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Ganoderic Acid and Exopolysaccharide Production by *Ganoderma Lucidum* from Semi-Solid-State and Submerged Fermentation

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ABSTRACT

Introduction: Production of Ganoderic acid (GA) and Exopolysaccharide (EPS) with using beneficial fermentation strategy has received great attention recently. The aim of present study is comparison of GA and EPS production by *G. lucidium* in submerge, Semi-Solid and Solid-State fermentation.

Materials and methods: Potato dextrose Agar (PDA) for cultivation of *G. lucidum* was used. A modified medium formulation for Semi-Solid-State fermentation was also used with both submerged and Solid-State cultivation advantages. The optimized media components and main effects, such as carbone source, inducers, and aeration were studied with using Taguchi orthogonal array design. Thin Layer Chromatography (TLC) was used to detect GA in mycelium and fruiting body of *G. lucidum* and Fourier transform infrared (FTIR) spectroscopy was used to detect EPSs in submerged fermentation.

Results: Findings showed that the increase of GA in Semi-Solid-State fermentation (256 µg/g) with a combination of wheat bran 9g/L, oak chips 13g/L but without aeration. Findings showed that EPS production in submerged fermentation was more noticeable than Semi-Solid-State and Solid-State fermentation. In submerged fermentation with a combination of malt 20%, glucose 4%, sucrose 2% and with aeration 98.3±3.78mg/g EPS were observed. FTIR band in 890 Cm^{-1} indicated the presence of polysaccharides.

Discussion and conclusion: Among the three sets of formulations, results showed that Semi-Solid-State fermentation is the most appropriate culture for GA production and submerged fermentation is the most appropriate culture for EPS production. Finally, we suggest Semi-Solid-State fermentation for both GA and EPS production using wholly submerged glucose and oak chips enriched solid particle.

Key words: Exopolysaccharide, Ganoderic Acid, *Ganoderma Lucidum*, Semi-Solid-State Fermentation

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Introduction

G. lucidum (Red Reishi) is currently the most popular among all mushroom species, because of its high medicinal values. Even today the basidiocarp (fruiting body) of *G. lucidum* is a popular remedy to treat many diseases. Various *Ganoderma* species are widely used in the synthesis of several drugs and also act as functional foods. *G. lucidum* produces several metabolites with biological activity, such as terpenoids and exopolysaccharides which might be responsible for medicinal properties (1-5). In recent years, submerged fermentation of *G. lucidum* has received great attention in Asia regions as a promising alternative for efficient production of its valuable metabolites, especially exopolysaccarides and Ganoderic acid. Ganoderic acid has been reported to possess hepatoprotective, anti-hypertensive, anti-HIV, hypocholesterolemic, anti-histaminic effects, anti-tumor, anti-engiogenic activity and many effects on platelet aggregation and complement inhibition. Exopolysaccharides have been known to possess anti-tumor effects through immunomodulation and anti-angiogenesis. In addition, polysaccharides have a protective effect against free radicals and reduce cell damage caused by mutagens (6-8).

In Asia regions basidiocarp production of *G. lucidum* is common. The advantages of submerged fermentation over traditional basidiocarp cultivation are the reduction in the time spent to obtain the product of interest. The production of basidiocarp takes at least 3 to 5 months, while reasonable amounts of GA and EPS can be obtained by submerged fermentation after only 2 to 3 weeks. Submerged fermentation advantages are less production with high quality, high control on environmental condition; and its important disadvantage is less GA production (9).

As mentioned, *G. lucidum* is traditionally cultivated in solid culture. In contrast, with submerged culture, in the traditional cultivation technique of *G. lucidum*, it takes at least several months until the fruit bodies are developed and this culture technique is used to obtain only basidiocarp. The advantage of solid-State fermentation (SSF) over other techniques is high production of GA; and its important disadvantages are the long time required to cultivate fruiting body, the difficulty of controlling the quality of the product, unsterile production process, and the ease with which the culture is affected by the environmental changes. There is a great need to supply the market with a large amount of high-quality *G. lucidum* products. In this study the advantages of both submerged and Solid-State fermentation were accumulated in Semi-Solid-State fermentation**.** It is obviously necessary and important to develop a process for the production of these valuable metabolites. However, as far as we know, until now there are no reports on Semi-Solid-State fermentation for high production of GA and EPS in biomass instead of fruiting body (9, 10). In addition, present work aimed to evaluate the effect of different medium components on the production of GA and EPS.

Material and Methods

Microorganism preparation: *G. lucidum (Curtis) P. Karst*. Gilan, Gorgan was obtained from National Center for Genetic and biological reserves in Iran, It was characterized and identified as *G. lucidum* according to the key of Iranian *Ganoderma* species (11, 12).

Media: *G. lucidum* were maintained by an occasional transfer on PDA with 2.5% wheat bran extract and incubated at 30°C for 8 days.

Pre-culture medium: For inoculation in pre-culture medium, 1cm×1cm block of fresh growing mycelia on PDA were inoculated into 500mL cotton-plugged Erlenmeyer flasks that contained 150mL of pre-culture medium. It consisted of the following components (g/L): sucrose 35, peptone 5, yeast extract 2.5, $KH_2PO_4.H_2O$ 1 and $MgSO_4.7H_2O$ 0.5 with initial pH of 5.5 (13). Flask cultivation was carried out at 30°C±2, 150 rpm for two days.

