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PRODUCTION OF FRAGRANCE ESTERS FROM FUSEL OIL BY MYCELIAL LIPASE OF *RHIZOPUS ARRHIZUS*

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The properly dried and defatted mycelium of *Rhizopus arrhizus* constitutes a naturally immobilized lipase. The mycelium was used to synthesize flavouring esters from fusel oil by esterification in organic solvents. Different parameters like reaction medium, temperature, substrate concentration and moisture level were optimized for the synthesis of butyrate esters of fusel oil. The mycelium was repeatedly used in batch reactions and was found to have high efficiency and good operational stability. The half life of the mycelial lipase was greatly influenced by the concentration of substrates, i.e. the residual activity remained about the same after 20 cycles with low concentrations of substrates (0.1 M each). However, when concentrations of butyric acid and fusel oil were high, the mycelium rapidly lost its activity.

Key words: Esters, Esterification, *Rhizopus arrhizus.*

Introduction

Lipases are extremely versatile enzymes. Their properties allow widespread application in the field of biotechnology [1,2]. Lipases from different sources can catalyze hydrolysis, synthesis and interesterification of various classes of esters [3-6]. To apply lipase for ester synthesis rather than hydrolysis, it is essential to carry out the reaction in an organic solvent [7-9]. Although the enzymes are, in general, unstable in organic solvents, immobilized or cell bound lipases are resistant to denaturation by organic solvents [10,11]. The lipase mediated synthesis reactions are receiving much attention because of their use in production of flavouring esters, sugar esters, glycerides, ester oligomers and lactones [12-15]. There are many well known flavour esters forming part of natural aroma that have been obtained either from their natural sources or by chemical synthetic methods. The natural sources are limited and traditional chemical methods of ester synthesis involve refluxing the reaction mixture at high temperature and many steps of purification thus resulting in expensive final product. The enzymic esterification takes place under ambient conditions of temperature and pressure [16-17]. Moreover, specificity of the enzyme prevents side reactions, thus, resulting in purer product at much lower capital and energy cost [18-20].

The present work provides knowledge of some of the factors affecting the synthesis of short chain esters from fusel oil using mycelial lipase or *R. arrhizus.* The fusel oil, a by-product of the alcohol industry is composed of a large number of alcohols (mainly C_3 to C_6). Esters of these alcohols with short chain fatty acids are flavouring in nature and find application in food additives industry [21]. The process is of great interest being safe and economical.

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Materials and Methods

The biocatalyst, used for ester synthesis was mycelial lipase of *Rhizopus arrhizus* (activity 250 units/g mycelium). The enzyme was produced as reported earlier [22] in 10 L glass stainless steel fermenter at 30°C in a medium consisting of (g/L) olive oil:20, egg yolk: 10; NH_4Cl:5.0, $MgSO_4.7H_2O$:0.25; K_2HPO_4:0.5; $CaCO_3$:5.0. Two days after inoculation the mycelium was harvested, freez dried and treated with (i) chilled acetone, (ii) acetone-ether and (iii) n-hexane. The dried and defatted mycelium, thus obtained, was kept in desicator over $CaCl_2$. Lipoloytic activity of the mycelial powder was determined titerimetrically on the basis of olive oil hydrolysis [23,24]. The moisture content of the mycelial powder was determined by drying at 105°C to constant weight.

Fusel oil was supplied by Ravi Rayon Limited, Pakistan. The fusel oil used in these experiments was composed of a large number of alcohols. The three main alcohols and their mole fractions were, propanol. 0.032, isobutanol 0.203 and isopentanol 0.764.

All other chemicals were of analytical grade (BDH Chemicals Ltd., Poole, England). The organic solvents were distilled before use.

Esterification. Ester synthesis was carried out in 100 ml stoppered conical flasks in which 500 mg mycelial powder was mixed with 25 ml organic solvent. Fusel oil and fatty acid were used at concentrations between 0.1 M and 0.2 M. The reaction mixture was incubated, unless otherwise stated, at 30°C using orbital shaker at 100 rpm. The samples were drawn from time to time and titrated against 0.1 N alcoholic KOH to determine residual fatty acids. Ester production was expressed in terms of moles of fatty acids converted to esters in 48 hr.

Effect of fatty acid chain length on ester yield was studied by reacting 0.4 M fatty acids (C_1-C_{10}) with 0.4 M fusel oil in

n-hexane. The reaction temperature, substrate concentrations and organic solvents were optimized for butyrate ester synthesis. The mycelium was recovered from the reaction mixture, 48 hr. after incubation and was repeatedly used to catalyze the reaction.

