International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol. 3, No.3, pp 1563-1570, July-Sept 2011

Nutrient Composition on the Effect of Extracellular Peroxidase Production by *Lentinus kauffmanii* - under Submerged Culture Condition

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Abstract: An indigenous wood rotting edible *Lentinus kauffmanii* isolated from Keeriparai forest of Western Ghats was studied for extracellular peroxidase production. Peroxidases are major extracellular lignolytic enzymes involved in lignocellulosic degradation. Physical factors such as pH, temperature and nutritional factors such as carbon, nitrogen sources and amino acids were standardized for the growth and enzyme production of extracellular peroxidase by *L. kauffmanii*. The optimum pH for both the biomass and the peroxidase production was found to be pH 6.5. Of the different temperature (20°C, 25°C, 30°C, 35°C and 40°C) tested optimal peroxidase activity (44.595 ± 0.674 UmL^{-1}) was recorded at 25°C. Among the different carbon sources, fructose supported maximum peroxidase activity (53.516 ± 0.952 UmL^{-1}), while peptone supported the optimal activity (54.454 ± 0.454 UmL^{-1}) among the different nitrogen sources tested. Of the different amino acids tested, alanine (54.981 ± 0.776 UmL^{-1}) enhanced the maximum enzyme activity.

Key words: Peroxidase, *Lentinus*, Physiological regulation, Lignocellulosic.

1. Introduction

Basidiomycetes comprise very different ecological groups that may ensure their nutrition in different ways. Some of them are edible and medicinal fungi; some have biotechnological and environmental applications. Of which, white-rot basidiomycetes are the only group of organisms that are capable of degrading all basic wood polymers due to their capability to synthesize the relevant hydrolytic (cellulases and hemicellulases) and the unique oxidative (ligninolytic) extracellular enzymes, which are responsible for the degradation of major components in the substrate (cellulose, hemicelluloses) and lignin into low-molecular weight compounds so as to assimilate by fungi and higher plant for their nutrition. [1] The lignin degrading system of this fungi have potential applications in biopulping and bioleaching [2] and the biodegradation of xenobiotic pollutants.[3] The extracellular enzymes that are involved in the initial steps of lignin degradation by white-rot fungi are lignin peroxidases (LiP), manganese-dependent peroxidase (MnP), laccase and H_2O_2 producing oxidases (e.g. aryl alcohol oxidase and

glyoxal oxidase). [4, 5] The potential application of ligninolytic enzymes in biotechnology has stimulated the investigation of selecting the most promising enzyme producers.

The understanding of physiological mechanisms regulating enzyme synthesis in lignocellulose bioconversion could be useful for improving the technological process of edible and medicinal mushroom production. The physiology of ligninolytic enzymes have been extensively studied using submerged and solid-state fermentation of lignocellulosic substrates. [6, 7, 8, 9] It is worth noting that the effect of these enzymes depends not only on the fungal physiology but also on the composition of cultivation medium. Many previous studies have proved that both the nature and the concentration of nitrogen, carbon, amino acids and vitamin sources are powerful nutrition factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes. [2, 10, 11, 12] The structural and catalytic properties, molecular genetics and biotechnological applications of lignin-degrading oxidases have recently been comprehensively reviewed [13, 14, 15, 16] whereas an exhaustive overview of the basic aspects of the microbiology of ligninolytic enzyme production is still lacking in the literature. The effects of complex nitrogen sources on laccase and MnP production have been investigated in numerous white-rot fungi such as *Trametes versicolor, T. pubescens, T. gallica*[10, 17, 18.]

In the present study, unique properties of aboriginal *L. kauffmanii* as well as several approaches providing enhanced secretion of peroxidase under submerged culture conditions were documented.

2. Materials and methods

2.1. Organism and inoculum preparation

Fruiting body of the *L. kauffmanii* was isolated from Keeriparai forest of Western Ghats, Tamil Nadu, India, and the culture was maintained on malt-extract agar medium at 4°C. Inoculum of *L. kauffmanii* was prepared from mycelia grown on the same media incubated at 25°C for 4–6 days. From the plate 7 mm diameter mycelial disc were used as the inocula.

