BIODEGRADATION OF SOME ENVIRONMENTAL POLLUTANTS BY LACCASE PRODUCED FROM *TRICHODERMA HARZIANUM* USING SOLID STATE FERMENTATION

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ABSTRACT

Trichoderma harzianum **produced 5.383 U/mg of laccase in solid state fermentation (SSF) using 10gm of wheat bran as substrate with moisture content 1:0.7 w/v, using 4 plugs×5mm as inoculums size, for 7 days at 28C°. Laccase was partially purified with 7.260 fold and yield 84.01% by ion exchange chromatography DEAE-cellulose. Partially purified laccase had an optimum pH of 4 and was stable in pH range from 3.5 to 5.5. The optimum temperature for laccase activity was 35C° and stability was range from 15 to 35C° with 1h of incubation. The ability of *T. harzianum* isolate to decolorize textile dyes on solid media appears completely decolorization of Blue dye with concentrations 50,100 and partially decolorized with concentration 150,200 and 250 ppm, while the Yellow dye and Red dye were completely decolorized at 50 ppm concentration. The ability of partial purified laccase to degradation of phenol was completely occurs in concentration 5, 10 but at 20, 50 and 100 ppm about 90.94%, 93.93%and 36.78% respectively, while furfural degradation at 5 and 10 ppm was about 86.84% and 68.66% respectively which detected using HPLC technique.**

Key words: *Trichoderma harzianum*, Laccase, optimization, biodegradation, pollutants.
***Part of M.Sc. thesis of the 1st author.**

التحلل الحيوي لبعض الملوثات البيئية بوساطة اللاكيز المنتج من الفطر *Trichoderma harzianum* باستخدام تخمرات الحالة الصلبة

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المستخلص

تم انتاج 5.383وحدة/ملغم من اللاكيز للفطر *Trichoderma harzianum* باستخدام تخمرات الحالة الصلبة ، باستخدام 10غم من نخالة القمح , بنسبة رطوبة 0.7:1(وزن/حجم) , حجم اللقاح4 اقراص بحجم 5ملم بعد فترة حضن 7 ايام بدرجة 28م°. تمت تنقية الانزيم وكانت عدد مرات التنقية 7.260 بحصيلة انزيمية مقدارها 84.01% باستخدام تنقية كهروموتغرافيا التبادل الايوني باستعمال المبادل الايوني DEAE–cellulose وكانت نتائج توصيف الانزيم المنقى ان الرقم الهيدروجيني الامثل لفعالية الانزيم هو 4 و الرقم الهيدروجيني الامثل لثباتيته من3.5الى5.5 بينما درجة الحرارة المثلى لفعالية الانزيم هي 35م° ولثباتيته الانزيم تراوح من15الى35م° عند حضنه لمدة ساعة واحدة. اظهر الفطر *T. harzianum* فعاليته على تحللا لصبغات النسيجية بدرجات متفاوتة حيث اظهر تحلل كاملا لصبغة الزرقاء بتركيز 50 و 100 جزء بالمليون وتحلل جزئيا لنفس الصبغة بالتركيز 250,200,150 جزء بالمليون . بينما اظهر تحللا كاملا لصبغات الحمراء والصفراء بتركيز50 جزء بالمليون.اظهر الانزيم المنقى جزئيا قابليته لتحلل الفينول بشكل كامل عند تركيز 5 و10 جزء بالمليون وكانت نسب التحلل لتراكيز 20, 50 و100 جزء بالمليون هي 90.94 ,93.93 و36.78% على التوالي.ان نسبة تحلل الفورفرال بالتراكيز 5 و10 جزء بالمليون كانت 88.84 و68.66% على التوالي, حيث تم الكشف عن نسبة التحلل للمواد المذكور باستخدام تقنيةالكهروموتوغرافي السائل العالي الاداء.

الكلمات المفتاحية: *Trichoderma harzianum* ، انزيم اللاكييز ، الظروف المثلى،الملوثات البيئية، التحلل الحيوي.
*** جزء من رسالة ماجستير للباحث الأول**

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INTRODUCTION

In the recent years the pollution increase day by day such as a physical, chemical and biological substance which directly or indirectly harmful to humans and other living organisms, one of these pollution that study in this work are synthetic dyes , phenol compounds and furfural. Synthetic dyes have range of application in various types of industry including food, pharmaceutical, textile, printing, paper or chemical but the release of dyes into the environment is harmful due to toxicity, carcinogenic and mutagenic effects on living organisms (23). Also phenol compounds produced as wastes of several industrial and agricultural activities. Phenolic compounds and their derivatives are considered priority pollutants because they are harmful toward living organisms, even at low concentrations (13). Oxidoreductases enzymes are able to catalyze the transformation of several phenolic compounds through an oxidative coupling reaction. This results in the formation of less soluble, high molecular weight compounds that may be easily removed from water by sedimentation or filtration (9). Furfural residues (FRs) are industrial wastes produced during the production of furfural from corncobs. They are composed mainly of cellulose and lignin, with about 45% of their weight being cellulose (36). From the food technology perspective, furfurals are potential carcinogenic compounds used as a marker of honey adulteration, generated by acid-catalyzed dehydration of carbohydrates of food-containing sugars (28). Also Furfural toxic activity against three biocontrol agent *Trichoderma harzianum*, *Trichoderma viride* and *paceilomyces lilacinus* (17).Several physicochemical methods have been used in the treatment of to achieve decolorization, detoxification and degradation but these treatments require enormous cost and continuous input of chemicals which becomes uneconomical and can also cause further environmental damage (7) so replacement with the economical and eco-friendly techniques using microorganisms and their enzymes. Some of this enzyme peroxidase produced by soya bean ability to decolorization textile dyes and degradation of phenolic compound (20). Laccase is one such enzyme that is well studied and currently forays into the various aspects of remediation. It was first described by Yoshida in 1883, during the extraction of exudates of Japanese lacquer tree *Rhus vernicifera* (37) Laccases have many biotechnological applications because of their oxidation ability towards a broad range of phenolic and non-phenolic compounds (25). Laccases (EC.1.10.3.2 parabenzene diol oxido reductase) are enzymes belonging to the family of blue multi copper oxidases. They catalyze the oxidation of a broad range of substrates such as ortho and para-diphenols, methoxy-substituted phenols, aromatic amines, phenolic acids and several other compounds coupled to the reduction of molecular oxygen to water with one electron oxidation mechanism (3). There are diverse sources of laccase producing organisms like bacteria, fungi and plants (4). Fungi laccases have higher redox potential than bacterial or plant. Laccases are secreted out in the medium extracellular by several fungi during the secondary metabolism but not all fungal species produce laccase such as Zygomycetes and Chytridiomycetes (26). Fungi belong to Deuteromycetes, Ascomycetes as well as Basidiomycetes are known producers of laccase (14). For huge volume of synthesis of enzymes, the agricultural waste residues utilization by solid-state fermentation (SSF) is a fermentation process conducted in the absence of free flowing water, using either a natural support or an inert support as a solid material (24). *Trichoderma* species are free-living fungi and common in soil and root ecosystems. *Trichoderma* spp. is one of the essential biological agents used in the field of biological control; *T. harzianum* and *T. viride* have been introduced in controlling of plant pathogens (34). *Trichoderma* sp. are great interest because of their ability to produce cellulose, chitinases, glucanases, xylanases, protease and laccase enzymes (35).Therefore, the aim of present work is to assessment of degradation percentage of some environmental pollutants by laccase produced from *Trichoderma harzianum* in solid state fermentation by HPLC technique.

