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Versatile peroxidase production from *Ganoderma lucidum* RMK1: augmentation of production levels by medium optimization and lignocellulosic biomass amendment

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Abstract: **Ligninolytic enzymes, especially versatile peroxidase, have received much attention in recent times due to expanding application in bioremediation, biofuel production and in the production of other valuable chemical products. Choosing appropriate medium and optimizing the medium is important for economic and over-production of the valuable products. Versatile peroxidase has been reported to be present in various white rot fungi including *Ganoderma lucidum*. In this study, we optimized submerged fermentation medium for maximum versatile peroxidase production including amendment of lignocellulosic wastes. The basal medium, after optimization, has produced 5.5 times more versatile peroxidase. Improvised production of versatile peroxidase will find application in various industries.**

Keywords: **Versatile peroxidase, submerged fermentation, *Ganoderma lucidum* RMK1, lignocellulosic wastes, lignin.**

1. INTRODUCTION

Lignin is the most abundant renewable aromatic polymer and the second most abundant organic polymer on earth [01]. Naturally it is available in association with other carbohydrates such as cellulose and hemicellulose, forming a unit called lignocellulose. These lignocelluloses become potential feedstock for biofuel production only when the cell wall polysaccharides are converted into simple and fermentable monosaccharide. The presence of lignin in these materials impedes the economy and viability of saccharification and ultimately the biofuel production [2]. Hindrance is caused by its chemical recalcitrance coupled with the physical barrier it creates to the enzymes from accessing the cellulosic materials. Apart from biofuel production, lignin degradation is also important for carbon recycling in the biosphere, since its accumulation could cause serious environmental problems [3, 4]. In the industries that utilize lignocellulosic biomass for ethanol production, the lignin residues are sometimes used just for power generation to propel the fermentation reaction [5, 6]. Besides lignin have other applications such as polyurethane foams, emulsifying agent, and in phenolic resin.

Biologically lignin is degraded by various fungal and bacterial species. Fungi are the most efficient organisms in degrading lignin than the bacterial counterparts. Fungi hold a system of variety of enzymes than the bacteria, whose degradation is slower and limited [7, 8, 9, 10]. Lignin degrading fungi are primarily classified into white rot fungi, brown rot fungi, and soft rot fungi, based on the morphology of wood degradation. All these fungi are capable of lignin decomposition, but only the white rot fungi are capable of completely degrading the lignin into CO_2 and H_2O (11, 12). These white rot fungi comprise more than 90% of the wood rot fungi and the notable members of the group are *Ganoderma* spp., *Lentinula edodes*, *Phlebia radiata*, and *Pleurotus* spp. [13].

Lignin degradation by white rot fungi owes to its capability of producing various classes of lignin modifying enzymes (LMEs) such as laccase (Lac), manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) [14,

15; 16]. The efficiency of each enzyme on lignin degradation depends on their catalytic property. Laccases are capable of oxidizing non-phenolic components of lignin due to its low redox potential which ranges from 0.48 to 0.78V. Lignin typically contains only 10 – 15% of phenolic components and the rest is made of non-phenolic polymer and are non-reactive to laccases [17, 18]. Even with the addition of specific mediators, laccases are still incapable of acting on condensed structures [19, 20].

The high redox potential (0.8 – 1.2V) enzymes such as peroxidases are more suitable for lignin degradation in terms of efficiency [17]. Manganese peroxidase cannot penetrate the lignocellulosic biomass and hence produce low molecular diffusible oxidants. These oxidants diffuse to the lignin target and only end up with low yields [15]. Lignin peroxidase, another strong oxidant, interacts directly with the non-phenolic lignin component. But, it too is inefficient in terms of yield and penetrating low porous lignocellulosic substrates (21, 22, 23].

