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BIOTRANSFORMATION OF CARAWAY OIL BY LACCASE PRODUCED FROM *Pleurotussapidus*

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ABSTRACT

In this study, laccase produced and purified from Pleurotus sapiduswas studied. Production of laccase induced by four type of inducer ($CuSO_4$, ABTS, Xylidin, and ABTS+$CuSO_4$) as inducer. The maximum laccase activity observed while P. sapidusin the presence of xylidin as inducer (1.5 U/ml). Laccase purification was performed by precipitated the enzyme with 90% ammonium sulphate followed by gel filtration with sephadex G25. The optimum laccase activity was observed at acidic pH values (close to 4.6- 5.6), while the optimum temperature 40 C. The ability of enzyme to bio-transform Caraway oil was detected at different reaction medium. Biotransformation of caraway oil in presence of three mediators (40 mM HBT, 40 mM TEMPO, 10 mM ABTS) was more effective than others systems.

INTRODUCTION

Laccases are extracellular enzymes secreted into the medium by filamentous fungi (1). Laccases are generally produced during the secondary metabolism at different fungi. Several factors including type of cultivation (submerged or solid state), carbon limitation, nitrogen source, and concentration of microelements can influence laccase production (2).

Laccases are N-glycosylated multi copper oxidases belonging to the group of the blue copper proteins. They can catalyze the oxidation of many substances coupled to the reduction of molecular oxygen to water (3).

Production of laccase can stimulated extremely by the presence of inducers (mainly phenolic or aromatic compounds related to lignin or lignin derivatives) like veratryl alcohol (4).The production of laccase enhanced after xylidin as inducer; the xylidin at higherconcentrationshad a reduced effect, possibly due to toxicity (5).

With respect to other ligninolytic enzymes, laccase can oxidize only phenolic fragments of lignin due to the random polymer nature of lignin and to the laccase lower redox potential (6, 7). Small natural low molecular weight compounds with high redox potential than laccase itself (> 900 mV) called mediators may be used to oxidize the non-phenolic part of lignin (5). A mediator is a small molecule that acts as a sort of ‘electron shuttle’: once the enzyme generating a strongly oxidizing intermediate oxidizes it, the co-mediator (oxidized mediator), it diffuses away from the enzymatic pocket and in turn oxidizes any substrate that, due to its size could not directly enter into the active site(8).

Caraway (*Carum carvi* L.) is one of the most important medicinal plant cultivated in Poland on the area 8.000 ha (9).The main constituents of oil are monoterpenes: carvone and limonene, which usually make 95% of all, oil (10, 11, 12). According to (13) carvone content determines the quality of caraway fruit.

The aim of present study is production and purification of laccase. Detect its ability to transform caraway oil.

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MATERIALS AND METHOD

Materials:-

Mediators 2, 2'-azino bis (3-ethylthiazoline-6-sulfonate) (ABTS) and (1-hydroxybenzotriazole) (HBT) were purchased from Fluka Chemie AG (Buchs, Switzerland), while the 2,2,6,6–tetramethylpiperidine–1-oxyl) (TEMPO) was supplied by Himedia (India). All chemicals used as buffers and substrates were commercial product.

Microorganisms:-

P. sapidus obtained from Baghdad University, collage of sciencewas grown in potato dextrose agar (PDA) plate at 30 C for 7 days.

Methods:-

Buffers and reagent preparation

Phosphate Buffer Saline (PBS)

PBS prepared by dissolving the following ingredients in 1000 ml distilled water and pH adjusted to 7.2.

Ingredients	Weight /gm
Sodium Chloride	8
Potassium Chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium hydrogen phosphate	0.2

Acetate buffer (0.1 M)

A= 0.1 M acetic acid 2.9 ml/ 500 ml of distilled water.
B= 0.1 M Sodium acetate 4.1gm/ 500 ml of distilled water.
X ml of A +Y ml of B then add D.W. to reach 100 ml in volume.

X	Y	pH
46.3	3.7	3.6
25.5	24.5	4.6
4.8	45.2	5.6
1.2	48.8	6.6

Bradford reagent (Protein assay):-

Weight out 100 mg of Coomassie Brilliant Blue G -250 and dissolve in 50 ml ethanol (95%) in dark bottle with agitation for 1 hour. Then, 100 mL of phosphoric acid was added and completed to 1000 mL with Mili-Q water (or DD water) under continuous stirring. Filter twice times with filter paper and store the reagent solution in dark, as described byBradford (14).

Protein assay protocol:-

1. Bovine Serum Albumin (BSA), as a standard protein, was prepared in different dilutions (0.025-12 mg/ml) in PBS (pH 7).
2. Fifty microliter from each dilution was mixed thoroughly with 950 µl of Bradford reagent and let stand for 10 minutes at room temperature.
3. Read absorbance in spectrophotometer at 595 nm, and duplicate measurements were done.
4. A standard curve was plotted between the concentrations and absorbance.
5. The equation that obtained from standard curve was used to calculate protein concentration.

Production and purification of Laccase from fungal strain of *Pleurotus sapidus*

Culturing

Fungal strain reactivated on Potato Dextrose Agar (PDA) medium for three daysat 29 ºC, pH (5.5). Thereafter, the plates were maintained at 4ºC and inoculated once every 3 months.