MATERIALS AND METHODS

Fungal isolate: The isolate *Trichoderma harzianum* was obtained from department of

plant protection, collage of agriculture, Baghdad University. Fungal isolate was cultivated at 28 C° on potato dextrose agar (PDA) and stored 4C°.

Primitive detection for laccase production

Fungal isolate was culture on petri plate containing sterilized potato dextrose agar (PDA) and incubated for 3-5 days at 28C°. Then drops of syrinagaldazine was added (Sigma, USA) as substrate, incubated at room temperature for few minutes then formation pink color around the fungal colonies indicated positive result for laccase production (15).

Enzyme extraction

Laccase was extracted from wheat bran culture using 50ml of 0.1M cooled citrate-phosphate buffer, pH 5.6. The contents of the flasks were grind in a mortar for 15 min in ice bath. The crude extracts were filtered through gauze, and then centrifuged at 6000 rpm for 15 min using cooled centrifuge (16).

Enzyme and protein assays

Laccase activity was followed spectrophotometrically at 525 nm, through the oxidation of syringaldazine as substrate to its quinone form, using a molar absorptivity of 65,000 for the product. The reaction mixture contained 2 ml citrate- phosphate buffer (0.1 M, pH 5.6), 0.2 ml syringaldazine prepared in (0.5 mM in methanol solution), and 1 ml of culture filtrates. The enzymatic activities were expressed as international units (U), defined as the amount of enzyme required to produce 1µmol product/min. Protein concentrations were determined using standard curve of bovine serum albumin according to (6).

Factors effect production Laccase in SSF

Substrates: Ten grams of wheat bran, sawdust, barley bran and rice husk have been used separately for producing the fungal laccase. These substrates were humidified with 7ml (1:0.7) w/v of mineral salt solution containing (g/L) $(NH_4)_2SO_4$ 1, $CaCl_2$ 0.125, NaH_2PO_4-H_2O 1 and $MgSO_4$_$7H_2O$ 0.5 without glucose (4).The humidified medium was placed in 250 ml Erlenmeyer flasks and autoclaved at (121 C°, 20 min). The sterilized medium were inoculated with three mycelial plug (5mm) from 7 day old culture of *T.harzianum* separately (with triplicate for each substrate) then the flasks were incubated for 7 days at 28 C°.

Moisture ratio: Mineral salt solution was added to the 10gm wheat bran at different ratio 1:0.5, 1:0.7, 1:1, 1:2and 1:3 (w/v). The humidified medium was placed in 250ml Erlenmeyer flasks and autoclaved (121°C, 20 min). The autoclaved medium was inoculated with three mycelial plugs (5 mm) from 7-day-old cultures of *T. harzianum* in triplicate. Then flasks were incubated for 7 days at 28C°.

Inoculum size: Mineral salt solution has been added to the 10gm wheat bran at ratio 1:0.7 (w/v). The humidified medium was placed in 250ml Erlenmeyer flasks and autoclaved (121°C, 20 min). The sterilized medium was inoculated with different inoculums plugs of 1, 2, 3, 4and 5 mycelial plugs (5 mm) from 7-day-old cultures of *T. harzianum* in triplicate. The flasks were incubated for 7 days at 28C°.

Incubation period: Mineral salt solution has been added to the 10gm of wheat bran at ratio 1:0.7 (w/v). The humidified medium was placed in 250ml Erlenmeyer flasks and autoclaved (121°C, 20 min). The sterilized medium was inoculated with four mycelial plugs (5 mm) from 7-day-old cultures of *T. harzianum* in triplicate. The flasks were incubated for 4, 7, 10, 14 and 21 days at 28C°.

Temperature: To determine the effect of incubation temperatures in laccase production, 10gm of wheat bran with moisture ratio 1:0.7(w/v) have been inoculated with 4 mycelial plugs (5mm). The flasks were incubated for 7 day at 28, 32, and 37ºC with triplicate for each temperature.

Purification of enzyme: Purification of laccase was carried out by ion exchange chromatography after crude enzyme concentrated by dialysis against the solid sucrose. Then loaded onto a DEAE –cellulose anion exchange column 28.5×1cm, and equilibrated with 0.01 M citrate phosphate buffers pH 5.6 at flow rate0.5ml/min, with Linearly increasing NaCl concentration gradient (0- 1) M in the same buffer. The protein fractions were estimated at wavelength 280nm of both wash and elution fractions and the part of protein peaks were assayed for laccase activity and peaks contain enzymatic was collected. Enzyme activity and protein concentration were measured.

Laccase Characterization: Optimum temperature and pH were determined by

performing enzymatic assays at different temperatures (5–85) Cº and pH levels (3–8.5), respectively. The pH level was adjusted using the following buffers: 0.1 M citrate phosphate buffer pH (3–5.5), 0.1 M potassium phosphate buffer pH (6–7), and Tris-base buffer pH (7.5-8.5). The stability of the purified laccase at various temperatures was investigated by pre incubating the purified laccase at different temperatures between 15 and 85 Cº for 1 h, followed by determination of the residual activity. The effect of pH on the laccase stability was determined by incubating the purified enzyme at 25 Cº in different pH levels for 1 h and the residual activity was determined.

