

# Proximate analysis of lignocellulosic biomass and its utilization for production, purification and characterization of ligninolytic enzymes by *Aspergillus flavus*

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**Keywords:** *Aspergillus flavus*, acid detergent lignin, ammonium sulfate, ligninolytic enzymes, *Platanus orientalis*.

**Abstract:** Ligninolytic enzymes are employed for the production of second-generation biofuel to minimize fuel crisis. Additionally, they play a crucial role in global carbon cycle and a variety of applications in food, agriculture, paper and textile industries. On a large scale production of ligninolytic enzymes, microorganisms can be cultured on lignocellulosic wastes. In the present study, proximate analysis including acid detergent lignin (ADL), acid detergent cellulose (ADC), acid detergent fiber (ADF) and acid insoluble ash (AIA) were performed for *Platanus orientalis* (chinar), *Bauhinia variegata* (orchid tree), *Pinus roxburghii* (chir pine), wheat straw and wheat husk. *Platanus orientalis* was selected as substrate because of higher lignin contents for the production of ligninolytic enzymes by *Aspergillus flavus*. Solid State Fermentation was used and Response Surface Methodology was employed for optimizing various parameters and enzymes production. Maximum production was achieved at temperature 32°C, fermentation period 120 hours, pH 4.5, inoculum size 3.5 mL, substrate mesh size 80 mm, substrate size 7 g. Maximum purification of laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) was achieved with 50%, 60% and 40% ammonium sulfate respectively and it was enhanced by gel filtration chromatography. Characterization of enzymes shows that Laccase has 35°C optimum temperature, 4.5 pH, 0.289 mM *K<sub>m</sub>* and 227.27 μM/ml *V<sub>max</sub>*. Manganese peroxidase has 30°C optimum temperature, 5.5 pH, 0.538 mM *K<sub>m</sub>* and 203.08 μM/ml *V<sub>max</sub>*. Lignin peroxidase has 30°C optimum temperature, 3 pH, 2 mM *K<sub>m</sub>* and 2000 μM/ml *V<sub>max</sub>*. Protein concentrations found in crude extracts and partially purified enzymes are respectively: laccase 1.78 and 0.71 mg/ml, MnP 1.59 and 0.68 mg/ml. LiP, 1.70 and 0.69 mg/ml.

## Introduction

Lignin is an aromatic hydrocarbon which exists extensively in nature, particularly in all higher plants, whose membrane cells are made up of carbon, hydrogen and oxygen. Lignin provides rigidity and stiffness to the stem of plant and prevents plants from microbial attack. (Feldman 2002, Boudet 2000). In pulp and paper industries ligninolysis has got much importance. Chemicals are used to degrade lignin for pulp manufacturing. This technique releases lignin containing harmful effluents into the environment which may cause cancer. (Harazono et al. 1996). One method to overcome the above problem is the use

of wood fungi for lignin biodegradation. (Bumpus et al. 1985). Pulping with microorganisms has the potential to improve pulp quality and properties of paper to lessen energy cost and impact on the environment in contrast to old pulping strategies. Saving of energy can make it economically valuable. (Wesenberg, et al. 2003).

Dye stuff industries are the main dyes manufacturers and textile industries are the main dyes users. Highly-drained waste matter produced by such industries are unpleasant aesthetically, hinder penetration of light and upset life of aquatic system. For surface and underground water, it is proved dangerous. Color removal from such wastes is a major issue. Treatment of such

effluents is very difficult and costly by physical and chemical methods such as coagulation, filtration, flocculation, oxidation, and electrochemical methods. (Kapdan 2000). Treatment of these wastes by biological methods can be cost-effective and ecological alternative to physicochemical treatment strategies.

Ligninolytic enzymes degrade complex and recalcitrant polymeric lignin as well as associated complex compounds. These enzymes have large number of applications in various other fields, such as agriculture, chemical, food, fuel and cosmetics (Asghar et al. 2008)

Ligninolytic enzymes viz laccase, manganese peroxidase and lignin peroxidase are group of extracellular enzymes employed in various industries for the degradation of ligninolytic substrates. (Orth and Tien 1995). Laccases are copper containing enzymes which oxidize wide range of substrates such as mono, di and polyphenols with the reduction of oxygen to H<sub>2</sub>O (Gualhup et al. 2002). LiP is extracellular monomeric heme glycoprotein peroxidase extracted from *Phanerochaete chrysosporium* for the first time (Venkatadri, and Irvine 1993) with molecular weight from 37 to 50 kDa. It oxidize non-phenolic, polycyclic aromatic, phenolic compounds and depolymerize plant's cell wall lignin. MnP is extracellular glycoprotein classified as oxidoreductases, with molecular weight from 40 to 50 kDa. It produce phenoxy radical by the oxidation of phenolic compounds with H<sub>2</sub>O<sub>2</sub> as an oxidant. (Asgher et al. 2008, Hofrichter, 2002). MnP also oxidize nonphenolic compounds such as lignin and xenobiotic pollutants.

The ligninolytic enzymes are capable for the oxidation of a numerous toxic organic compounds that change these compounds to non-toxic or carbon dioxide and H<sub>2</sub>O metabolites. (Bumpus and Aust 1987).

Solid state fermentation appears more promising for enzymes production rather than other strategies as it has many advantages for fungal growth. Enzymes can be isolated and purified to different extent for various industrial application. Solid state fermentation (SSF) is an attractive method for the conversion of lignocellulosic biomass and ligninase production. SSF is a kind of fermentation which supports the growth of fungus over solid substrate under moist environment. Enzymes production is affected by pH and temperature of fermentation broth and the concentration of carbon and nitrogen sources. For optimization of enzymes production Response Surface Methodology (RSM) provides more reliable results. In contrast to conventional Classical Optimization Strategy (COS) of one varying factor at a time RSM can examine more than one factor at a time at different levels to determine interaction between two or more factors. (Asgher et al. 2013). RSM is a statistical technique used to design experiments and evaluate interactive effect among variables and optimize fermentation medium.

*A. flavus* is a saprotrophic fungus with universal occurrence, it belongs to phylum Ascomycota, family Trichocomaceae, was investigated for ligninase production during current study This fungus is generally found in soil and plants.