2.2. Culture conditions

Incubation was carried out statically at 25 ± 1°C in 125 ml Erlenmeyer flask containing 50 ml of the medium inoculated with 7mm agar plug from 6 day old mycelia grown on malt-extract agar. Periodic harvesting of the mycelia was performed using the filter paper. A aliquots of supernatant was collected aseptically and culture filtrates were used as enzyme sources.

Optimization of peroxidase production by *L. kauffmanii* was studied using different medium *viz.,* Potato-dextrose broth, Malt-yeast extract broth, Czapek-dox broth, Modified Melin Norkrans broth, Carrot-potato broth, Yeast-peptone broth and Glucose-malt extract salt medium. Various carbon sources such as fructose, sucrose, mannitol, lactose, starch; nitrogen sources such as beef extract, peptone, ammonium chloride, ammonium nitrate and ammonium tartarate; amino acids such as glycine, asparagine, alanine, tyrosine and phenylalanine were used. Optimization of physiological parameters such as pH (4.0–9.0) and temperature (20–40°C) were carried out. All chemicals used in this research were of analytical grade and were used without further purification.

2.3. Analytical determinations

The extracellular peroxidase activity of cell free filtrate was assayed spectrophotometerically from day 3 to day 21. Increase in the absorbance was measured at 414 nm using 1.7 mM ABTS, 2.5 mM hydrogen peroxide and 0.1 ml of culture filtrate. Reaction mixture without culture filtrate served as the blank. One unit of peroxidase enzyme activity was defined as the change in absorbance of 1.0 /ml/min at 414 nm [19].

The experiments were performed at least two times using three replicates. The data presented in the tables correspond to mean values with standard deviation.

3. Results and discussion

Plate assay method conducted on *L. kauffmanii* showed the maximum size of bluish green zone around the mycelium with ABTS and reddish brown colored zone appeared in Guiacol amended medium.

3.1. Effect of different types of media on peroxidase production *L. kauffmanii*

L. kauffmanii showed maximum enzyme production (39.39 ± 1.04 UmL^{-1}) between 17 and 19 days of incubation period in malt yeast extract broth than the other six types of media used. The results show that extracellular peroxidase secretion started from day 3 after inoculation and continued to increase till day 19 (**Table 1).** Later, the enzyme activity gradually diminishes at the end. Higher level of peroxidase enzyme secretion in malt yeast broth may be due to the presence of complete pool of amino acids required for enzyme synthesis. [20] Moreover, malt extract which is rich in the aromatic amino acids tryptophan and tyrosine, tryptophan act as a precursor for the synthesis of a large number of nitrogen substituted aromatic secondary metabolites of fungi [21].

Table 1. Effect of different nutrient medium on production of extracellular peroxidase by *L. kauffmanii*.

Sampling interval	MYA	PDA	MMN	CPA	YPG	GMSM	CDA
Day 3	2.74±0.073	2.24±0.015	2.20±0.70	2.051±0.06	1.08±0.23	2.29±0.059	1.36±0.13
Day 5	4.35±0.28	4.94±0.34	3.93±0.059	4.42±0.059	2.55±0.15	2.73±0.029	1.90±0.15
Day7	6.15±0.59	4.25±0.12	4.54±0.32	5.10±0.12	4.29±0.31	3.47±0.05	2.70±0.12
Day 9	10.11±0.12	5.41±0.51	8.38±0.47	7.06±0.29	4.73±0.25	5.45±0.59	5.19±0.47
Day 11	16.32±0.23	11.95±0.35	10.32±0.59	7.96±0.10	6.96±0.31	12.67±1.19	11.52±0.23
Day 13	27.79±0.66	20.80±1.08	15.08±0.45	17.18±1.22	9.57±0.75	21.71±1.70	14.43±1.34
Day 15	30.90±1.25	22.69±0.72	17.85±0.76	**31.65±0.29**	19.60±0.53	30.69±0.05	2.88±0.94
Day 17	**39.39±1.04**	28.34±0.67	24.95±1.22	30.06±0.10	30.43±2.77	**31.38±0.34**	**25.90±3.55**
Day 19	37.27±0.32	**28.53±0.48**	**28.26±2.07**	31.13±0.073	**31.53±0.35**	29.74±0.35	25.90±0.12
Day 21	35.13±0.41	28.58±1.04	24.58±1.49	27.38±0.13	29.71±0.38	28.06±0.37	24.77±0.57

Values are mean, standard deviation and standard error of three replicates and the bolded values represent high activity

Table 2. Effect of different temperature on production of extracellular peroxidase by *L. kauffmanii*.