APPLICATION OF LACCASE

Decolorization of textile dyes: To test ability of fungal culture to textile dyes decolorization, *T. harzianum* cultured on potato dextrose agar contain three types of reactive dyes separately, include blue, yellow and red in different concentration (50, 100, 150, 200 and 250) ppm, and incubated at 28Cº for 7 days. Dye decolorization noted by comparing with control (plates containing the medium and the dyes, without the fungus) (11).

Degradation of phenol and furfural by partial purified laccase: Phenolic compounds were analyzed by HPLC equipped with a pump and variable wavelength absorbance detector set at 280 nm. The mobile phase used was 0.1ml phosphoric acid in water versus methanol (41:59). The test contained mixing 1ml of partial purified laccase with 1ml of phenol with different concentration (5, 10, 20, 50 and 100) ppm. Furfural was analyzed by HPLC equipped with a pump and variable wavelength absorbance detector set at 277 nm. The mobile phase used was 5ml acidic acid in water versus methanol (80:20). One ml of partial purified laccase with 1ml of furfural with different concentration (5 and 10) ppm, then injected to HPLC at the first time of reaction after all samples have been filtered through a 0.45 syringe filter, with each concentration standard being read before treatment. Degradation was calculated after mixing reaction according to the following equation:

$$\text{Degradation \%} = \frac{\text{Area of standard - Area of treatment}}{\text{Area of standard}} \times 100$$

RESULTS AND DISCUSSION

Effect of substrate on *T. harzianum* on laccase production: The results indicate that wheat bran using as the potential substrate for production of laccase specific activity 4.786U/mg by *T. harzianum* comparison with other substrates on the 7th day of fermentation as shown in (Fig. 1).Wheat bran is recommended as a balanced substrate for microbial growth as it contains higher cellulosic, hemicellulosic, protein as well as lignin content, it is rich in growth factors and vitamins. It is an abundant by-product formed during wheat flour preparation (4). In agreement with results, wheat bran also showed the highest laccase activity using *Pleurotus ostreatus* (12).Other study used saw dust as substrate for laccase production by *Coriolopsis gallica* under solid-state fermentation (10). laccase production by solid state fermentation using combination sawdust and bran mixture (2:1) with 0.06% xylene the productivity of laccase produced from *Streptomyces sp*. reached 177.8, U\ g (18).

Effect moisture ratio on *T.harzianum* laccase production: Moisture is another key parameter to control the growth of microorganism and metabolite production in SSF. In present work, ten grams of wheat bran has been humidified by mineral solution with different moisture ratio (1:0.5, 1:0.7, 1:1, 1:2 and 1:3) (w/v). Laccase production increase with ratio 1:0.7 w/v with laccase specific activity 4.898U/mg, increasing the initial moisture content above the optimum also resulted in decreased enzyme yield due to the reduction in interparticle space and decreased porosity. Wheat straw with moisture ratio 60% was used to produce laccase from *pleurotus ostreasstus* (23).

Effect of inoculum size on laccase production: Different mycelial plugs (1, 2, 3, 4 and 5× 5mm) of *T. hrazianum* fungal isolate have been used. Laccase activity and protein concentration were determined after 7 day of incubation at 28ºC. Highest level of laccase specific activity was obtained by using 4 mycelia plugs as inoculum, and reached to 5.270U/mg. The enzyme yield has been reduced at lower and higher inoculum levels.

A very low inoculum size was found to be inadequate for enzyme production while the inoculums above optimum level cause lowering in the yield probably due to the competition for nutrients. Highest laccase specific activity 1.6U/mg was obtained with 3×5mm mycelial plugs by *pleurotus ostreasstus* (32).

Effect of incubation period on *T. harzianum* laccase production: The effect of incubation period on laccase production has been studied. Maximum level of laccase specific activity (5.296U/mg) was observed in 7 days of incubation and enzyme production was reduced after 7 days, the enzyme production decreased due to depletion of macro and micronutrients in production medium. Maximum laccase specific activity of (151.6) U/g was produced at the 5th day of solid-state fermentation by *Pleurotus ostreatus* (22).

Effect of incubation temperature on *T.harzianum* laccase production: Results of the present study suggested that an incubation temperature of 28ºC was the optimum for laccase production with specific activity (5.383U/mg). The incubation temperature is a crucial factor that affects the fermentation process because both the fungal growth and enzyme production are sensitive to temperature, when increase temperature lead to reduce growth and decreased laccase production (29). Optimum temperature was 30C° for laccase production by *Coriolopsis caperata* under solid state fermentation (27).

Purification of laccase : The result showed two peaks of protein without any activity in wash fractions, where as the elution step (fig.6) shows one peak of protein when it elution step gradiant salt with one peak for enzyem activity at fraction between (72-86). These results indicated that laccase has a negative charge because bind with anionic ion exchanger DEAE-cellulose. In this step the specific activity was 31.22U/mg with purifiction fold 7.260 and yield reached to 84.01% as showen in (table 1). Ion exchange chromatography used in many studies of laccase purification from fungi. (39) used DEAE-Cellulose in laccase purification from *Cerrena unicolor* 6884 and the purification fold was 24.15 with yeild 14.77%.

Table 1. Purification steps of laccase from *T.harzianum*.

Purifiction steps	volume (ml)	Activity (U/ml)	Protein Con. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yield %
Crude enzyme	50	0.301	0.0708	4.3	15.05	1	100
Concentrated with sucrose	22	0.496	0.082	6.04	10.912	1.404	72.50
Ionexchange DEAE-cellulose Elution step(72-86)	45	0.281	0.009	31.22	12.645	7.260	84.01

Laccase characterization

Effect of pH on partially purified laccase: The effect of pH on laccase activity was determined with buffers of different pH varying pH from (3 - 8.5) figure(7) The maximum activity was observed at pH 4 and the activity was found to be 0.247 U/ml for syringaldazin oxidation, while the activity decreased in pH below and above 4. Increase in pH resulted in decreased in the enzyme yield due to a higher pH which also inhibited laccase activity. (32) Were shows the optimum pH for laccase activity were 4.5 produced by *T. harzianum*. Other study (1) observed the optimum pH for *T. harzianum* laccase activity was pH 5. The partially purified laccase remained quite stable within the pH range of (3.5-5.5) after 1h incubation at 25C°, figure (8) citrate phosphate best buffer for laccase activity and stability. The study (8) on pH stability of laccase carried showed the laccase was stable at slightly acidic pH (4 and 5).

