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Separation of Ti(IV) and Fe(III) from Aqueous Sulphate Solution by Cyanex 272 [Bis(2,4,4-Trimethylpentyl) Phosphinic Acid] in Kerosene

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Abstract. Extraction and separation of Ti(IV) and Fe(III) from aqueous sulphate solution by Cyanex 272 [bis(2,4,4-trimethylpentyl) phosphinic acid] in kerosene was investigated. Extraction of Ti(IV) and Fe(III) increased with the increase of extractant concentration and decreased with the increase of aqueous phase acidity. About 95% Ti(IV) and 24% Fe(III) was extracted with 0.20 M Cyanex 272 and 0.25 M H₂SO₄. It was thought that Fe(III) was extracted in the organic phase by the formation of the species FeA₅ or FeA₆(H₄A) and Ti(IV) as TiO₂A₅. Data shows that Cyanex 272 can be used as a very effective extractant for Ti(IV) extraction from ilmenite leach solution at high acidity showing large separation coefficient for Ti(IV) from [Ti(IV) and Fe(III)] mixture. The maximum separation factor (β=116.50) was obtained at moderate acidity (0.90 M H₂SO₄) with high extractant (0.20 M Cyanex 272) concentration. The experimental data also suggested that the extraction of Ti(IV) and Fe(III) by Cyanex 272 did not follow simple extraction mechanism for all the acid ranges. It is likely that solvation mechanism may be operative at high acidity of the aqueous phase.

Keywords: solvent extraction, Ti-Fe separation, sulphate solution, Cyanex 272, kerosene

Introduction
Iron(III) coexists as an impurity with other valuable metals, such as titanium, cobalt, copper, nickel, manganese, zirconium etc in leach solutions. It is necessary to remove it before the recovery of pure metals from solutions. The removal of iron by solvent extraction is widely studied as an alternative to solid-liquid separation. Solvent extraction is one of the most effective extraction and separation techniques in hydrometallurgy which has been in use for more than three decades for its versatility and simplicity. The extractant Cyanex 272 is a proprietary technical grade item of Cytec Canada Inc. Its active component is bis [2,4,4-trimethylpentyl] phosphinic acid (Cyanex 272 = HA). The structure of Cyanex 272 is as follows:

This technical grade extractant has been used since 1983 and found to be very effective for the extractive separation of Co(II) from Ni(II) (Parhi et al., 2008a; Gandhi et al., 1993; Tait, 1993; Danesi et al., 1984; Preston, 1983; Chou and Beckstead, 1990). However, other metal ion pairs have also been investigated for separation (Awwad et al., 2009; Bari et al., 2009a,b; Parhi and Sarangi 2008; Da Silva et al., 2008; Deep et al., 2006; Natharsarma and Niharbala 2006). Even though Cyanex 272 is selective for cobalt in the presence of nickel, a variety of several cations such as Fe(III), V(IV), Ni(II), Zn(II), Co(II), Cd(II), Cu(II), Mg(II), Al(III), Ca(II), Mn(II) can also be extracted depending upon the solution pH, from both chloride and sulphate media (Shiau et al., 2005; Fontana et al., 2005; Mandar et al., 1999; Rickelton 1996; Kathryn and Sole 1995; Boyle and Rickelton 1990; Sastre et al., 1990).

Cyanex 272 is totally miscible with common aromatic and aliphatic diluents and is extremely stable to both heat and hydrolysis. There seems to be no report on Ti(IV)/Fe(III) separation by Cyanex 272 in kerosene. Although the extraction of Ti(IV) and Fe(III) by Cyanex 272 has been widely studied from an equilibrium point of view, so far, the system has not been studied from the separation point of view, though separation using carboxylic acids and di-2-ethylhexyl phosphoric acid was attempted in the past.

The extraction and separation of titanium and iron by carboxylic acids, carbolic acid and carpic acid (Islam et al., 1988) is very low. Though the reports on bis-(2-ethylhexyl) phosphoric acid (Islam et al., 1979) had been satisfactory, complete removal of iron was not achieved in a single operation. There are numerous reports on the extraction, separation
and study of mechanism of different metal ions by other Cyanex type extractants such as Cyanex 301, Cyanex 302 Cyanex 471 and Cyanex 923.

The present work reports the extractive behaviour of titanium and iron as well as their separation from the mixture by Cyanex 272, as most of the titanium ores are associated with iron in nature. One of the chief ore of titanium is ilmenite (FeTiO₃). During processing for the production of pure TiO₂ from ilmenite leach solution, the separation of iron from titanium is a great problem. High purity TiO₂ can be obtained if all iron can be removed from the leach solution. Many methods are available but only few solvent extraction processes are reported with commercial extractants. Present work examines the efficacy of Cyanex 272 for separation of Ti and Fe from the aqueous phase.

Materials and Methods

Standard solution of Ti(IV) was prepared by fusing 0.5 g of analytically pure TiO₂ with 10.0 g of KHSO₄ in a suitable platinum crucible with heating until the oxide is dissolved. It was then cooled and dissolved in 15% H₂SO₄ by gentle heating and diluted to 500 cc by 15% H₂SO₄. Another stock solution of Fe(III) was prepared by dissolving exactly 0.864 g of A.R grade ferric ammonium sulphate in distilled water to which 10 cc conc. H₂SO₄ was added. The resulting solution was then diluted to 1000 cc and mixed thoroughly. The solutions were standardized by the following methods.

The extractant Cyanex 272 having 85% purity was used without further purification. All other chemicals were of reagent grade and used without further purification. The diluent, kerosene, was purchased from the local market and distilled to collect the colourless fraction obtained in the range of 200-260 °C.

A stock solution of Cyanex 272 (1 M) was prepared by weighing out exactly 290 g of Cyanex 272 in a 1 litre volumetric flask and diluting with distilled kerosene. Extractant solutions of different concentrations were prepared by proper dilution of this stock solution by distilled kerosene.

A definite aliquot of an aqueous phase 20 ml was taken in a 125 ml reagent bottle and to it the same aliquot of organic phase, 20 ml, was added. The bottle was stopped and shaken vigorously for a definite time (40 min) period in a thermostatic water bath at 30 ± 1°C (otherwise stated in the temperature dependence study). After attainment of equilibrium, the phases were allowed to settle and were disengaged. The aqueous phase was used to measure the metal ion content. The equilibrium organic phase metal ion concentrations were estimated by the method of difference (the organic phase Ti(IV)/Fe(III) concentration was determined by the formula: aqueous phase initial concentration – aqueous phase concentration of Ti(IV)/Fe(III) after extraction). The distribution ratio (D) of a metal ion was calculated as the ratio of metal ion concentration in the organic phase to that in the aqueous phase at equilibrium. In the case of loading test, the organic phase was repeatedly contacted with fresh equal volume of aqueous solutions until the saturation of organic phase with the metal was attained. Metal contents [Ti(IV) and Fe(III)] in the aqueous phase were estimated by the hydrogen peroxide colorimetric method at 410 nm for Ti(IV) (Vogel, 1989a) and the thiocyanate method at 480 nm for Fe(III) (Vogel, 1989b) using spectrophotometer ANA-75 (Tokyo Photoelectric Company, Japan).

Results and Discussion

Effect of phase contact time on the extraction of Ti(IV) and Fe(III). Figure 1 represents contact time in minutes and the distribution ratio. It is evident from the figure that extraction ratio of Ti(IV) and Fe(III) increases continuously with the contact time. Near the equilibrium point the increase of extraction ratio with respect to time is much slower. No appreciable extraction of Ti(IV) and Fe(III) took place after 35 and 18 min, respectively. Therefore, it is concluded that the equilibrium is reached at 40 min, for Ti(IV) and at 20 mins for Fe(III) and there is no further extraction of metal ions. Previously, the phase contact time of Ti(IV) extraction was reported to be 180 min(Biswas and Begum, 1998) by D2EHPA in kerosene system. The equilibrium time for Fe(III) extraction was reported...
to be 210 min (Islam et al., 1979) by D2EHPA in benzene system. Therefore the equilibrium attainment was obtained much faster with Cyanex 272 in kerosene system than with D2EHPA for Ti(IV) and Fe(III) extraction. This may be advantageous as less time is required for the solvent extraction operation. Thus, in subsequent experiments, 40 min mixing have been used to ensure equilibrium for both the aqueous [Ti(IV) and Fe(III)] mixture.

**Effect of extraction of Ti(IV) and Fe(III) from Ti(IV)-Fe(III) mixture on aqueous phase acidity and extractant concentration.** The extraction data are plotted as log D vs. log [H$_2$SO$_4$], M in Figs. 2 and 3. These figures represent the variation of distribution ratio on its aqueous phase acidity at different extractant concentrations. The plot shows that the extraction decreases with increasing aqueous phase acidity both for Ti(IV) and Fe(III) extraction within the range of 0.25 to 0.90 M H$_2$SO$_4$. The extraction of Ti(IV) is higher than that of Fe(III). About 95% Ti(IV) is extracted at 0.25 M H$_2$SO$_4$ with 0.20 M Cyanex 272 and about 24% of Fe(III) is extracted at the same condition indicating that comparatively larger separation of the metal ions Ti(IV) and Fe(III) is possible at higher acidity. The extraction of Ti(IV) and Fe(III) is higher with 0.20 M Cyanex 272 than with 0.05 M Cyanex 272 extractant. At 0.90 M H$_2$SO$_4$ concentration, the extraction of Fe(III) is very low (about 1.82 %) and almost similar with different extractant concentrations (0.05, 0.10, 0.15 and 0.20 M Cyanex 272), and for 0.60 M H$_2$SO$_4$ concentration, the extraction of Fe(III) is also similar to the extractant concentrations of 0.10, 0.15 and 0.20 M Cyanex 272.

At low aqueous acidity, the extractant molecules ionize in the aqueous phase boundary or its ionizing tendency becomes predominant forming a neutral extractable species with the metal ions. In a highly acidic solution, the above tendency is suppressed due to common ion effect and metal ion extraction is decreased. At a very high acidic solution, ionization of the extractant does not occur at all and it behaves like a neutral phosphate ester. In such a case, if any extraction occurs, the solvation by the extractant becomes the principal governing factor. The distribution ratio decreases with the increase of hydrogen ion concentration in the aqueous phase (Peppard et al., 1962). It is observed that the distribution ratio of both Ti(IV) and Fe(III) increases with the increase of extractant concentration. In the case of Ti(IV) extraction, the slopes of acid dependence curves are 1.24, 1.58, 1.50 and 1.57 with the extractant concentrations of 0.05, 0.10, 0.15 and 0.20 M Cyanex 272; respectively; in the case of Fe(III) extraction, the acid dependence slopes are 1.29, 1.72, 1.94 and 2.15 with the extractant concentration of 0.05, 0.10, 0.15 and 0.20 M Cyanex 272, respectively. These values are much lower than the theoretical value of 3 as discussed below. Thus, it is concluded that simple ion exchange is not the principal extraction mechanism.

**Fig. 2.** Effect of extraction of Ti(IV) from Ti(IV) and Fe(III) mixture on aqueous phase acidity. [Ti(IV)]$_{ini}$ = 1.1 g/dm$^3$; [Fe(III)]$_{ini}$ = 1.1 g/dm$^3$; Temperature = (30±1)$^\circ$C; contact time = 40 min; phase ratio (A/O) = 1:1; □: 0.05 M HA; slope = 1.24; ■: 0.10 M HA; slope = 1.50; ○: 0.15 M HA; slope = 1.50; ◆: 0.20 M HA; slope = 1.57

**Fig. 3.** Effect of extraction of Fe(III) from Ti(IV) and Fe(III) mixture on aqueous phase acidity. [Ti(IV)]$_{ini}$ = 1.1 g/dm$^3$; [Fe(III)]$_{ini}$ = 1.1 g/dm$^3$; Temperature = (30±1)$^\circ$C; contact time = 40 min; phase ratio (A/O) = 1:1; □: 0.05 M HA, slope = 1.29; △: 0.10 M HA, slope = 1.72; ○: 0.15 M HA, slope = 1.94; ◆: 0.20 M HA, slope = 2.15

Fig. 4 and 5 represent the variation of distribution ratios of Ti(IV) and Fe(III) on extractant concentration using the same data. It is seen that the extraction of Ti(IV) increases linearly with the increase of extractant concentration. The slopes of the lines are 1.50, 1.31, 1.30 and 1.20 for the aqueous phase acidities of 0.25, 0.46, 0.60 and 0.90 M H$_2$SO$_4$, respectively. In the case of Fe(III), at low acidities of 0.25, 0.46 and 0.60 M
H₂SO₄, the extraction of Fe(III) increases linearly with the increase of extractant concentration. The slopes of the lines are 0.73, 0.42 and 0.15 for the aqueous phase acidities of 0.25, 0.46 and 0.60 M H₂SO₄, respectively. On the contrary, at 0.90 M H₂SO₄, the extraction of Fe(III) is constant with different extractant concentrations and distribution ratio is very low or constant for 0.10, 0.15 and 0.20 M Cyanex 272 concentrations. In other words, the extraction does not increase with the extractant concentration but with 0.05 M Cyanex 272 is much lower than with extractant concentration of 0.20 M Cyanex 272.

In the case of Ti(IV), the extraction reaction is represented as follows by assuming the presence of TiO₂ in solution.

\[
\text{TiO}_2 + 2\text{HA} = \text{TiO}_2\text{A}_2 + 2\text{H}^+ \quad (1)
\]

This extraction reaction suggested that the extractant dependence and inverse hydrogen ion dependence is 2. However, the failure to confirm the extraction equation as suggested for the above reaction points to the fact that other phenomena such as the existence of polymerized TiO₂ ion may be responsible for such discrepancy. Low hydrogen ion dependency suggests that solvation mechanism may be operative at high acidity.

The extraction of Fe(III) by Cyanex 272 is also noteworthy. The slope for extractant dependence decreases with increasing acidity. The maximum slope obtained in the acidity range studied is 0.72, which is much less than the theoretical value as given by the general equation:

\[
\text{Fe}^{3+} + 3(\text{HA})_{\text{org}} = \text{FeA}_3(\text{HA})_{\text{org}} + 3\text{H}^+ 
\]

\[
\text{Fe}^{3+} + 6(\text{HA})_{\text{org}} = \text{FeA}_6(\text{HA})_{\text{org}} + 3\text{H}^+ 
\]

(\text{HA} = \text{Monomer of Cyanex 272})

Similar observation was made by Miralles et al. (1992), wherein extractant dependence of 3 and inverse hydrogen ion dependence of 3 is suggested. Fe(III) does not follow the simple extraction mechanism for all the acid ranges. It is likely that solvation mechanism may be operative at high acidity of the aqueous phase.

**Effect of extraction of Ti(IV) and Fe(III) from [Ti(IV) and Fe(III)] mixture on temperature.** Figures 6 and 7 represent log \( D \) vs. inverse of absolute temperature, showing the variation of distribution ratio on temperature. It is seen that the extraction of Ti(IV) increases linearly with the increase of temperature at all acidities. On the contrary, the extraction of Fe(III) at different acidities show different extraction behaviour. At acidity 0.46 M H₂SO₄, the extraction rate increased with the increase of temperature up to 40 °C and then decreased with the increase of temperature. At acidities 0.60 and 0.90 M H₂SO₄ concentration, the extraction of Fe(III) increased with the increase of temperature and at 50 °C, it was maximum for these two acidities and then the extraction decreased. The extraction became equal at 60 °C for all the three acidities. The lower extraction above 50 °C at acidities 0.60 and 0.90 M H₂SO₄ may be explained on the basis of the formation of unextractable hydrolyzed Fe(III) species with the increase of temperature. The slope of the lines has been calculated by Van't Hoff equation (Islam et al., 1988) giving the average enthalpy change (\( \Delta H \)) as 5.34 kcal mol for Ti(IV) and 20.14 kcal/mol for Fe(III) for the portion at which the distribution ratio increases with the increase of temperature. The extrac-
tion of Ti(IV) is endothermic up to 60 °C and Fe(III) extraction is also endothermic up to 50 °C by 0.10 M Cyanex 272. Thus extraction of Ti(IV) and Fe(III) by 0.10 Cyanex 272 in kerosene system is strongly influenced by temperature.

**Loading capacity of Cyanex 272 for Ti(IV) and Fe(III).** The loading capacity is defined as the amount of metal content in grams extracted per 100 g of pure extractant. It is an important factor for the study of mechanism of extraction and also for the industrial evaluation of the extractant. High values of loading capacity are desirable for any particular extractant metal system for industrial applications. Loading capacity is influenced by a number of factors including aqueous phase acidity, diluents, temperature, ions present in the aqueous phase and nature of the extractant etc.

The organic phase (20 ml) was repeatedly contacted for 40 min at 30 ± 1°C with fresh equal volumes of aqueous solution containing fixed concentration of metal ions [3.50 g/dm³ Ti(IV) and 2.0 g/dm³ Fe(III)] mixture with 0.32 M H₂SO₄. After equilibration, the phases were disengaged and the aqueous phases were analysed for Ti(IV) and Fe(III) contents. The amount of metal ions [Ti(IV) and Fe(III)] transferred into the organic phase for each contact was then determined by subtracting the determined aqueous concentration after extraction from the initial aqueous concentration, and the cumulative concentration of Ti(IV) and Fe(III) in the organic phase after each stage of contact was estimated.

The plot of cumulative [Ti(IV)/Fe(III)] g/dm³ vs. contact number is given in Fig. 8; it is seen that the organic phase is saturated with Ti(IV) after the 9th contact and with Fe(III) after the 13th contact for the stated experimental condition [3.50 g/dm³ Ti(IV), 2.00 g/dm³, Fe(III), 0.32 M H₂SO₄ and 0.40 M Cyanex 272]. The loading capacity for Ti(IV) is 18 g/litre, for Fe(III) 3.67 g/litre at 0.32 M H₂SO₄ and 0.40 M Cyanex 272 concentration; 7.68 g/litre for Ti(IV) and 3.64 g/litre for Fe(III) at 0.32 M H₂SO₄ and 0.20 M Cyanex 272 concentration. It is observed that the maximum loading of Cyanex 272 for Fe(III) is almost same for both 0.40 and 0.20 M Cyanex 272. Therefore, 0.20 M Cyanex 272 is satisfactory for Fe(III) loading of Cyanex 272.

Loading data indicates Ti(IV) : Cyanex 272 ratio of 1:1.25 and
Fe(III): Cyanex 272 ratio of 1:2.86 at 0.20 M Cyanex 272, indicating almost 1:1 metal complexes in the organic phase saturation for Ti(IV) and 1:3 complex for Fe(III) if monomeric species for extractant is assumed. However, this is not supported by acidity dependence slopes.

In the case of Fe(III), the loading test ratio is nearly equal to the theoretical value and at 0.40 M Cyanex 272, the loading test ratio is 1:5.72, which shows that less Fe(III) is extracted than the theoretical value at higher extractant concentration; it indicates the difference of extraction mechanism at different extractant concentrations. It may be concluded that solvation may be responsible for this discrepancy for extra molecules of Cyanex 272 may be solvated to the central Fe(III) ion.

**Seperations study. Effect of aqueous phase acidity and extractant concentration on separation factor.** The separation factor ($\beta$) for [Ti(IV) and Fe(III)] system has been calculated for each extractant concentration of 0.05, 0.10, 0.15 and 0.20 M Cyanex 272. It is seen from the plots (Fig.9) that the separation factor increases with the increase of aqueous phase acidities at all concentrations of Cyanex 272. As the acidity increases, the separation factor differs for different extractant concentration in the following sequences: 0.20 M > 0.15 M > 0.10 M > 0.05 M Cyanex 272. The maximum separation factor is noted for extraction with 0.20 M Cyanex 272 from highly acidic (0.90 M H$_2$SO$_4$) Ti(IV)-Fe(III) mixed (1:1) solution. It is concluded that the maximum separation factor for [Ti(IV)-Fe(III)] system is achieved at high aqueous acidity with high extractant concentration (0.20 M Cyanex 272).

