Submerged cultivation of medicinal mushroom in hydrolysate of ligno-cellulosic material

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Abstract: *Ganoderma lucidum* belongs to the family Ganodermataceae and found in Japan, China and some other parts of Asia. Traditionally it is used in herbal medicine as anti-diabetic, cancer prevention agent, antitumor, an immunomodulatory, antimicrobial and antiviral agent. Due to difficulty in field cultivation, submerged fermentation was employed as a promising method for efficient and large-scale production of mycelia biomass and bioactive metabolites. Cellulose was used in the form of a lignocellulosic substrate. The *Ganoderma lucidum* which is medicinal and edible mushrooms were successfully grown in the form of mycelial biomass in static submerged culture in Petri plates and flasks. The present study is based on the utilization of hydrolyzates of lignocellulosic materials such as Peanut cort, Sugarcane bagasse, and Wheat Straw was used after hydrolysis. A Static Fermentation Technique was employed to investigate the mycelial growth, instead of Fruiting Body. *Ganoderma lucidum* was kept up on PDA (potato dextrose agar) medium in Petri dishes at 4^{o}C and brooded at 25 ◦C for 5 days for the development of *G. lucidum* and generation of Ganoderic Acid. Morphology of *G. lucidum* on various Hydrolysates was white and delicate like cotton unpredictable shape, Cloud-like appearance spread in general plate and multiple little sporadic white cotton-like shape with string-like projections. We got a Ganoderic Acid from the Hydrolysates of Peanut cort concentrate, Sugarcane bagasse concentrate and Wheat straw concentrate at a concentration of 0.006g/L, 0.011g/L and 0.017g/L respectively.

Keywords: *Ganoderma lucidum ,* hydrolysate of ligno-cellulosic material, static fermentation technique, mycelia growth

INTRODUCTION

Ganoderma lucidum is an organism, which is being used for advance wellbeing and life span for many years in the world especially in China and Japan (Wasser 2017). Apparently, it is an extensive, cloudy mushroom with a polished outside and a woody surface. *Ganoderma lucidum* is derived forma Latin word *Lucidus* which literal meaning is, "sparkly" or "astonishing". *G. lucidum* is known as lingzhi in China, whereas in Japan the term used for Ganodermataceae family is Reishi or Mannentake (Gill, Mehra *et al*. 2018). The name lingzhi shows a blend of worldly intensity and pith of eternality and is viewed as the "herb of profound strength "symbolizing achievement, prosperity, godlike influence, and life span in China. *G. lucidum* is novel due to its pharmaceutical usage among other developed mushrooms. An assortment of business *G. lucidum* items are accessible in different structures, for example; precipitates, nutritional complements, and tea, which are acquired from different fragments of mushroom, like mycelia, spores, and common item body. Credited therapeutic activities and exact applications of Lingzi fuse regulation of blood glucose levels, balance of invulnerable framework, hepatoprotective and bacteriostasis. Different convictions with respect to the medical advantages of *G. lucidum* are constructed a great extent in the light of recounted confirm, conventional utilization. Most probably, late reports give logical help to a portion of the old cases of the medical advantages of lingzhi. Lingzhi has perceived as therapeutic mushroom from round about 2000 years, its intense impacts have been archived in old contents (Wasser 2005). The increased pictures of G. lucidum in craftsmanship are started in 1400 Advertisement and they are connected with Taoism (McMEEKIN 2004).

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MATERIALS AND METHODS

Micro-organism and maintenance

The fruiting body of *Ganoderma lucidum* was provided by Dr. Memoona Ghafur Shahid, Assistant Professor, Department of Botany, GC University, Lahore and was maintained on PDA (potato dextrose agar) medium, in petri dishes, according to the methods described by (Zhang, Zhong *et al*. 2014) at 4^{o}C .

Inoculum preparation

A medium for mycelium and submerged cultivation of *Ganoderma lucidum* constituting (gl-1) 5.0(NH4)2 SO4; 0.2 MgSO4.7H2O 1.0, K2PHO4; 2.0, yeast extract; 1.0 peptone using 40.0 glucose was used in petri plates. The pH culture media was initially adjusted at 4.0 for the cultivation of *G. lucidum* to obtain mycelial pellets.