Effect temperature on partially purified laccase: The optimum temperature for partial purified laccase was 35Cº, laccase activity was reached to 0.297 U/ml, the activity was decreased with increasing temperature up to 35C˚ and minimum activity observed at 85 C˚ was 0.032 U/ml figure(9). The increase temperatures lead to decrease in the enzyme activity due to denature of the enzyme which breaking of bonds so the shape of enzyme change configuration or conformation. Similar results was obtained by (32) when they

reported that laccase from *T. harzianum* had 35C˚ as an optimum temperature. Other study (31) maximum activity was found to be at temperature 45C° for laccase activity from *T. harzianum*. The enzyme was stable in temperature between (15- 35) C˚, then the activity begun to decrease with increasing temperature at 85C˚, the enzyme retained only 5.44% of the initial activity (figure 10). The enzymes are sensitive at higher temperature lead to denature the enzyme and loss of three dimensional structure and then decline enzyme activity. Laccase from *Trichoderma harzianum* WL1 was stable for 24 h at 35 C° and had half-life of 60 min at 65 C° (32). The thermal stability of the *Trametes versicolor* crude laccase was followed within the temperature interval 30-60 C° (19).

Decolorization of textile dyes

The ability of *T. harzianum* to decolorize textile dyes was tested on solid media. The fungus grew well and completely decolorized Blue dye H3R with concentrations 50,100 and partial decolorized with 150,200 and 250 ppm concentration figure (11C) contrasting to the yellow dye figure (11A), and Red were very slowly processed figure (11B), dye decolorization was appeared only at 50 ppm concentration, this could be due to difference in chemical structures' of the dyes, as illustrated in (figure 11). Other study showed the ability of *Pleurotus ostreatus* to complete decolorized Blue dye H3R with concentration 50, 100, 150, 200 and approximately 98% with 250 ppm concentration while Yellow dye and Red dye were completely decolorized only at 50 ppm concentration (32).

Degradation of phenol and furfural by partial purified laccase: The peak of partial purified laccase analysis by HPLC (figure.14) used to compared after the treatments with pollutants, notice the peak of enzyme was appear for each treatment according the equation of enzyme: Enzyme + Substrate= Enzyme + Products. Degradation of phenol by partial purified laccase was detected using HPLC. The result indicated that complete degradation was occur at 5 and 10 ppm while at 20, 50 and 100 ppm the percentage of degradation partially degraded with 90.94, 93.93 and 36.78% respectively (tab. 2, fig.12). The ability of laccase degradation of phenol in low concentration best than the phenol in high concentration due to need increased incubation periods and increased of lacase activity to complete degradation percentage. Ability of the purified laccase from *Paraconiothyrium variabile* after 30 min treatment was removed of 80% of phenol and 59.7% of bisphenol A (2). Degradation of furfural by partial purified laccase was investigated; the result revealed that, partially degradation was obtained at 5 and 10 ppm with percentage 86.84 and 68.66% respectively (table.2, figure.13). Furfural produce by lignocellulose more complex contain cellulose, lignin and hemicellulose, the degradation not completely in low concentration as show in the table (2). Laccase produced by *T. versicolor* that the lignin content in the furfural residues decreased gradually with increasing laccase dosages. Approximately 10.5% of the initial lignin was removed following laccase-mediator pretreatment with 100 U/g laccases. However, the lignin removal rate rose only to 12.1% as the laccase loading was increased to 200 U/g (38).

Table2. Degradation of phenol and furfural by partial purified laccase production *T. harzianum* detection by HPLC

Pollutant concentration	R.T (mm:ss)	Area of standard (µAs)	Area of treatment with enzyme(µAs)	Degradation %
Phenol				
5	**3:23**	**348929.4**	**No peak**	**100**
10	**3:23**	**819062.6**	**No peak**	**100**
20	**3:25**	**1789214.1**	**162066.9**	**90.94**
50	**3:23**	**2463201.3**	**149530.3**	**93.93**
100	**3:23**	**1492383.1**	**943454.4**	**36.78**
Furfural				
5	**3:33**	**1165079.6**	**153382.3**	**86.84**
10	**3:48**	**3703446.6**	**1160697.3**	**68.66**

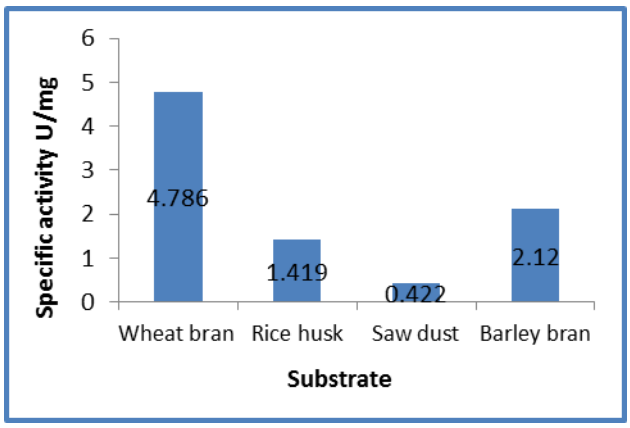


Figure1.Effect of substrate in laccase production from *T. harzianum* using SSF after incubation at 28C° for 7 days

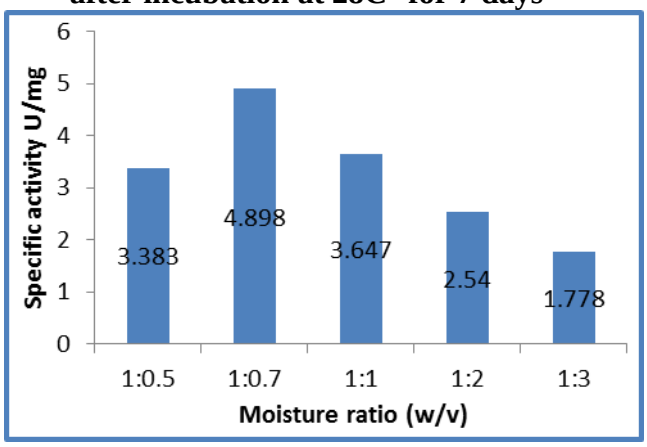


Figure2.Effect of moisture ratio in laccase production from *T. harzianum* using wheat bran; inoculate size 3×5mm at 28C° for7days