**Effect of temperature on separation factor.** The separation factor ($\beta$) at acidities 0.46, 0.60 and 0.90 M is graphically represented as a function of temperature (Fig.10). In all cases, the separation factor decreases with the increase of temperature, then it increased again with the increase of temperature. Maximum separation factor ($\beta \sim 116.50$) was obtained at higher aqueous acidity and higher temperature. The separation factor increases in the following order: 0.46 M > 0.60 M > 0.90 M H$_2$SO$_4$ at 0.10 M Cyanex 272. Thus the separation factor ($\beta$) is markedly temperature dependent. From the plots, it may be concluded that: (a) Ti(IV) can be separated from Fe(III) at all (0.46, 0.60 and 0.90 M H$_2$SO$_4$) acidities; (b) the maximum separation of Ti(IV) from Fe(III) is possible at 60°C with 0.46 M Cyanex 272 concentration.

**Fig. 9.** Effect of aqueous phase acidity on separation factor ($\beta$).

**Fig. 10.** Effect of temperature on separation factor ($\beta$).

**Conclusion**

Cyanex 272 is a very effective extractant for the extraction and separation of Ti(IV) and Fe(III) from an aqueous sulphate solution containing Ti(IV) and Fe(III). The equilibrium is reached within 40 min for Ti(IV) and 20 min for Fe(III). The loading capacity of Ti(IV) and Fe(III) are 18 g/litre and 3.65 g/litre, respectively, for 0.32 M H$_2$SO$_4$ concentration. Separation factor ($\beta$) of Ti(IV) with respect to Fe(III) is 116.50 at the experimental conditions [temperature = 30 ± 1°C, [HA] = 0.20 M Cyanex-272 in kerosene, phase ratio, A/O = 1:1; contact time = 40 min; □: 0.15 M HA; ○: 0.05 M HA; △: 0.20 M HA].

The extraction data suggests that Fe(III) is extracted in organic phase by the formation of the species Fe$_2$(HA)$_3$ and for Ti(IV) is TiO$_2$.A. The positive $\Delta H$ value...
suggested that the extraction process is endothermic and the average enthalpy change ($\Delta H$) values are 5.34 and 20.14 kcal/mol for Ti(IV) and Fe(III), respectively, for 0.10 M Cyanex 272. The temperature dependence data shows that Ti(IV)/Fe(III) separation is strongly influenced by the temperature of extraction.

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Synthesis and Characterization of Valero and Isovalero Hydroxamic Acids and their Complexes with Zn(II) And Al(III)

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Abstract. Valerohydroxamic acid (VAH) and isovalerohydroxamic acid (IVAH) were synthesized and characterized by m.p. and pKₐ determination, IR and ¹H NMR studies. The ligands were complexed with Zn²⁺ and Al³⁺ and the complexes were characterized by metal analysis, IR and conductance studies. Antimicrobial studies of all the compounds were carried out. The pKₐ of the ligands are 9.50 ± 0.01 (VAH) and 9.51 ± 0.01 (IVAH) at 25 °C and ionic strength is 0.1 mol/dm³, while their melting points are 77.8 °C and 76.8 °C, respectively. IR and ¹H NMR data are consistent with the proposed formula. The complexes are non-electrolytes in EtOH. Coordination mode (0,0) is consistent with the IR data of the complexes. The compounds exhibited no significant antimicrobial activity.

Keywords: hydroxamic acid, matrix metalloproteases, zinc, aluminum, valerohydroxamic acid

Introduction

Hydroxamic acids are compounds that have one or more CON(H)OH groups. These compounds play important biological role due to their metal complexing ability (Kurzak et al., 1992). They are also important due to their pharmacological, toxicological and pathological properties (Nielands, 1968). They are intimately associated with iron transport phenomena in microbes (Kehl, 1982) and are also able to chelate other metal ions. In fact, the only drug currently available for the treatment of aluminum overload is desferrioxamine B (a tris-hydroxamate) (Kaim and Schwederski, 1994).

The matrix metalloproteases (MMPs) are a class of zinc containing hydrolytic enzymes necessary for tissue remodeling and healing cascade (Levy et al., 1998). Malfunction in MMP activity can contribute to many diseased conditions (Holleran et al., 1997; Weckroth et al., 1996). Wounds possessing too much MMP activity may become ulcerated rather than heal properly (Chiu et al., 2005). Consequently, the inhibition of MMPs has received great attention. Prominent among the successful MMP inhibitors is the hydroxamate functional group as the zinc binding group (ZBG). The mode of coordination of hydroxamates to metal ions is also important. While most reports reveal coordination via the hydroxamate O atoms (Nwabueze, 1996; Brown and Roche, 1983; Brown et al., 1979), coordination via N atom is also known (Brown et al, 1982). Besides many reports available on metal complexes of some hydroxamic acids, a few reports are on those of VAH and IVAH (Porcheddu and Giacomelli, 2006; Katritzky et al., 2003).

This work reports the synthesis of VAH and IVAH, their characterization and complexation with Zn²⁺. An understanding of their mode of coordination will give more insight into the interaction of hydroxamates with Zn²⁺ and Al³⁺ in biological systems.

Materials and Methods

Preparation of hydroxamic acids. Hydroxamic acids were prepared by the adaptation of the method described in the literature (Nwabueze, 1996; Brown and Roche, 1983).

Valerohydroxamic acid (VAH): Sodium metal 11.5 g (0.5 mol) was dissolved in 250 ml of distilled MeOH and added to a solution of hydroxylamine hydrochloride 34.5 g (0.5 mol) in 250 ml of MeOH. The mixture was cooled to room temperature, 75 ml (65.6 g; 0.5 mol) of ethylvalerate was added and stirred for one h. A solution of 11.5 g (0.5 mol) of sodium metal in 250 ml MeOH was further added and stirred for 20 min. The mixture was filtered to remove the precipitated NaCl and the filtrate was acidified with conc. HCl. The precipitated NaCl was removed by filtration and the filtrate was left in a deep freezer for two weeks. The resulting crystals were filtered and recrystalized in ethyl acetate (yield 72%).

 Isovalerohydroxamic acid (IVAH) was prepared similarly using 75 ml (65.6 g, 0.5 mol) of ethyl isovalerate (yield 68%).

Preparation of complexes. Zn (II) Complexes: A solution of ZnCl₂, 1.36 g (0.01 mol) in 25 ml of ethanol was added to a solution of valerohydroxamic acid 2.34 g (0.02 mol) in 10 ml
of ethanol. The mixture was well stirred and the pH was raised to 6 by the addition of sodium ethoxide. The mixture was filtered, the filtrate was concentrated and left in a deep freezer for 48 hs. Cream coloured crystals were obtained which were filtered and dried over CaCl₂ in a vacuum desiccator (yield 53%). The same procedure was used for the preparation of Zn-IVAH complex.

**Al (III) complexes:** A solution of AlCl₃·6H₂O 2.41 g (0.01 mol) in 20 ml of ethanol was added to a solution of valerohydroxamic acid 3.51 g (0.03 mol) in 10 ml of ethanol. The mixture was stirred and the pH was adjusted to about 6 using sodium ethoxide. The mixture was filtered and the filtrate was left in a deep freezer for 48 hs to crystallize. The crystals were filtered and dried over CaCl₂ in vacuum desiccator (yield 47%). Al-IVAH complex was similarly prepared.

**Results and Discussion**

The ligands were prepared as reported in the literature (Nwabueze, 1996). The ligands reacted with Zn²⁺ and Al³⁺ according to the equation:

\[ M^{n+} + nRCON(H)OH \rightarrow [MRCO(NH)O]_n \times H_2O \]

where \( n = 2 \) or 3 and \( \times = 1 \) or 2

Crystallization of the ligands was achieved after concentration under laboratory conditions for 2 weeks and afterwards refrigeration in deep freezer for further two weeks. The ligands were recrystallized in 10 cc of dry ethanol after filtration. Their relatively low melting points (Table 1) are an indication of weak intermolecular force between the molecules. Literature search confirms that these ligands are being reported for the first time. Both ligands show similar basicity indicated by their pKₐ of 9.50 ± 0.01 and 9.51 ± 0.01 for VAH and IVAH, respectively, at 25 °C and ionic strength of 0.10 mol/dm³. These values imply that they can act as good Lewis bases.

**Spectrophotometric determination of the pKₐ of the ligands.** The pKₐ values for the ligands were determined spectrophotometrically by the method of Albert and Serjeant (1971) using phosphoric acid and tris (hydroxymethyl) methyl amine buffer. In each case, the ligand stock solution was 5.0x10⁻⁴ M, which was diluted five fold in the buffer solution. The optical densities of the solutions were measured at the analytical wave length of 210 nm. Seven buffer solutions were used for each ligand.

**Antimicrobial studies.** Antimicrobial screening of the ligands and complexes in aqueous EtOH was carried out using nutrient agar. Petri dishes were inoculated with microorganisms viz - *Staphylococcus aureus* (Sa), *Bacillus subtilis* (Bs), *Eschericia coli* (Ec), *Pseudomonas aeruginosa* (Ps) and *Candida albicans* (Ca). Petri dishes were then impregnated with the discs containing solutions of the ligands and the complexes and incubated for 24 hs at 37 °C and tests were carried out in duplicate.

**Table 1. Analytical data and some physical constants**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Formula</th>
<th>Yield wt</th>
<th>M.P. (%)</th>
<th>M (°C)</th>
<th>Ω/cm²/mol (%)</th>
<th>H</th>
<th>C</th>
<th>N</th>
<th>Colour</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAH</td>
<td>C₅H₁₁NO₂</td>
<td>117</td>
<td>58</td>
<td>77.78</td>
<td></td>
<td>0.855</td>
<td>(0.855)</td>
<td></td>
<td></td>
<td>White</td>
<td>MeOH,CHCl₃H₂O</td>
</tr>
<tr>
<td>IVAH</td>
<td>C₅H₁₁NO₂</td>
<td>117</td>
<td>62</td>
<td>76.77</td>
<td></td>
<td>0.856</td>
<td>(0.855)</td>
<td></td>
<td></td>
<td>White</td>
<td>MeOH,CHCl₃H₂O</td>
</tr>
<tr>
<td>Al(IVAH)₂H₂O</td>
<td>C₁₅H₃₄N₃O₈Al</td>
<td>411</td>
<td>32.50</td>
<td>194</td>
<td>6.61 (6.57)</td>
<td>0.051</td>
<td>0.245</td>
<td>(0.243)</td>
<td>2.93</td>
<td>3.42</td>
<td>Crystal</td>
</tr>
<tr>
<td>Zn(IVAH)₂H₂O</td>
<td>C₁₅H₃₄N₃O₈Zn</td>
<td>315</td>
<td>53.20</td>
<td>145</td>
<td>19.48 (19.52)</td>
<td>0.0125</td>
<td>0.322</td>
<td>(0.317)</td>
<td>3.84</td>
<td>4.46</td>
<td>Creamy</td>
</tr>
<tr>
<td>Zn(IVAH)₂H₂O</td>
<td>C₁₅H₃₄N₃O₈Zn</td>
<td>315</td>
<td>61.88</td>
<td>165</td>
<td>19.43 (19.52)</td>
<td>0.0150</td>
<td>0.320</td>
<td>(0.310)</td>
<td>3.86</td>
<td>4.45</td>
<td>Creamy</td>
</tr>
</tbody>
</table>
In the spectrum of VAH, the signal due to the methyl protons appear as a triplet centered at $\delta_{0.8}$. A sextet centered at $\delta_{1.3}$ is due to the -CH$_2$ protons adjacent to the methyl group while a quintet at $\delta_{1.5}$ is due to the methylene group next to it. A triplet at $\delta_{2.5}$ is due to the -CH$_2$ group adjacent to the carbonyl group. The appearance of this signal down field is due to anisotropy of the neighbouring carbonyl group. The mass spectrum of VAH and IVAH recorded at 70 eV electron energy shows a well defined peak at m/z=116 (M$^+$) with relative intensities of 98% and 95%, respectively. This data is consistent with the formula of VAH i.e. CH$_3$CH$_2$CH$_2$CON(H)OH.

In the spectrum of IVAH, there are two chemically equivalent -CH$_3$ groups whose signals appear as a doublet centered at $\delta_{0.9}$. A doublet at $\delta_{2.1}$ is due to the methylene protons, while a nanonet centered at $\delta_{2.3}$ is due to the C-H proton. The spectrum is consistent with the structure (Katritzky, 2003).

### Table 2. $^1$H NMR data for the ligands in CDCl$_3$ ($\delta$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>NH</th>
<th>CH</th>
<th>CH$_2^a$</th>
<th>CH$_3^b$</th>
<th>CH$_2^c$</th>
<th>CH$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAH</td>
<td>7.2 (si,1H)</td>
<td>2.5 (t,J6.5Hz2H)</td>
<td>1.5 (q,J6Hz2H)</td>
<td>1.3 (s,J4Hz2H)</td>
<td>0.8 (t,J6Hz3H)</td>
<td></td>
</tr>
<tr>
<td>IVAH</td>
<td>7.6 (s,1H)</td>
<td>2.3 (n,J6Hz, 1H)</td>
<td>2.1 (d,J6.8Hz, 2H)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$a$ = adjacent to carbonyl and b and c, to alkyl chain; s = singlet; d = doublet; t = triplet; q = quintet; si = sextet; n = nanonet

### Infrared

The diagnostic IR bands for the ligands and complexes are shown in Table 3. All the bands and nature of the spectra are consistent with the common features reported for hydroxamic acids (Nwabueze, 1996; Brown and Roche, 1983; Brown et al., 1982). The $\nu$(C=O) band, which is located in the spectra of the ligands at ca 1650 cm$^{-1}$, is lower in the spectra of the complexes by between 24-41 cm$^{-1}$. This observation together with an increase in the $\nu$(C-N) band from about 1365 cm$^{-1}$ in the ligands to above 1380 cm$^{-1}$ in the complexes is consistent with the coordination via the carbonyl oxygen (Mandlik and Aswar, 2003; Nwabueze, 1997).

Bands located around 3200 cm$^{-1}$ in the ligands are assigned to $\nu$(N-H) vibrations. Bands located above 3400 cm$^{-1}$ in the complexes are assigned to $\nu$(OH) of water and $\nu$(N-H) of the ligands. It is, therefore, difficult to assign these bands unambiguously and establish the involvement of amino N in bonding. Non-ligand bands below 600 cm$^{-1}$ have been assigned to M-N and M-O vibrations (Mandla and Aswar, 2003).

### Table 3. IR diagnostic bands (cm$^{-1}$) for the ligands and complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\nu$(OH,NH)</th>
<th>$\nu$(C=O)</th>
<th>$\Delta\nu$(C=O)</th>
<th>$\nu$(C-N)</th>
<th>$\Delta\nu$(C-N)</th>
<th>$\nu$(M-O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAH</td>
<td>3300, 3187</td>
<td>1650</td>
<td>-24</td>
<td>1365</td>
<td>+17</td>
<td>440</td>
</tr>
<tr>
<td>Al(VA)$_2$H$_2$O</td>
<td>3420</td>
<td>1626</td>
<td>-37</td>
<td>1390</td>
<td>+25</td>
<td>462</td>
</tr>
<tr>
<td>Zn(VA)$_2$H$_2$O</td>
<td>3430</td>
<td>1613</td>
<td>-41</td>
<td>1385</td>
<td>+15</td>
<td>409</td>
</tr>
<tr>
<td>IVAH</td>
<td>3198</td>
<td>1650</td>
<td>-28</td>
<td>1385</td>
<td>+15</td>
<td>440</td>
</tr>
<tr>
<td>Al(VA)$_2$H$_2$O</td>
<td>3420</td>
<td>1609</td>
<td>-41</td>
<td>1385</td>
<td>+15</td>
<td>440</td>
</tr>
<tr>
<td>Zn(IVA)$_2$H$_2$O</td>
<td>3420</td>
<td>1612</td>
<td>-28</td>
<td>1385</td>
<td>+15</td>
<td>440</td>
</tr>
</tbody>
</table>

$+$ = mild activity; $++$ = partial activity; $-$ = no activity; Sa = Staphylococcus aureus; Ec = Echericia coli; Ps = Pseudomonas aeruginosa; Bs = Bacillus subtilis; Ca = Candida albicans.

### Table 4. Microbial screening of the compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sa</th>
<th>Ec</th>
<th>Ps</th>
<th>Bs</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAH</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Al(VA)$_2$H$_2$O</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Zn(VA)$_2$H$_2$O</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IVAH</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Al(VA)$_2$H$_2$O</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Zn(IVA)$_2$H$_2$O</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

$+$ = mild activity; $++$ = partial activity; $-$ = no activity; Sa = Staphylococcus aureus; Ec = Echericia coli; Ps = Pseudomonas aeruginosa; Bs = Bacillus subtilis; Ca = Candida albicans.
Biological activity. The degree of antibacterial activity exhibited by the ligands and the complexes is shown in Table 4. Mild activities of the ligands were enhanced on complexation as revealed by the partial activity for isovalerohydroxamates against *Bacillus subtilis* and *Eschericia coli*, organisms. While in the case of the valerohydroxamates, there is no noticeable difference from that exhibited by the ligand against the tested organisms.

Conclusion
The IR spectra of the complexes are consistent with the observed trend in the reported cases of hydroxamates. Non-activity of the compounds is an indication of their lack of usefulness as antimicrobial agents.

References
Synthesis and Anti-inflammatory Activity of 4-Substituted-2,5-Disubstituted Indolyl Azetidine-3-yl/Thiazolidin-1-yl-Substituted Triazoles

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Introduction
Non steroidal anti-inflammatory drugs (NSAIDS) are a heterogeneous family of pharmacologically active compounds used in the treatment of acute and chronic inflammation, pain and fever. Heterocyclics, bearing a symmetrical 1,2,4-triazole moiety, are reported to possess a broad spectrum of pharmacological properties such as anti-inflammatory (Braccio et al., 2008; Metwally et al., 2008), analgesic (Goksen et al., 2007), antimicrobial (Kavegoudae et al., 2008) and anticonvulsant (Srivastava et al., 2002). A survey of literature revealed that 1,2,4-triazole has received much attention during recent years on account of their prominent utilization as antifungal (Reddy et al., 2008), analgesic (Mohd et al., 2007) and anti-inflammatory agents (Dunder et al., 2007). Substitution at third and fourth position of 1,2,4-triazole heterocyclic ring by aromatic/heterocyclic moieties plays a pivotal role in modulating the anti-inflammatory activity. Moreover, the substitution of indolyl/azetidinoyl/thiazolidinoyl moieties at different heterocyclic nuclei remarkably change the anti-inflammatory activity. Hence, synthesis of some new derivatives of 1,2,4-triazole was undertaken by incorporating indolyl/azetidinoyl/thiazolidinoyl moieties in a singular frame in the hope of finding better anti-inflammatory agents.

Chemistry
The target 1,2,4-triazole derivatives were synthesized according to Scheme 1. The reaction of substituted acid hydrazides with hydrazine hydrate in ethanol afforded the corresponding substituted potassium di-thiocarbazinates in high yields (85-90%). The di-thiocarbazinates were converted to 1-amino-5-mercapto-1,2,4-triazole (3a-d) using hydrazine hydrate in water (63-70%). 4-((Amino methylene)-(2’-substituted phenyl)-5’-methoxy indol-3’-yl)-3-(substituted phenyl)-5-mercapto-1,2,4-triazole (4a-h) were prepared by the reaction of substituted 1,2,4-triazoles with 2-substituted phenyl-5-methoxy indol-3-aldehydes in absolute ethanol (50-60%). To reaction mixture of compounds (4a-h) in dry benzene and chloroacetyl chloride triethylamine was added and 4-((5’-methoxy-2’-substituted phenylindole-3’-yl)-(3’-chioro-2’-oxoazetidin-1’-yl))-3-substituted phenyl-5-mercapto-1,2,4-triazoles (5a-h) (41-48%) was obtained. 4-(5’-methoxy-2’-substituted phenyl indole-3’-yl)-(2’-oxothiazolin-1’-yl)-3-substituted phenyl-5-mercapto-1,2,4-triazoles (6a-h) were synthesized by the mixture of compounds 4a-h and thioglycolic acid in the presence of a pinch of anhydrous zinc chloride in methanol (35-45%). The purities of all synthesized compounds were determined by thin layer chromatography using several solvent systems of different polarity.