Production of Laccase

Production of laccase was carried out after reactivation of *P. sapidus*. The medium (09 CBZ 6) was used to support laccase production (15). The following components of medium dissolved in 500 ml of distilled water:

Ingredients	Weight (gm)
Ammonium citrate	1
Glucose	5
Potassium hydrogen phosphate	0.5
Yeast extract	0.5
Magnesium Sulfate	0.25
Potassium chloride	0.25

The medium was divided into five flasks, each contain 100 ml of media with different inducers as following:

1. Flask (1) without inducer +10 gm sunflower husk.
2. Flask (2) contain 5 mg (1mM) $CuSO_4$.
3. Flask (3) contain 5 mg (1mM) ABTS.
4. Flask (4) contain 5 ml (25 μM) Xylidin.
5. Flask (5 contain 5 mg (1mM) $CuSO_4$& 5 mg (1mM) ABTS.

Ten block (4 mm diameter) of 7 days incubation was inoculated into 100 ml of production medium in 250 ml flask volume and incubated with shaking(150 rpm) at 29-30 ºC and pH 5.5 for 12 days . During fermentation process, the samples were collected every 24 h under sterile conditions and enzymatic activity was determined.

Extraction and purification of laccase

The culture fluid for the enzyme purification was first filtered through six layers of sterile gauze. The culture filtrate containing laccase activity was concentrated as in the following:

a) Salt fractionation:-

1. Gradually, add 90% of ammonium sulfate (15.2 gm) to each 25 ml of supernatant in ice bath with stirring for 18-24 h.
2. Centrifuge for 15 min. at 5000 rpm.
3. Dissolved the precipitate in 2.5 ml of 0.1 M acetate buffer pH (4.6).
4. Protein concentration should be determined by Bradford method.

b) Gel filtration:-

1. A packaged column of 1*5 cm of sephadex G-25 (Pharmacia, Biotech, Uppsala, Sweden) washed 3-4 times with D.W. (5 ml of each time).
2. Equilibrate the column with the 0.1 M acetate buffer pH (4.6) 3-4 times (5 ml of each time).
3. Add 2.5 ml of sample and leave it move down inside the column.
4. Thereafter, 3.5 ml of buffer was added and collect the same amount of elution.
5. Protein concentration should be determined by Bradford method.

DETERMINATION OF ENZYMATIC ACTIVITY

Laccase activity was assayed by measuring the rate of ABTS oxidation at room temperature. The activity was investigated in aqueous system. The reaction volume (0.2mL) consisted of the appropriate amounts of acetate buffer (0.1M and pH 4.6) containing 0.02 U of laccase and 1 mM ABTS. The oxidation of ABTS was followed by an increase of absorbance at 405 nm ($\varepsilon_{405} = 36\ 000\ M^{-1}cm^{-1}$). One unit of activity was defined as the amount of laccase that oxidized 1μmol of substrate per minute. (16).

STABILITY OF HOMEMADE AND COMMERCIAL LACCASES

The effect of pH

Laccase activity was estimated by using 0.02U from *P. sabidus,* .Acetate buffer 0.1M was used to study the effects of four pH degrees (3.6, 4.6, 5.6 , 6.6). The enzymes samples were incubated at 40 ºC, and samples withdraw at different incubation times. Oxidation activity of laccases against 1mM ABTS, as substrate, was determined by measuring the absorbance at 405 nm in ELISA reader.

The effect of temperature

The optimum temperature for laccases activity were determined using 0.02Uof enzyme in 0.1M acetate buffer (pH 4.5). Five temperatures were tested (20- 30- 40- 50- 60°C) and samples were submit to the same protocol as described previously.

The effect of reaction systems

The effect of the reaction systems on laccase stability was investigated through comparison of aqueous system with two nonconventional systems as in following:

- System one: include 16 % α-pinene, 65.1 % *tert*-butanol and 18.9% of 0.1 M Acetate buffer, pH 4.6 (PT system). (17)
- System two: include the same gradients as in system one, but acetonitrile was Used instead of *tert* – butanol (PA system). (17)

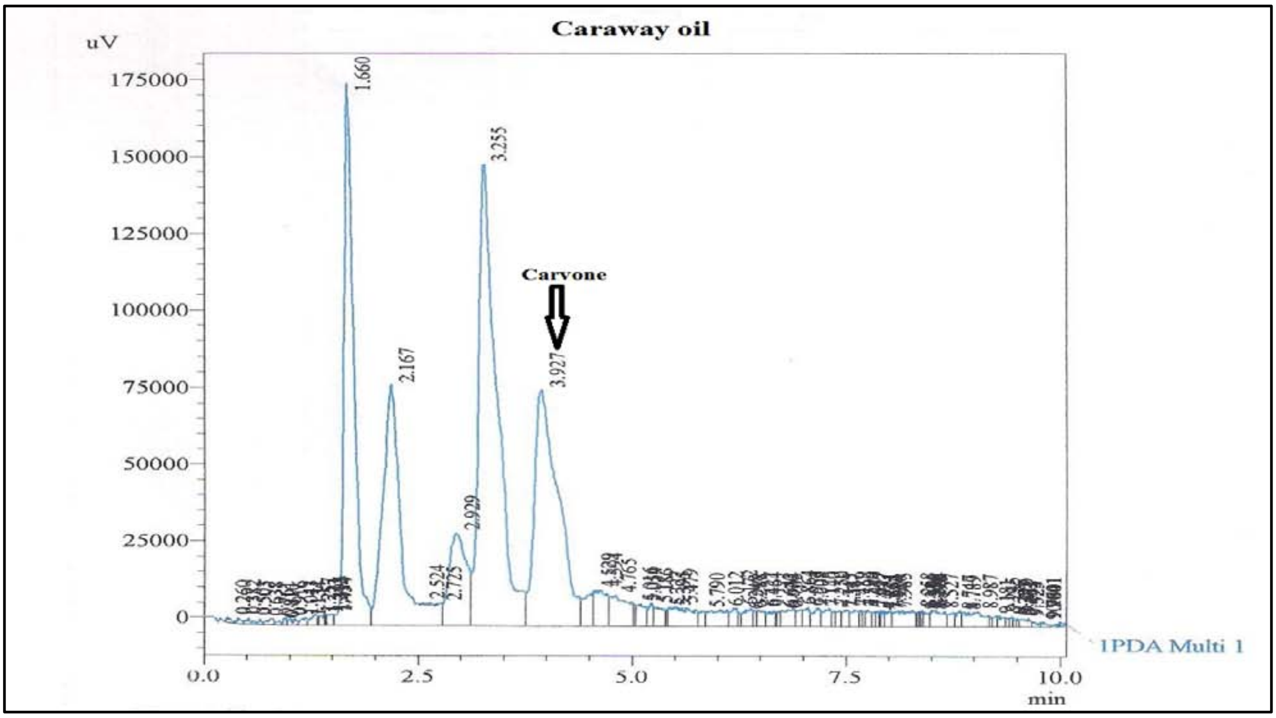
After preparing the systems, the appropriate amount of 0.1M acetate buffer, pH 4.6 containing laccase was added. The mixtures were then incubated at 40 °C and laccase samples were withdraw at different time (final laccase activity should be 0.02U/ml). The residual oxidation activity of laccases (assayed by oxidation 1mM ABTS at room temperature) was measured at 405 nm by ELISA reader. Experiments were carried out in triplicate.