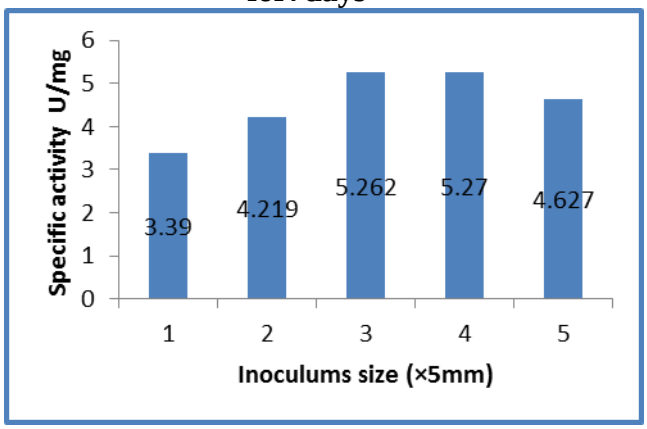


Figure3.Effect of inoculum size in laccase production from *T .harzianum* using wheat bran, moisturizing ratio 1:0.7 w/v at 28C° for 7 days

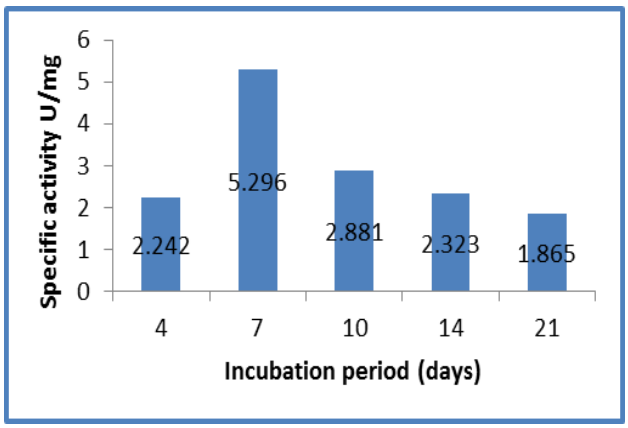


Figure4.Effect of incubation period in laccase production from *T .harzianum* using wheat bran as substrate, inoculums size 4×5mm, moisturizing ratio 1:0.7 w/v after incubation at 28C° for 7 day

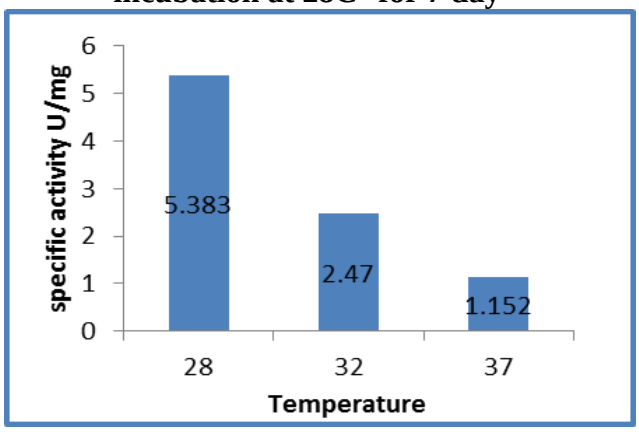


Figure 5.Effect of incubation temperature in laccase production from *T.harzianum* using wheat bran as substrate with inoculums size 4×5mm,moisturizing ratio 1:0.7 w/v for 7 days

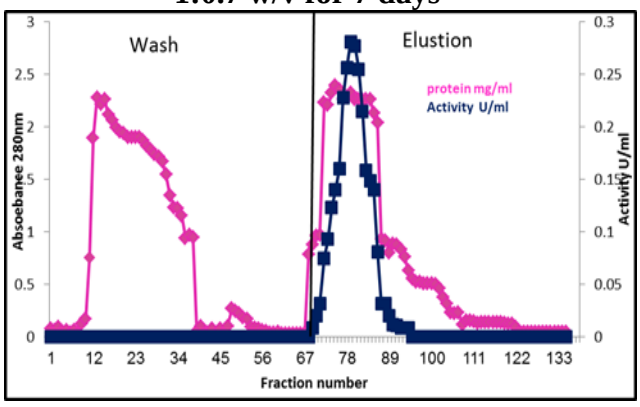


Figure6. Ion exchange chromatography for laccase purification from *T.harianum* by using DEAE-Cellulose column (28.5×1cm) equilibrate with citrate phosphate buffer (0.01 M, pH 5.6), eluted with citrate phosphate buffer with NaCl gradient (0-1) M in flow rate 30ml/ h. 3ml for each fraction

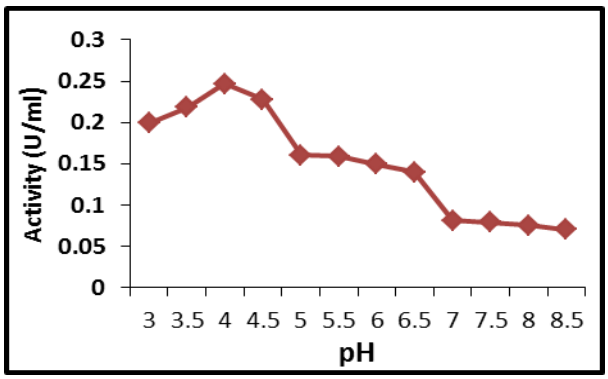


Figure 7.Effect of different pH values (3-8.5) on *T .harzianum* Optimum purified laccase activity using syringaldazine as substrate

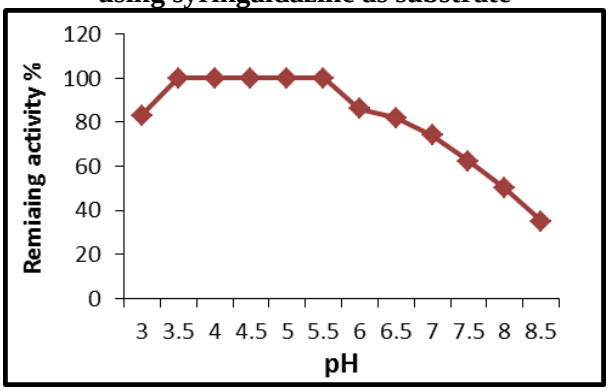


Figure 8.Effect of different pH values (3-8.5) on *T. harzianum* partial purified laccase stability at 25C°