Keeping in view the importance of ligninolytic enzymes, the present study was carried with the following objectives: optimization of the production of ligninolytic enzymes of *Aspergillus flavus* through SSF by using Response Surface Methodology (RSM), proximate analysis of different lingo-cellulosic substrates and selection of most suitable for

enzymes production, partial purification and characterization of ligninolytic enzymes produced under optimized conditions.

## Materials and methods

### Selection of substrate

Various ligninolytic substrates i.e. *Platanus orientalis* (chinar), *Bauhinia variegata* (orchid tree), *Pinus roxburghii* (chir pine), wheat straw and wheat husk were collected. The moisture content in these substrates was determined. These were sun dried for 8 days and dried at 70°C in oven for 24 hours to remove any remaining moisture, and then weight loss was measured. These were then ground through plant grinder having mesh size 40 mm and 80mm at room temperature and packed in air tight plastic jars, and proximate analysis were carried out.

### Proximate analysis

Five different agricultural wastes were analyzed for percentage nitrogen, crude protein, crude fiber, ether extract, total ash contents, and moisture contents as described by (AOAC 1990, AOAC 1995).

### Determination of ADF, ADL, ADC and AIA

For proximate analysis of each lignocellulosic substrate, acid detergent fiber (ADF), acid detergent lignin (ADL) acid detergent cellulose (ADC) and acid insoluble ash (AIA) contents were estimated by (AOAC 1995, Goering and Van Soest 1970, Van Soest 1963b).

### Preservation of fungus

Culture of *Aspergillus flavus* was obtained from Industrial and Environmental Biotechnology Laboratory (IEBL), Department of Biochemistry, PMAS-Arid Agriculture University Rawalpindi. It was grown on Potato Dextrose Agar (PDA) media for further use (Mahmood et al., 2013). The pH of the medium was maintained at 5.5, incubated at 30°C for 72 h.

### Preparation of fungal inoculum

Inoculum of *A. flavus* was prepared in Potato Dextrose broth using 250 mL Erlenmeyer flask with composition: Glucose (2.0 gm), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.02 gm), CaCl<sub>2</sub> (0.005 gm), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.005 gm), KH<sub>2</sub>PO<sub>4</sub> (0.02 gm) and distilled water was added to make the final volume 100 mL. The pH was adjusted at 5.5 and broth was autoclaved at 121°C for 20 minutes at 15 psi for sterilization. *A. flavus* was transferred to the flasks with Potato Dextrose broth aseptically. These flasks were kept in shaker incubator at 30°C for 72 hours and 120 rpm (Krishna, 1999).

### Solid state fermentation (SSF)

The ground *Platanus orientalis* was poured in 500 mL Erlenmeyer flasks. Flasks were autoclaved at 121°C for 20 min and specific amount of fungal inoculum (1–6 mL) was transferred aseptically. These flasks were incubated at various temperatures. The physical factors such as temperature (10 to 40°C), pH (3 to 6), inoculums size (1 to 6 ml) and substrate size (2 to 10 g) were optimized through response surface methodology (RSM) using five factors, five level central composite design (CCD) having 2 central points with a total of 28 runs each for both meshes sizes (40 mm and 80 mm).

Glucose, fructose and sucrose (0.1 to 0.5%) of Sigma Aldrich, were used as additional carbon sources while ammonium sulfate and urea (0.1 to 0.5%) of Sigma Aldrich were added as additional nitrogen sources in SSF medium to check their effect on the production of ligninolytic enzymes. To optimize ligninolytic enzymes production, RSM was employed under Central Composite Design (CCD) by using JMP software. Final SSF period was 120 hours, pH 4.5, inoculum size 3.5 ml, temperature 32°C, substrate size 7 g and substrate mesh 80.

### Crude enzyme extraction

The flasks were harvested for the extraction of ligninolytic enzymes by contact method after specific hours as described by Mahmood et al., (2013). In each of the flasks, 50 ml distilled water was added and shaken in WIs-20R incubator at 120 rpm for 1 h at room temperature. In this way, extra cellular enzymes were dissolved in water and filtered through filter paper. Filtered extract was then centrifuged in Centurion Scientific K241R centrifuge at 1000 rpm for 15 min at 4°C to remove spores and impurities. Supernatants were stored as crude extract at 4°C for enzyme assay.

### Enzymes Assay

All enzyme assays were carried out spectrophotometrically at room temperature. The activities were expressed in unit per ml.

Activity of laccases was measured by oxidation of Azino-bis ethylbenzthiazoline-6-sulfonic acid (ABTS). 1 mL each of 1 mM ABTS and 50 mM sodium malonate (pH 4.5) were mixed, 100 µL of supernatant culture was poured in it and absorbance was taken at 436 nm. Laccase activity was measured by

$$A = \epsilon_{436} c l \quad (\epsilon_{436} = 36,000 \text{ M}^{-1} \text{cm}^{-1}) \quad (\text{Shin and Lee 2000}).$$

LiP assay was performed by pouring 100 µL crude enzyme in a mixture of 1 mL 100mM Tartarate buffer (pH 3), 1 mL of 4 mM veratryl alcohol, 500 µL of 0.2 M H<sub>2</sub>O<sub>2</sub>. The absorbance was measured at 310 nm. Enzyme activity was calculated by

$$A = \epsilon_{310} c l \quad (\epsilon_{310} = 9,300 \text{ M}^{-1} \text{cm}^{-1}) \quad (\text{Tien and Kirk, 1983}).$$

MnP assay was carried out by the addition of 500 µL of 0.1 mM H<sub>2</sub>O<sub>2</sub> as oxidizing agent in 1 mL of 1 mM MnSO<sub>4</sub> and 1 mL of 50 mM sodium malonate buffer (pH 4.5). 100 µL of culture supernatant was added in the above mixture and absorbance was read at 270 nm. MnP activity was calculated by

$$A = \epsilon_{270} c l \quad (\epsilon_{270} = 11,590 \text{ M}^{-1} \text{cm}^{-1}) \quad (\text{Wariishi et al. 1992}).$$

Where:

ε = coefficient;

A = Absorbance;

c = concentration;

l = path length

### Protein estimation

Protein contents in crude and partially purified samples were estimated by adding 0.2 mL enzyme in 2 mL of Lowery reagent and incubated for 30 minutes in dark at 25°C. After 30 minutes 0.2 mL of Foline and Ciocalteu's phenol reagent was added

and again incubated at 25°C in dark for 30 minutes. Then the mixture was subjected to enzyme assay at 660 nm. Bovine serum albumin (BSA) was used as a standard. (Lowry et al. 1951). Simple linear regression equation was used to calculate the protein of unknown sample.