Biotransformation of natural compounds

The ability oflaccases to catalyseoxidations of natural compounds, such as hydrophobic substrates, in PT and PA systems was investigated. In a typical reaction, the substrates (1% of Caraway oil) was added to vial containing the appropriate amounts of system and 0.15 U/ ml of laccase. The reaction was initiated with the addition of 40 , 120 and 480 mM of HBT as a single mediator or in combination with 40 mM TEMPO and 10 mM ABTS. The reaction mixture was incubated at room temperature. Samples were withdrawn periodically and analysed by HPLC. Controls were performed in the absence of the enzyme or the mediator.Figure (1) signifies peaks of standards terpenes analysed by HPLC.

HPLC analysis

HPLC analysis of biotransformed products was performed on an Agilent 1100 liquid chromatograph, equipped with a diode array detector, using a Hypersil BDSC18 (5 µm, 250× 4.6 mm) column; the mobile phase was methanol -water (90:10), 0.8 mL /min and at 24ºC. (18).



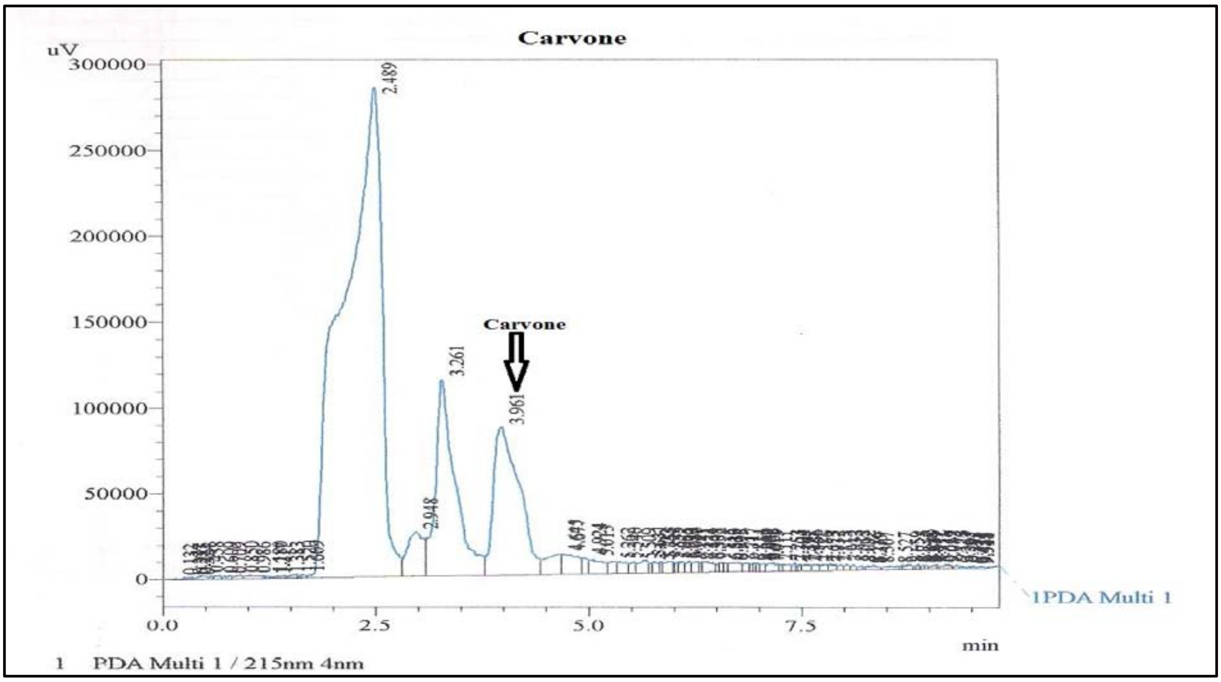


Figure-1: Peaks of standards terpenes analyzed by HPLC.

RESULT AND DISCUSSION

Production and purification of laccase from *P. sapidus*

P. sapidus was cultured on potato dextrose agar (PDA) medium to reactivate the fungal strain and for inoculum preparation. The medium (09-CBZ6) was used as a laccase production medium at pH (5.5) and temperature 29-30 ºC (15). The enzyme production was enhanced by addition of different inducers into five separate media (Fig 2).

Figure-2: Day 11 of ***P. sapidus*** fermentation in different production media at pH 5.5 and temperature 29-30 ºC. (1)10 gm sun flower husk. (2) 5 mg $CuSO_4$ (3) 5 mg ABTS (4) 25 % Xylidin (5) 5 mg $CuSO_4$ + 5 mg ABTS.

