

Synthesis and Characterization of Some Inorganic Compounds and Study their Effect in activity of Laccase Produced by Fungal isolate *Pleurotus ostreatus*

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Abstract

The new ligand(OBTMRZ), *Bis*[4-phenyl-3(2-propenyl)thio-1,2,4-triazole -5yl] methane was prepared and characterized by vibrational spectra and physical methods, selected metals, which were Cu^{II} , Zn^{II} , Mn^{II} , Co^{II} , Fe^{III} and Hg^{II} were reacted with ligand to produce new complexes. Solid compounds were isolated and identified through metal analysis, vibrational and electronic spectra, molar conductivity magnetic susceptibility measurements and other techniques depending on the data obtained, chemical formula geometry of complexes were suggested.

Other ligands(OXH, TRZH, OTRZ, BTMRZH₂) were prepared and select metals(Cu^{II} , Zn^{II} and Mn^{II}) reacted with them to produce complexes. These complexes were characterized by infrared spectroscopy and physical methods .

Laccase was produce from fungal isolate *P .ostreatus* using submerge culture at 30C° pH =6.5 after 96 hour of incubation .The analysis of fungal filtrate using (HPLC) technique shown the presence one peak which indicated , that was partial purified , the flam atomic absorption technique indicated that the enzyme was contains (2Zn,

1Cu,1Fe atom) .The optimum conditions for enzyme activity were pH=6 at 60C° and for stability pH=5-6 at 35-60C°,the operational activity of enzyme were pH=6 at 60 C° for 30 min .

The effect of salts,indicator (ligands) and their complexes in enzyme activity were studied as following :

- Zn complexes were act as enzyme inducer in case of OXH,TRZH, OTRZligands in comparison with Cu complexes but the complexes (BTMRZH2) were reveled opposite behavior with previously ligands.
- Enzyme activity was increase with Co ,Fe and Zn complexes by using new ligand OBTMRZ while the activity was inhibited with Hg,Cu and Mn complexes .
- Low activation was appeared with organic indicators (ligands) .
- High activation in enzyme activity has been shown with present of Fe,Zn and Co salts,and the highest inhibition was occurred in presence of NaN₃ and HgCl₂ .

The anion part of salts played animported role in enzyme activity ,the activity was increase in presence of NO₃⁻¹group in comparison with chloride group for the same metal .

Introduction

The chemistry of heterocyclic compounds and a particular biheterocyclic which have been contain five member ring such as 1,3,4-oxadiazole and1,2,4-triazole receive more attention during the last decades,as bioactive molecules and of industrial applications,

($\overset{|}{-N} - \overset{|}{C} - S$) especially thio derivatives of this ring (1-3) . While by virtue of in incorporating the grouping toxophorically important in many drug (4,5).It was suggested that the -SH group attached to heterocyclic nucleas many induce fungicidal activity (6).Although some derivatives show a broad spectrum of activity to be as nsectic,a caricidal and nematocidal as well .

Heterocyclic molecules with olefinic group is considered to be good coordination compounds because they contain the hard N besides soft S atoms and presence of unsaturated group in this ligands ,make the capable to form organometallic compounds ,and because of their established biological activity with structural features and behavior(7) .The interaction of triazole ligands containing unsaturated group with transition metals gave a great interest for versality and(8,9)

diversity from the point of structural, biological and catalytical aspects, and its metal complexes it can be postulated that chemotherapeutic area is the main and widely vast field in triazole applications .

In the past several years the use of laccase enzyme as catalysts on organic synthesis as example there is one step synthesis of substituted -1,2,4-triazole(1,2,4)benzothiadiazine-8-ones by laccase enzyme ,the mediated reaction in various -5-substituted-4-amino-3-mercapto-1,2,4-triazole and hydroquinone in aqueous solution (10).

Present paper describes the new olefinic *bis* triazole and other derivatives and coordination behavior toward some select metals.

Laccase enzyme was produce from local isolate *P. ostreatus* and purified , the optimum conditions for enzyme activity were pH=6 at 60C° and for stability pH= 5-6 at 35-60 C° ,the operational activity of enzyme were pH=6 at 60C°for30 min.

We have been studied the effect of the preparing ligands and their metal complexes in addition to the salt in enzyme activity .

Experimental

Materials and methods

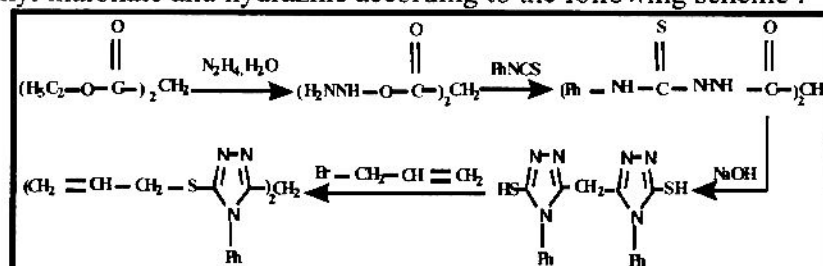
The chemicals used in this work were obtained from B.D.H and they were all pure grade reagents.Flame atomic absorption of elemental analyzer AA608 flame Emission spectrophotometer was used for metal determination,FTIR spectra were recorded using shimadza-8000 spectrophotometer,for the rang4000-200cm⁻¹.Electronic spectra were recorded using shimadza-uv-visible detector(SPD-10AVP) was used for purification determination of enzyme.