.Experimental design of Submerged, Semi-Solid-State. and Solid-State fermentation medium: The experimental designs were applied to extract independently the main effects of the factors; the analysis of variance technique was then applied to determine which factors were statistically significant. All calculations were performed using Qualitek-4 software. Table1 presents the important variables to be optimized and their trial levels. Standard Taguchi orthogonal array of L_8 (2×4) for submerged fermentation and L_9 (3×3) for Solid-State and Semi-Solid-State fermentation were used (Table 2, 3 and 4), respectively, *L* and the subscript 8 represent Latin square and the number of experimental formulation runs. Concentrations of other ingredients in the medium and inoculation were fixed.

100mL fermentation medium was prepared in 500mL cotton-plugged Erlenmeyer flasks that were composed of the following components with some modification (g/L): glucose and sucrose with substitution 40, peptone and malt with substitution 5, yeast extract 5, $KH_2PO_4.H_2O$ 1 and $MgSO_4.7H_2O$ 0.5. Before sterilization, the medium pH was adjusted to 6.5 by adding 1N HCl or 1N NaOH. Fermentation medium was inoculated with 10mL pre-culture broth (with ca. 100mg dry weight of cells/L), then followed by a 7-day incubation at 30°C±2 on a rotary shaker with150 rpm and some flasks were incubated without agitation (14)**.**

.Solid medium contained 100g of dry **.**substrate that were dispensed equally into each cotton-plugged Erlenmeyer flasks with 500mL capacity. Flasks were sealed with cotton plugs to facilitate air transfer, autoclaved for 20min at 121°C. Flasks contained wheat bran and oak sawdust as carbon source and tea slag as an inducer. Mentioned flasks were inoculated with 10mL pre-culture broth (with ca. 100mg dry weight of cells/L), then followed by 7-weeks incubation at 30°C±2 without agitation (Table 1 and 2).

Semi-Solid-State fermentation was conducted in 500mL cotton-plugged Erlenmeyer flasks contained oak chips and wheat bran according to Table 1 and 3. The composition of the culture medium was same as Solid-State fermentation but with duplicate volumes of submerged medium without carbone source. Then flasks were incubated in static and agitated conditions at 30°C±2 for 21 days. All experiments were happened in triplicates.

Table 1- *Definition and trial levels of factors*

Culture condition	Factors	Variables	Low level	Medium level	High level
Solid-State fermentation	A	Oak chips (g)	0	18	26
	B	Wheat bran (g)	0	18	26
	C	Tea slag (g)	0	1.25	2
Semi-Solid-State fermentation	A	Oak chips (g)	0	9	13
	B	Wheat bran (g)	0	9	13
	C	Tea slag (g)	0	1.25	2
Submerged fermentation	A	Malt (%)	0	-	4
	B	Glucose (%)	0	-	2
	C	Sucrose (%)	0	-	20
	D	RPM	0	-	150

Table 2- *Design and experimental formulation according to Taguchi*

Formulation in Solid-State fermentation			
No	A (Oak chips (g))	B (Wheat bran (g))	C (Tea slag (g))
1	0	0	0
2	0	18	1.25
3	0	26	2
4	18	0	1.25
5	18	18	2
6	18	26	0
7	26	0	2
8	26	18	0
9	26	26	1.25

Table 3- *Design and experimental formulation according to Taguchi*

Formulation in Semi-Solid-State fermentation			
No	A (Oak chips (g))	B (Wheat bran (g))	C (Tea slag (g))
1	0	0	0
2	0	9	1.25
3	0	13	2
4	9	0	1.25
5	9	9	2
6	9	13	0
7	13	0	2
8	13	9	0
9	13	13	1.25

Table 4- *Design and experimental formulation according to Taguchi*

Formulation in Submerged fermentation				
No	A (Malt (%))	B (Glucose (%))	C (Sucrose (%))	D (RPM)
1	0	0	0	0
2	0	0	2	150
3	0	4	0	150
4	0	4	2	0
5	20	0	0	150
6	20	0	2	0
7	20	4	0	0
8	20	4	2	150

Crude GA and EPS Extraction: For extraction of crude GA, 50mL of distilled water were added to flasks that contained fermented solids for 24h on rotary shaker at 160rpm. Then broth was filtrated with Whatman paper (Ashless, 42, 12.5 cm, England). After that, filtrates were further separated by centrifugation at 6000×g for 20min. Submerged and Semi-Solid-State fermentation broth also were filtrated with Whatman paper. All filtrates were lyophilized and powders were used to determine the amounts of GA and EPS production.