### Purification of ligninolytic enzymes

The ligninolytic enzymes produced under optimized conditions were purified for characterization using the following techniques.

#### Ammonium sulfate precipitation

The partial purification of ligninolytic enzymes was carried out by adding different concentrations of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> i.e. 10, 20, 30, 40, 50, 60, 70 and 80% in 2 ml crude sample each and left overnight at 4°C. These samples were then centrifuged at 10,000 rpm for 15 min at 4°C. Both pellet and supernatant were subjected to enzyme assay and protein estimation (Mahmood et al. 2013).

#### Gel filtration chromatography

Partially purified ligninolytic enzymes were subjected to gel filtration chromatography for further purification. 5% silica gel column was dissolved in malonate buffer of pH 4.5 for laccase and MnP, in tartarate buffer of pH 3 for LiP purification. After the addition of 1 ml enzyme, elutions were maintained at flow rate of 30 cm/h. Enzyme assay was carried out for all 20 elutions. The elution having maximum activity was subjected to characterization of different kinetic parameters.

### Characterization of ligninolytic enzymes

Purified ligninolytic enzymes were subjected to characterization to study optimum pH, temperature and kinetic parameters.

#### Optimization of pH for ligninolytic enzymes

Laccase and MnP were subjected to enzyme assay at different pH, i.e. 2.5, 3.5, 4.5, 5.5 and 6.5. Sodium malonate buffer was employed in the reaction mixture to maintain pH. LiP assay was done from pH 1, 2, 3, 4 and 5 and to maintain pH tartarate buffer was used.

#### Optimization of temperature for ligninolytic enzymes

Enzyme assay was performed for laccase, MnP and LiP activity at different temperatures from 25, 30, 35, 40 and 45°C.

#### Effect of substrate concentration ligninolytic Enzymes

The effect of substrate concentration on ligninolytic activity was studied by making different concentrations of substrates, i.e. 2, 4, 6, 8 and 10 mM to determine *K<sub>m</sub>* and *V<sub>max</sub>*. ABTS, manganese sulfate and veratryl alcohol were used as substrates for lignin peroxidase, manganese peroxidase and laccase respectively. Activities found at each concentration were used to draw double reciprocal graph between inverse of concentrations and rate of reaction. The linear regression equations obtained from graph were used to calculate *K<sub>m</sub>* and *V<sub>max</sub>*.

## Results and discussions

### Selection of substrate

Proximate analysis gives us the information of chemical composition of the agricultural biomass and is a scheme

for the description of animals feed stuffs. It allows us to make a comparison of feeds based on specific nutrients. The outcomes of proximate composition given in Table 1. shows that *Platanus orientalis* has more lignin contents among five analyzed samples and selected as substrate for ligninolytic enzymes production. Crude fiber contains lignin, cellulose, hemicelluloses, and other soluble fibers (Parish 2007). Based on the finding, the highest crude fiber contents were observed in *Platanus orientalis* which confirm it as suitable substrate among others. Crude proteins comprise both true protein and non protein nitrogen and *Bauhinia variegata* had the highest levels of crude protein followed by wheat straw. Crude fat is an estimate of the total fat contents and is very high in *Pinus roxbergii* followed by *Bauhinia variegata*. Low moisture content in a sample is an indication that microbe's activities would be reduced and thereby increases the shelf life of sample and vice versa. The lowest moisture content was observed in *Bauhinia variegata* and *platanus orientalis* respectively. Ash is a residue that contains inorganic mineral element of feed sample and is determined by burning the sample at high temperature. Ash content for the leaves and stems of *Bauhinia variegata* is highest among all samples. ADF is a measure of the plant components in forages that are the least digestible by livestock, including lignin and cellulose. More ADF content decreases the digestibility, so forages with high ADF percentage are typically lower in energy. ADF content determination is a preparatory step for lignin, cellulose and AIA determination in present study; ADF content was found highest in *platanus orientalis* which further confirms that it is a good substrate for ligninase production.

### Optimization ligninolytic enzymes production by *A. flavus*

#### Optimization of physical and nutritional conditions through RSM

Response Surface Methodology (RSM) was used with a central composite design. The interactions studied among independent variables were fermentation period, pH, inoculum size, incubation temperature, substrate size and substrate mesh. Comparison of SSF with 40 and 80 meshes reveals that 80-mesh size was more suitable. For enzymes production particle size of the substrate plays an important role in fungal growth and enzymes production in SSF. In the SSF growth of fungi, the availability of surface area is vital for fungal attachment, sufficient transference of nutrients and substrates, consequent fungal growth and enzyme production that ultimately depends on substrate particle size (Sindhu et al. 2009, Beeraka et al. 2008). Smaller mesh, i.e. 80, satisfies these conditions for fungal growth.

#### Interaction among variables

The interaction between two factors provides a better understanding of the overall process analysis. One factor may interact with any or all the other factors making the possibility of existence of many interactions.

#### Inoculums size vs pH

Inoculum size and pH play an important role in fungal growth and enzymes production. Response surface study shows that there is a positive interaction between inoculums size and pH. Maximum laccase, MnP and LiP production was observed

**Table 1.** Proximate analysis, ADF, ADL, ADC and ADL contents

| No | Name of biomass      | Moisture % | Crude protein % | Crude fat % | Crude fiber % | Total ash % | ADF % | ADC % | ADL % | AIA % |
|----|----------------------|------------|-----------------|-------------|---------------|-------------|-------|-------|-------|-------|
| 1  | Wheat straw          | 10.98      | 3.68            | 4.64        | 43.90         | 6.57        | 34.60 | 29.10 | 4.5   | 1.50  |
| 2  | Wheat husk           | 10.19      | 3.50            | 4.18        | 25.00         | 6.93        | 30.60 | 29.61 | 1.02  | 0.04  |
| 3  | <i>P. orientalis</i> | 12.64      | 2.80            | 8.58        | 49.10         | 5.40        | 37.20 | 27.40 | 8.9   | 0.90  |
| 4  | <i>P. roxbergii</i>  | 8.11       | 3.15            | 10.74       | 43.78         | 3.00        | 29.50 | 25.05 | 4.9   | 0.10  |
| 5  | <i>B. variegata</i>  | 5.82       | 4.02            | 9.29        | 32.65         | 12.45       | 18.20 | 17.67 | 0.77  | 0.80  |

