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SCREENING OF FIBRINOLYTIC PROTEASE FROM SOUTH INDIAN ISOLATES OF GANODERMA LUCIDUM

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ABSTRACT

Intravascular thrombosis due to fibrin aggregation in arteries is one of the main causes of cardiovascular disease in humans. Thrombolytic agents such as heparin and Streptokinase have been extensively used in the therapeutic treatment of thrombosis. Search for potent and effective thrombolytic agents from various sources such as bacteria and fungi continue around the world. In the present study, *Ganoderma lucidum* a higher fungus has been studied for the optimal production of fibrinolytic protease. The physical parameter been identified and standardized for optimal production of biomass and intracellular fibrinolytic protease. The medium composition and design of fermentation condition play vital role in enhancing the efficiency of biomass and by-product production. Out of eight media tested the maximum mycelial biomass of 15 g/L was obtained on day 21 of incubation when the test fungus *G. lucidum* was grown on fibrin amended potato dextrose broth. Of all the 24 isolates grown on fibrin amended PDA, it was found that the strain VK 12 isolated from *Tamarindus indica* showed faster growth rate and maximum protease production.

KEYWORDS

Ganoderma lucidum, fibrinolytic protease, fibrin, Cardiovascular diseases

INDRODUCTION

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels, and include coronary heart disease, cerebrovascular disease, hypertension,

peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. According to World Health Organization statistics, about 17.5 million people died from CVDs in 2005, which represented 30% of all global deaths. By 2015, almost 20 million people will die from CVDs, mainly from heart disease and stroke. Intravascular thrombosis due to fibrin aggregation in arteries is one of the main causes of CVDs. Thrombolytic agents have been extensively used in the therapeutic treatment of thrombosis. The major thrombolytic agents are plasminogen activators, such as tPA (tissue-type plasminogen activator), urokinase and streptokinase. Despite their widespread use, all these agents have undesirable side effects, and are also relatively expensive[1]. Therefore, the search for other thrombolytic agents from various sources continues.

More effective thrombolytic agents have been identified and characterized from snake venoms[2], earthworms[3], insects[4] and microorganisms[5] , marking a new era in the early treatment of CVDs.

Mushroom extracts are widely used as nutritional supplements and medicines, with claimed human health benefits[6]. In the East Mediterranean countries, *Ganoderma* species are regarded as the herb of longevity. These fungi have been used in folk medicine for hundreds of years and stains are commercially cultivated for preparation of health tablets[7]. Medicinal benefits of *Ganoderma* spp.. have been reviewed by Jong and Birmongham[8] . Six novel triterpenoids, i.e ganoderenic acid, furano ganodermic acid, ganodericacid derivatives, were isolated from the fruit body of the fungus *Ganoderma applanatum*[9]. Choi and Sa, has reported presence of fibrinolytic enzyme from a Chinese isolate[10].

Ganoderma lucidum preferentially attacks old and declining trees and decayed dead and stumps[7]. In India, *G. lucidum* occurs on coconut, *Casuarina* sp, *Eucalyptus* sp, Delonix *ragia*, Fig (*Ficus racemosa*), Palm (*Cocos nucifera*), Rubber (*Heave brasilensis*), Mango (*Mangifera indica*), *Acacia* sp and other economically important forest trees[11]. Physiological aspects of lignin degradation by *G. lucidum* have been investigated[12]. Terpenoids and bioactive compounds extracted from *G. lucidum* are extensively used in the pharmaceutical preparations[7].

Since the isolates of *Ganoderma lucidum* obtained from different regions of the biosphere exhibited different responses in cultural conditions for the maximum production of biomass and secondary metabolites, the present study was aimed at optimization of cultural conditions for the maximum production of fibrinolytic protease from a strain *G. lucidum* (VK12), that produced highest fibrinolytic protease among the 24 South Indian strains tested. The optimized was purified and characterized in the present study.

MATERIALS AND METHODS

Chemicals

The chemicals namely, thrombin, bovine fibrinogen, human fibrinogen, fibrin, urokinase and protein molecular mass standards were purchased from Sigma Chemical Co. USA. The other chemicals and analytical regents were purchased from Sisco Research Laboratory, Bombay, India.