The product mixture was treated with saturated solution of $NaHCO_3$ to remove unused butyric acid. After washing with distilled water the organic layer was dried over anhydrous Na_2SO_4. The impure mixture of esters and fusel oil was purified by column chromatography. The mixture was charged to a silica gel (72-230 mesh, merk) column and was eluted with n-hexane: ether (95:5) mixture and methanol. Eluted fractions were monitored by thin layer chromatography and infra red spectroscopy.

Results and Discussion

Effect for fatty acid chain length. The chain length of fatty acids had great influence on ester formation. (Figure 1) shows the effect of carboxylic acid with different chain length (C_2-C_{10}) on ester formation with fusel oil. The production of esters was increased with the increase in chain length of carboxylic acids. It may be due to decrease in ionization and increase in hydrophobicity with the increase of chain length. This work is similar to the work of Miller *et al.* [7] who studied lipozyme catalyzed esterification of n-octanol.

Effect of substrate concentration. The effect of fusel oil concentration (0.05 to 1.2 M) on esterification with butyric acid (0.4 M) was observed. Maximum ester production and yield was noted between 0.4 to 0.6M fusel oil concentration (Fig.2). Further increase in its concentration resulting in lowering both the % yield and total production of esters. It may be due to dehydration of the enzyme by fusel oil. Thus lowering the activity of lipase.

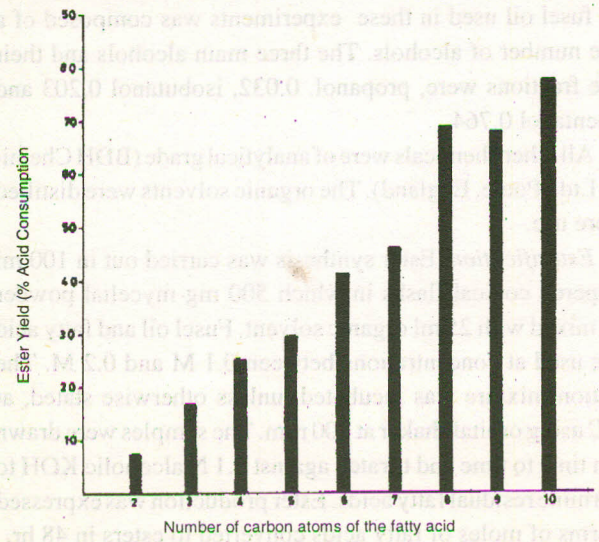

Fig. 1. Effect of fatty acid chain length on ester synthesis.

Similarly effect of changes in butyric acid concentration (0.05 to 1.2 M) on esterification was studied (Fig. 3). Maximum ester production (0.1 mol/L) was achieved at 0.4 M butyric acid concentration, beyond that there was decrease in acid consumption due to acidification of the reaction mixture. Although the naturally immobilized lipase of *Rhizopus arrhizus* is less sensitive to adverse conditions of pH and water scarcity as compared to free enzymes, the resistivity to such effects decreases at higher acid concentration.

It is evident from the above data that at lower concentrations of substrate (up to 0.4M, ester synthesis increases with increase in fusel oil concentration or butyric acid concentration. However, due to enzyme inactivation, increase in ester

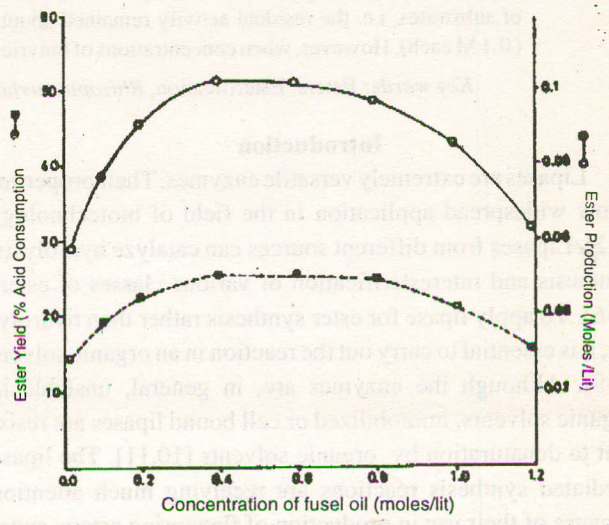

Fig. 2. Effect of fusel oil concentration on butyrate esters synthesis using 1% (w/v) mycelial powder of *Rhizopus arrhizus.*

