Effect of process variables on lignin peroxidase production

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Abstract
Lignin, one of the three major and inseparable components of lignocellulose, is a polymer of aromatic subunits. Although it is highly recalcitrant that resists biological activities but can be used to produce high-value-added products if exploited properly. After evaluation of different approaches for the lignin depolymerization by various researchers in past, use of lignin-degrading/modifying enzymes has been proved to be an efficient and eco-friendly approach. Microbes have a versatile set of lignin degrading enzymes that can be utilized as per the process requirement such as biorefinery, textile, energy, bioremediation, cosmetology and dermatology industries. Lignin peroxidase (LiP) is one of such enzymes that catalyzes hydrogen peroxide dependent oxidative degradation of lignin.

In present work, Pseudomonas sp. LiP 22, procured from RL-5 Department of Biotechnology, Himachal Pradesh University Shimla-05 was used for lignin peroxidase production. Further, the effects of various process parameter and growth factors were also evaluated by one variable at a time (OVAT) approach. As a result, LiP activity was increased from 1.62 U/ml to 59.49 U/ml (36.72 folds). The work was also confirmed by dye decolorization method which also signifies its importance for various industrial applications and environment conservation.

Keywords: Lignin peroxidases, *Pseudomonas*, dye decolorization, environment conservation.

Introduction

Biomass especially plant-based lignocellulosic biomass mainly consisting of cellulose (40%), hemicellulose (30%) and lignin (26%) can be used as a source of energy besides other uses[2,4,16,21]. Lignin, one of the most abundant renewable biopolymers on earth after cellulose is the recalcitrant compound, synthesized by plants that is responsible for wood strength[11,13].

It is present within and between the cell walls of tracheid, vessels and fibers of xylem tissue[12]. The structure of lignin is complex due to C-C and C=O linkage which make its degradation difficult. However, degradation of lignin is desirable in the paper and pulp industries, which is usually achieved by using physical or chemical methods[6,20].

Lignin can be depolymerized by thermo-chemical methods such as pyrolysis (thermolysis), gasification, chemical oxidation, hydrogenolysis and hydrolysis under supercritical conditions[14]. However, these processes release hazardous, toxic and carcinogenic lignin-compounds in the effluent and deteriorate the environment. Lignin degradation is also very important for the carbon cycle because most of renewable carbon either comes from lignin or related compounds i.e. cellulose and hemicellulose[8].

The most efficient way to degrade lignin is biodegradation i.e. using microorganisms which release enzymes like lignin peroxidases, manganese peroxidases and laccases[18]. The use of lignin degrading enzymes has proved attractive alternate to chemical degradation since this is an environmental friendly approach[7]. Besides degrading lignin, these enzymes are also used in the biodegradation of remaining xenobiotic compounds.

Among all the microorganisms, bacteria are preferred due to their added advantages including better separation of lignin from cellulose and hemicellulose[19]. Among lignin degrading enzymes, lignin peroxidase (EC 1.11.1.14) has gained more attention in comparison to laccases and manganese peroxidases[3]. Lignin peroxidase is an extracellular heme-protein, susceptible and dependent to H_2O_2 with a high redox potential and low optimum pH. Due to their high redox potential and broad substrate specificity, this enzyme has great application in various industrial processes[15]. Extracellular lignin peroxidase finds their use in various industrial applications.

The potential lignin peroxidase is attributed to its versatility in the degradation of xenobiotics compounds with both phenolic and non-phenolic constituents and delignification of lignocellulosic feedstock for ethanol production. Other applications are: textile effluent treatment and dye decolourization, coal depolymerization, treatment of hyper pigmentation and skin-lightening through melanin oxidation, bio-refinery, textile, energy, bioremediation, cosmetology and dermatology[5], in removal of recalcitrant organic pollutants and in the enzymatic polymerization in polymer industries[17].

Material and Methods

Culture collection and crude enzyme preparation: Bacterial isolate *Pseudomonas* sp. LiP 22 procured from RL-5 Department of Biotechnology, HP University Shimla was maintained on nutrient agar and further used for LiP production under optimized growth conditions. LiP activity

was determined by following methodology given by Tien and Krik[22].