The activity of produced laccase in each medium tasted after 48 h during fermentation process. ELISA reader measured Laccase activity determined as oxidation activity absorbance at 405 nm. Higher enzyme activity (1.5 U / ml) was observed in medium no.4 that contain 25 % of 2, 5- Xylidin, as inducers, on day 7 (Fig. 3).Lower enzyme activity of 0.25, 0.39 and 0.31 U/ml in the presence of $CuSO_4$, ABTS and their combination were revealed after 7 days of incubation, respectively.

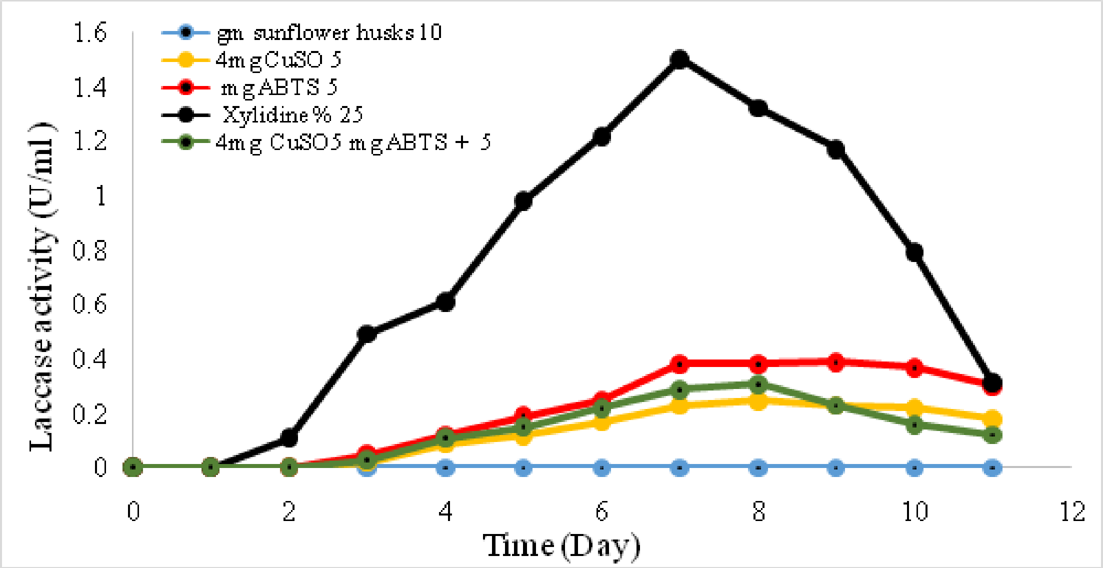


Figure-3: laccase production in medium 09-CBZ6 in the presence of inducers at pH 5.5 and incubation temperature 29-30 ºC.

Xylidin has been reported as an important laccase inducer under semi-solid-state condition (19). The effect of inducers on laccase production differs from fungus to fungus (20). The selection of 2, 5-xylidene as inducer was done in submerged fermentation with *P. ostreatus* for laccase expression. 2, 5-xylidene resembles phenolic structure of lignin molecule and it helped to increase the rate of biosynthesis of laccase during the fermentation of white rot fungi. The resemblance in phenolic structure of lignin molecule might trigger the preferential synthesis of one of the metabolites of the ligninolytic enzymes (21, 22).

Partial purification of laccase from *P. sapidus* supernatant (0.23 mg /ml) was performed by precipitated the enzyme with 90% ammonium sulphate followed by gel filtration chromatography using Sephadex-G25. The fold of purification changes depending on the protein that submit to the purification steps. There is no good or bad value. However, this number along with the percent yield indicates if a step was worthwhile or not. A poor fold purification with a low yield is a step to avoid in the future. Decreasing in the specific activity, from 5.2 to 2.7 U/mg during purification steps (Table 1), need more optimization and investigation.

Table-1: Purification of laccase produced by *P. sapidus*

Steps	Volume (ml)	Activity (u/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Fold purification
Crude	125	1.5	0.23	5.2	187.5	100	1
Precipitation	17.5	5.2	12	0.44	91	48.5	0.08
Gel filtration	24.5	0.8	0.3	2.7	19.6	10.4	0.51

– Initial reaction temperature

–

When laccase activity was studied as a function of temperature, laccases was found to be active in a temperature range of 30– 80°C, with the maximum activity 80 °C (Fig.4). Increasing the temperature increases reaction rates because of the disproportionately large increase in the number of high-energy collisions. It is only these collisions (possessing at least the activation energy for the reaction) which result in a reaction. Above the maximum initial reaction temperature, the activity was decreased. This is in accordance with reports where the maximum laccase activity was obtained at 80ºC, while further increase in the temperature lead to gradually inactivation (23, 24, 25).

