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Growth Optimization Conditions for the Production of Fibrinolytic Enzyme from *Ganoderma lucidum*

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Abstract

Ganoderma lucidum collected and isolated from south eastern part of India screened to produce fibrinolytic enzyme was conserved under laboratory condition to stablize or enhance the enzyme production. The fibrinolytic enzyme producing mushroom *Ganoderma lucidum* was grown under different pH, temperature, carbon and nitrogen sources to optimize the growth conditions required for enzyme production. The optimized conditions for fibrinolytic enzyme production was found to be pH at 5.5 (106 U/g. w.wt), Temperature at 30°C (88.17 U/g. w.wt), Carbon source - Glucose (143.43 U/g. w.wt) and Nitrogen source- Peptone (162.17 U/g. w.wt).

1. Introduction

Fibrinolytic enzymes are agents that dissolve fibrin clots extracted from microbes were considered to be a potential thrombolytic agent for the treatment of clot-dissolving in the cases of Myocardial infarction [1]. Over the past decades, many derived microbial fibrinolytic enzymes have been discovered in various traditional fermented foods [2, 3 and 4], in which mushrooms have captured the attention of a large number of investigators, because of their nutritional and economic value. In addition to that they have high protein content and a variety of proteins that are yet to be discovered. Lectins [5], Laccase [6] and Haemolysin [7] were some examples of biologically active mushroom proteins that have potential application in both medicinal and industrial sectors. Fibrinolytic enzyme from mushrooms are reported from a few medicinal and edible mushrooms such as, *Flammulina velutipes* [8], *Cordyceps militaris* [4], and *Pleurotus ostreatus* [9], *Grifola frondosa* and *Armillaria mellea* [10] respectively. Thus, it would be worthwhile when undertaking a work to isolate fibrinolysin from another species like *Ganoderma lucidum*. *Ganoderma lucidum* is an important non-toxic medicinal mushroom that has been used in folk medicine for hundreds of years and commercially cultivated for preparation of health tablets [11]. The present investigation would add up biological data to this medicinal mushroom that increase its economic importance. Hence, in the present study *Ganoderma lucidum* isolated from south eastern part of India was optimized for its fibrinolytic enzyme production under the laboratory condition could be used for characterization in detail.

2. Materials and Methods

Mushroom

The basidiocarps of *Ganoderma lucidum* collected from south eastern part of India, Chennai. Identification and classification were carried out and the specimens were deposited at the laboratory, CAS in Botany, University of Madras, Guindy Campus, Chennai - 25.

Assay of fibrinolytic activity

Fibrinolytic protease activity was carried out according to the method described by Greenberg [12]. 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 [13]. 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.

Effect of different ph and temperature on fibrinolytic activity

Fibrinolytic activity of *Ganoderma lucidum* grown under different pH and temperature in Modified

Czapek's Dox Broth with Fibrin (MCDBF) were assayed. The different pH levels from 3.5 to 8.5 at varying intervals of 0.5 pH and different temperatures ranging from 20°C to 45°C at varying intervals of 5°C were inoculated with Ganoderma lucidum culture and incubated for 28 days and their fibrinolytic activity was assayed at every 7 days interval.

Effect of different carbohydrate and nitrogen sources on fibrinolytic activity

Ganoderma lucidum grown on Modified Czapek's Dox Broth with Fibrin (MCDBF) amended with different carbon sources (Glucose, Fructose, Sucrose, Maltose, Lactose, Starch and Cellulose) and nitrogen sources (Yeast Extract, Beef Extract, Malt Extract, Peptone, Casein and Gelatin) prepared were inoculated with *Ganoderma lucidum* and and incubated for 28 days and their fibrinolytic enzyme activity was assayed at every 7days interval.

Statistical analysis

The values were expressed as mean ± standard deviation and one-way analysis of variance (ANOVA) using SPSS/12.0 student software followed by least significant difference (LSD) test. Difference between means at the 5% (P- values <0.05) were considered to indicate a statistical significance.

3. Results and Discussion

The mycelia homogenate obtained in different parameters were used to estimate the fibrinolytic protease activity.