Lignin peroxidase (LiP) assay: Lignin peroxidase activity was measured by the veratryl alcohol method: the initial rate of oxidation of veratryl alcohol to veratraldehyde was followed by absorption at 310 nm. The assay mixture contained in a final volume of 1 ml: 50 µL of the diluted supernatant, 250µL of 50 mM veratryl alcohol, 0.6 ml of 0.1 M citrate buffer (pH 5.5) and 0.1 ml of 10 mM H_2O_2. One unit of enzyme activity was considered as the amount of enzyme which oxidizes one micromole of veratryl alcohol per minute.

Effect of process variables on Lignin Peroxidase production

Media selection: Selected lignin peroxidase producing isolate was cultured in seven different media. 50 ml of nutrient broth (pH 7.0) was used as seed media and culture was incubated at 30ºC for 24 h in a rotatory shaker (150 rpm). Subsequently 1 ml of inoculum was transferred to 250 ml Erlenmeyer flask containing 50 ml of each medium represented (M1- M7 as production media) and incubated at 30ºC in incubator shaker for 24 h. The content was centrifuged at 10,000 rpm for 10 min and supernatant was used for LiP assay a discussed earlier.

Optimization of carbon source: The effect of different carbon sources on production of lignolytic enzyme was determined by adding various carbon precursors such as glucose, fructose, xylan, sucrose, galactose, lactose, CMC, xylose and starch (0.25%) while rest of composition of optimized production media was kept unchanged. After 24 h incubation, cell centrifugation and supernatant were assayed for lignin peroxidase.

Concentration of carbon source: Since glucose was observed as best carbon source, its varied concentration (w/v) 0.25%, 0.5%, .25%, 1.0%, 1.25%, 1.50%, 1.75% and 2% were used to see the resulted effects on the enzyme activity. After incubation, supernatant was collected and analyzed for lignolytic activity.

Optimization of nitrogen source: Various organic and inorganic nitrogen sources were used to check their effect on LiP production. The nitrogen sources used were ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate, meat extract, malt extract tryptone, yeast extract and peptone (0.25%).

Concentration of nitrogen source: Among all the sources of nitrogen used, peptone was found the best for LiP, its concentration was varied from 0.25%- 2% in production medium to see the relative yield of enzyme with supernatant.

Optimization of inoculum age: For this purpose, the loop full of bacterial culture was inoculated in 50 ml seed medium and incubated at 30°C in incubator shaker. 1 ml of sample collected from the seed medium after 3, 6, 9, 12, 15, 18, 21 and 24 h was inoculated to production medium and incubated at 30°C for 24 h. The culture was harvested and enzyme assay was carried. The inoculum that gave highest activity was used for further studies.

Inoculum size: Different inoculum size ranging from 1-10% was used to see the effects of inoculum volume on enzyme activity. For this purpose, bacterial culture was inoculated in Erlenmeyer flasks containing 50 ml of the medium and incubated at 30°C for 24 h in a rotatory shaker at 150 rpm. After 24 h incubation, cells were separated by centrifugation and lignin peroxidase activity was determined as discussed earlier.

Substrate concentration: Concentration of lignin was varied from 0.001-0.10% in order to optimize the best one.

Incubation temperature: For this purpose, 250 ml Erlenmeyer flasks, each containing 50 ml of the production medium were inoculated with seed culture. The flasks were incubated for 24 h at different temperatures ranging from 30, 35, 40, 45, 50, 55 and 60°C. After incubation, lignin peroxidase activity was determined as discussed earlier.

pH value: pH value of the fermentation medium was adjusted to different values ranging from 5.0 to 9.5 before sterilization using 0.1 N NaOH or 0.1 N HCl to see the effect of pH on bacterial growth. Flasks containing 50 ml of the medium were adjusted to various pH values and inoculated with seed culture and incubated for 24 h at 30°C. After incubation, lignin peroxidase activity was determined.

Production time: Different parameters optimized earlier were kept constant and 50 ml of production medium was inoculated with bacterial seed culture. Erlenmeyer flask was then incubated at 30°C and sample was taken after interval of 4h up to 52 h; simultaneously culture was harvested and lignin peroxidase activity was assayed.