Materials and Methods
All reagents and solvents were generally used as received from the commercial supplier. Reactions were routinely performed in oven dried glassware. Melting points were determined with an electro-thermal melting point apparatus and are uncorrected. The homogeneity of all newly synthesized compounds was checked by thin layer chromatography (TLC) on silica gel G coated plates. The eluent was a mixture of different solvents in different proportions and spots were visualized under iodine chamber.

General procedure for the preparation of substituted acid hydrazides (1). The ester of substituted acids (0.1 mol) was dissolved in ethanol (10 ml) and hydrazine hydrate (0.1 mol)
was added drop wise to the solution with stirring. The resulting mixture was allowed to reflux for 6 hs; the excess ethanol was distilled off and the contents were allowed to cool. The crystals formed were filtered, washed thoroughly with water and dried. Progress of the reaction was monitored on TLC using silica gel G coated plates while ethyl acetate and petroleum ether (1:1) were used as eluent. The plates were observed in UV light and substituted acid hydrazides were obtained.

**General procedure for the preparation of substituted acid potassium dithio-carbazinates (2).** Potassium hydroxide (0.15 mol) was dissolved in absolute ethanol (200 ml). To the
above solution, substituted acid hydrazides (0.2 mol) were added. The solution was cooled in ice and carbon disulfide (0.15 mol) was added to it in small quantities with constant stirring. The reaction mixture was stirred continuously for a period of 15 hs. It was then diluted with anhydrous ether. The precipitated potassium dithiocarbazinate was collected by filtration. The precipitate was further washed with anhydrous ether (100 ml) and dried under vacuum. The potassium salt thus obtained was in quantitative yield and was used in the next step without further purification. Other substituted compounds (2a-2h) were prepared similarly.

General procedure for the preparation of 3-substituted-4-amino-5-mercapto-1,2,4-triazole (3a-h). Suspensions of potassium dithiocarbazinates of respective aromatic esters (0.1 mol) in water (5 ml) (0.3 mol) were refluxed for 6-7 hs with occasional shaking. The colour of the reaction mixtures changed to green with the evolution of hydrogenous reaction mixtures which were obtained during the reaction process. The reaction mixtures were cooled to room temperature and diluted with concentrated hydrochloric acid. The triazoles formed were precipitated, filtered, washed thoroughly with cold water and recrystallized from ethanol. Progress of the reaction was monitored on TLC by using silica gel G coated plates while ethyl acetate and petroleum ether (1:1) were used as the eluent. The plates were observed in UV light.

4-Amino-3-(2-chlorophenyl)-5-mercapto-1,2,4-triazole (3c). Yield: 67%; mp: 238-239 °C; IR (KBr): ν (cm⁻¹) 3215 (NH stretching), 2970, 2810 (methyl C-H str), 1608(C=N), 1540 (C=C aromatic ring), 1280(N-N); 1H NMR: δ (ppm) 2.98 (s, 6H, N(CH₃)₂), 3.40 (s, 3H, OCH₃), 6.60 (s, 1H, CH=N-CH=N), 6.90-7.90 (m, 11H, Ar-H), 9.10 (s, 1H, NH, indolic exchangeable), 10.96 (s, 1H, phenolic); 13C NMR(CDCl₃) δ:56.1, 116.2, 116.8, 121.5, 129.7, 131.8, 148.4, 157.5, 167.1. Mass, M⁺ at m/z 250 Anal. Calcd. for C₁₇H₁₉N₅SO: C, 45.70; H, 3.87; N, 26.76. Found: C, 45.82; H, 3.72; N, 26.76.

4-Amino-3-(4-N,N-dimethylaminophenyl)-5-mercapto-1,2,4-triazole (3d). Yield: 58%; mp: 170 °C; IR (KBr): ν (cm⁻¹) 3215 (NH stretching), 3132(acromatic C-H stretching), 3350(NH stretching), 2580 (S-H), 1618 (C=N, 1560 C=C stretching), 1280 (N=N stretching); 1H NMR: δ (ppm) 2.93 (s, 3H, OCH₃), 3.40 (s, 3H, 3H), 6.72 (s, 1H, CH=NC-NC=N), 6.80-7.90 (m, 11H, Ar-H), 9.10 (s, 1H, NH; indolic exchangeable), 10.96 (s, 1H, phenolic); 13C NMR(CDCl₃) δ:55.8, 102.2, 104.5, 112, 112.4, 118.5, 123.7, 123.9, 129.9, 131.1, 134.3, 148.2, 148.5, 154, 156.5. Mass, M⁺ at m/z 245 Anal. Calcd. for C₂₄H₂₃N₆SOCl: C, 60.56; H, 3.81; N, 14.71. Found: C, 60.62; H, 3.76; N, 14.76.
stretched); $^1$H NMR (ppm): 3.41 (s, 3H, OCH$_3$), 6.74 (s, 1H, CH=N-N), 6.85-7.95 (m, 11H, Ar-H), 9.13 (s, 1H, NH indolic exchangeable), 13.52 (s, 1H, SH). $^{13}$C NMR (CDCl$_3$) $\delta$: 55.8, 102.6, 104.6, 112.6, 112.9, 116.7, 118.5, 119, 123.7, 123.9, 129.5, 131.1, 132, 138.8, 148, 148.5, 152.2, 156.6. Mass, M$^+$ at m/z 618. Anal. Caled. for C$_{24}$H$_{17}$N$_5$SOClBr: C, 56.33; H, 3.70; N, 15.14. Found: C, 56.26; H, 3.70; N, 13.16. 

4-(Aminomethylene)-2-(4"-bromophenyl)-5"-methoxy indol-3-yl)-3-(2-methoxyphenyl)-5-mercapto-1,2,4-triazole (4h). Yield: 53%; mp: 220 °C; IR (KBr): ν (cm$^{-1}$) 3345 (N-H stretching), 1712 (C=O), 1645 (N-C=O), 1610 (C=O), 1285 (N=N stretching); $^1$H NMR (ppm): 2.90 (s, 6H, N(CH$_3$)$_2$), 3.32 (s, 3H, OCH$_3$), 4.62 (s, 1H, CH=NN), 6.82, 7.805 (m, 11H, Ar-H), 9.13 (s, 1H, NH indolic exchangeable), 13.50 (s, 1H, SH). $^{13}$C NMR (CDCl$_3$) $\delta$: 102.6, 104.6, 112.8, 112.9, 116, 116.7, 118.5, 119, 123.7, 123.9, 129.3, 131.1, 132.1, 132.2, 138.5, 148.5, 154, 155, 156.9. Mass, M$^+$ at m/z 534. Anal. Caled. for C$_{23}$H$_{18}$N$_5$OClBr: C, 56.19; H, 3.77; N, 13.10. Found: C, 56.26; H, 3.70; N, 13.16.
Yield: 44%; mp: 248 °C; IR (KBr): ν\text{cm}^{-1} 3040, 2852 (methylene C-H str), 2586 (S-H), 1712 (C=O), 1652 (N-C=O), 3345 (N-H stretching), 3142 (aromatic C-H stretching), 3045, 2840 (methylene C-H str), 2570 (S-H), 1715 (C=O), 1615 (C=N), 1280 (N-N stretching); 1H NMR (CDCl₃) δ (ppm) 3.32 (s, 3H, OCH₃), 4.60 (s, 1H, COCHCl), 6.60 (s, 1H, CH=N-N), 6.82-7.85 (m, 11H, Ar-H), 9.05 (s, 1H, NH indolic exchangeable), 13.52 (s, 1H, SH). 13C NMR (CDCl₃) δ 55.5, 56.2, 104.2, 112.9, 116.9, 116.5, 123, 123.4, 127.3, 129.4, 129.7, 131.1, 132.4, 132.2, 138.8, 148.2, 154, 154.6. Mass, M+ at m/z 570. Anal. Calcld. for C₂₇H₂₁N₅SO₃Cl₂: C, 58.02; H, 4.17; N, 14.50. Found: C, 58.10; H, 4.13; N, 14.57.

**General procedure for the preparation of 4-((5'-methoxy-2'-4'-chlorophenylindole-3'-yl)-(3'-chloro-2'-oxoazetidin-1'-yl))-3-(2-methoxyphenyl)-5-mercapto-1,2,4-triazoles (6a-h).**

The mixtures of compounds 4(a-h) (0.01 mol) and thioglycolic acid (0.01 mol) in the presence of a pinch of anhydrous zinc chloride were refluxed and poured in ice cold water, the products were filtered and recrystallized from appropriate solvent.

4-((5'-methoxy-2'-4'-bromophenylindole-3'-yl)-(3'-chloro-2'-oxoazetidin-1'-yl))-3-(2-methoxyphenyl)-5-mercapto-1,2,4-triazoles (6a). Yield: 43%; mp: 215 °C; IR (KBr): ν\text{cm}^{-1} 3330 (N-H stretching), 3135 (aromatic C-H stretching), 3040, 2852 (methylene C-H str), 2586(S-H), 1712(C=O), 1652(N-C=O), 1615(C-N), 154, 156, 157.3, 157.2, 154.8, 154.9. Mass, M+ at m/z 615. Anal. Calcld. for C₂₇H₂₁N₅SO₃Cl₂ Br: C, 50.74; H, 2.95; N, 11.38. Found: C, 50.82; H, 2.90; N, 11.42.

4-((5'-methoxy-2'-4'-bromophenylindole-3'-yl)-(3'-chloro-2'-oxoazetidin-1'-yl))-3-(2-methoxyphenyl)-5-mercapto-1,2,4-triazoles (6b). Yield: 46%; mp: 233 °C; IR (KBr): ν\text{cm}^{-1} 3330 (N-H stretching), 3135 (aromatic C-H stretching), 3040, 2852 (methylene C-H str), 2586(S-H), 1712(C=O), 1652(N-C=O), 1615(C-N), 154, 156, 157.3, 157.2, 154.8, 154.9. Mass, M+ at m/z 624. Anal. Calcld. for C₂₇H₂₁N₅SO₃Cl₂ Br: C, 50.74; H, 2.95; N, 11.38. Found: C, 50.82; H, 2.90; N, 11.42.

4-((5'-methoxy-2'-4'-bromophenylindole-3'-yl)-(3'-chloro-2'-oxoazetidin-1'-yl))-3-(2-methoxyphenyl)-5-mercapto-1,2,4-triazoles (6c). Yield: 42%; mp: 240 °C; IR (KBr): ν\text{cm}^{-1} 3330 (N-H stretching), 3135 (aromatic C-H stretching), 3040, 2852 (methylene C-H str), 2586(S-H), 1712(C=O), 1652(N-C=O), 1615(C-N), 154, 156, 157.3, 157.2, 154.8, 154.9. Mass, M+ at m/z 624. Anal. Calcld. for C₂₇H₂₁N₅SO₃Cl₂ Br: C, 50.74; H, 2.95; N, 11.38. Found: C, 50.82; H, 2.90; N, 11.42.

4-((5'-methoxy-2'-4'-bromophenylindole-3'-yl)-(3'-chloro-2'-oxoazetidin-1'-yl))-3-(2-methoxyphenyl)-5-mercapto-1,2,4-triazoles (6d). Yield: 46%; mp: 233 °C; IR (KBr): ν\text{cm}^{-1} 3330 (N-H stretching), 3135 (aromatic C-H stretching), 3040, 2852 (methylene C-H str), 2586(S-H), 1712(C=O), 1652(N-C=O), 1615(C-N), 154, 156, 157.3, 157.2, 154.8, 154.9. Mass, M+ at m/z 624. Anal. Calcld. for C₂₇H₂₁N₅SO₃Cl₂ Br: C, 50.74; H, 2.95; N, 11.38. Found: C, 50.82; H, 2.90; N, 11.42.

4-((5'-methoxy-2'-4'-bromophenylindole-3'-yl)-(3'-chloro-2'-oxoazetidin-1'-yl))-3-(2-methoxyphenyl)-5-mercapto-1,2,4-triazoles (6e). Yield: 45%; mp: 215 °C; IR (KBr): ν\text{cm}^{-1} 3330 (N-H stretching), 3135 (aromatic C-H stretching), 3040, 2852 (methylene C-H str), 2586(S-H), 1712(C=O), 1652(N-C=O), 1615(C-N), 154, 156, 157.3, 157.2, 154.8, 154.9. Mass, M+ at m/z 624. Anal. Calcld. for C₂₇H₂₁N₅SO₃Cl₂ Br: C, 50.74; H, 2.95; N, 11.38. Found: C, 50.82; H, 2.90; N, 11.42.
1645(N-C=O), 1600(C=N), 1282(N-N), 770(C-S); 1H NMR: \(\delta(ppm)\) 3.25(s, 3H, OCH3), 4.05(s, 2H, CH2 of thiazolidenone ring), 6.65(s, 1H, CH=N-N), 6.75-7.70(m, 11H, Ar-H), 9.08(s, 1H, N-H of indole exchangeable), 11.24(s, 1H, OH exchangeable), 13.42(s, 1H, SH). 13C NMR (CDCl3) \(\delta\): 31.8, 5.8, 100.5, 112.4, 112.9, 121.5, 123.4, 128.5, 129.1, 130.1, 131.1, 134.2, 154.156, 167.9, 171. Mass, M' at m/z 550 Anal. Calcd. for C29H24N6S2O4Cl: C, 56.77; H, 3.66; N, 12.73. Found: C, 56.82; H, 3.60; N, 12.78.

4-(5′-methoxy-2′-(4″-bromophenylindole-3′-yl)-(2′-oxothiazolin-1′-yl)-3-(4,N-dimethylamino-phenyl)-5-mercapto-1,2,4-triazoles (6b). Yield: 46%; mp: 316-318 °C; IR (KBr): v(cm\(^{-1}\)) 3322(N-H stretching), 3130(aro. C-H stretching) 3035, 2840(methylene C-H str), 2567(s-H), 1690(C=O), 1642(N=C-O), 1602(C=O), 1281(N-S); 1H NMR: \(\delta(ppm)\) 2.72(s, 6H, N(CHO)); 3.22(s, 3H, OCH3), 4.00(s, 2H, CH of thiazolidene ring), 6.62(s, 1H, CH=N-N), 6.75-7.72(m, 11H, Ar-H), 9.06(s, 1H, N-H of indole exchangeable), 13.40(s, 1H, SH); 13C NMR (CDCl3) \(\delta\): 4.24, 44.1, 55, 100.2, 102.2, 104, 112, 112.4, 121.5, 123.6, 128.2, 129.2, 130.4, 134.5, 154.5, 156, 167.9, 171.8. Mass, M' at m/z 545. Anal. Calcd. for C29H26N6S2O4Cl: C, 61.67; H, 4.62; N, 15.42. Found: C, 61.64; H, 4.67; N, 15.54.

4-(5′-methoxy-2′-(4″-chlorophenylindole-3′-yl)-(2′-oxothiazolin-1′-yl)-3-(2-chlorophenyl-5-mercapto-1,2,4-triazoles (6b). Yield: 44%; mp: 320 °C; IR (KBr): v(cm\(^{-1}\)) 3324(N-H stretching), 3132(aro. C-H stretching) 3036, 2840(methylene C-H str), 2569(S-H), 1700(C=O), 1644(N=C-O), 1605(C=O), 1285(N-N), 765(C-S); 1H NMR: \(\delta(ppm)\) 3.25(s, 3H, OCH3), 4.08(s, 2H, CH of thiazolidene ring), 6.60(s, 1H, CH=N-N), 6.78-7.76(m, 11H, Ar-H), 9.10(s, 1H, NH exchangeable), 13.42(s, 1H, SH); 13C NMR (CDCl3) \(\delta\): 55.8, 100.6, 102.2, 104, 112, 112.4, 121.5, 123.6, 127.3, 128.2, 129.2, 130.4, 130.3, 134.5, 138, 154.5, 156, 167.4, 171.6. Mass, M' at m/z 568. Anal. Calcd. for C29H26N6S2O4Cl: C, 54.62; H, 3.37; N, 12.32. Found: C, 54.67; H, 3.31; N, 12.25.

4-(5′-methoxy-2′-(4″-bromophenylindole-3′-yl)-(2′-oxothiazolin-1′-yl)-3-(2-hydroxyphenyl)-5-mercapto-1,2,4-triazoles (6e). Yield: 44%; mp: 320 °C; IR (KBr): v(cm\(^{-1}\)) 3324(N-H stretching), 3132(aro. C-H stretching) 3036, 2840(methylene C-H str), 2567(S-H), 1690(C=O), 1642(N=C-O), 1602(C=O), 1281(N-S), 765(C-S); 1H NMR: \(\delta(ppm)\) 2.72(s, 6H, N(CHO)); 3.22(s, 3H, OCH3), 4.00(s, 2H, CH of thiazolidene ring), 6.62(s, 1H, CH=N-N), 6.75-7.72(m, 11H, Ar-H), 9.06(s, 1H, N-H of indole exchangeable), 13.40(s, 1H, SH); 13C NMR (CDCl3) \(\delta\): 6.318, 35.6, 40.2, 40.5, 50.4, 55.4, 100, 102.2, 104, 112, 112.4, 121.3, 123.4, 127.3, 128.2, 129.2, 130.2, 130.6, 131.8, 148, 153.2, 167.4, 171. Mass, M' at m/z 621 Anal. Calcd. for C29H26N6S2O4Br: C, 54.10; H, 4.05; N, 13.52. Found: C, 54.18; H, 4.07; N, 13.58.

4-(5′-methoxy-2′-(4″-bromophenylindole-3′-yl)-(2′-oxothiazolin-1′-yl)-3-(2-bromophenyl)-5-mercapto-1,2,4-triazoles (6g). Yield: 35%; mp: 304-306 °C; IR(KBr): v(cm\(^{-1}\)) 3324(N-H stretching), 3132(aro. C-H stretching) 3036, 2840(methylene C-H str), 2569(S-H), 1700(C=O), 1644(N=C-O), 1605(C=O), 1285(N-N), 765(C-S); 1H NMR: \(\delta(ppm)\) 3.25(s, 3H, OCH3), 4.08(s, 2H, CH of thiazolidene ring), 6.60(s, 1H, CH=N-N), 6.78-7.76(m, 11H, Ar-H), 9.10(s, 1H, NH of indole exchangeable), 13.42(s, 1H, SH); 13C NMR(CDCl3) \(\delta\): 35.8, 35.6, 50.8, 55.8, 100.7, 102.2, 104, 114.7, 118, 121.5, 123.4, 127.3, 128.2, 129.2, 130.2, 130.6, 131.8, 138, 148, 154.2, 167.4, 171. Mass, M' at m/z 612. Anal. Calcd. for C29H26N6S2O4Br: C, 50.94; H, 3.12; N, 11.42. Found: C, 50.82; H, 3.24; N, 11.48.
ring), 6.62(s, 1H, CH=N-N), 6.75-7.80(m, 11H, Ar-H), 9.00(s, 1H, N-H of indole exchangeable), 13.34(s, 1H, SH); 13C NMR (CDCl3) δ: 35.8, 50.8, 55.6, 100.5, 102.2, 104, 112, 112.4, 116.5, 116.6, 118, 121.5, 123.4, 125.4, 128.2, 130.2, 130.6, 131.8, 148, 154.2, 157.1, 167.4, 171. Mass, M+ at m/z 608. Anal. Calcd. for C27H22N5S2O3Br: C, 53.29; H, 3.64; N, 11.50. Found: C, 53.20; H, 3.60; N, 11.44.