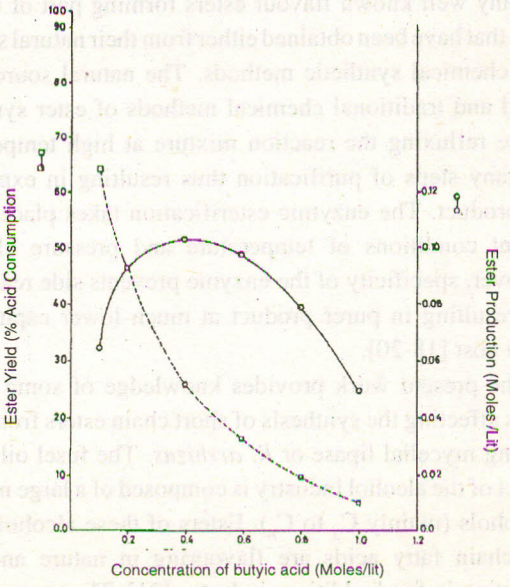

Fig. 3. Effect of butyric acid concentration on esterification of fusel oil using 1% (w/v) mycelyal powder of *Rhizopus arrhizus.*

production was not proportional to the increase in substrate concentration. Figure 2 and 3 show that the inactivation was more pronounced at higher concentrations (0.4M for butyric acid and 0.6M for a fusel oil) where increase in substrate concentration inhibits enzymic esterification.

Selection of solvent for esterification. The enzyme activity and the equilibrium of the reversible esterification reaction are greatly affected by the nature of the reaction medium. To shift the equilibrium towards ester synthesis rather than hydrolysis the reactions were performed in non-aqueous media. But complete removal of water from the system rendered the enzyme inactive. Intramolecular forces such as salt bridges and hydrophobic interactions are dependent upon water. So a small amount of water is necessary for active conformation of the enzyme protein. Figure 4 shows that tetrahydrofuran, dimethylformamide and acetone, which are hydrophilic solvents, inactivated the enzyme by extracting water essential for conformational integrity of the protein. Moreover, fatty acids ionized to greater extent in polar solvents thus lowering pH of the medium. On the other hand non-polar solvents such as n-hexane and n-octane proved to be good solvents due to their hydrophobic nature. In n-hexane the mycelial lipase of *Rhizopus arrhizus* showed maximum synthetic activity (relative activity 100). The n-hexane is compatible with the enzyme. It does not dehydrate the enzyme and both the substrates and products are soluble in it.

Effect of temperature. Effect of temperature on synthetic activity of the fungal lipase was determined for synthesis of butyrate esters in n-hexane. Maximum reaction rates were obtained at 46°C. But there is a little difference in reaction

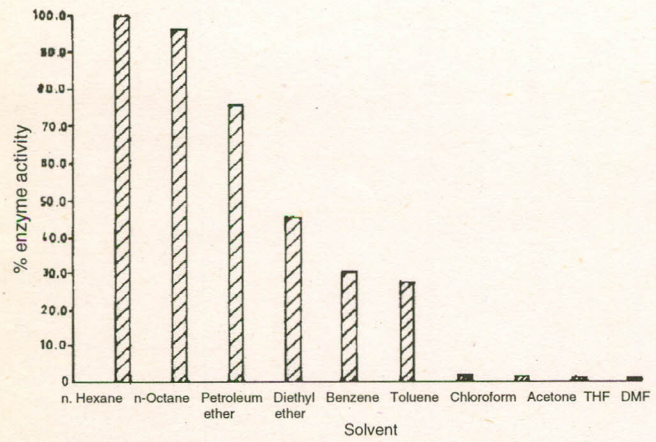

Fig. 4. Effect of organic solvents on the production of butyrate esters from fusel oil.

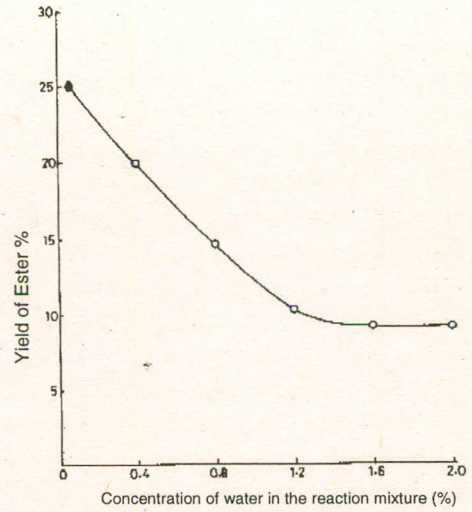

Fig. 5. Effect of water content on ester synthesis.