Sampling interval	20°C	25°C	30°C	35° C	40°C
Day 3	2.79±0.10	4.43±0.029	4.26±0.16	3.46±0.38	3.21±0.22
Day 5	4.63±0.38	6.46±0.48	6.54±0.59	3.46±0.38	5.48±0.29
Day7	6.39±0.12	7.78±0.16	9.33±0.66	5.70±0.45	6.56±.085
Day 9	9.96±0.33	11.79±0.66	12.32±0.30	8.45±0.044	10.07±0.34
Day 11	13.82±0.28	15.80±0.05	20.08±0.60	10.70±0.63	12.53±0.80
Day 13	16.28±0.78	19.53±1.10	23.35±0.82	11.75±0.38	18.17±1.58
Day 15	**30.56±0.94**	35.80±0.85	38.03±0.32	21.16±1.06	24.86±1.30
Day 17	29.77±1.61	**44.56±0.67**	**47.79±0.82**	34.24±4.12	**34.63±0.15**
Day 19	27.40±0.50	41.73±0.10	45.77±0.67	**38.74±0.29**	31.90±9.11
Day21	27.45±0.15	38.20±1.40	44.23±1.86	32.38±1.45	25.78±1.03

Values are mean, standard deviation and standard error of three replicates and the bolded values represent high activity

Table 3. Effect of different pH on production of extracellular peroxidase by *L. kauffmanii*.

Sampling interval	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.5	pH 9.0
Day 3	2.48±0.25	3.90±0.50	3.65±0.84	4.40±0.29	4.10±0.22	5.40±0.16	4.03±0.31	4.03±0.31	2.89±0.20	2.67±0.16	3.24±0.54
Day 5	4.54±0.15	6.83±1.23	6.46±1.16	6.92±0.38	8.59±0.15	9.89±0.98	7.06±0.82	4.80±0.32	5.35±0.43	4.22±0.91	3.84±0.10
Day 7	8.04±0.25	9.07±1.04	8.70±0.47	8.72±0.63	10.73±0.29	13.65±1.88	8.86±1.19	8.16±0.72	7.15±0.8	7.94±0.38	6.65±0.79
Day 9	17.11±0.38	17.10±1.63	9.50±1.86	20.35±0.51	22.46±1.77	24.12±2.46	17.86±2.65	14.40±2.13	12.41±0.93	12.88±1.28	11.21±0.07
Day 11	18.42±2.39	19.52±0.09	23.26±3.49	22.15±0.06	26.53±5.67	28.86±3.81	23.82±2.35	21.07±3.34	15.88±3.08	18.90±6.59	14.36±2.23
Day 13	20.47±2.42	23.19±3.18	30.28±0.98	25.83±0.54	32.46±4.54	37.10±4.07	28.25±3.14	25.21±3.03	15.88±3.08	21.42±6.95	20.83±5.66
Day 15	25.01±1.30	30.24±2.40	32.89±0.80	33.40±4.19	35.50±4.88	40.21±5.64	31.95±4.76	27.57±1.64	23.62±5.68	24.06±7.74	22.58±6.40
Day 17	**32.39±0.10**	**35.29±1.34**	**36.16±2.43**	37.33±3.80	**45.37±3.15**	**47.77±2.33**	**36.64±3.85**	31.19±7.10	**29.05±9.63**	**28.35±11.88**	**26.20±10.91**
Day 19	27.06±0.19	31.56±1.17	34.02±1.61	**39.15±5.57**	35.59±8.22	44.04±2.46	33.87±6.36	**33.34±9.76**	26.66±8.79	26.71±10.74	21.51±7.50
Day 21	23.40±0.13	26.33±0.57	27.37±0.59	32.57±0.19	32.70±8.09	36.64±6.31	27.51±3.69	30.75±6.31	21.64±5.03	22.85±10.37	21.70±7.81