.Analytical methods

.Determination of crude GA by Gravimetric technique: Previous extraction step filtrates, contained crude GA. Fang and Tang´s GA determination method with some modification was used: The lyophilized powder of samples (2±0.1g) was subjected to extraction using 75% (v/v) ethanol for 4 to 5 days at room temperature. The suspension which contained *G. lucidum* powder and ethanol was shook for overnight using a mechanical shaker (Model IRC-1-U kühner, German). After the removal of the solid particles by centrifugation at 7000×g for 10min, the supernatants were dried at 50^{o}C under vacuum (150 mbar) in a rotary evaporator (Lab Tech, EV311 series, Beijing, China) until all ethanol was vaporized. The residues were then suspended in distilled water (10mL) and later extracted with 10mL chloroform for 1 to 2 days. After chloroform removal by evaporation (at 50^{o}C for 35min.), GA in water extract were further extracted with 5% (w/v) $NaHCO_3$ for 12 hours. HCl (2N) was added to the solution to adjust the pH of the $NaHCO_3$ phase to lower than 3.0. Again GA in $NaHCO_3$ layer was further extracted by chloroform for 24 hours. After removal of chloroform by evaporation at 50^{o}C, crude GA was dissolved in absolute ethanol (99.4% v/v). Moreover, the absorbance was measured at 245nm in a spectrophotometer (Biochrom, Libra S12 Biowave II, UK) and the average readings were obtained (14).

Standard curve for 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.1mg/mL thymol (Merck, 108167, crystal) concentration was obtained and absolute ethanol was used as blank solution. Then crude GA content of each sample was analyzed according to standard curve.

TLC technique: In order to compare terpenoids band patterns between fruit body and mycelium in three kinds of fermentation formulation strategies, fruit body and mycelium of *G. lucidum* were used. Fruit body of *G. lucidum* finely were powdered, in 50mL of alcohol for 15min, centrifuged, withdrawer the supernatant, and evaporated at 50°C. The residues were dissolved in 2.0mL of alcohol, centrifuged, and supernatant were collected. Fruit body and mycelia extract were spotted on the plates as 2mm bands. For normal-phase TLC examination, pre-coated silica-gel TLC plates (Kieselgel 60 F254, DC-Alufolien, and Merck) were used. The developing reagents were composed of: chloroform, methanol and water in a volumetric ratio of 30:4:1. Visualization proceeded by spraying 10% sulfuric acid in alcohol and then heating sample to 105°C in order to evaporate solvents. Samples were initially examined under a UV lamp with a wavelength of 366nm and plates were further heated to 105°C for visualization (15).

Preparation of standard glucose curve: Lu´s gravimetric method with some modification was used: D-glucose anhydrous (0.1g) was accurately weighed and then dissolved in 100mL of distilled water, to produce corresponding stock standard solution (1mg/mL). Accurately glucose control solution 0.1, 0.2, 0.3, 0.4, 0.5, 1mL was draw to the test tubes and water was added to the volume of 20mL. Anthrone sulfuric acid solution (0.1g of anthrone was dissolved in 50mL concentrated sulfuric acid) was prepared. 3mL Anthrone solution was added to 1.5mL of D-glucose dilutions and mixture was heated for 10min, then was removed and put in room temperature for 15min, with the corresponding reagent as control. Absorbance was determined in 490nm wavelength and was made it as the ordinate, concentration as abscissa to establish a standard curve. For sample solution the method of establishing the standard curve was followed, as the "precisely Anthrone sulfuric acid 3mL was added" and 1.5mL of diluted sample solution was mixed then absorbance was determined; after that the content of the polysaccharide according to the standard curve was calculated (16).

FTIR Spectroscopy: FTIR spectroscopy of *G. lucidum* mycelia in submerged fermentation was tested in order to show glucans with using Nicolet FTIR Spectrometer (Nicolet 6700, Thermo Scientific). One part of sample was mixed with 99% of dried potassium bromide (KBr) powder and compressed to prepare a salt disc of 3mm diameter. These discs were subjected to FTIR spectrum measurement in the frequency range of 400 to 4000 cm^{-1} (17, 18).

Results

Production time: Results showed that, 21 days for growth in Semi-Solid-State fermentation was more appropriate in comparison with Solid-State fermentation growth time (49 days). However, submerged fermentation took place only in 7 days.

.Optimized medium composition for GA production: In each formulation effect of absence and presence of main effects were analyzed. The first formulation shows control level of medium that hadn't gotten any main effects. Fig. 1, 2 and 3 shows all medium formulation in submerged, Solid and Semi-Solid-State fermentation. As the histogram showed in formulation 1, GA wasn't detected. Medium formulation 6 in Solid-State fermentation and medium formulation 6 and 9 in Semi-Solid-State fermentation showed the highest amount of GA production. In Solid-State formulation 6 with 18g oak chips and 26g wheat bran 0.19±0.004 mg/g GA was detected and in Semi-Solid-State formulation 6 and 9 with 9g and 13g oak chips and 13g wheat bran respectively, 0.256±0.006 and 0.253±0.003 mg/g GA was detected.