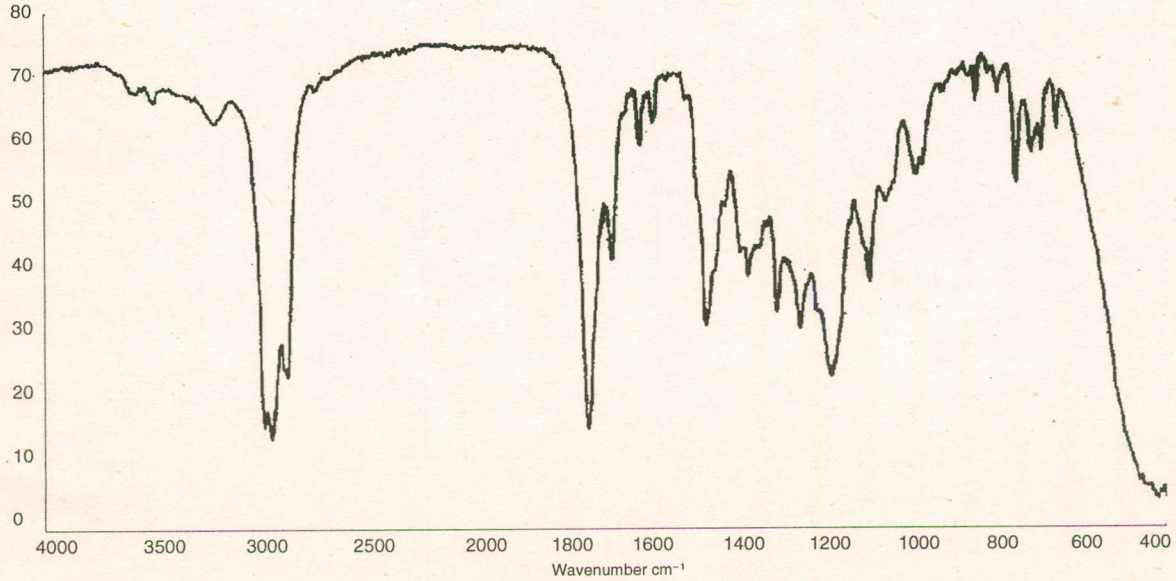

Fig. 6. I.R. spectrum of butyrate esters of fusel oil.

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Effect of temperature. Effect of temperature on synthetic activity of the fungal lipase was determined for synthesis of butyrate esters in n-hexane. Maximum reaction rates were obtained at 40°C. But there is a little difference in reaction

Fig. 5. Effect of water content on ester synthesis

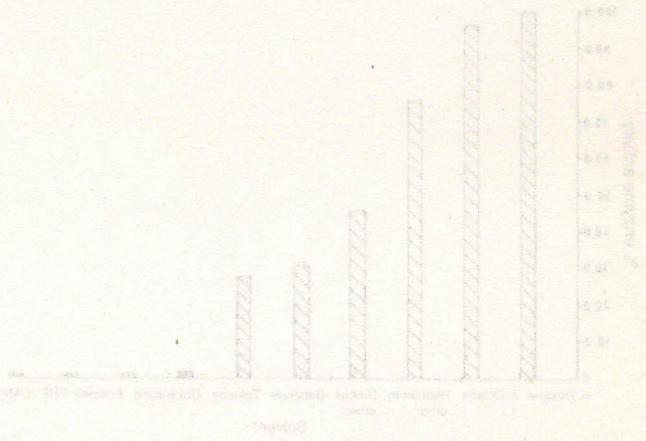
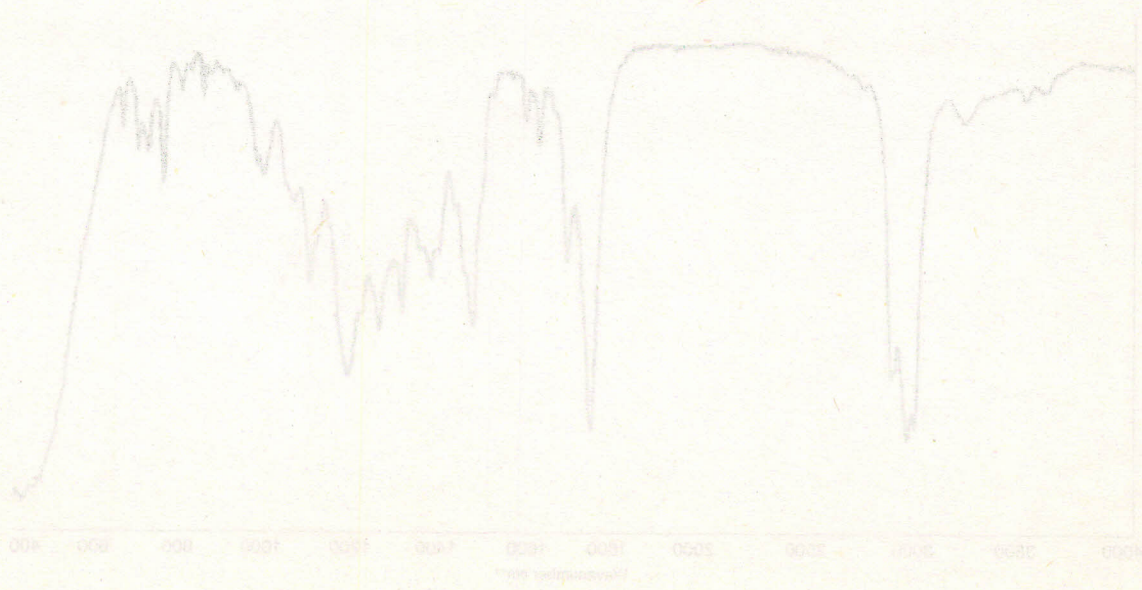