Effect of different pH and temperature on fibrinolytic protease activity of *Ganoderma lucidum*

Relatively higher intracellular fibrinolytic protease activity was observed on day 21 irrespective of the pH's used. The highest fibrinolytic protease activity was measured in mycelia grown in medium incubated at pH 5.5 (106 U/g. w. wt) followed by pH's 4.5 (90.83 U/g. w. wt), 6.5 (90.53 U/g. w. wt), 3.5 (63.13 U/g. w. wt), 7.5 (25.43 U/g. w. wt) and the least protease activity was recorded in medium incubated with a pH of 8.5 (20.43 U/g. w. wt). There was a decrease of enzyme activity by 50 and 20% when the cultures at pH 5.5 were incubated for day 28 and 14 respectively. The results were shown in Figure 1.

Figure 1. Effect of pH on the intracellular fibrinolytic protease activity (U/g.w.wt) of *Ganoderma lucidum*

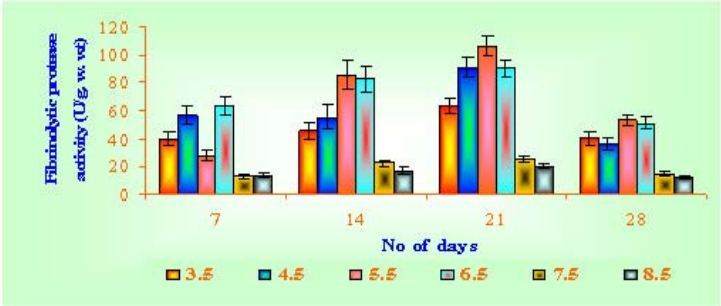
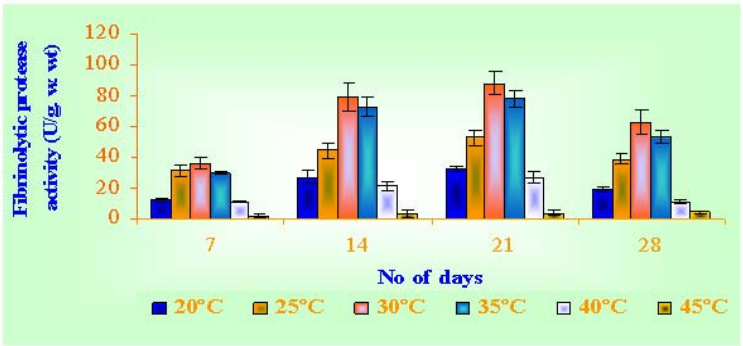


Figure 2 illustrates the fibrinolytic protease activity of mycelia grown at different temperatures. There was a relatively higher protease activity in mycelia grown at 30ºC when compared to the ones grown at 20,25,35,40 and 45ºC. The mycelia grown at 30ºC showed the highest enzyme activity (88.17 U/g w. wt) followed by 35ºC (78.20 U/g w. wt), and 25ºC (52.53 U/g w. wt). The least enzyme activity was observed in mycelium grown at 45°C (3.7U/g w. wt) which was about 88% decrease over the one observed at 30ºC.

Figure 2. Effect of temperature on the intracellular fibrinolytic protease activity (U/g.w.wt) of *Ganoderma lucidum*

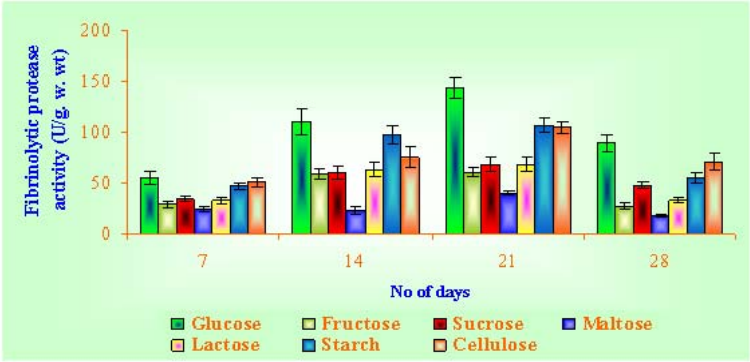


The effect of different pH on the enzyme activity (fibrinolytic protease) shown in Figure 1. clearly demonstrate that pH of 5.5 favors higher fibrinolytic activity. Similar to that Fang *et al.*, [14] have also observed that the initial pH of 5.5 was optimal for metabolite productions. Besides the pH, temperature also controls the metabolic activity, membrane stability and nutrient uptake. The optimum temperature recorded for fibrinolytic activity of *Ganoderma lucidum* was higher than soil inhabiting fungus *Fusarium pallidoroseum* showing maximum fibrinolytic activity at 25°C [15].