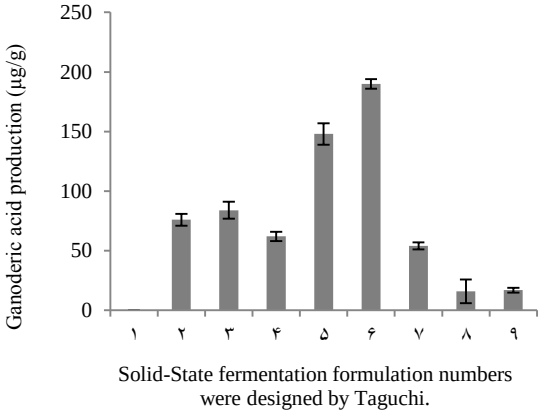

Fig.1- Ganoderic acid production by solid-state fermentation formulations

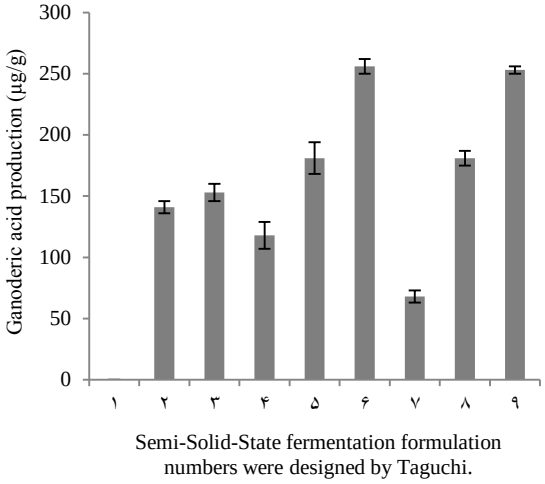

Fig.2- Ganoderic acid production by Semi-Solid-State fermentation formulations

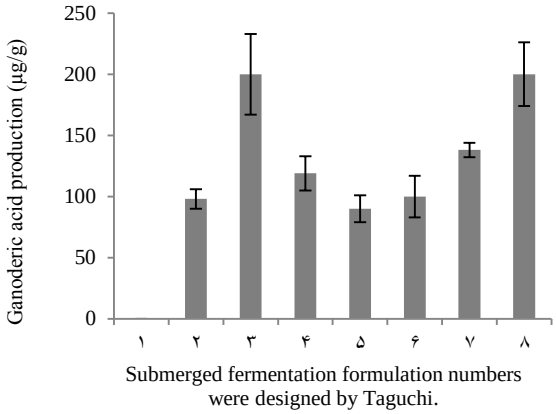

Fig. 3- Ganoderic acid production by submerged fermentation formulations

Analysis of data for determination of significant parameters on GA production was performed and results are shown in ANOVA Table 5. ANOVA Table of obtained results showed that, changing the carbone content of medium has been the most important factor in causing differences in obtained results. By the same way, the least important factor was the inducer. The experimental results suggested that these factors at optimum level strongly support the production of GA. In three kinds of fermentation formulation media, factors including: wheat bran, oak chips and glucose with P value ≤0.050 were significant factors for GA production.

Table 5- *ANOVA (Analysis of Variance) for Ganoderic acid production by G. lucidum*

Process	Factors	DOF (f)[1]	Sum of Sqrs. (S)[2]	Variance (V)	F-Ratio (F)	P Value	Pure Sum (Ś)	Percent P(%)
Solid-State fermentation	Oak chips (g)	2	0.012	0.006	120.034	0.003	0.011	35.916
	Wheat bran (g)	2	0.02	0.01	203.34	0.002	0.02	61.051
	Tea slag (g)	2	0	0	7.049	0.053	0	1.825
	Other error	2	-0.001	-0.001	-		-	1.208
	Total	8	0.033	-	-		-	100
Semi-Solid-State fermentation	Oak chips (g)	2	0/012	0.006	78.53	0.012	0.012	23.152
	Wheat bran (g)	2	0.039	0.019	242.308	0.004	0.038	72.06
	Tea slag (g)	2	0.002	0.001	13.031	0.067	0.001	3.592
	Other error	2	0	0	-		-	1.196
	Total	8	0.054	-	-		-	100
Submerged fermentation	Malt (%)	1	0.001	0.001	1.507	0.322	0	2.141
	Glucose (%)	1	0.014	0.014	11.849	0.025	0.013	45.745
	Sucrose (%)	1	0	0	0.616	0.423	0	0
	RPM	1	0.008	0.008	6.743	0.081	0.007	24.216
	Other error	3	0.003	0.001	-		-	27.898
	Total	7	0.029	-	-		-	100

1. Degrees of Freedom 2. Sum of Squares

Optimized medium composition for EPS production: In each formulation, the effect of absence and presence of main effects were analyzed. The first formulation shows the control level of medium that hadn't gotten any main effects. Fig. 4, 5 and 6 shows all medium formulation in submerged, Solid and Semi-Solid-State fermentation. As histogram showed in formulation 1, EPS wasn't detected. Medium formulation 3 and 8 in submerged fermentation showed the highest amount of EPS production.

Analysis of data for determination of significant parameters on EPS production was performed and results are shown in ANOVA Table 6. ANOVA Table of obtained results showed that, carbone content of medium and aeration have been the most important factors in causing differences in obtained results. By the same way, the least important factor was inducer. The experimental results suggested that these factors at optimum level strongly support the production of EPS. The production of 98.33±3.78mg/g EPS was observed in submerged fermentation with a combination of malt 20%, glucose 4%, sucrose 2% and with aeration. It can be observed that wheat bran and oak chips in Semi-Solid-State and Solid-State fermentation, and glucose in submerged fermentation respectively contributing of 67.506, 32.673 and 47.628%, have shown the highest positive impact on the EPS production. In three kinds of fermentation formulation media, factors including: wheat bran, glucose and aeration with P value ≤0.050 were significant factors for EPS production.