Versatile peroxidase is unique that it can oxidize both low redox and high redox compounds (24, 25]. Despite its high value for lignin valorization, it has not yet been extensively studied due to its very low amount of production. The occurrence of versatile peroxidase was initially reported from two white rot fungi *Pleurotus eryngii* and *Bjerkandera adusta* as a manganese-independent manganese peroxidase [26. 27, 28]. Later the presence of versatile peroxidase was reported from various fungi across different genera that include as *Dichomitus squalens*, *Irpex lacteus*, *Lepista nuda*, and *Panus tigrinus* [29]. Further it was also discovered from Cerrena spp., *Spongipellis* sp., *Ganoderma lucidum*, and *Trametes versicolor* [30]

Majority of the versatile peroxidase producing genera belongs to the white rot fungi group (efficient lignin degraders) emphasizing its origin, occurrence and significance in lignin degradation. Though the presence of versatile peroxidase has been reported in several genera, optimization of its production levels has not been carried out yet. Hence in this study, we attempted to optimize the versatile peroxidase production from *Ganoderma lucidum* RMK1 using submerged fermentation and lignocellulosic biomass amendment.

2. MATERIALS AND METHODS

Materials and cultures

The *Ganoderma lucidum* RMK1 (Genbank Accession: MH553170) was isolated from Kumarakom Bird Sanctuary, Kottayam, Kerala, India. All the chemicals used were of analytical grade. Phenol red and BSA (bovine serum albumin) were purchased from Sisco Research Laboratoty, Mumbai. The standard protein markers were purchased from Genei Laboratories, Bangalore.

Composition of Basal medium

The basal medium for optimization of versatile peroxidase production using submerged fermentation was adapted from Simonić et al., 2010 [31] with required modifications. The modified media composition (g/L) is as follows: glucose (10.0), K_2HPO_4 (1.0), $NaH_2PO_4 \cdot H_2O$ (0.4), $MgSO_4 \cdot 7H_2O$ (0.5), yeast extract (2.0), $CaCl_2$ (0.05), $FeSO_4 \cdot 7H_2O$ (0.05), and pH 5.5.

Analytical methods

The crude culture filtrates were centrifuged at 12,000 rpm for 15 minutes at 4°C for enzyme assay and protein estimation. Oxidation of phenol red (A_{610} ε = 22.0 mM $^{-1}$ cm $^{-1}$) was measured at Mn^{2+} - dependent and Mn^{2+} - independent conditions for the determination manganese-dependent (MnP) and manganese-independent (VP) peroxidase activity. The 1.5 mL reaction mixture contained 0.001 % (w/v) phenol red, 2.5 mM lactate, 0.01% (w/v) bovine serum albumin in 20 mM sodium acetate buffer (pH 4.5). The oxidation reaction was stopped by the addition of NaOH at the final concentration of 8 mM. The measurements were taken in the presence and absence of H_2O_2 for measuring the peroxidase-specific activity. Boiled samples of culture filtrate at the same conditions were used as blanks. One unit of enzyme activity is equivalent to amount of enzyme that catalyzed the production of 1 µM of oxidation product per mL of culture filtrate.

Protein estimation was carried out according to Bradford, 1976 [32]. SDS-PAGE [33] and Native-PAGE [34] were carried out according to previous reports. The polyacrylamide gels were removed from the plates after desired time and stained with silver nitrate following the method of Blum et al (1987) [35]. After staining the gels to the desired intensity, the gels were stored in acetic acid (7 % v/v).

Effects of different pH and temperature

Initially the pH of basal medium was optimized followed by temperature optimization. The pH of basal medium was adjusted using 1N HCl or 1N NaOH for achieving different pH conditions with the final pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The basal medium was inoculated with 6 mycelial discs of 6mm diameter. The protein concentration and versatile production (in terms of manganese-independent manganese peroxidase activity) was analyzed using appropriate methods.

The pH-optimized basal medium was prepared in 50 ml batches in different flasks and incubated at 25, 30, 35, and 40 °C. The cell-free culture filtrate (supernatant after centrifugation) was used for the estimation of protein concentration and versatile peroxide production.

Effect of different carbon sources

Different carbon sources such as glucose, fructose, galactose, maltose, sucrose, mannitol, and starch were amended in the basal medium, where each sugar is added in separate flask at the fixed concentration of 1% (w/v). After the addition of sugars, the flasks were sterilized and inoculated with G. lucidum discs and incubated at 30°C for 18 days. The supernatant were used for the analysis such as protein concentration determination and versatile peroxide production level determination.

Effect of different nitrogen sources

In order to determine the best nitrogen source that supports the maximal versatile peroxidase production, different nitrogen sources such as ammonium chloride, ammonium sulphate, ammonium nitrate, yeast extract, beef extract, and peptone were individually amended (0.25 % w/v) in separate flasks containing the basal medium. The flasks were incubated at 30°C for 18 days and the supernatant was collected for protein and enzyme concentration determination.