Organisms and inocula

The basidiocarps of *Ganoderma lucidum* were collected from the host belonging to gymnosperms and angiosperms representing varied climate regions from several places in and around Chennai, Kodaikkanal and Udakamandalum. Twenty

four *Ganoderma lucidum* specimens were collected from parts of economically important trees including mango, teak, Eucalyptus, pine and apple. *Ganoderma* occurred on living, dead and declining trees from different regions such as root, trunk and base of the host. The cortex tissue of the basidiocarps was used for raising *G. lucidum* cultures on agar plates. Cultures were identified by following the manual "Polypores of Kerala" written by Manoharan and Leelavathi[13]. *Ganoderma lucidum* was maintained on potato dextrose agar (PDA) medium and was transferred to Czapek's Dox agar (CDA) medium and allowed to grow for a period of 5 days. Mycelial discs of 5 mm diameter from 5 day old culture were taken as initial inocula for the following experiments.

Culture media

Potato dextrose agar (PDA), Malt extract agar (MEA), Czapek's Dox agar (CDA), Modified Czapek's Dox agar (MCDA), Potato dextrose broth with fibrin (PDBF), Potato dextrose broth added with fibrin (1% w/v). Malt extract broth with fibrin (MEBF), Malt extract broth added with fibrin (1% w/v). Czapek's Dox agar (CDBF), Czapek's Dox broth added with fibrin (1% w/v). Modified Czapek's Dox agar (MCDBF) Modified Czapek's Dox broth added with fibrin (1% w/v) were used for the production of fibrinolytic activity.

(i) Measurement of mycelial dry and wet weights.

Mycelial mat of *G. lucidum* recovered from culture medium by filtering through Whatman No.1 filter paper, washed with distilled water, the excess water was removed with blotting paper for 10 min and the fresh weights were weighed. The mycelial mat was dried on a pre-weighed filter paper at 50ºC for 48 h and the dry weight was recorded.

(ii) Protein estimation

The total protein content of the mycelial extracts and culture filterates were estimated by following the method of Bradford[14].

(iii) Protease assay

The protease assay was performed according to the method of Mc Donald and Chen[15] (1965). The enzyme activity was calculated from the standard graph constructed with tysosine. One unit of protease activity was calculated as the amount of enzyme required to produce one µmole of tyrosine equalent/min under the experimental conditions.

(iv) Qualitative detection of protease by plate clearing technique

The extracellular protease produced by the *G. lucidum* was detected by the methods described by Rajamani and Hilda[16] (1987). Skim milk agar plate (2% (w/v) was prepared using sodium phosphate buffer (0.05 M, pH.7.0).

(v) Qualitative detection of fibrinolytic protease by plate clearing technique

Fibrinolytic activity was determined using the method described by Astrup and Mullertz [17](1952). The fibrin agarose plate was made to a 1 mm thickness, and contained agarose (1.2% w/v), human fibrinogen (0.4% w/v), and human thrombin (20 U/mL) in a petri disc, and the clot was allowed to stand for 1 h at room temperature. Then, 10 µL of sample enzyme solution was carefully placed onto the plate. The plate was incubated for 5 h at 37ºC and the diameter of the lytic zone was measured and the clear transparent region was observed in which fibrin is hydrolyzed. This diameter is directly proportional to the strength of the fibrinolytic activity.

(vi) Fibrinolytic protease assay

Fibrinolytic protease activity was carried out according to the method descried by Greenberg[18] (1957). The reaction mixture contained 8 mg bovine fibrin, 500 µl mycelial extract in phosphate buffer, (0.05 M, pH.6.8) in a total volume of 1 mL. This mixture was incubated for 30 min at 37°C in a water bath. The reaction was stopped by adding 0.5 mL of 15% cold trichloro acetic acid (TCA) in glass distilled water. The mixture was centrifuged at 3,000g for 10 min to remove precipitated fibrin. To 0.5 mL of acid soluble filtrate 2.5 mL of 0.3 N sodium hydroxide and 2.9% (w/v) sodium carbonate in glass distilled water was added, followed by 0.75 mL of Folin's phenol reagent (diluted 1:3 with glass distilled water). The mixture was incubated for 25 min at room temperature and the color developed was read at 650 nm. The above said procedure was followed with heat killed enzyme and kept as blank. One unit of enzyme activity was calculated as the amount of enzyme which releases 1 µmol of tyrosine/ min under the specified reaction conditions.