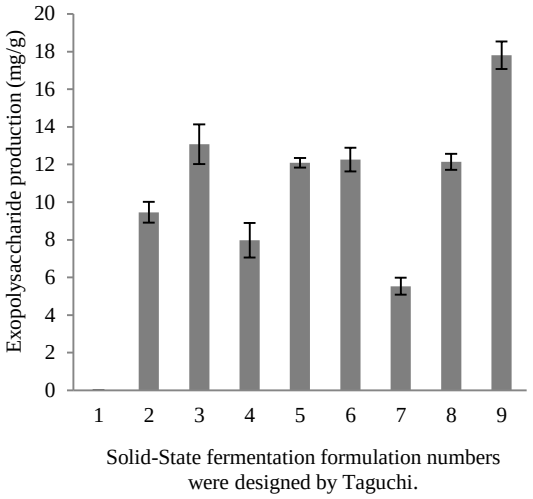

Fig.4- Exopolysaccharide production by solid-state fermentation formulations

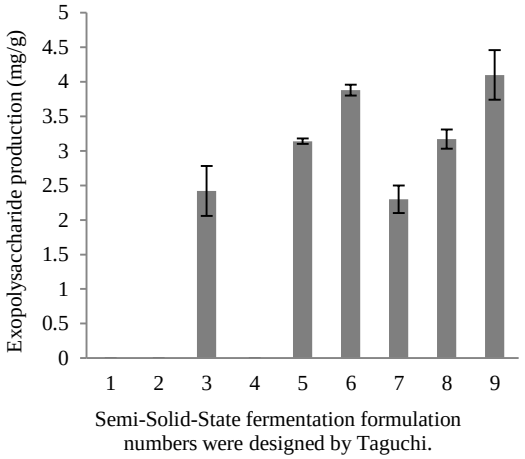

Fig.5- Exopolysaccharide production by Semi-Solid-State fermentation formulations

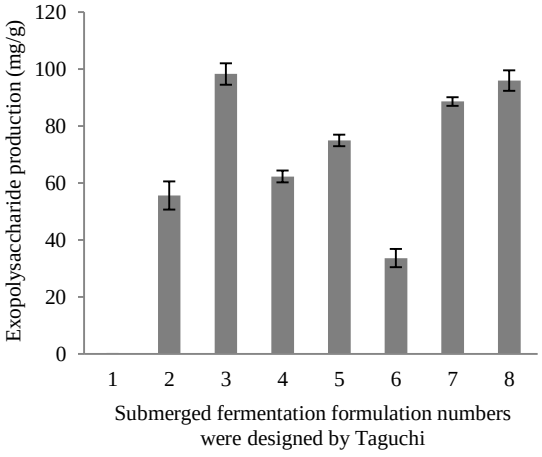

Fig. 6- Exopolysaccharide production by submerged fermentation formulations

Table 6- ANOVA (Analysis of Variance) for Exopolysaccharide production by G. lucidum

Process	Factors	DOF (f)[1]	Sum of Sqrs. (S)[2]	Variance (V)	F-Ratio (F)	P value	Pure Sum (Ś)	Percent P(%)
Solid-State fermentation	Oak chips (g)	2	35.318	17.659	4.128	0.195	26.764	11.801
	Wheat bran (g)	2	161.648	80.824	18.897	0.050	153.093	67.506
	Tea slag (g)	2	21.264	10.632	2.485	0.287	12.709	5.604
	Other error	2	8.553	4.276	-		-	15.089
	Total	8	226.784	-	-		-	100
Semi-Solid-State fermentation	Oak chips (g)	2	8.811	4.405	14.964	0.063	8.222	32.673
	Wheat bran (g)	2	13.599	6.799	23.096	0.042	13.01	51.697
	Tea slag (g)	2	2.167	1.083	3.68	0.214	1.578	6.27
	Other error	2	0.587	0.293	-		-	9.36
	Total	8	25.166	-	-		-	100
Submerged fermentation	Malt (%)	1	647.999	647.999	2.541	0.206	392.999	4.817
	Glucose (%)	1	4140.499	4140.499	16.237	0.026	3885.499	47.628
	Sucrose (%)	1	12.499	12.499	0.949	0.819	0	0
	RPM	1	259.1999	259.1999	10.164	0.048	3226.999	28.646
	Other error	3	764.999	254.999	-		-	18.909
	Total	7	8157.999	-	-		-	100

1. Degrees Of Freedom 2. Sum of Squares

TLC technique: Results in TLC technique were shown in Fig. 7. In red rejoin of TLC chromatogram, terpenoids bands were shown according to Ganoderic acids R_f (19). In comparison, between three kinds of fermentation formulation strategy, bands that were related to terpenoids region in Semi-Solid-State fermentation mycelia were noticeable than fruiting body and other fermentation formulation.