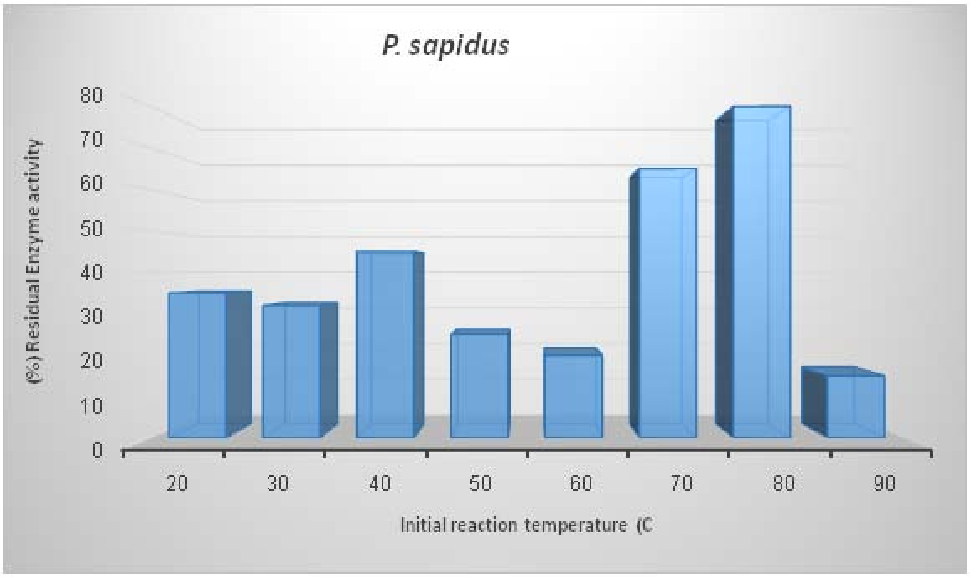


Figure-4: Initial reaction temperature of laccases in 0.1 M acetate buffer (pH 4.6) within 2 hours. 1mM ABTS oxidized by 0.02 U/ml as a final activity.

LACCASE STABILITY

The effect of pH on Laccase stability

Activity of laccases were determined at different pH. Specific effect of pH (ranging from 3.6-6.6) examined on enzyme activity. The optimal pH for laccases activity is observed at acidic environment, when ABTS used as substrate. Higher residual enzyme activity 54.16 and 52.86% observed at pH 4.6 and 5.6 after 48 hr. for both enzymes respectively (Fig. 5). At pH values larger than 5.6, the enzyme activity decreased gradually and completely inactivated at higher alkaline pH.

This phenomenon can be explained by the difference in redox potential between a reducing substrate and the type 1 copper in the active site of the enzyme and the inhibition of type 3 copper by hydroxide ion at higher pH (26). The state of ionization of amino acids in a protein and altered protein recognition or an enzyme might become inactive under different pH degrees. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or the properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis (27). Studies with laccases from *Coriolushirsutus*, *Trichoderma atroviride*, *Chalara (syn. Thielaviopsis) paradoxa CH32* and *Cerrena unicolor 059* showed that the optimal pH range for fungal laccase was from 4.0 to 6.0 (28, 29, 30).Stiolova *et al.*, (24) reported that crud laccase produced by *T. versicolor* have optimum pH at 4.5. Abdulah., (31) reported that laccase from *P.sapidus*was stable at pH 6.

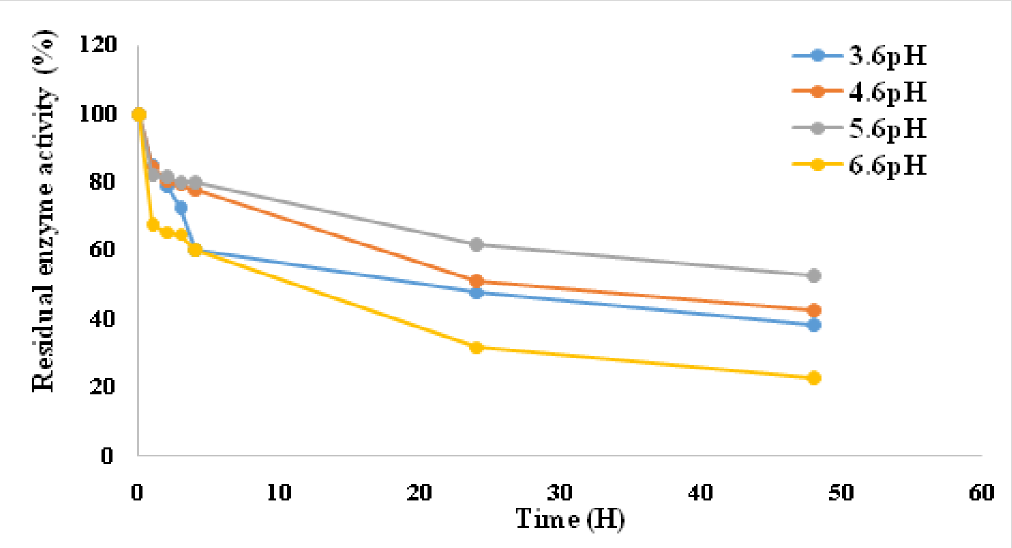


Figure-5: Stability laccases in 0.1 M acetate buffer incubated in˚C30 at different pH.1mM ABTS as substrate oxidized by 0.02 u/ml as a final activity.

Effect of incubation temperature on laccase stability

Activity of laccase also determined at different temperatures to identify the optimal temperature for enzyme activity. The optimal temperature for enzyme activity was 40 ºC of laccases (Fig.6). Studies of thermal effect on enzymatic activity showed different results. Gomes *et al*., (32) reported that the laccase from *Coriolosisbyrsina*, *Lentinusstrigillus*, *Lentinus*sp. And *Picnoporus* showed thermophilic properties and preserving 70-100% of initial activity between temperature ranges of (10-60) C˚ for 1 h. Abdulah, (31) showed that *P. sapidus*laccases retained its initial activity after 1h. when incubated at temperature ranged (15-35) C˚.