Biological methods. The compounds were tested for their anti-inflammatory and analgesic activities as well as for acute toxicity. The test compounds were suspended in 0.5% gum acacia in water and administered orally. The experiments were performed with albino rats of Charles-Foster strain of either sex, excluding pregnant females, 60 to 90 days old weighing 100 to 120 g. Food (chaw pallet) and water was given to the animals ad libidum. The tested compounds were dissolved in propylene glycol. Phenyl butazone and aspirin were used as reference drugs for the comparison of anti-inflammatory and analgesic activities.

Anti-inflammatory activity. Anti-inflammatory activity against carrageenan-induced rat paw oedema was determined by the method of Winter et al. (1962). This study was conducted on albino rats of either sex (100-150 g). The rats were divided into groups of five animals each. Compounds were screened for anti-inflammatory activity at 50 mg/kg p.o. The percentage of anti-inflammatory activity was calculated according to the following formula.

\[
\text{Anti-inflammatory activity} \% = \left(1 - \frac{V_t}{V_c}\right) \times 100
\]

Where, Vt and Vc are the volume of oedema in drug treated and control group, respectively.

Analgesic activity. This activity was determined by the method of Berkowitz et al. (1977), which is based on the property of the test compound to antagonize the phenyl quinone-induced pain syndrome in mice. Groups of five mice were injected intraperitonially with 0.25 ml of 0.02% solution of phenyl-quinone in ethanol (5%) one h after oral administration of the test compound. The number of writhes induced in each mice

| Table 1. Physical and analytical data of compounds 3a-3d, 4a-4h, 5a-5h and 6a-6h* |
|-----------------|-----------------|---------|-----------------|-----------------|-----------------|-----------------|
| Compound       | R1              | R2      | m.p. (°C)       | Yield (%)       | Molecular formula | Molecular weight |
| 3a              | 2-OH            | -       | 215             | 60              | C8H7N4O2S        | 208.17          |
| 3b              | 4N(CH3)2       | -       | 243-244         | 63              | C10H13N5S        | 235.30          |
| 3c              | 2-Cl            | -       | 258             | 70              | C8H7N4SCl        | 226.95          |
| 3d              | 2-OCH3         | -       | 238-239         | 90              | C8H8N4O2S        | 210.24          |
| 4a              | 2-OH            | 4-Cl    | 170             | 72              | C10H14N5S2Cl     | 475.99          |
| 4b              | 4N(CH3)2       | 4-Cl    | 184             | 92              | C10H14N5S2Cl     | 503.07          |
| 4c              | 2-Cl            | 4-Cl    | 206-208         | 68              | C10H14N5S2Cl     | 462.44          |
| 4d              | 2-OCH3         | 4-Cl    | 182             | 70              | C10H14N5S2Cl     | 490.02          |
| 4e              | 2-OH            | 4-Br    | 168             | 79              | C10H14N5S2Cl     | 534.39          |
| 4f              | 2-Cl            | 4-Br    | 180             | 75              | C10H14N5S2Cl     | 547.46          |
| 4g              | 4N(CH3)2       | 4-Br    | 200-201         | 60              | C10H14N5S2Cl     | 618.81          |
| 4h              | 2-OCH3         | 4-Br    | 174-175         | 70              | C10H14N5S2Cl     | 534.42          |
| 5a              | 2-OH            | 4-Cl    | 220             | 52              | C10H14N5S2Cl     | 552.53          |
| 5b              | 4N(CH3)2       | 4-Cl    | 245-246         | 54              | C10H14N5S2Cl     | 579.60          |
| 5c              | 2-Cl            | 4-Cl    | 248             | 55              | C10H14N5S2Cl     | 570.04          |
| 5d              | 2-OCH3         | 4-Cl    | 235             | 50              | C10H14N5S2Cl     | 566.56          |
| 5e              | 2-OH            | 4-Br    | 218             | 48              | C10H14N5S2Cl     | 596.93          |
| 5f              | 2-Cl            | 4-Br    | 238             | 45              | C10H14N5S2Cl     | 624.00          |
| 5g              | 4N(CH3)2       | 4-Br    | 240             | 42              | C10H14N5S2Cl     | 615.44          |
| 5h              | 2-OCH3         | 4-Br    | 215             | 40              | C10H14N5S2Cl     | 540.92          |
| 6a              | 2-OH            | 4-Cl    | 300-302         | 45              | C10H14N5S2Cl     | 550.08          |
| 6b              | 2-Cl            | 4-Cl    | 316-318         | 46              | C10H14N5S2Cl     | 545.10          |
| 6c              | 4N(CH3)2       | 4-Cl    | 320             | 44              | C10H14N5S2Cl     | 568.60          |
| 6d              | 2-OCH3         | 4-Cl    | 307             | 42              | C10H14N5S2Cl     | 564.11          |
| 6e              | 2-OH            | 4-Br    | 298             | 40              | C10H14N5S2Cl     | 594.48          |
| 6f              | 2-Cl            | 4-Br    | 294             | 30              | C10H14N5S2Cl     | 621.55          |
| 6g              | 4N(CH3)2       | 4-Br    | 304-306         | 35              | C10H14N5S2Cl     | 612.99          |
| 6h              | 2-OCH3         | 4-Br    | 282             | 32              | C10H14N5S2Cl     | 608.51          |

*Satisfactory analysis for C, H, N was obtained for all the compounds within ± 0.4% of the theoretical values
Table 2. Pharmacological evaluation of the synthesized compounds 3a-3d, 4a-4h, 5a-5h and 6a-6h

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<th>R'</th>
<th>Dose (mg/kg p.o.)</th>
<th>Antiinflammatory activity (% edema inhibition relative to control)</th>
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<th>Analgesic activity (% decrease of writhes in 60 min after treatment relative to control)</th>
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<td>50</td>
<td>45.5***</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>60.8***</td>
<td>100</td>
<td>59.3***</td>
<td></td>
</tr>
</tbody>
</table>
was counted for 5 min after injection of an irritant. The analgesic effect was expressed as percent protection in comparison to control.

Protection (%) = (1-mean no. of writhes in mice of test groups/ mean number of writhes in mice of control group) × 100

**Acute toxicity.** Acute Lethal Dose (ALD50) of all the compounds were investigated by the method of Smith (1960).

**Results and Discussion**

Physical and analytical data of all the newly synthesized compounds (3a-3d, 4a-4h, 5a-5h and 6a-6h are given in Table 1. All the synthesized compounds were screened for their anti-inflammatory activity and analgesic activities. All the compounds have shown anti-inflammatory activity ranging from 13.5-53.6% at the dose of 50 mg/kg, p.o. In addition to the anti-inflammatory activity, these compounds also exhibited analgesic activity ranging from 6.2-42% at the dose of 50 mg/kg i.p. (Table 2). When the compounds were substituted with 4-chlorophenyl group at the 2-position of indole nucleus, they showed better anti-inflammatory and analgesic activities than 4-bromophenyl group. The anti-inflammatory and analgesic activities of compounds 3a-d were 13.5-19.5% and 6.2-11.2%, respectively, while those of compounds 4a-h ranged between 19.1-25.8% and 12.3-18.5%, respectively. Among the compounds 3a-d and 4a-h, compound 4c which was substituted with 2-chlorophenyl at 3-position of triazole ring exhibited 25.8% protection against carrageenan-induced edema. In addition to anti-inflammatory activity, this compound exhibited 18.6% protection against phenyl quinine-induced analgesia. Cyclization of compounds 4a-h into azetidinones 5a-h and thiazolidinones 6a-h have shown better anti-inflammatory and analgesic activities than their corresponding parent compounds. Azetidinones (5a-h) exhibited anti-inflammatory and analgesic activities ranging from 24.2-51.3% and 23.2-38.2%, respectively. Among the azetidinones (5a-h), compound 5c showed potent anti-inflammatory (51.3%) and analgesic (38.2%) activities. However, compound 5f exhibited lesser degree of inhibition of oedema 24.2% as well as analgesia 23.2% due to the presence of N, N-dimethyl group at 4-position of phenyl ring. Thiazolidinones 6a-h, generally, showed better anti-inflammatory and analgesic activities than azetidinones 5a-h. Out of the eight synthesized thiazolidinones 6a-h, the compound 6c exhibited the most potent anti-inflammatory (34.3, 53.6, 72.2%) activity at the three graded doses of 25, 50 and 100 mg/kg, p.o., respectively. This compound was also associated with analgesic activity 25.6, 42.1 and 60.4% at the three graded dose, of 25, 50 and 100 mg/kg, p.o., respectively.

The compounds 5c and 6c were compared with reference drugs phenylbutazone and aspirin. At all the three doses, this compound elicited both activities better than the reference drugs.

**Conclusions**

- It can be concluded that the compounds 4a-h having an azomethyne (-N=CH-) group between the substituted triazole rings and substituted indoles show good anti-inflammatory and analgesic activities.
- The cyclization of compounds 4a-h into the four membered heterocyclic ring i.e. azetidinones 5a-h show better activity than the parent compounds.
- The conversion of compounds 4a-h into five membered ring thiazolidinone ring compounds 6a-h show much better activity than 5a-h and 4a-h.
- The substitution of fourth position of triazole nucleus by substituted indole azetidinonyl and substituted indole thiazolidinonyl moieties remarkably increase the activities.
- The results show that chloro-substituted analogues are more potent than the other derivatives.

**Statistical analysis.** Statistical analysis of the anti-inflammatory activity of the synthesized compounds by level of significance was determined using Student’s ‘t’ Method.

**Acknowledgement**

Authors are thankful to CDRI, Lucknow, India for providing spectral and analytical data of the compounds.

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Spatial Assessment of Polycyclic Aromatic Hydrocarbons in Streambed Sediments

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Abstract. The occurrence and seasonal changes of polycyclic aromatic hydrocarbons (PAHs) in oil-contaminated sediment from selected oil areas of Ondo State, Nigeria were studied using gas chromatography–mass spectrometry. Six PAHs were identified and quantified with phenanthrene and carbazole, recording the highest and the least concentrations during the dry and wet seasons. Mean PAHs content ranged from 0.06 - 4.42 ìg/g and 0.09 - 6.0 ìg/g during the dry and the wet seasons, respectively. Significant correlations were observed (α = 0.05) between the two seasons but without significant mean difference (p = 0.05). For anthracene and phenanthrene, the compound toxic units (TU) were >> 1 and far exceeded the available consensus-based guidelines about the expected adverse effects. Results of the study call for further investigations especially with aquatic species due to the transfer of PAHs to humans via food chain.

Keywords: sediments, hydrocarbons, polycyclic aromatic hydrocarbons, gas chromatography mass spectrometry, oil spill

Introduction

Increasing exploration and exploitation of petroleum in the Ondo coastal region of Western Nigeria has lead to greater risk of contamination from oil spill. Prominent among the hydrocarbon residue resulting from oil spill are the polycyclic aromatic hydrocarbons (PAHs) (Koh et al., 2004; Law et al., 2002). Sources of production of PAHs are both natural and human. Because of their low aqueous solubilities (0.003 to 0.34 mg/litre) and high octanol-water partition coefficient (log Kow = 3.4 – 7.6), PAH compounds in aquatic system tend to be associated with sediment and biota (Kannan et al., 2005; Koh et al., 2004; NOAA, 1998). Sediments, in general, have been employed to monitor the pollution of aquatic environments for the reason that PAHs, generally existing in low concentrations in the water column, have a propensity to associate preferentially with the sediments and attain considerable concentration (Kurunthachalam et al., 2008; German Ministry of Environment, 1993; Forstner and Wittmann, 1983).

Aromatic hydrocarbons are generally more toxic than aliphatic ones, with their toxicity increasing with the increase in the number of rings and degree of alkyl substitution. However, solubility decreases with increasing number of rings and alkyl groups. Thus, the most toxic petroleum hydrocarbons may be the compounds of 4-5 ring aromatics, although the most toxic contribution may be exerted by mono or dinuclear aromatics.

Research shows PAHs have negative effect on humans, mammals, invertebrates, birds and fish. As of 2001, about 17 PAHs have been registered as priority pollutants and with sufficient evidence to be considered toxic, carcinogenic and mutagenic (Zahodiakin, 2002; US EPA, 1993).

Studies have shown that each source of PAHs has a characteristics pattern and the usefulness of PAH isomer ratios, such as phenanthrene/anthracene (tricyclic aromatic, MW178) can be demonstrated for source identification (Ololade and Lajide, 2009; Ikenaka et al., 2005). Lower molecular weight PAHs e.g. phenanthrene and anthracene are known to be acutely toxic to aquatic organisms and are more easily degraded unlike the higher molecular weight compounds which tend to predominate in sediments where they are subjected to burial, resuspension, and degradation reactions (Villeneuve et al., 2002). The biodegradation of PAH probably occurs more slowly in aquatic system than in soil (Clement Associates, 1985).

Seasonal and historical changes on disposition of PAHs have been determined using sediment core in several studies (Ikenaka et al., 2005; Takeda et al., 2003). In fact, aquatic ecosystem remains one of the major sinks of PAH contamination, which has received most attention because of the lipophilic characteristics and bioconcentration effect of PAHs (Guzzella and Depaolis, 1994). Moreover, due to the environmental significance of PAHs, extensive studies of bottom sediments of Lorence Greek Lake and urban run off have been carried out.
(Ging et al., 2001). The increasing ubiquitous presence of these compounds, especially as those derived from petroleum in ocean, lakes and rivers in the form of spills necessitates the development of practical means of determining such pollutants in the natural environment. Sediment exposure to PAHs has been characterized by comparing sediment PAH concentration with sediment quality benchmarks empirically derived from 28-day toxicity tests using Hyalella azteca (HA-28). The database was constructed using data from several contaminated sites (MacDonald et al., 2000a, b; Ingersoll et al., 1996). These benchmarks represent the concentration below which toxicity is frequently observed (Effects Range Median, ERM).

Little environmental data is available for the coastal region of Ondo state located within the South-Western region of Nigeria (Ololade and Lajide, 2009; Ololade et al., 2009; Asaolu, 1998). The Ondo coastal region is known for biological production and is especially important for commercial fishing. Several oil wells having been located around the study area during the recent years have resulted in increased spills. The present study was undertaken as an attempt to create a friendly environment for recreational activities within the area. It is equally hoped that hydrocarbon source could be identified, which will help to establish the distribution pattern. Consequently, the most effective, hyphenated gas chromatography – mass spectrometry (GC-MS) was employed for the study based on literature guidance.

Materials and Methods

Study area. Ondo State occupies a total land area of 14 793 189 square kilometers. It lies entirely in the tropical area between longitudes 4° 30' and 6° 14' east, and latitudes 5° 45' and 8° 15' north. It is bounded to the south by the Atlantic Ocean. The research focuses upon the southern coastal area (Fig. 1). The climate is tropical with two distinct seasons: the rainy season (April-October) and the dry season (November-March), with a temperature range of 26.2 - 27.6 °C. Salinity increases in the rivers at high tide and decreases at low tide. The sampling sites are locations, previously noted for large biological production and are especially important for commercial fishing. However, the unfortunate state of affairs set in 1996 when the first oil spill occurred due to the activities of two foreign petroleum companies (Ondo State Environmental Protection Agency, 2000). Large areas of mangroves ecosystem have been destroyed within the area as a result of toxicity from persistent oil spillage. Consequent upon the negative impacts of this unpalatable experience, the present study was carried out.

Chemicals and apparatus. All chemicals were of analytical reagent grade. Dichloromethane (DCM) and hexane were all doubly distilled in all-glass apparatus while the alumina, silica gel, sodium chloride and sodium sulphate were Soxhlet extracted with n-hexane for 8 hs each. All glassware used were thoroughly cleaned with hot detergent, rinsed with distilled water, then methanol and dried at 120 °C for about 6 hs.

Sampling. Basically, two sites: Ayetoro and Awoye (Site A, Ayetoro; Latitude 06°6' 12.4" longitude 04°6' 36.0"; Site B, Awoye; latitude 05°55' 1.2", longitude 04°58' 49.8") with five locations at each, were selected within the ecological zone. Some of the variables that influenced the selected sites included proximity to oil well locations and high level of socio-economic activities, particularly fishing, within the area. Samples were collected during both the dry and the wet seasons. The exact positions of the sampling points were noted using the global positioning system (GPS) and the details are presented in Table 1. Samples were collected at each site, one from midstream and one from each bank in a cross section fashion and later homogenized into a composite sample from which representative samples were taken based on APHA standard method (Aderemi et al., 2003). Samples were collected and wrapped inside aluminum foil after proper labelling. They were subsequently placed inside coolers containing ice-bags and transferred to the laboratory where they were preserved in the freezer prior to analysis.

Saponification and extraction. About 100 g partially thawed sediment sample was weighed and transferred to a pre-cleaned extraction thimble which was then placed in the inner tube of

![Fig. 1. Map of the study area (inset is the area map of Nigeria and Africa showing the geographical location).](image-url)
the Soxhlet apparatus containing 140 ml DCM to which boiling chips had been added. Internal standards (anthracene, phenanthrene and pyrene) were used. The sediment sample was extracted for 8 hs and cooled. 20 ml of 0.7M KOH and 30 ml of pre-extracted water (with hexane) was added to the sediment solvent flask. The non-saponifiable lipids (containing the petroleum hydrocarbons) were carefully reduced to about 15 ml and then partitioned with hexane in 100 ml glass separatory funnel. 40 ml hexane was used and the mixture was thrice washed with 25 ml 0.1M Na2CO. The separated organic layer was filtered through glass wool and anhydrous sodium sulphate to dry the extract. The volume was reduced on a water bath maintained at 30 °C. Final evaporation of the extract was carried out under nitrogen gas and later chromatographed. The extracts were eluted through a silica-alumina glass column for the removal of polar lipids and other biogenic interferences and the samples were concentrated to a volume of 5 ml. The fractions were combined and dissolved in a known amount of dichloromethane prior to GC-MS measurement (Aderemi et al., 2003).

**Gas chromatography – mass spectrometric (GC–MS) analyses.** The capillary gas chromatography – mass spectrometry analysis were performed on a Hewlett–Packard (HP) 6890 GC series instrument coupled with a 5975 Hewlett–Packard mass spectrometer (MS). The capillary column used was of the Agilent 1909IS – 433 model with dimensions of 30 m x 0.25 mm ID x 0.25 im film thickness of HP- 5M5 (5% phenyl methyl siloxane). Full scan mass spectra between 35 and 500 m/z were acquired once every second. The peaks in the chromatogram were identified by comparison of the retention times and mass spectral data of reference compounds with those in the sample using MS Library Wiley and NIST (NIST, 2008). The peaks were quantified using the flame ionization detector (FID) through a five point calibration curve.

**Results and Discussion**

Two of the chromatograms obtained during dry and the wet seasons are presented in Figs. 2 and 3, respectively. All together, about six different PAH (anthracene, azuleno[2,1-b]thiophene, benz[a]anthracene, carbazole, dibenzothiophene and phenanthrene) were identified in the sediment extracts of the two sites studied. Total PAH concentration was the sum of the concentrations of individual identified compounds. The highest total PAH concentrations (Table 1) ranging from 0.11 to 6.00 ìg/g were detected at site-B during the wet season, while the least, ranging from 0.06 to 3.02 ìg/g was detected at
site-A during the dry season. Phenanthrene and anthracene were the compounds, most frequently detected at the highest concentrations. The data in Table 1 also shows that concentrations are equally higher for almost all the PAHs at site-B. This may be due to the closeness of the site to the Atlantic Ocean, through which anthropogenic oil spill enters the hinterland.