FTIR spectroscopy: A prominent band in the range of 1200 to 800 cm^{-1} indicated the presence of exopolysaccharides in *G. lucidum* mycelia. In the current study, peak in region 891cm^{-1} showed β-Dglucan (Fig. 8).

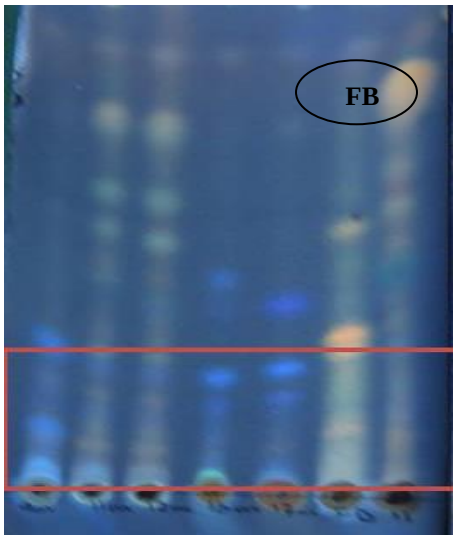

Fig. 7- TLC chromatogram of *G. lucidum* (Red rejoin in chromatogram shows Ganoderic acids band according to R_f) (19).
Lane 1, 7: *G. lucidum* mycelium biomass in submerged fermentation
Lane 2, 3: *G. lucidum* mycelium biomass in Semi-Solid-State fermentation
Lane 4, 5: *G. lucidum* mycelium biomass in Solid-State fermentation
Lane 6: *G. lucidum* Fruiting body

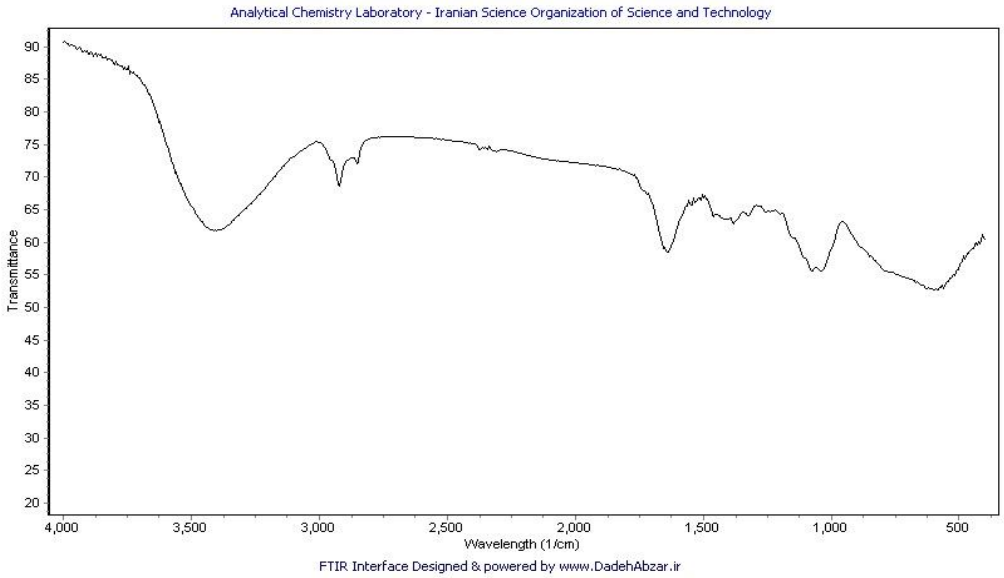

Fig. 8- FTIR spectra (4000-500 cm^{-1} region) of exopolysaccharides of *Ganoderma lucidum* obtained under submerged fermentation formulation.

Discussion and conclusion

This study investigated the formulation for the fermentation of *G. lucidum* in a shake-flask culture. The composition of the medium was initially screened to determine the suitable ingredients. The effects of various ingredients such as wheat bran, oak chips and glucose, on the efficiency of Solid, Semi-Solid-State and submerged fermentation were examined. Our objects were to find optimized formulation with short growth times and high production of GA and EPS. Variable growth time in many studies was reported, but there is less information about growth time in Semi-Solid-State fermentation. Fang and Zhong reported a high cell density through a 4-day shake-flask fermentation followed by a 12-day static culture. They saw an increase of GA production in the new two-stage process from 1.36 (control) to 3.19mg/100mg of DW (Cell Dry Weight). Although they mentioned 16 days for GA production in submerged fermentation, in our study 7 days submerged fermentation took and highest production of GA was 0.02mg/100mg of DW (Cell Dry Weight), and after 7 days the high viscosity of EPS didn't let us continue the process. Maybe in continuous fed batch system the amount of GA increases (13).