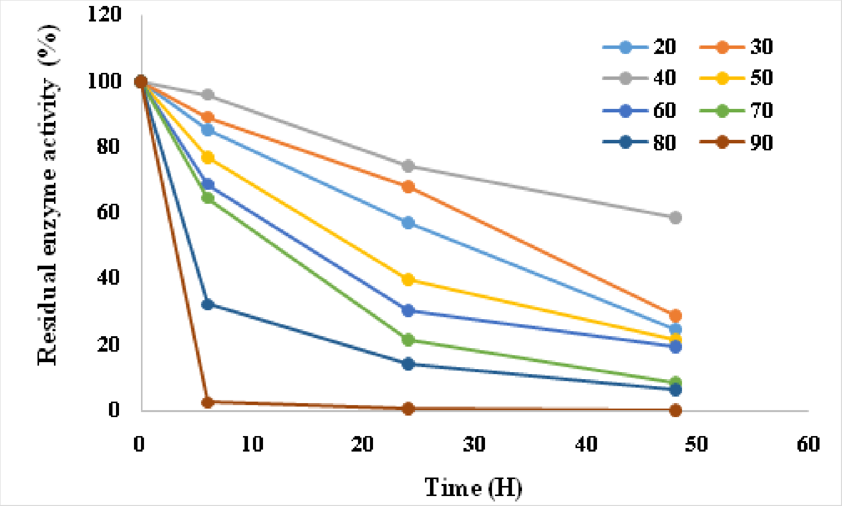


Figure-6: Stability of laccases under different temperatures incubated in 0.1 M acetate buffer 5.6. 1mM ABTS oxidized by 0.02 u/ml as a final activity.

Effect of reaction system on laccase activity

Activity of laccases had been tested at aqueous and two of organic systems (PT and PA) in incubation temperature 40 ˚C. Laccase activity was 58.64 and 47.81 % in PT and PAsystems whenexamined during 48 h. of incubation (Fig.7).

Generally, laccases are a prospective class of enzymes for biotechnological applications, such as biobleaching, detoxification, and food industry and for biosensing. However, in many cases, the interesting substrates are poorly soluble in water and the knowledge of the behaviour of laccases in various water-restricted media is necessary. The rate of the enzymatic reaction changes with the composition of the organic solvent and the general properties of reaction system can be change in the presence of organic solvent (such as dielectric constant, polarity, hydrophobicity, etc.). This extracellular and highly glycosylated enzymes retain their activity both in organic solvents and various water content systems. Thus, it is more interest to study the influence of organic solvents on laccases under this practically conditions, where the enzymes retain their native conformation and a high catalytic activity (33, 34, 35, 36, 37, 38, 39).

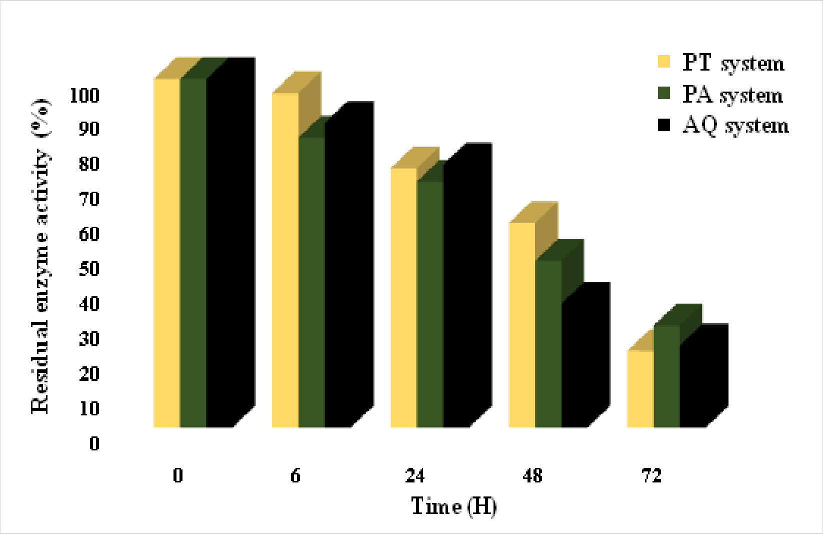


Figure-7: Stability of laccases in different reaction systems at incubation temperature of 30 and 40 ºC, respectively. Final enzyme activity 0.15 U/ml and 1mM ABTS as substrate. (AQ: Aqueous system).

– Biotransformation of Caraway oil.

According to previous studies caraway oil contain large amount of essential oil, limonene and carvone are the main constitute of caraway essential oil. According to this researches (40, 41, 42, 43), the percentage of carvone and limonene in essential oil ranges from 93.2 to 99.8%. Apart from monoterpenes. So that carvone has been produced from bio transforming caraway oil. Carvonecan be produced by bio transforming limonene and carvone that present in caraway oil to carvone. The mostspecific microbial biocatalysts described so far are the basidiomycete *Pleurotussapidus*, which converts d-limonene to *cis*- and *trans*-carveol and carvone at a low rate (44).Three systems used to transform caraway oil, different inducer and organic solvent used in each system. (figures 7, 8, 9).