**Table 2.** Interaction among variables for ligninolytic enzymes production

| Variables                          | Laccase      | MnP          | LiP          |
|------------------------------------|--------------|--------------|--------------|
| Inoculums size × pH                | 4 ml × 5     | 3.5 ml × 5.5 | 4 ml × 6     |
| Incubation period × temperature    | 120 h × 32°C | 100 h × 30°C | 100 h × 30°C |
| Incubation period × pH             | 120 h × 6    | 100 h × 6    | 100 h × 6    |
| Incubation period × substrate size | 120 h × 7 g  | 100 h × 7g   | 100 h × 7 g  |
| Substrate size × pH                | 7 g × 5      | 6 g × 6      | 7 g × 6      |
| Inoculums size × substrate size    | 3 ml × 7 g   | 3.5 ml × 5 g | 3.5 ml × 7 g |
| Glucose × sucrose                  | 0.3 × 0.3%   | 0.3 × 0.4%   | 0.3 × 0.3%   |
| Sucrose × fructose                 | 0.3 × 0.4%   | 0.4 × 0.4%   | 0.4 × 0.3%   |
| Ammonium sulfate × fructose        | 0.3 × 0.4%   | 0.3 × 0.4%   | 0.4 × 0.3%   |
| Urea × glucose                     | 0.3 × 0.3%   | 0.3 × 0.3%   | 0.3 × 0.2%   |

with 4, 3.5, 4 ml inoculum and at 5, 5.5, 6 pH respectively (Table 2). Small inoculum size may be unable to start growth while higher inoculum size may start competitive inhibition. At any pH the intact enzyme may have both cation or anion groups. These groups are often apparently part of the active site. Changing the pH of medium will change the ionic form and ultimately enzyme activity and reactions rate. Three-dimensional structure of the enzymes may alter due to change in pH.

#### *Incubation period vs temperature*

The interaction between incubation period and temperature to produce ligninolytic enzymes showed that enzymes yield increased with incubation period and temperature. (Table 2). Fungal growth and enzymes production increase up to certain fermentation period and after that it is decreased probably because of nutrients depletion and waste accumulation. In SSF system temperature is also of much importance because of its effect on microbial growth and enzymes production. During fermentation, the temperature of the fermenting mass is generally increased due to respiration. At higher temperature low enzymes production was seen because of substrate drying.

#### *pH vs incubation period*

Response surface finding of pH against incubation period is shown in Table 2. The result predicted that both pH and incubation period had a positive effect on *A. flavus* growth and enzymes production. Higher levels of the said parameters predict low enzyme production because higher pH retards fungal growth. pH offers an important role in fermentation through maintaining a suitable ionic strength. Enzymes production is favored either by acidic or alkaline pH which was observed acidic in present study.

#### *Incubation period vs substrate size*

Positive interactive influence is observed between incubation period and substrate size. Enzymes production increases with substrate size and incubation period. More amount of substrate in a container makes a thick substrate bed that lessens the pore size of the substrate and the transfer of oxygen is reduced among the substrate particles, particularly at the bottom of the flask where the substrate was not completely fermented out. A thinner bed height facilitates better oxygen transfer and heat removal than a thicker bed height.

#### *Substrate size vs pH*

RSM shows that pH and substrate have positive interaction for ligninase production. Table 2 shows enhanced production of ligninolytic enzymes with increase in substrate size and pH. Higher pH beyond optimum hinders fungal growth and thick bed of substrate makes it less available for fungus.

#### *Inoculum size vs substrate size*

RSM results show synergistic and antagonistic effect on ligninolytic enzymes production. Higher ligninolytic activities were observed as inoculum and substrate size were increased up to a certain level (Table 2) and then enzyme activity became low because of competitive inhibition and non-availability of the substrate in the case of inoculum and substrate respectively.

#### *Glucose vs sucrose*

The interaction between glucose and sucrose shows enhanced growth of microbe and production of enzymes (Table 2). *A. flavus* growth and ultimately enzymes production are increased more because of readily available carbon sources in the form of glucose and sucrose than substrate. Glucose proved a better carbon source as sucrose being disaccharide would first be converted to glucose and then used by fungus. Glucose is readily available to fungus.

#### *Fructose vs sucrose*

Both fructose and sucrose have a positive impact on microbial growth and ligninase production. As mentioned earlier, those external carbon sources enhanced microbial growth and enzymes production because of their readily availability (Table 2).

#### *Fructose vs ammonium sulfate*

An optimum C:N is necessary for fungal growth and enzymes production. Being a part of cellular components and proteins, easily available nitrogen favored microbial growth and ligninase production. A positive interaction was observed for ligninase production with fructose and ammonium sulfate (Table 2).

#### *Glucose vs urea*

Enhanced ligninolytic enzymes production was observed by interaction between glucose and urea. (Table 2). Carbon and nitrogen are important variables in ligninase production. At low C:N the fungus is carbon starved and higher nitrogen concentration makes the substrate unfavorable for fungus by changing its texture.

#### *Protein Estimation*

Protein in crude extracts and partially purified ligninolytic samples were estimated by method of Lowery et al., (1951). A standard curve is obtained by making a graph of absorbance against various concentrations of BSA (Fig. 1). By using regression equation, protein in laccase crude extract and partially purified sample were found to be 1.78 and 0.71 mg/ml respectively. MnP crude extract had 1.59 and 0.68 mg/ml protein in purified sample. Average protein in LiP crude sample was 1.70 mg/ml and 0.69 in partially purified enzymes. High protein amount in crude samples indicate the presence of enzymes other than ligninase. Low protein contents in purified samples confirm the purification of ligninase excluding unwanted enzymes.

#### *Purification of ligninolytic enzymes*

##### *Ammonium sulfate precipitation*

Ammonium sulfate is used for salting out preferentially because it is soluble in water and high ionic strength is attained. It precipitates out ligninase by decreasing its solubility.