RESULTS

(i) Collection and isolation of G. lucidum

The basidiocarps of *G. lucidum* were collected from the host trees belonging to gymnosperms and angiosperms representing varied climatic regions of Kodaikkanal, Udagamandalum and several places in and around Chennai. Twenty four *G. lucidum* specimens were collected from economically important trees including mango (*Mangifera indica*), teak (*Tectona grandis*), *Eucalyptus*, pine (*Pinus roxburghii*) and apple (*Malus domestica*). The fruit bodies were collected from different parts of living, declining and dead trees such as root, trunk and base of the trees. The observed color of the basidiocarps ranged from white to brown. Among the twenty four isolates, twelve were stipitate and the remaining twelve were sessile in habit. The occurrences of *G. lucidum* on some important trees are listed in Table 1.

Table 1.
Location, habitat and sources of G. lucidum isolates collected for this study

Isolates	Hosts	Habit	Color	Locality	District	Infection site
VK01	*Boswellia serrate*	Stipitate	Brown	Indian Institute of Technology	Chennai	Trunk
VK02	*Pinus taeda*	Stipitate	Brown	Indian Institute of Technology	Chennai	Base
VK03	*Prosopis spicigera*	Sessile	Brown	Indian Institute of Technology	Chennai	Trunk
VK04	*Ficus bengalensis*	Stipitate	Brown	Bombay chola	Kodaikkanal	Stump
VK05	*Citrus medica*	Stipitate	Brownish	Lake area	Kodaikkanal	Root

			white			
VK06	*Enterolobium saman*	Stipitate	Brownish white	Brayan park	Kodaikkanal	Branches
VK07	*Cinnamomum camphora*	Sessile	Brownish white	Bombay chola	Kodaikkanal	Branches
VK08	*Pinus longifolia*	Stipitate	Brown	Bombay chola	Kodaikkanal	Root
VK09	*Crotolaria formose*	Sessile	Brown	AC tech	Chennai	Whole tree
VK10	*Cryptacarya stocksii*	Sessile	Brown	AC tech	Chennai	Base and Root
VK11	*Pongamia glabra*	Stipitate	Brown	AC tech	Chennai	Root
VK12	*Tamarindus indica*	Stipitate	Brown	AC tech	Chennai	Base
VK13	*Albizzia lebbeck*	Sessile	Brown	Velacherry	Chennai	Root
VK14	*Enterolobium saman*	Sessile	Brown	Velacherry	Chennai	Base
VK15	*Tectona platyphylla*	Sessel	White	Dhoddabetta	Udagamandalam	Stump
VK16	*Populus tremuloides*	Stipitate	Brown	Dhoddabetta	Udagamandalam	Base
VK17	*Fragaria elatior*	Sessile	Brown	Lake	Udagamandalam	Trunk
VK18	*Albizzia marginata*	Sessile	Brown	Dhoddabetta	Udagamandalam	Trunk
VK19	Decomposing soil	Stipitate	Brown	Sims park	Udagamandalam	Root
VK20	Decomposing soil	Stipitate	Brown	Sims park	Udagamandalam	Root
VK21	*Pinus ovium*	Stipitate	Brown	Sims park	Udagamandalam	Root
VK22	*Prunus communis*	Stipitate	Brown	Sims park	Udagamandalam	Root
VK23	Decomposing soil	Stipitate	Brown	Sims park	Udagamandalam	Soil
VK24	*Eriobotrya japonica*	Stipitate	Brown	Indian Institute of Technology	Chennai	Base

(ii) Screening of G. lucidum isolates for intracellular fibrinolytic protease

The isolates of *G. lucidum* listed in Table-1 were screened for protease activity. All the isolates were screened for intracellular

caseinolytic and fibrinolytic activities in terms of measuring the lytic zone in the medium incorporated with skim milk powder as the substrate using the methods described by Astrup and Mullertz and. Rajamani and Hilda. Among the twenty four isolates, one isolate VK12 collected from *Tamarindus indica* gave a very strong fibrinolytic activity followed by VK06 isolated from *Enterolobium saman* and VK07 isolated from *Cinnamomum camphora* (Table 2). Interestingly, the isolate VK12 exhibited faster growth rate among the three fibrinolytic positive isolates such as VK12, VK06 and VK07. There fore, this isolate was used for the entire study.

Table 2.