Inoculum development

A viable strain of *Ganoderma lucidum* was isolated from a small piece of tissue collected from the fruiting body and placed on the sterilized Potato Dextrose Agar (PDA) medium on petri plates under strict sterilized conditions using Robus Technologies Laminar Flow Cabinet (Model: RTVL-1312). The inoculated medium was incubated at 25-30^{o}C for 7 days in an incubator (Model: DSI-800D) to obtain sufficient mycelium growth which was further morphologically recognized on the basis of basidiocarp and basidiospore by microscopic examination. The mycelium was further inoculated onto PDA slants for subsequent use.

Submerged-static cultivation technique

Submerged-Static Cultivation Technique was conducted in a 9cm Petri plates containing 20ml homogenized fermentation media. A cube of 1x1cm mycelium of *G. lucidum* grown on PDA was used as inoculum, transferred aseptically to Petri Plates containing liquid fermentation medium and incubated at 25◦C for 5 days for the growth of *G. lucidum* and production of Ganoderic acid. The mycelial biomass from liquid culture was harvested by using filtration technique and suspended in 0.9% saline solution and stored in Pre-sterilized cotton wool plugged culture tubes prior to use for the determination of dry weight.

Ligno-cellulosic material

Ligno-Cellulosic material such as Peanut cort, sugarcane bagasse and Wheat Straw was chopped to .5mm size prior to proceeding for pre-treatment process then ground the cellulosic material to 2mm size and soaked the material over nightly in 0.5% H_2SO_4 and further this material is autoclaved at 20psi for 1 hour and cooled the material at ambient temperature and filtered through cheesecloth and then filtered again through glass filter paper to remove further impurities, then measured PH of filtrate by using PH meter. Initially, the pH of solution was 1.5, (Acidic) then maintained the PH by adding base, (NaOH) drop by drop until PH Of filtrate reached up to 5-6. Then again autoclaved it at 120^{o}C for 1 hour. Then cooled and hence this formulation was used as a media for the inoculation of viable spores of *Ganoderma lucidum* (fig. 2 and 3).

Analytical method

Separation of biomass

G. lucidum mycelium was recovered by centrifugation of fermented broth at 12,000 rpm for 4 min. Cells were then collected and washed twice by utilizing sterile distilled water, trailed by drying in the oven at 105^{o}C until the constant weight. The dry weight was measured gravimetrically as described by (Tang and Zhong 2003).

Extraction of ganoderic acid from solution

The extracellular GA was extracted from fermentation broth filtrate was added with 4 volumes of ethanol (95%) and left overnight at 4^{o}C for precipitation of crude GA. The precipitated GA was collected by centrifuging at 10,000 rpm for 10 min, dried at 60^{o}C to remove the residual ethanol according to the methodology described by (Baskar, Sathya *et al*. 2011).

STATISTICAL ANALYSIS

Data were subjected to ANOVA according to the method of Snedecor and Cochran, 1980.

RESULTS

Table 1 shows different morphology of the Strains. It shows that the GL1 has filaments twisted, white cotton-like appearance with fine thread-like appendages on the surface as shown in fig. 2. The GL2 has filaments twisted, white cotton or clouds like appearance with fine spikes like projection on the surface as shown in fig. 3. The GL3 has filaments twisted, white cotton-like appearance with fine thread-like projections on the surface as shown in fig. 4.The GL4 has filaments twisted, white cotton-like appearance with covered by thread-like projection on the surface as shown in fig. 5.