Values are mean, standard deviation and standard error of three replicates and the bolded values represent high activity

This medium then act as inducers for MnP in a way similar to veratryl alcohol and guaiacol, which are substrates as well as inducers for ligninase and laccase. The result obtained are *on par* with earlier studies where the addition of tryptophan to culture of some white-rot fungi increases the production of lignin peroxidise. [22] Studies on the production of ligninolytic enzymes have been carried out with defined media indicate that basidiomycetes display a wide diversity in their response to carbon sources and their concentration in nutrient medium.[9,10,23,24, 25] In *Phanerochaete chrysosporium*, the ligninolytic gene expression is triggered only by the depletion of nutrient carbon.[25]

3.2. Effect of pH and temperature

To determine the optimal pH and incubation temperature for maximum production of peroxidase, the test fungus was incubated at different pH such as 4.0, 4.5, 5.0 up to 9.0 and at various temperatures of 20°C, 25°C, 30°C, 35°C and 40°C. These two experiments were carried out separately.

L. kauffmanii showed highest level of peroxidase enzyme activity at 25ºC on day 17 (44.595 ± 0.674 UmL^{-1}). The lower level of peroxidase enzyme activity (30.56 ± 0.938 UmL^{-1}) was obtained at 20ºC (**Table 2**) which is in consensus with earlier findings *Phellium* sp. *Pleurotus* sp. *and Coprinus oinereus*.[26-29] Similarly, according to Gill and Arora,[30] in *Coriolus versicolor*, and *P. chrysosporium* the optimal activity of the enzyme was expressed at 25–30ºC and was completely lost above 35ºC except with *Polyporus sanguineus* where no activity was observed above 30ºC.

Maximum enzyme activity was observed in pH 6.5 on day 17 (47.77 ± 2.33 UmL^{-1}), while in the following pH 5.0, 5.5 6.0, 6.5, 7.0, 7.5 and 8.0 (Table. 3) moderate activity was observed. The lower level of enzyme activity (26.15 ± 10.92 UmL^{-1}) was observed in pH 9.0. Optimum enzyme production occurred at pH 6.5 by the test organism, however, under more acidic pH like 4.0 – 5.0 the enzyme was less active. Interestingly the test organism produces large amount of enzymes in alkaline condition at late phase of growth and the enzyme activity persisted up to day 17 and 19.This is an advantageous character and can be applied in the detergent industry. But this phenomenon did not occur at optimal pH condition (pH 6.5).

Similar observation was made by Stajic *et al*.[31] in *Pleurotus* sp. that produced maximum peroxidase and laccase activity at the pH of 6.0. While in *Bjerkandara* sp. strain B055, the maximum enzyme activity was reported at pH 5.2 (60 U/L)[32]. Similar results were observed with *Coprinus cinereus* Sakurai *et al*.[27] where maximum enzyme production was at neutral pH (7.0). *Pleurotus dryinus, Phellinus robustus and Ganoderma adspersum* also secrete laccase and MnP in the medium at pH 6.5[1,24].

3.3. Effect of carbon and nitrogen

The peroxidase production was found to vary with the different carbon sources. The maximum enzyme production (53.516 ± 0.952 UmL^{-1}) was recorded on 19th day of incubation with fructose at the concentration of 0.1% in the medium. Moderate levels of enzyme activities were obtained with mannitol, lactose, sucrose and starch (**Table 4**). On the other hand, various monosaccharide carbon sources and carboxyl methyl cellulose did not favour for peroxidase production. Glucose supported only 10-14 UmL^{-1} enzyme (by *Coprinus* sp. UAMH10067) and 20-28 UmL^{-1} (by *Coprinus cinnereus* UAMH 4103). Similar trend was also noted by Ikehata & Buchanan[26] and Ikehata *et al*.[28]. On the other hand, Tuncer *et al*.,[33] reported the enhancement of peroxidase production in *T. fusca* by xylan (5.6 UmL^{-1}). The lignolytic enzyme activity obtained in cultivation of *Pleurotus sajor-caju* in media containing 0.5 gl^{-1} fructose or glucose (37 UmL^{-1} and 36 UmL^{-1}, respectively) were significantly higher than those obtained with lactose[34]. Quite the contrary, lactose appeared to be the best carbon source for the lignocellulosic enzyme secretion by *P.gibbosa*. Lignolytic enzyme activity of white-rot fungus WR-1 varied from 44 UmL^{-1} with fructose and 170 Uml^{-1} with starch as sole carbon source[35]. These observations indicate that specific carbohydrates appear to regulate the peroxidase production in particular white-rot basidiomycetes maximally.