Electrical conductivity was measured by WTW conduct meter and magnetic susceptibility was measured by Brukar magnetic using faraday method , potentiometer was measured by HF-98150-GippH or Demeter type Hanna.Also cooling centrifuge Beckman ,Autoclave and shaker incubator were used in this work .

Preparation of ligands

The methods that were used to prepare the{ (OXA) 5-phenyl-2-mercapto-1,3,4-oxadiazole(TRZH)4,5-diphenyl-3-thiol-1,2,4-triazole, (BTMRZH₂)*Bis*[4-phenyl-3-thiol-1,2,4-triazole-5yl]methane, (OTRZ) 4,5-diphenyl-3(2-propenyl)thio-1,2,4- triazole}as reported in

the literature(11-13) .The new ligand (OBTMRZ) Bis[4-phenyl-3(2-propenyl) thio-1,2,4-triazole-5yl] methane was prepared starting from ethyl malonate and hydrazine according to the following scheme :



The physical properties of the product are shown in the table (1) ,characterization by physical method and FTIR spectra are given in table (2) .

Preparation of complexes

The methods that were used to prepare the metal complexes of OXA,TRZH,BTMRZH₂ andOTRZ as reported in the literature(8,12,13) .

Their is one general procedure was adopted as follows for preparation of OBTMRZ complexes :

A mixture of ethanol solution of metal salt with a certain ligand in (2:1) mole ratio was stirred under reflux for 10 min. , the precipitate which was formed after addition diethyl ether in all cases , isolated by filtration and dried under vacuum . The physical data of the prepared complexes are shown in table (1) .

Enzyme isolation

Fungal laccase was produced from *P .ostreatus* using submerged culture(70) at 30 C° and 120 rpm .Fungal filtrated was separated using cooled centrifuge at 5000 rpm for 15 min .

Estimation of Enzyme activity

The activity of laccase was measured using syringaldazine as substrate at different pH values (4-7). Laccase activity U/ml was calculated(14) according the following :

$A=10^6 \Delta E / c \Delta t$; A=Enzyme activity(U/ml), ΔE =Absorbance at 525 nm , Δt =The reaction time (min), C = 65000

Protein was determined by Bradford method(15)using BSA as standard protein and the purity of enzyme was determined by HPLC

using 0.1% trichloroacetic acid with acetonitrile as mobile phase and 20 μ L of enzyme was used .

Enzyme characterization

*** Determination the optimal pH for enzyme activity and stability :**

Laccase activity was measured in different pH values (4-8) using phosphate-citrate buffer. Enzyme stability was measured using the same buffer by incubation the enzyme for 10 min at different pH values .

*** Determination the optimal temperature for enzyme activity and stability :**

Laccase activity was measured at different temperature (20-80) C° at optimal pH , then the stability of enzyme was measured using the same range of temperature but after incubation for 10 min .

*** Operational activity :**

The operational activity of fungal laccase was measured by incubation it in optimum pH and temperature from 10 to 210 min .

The effect of copper chloride in enzyme activity

2ml of CuCl₂ at different concentrations (0.05-0.5) μ M than the mixture was incubated at room temperature for 10 min .The reaction was stopped using ice bath and the remained activity % was measured.

The effect of oxadiazole ,triazole and their complexes in enzyme activity

2ml of ligands solutions and their complexes (0.2 and 0.3) μ M were dispensed in test tubes ,then (1ml) of enzyme was added to each tube .The mixture was incubated at room temperature for 10 min ,the reaction was stopped using ice bath and the remained activity % was measured .

The effect of metal salts in enzyme activity

2ml of metal salts solutions (0.2 and 0.3) μ M which included , Cu(NO₃).3H₂O, ZnCl₂, Zn(NO₃).6H₂O, MnCl₂.4H₂O, HgCl₂, Fe(NO₃).9H₂O ,CoCl₂.6H₂O and NaN₃ were dispensed in test tubes, 1ml of enzyme was added to each tube, then the mixture incubated at room temperature for 10 min ,the reaction was stopped using ice bath and the remained activity % was measured .

Result and Discussion

Metal Analysis

The physical and analytical data of the metal complexes are given in table(1).The result obtained from metal analysis in satisfactory agreement with the calculated values .

The suggested molecular formula was also supported by spectral as well as magnetic moments .