GA production strategy: GA productions in Semi-Solid and submerged fermentation were close. However, cost beneficial carbone source and short growth time is valuable as well. Yanru et al. tested all chemical components that might be possibly from the wood decaying to GAs induction in the submerged liquid fermentation. They found that GAs production increased 85.96% by 1.5% microcrystalline cellulose and 63.90% by 0.5% D-galactose. In comparison with this study, oak chips and wheat bran with synergistic properties in Semi-Solid and Solid-State fermentation were effective carbone sources in induction of GA production. In Yanru's study, though adding L-arabinose and D-xylose increased the biomass of *G. lucidum* significantly, they had no contribution to GAs biosynthesis. On the other hand, D-

galactose significantly induced GAs biosynthesis and it promoted *G. lucidum* growth with an increase of 58.9% in the dosage of 0.5%. Lignin, another important compound of wood degradation, could increase GAs content significantly. Additionally, they found comparably high triterpenoid background absorbance in lignin. In their study, only D-galactose significantly increased both the biomass and GA's yield. Other sugars had little improvement in the GAs production or even adversely affected its yield. In addition, the biomass did not increase with the concentration of galactose and they indicated that it was not a main carbon source to support the mushroom's growth and wood ingredient could help it, because there are some chemical residues other than glucose in the microcrystalline cellulose that made a contribution to the GAs over production. The assumption is based on the fact that the glycosidic bonds in microcrystalline cellulose and cellobiose are the same and so their final products as well. This report supports our significance in results of oak chips and wheat bran in synergism (20, 21).

In the current study, enhanced production of GA, an important bioactive triterpene, by *G. lucidum* in three formulation culture were studied. GA production occurred with 251% increase in Solid-State, 271% increase in Semi-Solid-State and 122% increase in submerged optimization formulations. It is evident that the use of a statistical culture condition optimization approach and Taguchi methodology was helpful to locate the optimum levels of the significant conditions with minimum effort and time. Gao et al. reported a model that predicts the maximum GA yield of 12.4 mg/L with glucose 44.4 g/L. In their study, the optimization of the culture conditions resulted in a 129.6% increase in GA production (22).

In comparison, among three kinds of fermentation formulation strategy, TLC bands related to terpenoids region in Semi-Solid-State fermentation were noticeable than fruiting body and other fermentation formulation. TLC chromatograms of *G. lucidum* in this study were in pattern of terpenoids isolated from the fruiting bodies and mycelium of *G. lucidum* as mentioned in American Herbal Pharmacopeia. Previously, it has been found that different species of *Ganoderma* have shown quite varied patterns in the TLC chromatograms of terpenoids. In literature, a pattern was disclosed that was peculiar to the strain for several terpenoids isolated from the fruiting bodies of *G. lucidum***.** Lin and Shiao suggested a chromatographic method that has been developed to not only resolve each pair of stereoisomers but also to identify certain triterpenoids present in the crude extract of *G. lucidum*. Changes in the triterpenoid patterns during formation of the fruiting body in *G. lucidum* have been reported as well (23-25). It was thought that those secondary metabolites of triterpenoids from *G. lucidum* could possibly be used as a way of identification.

EPS production strategy: In this study, we used 40g/L glucose in our formulation that due to the highest amount of EPS production. Although, in many studies, malt for EPS production was mentioned, in our study malt wasn't as efficient as glucose. Yuan et al. found that the optimum medium for EPS production is 70 g/L glucose, 5 C/N ratio, 2.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4 \cdot 7H_2O$. When they cultivated in

the obtained optimal media in 3L shake flask, compared to the basal medium, EPS yield increased markedly from 1.003 to 1.723 g/L. Erkel in his study investigated effects of different dosages of molasses as a carbohydrate source and corn-gluten meal as a protein source on the yield of *G. lucidum* to improve yield on Solid-State fermentation medium. Three dosages (1, 2 and 3%) of gluten meal or molasses were used to sawdust and bran based medium. His results showed that the yield increased significantly when 1% molasses and gluten meal were added to substrate media. Molasses was found to be the best supplement as a carbohydrate source compared to gluten which is rich in protein for Solid-State fermentation (26, 27).

Results of the present study showed that carbon content of medium and aeration have been the most important factors in causing differences in EPS obtained results. Shi et al. determined the optimal fermentation conditions for *G. lucidum* polysaccharide production to be the following: 14.53% of the inoculum size, 10.49 of the C/N ratio, and 21.18 days incubation. The maximum polysaccharide yield of 48.14±1.47 mg/g was obtained in the verification experiment. Montoya et al. reported among 10 species screened for production of EPSs in submerged cultures with glucose, soy oil, and yeast extract, the best results obtained with *G. lucidum* (0.79 g/L EPS). They showed that agitation strongly improves EPS production in most of the studied strains as we found the importance of this factor (28, 29).

In this study, after 7 days in submerged fermentation medium, the highest amount of EPS produced but in Semi-Solid and Solid-State fermentation after 49 days the amount of EPS reduced. It might be due to water mass fraction fall. Habijanic and Berovic reported that the content of EPS in the solids increased rapidly during the first 7 days, remained relatively constant until 21 days and then decreased, suggesting that the polysaccharide was actually degraded in the latter stages of the process. The period during which the polysaccharide content decreased corresponded with the period in which the water mass fraction was falling rapidly, from the values of 70–80% that were maintained during the first 21 days, to 20% at 35 days. However, it is not clear whether there is a direct cause-and-effect relationship between these two observations. The mechanisms of the processes controlling EPS production and consumption are not known, however, the authors suggested that EPS serve to fasten the hyphae to the surface of the solid particle, and to protect the hyphae both from mechanical damage during agitation and from desiccation at low moisture contents (30).