Fig. 4. Effect of organic solvents on the production of butyraldehyde from fusel oil.



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in the same solvent. For VO^{2+} or Pd^{2+} complexes, aqueous solutions of vanadyl sulphate and methanolic solution of palladium (II) acetate were added to the ligand solution respectively. The reaction mixture was stirred for 20-30 mins and then subjected to refluxing for one hour during which the complex precipitated. The reaction mixture was cooled and the complex was separated, washed several times with methanol, finally with ether and air dried.

Analytical techniques. Elemental analyses of these complexes were obtained from Chemistry Department, School of Pharmacy, University of London, England and University of Stuttgart, Germany. The metal contents, V^{4+}, Co^{2+} and Ni^{2+} were estimated by spectrophotometric methods [16]. The complexes were first decomposed with concentrated nitric acid and evaporated to dryness. The residue was dissolved in distilled water containing a few ml of dilute nitric acid and used for estimation of metal ions.

The melting points were determined on Mettler's model MP-D apparatus using sealed capillary method. A single beam Shimadzu model (120-01) spectrophotometer was used for estimation of metal ions. The infra red spectra of solid complexes and ligand as KBr discs were measured on Hitachi model 270-50 spectrophotometer.

The magnetic moments of solid complexes were determined on a Gouy's balance consisting of an electromagnet, a stabilized DC source and Mettler H-54 balance. The sample tube was calibrated with a standard $HgCo(SCN)_4$. Diamagnetic corrections for the ligand and metal ions were determined from Pascal's constants. Magnetic moments accurate to ± 0.05 B.M. are reported in Table 1.

Results and Discussion

Five complexes of VO^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Pd^{2+} ions with a new ligand 4-(4-methoxyphenyl)-1-(2-pyridyl)thiosemicarbazide are synthesized by mixing methanol solutions of the respective metal salts with that of the ligand in 1:2 molar ratio. These compounds are characterized by elemental analyses, I.R. and magnetic moments studies. These are green, dark brown, amorphous powders and decompose between 205-

Table 1. Magnetic Moments or Molar Conductivities of MPT

Complex	Mol.wt (calcd)	Gram susceptibility $\chi_g \times 10^6$ c.g.s	Molar susceptibility $\chi_M \times 10^6$ c.g.s (corrected)	Molar susceptibility $\chi_M \times 10^6$ c.g.s	μ_{eff} (corrected) B.M.
1. $VO(MPT)_2SO_4.3H_2O$	821.77	1.613	1531.82	1062.0	1.92
2. $Co_2(MPT)_4.2H_2O$	1445.32	3.18	4829.7	3511.84	2.79
3. $Ni_2(MPT)_4.H_2O$	1440.76	4.845	6456.6	2061.14	2.03
4. $Co_2(MPT)_4Cl_2$	800.66	3.10	2517.42	2554.5	1.29

385°C, $[Ni_2(MPT)_4H_2O]$ being the least stable. Their elemental analyses (Table 2) show that two ligands are coordinated with each of VO^{2+}, Co^{2+}, and Ni^{2+} ions while Cu^{2+} and Pd^{2+} interact with only one ligand to form complexes of formula ML_2X_2. The ligand behaves as a monomeric in most of these complexes except $[VO(MPT)_2]SO_4.3H_2O$ in which it acts as a neutral species. These complexes are insoluble in water as well as most of the common organic solvents. However, these are partially soluble in DMF or DMSO while $[Pd_2O(MPT)_4]$ is insoluble even in these two solvents.