Maximum laccase activity in supernatant was 77.78 U/ml with 50% and 36.11 U/ml in pellet with 40%  $(\text{NH}_4)_2\text{SO}_4$  (Fig. 2a). (Sahay et al., 2009) used 30% to 80% ammonium sulfate for laccase purification. MnP maximum activity in supernatant was 224.33 U/ml with 60% and 77.65 U/ml in pellet with 40%  $(\text{NH}_4)_2\text{SO}_4$  (Fig. 2b). (Sylvia et al., 2015) used 60% ammonium sulfate for MnP purification. The maximum LiP activities were 301.07 and 139.78 U/ml

in supernatant and pellet respectively with 40%  $(\text{NH}_4)_2\text{SO}_4$  (Fig 2c). Comparison of activities of supernatant and pallet reveals that supernatant has got more activity than pallet. Supernatant contains more enzymes than pellet therefore higher activities were achieved in supernatants. As mentioned earlier, partially purified samples have lesser protein content than crude extract which confirms the removal of unwanted enzymes and purification of ligninase. The results of current study are within the international range.

#### Gel filtration chromatography

After gel filtration chromatography with 5% Silica gel, the analysis results revealed increase in enzyme activity. The maximum activities were observed at elutions 7 (97.22 U/mL), 11 (276.10 U/ml) and 9 (483.87 U/ml) for laccase, MnP and LiP respectively. (Fig 3 a, b, c). Increase in enzymes activities was observed because of reduction in total protein concentration. This is due to purification of our desired protein and exclusion of unnecessary proteins after purification. Higher enzymes

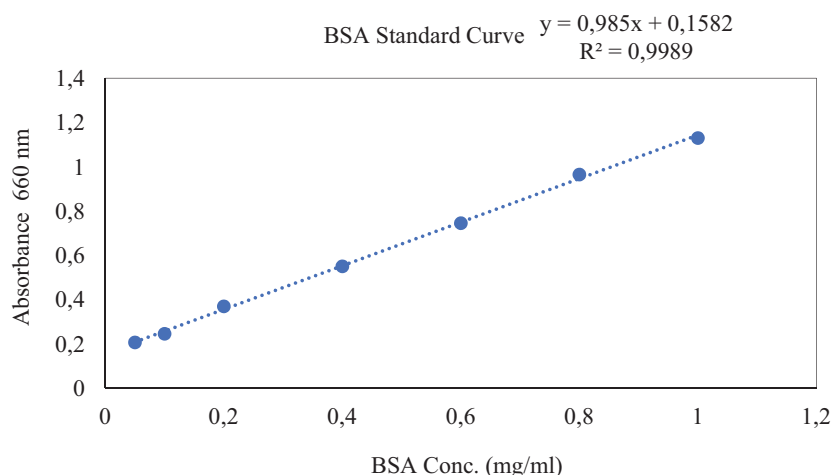


Fig. 1. Standard curve drawn using various concentration of BSA to determine protein in crude and partially purified enzymes

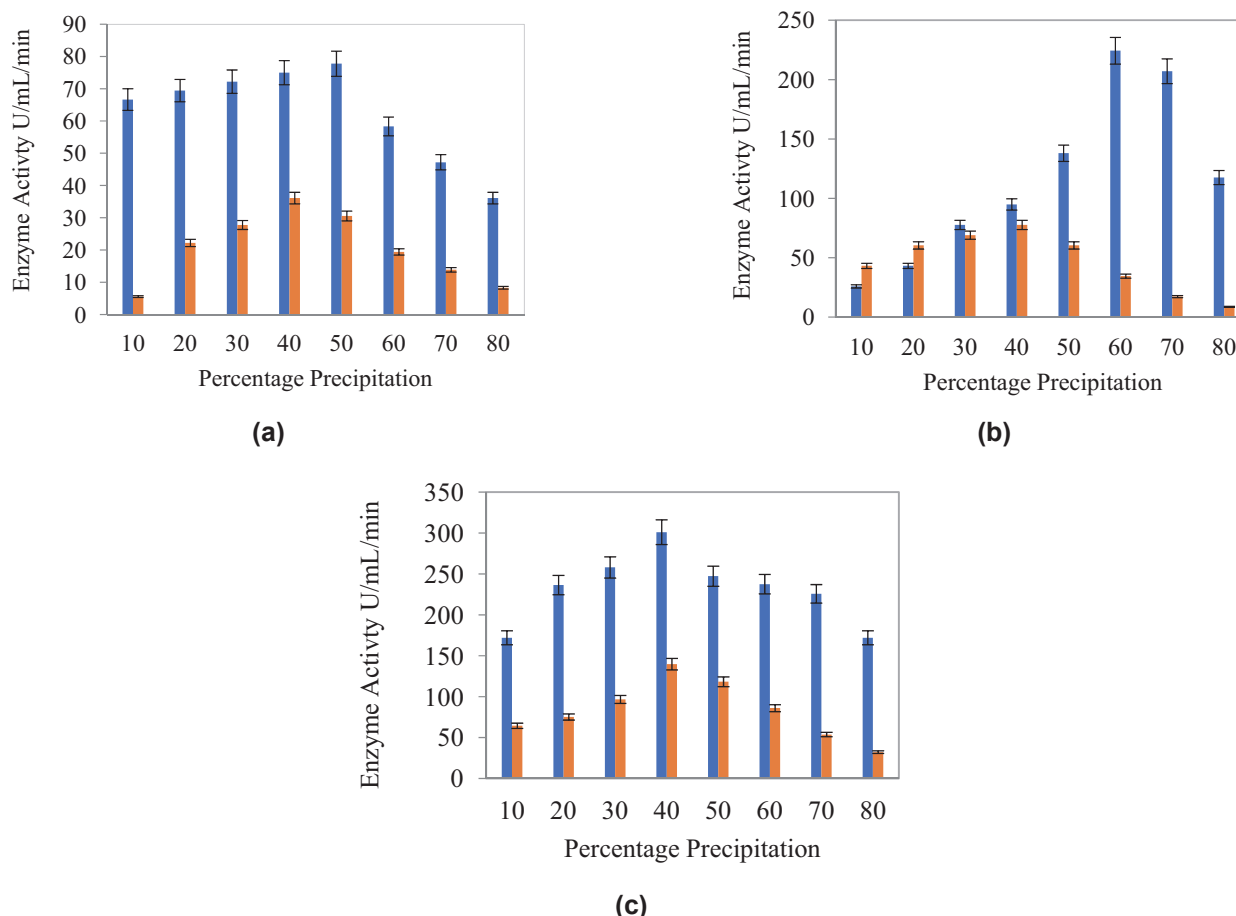


Fig. 2. Comparison of enzyme activities in ammonium sulfate precipitated supernatant (blue) and pallet (red), Laccase (a), MnP (b), LiP (c)

activities after gel filtration chromatography confirm complete purification of ligninolytic enzymes.