Infrared spectroscopic study

OBTMRZ is a potential ligand which may act as mono or bidentate as illustrated in scheme (1).So it is expected that i.r measurement are highly information with respect to the complication behavior with various metal ions .The characteristic frequencies of the free ligand and its metal complexes were readily assigned based on comparison with literature references (16) .The spectrum of OBTMRZ is characterized by the presence of an essential band which belong to the olefinic group at 1630 cm^{-1} and other bands at 965 and 865 cm^{-1} which originate from the out of plane bending vibration of vinylic group .Table (2) shows the position and assignment of the main absorption bands for the ligand and their metal complexes .The spectra i.r absorption peaks of the complexes were compared with pure ligand , were adopted as follows :

- All the complexes exhibited a considerable lowering in the olefinic bands absorption $\nu\text{C}=\text{C}$ str.by($131.2,36.7,129.3,129.3,129.3$ and 131.2) cm^{-1} respectively and by ($29.6,18.4- 25.8,22.3-16.1,15.4-18, 29.7-16.1,54-18$ and 1.9) cm^{-1} respectively,for the double of the vinylic bonding of CH_2 group, indicating that the coordination of metals are through the olefinic π - bonding(16),and the spectra of the complexes show also a red shift for $\nu\text{C-S}$ band by($1,10,13,13,6$ and 9) cm^{-1} respectively,indicating the participation of sulfur atom in coordination (16,17) .
- These absorptions were further supported by the appearance $\nu\text{M-S}$ and $\nu\text{M-O}$ (16) ,there for the ligand may be considered to behave as bidentate .
- The nitrate group was found to coordinates (T_1, T_2 and T_5) metals through the oxygen atom (16) .
- A broad band was observed around ($3400-3462$) cm^{-1} spectra of ($\text{T}_1, \text{T}_3, \text{T}_4$ and T_5) complexes,assigned to a νOH and suggested the

presence of a water molecules in the complexes (18).

Electronic spectra and electrical conductance

The electronic spectra of the metal complexes were recorded for their solution in ethanol and DMF .

T_1 : The electronic spectra of dark green complex showed one broad absorption band in the region around $12,626 \text{ cm}^{-1}$ which has been attributed to $Eg^2 \longrightarrow {}^2T_2g$ transition .

This position of this band is in agreement with reported for highly distortion octahedral geometry(19,20).The electronic spectra coupled with magnetic moment(2.01)BM studies indicate square planer geometric around Cu(II)complex(21),conductivity measurement showed that the complex was non ionic, table (3) .

T_2, T_6 : The prepared complexes are colorless and diamagnetic which is expected for d^{10} ion.The uv-vis spectra of the compounds show relative change in the band position compared to that of the free.

The conductivity measurement for these complexes in ethanol at $25C^\circ$ showed to be non conducting for zinc(II)complex ($22 \mu s .cm^{-1}$) and conducting for mercury complex ($60 \mu s .cm^{-1}$) .

T_3 : The electronic spectra of Mn(II)complex was recorded for the ethanol solution .Spectra assignments were obtained by fitting the observed spectra to Tanaba-Sugano diagram for d^5 configuration values of β , and $10Dq$ computed there form (22) .

Two observations have to be pointed out:(I) a series of very weak ,some were narrow,bands have been observed in manganese spectra, this is expected because the only sextet term of the d^5 configuration in octahedral stereochemistry is the ${}^6A_{1g}$,consequently , there can be no spin-allowed transitions (22,23) .

Furthermore the energies of the ${}^4Eg_{(G)}$ and ${}^4A_{1g_{(G)}}$ terms do not change much relative to the ${}^6A_{1g_{(G)}}$ ground term as $10Dq$ change , as seen from the Tanaba-Sugano diagram ,therefore bands corresponding to transitions between the ground term and these two term are not appreciably broadened by vibronic coupling (22) .

(II) the values of the nephelauxetic ratio, β , evaluated as the ratio of the term separation in the complex , B' , to that in the free ion ,are high .

This is in keeping with the general opinion that manganese(II) complex was highly ionic(23) .The electronic spectra coupled with magnetic(4.87) BM and conductivity measurement showed that the complex was non ionic indicate octahedral geometric around Mn(II) .

T_4 : The blue cobalt (II) complex with ligand showed a magnetic moment of (4.78) BM ,which indicates a high-spin type complex . Electronic spectrum in ethanol solution exhibited a split band in the range of(17,000-14,000) cm^{-1} ,the split band is a typical tetrahedral spectra type and can be assigned as ${}^4A_{2g} \rightarrow {}^4T_{1g(P)} (\sqrt{3})$,and in addition there is band at(5181) cm^{-1} can be assigned to ${}^4A_{2g} \rightarrow {}^4T_{1g(F)} (\sqrt{2})$ transition (24,25) .

The blue color as well as the magnetic moment is further indicated tetrahedral geometry .

The($\sqrt{1}$)and various ligand field parameter have been calculated,by reference to Tanaba-Sugano diagram for d^7 configuration(26), table(3).