These complexes (except $Pd_2O(MPT)_4$ which is diamagnetic) are paramagnetic, their magnetic moments being 1.29-2.03 B.M. at room temperature. The magnetic moment of vanadyl(IV) complex closely resembles with that of simple monomeric species. The magnetic moments of $[Co_2(MPT)_4]$ $2H_2O$, $[Ni_2(MPT)_4H_2O]$ and $[Co_2(MPT)_4Cl_2]$ are considerably lower than those expected for monomeric complexes of these metal ions. The lower paramagnetic susceptibility of these complexes may be attributed to metal metal interactions.

The prominent infrared bands of the ligand and its complexes (Table 3) have been assigned to various vibrational modes of characteristic groups. Some of these vibrational bands are shifted in the complexes which may be used to identify metal bound binding sites.

The ligand has a couplet of sharp bands at 3200 cm^{-1} and 3220 cm^{-1} due to N-H stretching. In complexes these bands have merged into a weak broad band between 3104-3410 cm^{-1} due to N-H and O-H stretching. Another band at 1552 cm^{-1} assigned to N-H deformation in the ligand is shifted to lower frequency between 1512-1540 cm^{-1} in VO^{2+}, Co^{2+} and Ni^{2+} complexes while a second band at 1512 cm^{-1} due to N-H deformation in the ligand is not shifted in most of these complexes. Another band at 1170 cm^{-1} assigned to C-N stretching is also not affected upon complexation indicating that secondary amino nitrogen attached to phenyl ring is not coordinated. The shift in N-H vibrations indicates that one nitrogen of hydrazine is bonded to the metal ions.

in the same solvent. For VO^{2+} or Pd^{2+} complexes, aqueous solutions of vanadyl sulphate and methanolic solution of palladium (II) acetate were added to the ligand solution respectively. The reaction mixture was stirred for 20-30 mins and then subjected to reflux for one hour during which the complex precipitated. The reaction mixture was cooled and the complex was separated, washed several times with methanol, finally with ether and air dried.

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These complexes (except $Pd_2O(MPT)_2$ which is diamagnetic) are paramagnetic, their magnetic moments being 1.29-2.03 B.M. at room temperature. The magnetic moment of vanadyl (IV) complex closely resembles with that of simple, mononuclear species. The magnetic moments of $[Co_2(MPT)_4].7H_2O$, $[Ni_2(MPT)_2H_2O]$ and $[Cu_2(MPT)_2Cl_2]$ are considerably lower than those expected for mononuclear complexes of these metal ions. The lower paramagnetic susceptibility of these complexes may be attributed to metal-metal interactions.

The prominent infrared bands of the ligand and its complexes (Table 3) have been assigned to various vibrational modes of characteristic groups. Some of these vibrational bands are shifted in the complexes which may be used to identify metal ligand binding sites.

The ligand has a couplet of sharp bands at 3300 cm^{-1} and 3220 cm^{-1} due to N-H stretching. In complexes, these bands have merged into a weak broad band between 3104-3416cm^{-1} due to N-H and O-H stretching. Another band at 1552 cm^{-1} assigned to N-H deformation in the ligand is shifted to lower frequency between 1532-1540 cm^{-1} in VO^{2+}, Co^{2+} and Ni^{2+} complexes while a second band at 1512 cm^{-1} due to N-H deformation in the ligand is not shifted in most of these complexes. Another band at 1170 cm^{-1} assigned to C-N stretching is also not affected upon complexation indicating that secondary amine nitrogen attached to phenyl ring is not coordinated. The shift in N-H vibrations indicates that one nitrogen of hydrazine is bonded to the metal ions.

TABLE 1. MAGNETIC MOMENTS OF METAL COMPLEXES OF MPT.