### Characterization of ligninolytic enzymes

Ligninolytic enzymes were characterized by studying the effect of temperature, pH, kinetic parameters like  $K_m$  and  $V_{max}$ . Characterization of ligninolytic enzymes enhance their efficiency in industrial applications.

### Optimization of temperature of ligninolytic enzymes

Laccase showed maximum activity at 35°C (47.22U/ml), it increased from 25 to 35°C but after that it started to decline (25.0 U/ml) a° (Domenguez et al. 2007) have reported 30°C optimum temperature, (Sahay et al. 2008) 37°C (Kammoun et

al. 2009) 55°C, (Rogalski and Janusz, 2010) 55°C and (Sahay et al., 2009) 60°C.

Manganese peroxidase activity increases gradually up to at 30°C (327.8U/ml) and then decreases (138.0 U/mL/min) at 45°C. (Fig. 4b). Reported optimum temperature for the Mnp activity varies from 30°C to 40°C. (Aslam and Asgher 2011) have reported the optimum temperature to be 30°C (Irshad and Asgher 2011) 35°C.

Optimum temperature for LiP activity was 30°C (677.41 U/ml) and a gradual decrease was seen at higher temperatures (75.0 U/ml) at 45°C. (Fig. 4c). Reported optimum temperature for LiP activity varies from 22°C to 40°C. (Egwim et al. 2015) 35°C. (Fujian et al. 2001) 39°C, (Rai et al. 2016) 22°C.

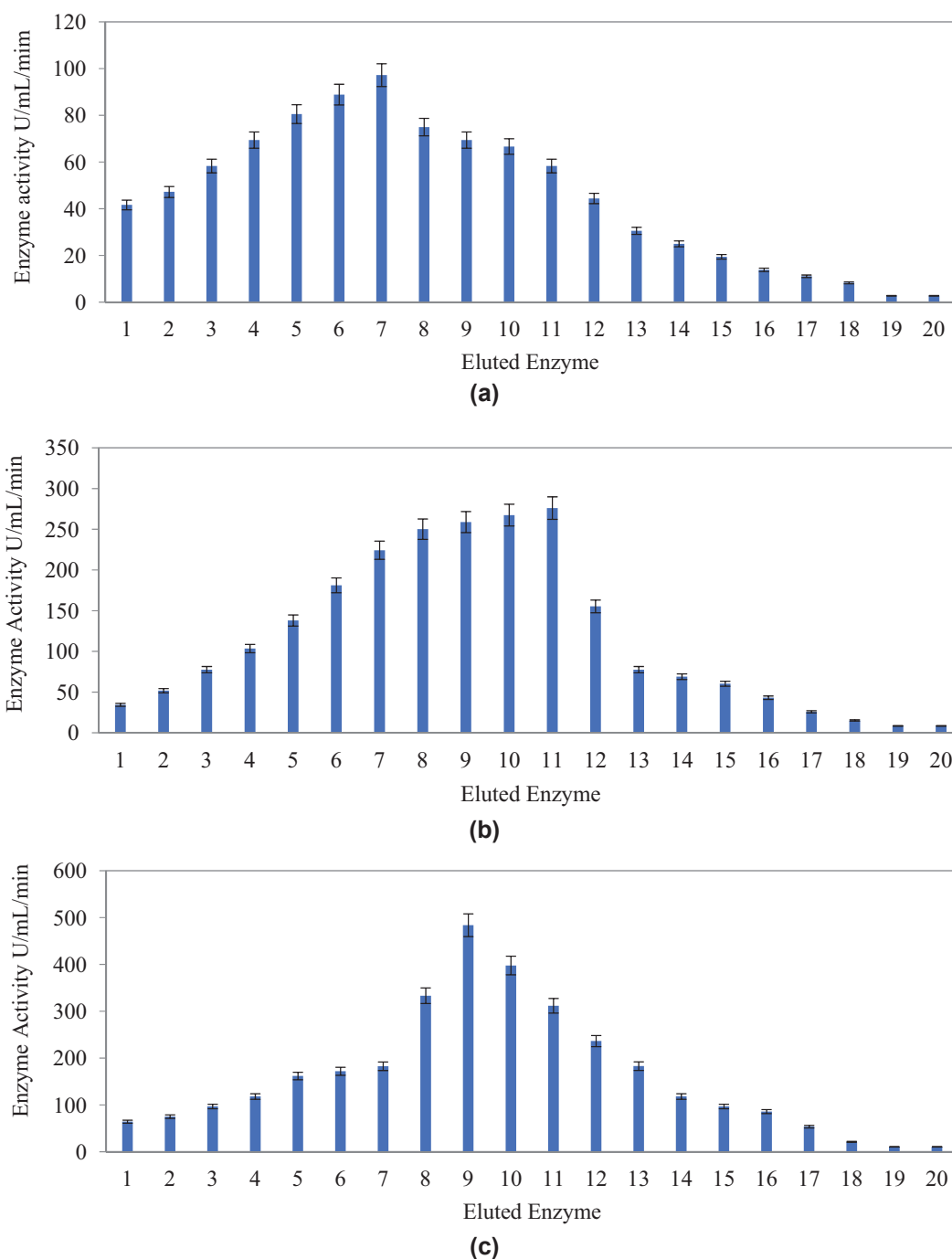


Fig. 3. Laccase (a), MnP (b), LiP (c) activities in eluted samples of gel chromatography

Ligninase activities decrease above optimum temperature because denaturation of enzymes starts above optimum and proteins lose their globular structure. These large variations of the optimum temperature of ligninolytic enzymes suggest that the optimum temperature for ligninase differ according to the type of fungus used and the type of enzymes produced. The results of current study are within the international range.

