0.5%) and ammonium sulphate (2mM). A comparison of cellulolytic and hemicellulolytic enzymatic activities at different concentrations of four nitrogen sources is mentioned in Table 3. Among the four nitrogen sources supplemented to wheat straw, maximum activities of CMCase, CBase and xylanase were observed on supplementation of Rice bran at a concentration of 20%. In case of CMCase, a maximum activity of 213µg/min/ml was observed in rice bran which was 2, 5.7 and 10 folds more than the activities observed with Peptone, Wheat Bran and Ammonium sulphate supplementation, respectively. CBase exhibited a maximum activity of 1200 µg/min/ml on 20% rice bran showing 3 fold increase as compared to the activity observed on wheat bran. On 20% rice bran supplementation, a maximum xylanase activity of 600µg/min/ml followed by 520 µg/min/ml (10% rice bran) was observed. Majid et al have reported maximum yield of *G. lucidum* in a combination of poplar sawdust supplemented with 5% malt extract and 10% wheat bran.[25] Han et al [26] also reported an improved α-amylase activity of *G. lucidum* on supplementation with yeast extract and peptone while ammonium sulphate and ammonium nitrate was not that effective. Malarvizhi et al also tested different agrowastes for xylanase production by *G. lucidum* and reported wheat bran to be a better substrate than sugarcane baggase and rice bran in solid state fermentation. [27] Irrespective of the nitrogen source, a very little or no FPase activity was observed. A comparative analysis of enzyme activities at optimum concentration of different nitrogen sources w.r.t control has been shown in Figure 1. All the enzymes exhibited a similar activity profile (data not shown) i.e. maximum enzyme activities were observed on the 8th day of incubation, followed by a decrease on the 12th day and very sharp fall was observed in the activity on 16th day. The enzyme activity corresponded well with the growth pattern of the mycelium. On the fourth day of the incubation low growth as well as low enzyme activity was observed. Maximum growth as well as enzyme activity was observed on the 8th day.

Table 3
Comparative enzyme activities on different concentration of inorganic and organic nitrogen sources

Rice bran (%)	Enzyme activity (µg/min/ml)				Wheat bran (%)	Enzyme activity (µg/min/ml)				Peptone (%)	Enzyme activity (µg/min/ml)				Amm. Sulphate (Mm)	Enzyme activity (µg/min/ml)			
	CBase	Xnase	CMCase	FPase		CBase	Xnase	CMCase	FPase		CBase	Xnase	CMCase	FPase		CBase	Xnase	CMCase	FPase
0 (C)	496	300	48	1.33	0 (C)	496	300	48	1.33	0(C)	496	300	48	1.33	0(C)	496	300	48	1.33
1.25	600	388	51	1.33	1.25	501	300	37	1.33	0.25	712	388	24	1.33	1	624	328	27	1.33
2.5	608	396	56	2.66	2.5	520	310	37	2.66	0.5	880	520	48	1.33	2	752	364	37	6.65
5	648	440	101	3.9	5	530	370	50	2.66	1	760	368	98	5.32	3	688	332	27	5.32
10	776	520	112	4.98	10	580	420	61	3.99	2	728	320	123	5.3	4	672	332	32	3.99
20	1200	600	213	5.4	20	440	280	37	1.33	4	688	292	106	2.6	5	592	312	21	1.33

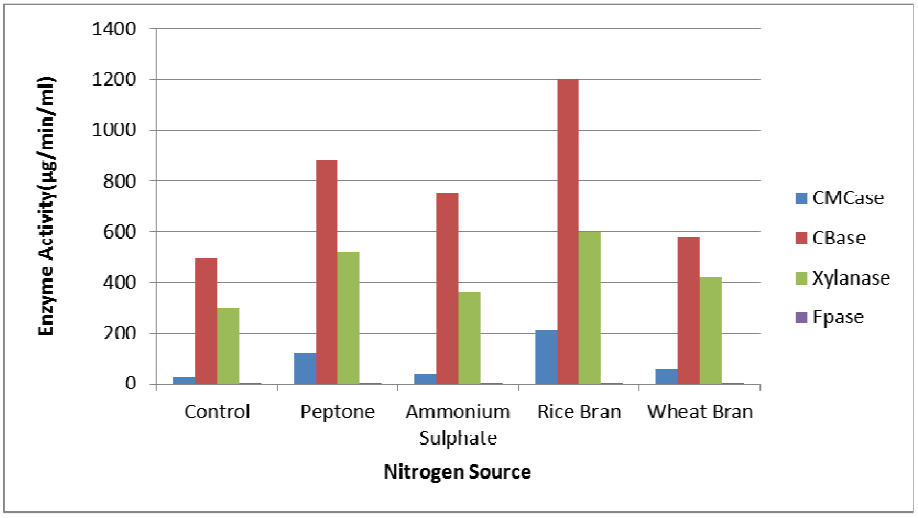

Figure 1
Comparative analysis of effect of selected nitrogen sources on enzyme activities

3. Protein Profile of the Crude Enzyme Extract

The protein profile of the extracellular enzyme extracts obtained from mycelial supplemented with optimum nitrogen sources was done by the means of SDS-PAGE. 62µl of sample was loaded into each well. The comparison of protein profile of enzyme extract obtained from different nitrogen sources as shown in Figure 2 revealed a common banding pattern with exception of a single dark band obtained in case of rice bran as nitrogen source. This band may be co-related with high cellulolytic potential of the crude extract obtained by using rice bran and may give an indication of elevated expression of a particular enzyme sub-unit.