Table 1: Strains isolated and their morphology

Isolated Strains	Morphology
GL1	Filaments twisted white cotton-like appearance with fine thread-like appendages on the surface
GL2	Filaments twisted, white cotton or clouds like appearance with fine spikes like projection on the surface
GL3	Filaments twisted white cotton-like appearance with fine thread-like projections on the surface
GL4	Filaments twisted, white cotton-like appearance with covered by thread-like projection on the surface

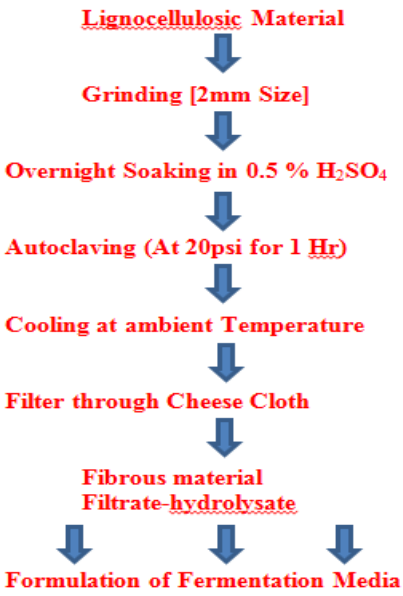

Fig. 1: Schematic representation of pre-treatment lignocellulosic materials

Fig. 2: Isolated strains of GL1

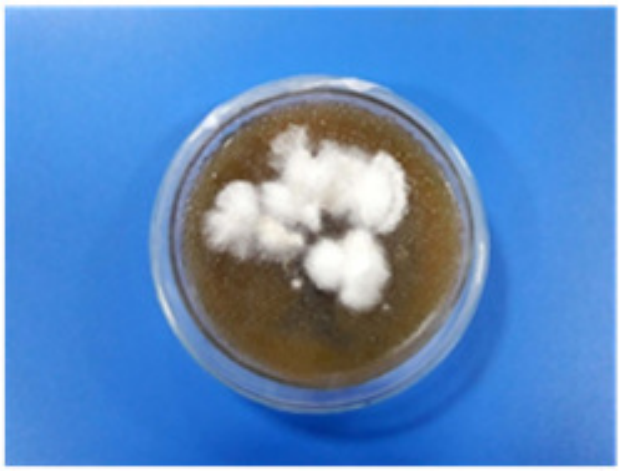

Fig. 3: Isolated strain of GL2

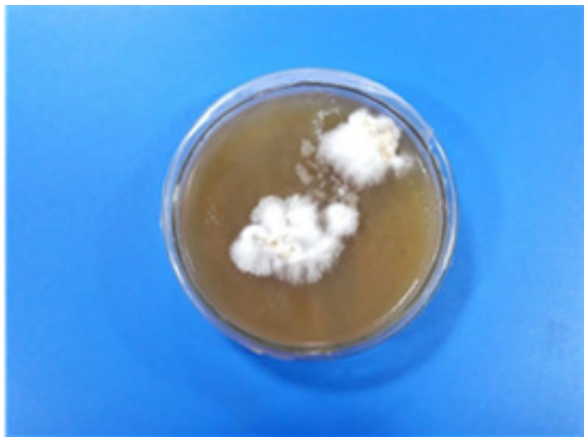

Fig. 4: Isolated strains of GL3

Fig. 5: Isolated strains of GL4

Table 2 shows the effect of temperature on different strains of *G. lucidum* after 7 days inoculation. The mean diameter of the strain GL1 at 10ºC was 12.6mm, at 15ºC it was23.3mm, at 20ºC it was 40.3mm, at 25º it was 70.3mm, at 30ºC it was 79.3 and at 35ºC the mean diameter was 45.2mm. The overall growth at 35ºC was slow, filaments twisted and white in appearance.