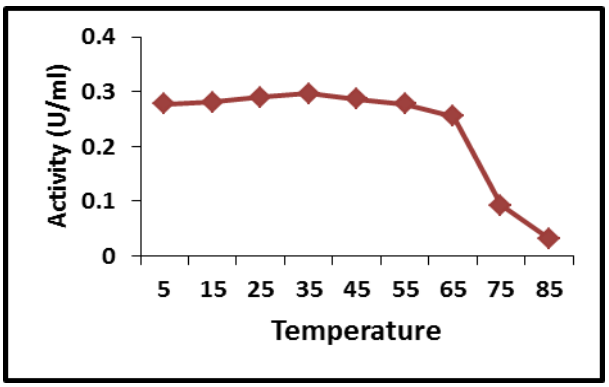


Figure 9.Effect of different range of temperature (5-85) C° on *T .harzianum* laccase activity at pH 4

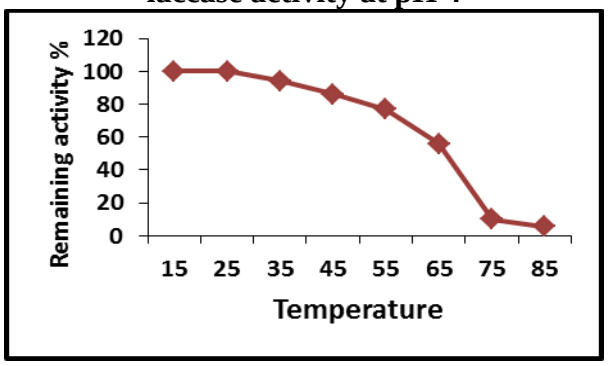


Figure10.Effect of different range temperature (15-85) C° on *T .harzianum* laccase stability using syringadazine as substrate

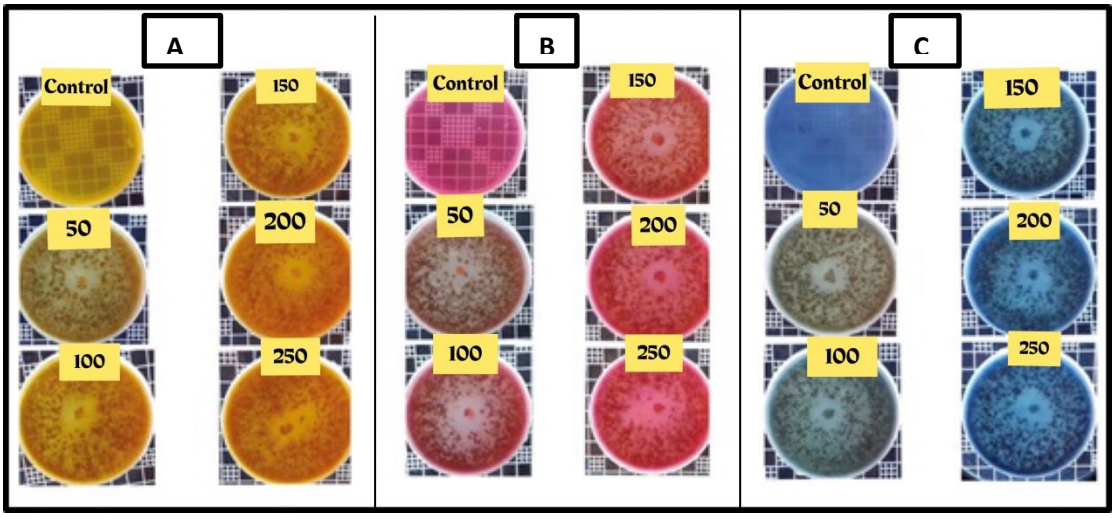
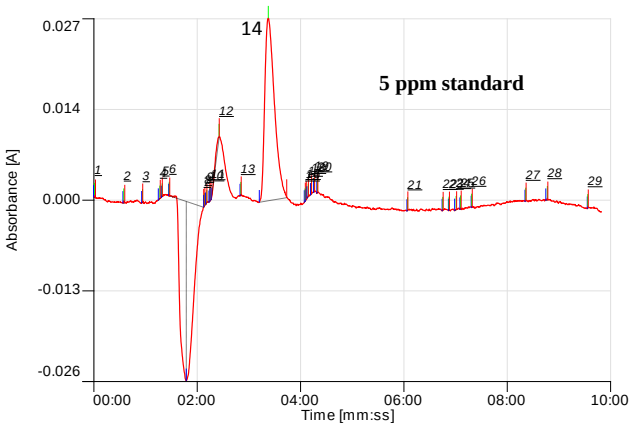
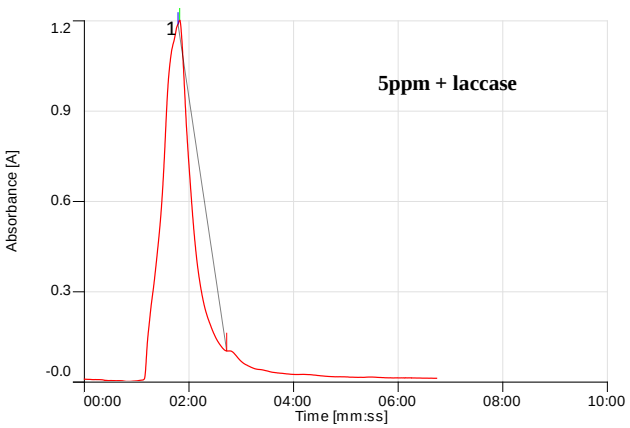
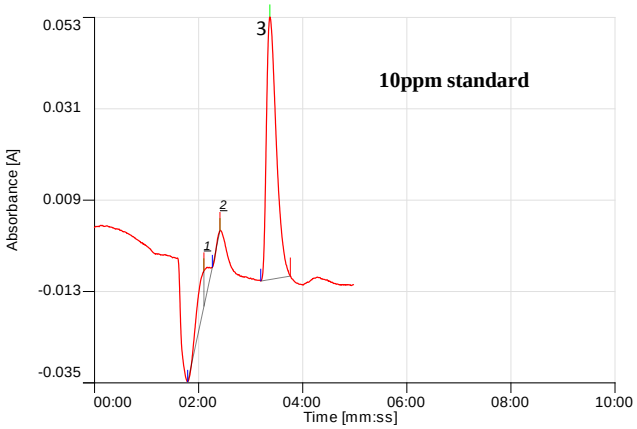
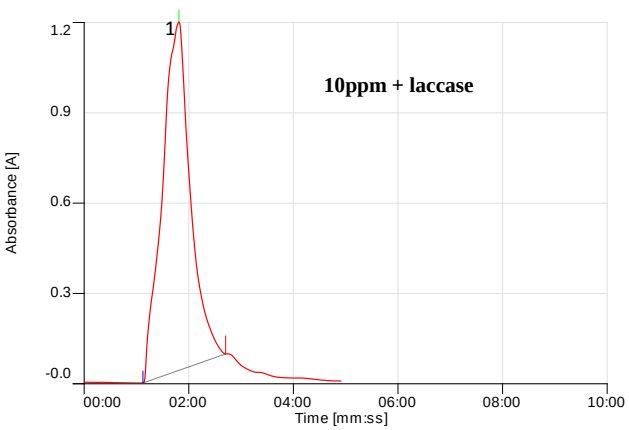