In current study, the presences of exopolysaccharides in *G. lucidum* mycelium was obtained under submerged fermentation formulation with FTIR in region of 890 Cm^{-1} that shows glucans existence. IR spectra of six water-soluble polysaccharides GTM1 to GTM6 in *G. tsugae* were observed by Peng et al. Mathlouthi and Koenig showed FTIR results for their *Ganoderma* sp.: GTM1exhibited the typical absorption peaks at 870 and 810 cm^{-1} for mannan. The appearance of obvious characteristic peaks both at 850 and 920 cm^{-1} for α-Dglucan and at 890 cm^{-1} for β-Dglucan in GTM3 and GTM4 implied the co-existing of α-and β-Dglucans. They observed that GTM5 and GTM6 exhibited the main absorption peak

at 890 cm^{-1} for the β configuration of D-glucan. Carbonero et al. observed a heteropolysaccharide, a fucomannogalactan, with a main chain of (1-6)-linked α-Dgalactopyranosyl units, partially substituted at O^{-2} by single-unit β-Dmannose or α-Lfucose side chains. He et al. studied IR characteristic peaks of *G. lucidum* from different places. They observed that there was obviously a wide and strong absorption peak in 3377.8 to 3396.5 cm^{-1}, a small acromion in 2924.2 to 2925.1 cm^{-1}, a medium intensity absorption peak in 1635.8 to 1650.3 and 1372.5 to 1375.2 cm^{-1}, a strong absorption bifurcate peak in 1074.8 to 1075.3 and 1043.2 to 1045.2 cm^{-1}, an obvious weak peak in fingerprint regions 891.0 to 894.8 cm^{-1}, and a medium intensity absorption peak in 563.10 to 574.7 cm^{-1} (31-34).

Finally, we suggest Semi-Solid-State fermentation for both GA and EPS production with wholly submerged glucose enriched solid particles. More studies on main factors on GA and EPS production in Semi-Solid-State fermentation by *G. lucidum* are in progress.

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تولید اسیدگانودریک و اگزوپلی‌ساکارید توسط *گانودرما لوسیدوم* در کشت نیمه جامد و غوطه‌ور

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چکیده

مقدمه: اخیرا تولید اسید گانودریک و اگزوپلی‌ساکارید با استفاده از استراتژی‌های مفید فرمانتاسیون مورد توجه قرارگرفته است. هدف از این مطالعه مقایسه شیوه کشت غوطه‌ور، نیمه‌جامد و جامد گانودرما لوسیدوم در تولید اسید گانودریک و اگزوپلی‌ساکارید می‌باشد.

مواد و روش‌ها: محیط عصاره سیب زمینی جهت تکثیر گانودرما لوسیدوم استفاده شد. یک فرمولاسیون جدید با عنوان کشت نیمه‌جامد که دارای مزایای کشت غوطه‌ور و جامد می‌باشد، استفاده شد. ترکیبات محیط‌های کشت استفاده شده، بهینه گردید و فاکتورهای اثرگذار مانند منبع کربن، القا کننده‌ها و هوادهی با استفاده از طراحی تاگوچی بررسی شدند. از کروماتوگرافی لایه نازک جهت شناسایی گانودریک اسید در میسلیوم و میوه‌بارده گانودرما لوسیدوم و از طیف سنجی تبدیل فوریه مادون قرمز جهت شناسایی اگزوپلی‌ساکاریدها در کشت غوطه ور استفاده شد.

نتایج: در کشت نیمه جامد با ۹g/L سبوس گندم، ۱۳g/L چیپس چوب بلوط و بدون هوادهی میزان تولید اسید گانودریک افزایش یافت (۲۵۶ µg/g). همچنین تولید قابل توجه اگزوپلی‌ساکارید در کشت غوطه‌ور نسبت به کشت نیمه‌جامد و جامد مشاهده شد. در کشت غوطه‌ور با ٪۲۰ مالت، ٪۴ گلوکز، ٪۲ سوکروز و هوادهی ۹۸/۳±۳/۷۸ mg/g اگزوپلی‌ساکارید بدست آمد. وجود اگزوپلی‌ساکاریدها توسط باند طیف سنجی تبدیل فوریه مادون قرمز در Cm^{-1} ۸۹۰ تعیین شد.

بحث و نتیجه‌گیری: در میان سه فرمولاسیون بهینه شده، نتایج حاصل نشان داد که کشت نیمه‌جامد جهت تولید اسید گانودریک و کشت غوطه‌ور جهت تولید اگزوپلی‌ساکارید مناسب است. در کل شیوه کشت نیمه‌جامد غنی شده با گلوکز و ذرات جامد تراشه چوب بلوط، جهت تولید اسید گانودریک و اگزوپلی‌ساکارید پیشنهاد شد.

واژه‌های کلیدی: اگزوپلی ساکارید، اسیدگانودریک، *گانودرما لوسیدوم*، کشت نیمه جامد

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