Screening of G. lucidum isolates for caseinolytic and fibrinolytic protease activities

Isolates	Hosts	Growth (mm radius)	Caseinolytic activity	Fibrinolytic activity
VK01	*Boswellia serrate*	14	-	_
VK02	*Pinus taeda*	8	-	-
VK03	*Prosopis spicigera*	14	-	-
VK04	*Ficus bengalensis*	16	++	+
VK05	*Citrus medica*	17	-	-
VK06	*Enterolobium saman*	8	++	++
VK07	*Cinnamomum camphora*	12	-	++
VK08	*Pinus longifolia*	13	-	+
VK09	*Crotolaria formose*	13	++	-
VK10	*Cryptacarya stocksii*	12	++	-
VK11	*Pongamia glabra*	6	-	-
VK12	*Tamarindus indica*	16	++	+++
VK13	*Albizzia lebbeck*	6	-	-
VK14	*Enterolobium saman*	16	+	-
VK15	*Tectona platyphylla*	16	-	-
VK16	*Populus tremuloides*	6	++	-
VK17	*Fragaria elatior*	5	++	-
VK18	*Albizzia marginata*	15	++	-
VK19	Decomposing soil	15	-	-
VK20	Decomposing soil	16	-	-
VK21	*Pinus ovium*	8	-	-
VK22	*Prunus communis*	15	-	-
VK23	Decomposing soil	15	-	-
VK24	*Eriobotrya japonica*	16	-	-

- No activity; + Weak activity; ++ Strong activity; +++ Very strong activity

(iii)Estimation of extracellular and intracellular fibrinolytic protease activity of G. lucidum.

Eight different liquid media such as Czapek's Dox broth (CDB), Modified Czapek's Dox broth (MCDB), Czapek's Dox broth with fibrin (CDBF), Malt extract broth (MEB), Modified Czapek's Dox broth with fibrin (MCDBF), Potato dextrose broth (PDB), Malt extract broth with fibrin (MEBF), and Potato dextrose broth with fibrin (PDBF) were used to test for intracellular and extracellular fibrinolytic protease activities. The cultures were grown for a period of 28 days and the mycelial mats were collected at an interval of seven days. The fresh and dry weights were measured. The culture filtrate and mycelial homogenate were used for the estimation of fibrinolytic protease activity and the data are presented in Table 5.

(iv)Effect of media on the mycelial growth of G. lucidum

Relatively higher mycelial growth occurred on day 21 irrespective of the media used (Table 3). However, the highest mycelial growth was obtained in potato dextrose broth (15552 mg/L) among the different media used followed by malt extract broth (14694 mg/L) and Czapek's Dox broth ((8239 mg/L). The least mycelial growth was observed in potato dextrose broth with fibrin (1420 mg/L; Table 3).

Table 3.

Effect of media on mycelial growth (mg dry wt/L) of G. lucidum

Media	Growth period (Days)			
	7	14	21	28
CDB	4494.31±627.66	7359.63±399.99	8174.5±547.38	8239.23±582.25
CDBF	1842.83±585.45	3829.33±319.62	6088.66±484.33	6242.6±572.09
MEB	6598.41±586.94	12728.27±653.31	14129.53±334.46	14694.83±618.44
MEBF	2058.93±229.31	3444.86±346.31	3819.5±347.50	4355.2±579.65
MCDBF	5305.5±495.41	7201.33±228.16	8524.76±308.32	8709±350.67
MCDB	787.33±32.37	1386.43±133.97	1450.26±330.64	1694.23±341.01
PDBF	689.2±41.27	1163.5±143.52	1129.33±142.17	1420.66±296.19
PDB	7471.15±823.30	13440.5±682.42	14423±575.90	15552.53±692.69

Czapek's Dox broth (CDB); Modifed Czapek's Dox broth (MCDB); Czapek's Dox broth with fibrin (CDBF); Malt extract broth (MEB); Modifed Czapek's Dox broth with fibrin (MCDBF); Potato dextrose broth (PDB); Malt extract broth with fibrin (MEBF); Potato dextrose broth with fibrin (PDBF)

(v) Effect of media on the intra and extracellular protein content of G. lucidum

The mycelium grown in malt extract broth amended with fibrin showed the highest amount of extracellular proteins (8.53 µg/g. w. wt), on day 14 closely followed by malt extract broth (8.43 µg/g. w. wt), and the least protein content was observed in Czapek's Dox broth (3.26 µg/g. w. wt). The highest extracellular proteins were recorded in malt extract broth amended with fibrin (9.3 µg/mL) followed by MEB medium (8.2 µg/mL), MCDBF (8.0 µg/mL) and MCDB (6.5 µg/mL). The least extracellular protein content was observed in CDB medium (3.2 µg/mL; Table 4)