#### Optimization of pH for ligninolytic enzymes

Optimum pH for laccase was 5.5, increase from pH 2.5 to 5.5 (80.55 U/ml) and then decrease were observed (Fig. 5a). (Dhong and Zhang 2004) have reported pH from 6 to 9, (Rotkova et al. 2009) have reported two optimum pH values depending on the type of substrate for laccase assay. These pH values are 3.5 and 5 for ABTS and SGZ respectively. (Kammoun et al. 2009) have reported the optimum pH of 8, (Sahay et al. 2009) 5, (Sahay et al. 2008) 4.5 and (Rogalski and Janusz, 2010) the optimum pH of 5.5 for laccase activity.

MnP showed maximum activity at pH 5.5 (448.66 U/ml) (Fig. 5b). (Yasmeen et al., 2011) have observed 4.5 optimum pH, (Asalam and Asgher 2011) 4, and (Rajan et al. 2010) the optimum pH of 5 for MnP activity. An optimal pH range from 4 to 7 has been observed by (de Oliveira et al. 2009, Baborova et al. 2006, Bermek et al. 2004).

LiP activity remains active up to pH 3 (545.16 U/mL/min) and then decreases (Fig 5c). Egwim et al. (2015) reported the

optimum pH of 6.5 for LiP activity, (Yasmeen et al. 2011) have reported the optimum pH of 4.5, (Dias et al. 2007, Garavaglia et al. 2004, Angel 2002) have reported the optimum pH from 3 to 4.5.

The decrease in enzyme activities was probably because of change in the ionic strength of the reaction mixture which caused instability of the proteins. The variations in optimum temperature of ligninolytic enzymes suggest that the optimum pH for ligninolytic enzymes varies with type of substrate used. The outcomes of our study agree with previously published results.

#### Effect of Substrate Concentration and Determination of $K_m$ and $V_{max}$

Substrate concentration plays an important role in the enzymatic activity. Low concentration may cause lower activity. Therefore, the determination of  $K_m$  and  $V_{max}$  is an important step in enzyme characterization.

Different concentrations from 2 to 10 mM of the substrates ABTS,  $MnSO_4$  and Veratyl alcohol were prepared for laccase MnP and LiP respectively. Increase in enzyme velocity was observed with increase in substrate concentration but the rate becomes constant after certain concentration. This is because of non-availability of binding site for substrate on enzyme. Line-weaver Burk plot of the  $1/V_o$  versus  $1/[S]$  was prepared to find out  $K_m$  and  $V_{max}$ , these were 0.289 mM and 227.27  $\mu M/ml$