The calculation of the spin-orbit coupling constant (λ') was

$$\mu_{obs} = \mu_{s.o} - 15.59 \lambda' / 10 Dq$$

Where μ_{obs} = The observed effective magnetic

$\mu_{s.o}$ = The electronic spin only magnetic moment

The resulting value($\lambda' = -206.8cm^{-1}$) shows the present complex to be distorted tetrahedral(12,23).The nephelauxetic factor β was calculated and found to be(0.613)indicating high degree of covalence in bonding of ligand-donor atoms with cobalt(II)ion .

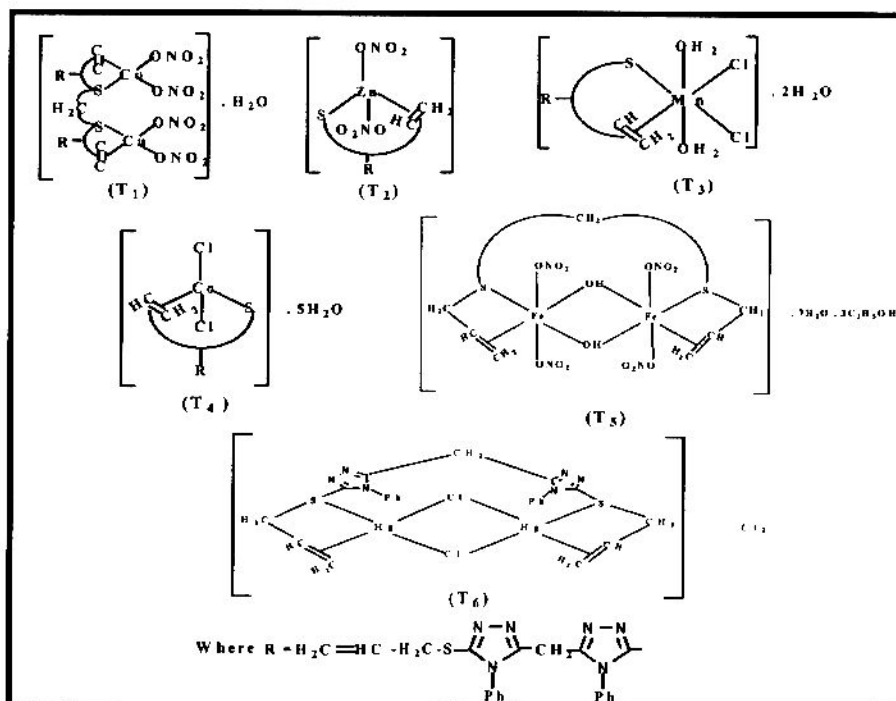
The molar conductance showed that the complex is non electrolyte .

T_5 :The magnetic measurement show the iron ion in its orange complex to be high spin paramagnetic(5.15) BM,of d^5 configuration .

This suggestion was supported by the number of maxima observed in the electronic spectrum of the complex , which show two maxima and may be assigned to transition ${}^6A_{1g} \rightarrow {}^4T_{2g(G)}$, ${}^6A_{1g} \rightarrow {}^4A_{1g} + {}^4E_{g(G)}$ as shown in(27,28) table (3).The $\sqrt{1}$ and racah parameter β ,and the value of $10Dq$,which calculated for d^5 configuration .

The conductivity measurements in ethanol show the complex to be non-electrolyte .

According to the results obtained from previous analysis the propose structure of the prepared complexes can be illustrated as follow :



Activity of enzyme :

a- Optimum condition for enzyme production:

* The optimal pH :

The optimal pH for laccase production from fungal isolate was 6.5, enzyme activity reached up to 0.717 U/ml. laccase activity decreased in acid values between 5-5.5 and there was no activity in pH 4,4.5, while it was reached to 0.2 U/ml in pH 7.5 .

The growth of *P. ostreatus* was in pH values between 5-7 but the optimal pH for enzyme activity was 6-6.5 (29-31). It was observed that the optimal pH for enzyme production was 6.5 and it was almost the same value for activity and this character is one of extracellular enzyme characters (32) , see the figure (1) .

* The optimal incubation period :

High level of enzyme production was 0.717 U/ml after 96 h. of incubated at 30 C°, figure(2).

Enzyme purification :

The analysis of fungal filtrate by HPLC technique appear one peak in retention time 2.9, this result was indicated that the enzyme almost partially pure ,figure(3).

Enzyme characterization :

-The optimal pH for enzyme activity and stability :

The result was showed that the optimum pH for enzyme activity was(6),enzyme activity reached up to 17.94 U/ml,and the enzyme was stabile at pH in the range between (5-6),figure(4& 5).

- The optimal temperature for enzyme activity and stability :

The results in figure(6&7)were showed that the optimal temperature for enzyme activity was 60C°,enzyme activity reached up to28.2 U/ml and the enzyme was stable at the rang between(35-60)C° .