Table 4

Effect of media on intra (µg/g. w. wt) and extracellular (µg/mL) protein content of mycelium of G. lucidum

Media		Growth period (Days)			
		7	14	21	28
CDB	IP	1.6±0.36	3.26±0.58	1.3±0.20	1.23±0.24
	EP	1.9	3.6	3.2	3.0
CDBF	IP	1.81±0.34	3.6±0.41	2.23±0.46	1.63±0.29
	EP	2.3	4.6	5.2	4.3
MEB	IP	4.4±0.55	8.43±0.49	7.43±0.54	3.63±0.86
	EP	4.3	6.1	8.2	5.3
MEBF	IP	4.46±0.46	8.53±0.58	6.83±0.60	5.46±0.48
	EP	5.6	7.9	9.3	8.7
MCDBF	IP	3.4±0.46	5.53±0.64	4.86±0.64	4.26±0.53
	EP	4.2	8.2	8	7.5
MCDB	IP	2.1±0.63	4.4±0.40	3.73±0.49	2.9±0.81
	EP	5.3	7.9	6.5	7
PDBF	IP	4.5±0.34	7.36±0.38	6.8±0.36	4.2±0.55
	EP	4.3	6.5	6.2	6
PDB	IP	2.56±0.52	5.56±0.70	4.2±0.72	1.83±0.17
	EP	3.1	5.2	4.1	3.0

IP-Intracellular protein content; EP-Extracellular protein content

Czapek's Dox broth (CDB); Modifed Czapek's Dox broth (MCDB); Czapek's Dox broth with fibrin (CDBF); Malt extract broth (MEB); Modifed Czapek's Dox broth with fibrin (MCDBF); Potato dextrose broth (PDB); Malt extract broth with fibrin (MEBF); Potato dextrose broth with fibrin (PDBF)

(vi)Effect of different media on the intracellular and extracellular fibrinolytic protease activity of G. lucidum

The highest intracellular fibrinolytic activity was observed on day 21 in MCDBF medium (112.26 U/g. w. wt) followed by MEB (56.26 U/g. w. wt), MCDB (31.73 U/g. w. wt), CDBF (31.16 U/g. w. wt), PDBF (28.16 U/g. w. wt), CDB (27.56 U/g. w. wt), MEBF (14.6 U/g. w. wt) and the least activity was observed in PDB (9.5 U/g. w. wt). The intracellular activity started decreasing when the cultures were grown beyond day 21 (Table. 5).

Table 5.

Intracellular (U/g. w. wt) and extracellular (U/mL) fibrinolytic protease activities of G. lucidum grown in different media

Media		Growth period (Days)			
		7	14	21	28
CDB	IF	14.6±1.4	23.5±1.75	27.56±1.86	16.33±0.67
	EF	-	0.7±0.07	2.3±0.2	-
CDBF	IF	10.33±0.35	23.76±0.78	31.16±0.68	13.43±0.53
	EF	0.7±0.06	1.2±0.1	4.8±0.52	0.5±0.04
MEB	IF	24.93±0.52	43.6±1.13	56.26±1.18	29.26±1.18
	EF	-	1.0±0.1	2.1±0.21	-
MEBF	IF	10.6±0.56	12.06±0.52	14.6±0.4	9.03±0.31
	EF	3.2±0.35	5.2±0.46	7.8±0.85	2.3±0.23
MCDBF	IF	43±1.52	79.5±0.55	112.26±5.21	75.26±0.43
	EF	4.6±0.41	22.5±2.0	25.9±2.33	18.3±2.0
MCDB	IF	13.5±0.76	22.13±0.4	31.73±0.63	15.23±0.50
	EF	0.9±0.08	1.7±0.2	4.9±0.49	0.9±0.08
PDBF	IF	13.7±0.73	23.1±0.81	28.16±0.60	14±0.79
	EF	0.3±0.03	1.0±0.12	3.2±0.32	-
PDB	IF	2.5±0.28	4.73±0.63	9.5±0.32	4.16±0.44
	EF	-	0.9±0.1	1.3±0.11	-