The trend of the concentrations during both the seasons was almost similar with the PAH concentrating more during the wet season. Significant positive correlation ($\alpha = 0.01$) was observed between PAHs concentrations during both the seasons ($r = 0.998$ and 0.923) at site-A and B, respectively, but without any significant mean difference ($p = 0.077$ and 0.118, respectively). Though, dry season samples were first collected, it was thought that concentrations during the wet season would be less due to the prevailing ecological conditions at that time. However, the increased levels during this period further implicate continuous release of hydrocarbon residues into the area. It also re-affirms that sediments are capable of adsorbing organic pollutants such as PAHs (Ololade et al., 2009; Aderemi et al., 2003).

Using the sediment quality benchmarks empirically derived from 28 days toxicity tests using *Hyalella azteca* (HA–28) (MacDonald et al., 2000a, b; USEPA, 1996) all the sediment samples taken during both seasons had their PAH concentrations exceeding the ERM for anthracene, benz[a]anthracene and phenanthrene. Thus, exposure of sediment-dwelling organisms to PAHs is likely at these sampling locations. In fact, anthracene and phenanthrene exceeds the consensus-based probable effect concentration (PEC) as indicated in Table 1. However, concentration of benz[a]anthracene was found to be $<\text{PEC}$, but $>\text{TEC}$. All these observations are indicative of serious health hazards for the ecosystem.

For each PAH identified in Ondo coastal sediment, a toxic unit (TU) was calculated by dividing the sediment PAH concentration by the HA–28 ERM based on the reported model (MacDonald et al., 2000a, b; Long and Morgan, 1991). The TUs for each identified PAH are included in Table 1 in parenthesis. However, guidelines for some of the identified compounds are unavailable; thus, their TUs could not be calculated. The summation of these TUs is indicative of the overall estimated PAH toxicity of the sample. As indicated in Table 1, the $\Sigma\text{TU}>>1$, thus, adverse effects are expected based on the literature guidance (MacDonald et al., 2000a, b). Phenanthrene (Phe) is generally more stable thermodynamically than
anthracene (Ant) and the ratio Phe/Ant is a reflection of the origin of PAHs; petrogenic or pyrogenic in the environment (Ikenaka et al., 2005). A high ratio of Phe/Ant (> 15) is characteristic of petrogenic source, while pyrogenic is characterized by lower ratio. In the present report, the ratio Phe/Ant was 1.28 ± 0.16 (average ± SD, n = 8) in the sediment. Thus, the PAHs are more from the pyrogenic than petrogenic source. The data further imply that the oil residues consist mainly of the heavier portion which tend to sink and become sedimented. This may be due to the fact that PAHs, because of their hydrophobic character, show strong adsorption affinity to solid particles in aqueous matrices (Lombas-Garcia et al., 1998). Thus, the extent of hydrocarbon adsorption was found to be positively correlated (r = 0.8335) with the organic carbon (OC) present in the sediment during both the dry and the wet seasons (Fig. 4).

The impact of the PAHs in Ondo coastal environment on populations of benthic organisms may be very devastating based on the fact that the concentration of PAHs measured in the sediments are comparable to other contaminated sites in which severe impacts have been observed (Ikenaka et al., 2005; Aderemi et al., 2003; Pavlova and Ivanova, 2003). Thus, sediment-dwelling or burrowing organisms such as crabs, periwinkles etc. are at the greatest risk especially during the wet season because PAHs tend to remain bound to organic sediment (Kannan et al., 2003, 2005; Koh et al., 2004). Aquatic organisms that reside on the sediment surface, restricted to the water column only, are likely to receive less concentration from the sediment-bound PAH, and are, therefore, at less risk. The occurrence of PAHs, even at the levels obtained in this report within the environment is of concern due to their carcinogenic properties, and their ability to exert toxic effects through the aryl hydrocarbon receptor (AhR) mediated mechanism, similar to those of dioxins (Villeneuve et al., 2002). Several workers have shown that most PAHs such as carbazole, phenanthrene etc. have shown to be mutagenic and carcinogenic in laboratory studies (Aderemi et al., 2003; Ghauch et al., 2000; Ericson et al., 1999; Qanh et al., 1999; MacCoy and Black, 1998; Robertson, 1998; Myers et al., 1987). It is hoped that some site-specific resident organisms and important marine species will be investigated to further establish the present findings.

**Table 1.** Mean PAH concentration (μg/g) in sediments from Ondo Coast, Nigeria

<table>
<thead>
<tr>
<th>PAH</th>
<th>SITE A (Ayetoro) Seasons</th>
<th>SITE B (Awoye) Seasons</th>
<th>Consensus-based TEC</th>
<th>Consensus-based PEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry</td>
<td>Wet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthracene</td>
<td>2.08±0.47 (12.24)*</td>
<td>2.30±1.20 (13.53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azuleno[2,1-b] thiophene</td>
<td>2.40±1.12</td>
<td>2.49±1.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benz[a]anthracene</td>
<td>0.14±0.12 (0.50)</td>
<td>0.16±0.09 (0.57)</td>
<td></td>
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<tr>
<td>Carbazole</td>
<td>0.06±0.02 (0.08)</td>
<td>0.09±0.04 (0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>0.18±0.04</td>
<td>0.25±0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3.02±0.12 (3.39)</td>
<td>3.44±1.04 (8.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ PAH (Σ TU)</td>
<td>7.88(20.13)</td>
<td>8.73(22.52)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values in parenthesis are Toxic Units (TU); means in the same row followed by the same superscript are not significantly different (α = 0.05) according to Duncan’s New Multiple Range Test. ERM: For anthracene (0.17 μg/g); phenanthrene (0.41μg/g); benz[a]anthracene (0.28 μg/g). TEC=threshold effect concentration (MacDonald et al., 2000); PEC: probable effect concentration (MacDonald et al., 2000); PEL–HA 28: probable effect level for *Hyalella azteca*: 28-day test, dry weight(USEPA,1996); NG: no guidelines for TEC and PEC.

**Fig. 4.** Σ PAH concentrations with sediment organic carbon content (p < 0.05).
Conclusion

Oil spills have occurred several times along the Ondo coast in Nigeria—which is at the Gulf of Guinea—as a result of upsurge in oil exploration and exploitation activities. The present study demonstrates the adsorbing capacity of sediments for the toxic pollutants such as PAHs. It was also observed that these chemical pollutants are capable of building up (bioaccumulate) within the system, which poses serious threat to sediment dwelling organisms, other aquatic biota and eventually the man-through the food chain system. The study showed that the PAHs are more of a pyrogenic than a petrogenic origin. The temporal increase during the rainy season is indicative of slow degradation and efficient adsorption into the sediment matrices. It is recommended that investigations on site-specific resident organisms should be carried out for their PAHs status. This will assist appropriate food regulatory agencies in policy formulation on sea-food products.

Acknowledgement

The authors are grateful to Dr. Stefan Louw and Désirée Prevoo of the Gas Chromatography– Mass Spectrometry Laboratory, Central Analytical Faculty of Stellenbosch University, South Africa for assistance in the hydrocarbon analysis. Thanks are due to Dr. Stephen and Shola Ololade for providing logistics in South Africa.

References


**Introduction**

*Ducrosia anethifolia*, *Bunium persicum*, *Bunium cylindricum* and *Ammi majus* are herbs of the family Umbelliferae. These plants abundantly grow wild in areas such as Gilgit, Swat, Hazara, Zargon and the province of Baluchistan, while *Ammi majus* has been successfully cultivated in the Punjab, Baluchistan and North West Frontier Province of Pakistan. These plants and their roots have been widely used in medicines as carminative, astringent, diuretic, expectorant, anthelmintic, in dental preparations, cosmetics and as insecticides. They are also used in many food products as condiments, component of various flavouring agents used in beverages and find extensive use in curry powders. The seeds are also reported to possess high-cough curing properties. The medicinal values of *A. majus* have also been described in the old Arabic literature, where the seeds of the plant have long been used for the treatment of leucoderma (Dymock, 1972).

The oils extracted from the cultivated species of *Celery, Daucus, Apium graveolens, Feniculum vulgare* and *Cuminum cyminum* have already been studied for their fatty acid compositions (Hilditch and Williams, 1964).

The seed oils of *D. anethifolia*, *B. persicum*, *B. cylindricum* and *A. majus* wild species have already been extracted and chemically evaluated. The fatty acid profiles of these seed oils are supposed to be similar to that of other cultivated members of this family as indicated in the earlier studies on *Daucus anethifolia, Bunium persicum, Bunium cylindricum* and *Ammi majus*.

**Materials and Methods**

Fresh seeds of *D. anethifolia*, *B. persicum*, *B. cylindricum* and *A. majus* were obtained from the market in the months of...
May and June for extraction of oil. The chemicals used for this study were of analytical grade and the reagents used were prepared according to the standard methods of AOAC (2000).

**Extraction of oil.** Dried seeds of *D. anethifolia* (Gwartkh), *B. persicum* (Zira siyah), *B. cylindricum* (Siah zira khar) and *A. majus*, after extraction of essential oils, 100 g each (steam distillation), were powdered and subjected separately to extraction of oil in Soxhlet extractor (AOAC, 2000) with normal hexane as the solvent. The oils so obtained were dried over anhydrous sodium sulphate and the solvent was removed under vacuum. Oil percentage was determined on dry weight basis.

**Composition of seed meals and physicochemical value of the oils.** The seed meals, obtained after oil extraction, were freed from the solvent at 70 °C under vacuum and were then analyzed for protein, moisture, ash, fibre and carbohydrate contents by the method of AOAC (2000) and Solelo *et al.* (1995). Physicochemical values such as refractive index, specific gravity, saponification value and iodine value of the oil were also determined by standard methods (AOAC, 2000). Mineral composition of ash was estimated by using atomic absorption spectrophotometric technique (El-Gendi, 1988; Chowdhury *et al.*, 1983).

**Chromatographic analysis.** The seed oils were separately saponified with 0.5 N alcoholic potash and extracted with diethyl ether to remove the unsaponifiable matter. Fatty acids were then obtained from the soap by acidification with 4 N sulphuric acid followed by extraction with petroleum ether. The ether extract was washed thrice with distilled water, and dried over anhydrous sodium sulphate (AOAC, 2000). Methyl esters of the liberated fatty acids were prepared by adding 1-2 ml of methylated mixture of boron trifluoride in hexane as the solvent. The oils so obtained were dried over anhydrous sodium sulphate and the solvent was removed under vacuum. Oil percentage was determined on dry weight basis. The esters obtained were oxidized by the modified Vonz-Rudloff’s method, (Hamilton and Raie, 1972). The short chain methyl esters of adipic and lauric acids were estimated by gas chromatography (GC). GC analysis was carried out with a GC-14A gas chromatograph (Shimadzu) fitted with hydrogen gas flame ionization detector and data processor. A PEG capillary column (25 m × 0.2 m.m. i.d.) was used and the column temperature was maintained at 180 °C for fatty acid methyl esters; for adipic and lauric acid methyl esters, the column was operated with temperature programming from 150 to 180 °C. The injection and detector temperatures were maintained at 250 °C and 300 °C, respectively. Flow rate of carrier gas (nitrogen) was 20 ml/min at split ratio of 1:50. Identification of components was based on their retention times as compared with those obtained for methyl esters of known fatty acids analysed under the same conditions.

### Results and Discussion

The seeds of *D. anethifolia*, *B. persicum*, *B. cylindricum* and *A. majus* species yielded about 8.7%, 16.1%, 15.4 % and 7.78% oil, respectively. Seed meals were subjected to chemical treatment for evaluation of protein, moisture, ash, fibre and carbohydrate contents (Table 1).

The physicochemical characteristics, refractive index, specific gravity, iodine value, saponification value, saponifiable and unsaponifiable matter of the four oils as determined experimentally are shown in Table 2. Data for the fatty acid composition of the four species is presented in Table 3. GC data of these esters revealed the presence of high percentage of petroselinic acid in the analysed four species. The results are almost similar to those reported for other species of the plants of the family Umbelliferae, (Klieman and Spencer, 1982; Hilditch and Williams, 1964). Ash content was further evaluated for the mineral profile; results are shown in Table 4.

Physicochemical characteristic of *D. anethifolia*, *B. persicum*, *B. cylindricum* and *A. majus* seed oils have been found to be similar in all the species studied with a little variation in the oil percentage. The residual meals obtained after extraction of the fixed oils were analysed to determine their chemical composition for protein, moisture, ash, fibre and carbohydrate contents (AOAC, 2000) and the results are shown in Table 1. Seed meals of *D. anethifolia*, *B. persicum*, *B. cylindricum* and *A. majus* contained 17.40%, 18.40%, 22.84% and 18.94% protein and 11.89%, 8.05%, 7.85% and 10.74% fibre, respectively, whereas carbohydrates were present in high amounts being 47.73%, 48.48%, 45.05% and 49.12%, respectively.

### Table 1. Chemical composition of seed meals of the plants

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Moisture</th>
<th>Ash</th>
<th>Fibre</th>
<th>Carbohydrate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. anethifolia</em></td>
<td>17.40</td>
<td>7.38</td>
<td>8.9</td>
<td>9.89</td>
<td>47.73</td>
<td>91.3</td>
</tr>
<tr>
<td><em>B. persicum</em></td>
<td>18.40</td>
<td>5.10</td>
<td>3.86</td>
<td>8.05</td>
<td>48.48</td>
<td>83.89</td>
</tr>
<tr>
<td><em>B. cylindricum</em></td>
<td>22.84</td>
<td>4.95</td>
<td>3.91</td>
<td>7.85</td>
<td>45.05</td>
<td>84.6</td>
</tr>
<tr>
<td><em>A. majus</em></td>
<td>18.94</td>
<td>5.02</td>
<td>8.39</td>
<td>10.74</td>
<td>49.12</td>
<td>92.21</td>
</tr>
</tbody>
</table>
Table 4 shows the mineral contents of these species determined by atomic absorption photometry, with high quantities of K, Mg and Ca, whereas other elements Na, Fe, Cu, Mn, Zn and P were present in moderate quantities. The results indicate that D. anethifolia, B. persicum, B. cylindricum and A. majus meals are rich sources of proteins, carbohydrates and minerals. These can also be used as cattle feed. These results are similar to those reported by the earlier workers (El-Gendi, 1988; Hans, 1969; Miller, 1951). Many of the Umbelliferae plants have already been cultivated for food and food additives and the wild species under study have also been cultivated successfully (Bhatt et al., 1977). These species have the potential for use as industrial crops as they have appreciable amounts of oil contents with high petroselinic acid content.

Seed oils of the four species contained 69.72-84.50% unsaturated, 11.48-21.96% saturated and 4.01-5.29% unidentified fatty acids. Petroselinic acid (PA) is the general characteristic acid of the seed oils of species of Umbelliferae, as reported by Mallet et al. (1990) and Prasad et al. (1987). These acids were separated on thin layer chromatographic plates into unreacted methyl esters of monoenoic acid and short chain fatty acids; nonanoic and dodecanoic acids gave percentages of positional isomers as petroselinic acid (PA) and oleic acid (Table 3). It is indicated that the amounts of monoenoic acid 66.89%, 55.05%, 44.7% and 61.46% in the seed oil of D. anethifolia, B. persicum, B. cylindricum and A. majus, respectively, are the sum of petroselinic and oleic acids, being 58.8% and 8.09%, 44.2% and 10.85%, 33.3% and 11.4% and 38.56% and 22.9% in their seed oils, respectively. These results are almost similar to those reported in the literature determined by other techniques (Mallet et al., 1990). Linoleic acid is present in significant amounts in the four species (13.37%, 15.02%, 22.02% and 21.27%, respectively), while in three species linolenic acid is present in small amounts (1.67%, 2.67% and 1.77% in D. anethifolia, B. persicum and A. majus, respectively). But an appreciable amount of linolenic acid (11.0%) was present in B. cylindricum. These results are very close to the results reported earlier by Klieman et al. (1969).

Significant amounts of palmitic acid and other short chain saturated fatty acids were present in all the seed oils of these four species, but distribution is different in all the individual species (Table 3). In the earlier studies, C16:1 was reported in

### Table 2. Physicochemical evaluation of seed oils

<table>
<thead>
<tr>
<th>Species</th>
<th>Oil content (on dry weight basis)</th>
<th>Refractive Index</th>
<th>Specific gravity</th>
<th>Iodine value</th>
<th>Saponification value</th>
<th>Unsaponifiable matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. anethifolia</td>
<td>8.70</td>
<td>1.4693</td>
<td>0.9075</td>
<td>92.50</td>
<td>138.9</td>
<td>1.99</td>
</tr>
<tr>
<td>B. persicum</td>
<td>16.10</td>
<td>1.4738</td>
<td>0.8992</td>
<td>112.1</td>
<td>152.5</td>
<td>2.15</td>
</tr>
<tr>
<td>B. cylindricum</td>
<td>15.40</td>
<td>1.4694</td>
<td>0.9199</td>
<td>120.6</td>
<td>137.85</td>
<td>2.51</td>
</tr>
<tr>
<td>A. majus</td>
<td>7.78</td>
<td>1.4660</td>
<td>0.9087</td>
<td>98.6</td>
<td>175.5</td>
<td>2.01</td>
</tr>
</tbody>
</table>

### Table 3. Fatty acid composition of seed oils obtained by GC

<table>
<thead>
<tr>
<th>Species</th>
<th>C10:0</th>
<th>C12:0</th>
<th>C14:0</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1:6*</th>
<th>C18:1:9**</th>
<th>C18:2</th>
<th>C18:3</th>
<th>Unidentified</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. anethifolia</td>
<td>—</td>
<td>3.34</td>
<td>2.17</td>
<td>7.36</td>
<td>1.00</td>
<td>58.8</td>
<td>8.09</td>
<td>13.37</td>
<td>1.67</td>
<td>4.21</td>
</tr>
<tr>
<td>B. persicum</td>
<td>2.25</td>
<td>3.38</td>
<td>5.63</td>
<td>9.01</td>
<td>1.69</td>
<td>44.2</td>
<td>10.85</td>
<td>15.02</td>
<td>2.67</td>
<td>5.25</td>
</tr>
<tr>
<td>B. cylindricum</td>
<td>2.58</td>
<td>2.92</td>
<td>2.75</td>
<td>7.34</td>
<td>1.83</td>
<td>33.3</td>
<td>11.4</td>
<td>22.02</td>
<td>11.0</td>
<td>4.85</td>
</tr>
<tr>
<td>A. majus</td>
<td>1.18</td>
<td>1.77</td>
<td>1.44</td>
<td>5.91</td>
<td>1.18</td>
<td>38.56</td>
<td>22.9</td>
<td>21.27</td>
<td>1.77</td>
<td>4.01</td>
</tr>
</tbody>
</table>

* = petroselinic acid; ** = oleic acid

### Table 4. Mineral contents of seed meals

<table>
<thead>
<tr>
<th>Elements on dry weight basis (%)</th>
<th>K (g)</th>
<th>Mg (g)</th>
<th>Ca (g)</th>
<th>Na (mg)</th>
<th>Cu (mg)</th>
<th>Fe (mg)</th>
<th>Mn (mg)</th>
<th>Zn (mg)</th>
<th>P (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. anethifolia</td>
<td>1.21</td>
<td>1.30</td>
<td>0.41</td>
<td>33.85</td>
<td>28.03</td>
<td>11.67</td>
<td>0.15</td>
<td>3.12</td>
<td>0.43</td>
</tr>
<tr>
<td>B. persicum</td>
<td>0.99</td>
<td>1.13</td>
<td>0.38</td>
<td>37.78</td>
<td>21.49</td>
<td>13.04</td>
<td>0.22</td>
<td>2.6</td>
<td>0.26</td>
</tr>
<tr>
<td>B. cylindricum</td>
<td>1.38</td>
<td>1.24</td>
<td>0.43</td>
<td>29.07</td>
<td>19.26</td>
<td>12.29</td>
<td>0.28</td>
<td>3.01</td>
<td>0.48</td>
</tr>
<tr>
<td>A. majus</td>
<td>1.02</td>
<td>1.39</td>
<td>0.32</td>
<td>0.36</td>
<td>20.12</td>
<td>10.98</td>
<td>0.31</td>
<td>2.9</td>
<td>-</td>
</tr>
</tbody>
</table>
trace amounts in some Umbelliferae species by Rankova et al. (1957) and Pearson (1970), but the present study did not show its presence in any of these species. The presence of higher percentage of petroselinic acid (PA) as compared to oleic acid is in agreement with the earlier investigations showing PA as a characteristic fatty acid of this family (Hamilton and Raie, 1972). The percentages of petroselinic acid (PA) in seed oils of these species as determined here are lower than those claimed by Kleinman and Spencer (1982), which may be attributed to the differences in climatic conditions. However, it is observed that, by and large, the occurrence of PA is the dominant feature of the fixed oil of members of Umbelliferae. Minor variations in its content as the constituent of the glycerides is attributed to the climatic as well as soil conditions of various regions. It can be thus inferred that these plants with high quantity of petroselinic acid may be used as potential raw material for the industry (Merck, 2006; National Research Council US, 2000).