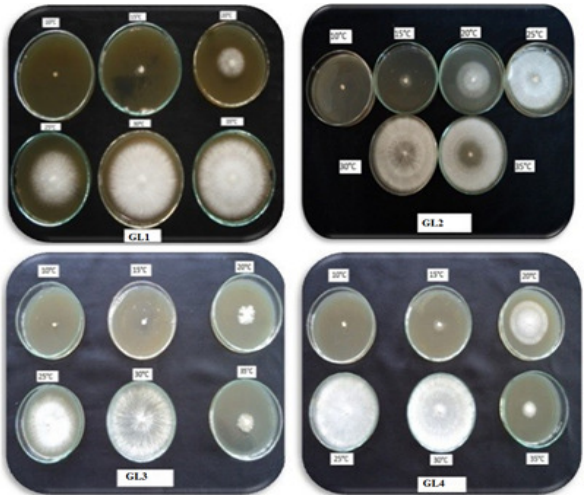

Fig. 6: Effects of Temperature on different strains of GL

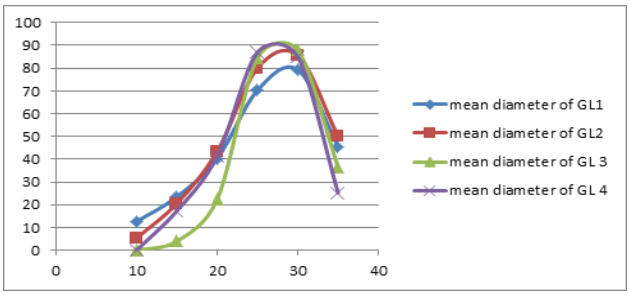

Fig. 7: Graphical representation of the effects of Temperature on different strains of GL

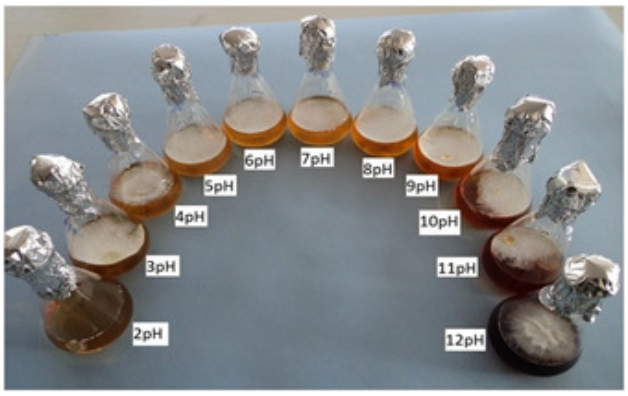

Fig. 8: Effects of pH on mycelial growth of strain GL3

The mean diameter of the strain GL2 at 10ºC was 5.3mm, at 15ºC it was 20.6, at 20ºC it was 43.5mm, at 25ºC it was 80.0mm, at 30ºC it was 85.6mm and the mean diameter of GL2 at 35º was 50.0mm while at 30ºC the growth rate was fast, filaments twisted and white in appearance.

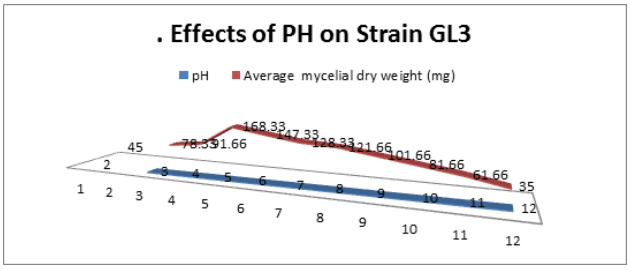

Fig. 9: Graphical representation of the effect of pH on Strain GL3

Fig. 10: White and soft cotton-like irregular shape

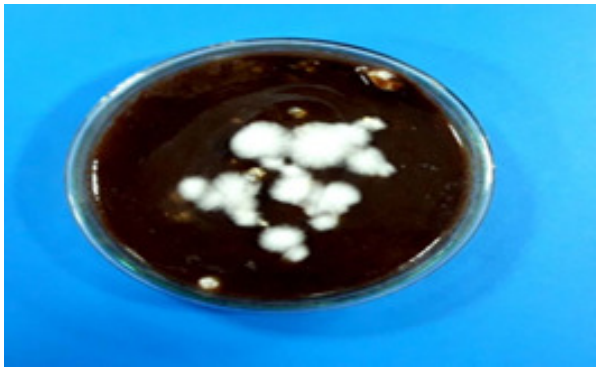

Fig. 11: Multiple small irregular white cotton-like shapes with thread-like projection

There was no growth observed of Strain GL3 at 10ºC while at 15ºC a little growth of 4.0mm mean diameter was noted, at 20ºC the mean diameter was 22.3mm, at 25ºC it was 84.6mm, at 30ºC it was 88.0 while at 35ºC the mean diameter was 36.6ºC and the overall growth rate at 30ºC was noted fast, filaments twisted and white in appearance.