Among the various organic and inorganic nitrogen sources, the maximum enzyme activity (54.454 ± 0.454 UmL^{-1}) was recorded when peptone was used in the medium at the concentration of 0.1%, followed by beef extract (**Table 5**). Previous studies have proved that both the nature and the concentration of nitrogen sources are powerful factors regulating lignolytic enzyme production by wood-rotting basidiomycetes[6,9,10]. Glutamic acid followed by peptone, resulted the best nitrogen sources for laccase and MnP production under submerged culture[12]. While various inorganic nitrogen sources were tested, the peroxidase production was found repressed than organic sources. Similar results were also obtained by Stajic *et al*.[31] with *P. eryngii* 616, *P. ostreatus* 493 and *P. ostreatus* 494 and also by Ikehata *et al.*[28] with the *Coprinus* sp. On the other hand, Stajic *et al*.[31] reported that enhancement of peroxidase production in *P. pulmonarious* by inorganic nitrogen sources like KNO_3 and $NH_4H_2PO_4$.

Table 4. Effect of different carbon sources on production of extracellular peroxidase by *L. kauffmanii.*

Sampling interval	Control	Fructose	Maltose	Lactose	Starch	Sucrose
Day 3	1.63 ± 0.07	3.88 ± 0.07	4.38 ± 0.31	4.59 ± 0.31	4.41 ± 0.43	2.67 ± 0.26
Day 5	4.35 ± 0.28	5.17 ± 0.25	7.78 ± 1.48	6.65 ± 1.35	6.59 ± 1.00	7.93 ± 0.37
Day 7	5.04 ± 1.70	9.77 ± 1.33	10.33 ± 1.89	8.81 ± 0.92	9.86 ± 1.60	8.51 ± 0.48
Day 9	10.11 ± 0.12	13.39 ± 0.9	41.87 ± 1.76	13.51 ± 0.70	11.94 ± 1.98	13.92 ± 2.55
Day 11	16.32 ± 0.23	24.06 ± 1.90	23.35 ± 4.92	23.13 ± 2.18	23.04 ± 2.89	21.33 ± 5.25
Day 13	27.79 ± 0.66	28.93 ± 3.65	24.03 ± 3.93	30.36 ± 6.21	26.72 ± 2.96	25.29 ± 2.34
Day 15	30.90 ± 1.25	40.86 ± 4.56	34.78 ± 3.31	43.14 ± 2.15	38.27 ± 3.05	32.66 ± 2.51
Day 17	**39.9 ± 1.04**	50.35 ± 22.24	**42.10 ± 3.81**	49.75 ± 5.04	**43.12 ± 2.89**	39.31 ± 2.39
Day 19	37.27 ± 0.32	**51.00 ± 1.19**	39.03 ± 2.40	**53.52 ± 0.95**	38.53 ± 1.88	**39.91 ± 3.02**
Day 21	35.13 ± 0.41	50.70 ± 0.98	36.63 ± 6.56	48.16 ± 1.16	38.87 ± 2.36	36.96 ± 2.97

Values are mean, standard deviation and standard error of three replicates and the bolded values represent high activity

Table 5. Effect of different nitrogen sources on production of extracellular peroxidase by *L. kauffmanii.*