Results and Discussion

Isolation and screening of bacteria: 50 ml broth enriched with lignin was used to isolate lignin peroxidase producing microorganisms on agar plates by the serial dilution of all the collected samples. Maximum lignin peroxidase activity is 1.62 ± 0.035 U/ml was recorded with bacterial isolate LiP 22. Thus, bacterial isolate LiP 22 was selected for further investigation for characterization.

Effect of process variables on Lignin Peroxidase production

Media: The level of enzyme production and microbial growth depends on the composition of the fermentation medium. The bacterial isolate i.e. *Pseudomonas sp.* LiP 22 was grown on seven different growth media (Fig. 1). Among different media used, this organism showed maximum activity (2.85 ± 0.025U/ml) in media M4 (K_2HPO_4-4.55g/l;

KH_4PO_4-0.53g/l; $MgSO_4$-0.5g/l; NH_3NO_3-0.5g/l; Glucose-5.0g/l; meat extract-10g/l) followed by M3.

Effect of carbon source: Various carbon sources mono, di and polysaccharides were added to the basal medium in order to rate resulted effect on enzyme production. Out of these, glucose was found to be an effective carbon source for the production of lignin peroxidase with 4.43 ± 0.032 U/ml enzyme activity (Fig. 2). Starch showed negative effect on the activity of enzyme.

Concentration of carbon source: The concentration of optimized carbon source i.e. glucose was varied from i.e. 0.25% to 2% in order to see the effect of varied glucose concentration on enzyme activity. The enzyme activity increased with increasing concentration upto 0.5% and maximum activity was obtained 16.10±0.051U/ml. However, further increase in glucose concentration resulted into decreased enzyme activity (Fig. 3).