At 10ºC there was no growth observe of the strain GL4 while at 15ºC the mean diameter of GL4 was 17.3mm, at 20ºC it was 41.3, at 25ºC it was 87.0, at 30ºC it was 85.0 while at 35º the mean diameter was noted 25.3mm. The overall growth rate was observed fast, filaments twisted and white in appearance.

Table 2: Effects of Temperature on different strains of GL

S. No.	Isolates/ Strains	Mean Diameter in (mm)* Growth Type at						Growth Type at 30°C
		10°C	15°C	20°C	25°C	30°C	35°C	
1.	GLI	12.6	23.5	40. 3	70.3	79.3	45.6	Growth slow, Filaments twisted, white
2.	GL2	5 .3	20. 6	43.5	80.0	85.6	50.0	Growth fast, filaments twisted, White
3.	GL3	0.0	4. 0	22.3	84.6	88.0	36.6	Growth fast, filaments twisted, white
4.	GL4	0 .0	17.3	41.3	87.0	85.0	25.3	Growth fast, filaments twisted, white

Fig. 12: Cloud-like appearance spread on the whole plate

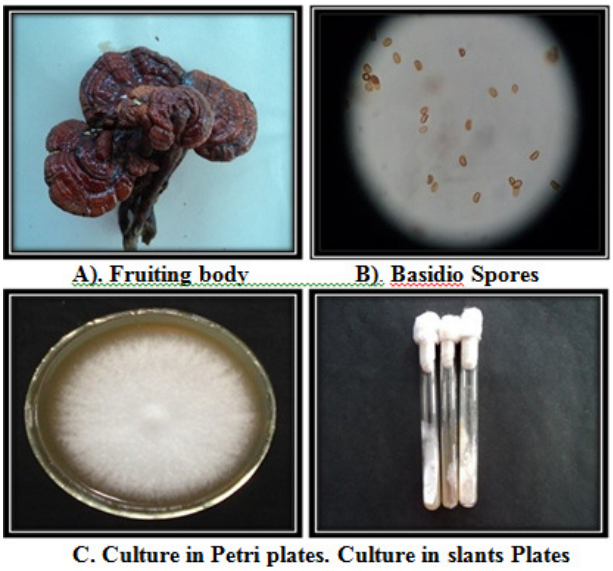

Fig. 13: Microscopic and Morphological characteristics of *G. lucidum*.

Fig. 7 show the effect of Temperature on the GL. By the increase in the temperature, the mean diameter of all the strains has increased. By reaching the optimum temperature i.e. 25-30ºC the maximum growth of all strains was obtained. By increasing further up to 35ºC decline has observed.

Table 3 shows the effect of pH on strain GL3 which shows that the average mycelial dry weight at pH2 is 45mg, at pH3 the weight is 78.33mg, at pH 4 the weight is 91.66mg, at pH 5 the weight is 168.33mg, at pH 6 the weight is 147.33mg, at pH 7 the weight is 128.33mg, at pH 8 the weight is 121.66mg, at pH 9 the weight is 101.66mg, at pH 10 the weight is 81.66, at pH 11 the weight is 61.66 while the average mycelial dry weight at pH 12 is 35.00. fig. 8 and 9 shows the different concentration of pH used and in the resulting amount of mycelial obtain in mg.

Table 4 shows the amount of Ganoderic Acid extracted from different Hydrolysates like, from Peanut cort Extract the amount of Ganoderic acid extracted is 0.006g/L, from wheat straw extract the amount is 0.011g/L and from Sugarcane bagasse extract the amount is 0.017g/L.