Effect of different concentrations of Mn^{2+} ion

The influence of Mn^{2+} ions on the production of versatile peroxidase production using G. lucidum was studied at different concentrations of $MnSO_4$ (such as 10, 20, 30, 40, 50, and 60 µM). After addition of different concentrations of Mn^{2+} ions to individual flasks, the flasks were incubated at 30°C for 18 days under shaking condition. The enzyme and protein concentrations were determined with appropriate methods mentioned.

Effect of different aminoacids

Different amino acids were amended in the basal medium at the concentration of 100mg/L and the flasks were incubated at 30°C for 18 days under shaking condition. The amino acids added were glycine, alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, tyrosine, phenylalanine, proline, serine, tryptophan, threonine, and cystein. The flask without addition of any amino acid served as control. The total protein and versatile peroxidase were quantified from the supernatant after incubation and centrifugation.

Effect of different vitamins

Different vitamins such as ascorbic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, and biotin were amended in the basal medium at the concentration of 100mg/l to study their effect on the production of versatile peroxidase by *G. lucidum*. The basal medium without addition of any vitamin source served as the control.

Effect of different aromatic compounds

The influence of various aromatic (inducer) compounds on versatile peroxidase production was studied using varatryl alcohol, ferrulic acid, gallic acid, vanillin, and 2,5 xylidine. The inducers were amended in the basal medium in separate flasks at the concentration of 1mM. The inducers were added as liquid, where they were dissolved in 95% (v/v) ethanol already. The basal medium added with 2ml of 95% (v/v) ethanol served as control. Flasks were incubated at 30°C for 18 days, after which the supernatants were collected for protein and enzyme quantification.

Effect of different lignocellulose biomasses

Accurately measured ground biomass (paddy straw, corn stover, wheat bran, rice bran, sugarcane bagasse, cassava stem) were amended to the basal medium at the concentration of 1% (w/v) to study their ability to support the production of

versatile peroxidase production by G. lucidum. The basal medium without addition of any of the biomass served as the control. The protein and enzyme concentrations were determined after 18 days from the flasks incubated at 30°C under shaking conditions.

Versatile peroxidase production in bioreactor

The production of versatile peroxidase using optimized basal medium was carried out in lab-scale bioreactor (Lark Pvt. Ltd., Chennai, India). The bioreactor used in this study contained *ex situ* sterilizable glass vessel with dimensions 14 cm w × 32 cm h and total volume of 5 L (inclusive of working volume of 3 L). The bioreactor contained in-built probes for pH and temperature sensing. Additionally the bioreactor had itself a motor-driven agitator with three impellers for through mixing and ensuring even oxygen supply. Three liters of basal medium prepared with optimized components (and pH 5.5) was added to the bioreactor vessel and sterilized at 121°C and 15 lbs pressure for 30 minutes. The *G. lucidum* was already grown separately in a liquid medium added with glass beads for homogenization of the fungal mycelium. Six days old mycelium (10% inoculums) was used to aseptically inoculate the sterile production medium in the bioreactor using inoculation port. The temperature of the vessel components was constantly maintained at 30°C ± 0.05 and the pH was maintained at 5.5 ± 0.05 using 0.5 M NaOH or 0.5 M HCl. The agitator speed was constantly set at 80 rpm. The filter-sterilized air was continuously supplied to the glass vessel at the rate of 1 vvm. At three days interval, five milliliters of culture was extracted aseptically from the fermentation vessel to determine the versatile peroxidase quantity and to estimate the extracellular protein production.

3. RESULTS AND DISCUSSION

Optimization of different pH for versatile peroxidase production

Influence of pH of the production media on versatile peroxidase production was carried out at different pH ranging from pH 4.0 to 8.0. The *Ganoderma lucidum* RMK1 produced maximum amount of versatile peroxidase (39 UL^{-1}) at the pH 5.5 on the 18^{th} day of incubation, followed by pH 5.0 (26 UL^{-1}) and pH 5.0 (25 UL^{-1}). At other pH conditions, the versatile peroxidase production declined to the near half of the maximum production levels. The pH 5.5 supported the maximum extracellular protein production to the level of 4.82 µg/ mL and maximum mycelia biomass of 3.54 g/L on 18^{th} day of fermentation.