Complex	Mol.wt (calcd)	Gram susceptibility $\chi_g x10^6$ c.g.s.	Molar susceptibility $\chi_M x10^6$ c.g.s.	Molar susceptibility (corrected) $\chi_M x10^6$ c.g.s.	$\mu_{eff/metal}$ B.M.
1. $VO(MPT)_2SO_4.3H_2O$	821.77	1.623	1333.82	1682.0	1.98
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3. $Ni_2(MPT)_4.H_2O$	1340.76	4.845	6496.6	7031.14	2.03
4. $Cu_2(MPT)_2Cl_2$	800.66	3.16	2537.42	2854.5	1.29

The stretching vibration of C=S observed at 1246 cm^{-1} in the ligand remains unchanged in all of these complexes. It indicates that sulpher of the thiosemicarbazide is not coordinated with the metal ions. However, a strong band at 1662 cm^{-1} assigned to carbonyl stretching is shifted to lower frequency in most of these complexes while it is shifted to 1728 cm^{-1} in the Ni^{2+} complex. These changes in frequency of carbonyl stretching indicates that oxygen attached to amide

TABLE 2. PHYSICAL CONSTANTS AND ELEMENTAL ANALYSES OF METAL COMPLEXES OF MPT.

Complex	Colour/ physical state	Decomposition point °C	Elemental Analysis (Percentage) C Found (calcd)	H Found (calcd)	N Found (calcd)	S Found (calcd)	M Found (calcd)
1. $[VO(C_{14}H_{14}N_4O_2S)_2]\ SO_4 \cdot 3H_2O$	Brown amorphous	315	41.06 (40.93)	4.04 (4.17)	13.69 (13.64)	—	5.94 (6.20)
2. $[Co_2(C_{14}H_{13}N_4O_2S)_4] \cdot 7H_2O$	Light brown amorphous	340	46.57 (46.40)	3.76 (4.59)	15.01 (15.46)	—	9.21 (8.14)
3. $[Ni_2(C_{14}H_{13}N_4O_2S)_4] \cdot H_2O$	Pea green powder	305	50.26 (50.16)	4.14 (4.06)	16.26 (16.17)	—	8.51 (8.76)
4. $[Cu_2(C_{14}H_{13}N_4O_2S)_2Cl_2]$	Dull green powder	380-3	41.85 (42.00)	3.03 (3.27)	13.99 (13.99)	8.15 (8.00)	—
5. $[Pd_2O(C_{14}H_{13}N_4O_2S)_2]$	Reddish brown powder	330-3	40.73 (40.44)	3.25 (3.15)	13.46 (13.48)	8.04 (7.71)	—

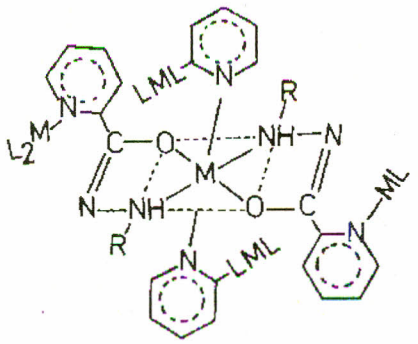
TABLE 3. PROMINENT INFRA-RED BANDS OF MPT AND ITS METAL COMPLEXES MEASURED IN KBr DISC (BANDS REPORTED IN cm^{-1}).

MPT	$[VO(MPT)_2]$ $SO_4 \cdot 3H_2O$	$[Co_2(MPT)_4]$ $7H_2O$	$[Ni_2(MPT)_4]$ H_2O	$[Cu_2(MPT)_2]$ Cl_2	$[Pd_2O(MPT)_4]$	Assignment
3300(s) 3220(s)	3416(b)	3416(w,b)	3416(b)	3104(b)	3396(b)	υN-H, υOH, υCH,
1662(s)	1608(s)	1650(s)	1728(m)	1650(s) 1602(s)	1628(s)	υC=O
1598(s)	1586(s)	1590(m)	1598(s)	1566(s)	1566	υC=C + υC=N
1552(s)	1530(s)	1540(s)	1532(s)	--	--	δN-H
1512(s)	1512(s)	1512(s)	1510(s)	1512(s)	1510(s)	δN-H +υC-N
1454(s)	--	1468(m)	1466(m)	1468(m)	1470(w)	υC-H (methyl)
1430(s)	1410(m)	1428(b,m)	1394(m)	1404(m)	1410(m)	υC-H
1300(m)	1298(w)	1298(m)	1298(m)	--	1294	
1246(s)	1244(s)	1244(s)	1242(s)	1250(s)	1242(s)	υC=S
1170(m)	1176(m)	1174(m)	1170(m)	1172(m)	1150(m)	υC-N
1028(m)	1028(m)	1030(m)	1032(w)	1032(w)	1028(m)	C-H defor (pyridine ring)
	970(m)	--	--	--	--	υV=O
924(w)	934(w)	962(w)	--	--	938	Phenyl ring bending
830(w)	828(w)	830(w)	828(w)	816(w)	830	C-H out of plan bending
710(w)	724(w)	716(w)	714(w)	716(w)	--	C-H bending (pyridine)
694(w)	688(w)	676(w)	688(w)	678(w)	668(w)	C=S rocking
	590(w)	588(w)	590(w)	588(w)	--	υM=O
	528(w)	522(w)	522(w)	--	--	υM-N
	360	--	--	--	394	υM-N