References
Comparative Study for the Effect of Biofertilizers and Chemical Fertilizers on Soybean Oil Content and its Potential for Biodiesel Production

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(received October 15, 2008; revised August 15, 2009; accepted August 31, 2009)

Abstract. The present study makes comparative evaluation of biofertilizers (brands Biopower and Biozote) and chemical fertilizers (urea and diamonium phosphate (DAP)) on yield and the quality of soybean cv.NARC-1. Significant increase in number of pods per plant, seed oil content and specific gravity of oil was observed in case of chemical fertilizer treatment. All the treatments decreased the acid value and free fatty acid (oleic acid) content of oil, maximum reduction being in the case of Biopower treatment. Biopower treated plant seed oil exhibited higher refractive index and maximum conversion to methyl esters/biodiesel.

Keywords: biodiesel, biofertilizers, soybean oil, chemical fertilizers

Introduction

Alternative fuels produced from renewable feedstock resources are gaining popularity these days. Biodiesel is one of such alternative fuels produced from vegetable oils. The commercial scale production of biodiesel has many socio-economic benefits. The main advantages of biodiesel is its biodegradability, and emission of better quality of exhaust gases, given that all the organic carbon present is photosynthetic in origin (Barnwal and Sharma, 2005).

Chemical fertilizers are unavoidable in increasing crop yield but they have been found to adversely affect ecosystem and are rather expensive with limited resources. On the other hand, biofertilizers are sustainable and environment friendly. Biofertilizers have emerged as a promising component of integrating nutrient supply system in agriculture (Bloemberg et al., 2000). They mainly include nitrogen fixing, phosphate solubilizing and plant growth promoting microorganisms (Goel et al., 1999). Chauhan et al. (1995) found that application of biofertilizers markedly increased the pod number and seed yield of Brassica juncea L. plants over the non-inoculated ones. Goel et al. (1999) reported that inoculation of plants with growth promoting rhizobacteria (PGPR) enhances crop productivity either by making the other nutrients available or protecting plants from pathogenic microorganisms. Zodape (2001) reported that increase in productivity with biofertilizer application is due to microelements and plant growth regulators contained in the fertilizer. Shehata and El-Khawas (2003) reported that oil contents and seed yield in sunflower significantly increased in response to biofertilizer application as compared to the control.

Soybean (Glycine max L.) Merrill is among the most important oilseed crops containing 18 to 22 percent oil with 85% unsaturated fatty acids and is widely used for biodiesel production. In view of the limited availability of biodiesel resources, the present investigation was carried out to compare the effects of chemical fertilizers and biofertilizers on the yield and quality of soybean oil pertaining to biodiesel production.

Materials and Methods

The experiment was carried out in complete randomized design in green house at the Department of Plant Sciences, Quaid-e-Azam University, Islamabad. Seeds of soybean cv. NARC-1 were obtained from National Agriculture Research Centre (NARC), Islamabad. The seeds were sown in earthen pots measuring 27 x 30 cm² filled with clay loam and farm yard manure (FYM) in the ratio of 7:1 under natural environmental conditions. Following treatments were made.

T1 = Control
T2 = Biopower (Rhizobium + phosphate solubilizing microbes)
T3 = Nitrogen and phosphorus fertilizers
T4 = Biozote (Rhizobium)

Biopower and Biozote are trade names of biofertilizers prepared by National Institute of Biotechnology and Genetic Engineering (NIBGE) Faisalabad and National Agriculture
Research Centre (NARC), Islamabad, respectively. Both biofertilizers were purchased from the respective institutes.

**Method of seed inoculation.** The seeds of soybean were surface sterilized with 0.1% mercuric chloride (HgCl₂) solution for 2 min and subsequently washed thrice with sterilized water. The sterilized seeds were moistened in 45% sugar solution and inoculated with Biozote and Biopower separately and were thoroughly mixed to get a thin, uniform coating of inocula on the seeds. Inoculated seeds were air dried before sowing (Samasegaran et al., 1982).

**Application of chemical fertilizers.** The chemical fertilizers viz. nitrogen in the form of urea and phosphorus as diammonium phosphate (DAP) were applied at the rate of 50 kg/ha. The whole dose of chemical fertilizers was split into four equal sub-doses. The first sub-dose of DAP was applied at the time of sowing while the first dose of urea was applied 10 days after sowing. The remaining 3 sub-doses of both fertilizers were applied subsequently at 10 days interval.

**Parameters studied.** Number of pods/plant, number of seeds/pod and 100 seed weight were determined. Seed oil content was determined by nuclear magnetic resonance (Robertson and Morrison, 1979). The seed samples were rendered into powder form with grinding mill. The oil was extracted using petroleum ether with Soxhlet (AOAC, 1960) and stored at 4 ºC in the refrigerator. The oil sample (2 g) was poured into a clean dry beaker and 25 mL of hot distilled water was added to the sample in the beaker and stirred slowly. It was cooled in a water bath to 25 ºC and pH of the sample was recorded.

**Specific gravity (g/cc).** Specific gravity of oil was determined using density bottle according to the method described by Pearson (1980).

**Acid value (mg KOH/g).** Acid value was determined according to the method described by Akubugwo and Ugboagu (2007) and calculated according to the following formula.

\[
\text{Acid value} = \frac{56.1 \times N \times V}{W}
\]

where \(N\) = normality of NaOH used; \(V\) = volume (ml) of NaOH used, \(W\) = weight of the sample used

**Free fatty acids (as oleic acid).** Percentage of free fatty acids was estimated by multiplying the acid value with the factor 0.503.

**Refractive index.** The refractive index was determined using refractometer according to the method described by AOAC (1990).

**pH value.** The oil sample (2 g) was poured into a clean dry beaker and 25 mL of hot distilled water was added to the sample in the beaker and stirred slowly. It was cooled in a water bath to 25 ºC and pH of the sample was recorded.

**Biodiesel production.** Crude oil was processed to methyl esters (biodiesel) using transesterification method utilizing basic catalyst (Freedman et al., 1986). Biodiesel was washed by spraying of water on top of the column at low velocity. Thereafter it was dried in rotary evaporator at 120 rpm at 60 ºC for 40 min. Methyl ester so produced was determined on w/w (%) basis of ester to soybean oil content.

**Statistical analysis.** The data was analysed statistically by Analysis of Variance technique (Steel and Torrie, 1980) and through Duncan’s Multiple Range Test (DMRT), comparison was made among treatment means (Tables 1 and 2).

**Results and Discussion**

**Number of pods per plant, number of seeds per pod and 100 seed weight (g).** The results (Table 1) revealed that chemical fertilizers were highly effective in increasing the number of pods per plant. However, Biopower and Biozote treated plants also showed significant increase in the number of pods per plant as compared to the control. All the treatments resulted in non-significant increase in the number of seeds per pod and 100 seed weight as compared to the control. Asad et al. (2004) reported that application of Biopower and Biozote increased the number of pods, number of seeds per pod and seed weight in mung bean as compared to the control. This may be due to the increased rate of nitrogen fixation by biofertilizers which resulted in more nitrate supply to reproductive parts causing dry matter accumulation in seeds leading to increased seed weight in soybean. Shehata and El-Khawas (2003) reported that yield characters such as number of seeds per head, weight of seed per head and weight of 1000 seed in sunflower were significantly increased by the application of each of the two biofertilizers, Biogien and Microbien (trade names), either separately or in combination. Urea has also been reported to increase the yield in sesame (Paul and Savithri, 2003). It was found that the chemical fertilizers and the biofertilizers both have significant effect on the increase in soybean plant growth. It had been estimated previously that biofertilizers could replace utilization of 50% of the chemical fertilizers (El-Kholy and Gomaa, 2000) without decreasing the green and dry fodder; this could be attributed to the plant growth-promoting substances produced by the biofertilizers (Gomaa, 1995; Bottini et al., 1989).

**Seed oil content.** All the treatments had stimulatory effect on the seed oil yield. Chemical fertilizer and Biozote treatments significantly increased seed oil content by 32% and 22%, respectively, as compared to the control. Biopower treatment exhibited non-significant increase in the seed oil content (Table 1). Similar results were obtained by Sawan et al. (2006) who reported that application of chemical fertilizers such as phosphorus increased the seed oil content and oil yield per hectare over the control in cotton. This may be attributed to
the role of phosphorus as coenzyme involved in energy transfer reaction so that energy is trapped during photosynthesis in the form of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADP), which is subsequently used in the photosynthetic fixation of CO₂ and the synthesis of lipids and other essential organic compounds (Taiz and Zeiger, 1991). Mekki and Ahmed (2005) reported that plants treated with biofertilizers singly or in combination with organic manure + yeast resulted in an increase in the seed oil contents of soybean.

**Specific gravity of oil.** Chemical fertilizers significantly increased specific gravity of oil by 6% as compared to the control (Table 2). Biopower and Biozote exhibited no significant effect on oil specific gravity. Cetane number, heating value, fuel storage and transportation are important qualities of biodiesel, closely related to the specific gravity (Yuan et al., 2004). Fuel density is correlated with particulate emissions and increase in the density gives increased particulate emissions (Mulin, 1994). It was found that chemical fertilizers increased oil specific gravity but biofertilizers had no effect. Oil obtained from the seeds of biofertilizer treated plants can be utilized successfully for production of good quality biodiesel with low particulate emissions of lighter quality.

**Acid value (mg KOH/g) and free fatty acid contents (% FFAs as oleic acid).** Results presented in Table 2 reveal that biofertilizers significantly decreased the oil acid value and free fatty acid content as compared to the control. Maximum reduction in oil acid value and free fatty acid content was recorded in the Biopower treatment. Biofertilizers have been reported to be involved in the production of phytohormones such as indole-3-acetic acid (IAA), gibberillic acid (GA3) and ethylene (Bashan et al., 2004), zeatin (Tien et al., 1979), abscisic acid (ABA) and production of plant growth regulatory substances such as polyamines (Thuler et al., 2003). Release of these growth-promoting substances in the soybean plants by biofertilizers may be responsible for altering the physiology of fat metabolism leading to decrease in acid value. Oil-seed crops require large amounts of nitrogen as the component of plant proteins, amino acids, nucleotides, nucleic acids and chlorophyll. Adequate supply of nitrogen and phosphorous may have led to increase the supply of nutrients to reproductive parts that led to decrease the seed oil acid value. The higher the acidity of the oil, the smaller is its conversion efficiency to biodiesel. These free fatty acids react with the alkaline catalyst to produce soaps instead of esters. For the completion of alkali-catalysed reaction, free fatty acid (FFA) content lower than 3% is needed (Dorado et al., 2002). At

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of pods/plant</th>
<th>No. of seeds/pod</th>
<th>100 seed weight (g)</th>
<th>Seed oil content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.00 c</td>
<td>2.183 a</td>
<td>1.500 a</td>
<td>16.29 c</td>
</tr>
<tr>
<td>Biopower</td>
<td>16.67 b</td>
<td>2.340 a</td>
<td>1.563 a</td>
<td>17.75 bc</td>
</tr>
<tr>
<td>Chemical fertilizers</td>
<td>26.67 a</td>
<td>2.287 a</td>
<td>1.320 a</td>
<td>21.56 a</td>
</tr>
<tr>
<td>Biozote</td>
<td>17.00 b</td>
<td>2.200 a</td>
<td>1.600 a</td>
<td>19.92 ab</td>
</tr>
<tr>
<td>LSD (5%)</td>
<td>3.216</td>
<td>1.882</td>
<td>1.082</td>
<td>1.8822</td>
</tr>
<tr>
<td>F-value*</td>
<td>35.124**</td>
<td>0.0164 ns</td>
<td>0.04629 ns</td>
<td>16.249***</td>
</tr>
</tbody>
</table>

*F-values are from one-way ANOVA; **significant at p<0.001; ***significant at p<0.01; ns= non-significant

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Specific gravity (g/cc)</th>
<th>pH</th>
<th>Acid value (mg KOH/g)</th>
<th>Free fatty acid (% oleic acid)</th>
<th>Refractive index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.92 b</td>
<td>5.00 a</td>
<td>2.63 a</td>
<td>1.31 a</td>
<td>1.46 a</td>
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<tr>
<td>Biopower</td>
<td>0.93 b</td>
<td>4.70 a</td>
<td>1.45 c</td>
<td>0.61 b</td>
<td>1.47 a</td>
</tr>
<tr>
<td>Chemical fertilizers</td>
<td>0.98 a</td>
<td>4.63 ab</td>
<td>1.99 b</td>
<td>1.00 a</td>
<td>1.47 a</td>
</tr>
<tr>
<td>Biozote</td>
<td>0.94 b</td>
<td>4.30 b</td>
<td>1.46 c</td>
<td>0.73 a</td>
<td>1.46 a</td>
</tr>
<tr>
<td>LSD</td>
<td>0.0595</td>
<td>0.3812</td>
<td>2.656</td>
<td>0.05954</td>
<td>0.3812</td>
</tr>
<tr>
<td>F-value*</td>
<td>3.763**</td>
<td>6.061**</td>
<td>12.096**</td>
<td>3.856**</td>
<td>6.102 ns</td>
</tr>
</tbody>
</table>

*F-values are from one-way ANOVA; **significant at p < 0.05; ns = non-significant
higher temperatures, free fatty acids react with metals like zinc, lead, manganese and cobalt etc. This could lead to increased engine wear (Romano, 1982). Addition of more sodium hydroxide catalyst compensates for higher acidity but the resulting soap causes an increase in viscosity or formation of gels that interfere in the reaction as well as with separation of glycerol (Freedman et al., 1984). Reduced oil acid value in biofertilizers is favourable for the production of good quality biodiesel at lower catalyst cost with higher yield.

**pH.** Biozote treatment significantly decreased the pH of oil as compared to the control. Chemical fertilizers and Biopower were ineffective in altering the pH. Acidity of vegetable oil is correlated with free fatty acids (Kusdiana and Saka, 2001). In the base-catalyzed method of biodiesel production, the quantity of catalyst used depends very much on the acidity of the vegetable oil. More quantity of basic catalyst is required in case of more acidic oil to neutralize the pH. Alkaline catalysts are deactivated by the presence of large amounts of free fatty acids and then excessive amount of alkali is required for the formation of emulsions; and increase in the viscosity ultimately leads to the formation of gels and is associated with problems in glycerol separation and loss in the yield of methyl esters (Crabble et al., 2001).

**Refractive index.** All the treatments exhibited non-significant variations in the refractive index of seed oil. Nonetheless, maximum increase in the refractive index occurred with chemical fertilizer and Biopower treatments. It has been found that refractive indices of natural fats and oils are related to their unsaturation in an approximately linear way (Rudan-Tasic and Klofutar, 1999). This increase in refractive index of soybean seed oil may be due to the increase in concentration of unsaturated fatty acids by Biopower treatment. Biopower exhibited stimulatory effects on the refractive index of the oil and thereby improving the oil quality, which can be utilized for production of good quality biodiesel, particularly for usage in colder regions.

**Methyl ester content.** Maximum ester content (82%) was obtained from the Biopower-treated oil while 78% from Biozote-treated and 79% from chemical fertilizer-treated oil (Fig. 1). In the transesterification of vegetable oils, a triglyceride reacts with a short chain alcohol in the presence of a catalyst (base or acid), producing a mixture of fatty acid alkyl esters and glycerol (Schuchardt et al. 1998). The methyl ester content produced after transesterification depends upon the type of feedstock, catalyst formulation, catalyst concentration, alcohol to oil ratio and reaction temperature. Free fatty acid content in the reactant mixture plays important role in the biodiesel yield (Refaat et al., 2008). The higher yield of methyl esters from Biopower treated seeds might be due to decrease in free fatty acid content by Biopower. Therefore, it can be inferred that application of biofertilizers and chemical fertilizers significantly increase the overall yield, seed oil content and quantity and quality of biodiesel from soybean. Although chemical fertilizers lead to increase in the yield and soybean oil content but the oil quality decreases due to increase in the specific gravity of oil. On the contrary, biofertilizers not only improve the oil content but also the quality of soybean oil. Thus it can also be inferred that biofertilizers can be supplemented with chemical fertilizers to improve the yield, content and quality of oil, which can be utilized for production of good quality biodiesel on commercial scale. This approach of using microbes is economical, sustainable and environment friendly as biofertilizers have been reported to replace 50% chemical fertilizers (El-Kholy and Gomaa, 2000).

References


Effect of Different Humidity Levels on the Biology of Longtailed Mealy Bug

_Pseudococcus longispinus_ (Targioni and Tozzetti)

(Homoptera: Pseudococcidae)

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**Abstract.** On determining the effects of different humidity levels on the biology of mealy bug _Pseudococcus longispinus_ (Targioni and Tozzetti), it was found that the relative humidity (RH) at 35%, 55% and 75% had no effect on pre-adult development, adult longevity, life span and fecundity of _P. longispinus_. The survival of pre-adult stages was minimal at 35% RH. Sex ratio was male-biased at 35% RH and female-biased at 75% RH.

**Keywords:** Mealy bug; humidity levels; _Pseudococcus longispinus_

**Introduction**

Mealy bugs are mainly of tropical and subtropical origin and many of them have become established as pests. They attack a wide range of plants including fruits, vegetables and ornamentals. The very broad host range of mealy bugs in part explains their success. As sap feeders, they have the potential to be vectors of various viral diseases (Golino et al., 2002; Campbell, 1983; Harris, 1981) and some species are known to inject potent phytotoxins during feeding (Lema and Herren, 1985). Their direct damage takes the form of distortion, stunning and yellowing of foliage, early dropping of the flowers and fruits, sometimes followed by defoliation. Indirectly, their copious secretion of honeydew promotes the growth of sooty moulds which can detract from the aesthetic and economic value of the plants (Hattingh, 1993; Copland et al., 1985; Pritchard, 1949).

The longtailed mealy bug _Pseudococcus longispinus_ (Targioni-Tozzetti) is widely distributed in tropical and sub-tropical regions and in glasshouses in the temperate zone. It is found in the Mediterranean basin, Africa, Southern Asia, Far East, Australia, New Zealand, Pacific Islands, USA and Central and South America (Pantoja et al., 2002; Anon, 1958). _P. longispinus_ is polyphagous and has been recorded as an economic pest of varying importance on citrus, grapevines, avocado, palms, coffee, cocoa, peaches, plums and other horticultural field crops in various parts of the world, especially the southern USA, Australia and New Zealand (Charles, 1981). In Israel, the longtailed mealy bug was recorded on 36 host plants belonging to 29 different botanical families (Wysoki et al., 1977). It was reported as one of the six mealy bug species from citrus pests in Mediterranean basin (Franco et al., 2004).