Optimization of temperature for the maximum versatile peroxidase production

The *Ganoderma lucidum* RMK1 was capable of growing in all the temperatures tested viz. 25, 30, 35, and 40 °C as evident from the biomass growth and protein secretion. The enzyme maximum versatile peroxidase production occurred at 30°C with 39 UL^{-1}. At 25°C, *Ganoderma lucidum* RMK1 produced 22 39 UL^{-1} and at higher temperatures, the production reduced further to about 19 UL^{-1} at 35°C and 15 UL^{-1} at 40°C. At 30 °C, the extracellular protein and mycelia biomass attained the maximum of about 5.85 µg/ mL and 3.54 g/L on 18^{th} day of fermentation.

Optimization of carbon source for versatile peroxidase production

Different carbon sources (vide, Materials and Methods) were added to the optimization media at the concentration of 1% w/v to determine their ability to support versatile peroxidase production. Among all tested, the monosaccharide glucose and fructose supported maximum versatile peroxidase production to about 39, 33 UL^{-1}, respectively. But Graz and Jarosz-Wilkołazka (2011) reported 1800 UL^{-1} of versatile peroxidase production by *Bjerkandera fumosa* in glucose-peptone based media [36]. The other carbon sources such as mannitol, starch, maltose, and galactose did not support versatile peroxidase production significantly and their production levels fall in the range between 24 and 27 UL^{-1}. Sucrose, as a carbon source, resulted in the production of least level of versatile peroxidase to about 18 UL^{-1} on 18^{th} day of incubation.

Optimization of nitrogen source for versatile peroxidase production

Six different nitrogen sources, three organic and three inorganic nitrogen sources, were amended to the optimization medium. Among the three organic nitrogen sources, peptone and yeast extract supported maximum production of versatile peroxidase to about 57 and 39 UL^{-1} on the 18^{th} day of fermentation. The inorganic nitrogen sources resulted in the poor production of versatile peroxidase and the least supporting nitrogen source was beef extract with the production of 13 UL^{-1}. Similarly, Simonić et al., 2010 reported 35 UL^{-1} in peptone medium and 12 UL^{-1} for control using *Ganoderma carnosum* [31].

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Effect of amino acids on versatile peroxidase production