- The effect of incubation time in enzyme activity at optimal conditions:

The result in figure(8),indicated that,enzyme was appeared100% of it intial activity after incubation period reached up to30 min in pH=6 and temperature 60C° .

The effect of copper chloride in laccase activity :

The activity of enzyme was enhanced gradually by copper chloride ,and the highest activity was 1.35 U/ml and concentration of 0.2 mM and then decreased .

The decreasing of enzyme activity was because of the ability of copper to form binding with the protein His-Cu-His or His-Cu-Cys in the active side or between enzyme molecules (33,34) .

The effects of OXH , TRZH , OTRZ, BTMRZH₂ andO BTMRZ complexes in enzyme activity :

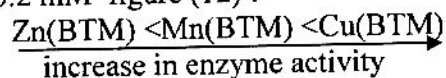
-The effects of OXH,TRZH andOTRZcomplexes in enzyme activity:

Figure(9,10&11) were indicated that the zinc complexes for each indicators were act as inducers for enzyme activity in concentration 0.2 and0.3mM .

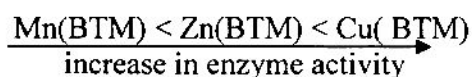
-The effects of BTMRZH₂ complexes in enzyme activity :

The BTMRZH₂complexes were appeared a different effect in enzyme activity in

concentration 0.2 mM figure (12) :



This result was indicated to the ability of copper to enhanced laccase activity^(35,36), while the same complexes in concentration 0.3mM were showed this effect :



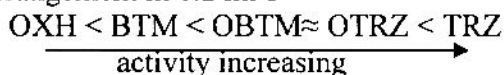
The inhibition of laccase activity in presence of manganese belonged to the ability of Mn^{II} to occupied the side of metals which presence active side of enzyme (37) .

- The effect of OBTMRZ complexes :

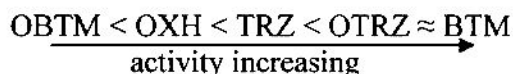
Co,Zn,Fe&Mn of OBTMRZ complexes were enhanced laccase activity in 0.2&0.3 mM concentration , while Cu & Hg OBTMRZ complexes were inhibited laccase activity figure (13) .

The effects of OXH , TRZH , OTRZ, BTMRZH2 and OBTMRZ in enzyme activity :

The effect of the prepared indicators in laccase activity were reflected this arrangement in 0.2 mM



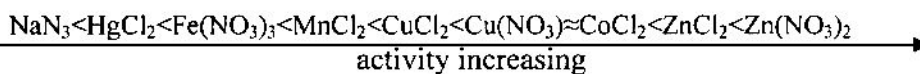
While in 0.3 mM case was appeared this effect :



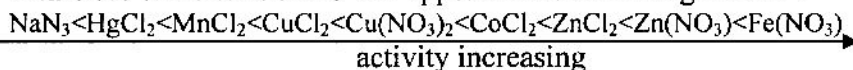
These ligands were appeared trace activation in enzyme activity because there contain olefinic groups and thioamid which have ability to form organometallic and Werner 's complexes in the same time figure (14) .

The effect of metal salts in laccase activity :

The effect of metal salts in enzyme activity was studied , the results in figure (15) showed following effect in 0.2 mM



While the same salts at 0.3 nM appeared the following effects :



The highest effect in enzyme activity was appeared in case of using Zn and Fe complexes , while sodium azide was appeared the highest inhibition .

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Table (1) : The physical properties of OBTMRZ and its complexes

Compound	Color	M.P.C Or Dec.	Yield%	Solvent	Metals analysis%		Suggestion formula
					Calculate	Found	
OBTMRZ	ابيض	120-118	92	الإيثانول	—	—	$C_{21}H_{17}N_6S_2$
Cu(OBTM)	خضر	137-135	60	الإيثانول	14.80	15.80	$[Cu_2L(ONO)_2] \cdot 11H_2O$
Zn(OBTM)	ابيض	105-103	72	الإيثانول	10.30	11.00	$[Zn(NO_3)_2L]$
Mn(OBTM)	بيج	76-74 D	52	الإيثانول	8.54	8.22	$[Mn(H_2O)_2Cl_2] \cdot 2H_2O$
Co(OBTM)	ارزق	92-90 D	60	الإيثانول	6.94	6.46	$[CoCl_2] \cdot 5H_2O$
Fe(OBTM)	مخضر	62-60 D	65	الإيثانول	10.11	9.90	$[Fe_2L(OH)_2(NO_3)_4] \cdot 7H_2O$
Hg(OBTM)	برنقالي ابيض	114-112	50	الإيثانول	—	—	$3EtOH$ $[Hg_2LCl_2]Cl_2$

Table (2) : Characteristic stretching vibration frequencies located in FTIR of the ligand and their metal complexes in cm^{-1} units