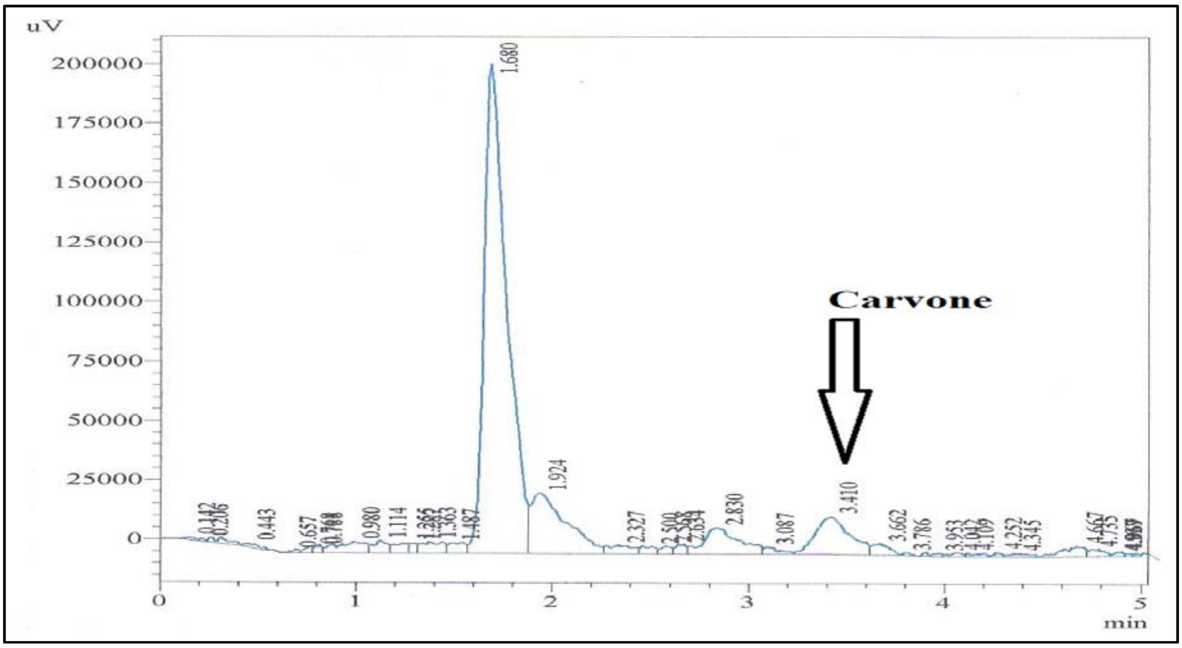


Figure-7: Biotransformation of Caraway oil by Laccase by three mediators.

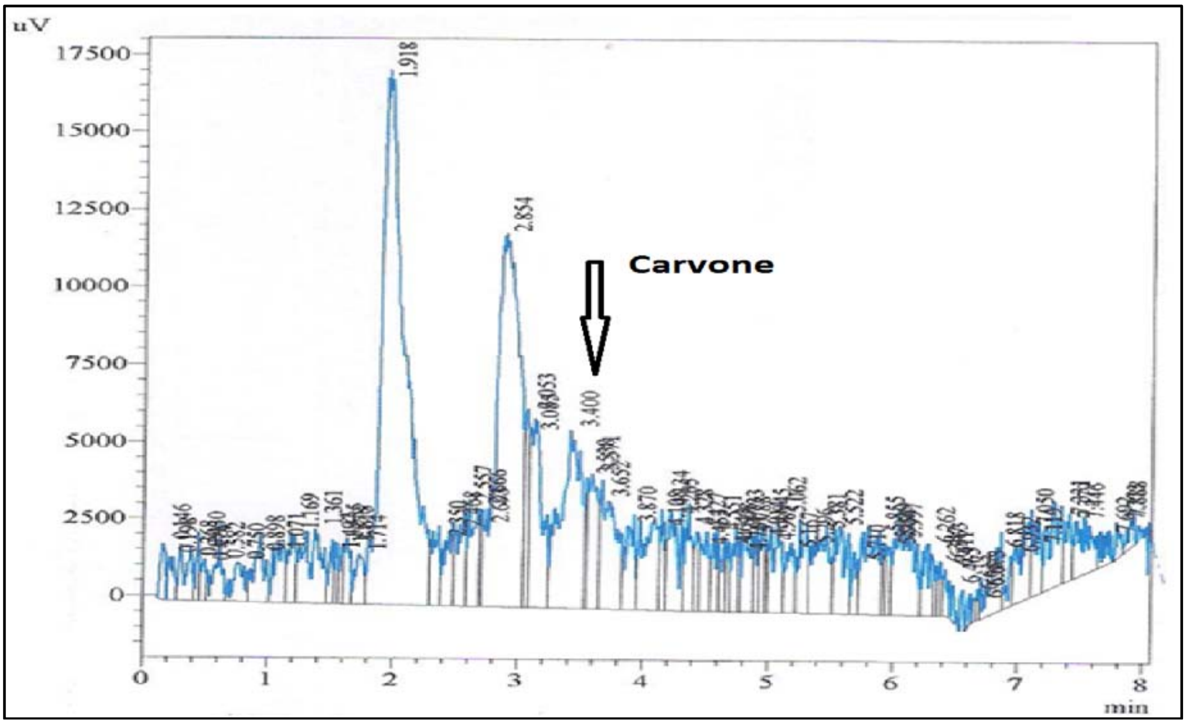


Figure-8: Biotransformation of Caraway oil by Laccase in PA. System.