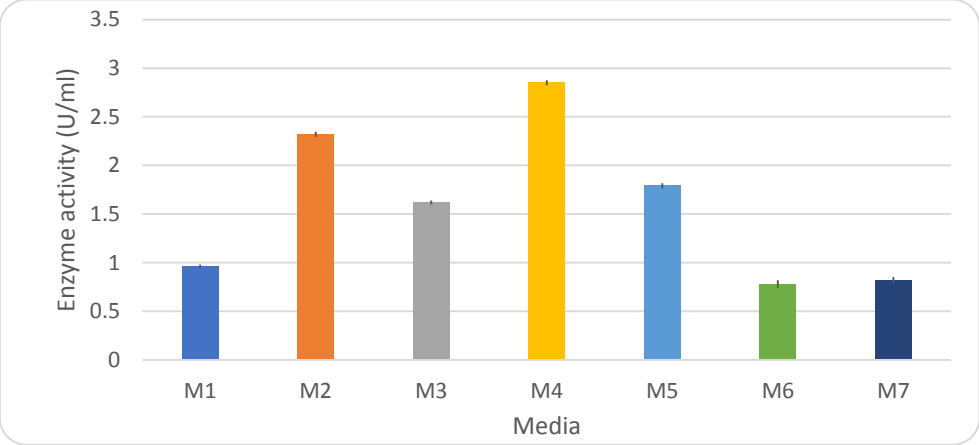

Fig. 1: Effect of media on enzyme production

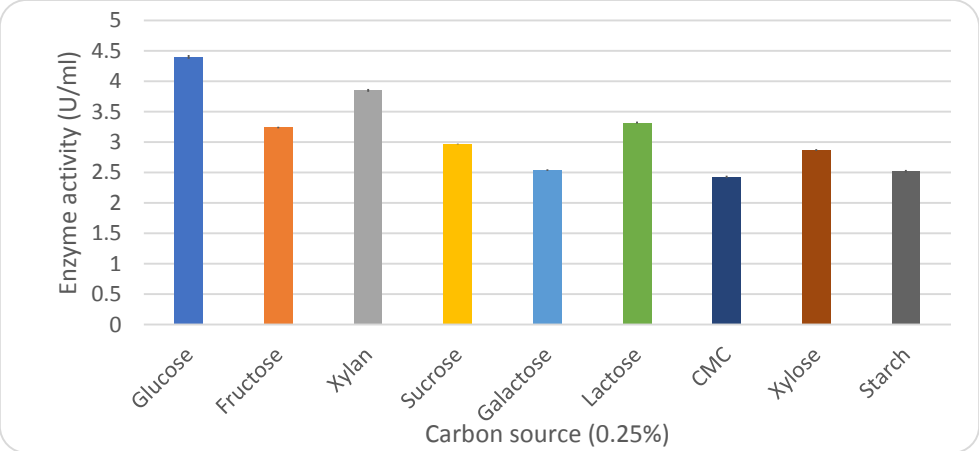

Fig. 2: Effect of different carbon sources on Lignin peroxidase production

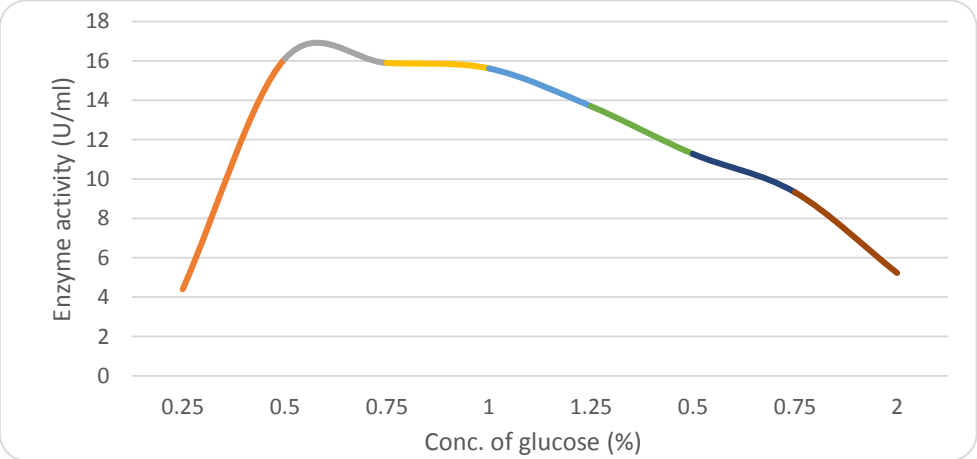

Fig. 3: Effect of concentration of glucose on enzyme production

Effect of nitrogen source: Lignolytic enzyme production is greatly influenced by the nitrogen concentration in the culture medium. To see the resulted effect of nitrogen source on LiP activity, various organic and inorganic sources of nitrogen were used and enzyme activity was recorded in each case. Out of these, meat extract was found to be best one, which showed increased enzyme activity (17.82 ± 0.023U/ml).

Effect of concentration of nitrogen source: In order to optimize concentration of meat extract, its concentration was varied from 0.25% to 2% and activity of LiP was recorded in each case. The activity of enzyme increased with increasing the concentration and maximum activity (32.3 ± 0.030U/ml) was observed at 1% of meat extract in the medium (Fig. 5).