Clausen (1915) experienced considerable difficulty in measuring the rate of larviposition of _P. longispinus_ due to the disturbing effect it had upon the female. He observed that the young remained clustered under the body of the parent for one or more days after birth. The time required for different stages was variable. Mating took place largely during the third instar and larviposition began within 10 to 15 days after the third moult. James (1937) found three nymphal instars in the female and four in the male. The sexes were indistinguishable externally in the first instar but sexual dimorphism was apparent in the second instar. Gullan (2000) provided identification guide to most of the immature stages of _P. longispinus_ collected from citrus in Australia. A key and table based on microscopic features allow separation of different instars.

Browning (1959) showed that the number of long-tailed mealy bugs on irrigated orange trees in South Australia rose and fell in a fairly regular sequence throughout the year. Panis (1969) described _P. longispinus_ as a viviparous species and showed that light, gravity and host plant quality had a great effect on adult orientation and distribution on plant leaves as well as on its sex ratio. Mating was obligatory for production of eggs and the development of ovaries. Males were capable of several matings.

Furness (1976) showed that dispersion of mealy bugs changed with age. The first-instar crawler dispersed over the whole tree; some second-instar were found in exposed positions but most second- and all third-instar larvae sought sheltered
living sites. Adults reproduced in protected sites. El-Minshawy et al. (1974) found that the duration of all pre-adult stages was highly affected by temperature. In New Zealand, the longtailed mealy bug has three discrete generations on grapevines in a year (Charles, 1981).

A complete biological knowledge of a pest is the pre-requisite for its successful biological control. Very little information is available regarding longtailed mealy bug’s bionomics. Flanders (1940) concluded that longtailed mealy bug is native to Australia. Furness (1976), El-Minshawy et al. (1974), Browning (1959) and James (1937) studied the biology of its different stages. In Auckland (New Zealand), the longtailed mealy bug has three discrete generations on grapevines in a year (Charles, 1981). Wakgari and Giliomee (2003) reported that the natural enemies of longtailed mealy bug were found on citrus. Mani and Krishnamurthy (2004) explained the role of predators in the control of this pest on temperate and tropical fruits. Recently, it was found that limonene, a citrus extract, has promising role in controlling the mealy bugs on tolerant plants (Hollingsworth, 2005).

Keeping in view the extensive importance, the effect of different levels of relative humidity (RH) on the biology of longtailed mealy bug has been investigated.

Materials and Methods

The experiments were carried out in the Department of Environment, Wye College, University of London. The studies were conducted in plastic boxes (27 × 15 × 10 cm) containing one butternut squash, at a constant temperature of 27 °C in an incubator with a continuous photoperiod and light intensity of 7.5 watts/m². Three different RH levels, 35%, 55% and 75%, were produced in these boxes by placing saturated salt solutions of magnesium chloride, magnesium nitrate and sodium chloride, respectively, in small plastic boxes of 75 × 45 × 20 mm dimension, covered with mesh cloth lids. At each RH level, five reproducing female mealy bugs were transferred, one to each butternut squash, within a 25 mm diameter ring cage fastened to the squash with a rubber band. These mealy bugs were removed after 24 hs. The crawlers laid by them were allowed to develop within the cages and their positions were noted. After the first moult, differentiation between the male and female became evident. Observations were made every 24 hs to ascertain the time of moulting and the longevity of each instar.

Ten virgin females were isolated soon after the third moult and each was confined with a male for fertilisation. These fertilised females were released singly on butternut squashes in the ring cages to study the pre-larviposition and the larviposition periods, fecundity total life span. For sex ratio, ten fertilised, newly-emerged 4th-instar females were released singly in the cages on butternut squashes. Their progeny was raised until the determination of gender. Number of males and females was determined after the fourth moult of males. The experiments were conducted in a randomised complete block design with ten replications. The results obtained for developmental stages, fecundity, survival and the total life span were analysed statistically through one-way ANOVA; means were compared and ranked through Fisher’s test at 5% level. The data for sex ratio was pooled separately into the males and females, which had emerged at different humidity levels for chi-squared tests.

Results and Discussion

The female passed through three and the male through four pre-adult instars. The data (Table 1) showed that RH had no effect on the developmental time of any of the pre-adult stages of male P. longispinus and on the overall total developmental period of all stages (P > 0.05, Table 1). Because of the larviposition behaviour of P. longispinus, no observations could be made on the egg incubation period.

<table>
<thead>
<tr>
<th>Humidity level</th>
<th>Mean developmental period in days ± S.E. at 27 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st instar</td>
</tr>
<tr>
<td>75%</td>
<td>13.49±0.15</td>
</tr>
<tr>
<td>n=33</td>
<td>n=33</td>
</tr>
<tr>
<td>55%</td>
<td>13.47±0.32</td>
</tr>
<tr>
<td>n=37</td>
<td>n=37</td>
</tr>
<tr>
<td>35%</td>
<td>13.60±0.14</td>
</tr>
<tr>
<td>n=40</td>
<td>n=40</td>
</tr>
</tbody>
</table>

P<sub>α</sub> value > 0.05 > 0.05 > 0.05 > 0.05 > 0.05

ANOVA one-way Fisher’s test showed no significant difference at P ≤ 0.05; n = number of individuals studied.
Table 2. Effect of three different humidity levels on the development of pre-adult stages of female *P. longispinus*

<table>
<thead>
<tr>
<th>Humidity level</th>
<th>1st instar (Mean ± S.E.)</th>
<th>2nd instar (Mean ± S.E.)</th>
<th>3rd instar (Mean ± S.E.)</th>
<th>Total pre-adult period (Mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75%</td>
<td>13.27±0.14</td>
<td>9.35±0.14</td>
<td>8.47±0.12</td>
<td>31.09±0.34</td>
</tr>
<tr>
<td></td>
<td>n=66</td>
<td>n=66</td>
<td>n=66</td>
<td>n=66</td>
</tr>
<tr>
<td>55%</td>
<td>13.40±0.16</td>
<td>9.50±0.14</td>
<td>8.61±0.11</td>
<td>31.51±0.37</td>
</tr>
<tr>
<td></td>
<td>n=43</td>
<td>n=43</td>
<td>n=43</td>
<td>n=43</td>
</tr>
<tr>
<td>35%</td>
<td>13.42±0.10</td>
<td>9.56±0.13</td>
<td>8.66±0.11</td>
<td>31.64±0.30</td>
</tr>
<tr>
<td></td>
<td>n=20</td>
<td>n=20</td>
<td>n=20</td>
<td>n=20</td>
</tr>
</tbody>
</table>

P < 0.05 for all comparisons. ANOVA one-way Fisher’s test showed no significant difference at P ≤ 0.05; n = number of individuals studied.

RH had no significant effect on the duration of pre-adult stages of the female. *P* values had no significant effect on the overall total developmental period of female *P. longispinus* (*P* > 0.05, Table 2).

RH showed no significant effect on the pre-larviposition and the larviposition periods, fecundity and life span of the female (*P* > 0.05, Table 3).

RH seems to have a significant effect on the sex ratio (Table 4). Primary sex ratio for *P. longispinus* is normally 1:1. The proportion of female significantly increased with the increasing RH (Table 4). At RH 70% the female proportion was significantly more than the expected proportion (c² = 135.08, *P* < 0.001, Table 4) and at RH 35%, significantly less than the expected value (c² = 66.02, *P* < 0.001, Table 4).

In Russia, Oganesyan and Babayan (1979) observed that the host, temperature and RH had a marked effect on the egg viability and duration of embryonic development of *Pseudococcus comstocki* (Kuw.) but in the present study, RH was found to have no significant effect on the developmental periods of either male or female. However, RH affected the survival and the sex ratio. The proportion of females was greater at higher RH compared to lower RH probably due to RH having a significant effect on the survival of all pre-adult stages (pooled sexes) of *P. longispinus* (*P* < 0.05, Fig. 1). The number of individuals which survived at 75% relative humidity level was significantly higher than those which survived at RH 55% and 35%. The lowest survival was observed at RH 35% (*P* < 0.05, Fig. 1).

In Russia, Oganesyan and Babayan (1979) observed that the host, temperature and RH had a marked effect on the egg viability and duration of embryonic development of *Pseudococcus comstocki* (Kuw.) but in the present study, RH was found to have no significant effect on the developmental periods of either male or female. However, RH affected the survival and the sex ratio. The proportion of females was greater at higher RH compared to lower RH probably due to RH having a significant effect on the survival of all pre-adult stages (pooled sexes) of *P. longispinus* (*P* < 0.05, Fig. 1). The number of individuals which survived at 75% relative humidity level was significantly higher than those which survived at RH 55% and 35%. The lowest survival was observed at RH 35% (*P* < 0.05, Fig. 1).

![Fig. 1](image-url). Effect of three different relative humidity levels on the age specific survival of pre-adult stages of *P. longispinus* at 27 °C. [within a group, bars with different letters are significantly different at 5% level (ANOVA)].
reduced survival of females at low RH. The survival of *P. longispinus* (pooled sexes) was significantly higher at higher RH in all the stages and lowest at lower RH. The findings agree with those of Gordon (1984) who stated that the first-instar of insects were more susceptible to lower RH as compared to other stages.

The effect of RH on insect development cannot be described by general rules similar to those that seem to govern temperature effects. However, variations in the RH can have marked effect on the life cycle of insects (Gordon, 1984). Some species show RH optima, from as low as 30% or less to as high as 90%, occasionally with a narrow range permitting high survival. Others show little effect until RH falls below a critical level that is temperature-dependent. The egg and pupal stages are often unaffected by humidity over a very wide range, except at temperature extremes, in contrast to the first-instar larvae which are less tolerant than either the eggs or the second-instar larvae to low humidity (Gordon, 1984).

### References


Flanders, S.E. 1940. Biological control of the long-tailed mealybug *Pseudococcus longispinus*. *Journal of Economic Entomology* **33**: 754-759.


Optimization of Substrate Concentration for Enhanced Citric Acid Production by *Aspergillus niger* M-101

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Citric acid, the most important organic acid used in food industry is produced chiefly by fermentation. Large number of microorganisms including fungi, yeast and bacteria have been employed for citric acid production (Grewal and Kalra, 1995) but most of them are not able to produce commercially acceptable yields except the fungus, *Aspergillus niger*. Citric acid is produced by *A. niger* by submerged fermentation process (Guilherme et al., 2008). Main advantages of using *A. niger* are its ease of handling, its ability to ferment a variety of cheap raw materials and high yields (Soccol et al., 2006). Various substrates like sugar beet molasses, sugar cane molasses, inulin, kurma, date fruit syrup and carob pod (Soccol et al., 2006) have been used for citric acid production by *A. niger*.

Worldwide demand of citric acid is about 6×10⁵ tons per year (Karaffa and Kubicek, 2003). According to an estimate, annually 500,000 tons of citric acid are produced almost exclusively through fermentation by *A. niger* and widely used in food, chemical, pharmaceutical and other industries (Wang and Liu, 1996) for applications such as acidulation, antioxidation, flavour enhancement, preservation as plasticizer and as a synergistic agent (Sarangbin et al., 1993).

At present, citric acid is imported into Pakistan to the tune of more than Rs. 1.5 billion, annually. Pakistan being an agricultural country is producing both cane and beet molasses approximately 2 million tons per year. About 3.5 million tons per year of the molasses are used for the production of ethanol using baker’s yeast. Beet molasses, being a rich source of sucrose, is employed for citric acid production. One of the major objectives of the present study was to optimize substrate (beet molasses) concentration for maximum citric acid production using *A. niger* strain M-101 through submerged fermentation.

Approximate analysis of beet molasses was done according to the method of Ranganna (1986). Composition of molasses used in the study is given in Table 1.

The following fermentation medium was employed for citric acid production (values are in (g/litre)): beet molasses, 150.0; NaNO₃, 4.0; KH₂PO₄, 1.0; MgSO₄ · 7H₂O, 0.23; FeCl₃, 0.02; ZnSO₄, 0.0012; MnCl₂ · H₂O, 0.0012 (pH 4±0.2).

### Table 1. Composition of beet molasses

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Dry weight basis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>19.4</td>
</tr>
<tr>
<td>Dry solids</td>
<td>81.6</td>
</tr>
<tr>
<td>Ash 9</td>
<td></td>
</tr>
<tr>
<td>Total reducing sugar</td>
<td>17.23</td>
</tr>
<tr>
<td>Total sugar</td>
<td>60.16</td>
</tr>
<tr>
<td>Sucrose (nonreducing sugar)</td>
<td>42.93</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.38</td>
</tr>
<tr>
<td>Metal ions (g/kg):</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>0.076</td>
</tr>
<tr>
<td>Cu</td>
<td>0.012</td>
</tr>
<tr>
<td>Mn</td>
<td>0.019</td>
</tr>
<tr>
<td>Zn</td>
<td>0.122</td>
</tr>
</tbody>
</table>

*Author for correspondence; E-mail: aftabnadim@gmail.com*
Pretreatment of beet molasses was carried out using sulphuric acid treatment (Mayilvahanan et al., 1996), after which it was centrifuged and the supernatant was used for citric acid production. Spores of A. niger M-101 were grown for 192 hs and used for fermentation. Citric acid in the filtrate of the fermentation broth was estimated gravimetrically, using pyridine-acetic anhydride (Marrier and Boulet, 1958).

Study of the effect of different sugar concentrations on citric acid production by A. niger M-101 using beet molasses (Fig. 1) showed that the production of citric acid was inversely proportional to the biomass growth up to 15 % sugar concentration but afterwards its production was directly proportional to the biomass growth. Maximum citric acid production (27.25±2.35 g/litre) was observed in the medium containing 150 g/litre initial sugar concentration while biomass was 35.20 g/litre, percentage yield being 21.57. Sugar level of 150 g/litre (15%) is thus optimal for citric acid production. Similar results for the production of citric acid were also reported by Papagianni et al. (2005).

According to Pazouki et al. (2000), high sugar concentration leads to the accumulation of oxalic acid in the culture broth. Kubicek-Pranz et al. (2000) found that triggering citric acid accumulation by placing A. niger in high concentrations of sucrose or glucose is paralleled by a rise in the intracellular concentration of fructose-2,6-diphosphate (Fru-2,6-P$_2$), the strongest activator of PFK1. Higher concentrations of Fru-2,6-P$_2$ were observed in mycelia cultivated on 15 % sucrose concentration, which allows higher yields of citric acid to be obtained (Kubicek and Rohr, 1977). The regulatory osmotic effect of high sugar concentration on citric acid accumulation is due to the possible role of tyrosine kinases in regulating the ion efflux pathways induced by hyper-osmotic stimulation. These kinases are required for restoring the osmotic gradient across the cell membrane in response to increased external osmolarity (Fiedurek, 1998). Facilitated diffusion and active transport, both, are characterized by a non-linear activity response to increasing substrate concentration, whereas simple diffusion has a linear response (Wayman and Mattey, 2000).

Kinetic parameters for citric acid production are shown in Table 2. Value of growth yield co-efficient ($Y_{Y_g}$) tends to decrease with increase in substrate concentration and reached to its minimum value (0.225 g/g). Values of specific product yield coefficient ($Y_{p_s}$) and product yield coefficient ($Y_{ds}$) also tend to increase with increase in substrate concentration and reached the maximum values of 0.810 and 0.215 g/g. Increase in $Y_{p_s}$ value with increase in substrate concentration was also reported by Peksel and Kubicek (2003) who obtained maximum yield (0.141 g/litre) of citric acid at 15 % substrate concentration.

Table 2. Kinetic parameters and coefficients of citric acid fermentation by A. niger at different substrate concentrations

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>10</th>
<th>12.5</th>
<th>15.0</th>
<th>17.5</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate uptake rate ($Q_s$)</td>
<td>0.419</td>
<td>0.498</td>
<td>0.657</td>
<td>0.628</td>
<td>0.586</td>
</tr>
<tr>
<td>Specific substrate uptake rate ($q_s$)</td>
<td>0.007</td>
<td>0.011</td>
<td>0.018</td>
<td>0.021</td>
<td>0.023</td>
</tr>
<tr>
<td>Product yield coefficient ($Y_{p_s}$)</td>
<td>0.098</td>
<td>0.212</td>
<td>0.215</td>
<td>0.187</td>
<td>0.182</td>
</tr>
<tr>
<td>Specific product yield coefficient ($Y_{p_s}$)</td>
<td>0.140</td>
<td>0.469</td>
<td>0.774</td>
<td>0.793</td>
<td>0.810</td>
</tr>
<tr>
<td>Growth yield coefficient ($Y_{ds}$)</td>
<td>0.698</td>
<td>0.452</td>
<td>0.278</td>
<td>0.236</td>
<td>0.225</td>
</tr>
<tr>
<td>Productivity ($Q_p$)</td>
<td>0.041</td>
<td>0.105</td>
<td>0.141</td>
<td>0.117</td>
<td>0.107</td>
</tr>
<tr>
<td>Specific productivity ($q_p$)</td>
<td>0.0007</td>
<td>0.002</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

$Q_s$ = g substrate consumed/litre/h; $q_s$ = g substrate/g cells/litre/h; $Y_{p_s}$ = g citric acid produced/g sugar consumed; $Y_{ds}$ = g citric acid produced/g cells; $Y_{ds}$ = gram cell mass/gram sugar consumed; $Q_p$ = g citric acid produced/litre/h; $q_p$ = g product/g cells/litre/h; all the values differ significantly at $p \leq 0.05$. 

![Fig. 1. Evolution of citric acid and biomass during the fermentation using different substrate concentration.](image-url)
References


Introduction

Recently, many of the South Asian countries are experiencing severe environmental problems due to their rapid industrialisation. This phenomenon is very common where the polluting industries like textile dyeing, leather tanning, paper and pulp processing, sugar manufacturing, etc. thrive as clusters.

Textile dyeing industry consumes large quantities of water and produces large volumes of wastewater at different steps of printing, dyeing and finishing processes, which is often rich in colour, containing residues of reactive dyes and chemicals, and requires proper treatment before being released into the environment. India produces dyestuff and pigments close to 80,000 tonnes and is the second largest exporter of dyestuffs and intermediates among developing countries, after China. The textile industry accounts for the largest consumption of dyestuffs, at nearly 80% (Mathur et al., 2005). During textile production, potentially hazardous compounds at various stages of operations are released with a negative impact on the ecosystem (Asamudo et al., 2005).

Treatment and disposal of effluents from the textile and dyeing industries is quite difficult by common physical and chemical methods, mainly because of the high BOD, COD, heat, colour, pH and presence of metal ions. Besides, they are highly expensive, emit toxic substances and form large amounts of sludge (Johnson et al., 1978), posing disposal problem (Banat et al., 1996). Several physicochemical decolourisation techniques have been reported, a few of which were accepted by the textile industries (DaSilva and Faria, 2003; Okazaki et al., 2002).

Microbial degradation and decolourisation is an environment friendly and cost-competitive alternative to chemical decomposition processes. Many microorganisms belonging to different taxonomic groups of bacteria, fungi, actinomycetes and algae have been reported for their ability to decolourise azo dyes (Khehra et al., 2005). Pure fungal cultures have been used to develop bioprocesses for the mineralisation of azo dyes (Zhang et al., 1999). However, the long growth cycle and moderate decolourisation rate limit the performance of fungal decolourisation system (Banat et al., 1996). In contrast, bacterial decolourisation is normally faster, but it requires a mixed community of bacteria to mineralise azo dyes through a combined metabolic mode of anaerobic-aerobic sequence (Chang et al., 2004). In previous studies, mixed bacterial cultures having the highest decolourisation activity under anaerobic conditions were grown on rich media supplemented with yeast extract or glucose (Chen et al., 2003; Oxspring et al., 1996; Nigam et al., 1996a).
Mixed culture studies may be more appropriate for decolourisation of azo dyes. About 80% of colour removal in an effluent sample containing mixture of azo and diazo reactive dyes was observed by He et al. (2004) using mixed bacterial culture. As the catabolic activities of microorganisms in mixed consortium complement each other, obviously syntrophic interactions present in the mixed communities can lead to complete mineralisation of azo dyes (Chang et al., 2004). Recently, different kinds of dye decolourising bacterial cultures under anaerobic conditions were isolated from textile effluents (Kapdan et al., 2000; Nigam et al., 1996b). In general, a single parameter method or single dimensional search which involves changing one variable while others are kept constant at a certain level, is laborious and time consuming especially when the number of variables is large. An alternative and effective approach is, therefore, increasingly being involved in the use of statistical methods (McDaniel et al., 1976). The Plackett-Burman (PB) experimental design (Plackett and Burman, 1946) was applied to maximise enzyme production and decolourisation efficiency.