Compound	ν_{OH}	$\nu_{C=C}$	δ_{CH_2}	$\nu_{C=N}$ $+C=S$	ν_{C-S}	δ_{OH}	ν_{M-S}	ν_{M-X}	ν_{M-C}	ν_{M-ONO_2}	ν_{M-O}
OBTMRZ	—	1631.7	949.6 860.2	1282.6	731	—	—	—	—	—	—
Cu(OBTM)	3442.7	1500.5	920 841.8	1240.1	720	—	441	—	419	1010.6 1310 1375	—
Zn(OBTM)	—	1595	923.8 837.9	1263.3	721	—	368.4	—	413	1400.2 1338.2 1002.9	—
Mn(OBTM)	3384	1502.4	933.5 844.8	1238.2	718	933.5	420	235	416	—	351
Co(OBTM)	3413.3	1502.4	931.6 830.5	1278	718	—	422	329	410	—	—
Fe(OBTM)	3406 3072	1502.4	933.5 806.2	1238.2	725	933.5	464.8	—	418	1018.3 1348.1 1467.3	450
Hg(OBTM)	—	1500.5	931.6 858.3	1269.1	722	—	370.3	314.4	419	—	—

Table (3) : Electronic spectra , conductivity and magnetic moment data of the prepared new complexes

Complex	Bands cm^{-1}	Assignments	B	Dq/B'	β	10Dq	15B'	B	Mag. Sus. B. M	μ_{eff} cm^{-1}
Cu(OBTM)	$\sqrt{12,62602}$	$t_{2g} \rightarrow e_g$	-	-	-	-	-	-	2.19	34
Mn(OBTM)	$\sqrt{11,049}$ $\sqrt{20,044}$ $\sqrt{125,789 \text{ cal}}$	${}^4A_{1g} \rightarrow {}^4E_{g(1)}$ ${}^4A_{1g} \rightarrow {}^4E_{g(2)}$ ${}^4A_{1g} \rightarrow {}^4A_{1g} + {}^4E_{g(2)}$	866	1.2CB	0.98	10236	12646	843	4.87	31
Co(OBTM)	$\sqrt{3450}$ $\sqrt{5181}$ 5002 cal $\sqrt{15,932}$ av 15,549 cal	${}^4A_{1g} \rightarrow {}^4E_{g(1)}$ ${}^4A_{1g} \rightarrow {}^4E_{g(2)}$ ${}^4A_{1g} \rightarrow {}^4E_{g(2)}$	1128	0.52CB	0.613	3490	10380	692	4.78	36
Fe(OBTM)	$\sqrt{10,226}$ cal $\sqrt{20,080}$ $\sqrt{27,173}$	${}^4A_{1g} \rightarrow {}^4E_{g(1)}$ ${}^4A_{1g} \rightarrow {}^4E_{g(2)}$ ${}^4A_{1g} \rightarrow {}^4A_{1g} + {}^4E_{g(2)}$	1300	1.9CB	0.85	11081	16575	1105	5.15	28

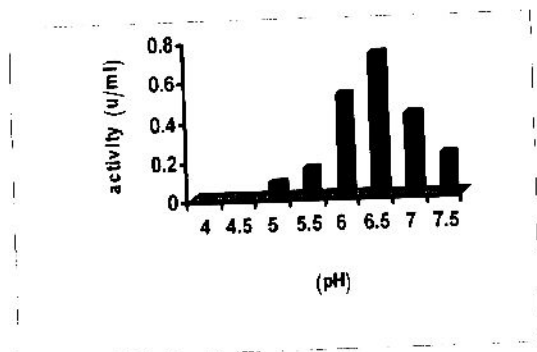


Fig (1) : Effect of different pH values in laccase production from *P. ostreatus*