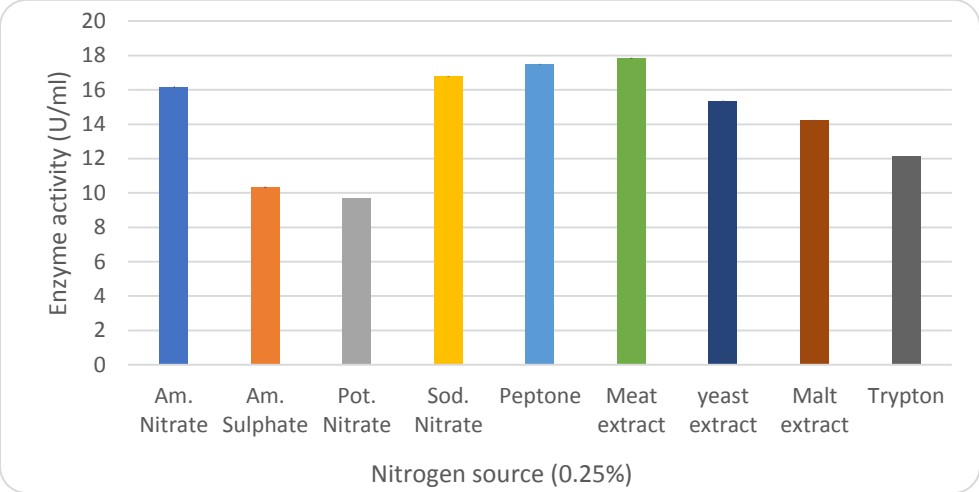

Fig. 4: Effect of different nitrogen sources on Lignin peroxidase production

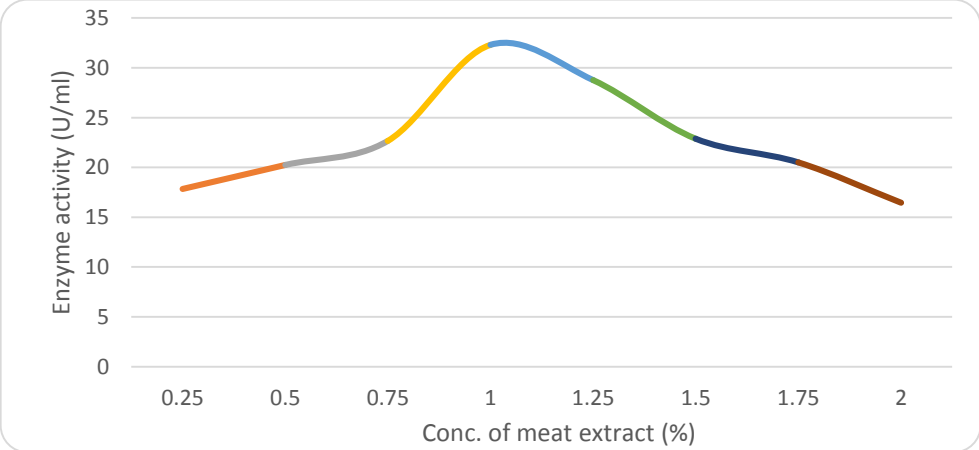

Fig. 5: Effect of concentration of meat extract on Lignin peroxidase production

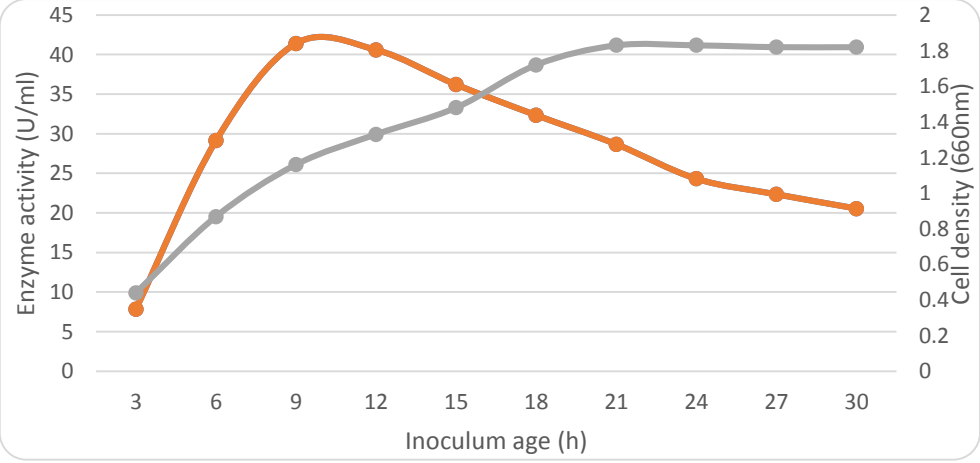

Fig. 6: Effect of inoculum age on lignin peroxidase production

Effect of inoculum age: The inoculum age and size directly affected microbial growth and resulted in enzyme production. To study the effect of inoculums age on the lignin peroxidase production, inoculums with age ranging from 3-24 h were used and enzyme activity was recorded in each case. Maximum activity (41.40 ± 0.073U/ml) was obtained with 9 h old culture (Fig. 6) and activity decreased afterwards.

Effect of inoculum size: Inoculum size has significant effect on enzyme production. In order to improve the activity of lignin peroxidase, varied volumes of inoculums ranging from 1-10 (% v/v) were used for inoculation into production medium and LiP activity was recorded in each case. Results obtained showed that the highest production of the lignin peroxidase was with 5% inoculum (48.53 ± 0.030U/ml) followed by 6% and least at 10% (Fig. 7).