Table 5 shows the morphology of different Hydrolysate. It shows that the peanut cort extract has a White and soft like cotton irregular shape as shown in fig. 10. The wheat straw extract has the morphology of multiple small irregular white cotton-like shapes with thread-like projection as shown in fig. 11. The sugarcane bagasse extract has the morphology of Cloud Like appearance spread on the whole plate as shown in fig. 12.

DISCUSSION

G. lucidum has been accounted for to develop on a wide range of host species. It develops close stumps of oak and expansive leaved trees species in autumn and summer in the wild at a temperature of 25-30°C. It might likewise develop parasitically or saprophytically on logs (Singh, Dhingra *et al*. 2014). Four strains/separates, GLI, GL-2, GL-3 and GL-4 were chosen as illustrative segregates for conduction of preliminary cultural studies.

Morphology

Different isolates accumulations were examined in points of interest and recognized after standard representation of the species as under Fruit bodies are normally extensive,

Table 3: Effects of pH on Strain GL3

PH	Average mycelial dry weight (mg)*	Mycelial characteristics
2	45.00	Very, thin, very poor growth, incomplete mycelial mat
3	78.33	Mat incomplete, filaments twisted, cottony, whitish growth, poor growth
4	91.66	Mat incomplete, filaments twisted, cottony, whitish growth, poor growth
5	168.33	Mat complete, filaments twisted, cottony, growth very good
6	147.33	Mat complete, filaments twisted, cottony, growth very good
7	128.33	Mat incomplete, thick, filaments twisted, cottony, whitish growth, growth good
8	121.66	Mat incomplete, thick, filaments twisted, cottony, whitish growth, growth good
9	101.66	Mat incomplete, thin, filaments twisted, cottony, whitish growth, growth poor
10	81.66	Mat incomplete, thin, filaments twisted, cottony, whitish growth, growth poor
11	61.66	Mat incomplete, thin, filaments twisted, cottony, whitish growth, growth poor
12	35.00	Mat incomplete, thin, filaments twisted, cottony, whitish growth, poor growth

Table 4: Ganoderic Acid Concentration extracted from different Hydrolysates

Hydrolysates	Concentration (G.A)
Peanut cort extract	0.006 g/L
Wheat straw extract	0.011g/L
Sugarcane bagasse extract	0.017g/L

Table 5: Morphology of *G.lucidum* on different Hydrolysates

Hydrolysate	Morphology (*G. Lucidum*)
Peanut cort extract	White and soft like cotton irregular shape (Figure V)
Wheat straw extract	Multiple small irregular white cotton-like shape with thread-like projection (Fig. VI)
Sugarcane bagasse extract	A cloud-like appearance spread on the whole plate (Figure III)

reddish-brown, stipitate, rarely suborbicular, dimidiate, upper and lateral surfaces covered with hard gleaming substance resemble with sealing wax.

Pileus 2.0- 5.0 cm expansive, reddish-brown in color and pileal surface rough, length and thickness of stipe is 1.5-4.5 cm and 0.5- 2.0 cm respectively. Pileus surface regularly seemed varnished. Ovate, brown basidiospores with a rounded base and truncate to narrowly rounded apex; spore surface marginally too powerfully dimpled; wall containing several layers. Inter-wall pillars connect the outermost wall with the inner wall. Measurement of basidiospores10-12 x 6.5-8**.**0 µm.

All the segregate was observed to be typical of *G. lucidum*. The morphology of basidiospores and basidiocarp has additionally been measured in previous studies (Cao, Wu *et al*. 2012). The varnished appearance of the basidiocarp of numerous polypores is because of a shapeless substance emitted by the hyphae and the characteristic shiny cover is seen especially in segregate of *Ganoderma lucidum."*

Inoculation of Pure Culture

Pure culture of *G. lucidum* was inoculated on Potato Dextrose Agar following tissue culture technique. A good medium is Potato Dextrose Agar as suggested by earlier workers (Bailey, Otten *et al*. 2000).