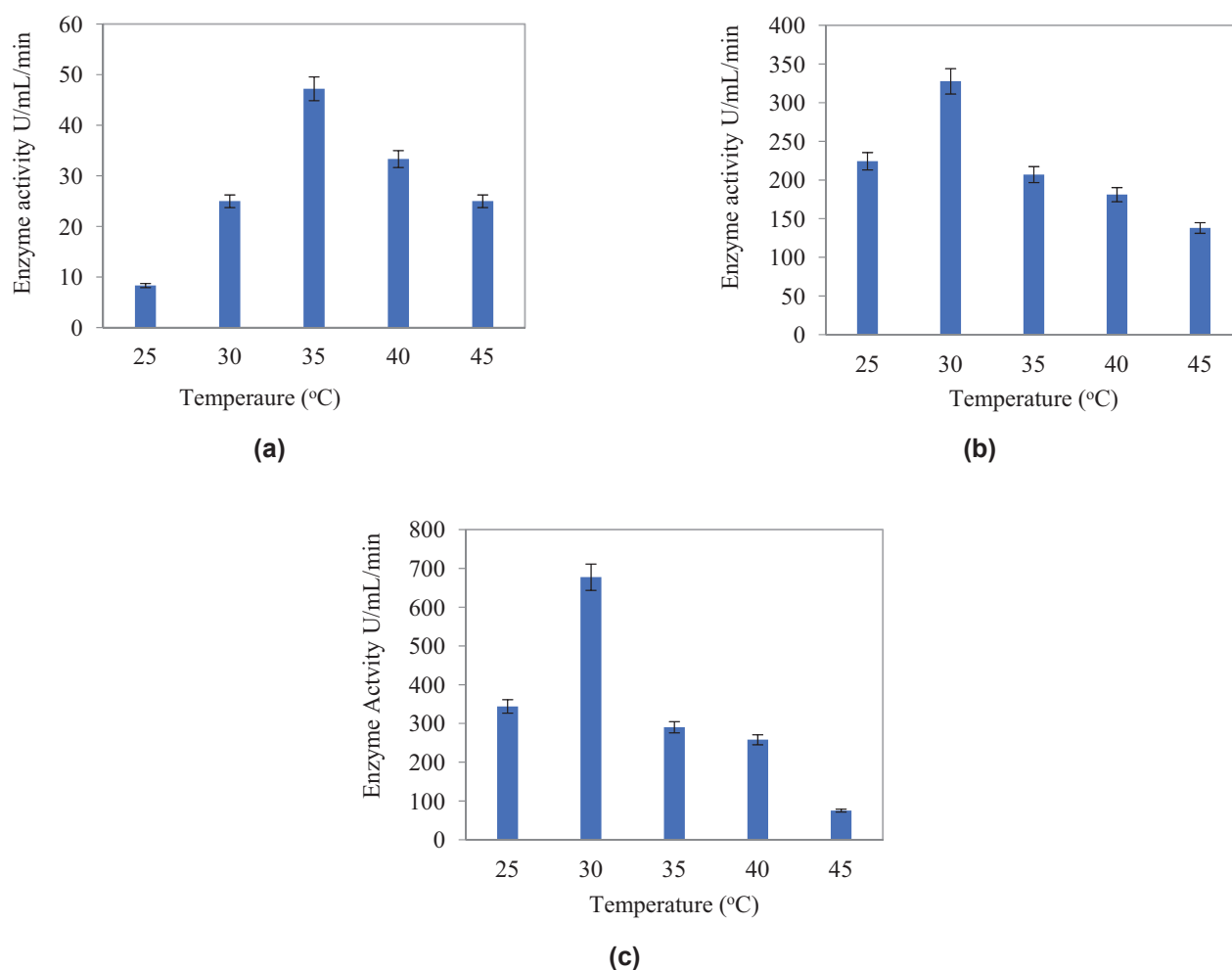


Fig. 4. Effect of Temperature on Laccase (a) MnP (b) LiP (c) activities



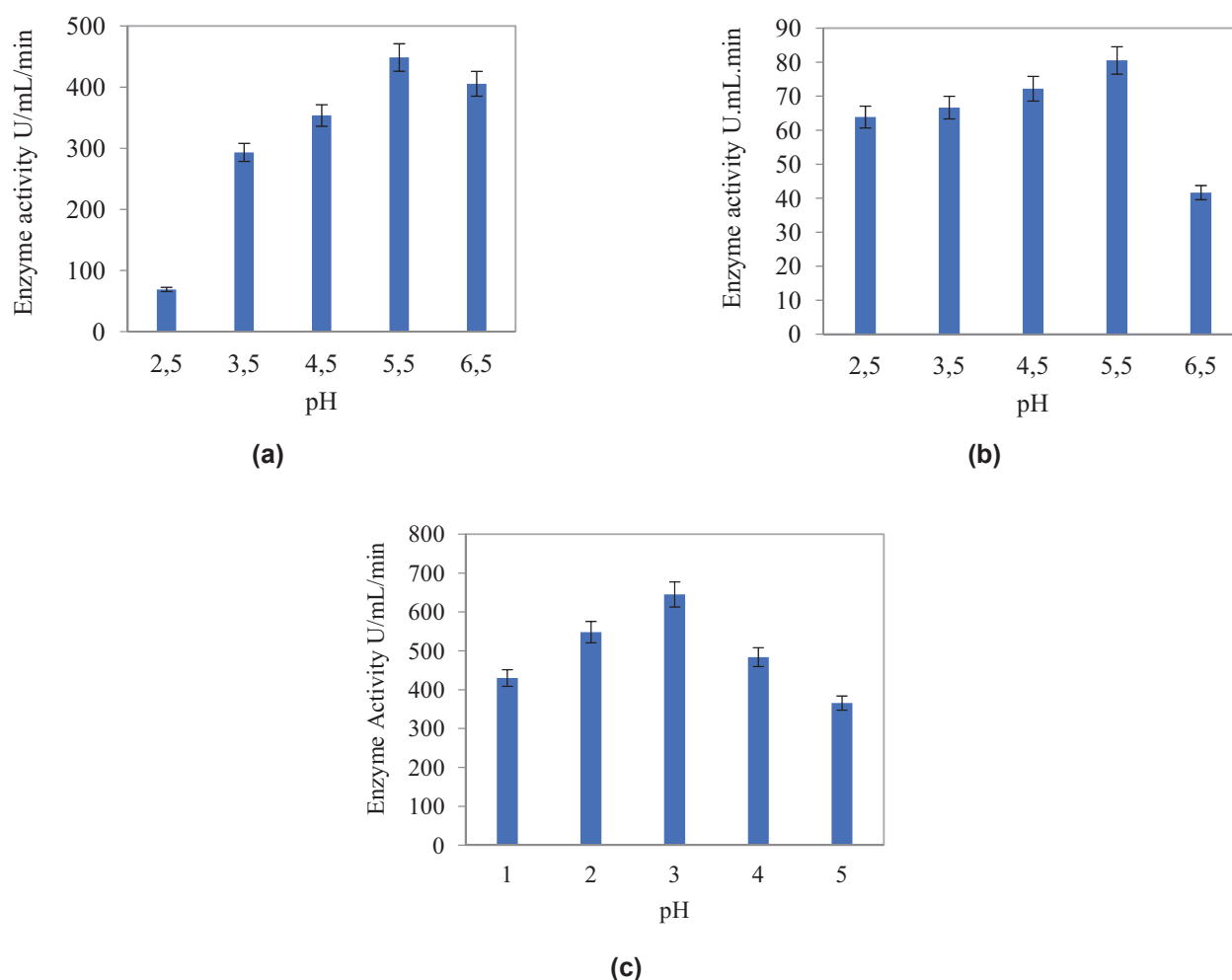


Fig. 5. Effect of pH on Laccase (a) MnP (b) LiP (c) activities

respectively for laccase, 0.538 mM and 203.08  $\mu\text{M}/\text{ml}$  for MnP, 2 mM and 2000  $\mu\text{M}/\text{ml}$  for LiP. Reported  $K_m$  and  $V_{max}$  varies in literature for ligninase. (Sahay et al., 2009) have reported five different  $K_m$  values using five different substrates. They have reported 480, 350, 320, 230 and 480  $\mu\text{M}$  values using 2,6-dimethoxyphenol, syringaldize, pyrogallol, catechol and m-cresol respectively as substrate for  $K_m$  of laccase. (Rotkova et al. 2009) have reported 0.0337 mM, (Dong and Zhange 2004) 0.001 mM  $K_m$  values for ABTS as a substrate. (Asgher et al. 2013) have reported 0.4 mM  $K_m$  and 410 mM  $V_{max}$  for MnP using  $\text{MnSO}_4$  as a substrate, (Sylvia et al. 2015) reported  $K_m$  27 mM and  $V_{max}$  0.3 mM, for MnP from *Bjerkandera* sp. whereas the  $K_m$  of MnP for  $\text{H}_2\text{O}_2$  and 2,6-Dimethoxyphenol were 71.4 mM and 28.57 mM respectively (Asgher et al. 2013). For LiP Yadav et al. 2009) have reported  $K_m$  and  $V_{max}$  58 and 83mM, (Bibi and Bhatti 2012) 16.67 mM  $K_m$  and 179 U/ml  $V_{max}$  values. Variations in  $K_m$  and  $V_{max}$  result from different substrates used and type of fungus. Low  $K_m$  values show high affinity of the enzymes for the substrates. Considering the experimental values for  $K_m$  and  $V_{max}$  already reported, our results are in accordance with published results.

## Conclusions

Proximate composition of substrates shows *Platanus orientalis* with more lignin content (8.9%) and used in SSF for ligninase

production. Optimization and characterization of ligninolytic enzymes by *Aspergillus flavus* was carried out. Higher values of ligninolytic activities show that these enzymes have potential in industrial applications.

## Acknowledgments

We acknowledge the Poultry Research Institute Rawalpindi Pakistan for providing their technical support.

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sector.

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