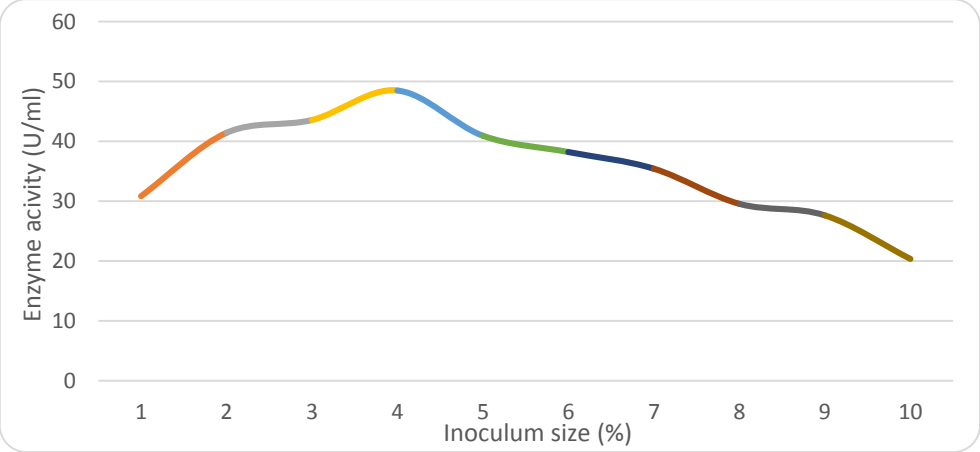

Fig. 7: Effect of inoculum on lignin peroxidase production

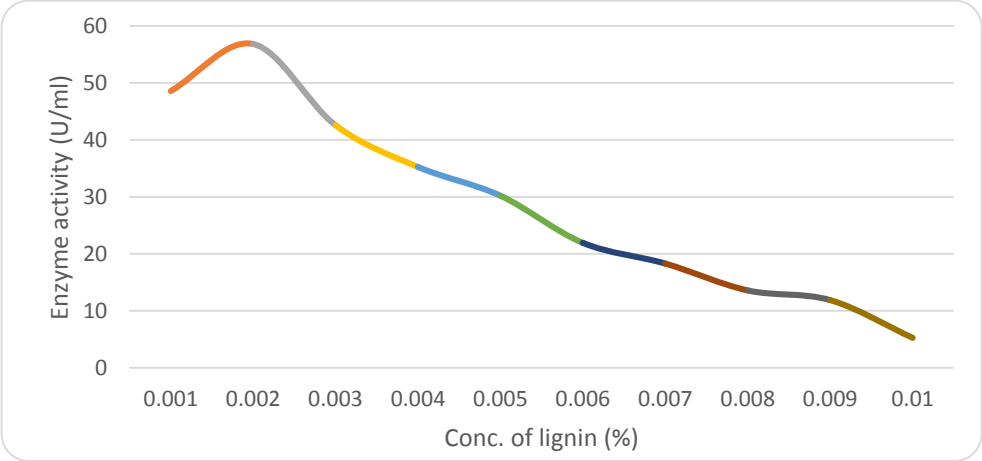

Fig. 8: Effect of substrate concentration on lignin peroxidase production

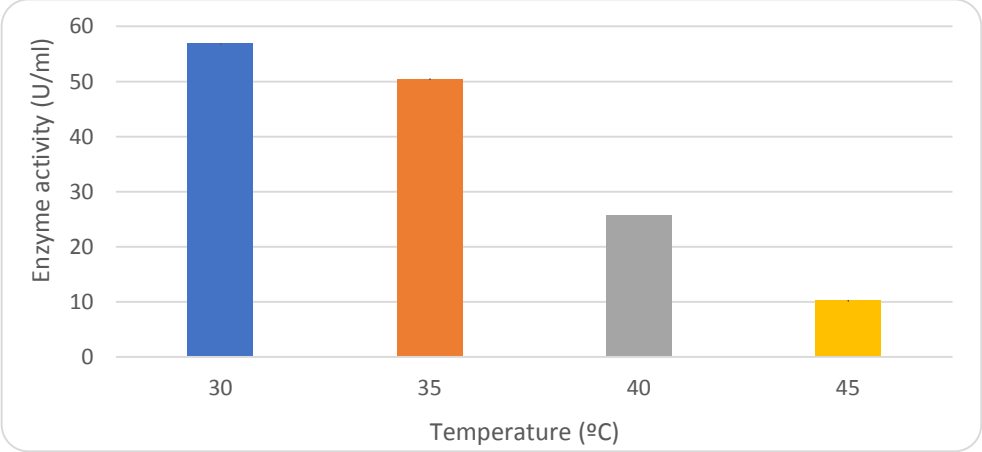

Fig. 9: Effect of temperature on lignin peroxidase production

Effect of substrate concentration: Concentration of lignin i.e. substrate was varied from 0.001 to 0.010% and maximum enzyme activity (56.32 ± 0.037U/ml) was recorded with 0.002% lignin concentration (Fig. 8).

Effect of incubation temperature: Temperature is one of the most important parameters for the growth of organism and activity/yield of any enzyme. The culture was inoculated in a flask having 50 ml production media and incubated at different temperature ranging from 30-60ºC. Results shown in fig. 9 indicate that the enzyme activity (56.32 ± 0.037U/ml) was maximum at 30ºC. Further increase in temperature led to decrease in enzyme activity.

Effect of pH: Enzymes are greatly affected by changes in pH. Optimization of pH is most favorable pH value at which the enzyme is most active. Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH also effects the stability of enzymes. Among different pH used in the present report, the enzyme activity (58.92 ± 0.026U/ml) was maximum at pH 7.5 after that it decreased gradually (Fig. 10).

Effect of Production time: Samples were taken after interval of every 4 h from the production medium up to 52 h in order to optimize the production time suitable for enzyme production, centrifuged at 10,000 rpm for 10 min and supernatant was assayed for lignin peroxidase activity. The maximum reduction was found after 44 h (59.49 ± 0.083U/ml). There was no significant increase in enzyme activity form 24 (58.92 ± 0.026U/ml) -40 h (59.49 ± 0.083 U/ml). However, a rapid decline was recorded after 48 h. Further increase in production time led to decrease in extracellular production of lignin peroxidase as shown in figure 11.

In view of their immense potential industrial applications, enzymes carry its own place in research as well market. Among them, major emphasis has been laid on use of LiP for their potential uses in biotechnology-based industries like textile, paper and pulp, pharmaceutical or in cosmetic. Lignin peroxidase has great utility as a substitute used in skin lightening and bioremediation of wastewater treatment[25]. Microorganisms provide one of the best feasible sources for the production of enzymes and can be isolated easily from various habitats including soil. Many bacteria, fungi and yeasts producing lignin peroxidase have been reported during last few decades[1].