Effect of different carbon and nitrogen sources on fibrinolytic protease activity of *Ganoderma lucidum*

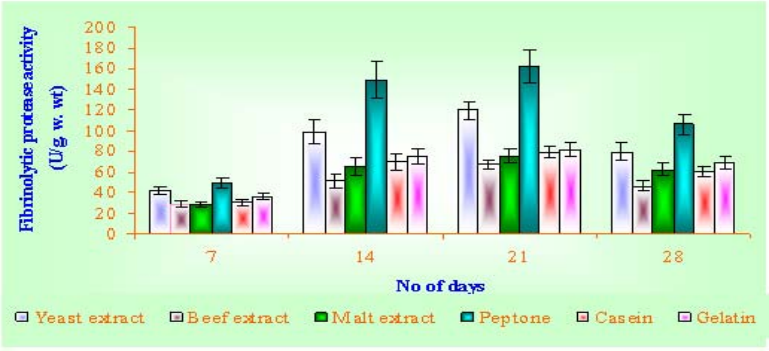
There was a relatively higher fibrinolytic protease activity measured in media amended with all the carbon sources on day 21 than the ones grown for 7, 14 and 28 days. However, the medium amended with glucose showed the highest intracellular fibrinolytic protease (143.43 U/g. w. wt) activity among all the carbon sources used which was followed by the media amended with starch (107.16 U/g. w. wt), cellulose (105.16 U/g. w. wt), lactose (68.43 U/g. w. wt), sucrose (68.3 U/g. w. wt) and fructose (61.16 U/g. w. wt). The least fibrinolytic activity was observed in medium amended with maltose (40.1 U/g. w. wt; Table 20). There was a 72% decrease in enzyme activity in medium amended with maltose when compared to the one medium with observed in glucose.

Figure 3. Effect of Carbon source on the intracellular fibrinolytic protease activity (U/g.w.wt) of *Ganoderma lucidum*



The intracellular fibrinolytic protease activity measured in mycelia grown in medium supplemented with different nitrogen sources was shown in the figure 4. The fibrinolytic protease activity reached its maximum on day 21 irrespective of the nitrogen sources used. The highest enzyme activity was observed in mycelia grown with peptone (162.17 U/g. w. wt) followed by yeast extract (119.5 U/g. w. wt), gelatin (80.7 U/g. w. wt), casein (78.8 U/g. w. wt) and malt extract (75.5 U/g. w. wt). The least enzyme activity was observed in mycelia grown in medium with beef extract (66.06 U/g. w. wt), which had 58% decrease in activity over what is observed with peptone (162.17 U/g. w. wt).

Figure 4. Effect of Nitrogen source on the intracellular fibrinolytic protease activity (U/g.w.wt) of Ganoderma lucidum



Carbohydrates are the important energy source for the higher fungi. Deficiency or excess of these sources directly influence the production of biomass. The test fungus showed more enzyme activity in the glucose amended medium. Kim *et al.*, [4] have recorded more fibrinolytic activity with *Cordyceps millitaris*, when glucose was used as carbon source. *Fusarium species* and *Penicillium chrysogenum* has

also preferred glucose for maximum fibrinolytic activity. Most of the higher fungi prefer organic and ammonical nitrogen sources than inorganic nitrogen sources [16]. In the present study, among the different nitrogen sources used, peptone supported more fibrinolytic activity [17].

The optimal conditions for fibrinolytic enzyme production by *Ganoderma lucidum* in this study clearly showed that the fibrinolytic activity is dependent on pH, temperature and nutritional sources like carbohydrate and nitrogen. In future, the developed optimum conditions showed fibrinolytic activity applied in fermentation technology, for mass production and characterization of the fibrinolytic enzyme that may reduce the cost and availability of the enzyme.

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