Termites, in this study were selected as the source of potent dye-degrading bacteria because they are assumed to harbour microorganisms responsible for digestion of lignocellulosic substances; a complex symbiotic community of prokaryotic and eukaryotic microorganisms has been found in the intestinal tract of termites. The gut flora consists of bacteria, archaea and archaezoa (Konig and Varma, 2006). In this respect, the use of a bacterial consortium has several advantages. Generally, such bacteria do not require sterile conditions, thus greatly reducing costs. Besides, they are usually more resistant to changes in pH, temperature and feed composition as compared to individual species (Forgacs et al., 2004). Finally, there is a higher possibility of complete mineralisation of the dye since few strains have been found which can metabolise such types of compounds (Pearce et al., 2003). Hence the present study is aimed at employing the termite-derived bacterial consortium for decolourisation of Reactive Dye. Sani and Banerjee (1999) found 92, 96 and 96% decolourisation rates of Magenta, Crystal Violet and Malachite Green dyes, respectively, using Kurthia sp. They found higher COD removal efficiency (56-85%) and decolourisation efficiency (19-33 mg/g).

Reactive Blue 222 is one of the reactive azo dyes and has large consumption in the textile dyeing processes. It is a well-known surrogate for non-biodegradable Reactive Azo dyes. In the present study, the decolourisation of Reactive Blue 222 was accomplished by a bacterial consortium isolated from the termite gut. The optimisation conditions were also assessed by the response surface methodology using Plackett-Burman design.

Materials and Methods

Chemicals and media. The dye powder used in this study was obtained from Hi Media, Mumbai. All the chemicals used were of the highest purity or analytical grade, obtained from recognised chemical suppliers. Decolourising medium (DM) was used for decolourisation and the composition (g/litre) was as follows: glucose, 2.0; peptone, 2.0; NaCl, 5.0; CaCl₂, 0.04; MgSO₄, 0.02; distilled water 1000 ml; pH of the medium was adjusted to 7.0. For solid media, agar was added at concentration of 20 g/litre.

Preparation of dye solution. The dyes were used in dissolved form throughout the experiment. One gram of dye was dissolved in deionised water and made up to 1000 ml to give 1000 mg/litre. From the stock solution, required concentrations were prepared. Whenever needed, dye solutions were filter sterilised in 0.45μ membrane filter and aseptically added to the medium.

Development of consortium from termites. Wood-eating termites (Macrotermes sp.), were collected from a soil mound of Western Ghats region, Coimbatore, Tamilnadu, India. Termites were collected in a sterile container and separated from soil and used for bacterial isolation. Collected termites were washed with sterile de-ionised water and surface sterilised with diluted ethanol under aseptic conditions. Ten grams of termites were homogenised in a sterile glass mortar with distilled water and made up to 100 ml. The entire process was carried out under aseptic conditions.

Preparation of inoculum of consortium. Individual bacterial strains (six in all) were grown in nutrient broth for 16-18 hs; one ml of culture was taken from each flask and mixed well. Optical density was adjusted to 1 OD containing the cell concentration of 32.0×10⁴ CFU/ml.

Dye concentration measurement (decolourisation assay). A standard solution of dye was taken and the absorbance was determined at its wavelength to obtain a plot of absorbance vs. wavelength. The wavelength corresponding to maximum absorbance (λ_max) was determined from this plot for the dyes (612 nm for Reactive Blue 222). These wavelengths were used for preparing the calibration curves between absorbance and the concentration of dye solution. During estimation, samples were pelletised by brief centrifugation at 10,000 rpm for 15 mins to prevent absorbance interferences from the cellular or other suspended debris. The absorption spectrum of the clear supernatant was recorded using a spectrophotometer (UV-Vis 3210, Hitachi, Japan). Dye solution incubated without
the inoculum was taken as positive control and uninoculated culture without dye was used as negative control (blank) for the dye and the rate of decolourisation was calculated. Samples with higher concentration of dyes were diluted, when necessary, for accurate determination of the dye concentration in the solution. The absorbance value obtained in each case was then used to calculate percentage decolourisation of the dye. Decolourisation of culture supernatant was measured at the absorbance maxima of dye, as follows:

$$\text{Decolourisation (\%)} = \left\{ \frac{A_i - A_f}{A_i} \right\} \times 100$$

where, $A_i$ and $A_f$ are the initial and the final absorbance values, respectively. Results were corrected according to the blank (dye free) samples.

Optimisation of the operational variables for decolourisation of the dyes. In order to optimize the conditions required for decolourisation, various operational variables were employed such as pH, agitation condition (shaking), static condition, temperature, incubation time, and dye concentration. Influence of sodium salts and phenols on colour removal was also monitored.

Medium optimization for decolourisation of Congo Red by the bacterial consortium. Process optimization could be carried out by empirical or statistical methods. The empirical method is time consuming and does not necessarily enable an effective optimization. A statistics-based procedure called the Response Surface Method (RSM) is a powerful experimental design tool to recognise the performance of composite systems (Kiran et al., 2007; Linder et al., 2005; Ravikumar et al., 2005). The RSM represents an assemblage of experimental design and multiple regression-based methods that can be applied to evaluate tribulations where several factors might influence a response (Gardiner and Gettinby, 1998).

For any microbial decolourisation process, media components such as carbon source, nitrogen source, dye concentration, inoculum size, pH and temperature are the most important parameters which affect the process. The main conventional strategy used is media engineering for which the optimal operating conditions of a process are optimized by changing one parameter at a time and keeping the others at a constant level. This method often does not yield reliable results, and is also laborious, time consuming and impractical. In this regard, the recently developed response surface methodology is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. This methodology consists of Plackett-Burman design as first optimization step and central composite design as a second step to optimize the factors that have significant effects on response surface analysis. This methodology was adopted for the study.

Screening of important nutrient components using Plackett-Burman design. Experimental design and statistical analysis. The Plackett-Burman experimental design was used to evaluate the relative importance of various nutrients for dye decolourisation by the bacterial consortium in static condition. This design presumes that there are no interactions between different media constituents, in the range of variables under consideration. A linear approach is considered to be sufficient for screening.

This study was carried out using Plackett-Burman design for screening medium components with respect to their main effects and not to their interaction effect (Plackett and Burman, 1946) on dye decolourisation by the bacterial consortium. The medium components were screened for eleven (11) variables at two levels, maximum (+) and minimum (-). According to the Plackett-Burman design, the number of positive signs (+) is equal to $(N+1)/2$ and the number of negative signs (-) is equal to $-(N-1)/2$ in a row. A column should contain equal number of positive and negative signs. The first row contains $(N+1)/2$ positive signs and $(N-1)/2$ negative signs; the choice of placing the signs is arbitrary. The next $(N-1)$ rows are generated by shifting cyclically one place $(N-1)$ and the last row contains all negative signs. The experimental design and levels of each variable are shown in Table 1.

The medium was formulated as per design and the flask culture experiments; dye decolourisation was assayed as described earlier. Response was calculated as percentage rate of the dye decolourisation. All experiments were performed in triplicate.

The effect of each variable was calculated using the following equation:

$$E = \left( \sum M^+ - \sum M^- \right) / N$$

where $E$ is the effect of tested variable, $M^+$ and $M^-$ are responses (dye decolourisation) of trials at which the parameter was at its higher and lower levels, respectively, and $N$ is the number of experiments carried out.

The standard error (SE) of the variables was the square root of variance and the significance level ($p$ - value) of each variable calculated by using Student’s $t$ - test.

$$t = E_{wi} / SE$$

where $E_{wi}$ is the effect of the tested variable. The variables with higher confidence levels were considered to influence the response or the output variable.
Optimisation of concentrations of the selected medium components using response surface methodology. Response surface methodology is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously. The screened medium components affecting dye decolourisation were optimized using central composite design (CCD) (Box and Hunter, 1957; Box and Wilson, 1951).

According to this design, the total number of treatment combinations is \(2^k + 2k + n_0\) where ‘\(k\)’ is the number of independent variables and \(n_0\) the number of repetitions of the experiments at the center point. For statistical calculation, the variables \(X_i\) have been coded as \(x_i\) according to the following transformation:

\[
x_i = X_i - X_o / \delta X
\]

where \(x_i\) is dimensionless coded value of the variables \(X_i, X_o\) the value of the \(X_i\) at the center point, and \(\delta X\) is the step change. A \(2^k\)-factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components.

The behaviour of the system was explained by the following quadratic equation:

\[
y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j
\]

where \(Y\) is the predicted response, \(\beta_0\) the intercept term, \(\beta_i\) the linear effect, \(\beta_{ii}\) the squared effect, and \(\beta_{ij}\) is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using Design Expert Version 7.1.5 (State Ease, Minneapolis, MN).

Validation of the experimental model. The statistical model was validated with respect to dye decolourisation under the conditions predicted by the model in flask conditions. Samples were withdrawn at the desired intervals and the dye decolourisation was determined as described above.

Extraction and analysis of the degraded products. After 48 hs of decolourisation, the entire decolourised medium was centrifuged at 5000 rpm for 20 mins. Supernatant of decolourised components of the dyes were extracted using equal volume of ethyl acetate, dried over anhydrous Na\(_2\)SO\(_4\) and evaporated to dryness in a rotary vacuum evaporator. The biodegradation was analysed by FTIR spectroscopy. Biodegradation of Reactive Blue 222 was characterised by Fourier Transform Infrared Spectroscopy (Shimadzu), compared with the control dye and changes in transmission (%) at different wavelengths were observed. FTIR analysis was performed in the mid IR region of 400-4000/cm with scan speed of 16. The pellets prepared using spectroscopic pure KBr (5:95) were fixed in sample holder and analyses were carried out.

Results and Discussion

Optimisation of bioprocess variables using factorial design and response surface methodology for decolourisation of the dye Reactive Blue by bacterial consortium. Plackett-Burman design. In the present study, the influence of eleven factors (A - L) namely pH, temperature, agitation, incubation time, dye concentration, glucose, yeast extract, KH\(_2\)PO\(_4\), chromium, para nitrophenol and trace salts on the dye decolourisation was investigated in 12 runs using Plackett-Burman design. Table 2 represents the Plackett-Burman design for the eleven (11) selected variables and the corresponding

<table>
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<tr>
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<th>pH</th>
<th>Temp (°C)</th>
<th>Agitation (rpm)</th>
<th>Incubation time (h)</th>
<th>Dye conc. (ppm)</th>
<th>Glucose (g/litre)</th>
<th>Amm. nitrate (g/litre)</th>
<th>Mag. sulphate (g/litre)</th>
<th>Chromium (μg/litre)</th>
<th>Phenols (μg/litre)</th>
<th>Trace salts (g/litre)</th>
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response for the dye decolourisation. Variations ranging from 5.5 to 79.4% in the dye decolourization were observed by Plackett-Burman design. Our reports concur with the results observed by Prouty (1990), Bumpus and Aust (1986) and Reid (1979) that dye decolourisation was up to 98.0% within 1-2 days which is similar to our report on Reactive Blue 222 decolourisation. They also reported that dye degradation decreased with the increase in the dye concentration.

The Pareto chart (Fig. 1) illustrates the order of significance of the variables affecting dye decolourisation. Among the variables screened, the most effective factors with high significance level indicated by Pareto chart were in the order of pH, temperature, agitation and glucose. They were identified as the most significant variables in dye decolourisation and selected for further optimization. A report of Sani et al. (1998) suggested that the agitated cultures have often been found more efficient in decolourisation of various dyes, compared to the static ones, presumably, because of an increased mass and oxygen transfer.

Statistical analysis of the Plackett-Burman design demonstrates that the model F value of 0.81 is significant. The values of p < 0.05 indicate that the model terms are significant (Table 3).

Regression analysis was performed on the results and the first order polynomial equation was derived representing dye decolourisation as a function of the independent variables.

Reactive Blue decolourisation (%) = +48.93 + 23.73 A + 9.84 B + 17.83 C + 6.93 F

The magnitude of the effect indicates the level of significance of the variable on the decolourisation of Reactive Blue. Consequently, based on the results from this experiment, statistically significant variables i.e. pH, agitation, temperature and glucose with positive effect were further investigated with central composite design to find the optimal range of these variables.

**Central composite design.** Results of 30 runs of Central Composite Design (CCD) in four variables, pH, agitation, temperature and glucose chosen for the optimisation of decolourisation of Reactive Blue dye by bacterial consortium are shown in Table 4. It shows decolourisation (%) corresponding to the combined effect of four components in their

---

**Table 2.** Plackett-Burman experimental design for evaluating factors influencing dye degradation by the bacterial consortium

<table>
<thead>
<tr>
<th>Run</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>J</th>
<th>K</th>
<th>L</th>
<th>Decolourisation (%)</th>
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<td>1</td>
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</table>

A: pH; B: temperature (°C); C: agitation (rpm); D: incubation time (h); E: dye concentration (ppm); F: glucose (g/litre); G: yeast extract (g/litre); H: KH2PO4 (g/litre); J: chromium (g/litre); K: paranitrophenol (μg/litre); L: trace salts (g/litre)

---

**Fig. 1.** Pareto chart for Plackett-Burman design for 11 factors on decolourisation of Reactive Blue 222 by bacterial consortium.
Decolourisation varied markedly with the conditions tested, in the range of 12.69-99.21%. Decolourisation values of 99.21% was observed at pH 7, agitation 0 rpm, temperature 20 °C and the glucose 6 g/litre (run 30). The experimental results suggested that these variables strongly affect the decolourisation of Reactive Blue. The results obtained were subjected to analysis of variance on Stat-Ease package, with the regression model given as:

\[ Y = 97.17 + 97.17 - 3.13 A - 3.96 B - 4.13 C + 2.96 D + 1.94 AB + 2.94 AC + 0.063 AD - 5.56 BC - 2.94 BD - 1.44 CD - 22.18 A^2 - 12.68 B^2 - 17.68 C^2 - 7.43 D^2 \]

where Y is the response value (percent decolourisation of Reactive Blue) and A, B, C and D are the coded levels of pH, agitation, temperature and glucose, respectively. The adequacy of the model was checked using analysis of variance (ANOVA) and the results are presented in Table 5. The analysis of variance of the quadratic regression model suggested that the model is very significant as was evident from the Fisher’s F-test. The model F value of 9.30 implies that the model is significant. The R² value (multiple correlation coefficient) closer to 1 denotes better correlation between the experimental and predicted values. In this case, the value of R² (0.89) indicates good correlation between the experimental and predicted values. It was also reported that in a study of the stability and kinetics of b-1,3-glucanase from Trichoderma harzianum, a very low value of R² was obtained.

The coefficient of variation (CV) indicates degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case, a low CV (3.14) denotes that the experiments performed are highly reliable. The p value denotes the significance of coefficients and is also important in understanding the pattern of mutual interactions between the variables.

The main goal of response surface is to efficiently hunt for the optimum values of the variables so that the response is maximised (Dey et al., 2001). The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The response surface contour plots are presented in Fig. 2. The contour plots showcase the

Table 3. Analysis of variance (ANOVA) for dye decolourisation

<table>
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<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F-value</th>
<th>p-value</th>
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Table 4. Experimental plan for optimisation of dye decolourisation using central composite design

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<th>Agitation (°C)</th>
<th>Temperature (°C)</th>
<th>Glucose (g/litre)</th>
<th>Decolourisation (%)</th>
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Fig. 2. Three dimensional response surface plot for the effect of (A) pH, agitation; (B) pH, temperature; (C) pH, glucose; (D) agitation, temperature; (E) agitation, glucose; (F) temperature, glucose on decolourisation of Reactive Blue by bacterial consortium.
O-H as functional groups etc. The FTIR spectra of the treated Reactive Blue 222 showed the specific peaks in fingerprint region for primary amine which is supporting the peak at 3469.70 cm⁻¹, 3384.84 cm⁻¹ and 1458.08 cm⁻¹ for stretching vibrations of the functional groups with N-H bonds and primary amines having two bonds. The group frequency region shows specific peaks for functional groups; the peak at 1635.52 cm⁻¹ for -N=N- stretching vibrations represents the presence of hydroxyl (-OH) and secondary amino (-NH₂).

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CV - 3.14; R² - 0.89

behaviour of response (decolourisation percentage) with respect to the simultaneous change in two variables. The roles played by various factors affecting the decolourisation percentage are highlighted by 3D graphs. The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables and combined effects of each independent variable on the response variable.

Validation of the model. The maximum experimental response for decolourisation of Reactive Blue was 99.21% whereas the predicted value was 97.16% indicating a strong agreement between them. The optimum values of the tested variables are pH 7.0, agitation 0 rpm, temperature 20 °C and glucose 6 g/litre as shown in perturbation graph (Fig. 3). The results are encouraging for optimization under pilot scale or industrial scale conditions. Similar R² values were reported by Levin et al. (2005) being 0.993 for Ponceau 2R decolourisation, 0.987 for Malachite Green and 0.968 for Anthraquinone Blue degradation. It suggests that the fitted linear plus interactions models could explain 99.3, 98.7 and 96.8%, respectively, of the total variation.

FTIR analysis. Results of FTIR analysis of the control and the sample obtained after decolourisation showed various peaks. The FTIR spectra of the control displays various significant peaks at 1135.99 cm⁻¹, 1045.35 cm⁻¹, 1018.34 cm⁻¹ and 617.18 cm⁻¹ which supports the presence of secondary amines CN stretch, alkyl substituted ether C-O stretch, thioethers, CH₃ -S -(C - S stretch), stretched vibrations with O-H as functional groups etc. The FTIR spectra of the treated Reactive Blue 222 showed the specific peaks in fingerprint region for primary amine which is supporting the peak at 3469.70 cm⁻¹, 3384.84 cm⁻¹ and 1458.08 cm⁻¹ for stretching vibrations of the functional groups with N-H bonds and primary amines having two bonds. The group frequency region shows specific peaks for functional groups; the peak at 1635.52 cm⁻¹ for -N=N- stretching vibrations represents the presence of hydroxyl (-OH) and secondary amino (-NH₂)
groups in the dye. Considerable difference in the FTIR spectrum of Reactive Blue 222 reveals biodegradation (Fig. 4).

**Conclusion**

The bacterial consortium exhibited greatest ability in decolourising Reactive Blue 222 which seems to be a practical approach. It can be concluded from the study that the bacterial consortium obtained from termites possesses higher colour removal efficiency due to synergistic activity among the strains. FTIR spectra of raw dye and treated degradation products proved that the dye was completely mineralised by the bacterial consortium. This study shows that the response surface methodology is a suitable system to optimise the excellent culture conditions for achieving the maximum decolourisation of dye. The experimental and the predicted values were very close which reflected the accuracy and the applicability of RSM. By applying central composite design and RSM to the optimization experiments, the process variables could be completely investigated and decolourisation values up to 99.21 could be achieved. The culture not only decolourised the dye but it also degraded it as seen in the FTIR spectra. The efficiency of the bacterial consortium supports the merits of using a compatible mixed strain which could be further exploited for treating similar dye-bearing waste waters. Thus biodecolourisation and biodegradation of textile dye Reactive Blue 222 by the isolated bacterial consortium from termite gut can be claimed as an environment friendly method of dye degradation. Further work on identification of the intermediates formed is in progress.

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**References**


Ravikumara, K., Pakshirajan, K., Swaminathan, T., Balu, K.