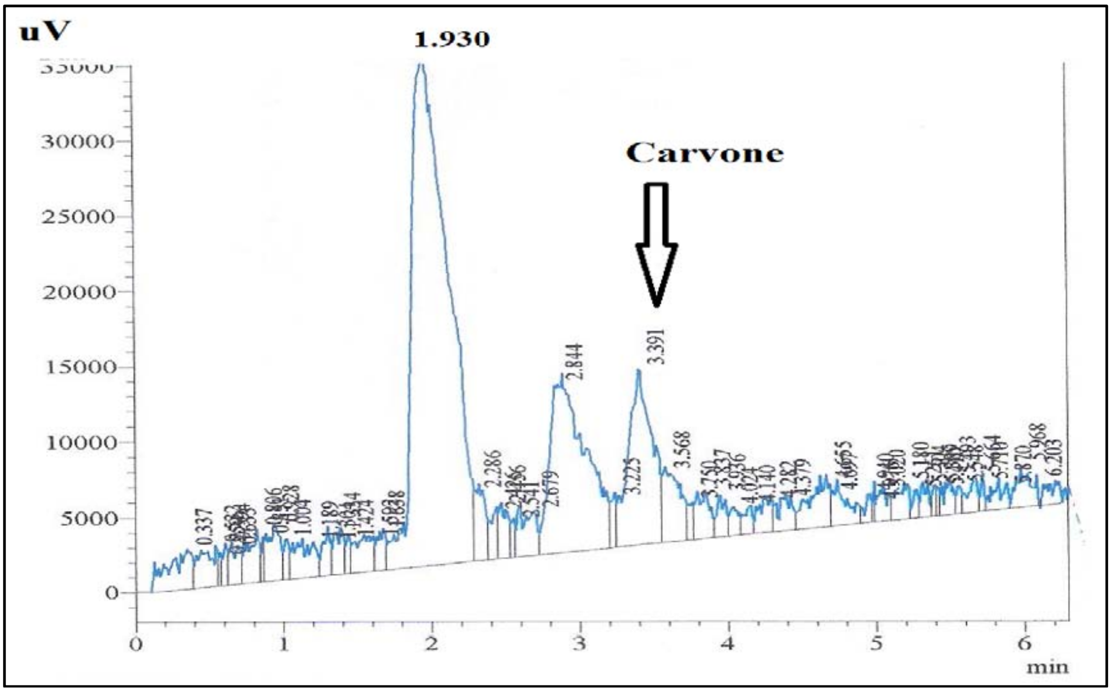


Figure-9: Biotransformation of Caraway oil by Laccase. In PT. System.

Biotransformation of caraway oil by laccase was more efficient in PA with three mediators (40 mM HBT, 40 mM TEMPO, 10 mM ABTS) and with one mediator.(Figure 10). Bourbonnais et al., 1997 (45) proposed that 1-Hydroxybenzotriazole (HBT) as one of the most effective organic mediators. The activity of HBT as mediator effected by organic solvent that used to dissolve it, so this explain the difference in laccase activity between PA and PT system. The effect of acetonitrile in PA system (solvent used to dissolve HBT) on laccase stability was maintain native conformation of laccase. Solvent with high values of partition coefficient between water and n- octanol types of solvent will be more favorable for preserving enzyme activity. Besides that, stability may not only effect through hydrophobicity but also by other characteristics of the solvents, such as hydrogen binding, anion stabilizing, and free energy of solvent (46).Kovrigin and Potekhin, 2000(47) Suggested to weaken the hydrophobic interaction and increased the stability of laccase in aqueous solutions.The t-butanol molecules are small, branching structure is rather special but it is not easy to penetrate into the internal structure of the enzyme protein folding, do not cause inactivation of the enzyme protein denaturation (48)**.**

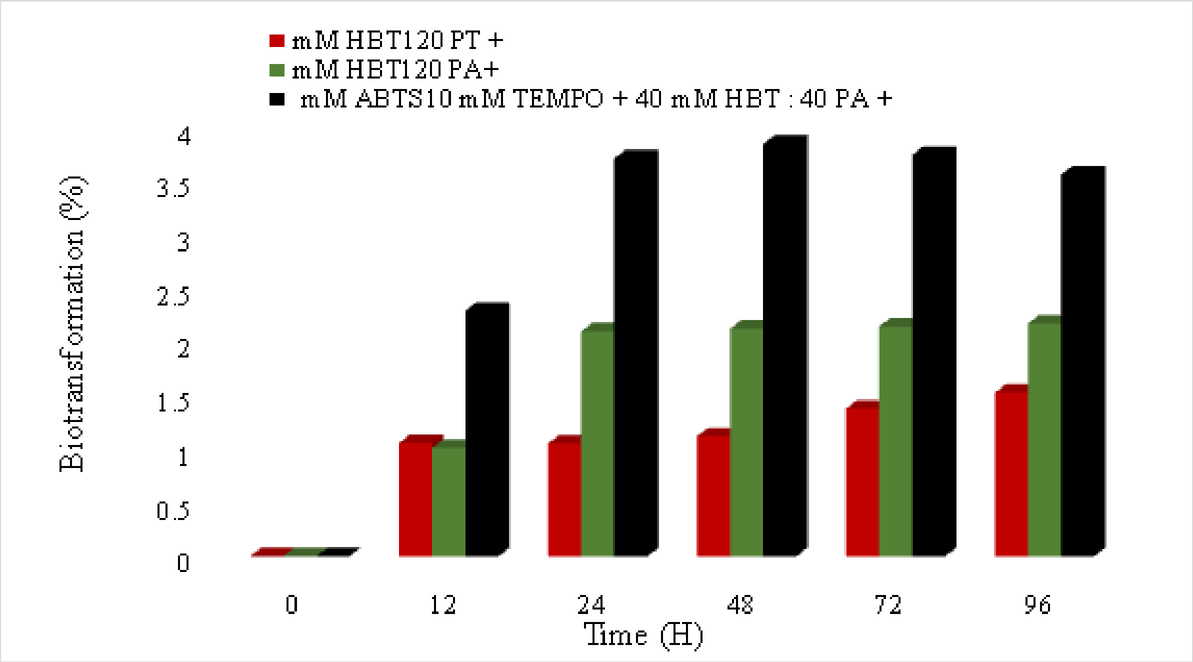


Figure-10: Biotransformation of caraway oil by Laccase.

CONCLUSION

Production of laccase demonstrated with four different inducer, but xylidin was the most effective one. The optimum pH and temperature for produced laccase was studied, the optimum pH was in acidic environment close to 5.6 and temperature close to 40 C. Biotransformation of caraway oil detected by produced laccase and the result present that organic solvents and mediators effect biotransformation process. Biotransformation of caraway oil by Laccase Mediator System (LMS) has industrial and environmental importance due to its ability to destroy different compounds.

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