b = Broad, m = Medium, s = Sharp, strong, w = weak.

group is the second donor site in present series of complexes. These band assignments closely resemble with those already reported for complexes of thiosemicarbazides [11,12].

A number of vibrational bands due to pyridine ring observed at 1598 cm^{-1}, 1028 cm^{-1} and 710 cm^{-1} are slightly shifted in these complexes indicating relatively weaker interaction of pyridine nitrogen with metal ions. It is, therefore, proposed that MPT is coordinated with these metal ions through its amide oxygen and hydrazine nitrogen forming five membered chelates. The pyridine nitrogen of the ligand is weakly bonded to the neighbouring metal ion as shown in II.



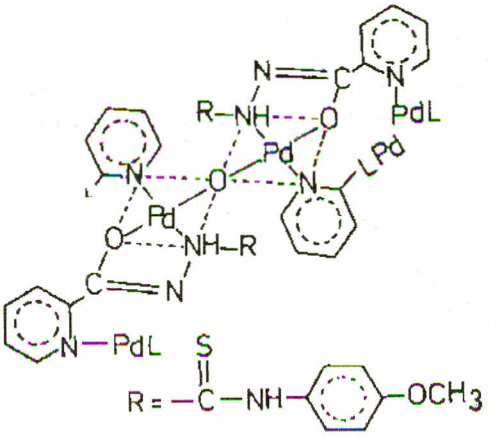
II

The vanadyl complex has a sharp I.R. band at 970 cm^{-1} due to V=O stretching. In the far infra red region, a weaker band at 590-588 cm^{-1} in these complexes may be assigned to M-O stretching while two bands at 528-522 cm^{-1} and 394-360 cm^{-1} (VO^{2+} and Pd^{2+} complexes) are assigned to M-N stretching vibrations. These band assignments are in agreement with the earlier reported values for M-O and M-N stretching vibrations [17].

The ligand behaves as a bidentate anion in these complexes except in $[VO(MPT)_2]SO_4 . 3H_2O$ where it is acting as a neutral moiety. The different behaviour in vanadyl complex is perhaps due to smaller size and weaker electron attracting tendency of the metal ion.

All of these complexes are polymeric as indicated by their low solubility and magnetic moments. In certain cases (particularly in Ni^{2+} and VO^{2+}) metal-metal interactions are very weak and their magnetic moments are closer to those expected for mononuclear species. In $[Co_2 (MPT)_4] . 7H_2O$ and $[Cu_2(MPT)_2Cl_2]$, the metal ions are closely situated effectively shielding spins of the individual ions and considerable reduction in magnetic moments is observed. The complexes $[(VO)_2 (MPT)_4](SO_4)_2 . 3H_2O$, $[Co_2(MPT)_4].7H_2O$ and $[Ni_2(MPT)_4] H_2O$ may be assumed as six coordinated octahedral species while $[Pd_2O(MPT)_2]$ and $[Cu_2(MPT)_2Cl_2]$ are four coordinated and square planer. In $[Pd_2O(MPT)_2]$ two corners of the square are occupied by oxygen bridge as in III.

It is concluded from these studies that 4-(4-methoxyphenyl)-1-(2-pyridoyl) thiosemicarbazide is a versatile ligand capable of forming complexes with a number of bivalent metal ions. The metal ions, VO^{2+}, Co^{2+} and Ni^{2+} are surrounded by six donor atoms leading to octahedral species while $[Cu_2(MPT)_2Cl_2]$ and $[Pd_2O(MPT)_2$ are four coordinated with square planar environment around the metal ion. As the ligand contains a number of donor atoms, polynuclear complexes are formed in which pyridine nitrogen is coordinated to a neighbouring metal ion. Since these complexes are insoluble in most of the solvents, antibacterials studies could not be carried out.



III

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