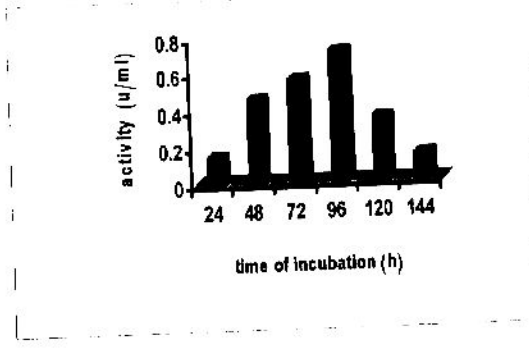


Fig (2) : Effect of incubation time in laccase production

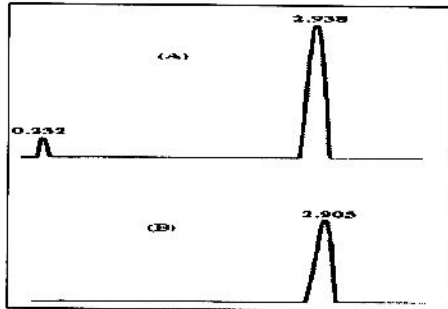


Fig (3) : HPLC technique (A) before dilution (B) after dilution

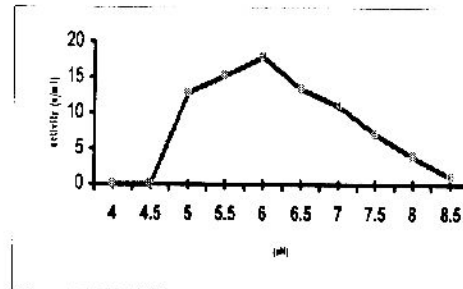


Fig (4) : Effect of pH in laccase activity

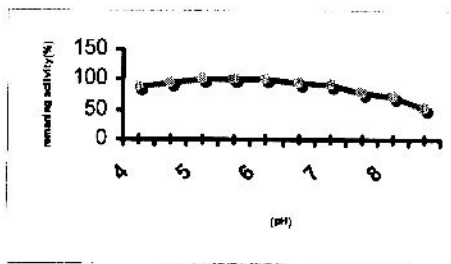


Fig (5) : Effect of different values of pH in laccase stability

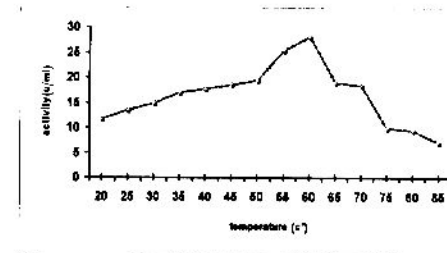


Fig (6) : Effect of temperature in laccase activity

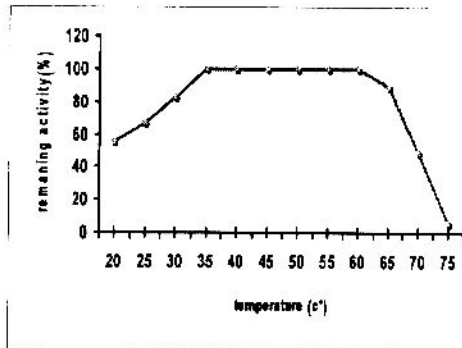
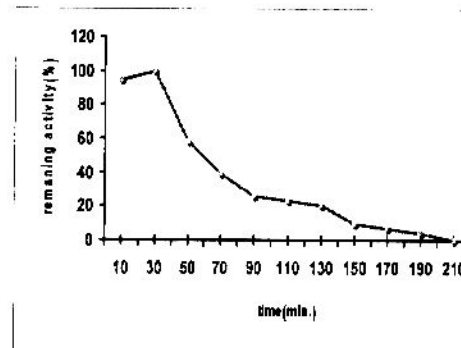