Effect of Temperature

Temperature is one of the essential variables for the development of organisms. The mycelial growth pattern of four strains/disconnects of *G. lucidum* was recorded at six distinct temperatures viz.10°C, 15°C, 20°C, 25°C, 30°C and 35°C for 7 days on Potato Dextrose Agar when the plate was totally colonized. The information demonstrates that a temperature scope of 25-30°C is ideal for the vast majority of the *G. lucidum* confines/strains which favored a temperature scope of 25-30°C, individually for its ideal mycelial development. The CRD examination of information at 5 percent level of relevance demonstrated that strain GL-4 displayed most extreme mycelial growth at temperature 30°C among all the strains at different temperature levels the ideal temperature of 25-30°C has been accounted (Bhardwaj and Misra 2018). Likewise, a temperature of 30-35°C has additionally been discovered appropriate for the development of *G. lucidum* (Kapoor and Sharma 2014). Prior discoveries are comparable to the temperature necessity for vegetative growth of the present confine/strains. The wide range of variation in temperature necessity can be credited to biological assorted variety of *G. lucidum*."

From our experiment, we observe that the optimum temperature for the growth is 25ºC to 30ºC because bellow 25ºC low amount of growth was observed and by reaching the optimum temperature the growth was maximum. When the temperature exceeds 35ºC we observe decline in growth.

Effect of pH

PH may be likewise a critical element for the growth of fungi. A trial was, therefore, led on fig. out the ideal pH to the Growth for GL-3 strain of *G. Lucidum* around potato dextrose broth medium. Separate pH levels going starting with 3. 0 will 12. 0 were tried for three. Replications each. That information was recorded as far as Normal dry mycelial weight then afterward 10. That information was recorded as far as Normal dry mycelial weight after 10. Days of incubation at 30°C and demonstrated in table no 3. Those CRD investigations for information in 5% level for. Hugeness demonstrated that pH 5. 0 will be best for that Growth for mycelium Furthermore on the alkaline side. There might have been n sudden passing drop. However, the test fungus might grow over a large vary of pH scale between 3.0 to 11.0 each lower and elevated levels of pH scale showed adverse impact on the mycelial growth. Rai, (2003) have investigated for mycelial Growth of the *G. Lucidum.* In acidic Ph, pH range claiming 4. 0- 6. 5 will make the best for Growth of *G. Lucidum***.** As per our experiment we observe that average mycelial dry weight obtains at pH 5 and 6 remain maximum. At pH 2-4 and 7-12 we observe less amount of average mycelial dry weight.

Concentration of Ganoderic Acid Extraction

The GA was extracted from fermentation broth filtrate of Peanut cort extract, Wheat straw extract Sugarcane bagasse extract was added with 4 volumes of 95% ethanol and left overnight at 4^{o}C to precipitate crude GA. The precipitated GA was collected by centrifuged at 10,000 rpm for 10 min, dried at 60^{o}C to remove the residual ethanol and Ganoderic Acid concentration obtained from the Hydrolysates of Peanut cort extract was 0.006g/L, the wheat straw extract was 0.011g/L and Sugarcane bagasse extract was 0.017g/L.

CONCLUSION

It is finished up from this investigation that waste material like peanut cort, sugarcane bagasse and wheat straw can be utilized as media for the development of *Ganoderma lucidum*. These waste materials were utilized as a part of this examination and development of *Ganoderma lucidum* was got. After physical and compound treatment Ganoderic acid was extricated from *Ganoderma lucidum*. This study will be additionally advanced to discover the impact of Ganoderic acid as a helpful operator (hostile to malignant). In show ponder, Morphology of *G. lucidum* on various Hydrolysates like peanut cort, sugarcane bagasse, wheat straw extract have white and delicate like cotton sporadic shape, Cloud-like appearance spread all in all plate and Multiple little unpredictable white cotton-like shape with string-like projections. According to our analysis, we watch that normal mycelial dry weight gets at pH 5 and 6 stays greatest. At pH 2-4 and 7-12 we watch less measure of normal mycelial dry weight.

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