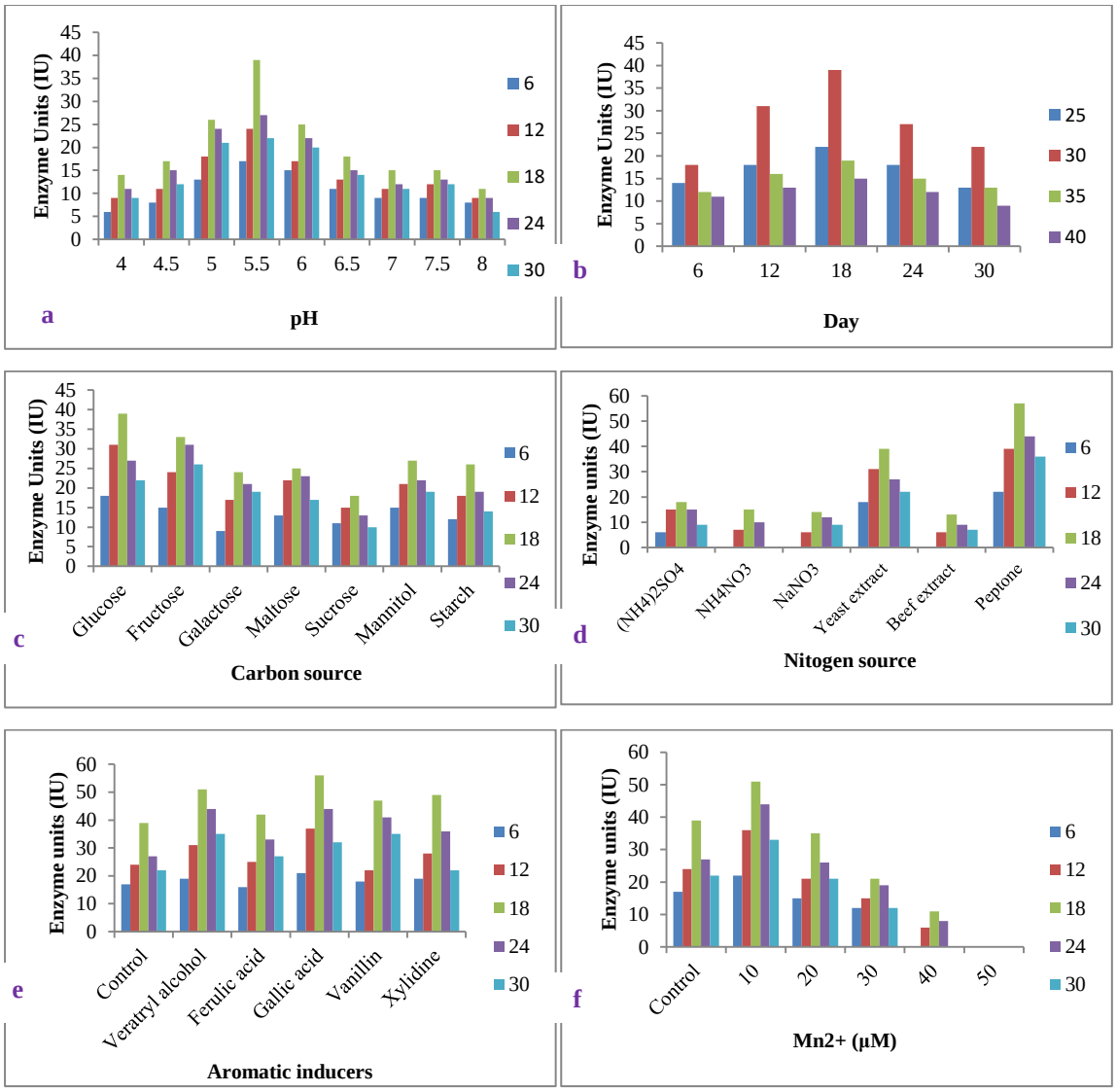
Among all the ten amino acids amended in the optimization medium, the aromatic amino acids increased the versatile peroxidase production to about 52, 51, 48 UL^{-1} for tryptophan, phenylalanine, and tyrosine, respectively. Other amino acids did not significantly influence the production of versatile peroxidase.

Effect of vitamins on versatile peroxidase production

Different vitamins such as ascorbic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, and biotin were amended in the basal medium at the concentration of 100 mg/L to study their effect on the production of versatile peroxidase by *G. lucidum* RMK1. Among them, thiamine hydrochloride increased the versatile peroxidase production to about 47 UL^{-1}, whereas both ascorbic acid and biotin resulted in the production of 44 UL^{-1}. Other vitamins also produced versatile peroxidase in the same titers.

Effect of aromatic inducers on versatile peroxidase production

Aromatic inducers are capable of inducing peroxidase production. In that line, five different aromatic inducers were tested for their ability to increase versatile peroxidase production. Gallic acid amended optimization medium produced 56 UL^{-1} of versatile peroxidase on 18th day of incubation. At the same time, addition of veratryl alcohol and xylidine resulted in the production of versatile peroxidase to about 51and 49 UL^{-1}. Vanillin and ferulic acid produced 47 and 42 UL^{-1} of versatile peroxidase respectively.