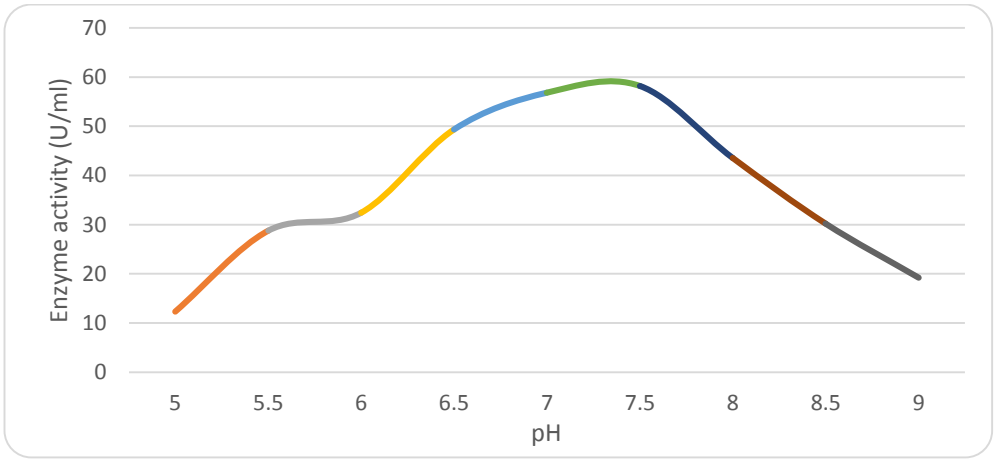

Fig. 10: Effect of pH on lignin peroxidase production

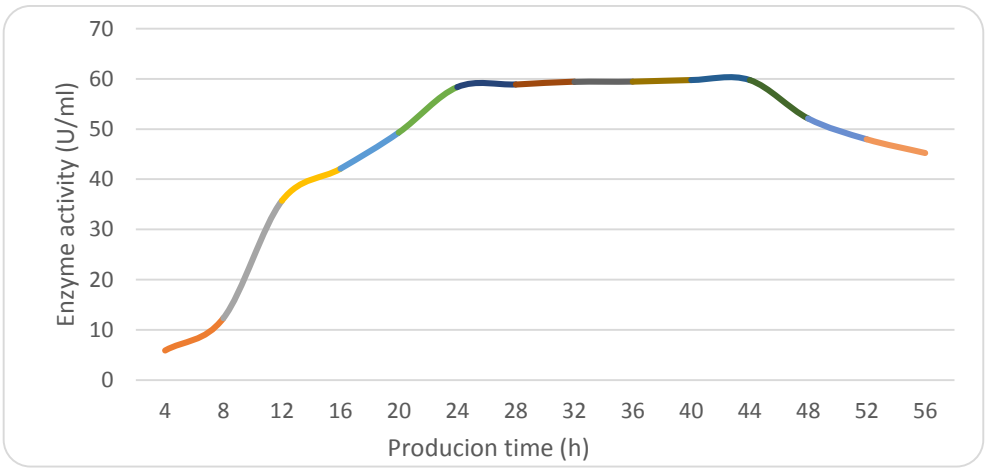

Fig. 11: Effect of production on enzyme production

However, there is always a need to explore nature for novel microbes that can produce extracellular lignin peroxidase, which can withstand varied stress conditions including higher temperature and wider pH.

In most of previous researches different fungal species have been used for LiP production. Kantelinen et al[10] developed a method to cultivate *Phlebia radiata* 79 (ATCC 64658) for the production of laccase and lignin peroxidase using carrier bound culture system in batch and semi-continuous mode. In order to enhance the enzyme production, 1.5 mM veratryl alcohol was added to production medium. It was reported that in presence of veratryl alcohol, laccase activity increased significantly, however both veratryl alcohol and tween 80 were required for lignin peroxidase production in submerged cultivations.

Under semi-continuous cultivations, lignin peroxidase activity further increased by fourfold in comparison to static cultivation system[10]. In another work, Zanirun et al[24] used statistical modeling for lignin peroxidase production from *Pycnoporus* sp. Several production factors were varied by factorial design approach and responses were recorded with respect to each combination. As a result of factorial modeling, maximum lignin peroxidase activity (51.1 U L^{-1}) was reported at 24 mM of nitrogen concentration, 110 rpm, pH 3.5, inoculum concentration of 6x106 spores mL^{-1} in presence of veratryl alcohol as inducer.

Later, Vandana et al[23] compared three isolates LPS1, LPS2 and LPS3 of white rot fungi for lignin peroxidase production using crop residues. Further LiP production optimized statistical modeling by screening of medium components by Plackett–Burman design followed by response surface methodology with only significant parameters to optimize the enzyme production. Among three, maximum lignin peroxidase was recorded from LPS1 (280 µmoles/min), followed by LPS2 (233 µmoles/min) and LPS3 (220 µmoles/min). In case of all three isolates, maximum LiP activity was also reported at 30ºC with pH 3 and in presence of veratryl alcohol[23].

Further optimization of lignin peroxidase production by LPS1 using Response Surface Methodology resulted in maximum LiP activity of 349.3 units at 30ºC, pH 4.2, 12 g glucose, 100mM veratryl alcohol and 0.1mM H_2O_2. The optimized activity was 21% higher in comparison to un-optimized growth condition.

In order to validate the role of LiP, lignin depolymerization using nine crop residues showed a reduction in lignin content ranging between 0.21 and0.94 and increase *in vitro* dry matter digestibility ranging between 0.45 and2.76[23].

Conclusion

Lignin peroxidase is of great significance for industries and research. Earlier researchers have revealed white rot fungi for LiP production but comparatively fewer literatures are available. In present work, bacterial isolate (*Pseudomonas* sp.) was used for LiP production.

Results from current work suggested the possible availability of more bacterial isolates that can potentially be used at industrial scale and further contribute to biomass utilization and environment conservation.

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