IF-Intracellular; EF- Extracellular fibrinolytic protease activity

Czapek's Dox broth (CDB); Modifed Czapek's Dox broth (MCDB); Czapek's Dox broth with fibrin (CDBF); Malt extract broth (MEB); Modifed Czapek's Dox broth with fibrin (MCDBF); Potato dextrose broth (PDB); Malt extract broth with fibrin (MEBF); Potato dextrose broth with fibrin (PDBF)

The highest extracellular fibrinolytic protease activity was recorded on day 21 in MCDBF (25.9 U/mL) followed by MEBF (7.8 U/mL), MCDB (4.9 U/mL), CDBF (4.8 U/mL), PDBF (3.2 U/mL), CDB (2.3 U/mL), MEB (2.1 U/mL) and the least enzyme activity was observed in PDB (1.3 U/mL). The extracellular protease activity decreased progressively when the cultures were incubated beyond day 21 (Table.5). One general observation is that the intracellular fibrinolytic activity was higher in all the media than the extracellular activity.

The **ANOVA** performed on mycelial growth, protein content and fibrinolytic activity of *G. lucidum* in different media was found to be statistically significant at p<0.05 level which was inferred from the Fisher's F-test (F model, mean square regression/mean square residual of 104.632 for mycelial growth, 14.873 for protein content and 255.276 for fibrinolytic protease activity with a very low probability value [(P > F) = 0.000] (Table 6).

Table 6.

One way ANOVA for mycelial growth, intracellular protein content and fibrinolytic protease activity of G. lucidum in different media tested.

Media	Mycelial growth (mg/L)	Protein content (µg/g w. wt)	Fibrinolytic activity (U/g w. wt)
CDB	8239.23±582.25^{b}	3.26±0.58^{c}	27.56±1.86^{c}
CDBF	6242.6±572.09^{c}	3.6±0.41^{c}	31.16±0.68^{c}
MEB	14694.83±618.44^{a}	8.43±0.49^{a}	56.26±1.18^{b}
MEBF	4355.2±579.65^{d}	8.53±0.58^{a}	14.6±0.4^{d}
MCDBF	8709±350.67^{b}	5.53±0.64^{b}	112.26±5.21^{a}
MCDB	1694.23±341.01^{e}	4.4±0.40^{bc}	31.73±0.63^{c}
PDBF	1420.66±296.19^{e}	7.36±0.38^{a}	28.16±0.60^{c}
PDB	15552.53±692.69^{a}	5.56±0.70^{b}	9.5±0.32^{d}

Means sharing a common letter within the same column are not statistically significant at P<0.05 level (Duncan New multiple Range Test) Means followed by ± S.D

DISCUSSION

Higher fungi are abundant resources of a wide range of useful products and new compounds with interesting biological activities. There is a lack of investigation on the development of higher fungi bioprocess when compared to many of the filamentous fungi

such as *Penicillium* and *Aspergillus* spp[19]. The slow growth rate, and high viscosity resulted from the production of extracellular polysaccharide (EPS), are some of the factors which retards the study of large scale fermentation of higher fungi. In the present study, *Ganoderma lucidum* higher fungus has been studied for the optimal production of fibrinolytic protease, a key product/drug for the prevention of cardiovascular disease. The physical parameters like temperature, pH and nutritional factors such as carbon, nitrogen sources have been identified and standardized for optimal production of biomass and intracellular fibrinolytic protease. The results obtained have been discussed below.

The medium composition and design of fermentation condition play vital role in enhancing the efficiency of biomass and by-product production[20,21]. Out of eight media tested the maximum mycelial biomass of 15 g/L (Table 3) was obtained on day 21 of incubation when the test fungus *G. lucidum* was grown on potato dextrose broth. Nasreen *et al.* have also obtained a maximal mycelial biomass of 1.59/100 mL obtained on PDB medium[22].

All the 24 isolates were grown on PDA and found that the strain VK 12 isolated from *Tamarindus indica* showed faster growth rate and maximum protease production (Table 2). Further it has been studied that the intracellular enzyme activity (112.26 U/g. w. wt) was more when compared to extracellular activity (25.9 U/mL) on Modified Czapek's Dox agar with fibrin amended medium (Table 5). It has been suggested that the lesser extracellular activity may be due to certain protease inhibitors produced by *G. lucidum* itself [23]. Therefore, in the present study, intracellular enzyme production has been studied and characterized in detail.

CONCLUSION

The result of this study clearly indicates that the optimal production of the active enzyme was obtained by using fibrin amended PDA and this media can be used for further large scale production and purification of this potential enzyme.