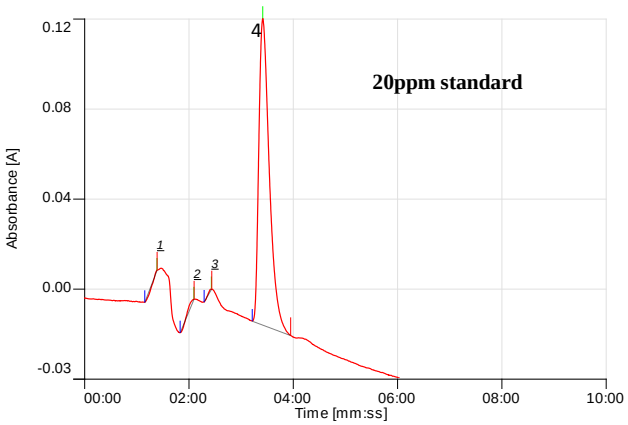
Figure11. Decolorization of three textile dyes by *T .harzianum* after incubation 7 days at 28C° using potato dextrose agar supplement with each dye at different concentration (50-250) ppm. (A) Yellow (B) Red (C) Blue dyes.

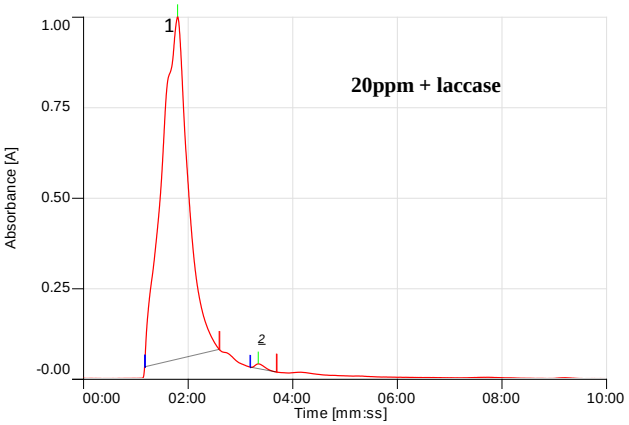

5 ppm standard
Absorbance [A]
Time [mm:ss]

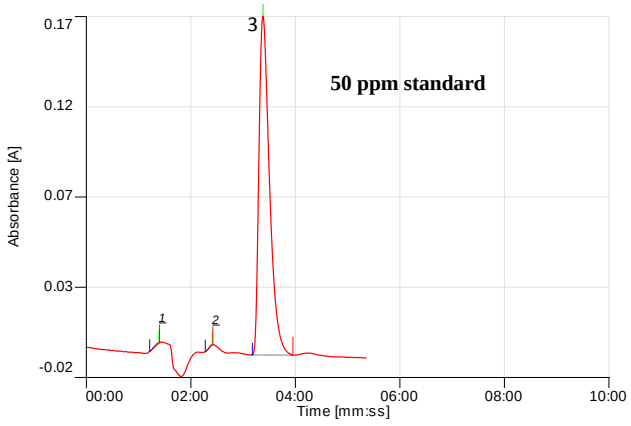

5ppm + laccase
Absorbance [A]
Time [mm:ss]

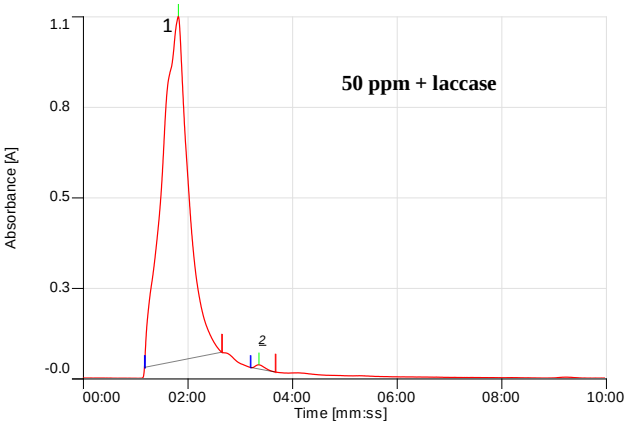

10ppm standard
Absorbance [A]
Time [mm:ss]


10ppm + laccase
Absorbance [A]
Time [mm:ss]


20ppm standard
Absorbance [A]
Time [mm:ss]


20ppm + laccase
Absorbance [A]
Time [mm:ss]


50 ppm standard
Absorbance [A]
Time [mm:ss]


50 ppm + laccase
Absorbance [A]
Time [mm:ss]