Fig (7) : Effect of different values of temperature in laccase stability



(8) : Effect of incubation time in laccase activity at pH 6 and 60C°

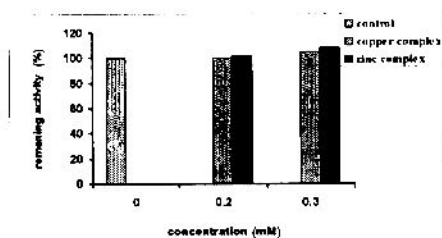


Fig (9) : Effect of OXH liga activity

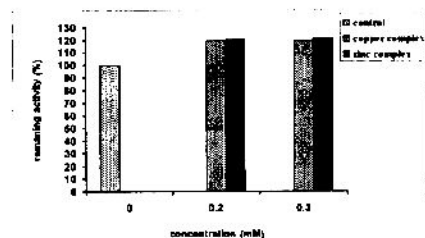


Fig (10): Effect of TRZH ligand in laccase activity

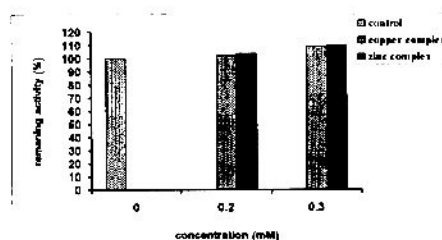


Fig (11) : Effect of OTRZ ligand in laccase activity

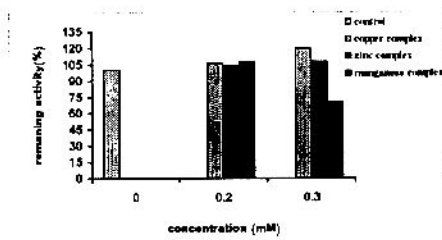


Fig (12) : Effect of BTMRZH₂ ligand in laccase activity

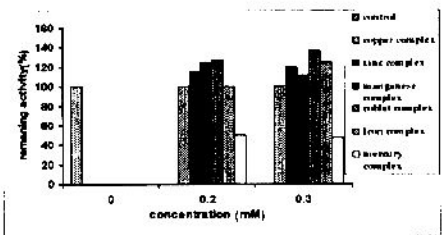


Fig (13) : Effect of OBTMRZ ligand in laccase activity

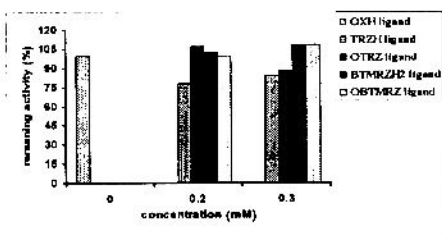


Fig (14) : Effect of ligand in laccase activity

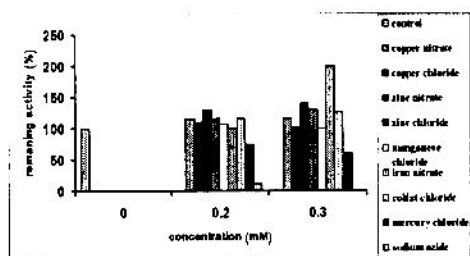


Fig (15) : Effect of metal salts in laccase activity

فعالية انزيم Iaccase

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الخلاصة

حُضر الليكاند الجديد

Bis[4-phenyl-3(2-propenyl)thio-1,2,4-triazole-5yl] (OBTMRZ)

methane. وشخص باستعمال الطرائق الطيفية والفيزيائية المناسبة، حُضرت معقداته الجديدة من مفاعلة الليكاند مع الايونات الفلزية Hg^{II} ، Fe^{III} ، Co^{II} ، Mn^{II} ، Zn^{II} ، Cu^{II} . ثم دراسة البنية التركيبية لمعقداته الجديدة المحضرة بالحالة الصلبة باستعمال الطرائق الفيزيائية المناسبة، مثل تقنية الامتصاص الذري اللهبى، و اطيفاف الاشعة تحت الحمراء و فوق البنفسجية - المرئية، و قياسات التوصيلية الكهربائية فضلاً عن الحساسية المغناطيسية بدرجة 25 م° ودرجة الانصهار او التفكك ، وبهذا اتضح عدد من الخصائص التركيبية والسلوكية عند دراسة هذه المعقدات. حُضرت الليكاندات (OBTMRZ، OTRZ، TRZH، OXH) وتتم مفاعلتها مع الايونات (Cu، Zn، Mn) المنتجة لتكوين معقدات مختلفة، شُخصت الليكاندات والمعقدات المحضرة بالحالة الصلبة بالطرائق الفيزيائية المناسبة باستعمال تقنية الاشعة تحت الحمراء ودرجة الانصهار . أنتج انزيم اللاكيز من العزلة الفطرية لـ *p. ostreatus* في المزارع المغمورة عند الظروف المثلى من $pH = 6.5$ ودرجة حرارة 30 م° ومدة حضارة 96 ساعة. حُددت مدى نقاوة اللاكيز باستعمال تقنية الـ HPLC. وظهرت النتائج ان الانزيم المستخلص نقي جزئياً، كما حدد تركيز ايونات العناصر (النحاس والخرصين والحديد) في الانزيم باستعمال تقنية المطياف الذري اللهبى. وقد اظهرت النتائج ان الانزيم يحوي ذرتي خارصين وذرة نحاس وذرة حديد في تركيبه الداخلي، وكانت الظروف المثلى لفعالية الانزيم من $pH = 6$ عند درجة حرارة 60 م° ولثباتية $pH = 5-6$ عند درجات الحرارة 35-60 م° واحتفظ الانزيم بكامل فعاليته عند $pH = 6$ ودرجة حرارة 60 م° مدة 30 دقيقة . ودرس تأثير كل من الاملاح والكواشف (الليكاندات) ومعقداتها المحضرة في فعالية الانزيم المستخلص وكما يأتي:

- ❖ تعمل معقدات الخارصين مستحضات جيدة لزيادة فعالية اللاكيز باستعمال الكواشف OXH، TRZH، OTRZ مقارنة بمعقدات النحاس، في حين تسلك معقدات الكاشف $BTMRZ_2$ سلوكاً مغايراً لسلوك معقدات الكواشف السابقة.
- ❖ لوحظ ازدياد فعالية الانزيم المستخلص بوجود معقدات الكوبلت والحديد والخارصين باستعمال الكاشف OBTMRZ، في حين ثبطت فعاليته بوجود معقدات الزنبق والنحاس والمنغنيز.
- ❖ عند مقارنة تأثير الكواشف العضوية (OBTMRZ، OTRZ، TRZH) في فعالية الانزيم لوحظ تأثير طفيف من حيث التنشيط .
- ❖ اعلى قدرة تنشيطية للاكيز كانت باستعمال املاح الحديد والخارصين والكوبلت، واعلى قدرة تثبيطية له بوجود ملح ازيد الصوديوم وكلوريد الزنبق، كما يؤدي الجزء السالب من الاملاح المستعملة دوراً كبيراً في فعالية الانزيم اذ ينشط الانزيم بوجود مجموعة النترات ويثبط بوجود مجموعة الكلورايد للفلز نفسه .