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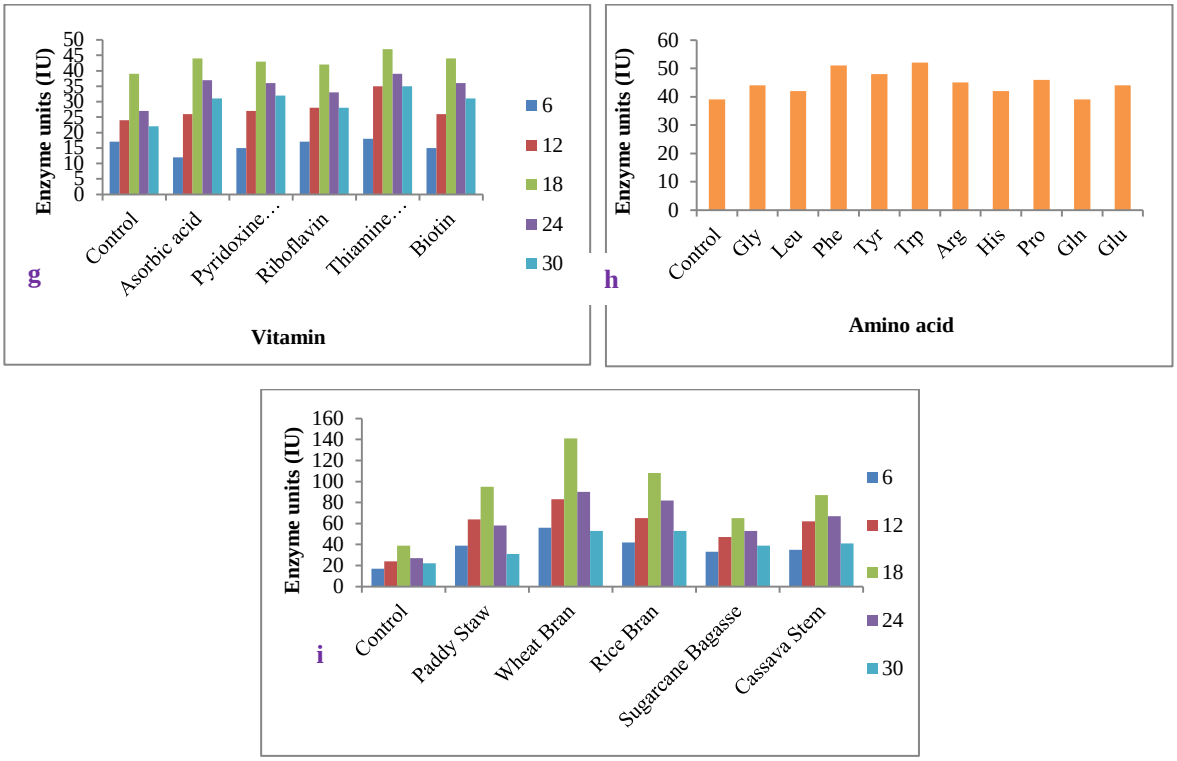

Figure 1. Optimization of versatile peroxidase production in submerged fermentation at different pH (a), temperature (b), using different carbon (c), nitrogen (d), aromatic inducers (e), and at different concentrations of manganese ion (f), and using different vitamins (g), amino acids (h), and lignocellulosic biomass (i). Effect of Mn^{2+} on versatile peroxidase production

The effect of Mn^{2+} ion on the production of versatile peroxidase was studied by the addition of $MnSO_4$ to the optimization medium at the concentrations ranging from 10 to 60 mM. At 10 mM concentration of $MnSO_4$ the production of versatile peroxidase achieved the maximum of 51 UL^{-1} on the 18th day of incubation. But the versatile peroxidase production decreased at 10 mM concentration of $MnSO_4$ to about 35 UL^{-1}. At further increasing concentrations the versatile peroxidase production was completely repressed or insignificant.

Effect of selected lignocellulosic biomasses on versatile peroxidase production

Different lignocellulosic biomasses were amended to the production medium at the concentration of 1% (w/v) to study their influence on versatile peroxidase production. Wheat bran amended media produced the maximum of versatile peroxidase to about 141 UL^{-1}, followed by rice bran, which produced 108 UL^{-1} of the enzyme. The paddy straw, sugarcane bagasse, and cassava stem resulted in the production of 95, 68, 89 UL^{-1} of versatile peroxidase on 18th day of incubation. Stajic et al. (2010) repoted 80.80 UL^{-1} of versatile peroxidase production in the grape wine sawdust medium [37].

Versatile peroxidase in lab scale fermentor using optimized medium

The *G. lucidum* RMK1 produced maximum of 217 UL^{-1} of versatile peroxidase and 131.45 mg/L of total extracellular protein as recorded on the 18th day of incubation. Hence, the optimization of versatile peroxidase production has resulted in the 5.5 times more yield of the enzyme.

4. CONCLUSION

Valuable enzymes and some secondary metabolites of pharmaceutical importance are produced in minimum quantity by microorganisms. Optimization of various factors leads to over-production of such valuable products. Here, we optimized the production of versatile peroxidase by *Ganoderma lucidum* RMK1. The optimization of various factors has led to 5.5 fold increase in the production levels of the enzyme. Hence, this helps in the purification of versatile peroxidase in more volumes for further applications.

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