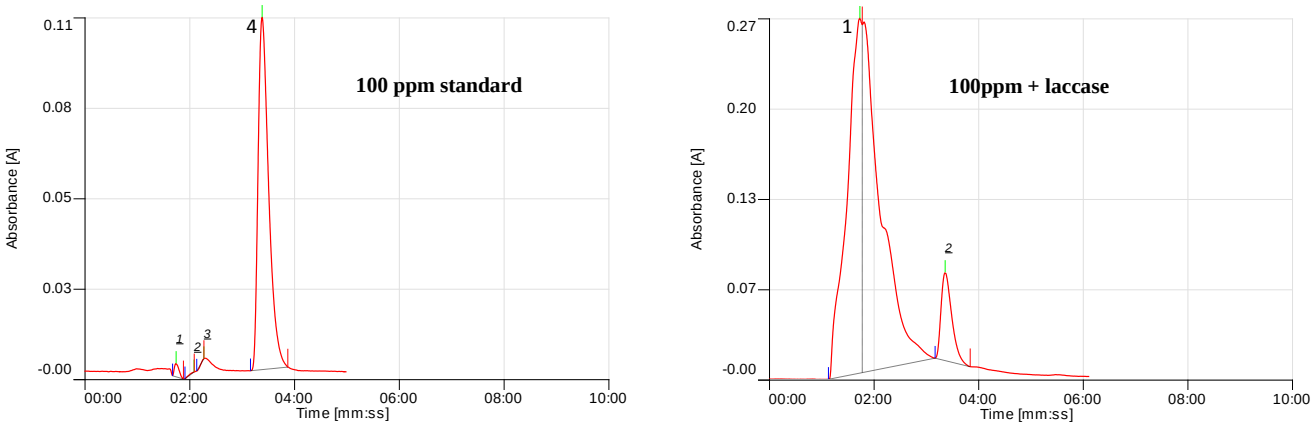


Figure12. HPLC analysis for degradation of phenol at different concentration (5-100) ppm by partial purified laccase produced from *T. harzianum*

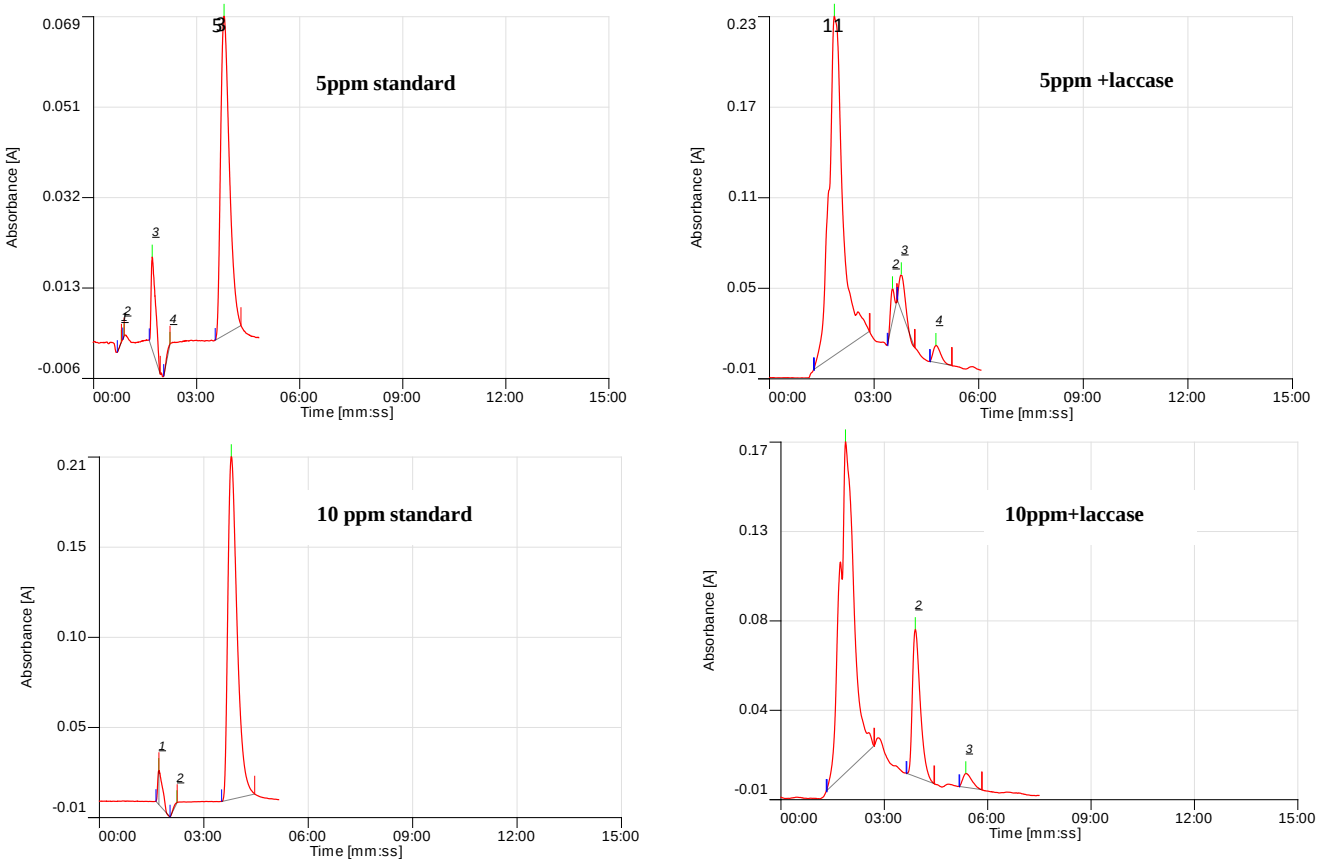


Figure13. HPLC analysis for dergradation of furfural at different concentration (5-10)ppm by partial purified laccase produced from *T. harzianum*.

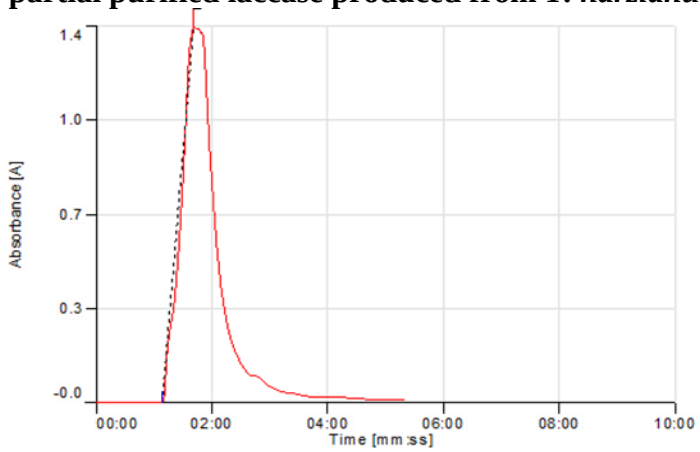


Figure14.HPLC analysis for partial purified laccase

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