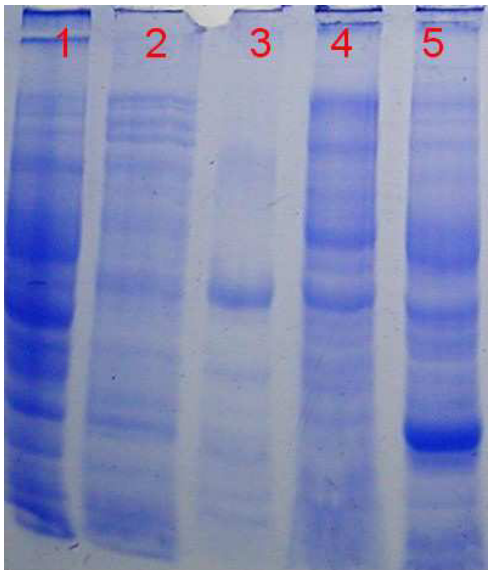

Figure 2
20 % SDS PAGE showing the Protein Profile of Crude Enzyme Extracts. Lane 1: Control Lanes 2: Ammonium sulphate (2mM) Lane 3: Wheat Bran (2%) Lane 4: Peptone (0.5%) and Lane 5: Rice Bran(20%).

4. *Ammonium Sulphate Precipitation and Zymogram analysis of partially purified enzyme*

The Partial purification of enzyme was carried by ammonium sulphate precipitation. 25ml of crude enzyme solution was brought to 40-80% saturation with solid ammonium sulphate with stirring at 4°C. The modified method of Teunissen et al [24] was followed. Partially purified enzyme was resolved in 10% polyacrylamide Native gels containing 1% cellobiose (as CBase activity was maximum in crude extract) and the characteristic CBase profile for the samples is shown in Figure 3. As evident from the zone of clearance formed by 40% fraction and 80% fraction that the activity of CBase increased after partial purification. Comparison with control revealed that addition of 20% rice bran lead to a 2.5 folds increase in the CBase activity, 2 folds increase in xylanse activity and a 4.5 folds increase in CMCase activity. Also the enzyme profile showed that maximum enzyme activity was achieved on the 8th day of incubation. This result indicates that the type and concentration of nitrogen source is directly related to the mycelial colonization efficiency and cellulolytic potential. Therefore, it can be inferred from this study that a combination of wheat straw and rice bran is a good substitute to the conventionally used saw dust and wheat bran combination for cultivation of *Ganoderma lucidum* in areas with huge availability of wheat straw.

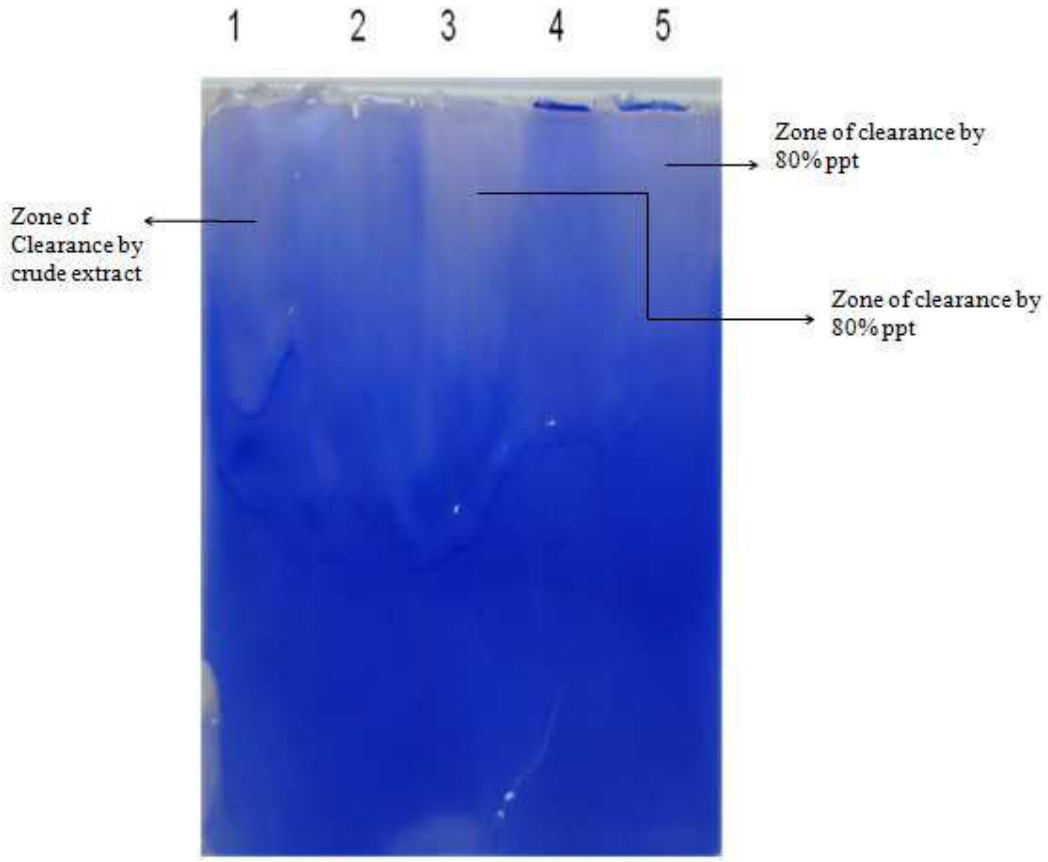

Figure 3

10 % PAGE containing 1% Cellobiose showing characteristic CBase (Ricebran 20%) profile. Lane 1: Crude Extract, Lane: 2 Empty, Lane 3: 80% ppt fraction, Lane 4: empty and Lane 5: 40 % ppt fraction

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