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REFERENCES

1. Blann, A.D., Landray, M.J., Lip, G.Y., ABC of antithrombotic therapy: an verview of antithrombotic therapy. BMJ 325, 762–765. (2002).
2. Bortoleto, R.K., Murakami, M.T., Watanabe, L., Soares, A.M., Arni, R.K.,. Purification, characterization and crystallization of Jararacussin-I, a fibrinogen-clotting enzyme isolated from the venom of Bothrops jararacussu. Toxicon 40, 1307–1312, (2002).
3. Hrzenjak, T., Popovic, M., Bozic, T., Grdisa, M., Kobrehel, D., Tiska-Rudman, L.,.Fibrinolytic and anticoagulative activities from the earthworm Eisenia foetida. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 119, 825–832,(1998).

4. Ahn, M.Y., Hahn, B.S., Ryu, K.S., Kim, J.W., Kim, I., Kim, Y.S., Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, *Catharsius molossus*. Thromb. Res. 112, 339–347, (2003).
5. Hua, Y., Jiang, B., Mine, Y., Mu, W., Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste. J. Agr. Food Chem. 56, 1451–1457, (2008).
6. Borchers, A.T., Stern, J.S., Hackman, R.M., Keen, C.L., Gershwin, E.M.,. Mushrooms, Tumors, and Biologically Active Substances. Pergamon Press, New York, USA. (1999).
7. Hseu, R.S., Wank, H.H., Wang, H.F., and Moncalvo, J.M., Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by Random Amplified Polymorphic DNA-PCR compared with grouping on the basis of International Transcribed Spacer Sequences. Appl. Environ. Microbiol. 62; 1354-1363, (1996).
8. Jong, S.C., and Brimogham, J.M., Medicinal benefits of the mushroom *Gaoderma.* Adv. Appl. Microbiol. 37; 101-134, (1992).
9. Nishitoba, T., Sato, H., Sakamura, S., Triterpenoids from the fungus *Ganoderma lucidum*. Phytochemistry 26, 1777–1784, (1987).
10. Choi, H. S., Sa, Y. S. Fibrinolytic and antithrombotic protease from *Ganoderma lucidum.* Mycologia 92; 545–552, (2000).
11. Samiyappan, R., Bhaskaran, R., and Retinan, P. Diagnosis for early detection of *Ganoderma* diseases in perennial crops: a approaches and prospects. J. Plant. Dis. Phol. 103 (1); 85-93, (1996).
12. Adaskaveg, J.K and Glibertson, R.L., and Blanchette, R.A., Comparative studies of delignification caused by *Ganoderma* species. Appl. Environ.Mycrobiol. 56; 1932-1943, (1990).
13. Manoharan and Leelavathi "Polypores of Kerala" (1952)
14. Bradford, M.M., A rapid and sensitive method for the microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72; 248–254, (1976).
15. McDonald, C.E., and Chen, L.L., The lowry modification of the folin reagent for determination of protease activity. Anal.Biochem.10; 175-177, (1965).
16. Rajamani, S., and Hilda, A., Plate assay to screen fungi for proteolytic activity. Current Sci. 20 (56); 1179-1181, (1987).
17. Astrup, T., Mullertz, S., The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. Biophys. 40, 346–351, (1952).
18. Greenberg, D.M., Methods in Enzymol. 2; 54-64, (1957).
19. Tang, Y.J., Zhong, J.J., Submerged fermentation of higher fungi for production of valuable metabolites. In: Zhong J-J, editor. Advances in applied biotechnology (in English). Shanghai: East China University of Science and Technology . 104–116, (2000).
20. Yang, F.C, Liau, C.B. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged fermentations. Process Biochem. 33; 547–53, (1998).
21. Fang, Q.H., Tang, Y.J., and Zhong, J.J., Significance of inoculation density control in production of polysaccharide and ganoderic acid by submerged culture of *Ganoderma lucidum.* Process Biochem. 37; 1375–1379, (2002).
22. Nasreen, Z., Kausar, M., Nadeem and Bajwa, R. Study of different growth parameters in *Ganoderma ludium*.

Micologia. Aplicada. International. 17(1); 5-8, (2005).

23. Tian, Y.P., and Zhang, K.C.. Purification and characterization of a novel protease A inhibitor from *Ganoderma lucidum* by submerged fermentation. Enzyme and Microbiol. Techonol. 36; 357-361, (2005).