Sampling interval	Control	Peptone	Amm.chloride	Amm.tartarate	Beef extract	Amm.nitrate
Day 3	1.63 ± 0.07	5.05±0.43	3.87±0.47	4.15±0.60	4.60±0.12	3.7±0.26
Day 5	4.35 ± 0.28	7.75±1.22	5.95±0.65	6.12±0.53	5.67±1.36	5.49±0.37
Day 7	5.04 ± 1.70	11.81±1.82	9.13±0.69	9.87±1.38	15.35±4.16	8.32±0.15
Day 9	10.11 ± .12	16.31±3.06	15.81±3.85	20.63±2.32	20.01±2.32	17.29±4.25
Day 11	16.32 ± .23	23.25±4.59	19.62±3.91	23.43±1.71	24.98±2.71	22.63±3.30
Day 13	27.79 ±0.66	28.35±4.59	27.53±1.51	30.18±2.26	28.79±2.12	27.40±2.11
Day 15	30.90 ±1.25	40.24±0.86	34.71±2.42	36.63±1.41	42.73±2.89	41.08±4.57
Day 17	**39.9 ± 1.04**	46.97±1.82	43.42±2.20	**41.92±2.42**	**51.82±1.95**	41.14±4.28
Day 19	37.27 ±0.32	**54.45±0.45**	**45.97±2.93**	39.97±1.35	51.06±1.25	39.41±3.52
Day 21	35.13 ±0.41	48.64±1.55	42.16±3.55	38.41±1.85	48.96±2.55	**48.42±0.45**

Values are mean, standard deviation and standard error of three replicates and the bolded values represent high activity

Table 6. Effect of different amino acids on production of extracellular peroxidase by *L. kauffmanii.*

Sampling interval	Control	Alanine	Glycine	Asperagine	Tyrosine	Phenylalanine
Day 3	1.63 ± 0.07	3.78±0.41	2.68±0.63	2.78±0.56	3.68±0.80	2.73±0.26
Day 5	4.35 ± 0.28	4.63±0.56	4.79±0.43	5.12±0.31	4.53±0.72	4.26±1.16
Day7	5.04 ± 1.70	7.25±0.48	7.08±0.87	6.23±1.34	6.73±1.25	6.71±0.41
Day 9	10.11 ± 0.12	20.01±3.64	13.05±1.16	12.15±0.66	14.33±2.12	11.34±0.12
Day 11	16.32 ± 0.23	24.92±4.32	22.42±2.12	23.69±2.68	24.19±4.47	21.49±1.28
Day 13	27.79 ± 0.66	34.37±1.11	34.39±2.42	26.56±2.64	31.98±4.56	33.84±2.99
Day 15	30.90 ± 1.25	45.14±0.80	39.921±1.42	36.85±1.16	40.19±2.68	42.93±4.34
Day 17	**39.39 ± 1.04**	**54.98±0.77**	46.85±0.58	**51.48±0.88**	47.25±1.83	49.64±1.73
Day 19	37.27 ± 0.32	51.82±0.22	**48.48±4.38**	47.54±0.89	**52.19±1.57**	**50.63±0.32**
Day 21	35.13 ± 0.41	49.45±0.34	43.29±2.36	45.04±0.62	46.85±1.73	48.13±1.60

Values are mean, standard deviation and standard error of three replicates and the bolded values represent high activity

Addition of various amino acids in the medium affect the peroxidase production. The higher enzyme production (54.98 ± 0.78 UmL^{-1}) was recorded on 17th day of incubation with alanine at the concentration of 0.01% in the medium (Table 6). Moderate to good level of enzyme activities were obtained with glycine, asparagine, tyrosine and phenylalanine (Table 6). Amino acids such as glycine, tryptophan and methionine increased lignolytic enzyme production by *Ganoderma* sp.kk-02 up to 3.5-fold. Dhawan and kuhad[36] reported maximum lignolytic enzyme production by *Cyathus bulleri* in the presence of methionine. The addition of various amino acids showed stimulating effects on laccase production by *C. buller* [36] and *Ganoderma* sp. kk-02[37].

4. Conclusion

In conclusion, this study emphasised the need to explore more organisms and to evaluate the real potential of fungi producing lignolytic enzymes. By proper selection of the initial pH, temperature, different carbon, nitrogen and amino acid sources for fungus growth and target enzyme synthesis may play an important role in the development of an efficient technology. The results obtained here allow as to conclude the *L. kauffmanii* is a good candidate for scale up lignolytic enzyme production. However, further studies are required to elucidate the reasons for stimulation of enzyme production by some complex substrates.

Acknowledgement

The authors thank the Director, CAS in Botany, University of Madras, India, for providing lab facility and the University Grant Commission, Government of India, for providing a research grant.

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