

Physical Sciences

- Extractive Separation of Al(III) and Ni(II) by Di-2-Ethylhexyl Phosphoric Acid -Kerosene System from Aqueous Fluoride Medium
Muhammad Fakhru Islam, Dil Afroz Begum, Muhammad Matiur Rahman and Muhammad Saidur Rahman 59
- Studies on the Lipolytic Enzymes of *Sesamum indicum* Seed Powder
Nusrullah Akhtar, Salma Rahman and Abdul Jabbar 66
- An Ecofriendly Synthesis of 4-Thiazolidinone Derivative Using Tributylammonium Bromide Under Microwave Irradiation
Muhammad Naeem, Muhammad Nawaz Chaudhry and Rana Amjad 70

Biological Sciences

- Salicylic Acid Induced Physiological and Biochemical Changes in Wheat Under Drought Stress Conditions
Sami Ullah Khan, Asghari Bano, Jalal Ud Din and Suba Sadiq Tahir 75
- Micronutrient (Zn) Role in Stimulating Root Nodules and Yield of Chickpea
Abdur Rashid 80
- Status of Plant Available Sulphur and its Relationship to Other Soil Characteristics in Pothwar Soils
Rizwan Khalid, Khalid Saifullah Khan, Ghulam Shabbir, Muhammad Yousaf and Shahid Yaqub Naz 84

Technology

- A Weak Current Amperometric Technique in Physiological and Bioelectromagnetic Measurements
Masroor Hussain Shah Bukhari, John H. Miller Jr. and Zahoor Hussain Shah 91
- Heterologous Expression of *Chaetomium thermophilum* Xylanase 11-A (CtX 11-A) Gene
Saiqa Wajid, Shafiq Shahid, Farooq Latif, Zahid Mukhtar, Sher Afzal and Shahid Mansoor 100

Review

- Lobsters from Northern Arabian Sea (Pakistan Coast)
Razia Sultana, Quddusi Begum Kazmi and Shahid Amjad 107

Extractive Separation of Al(III) and Ni(II) by Di-2-Ethylhexyl Phosphoric Acid -Kerosene System from Aqueous Fluoride Medium

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Abstract. In the study of the extractive separation of Al(III) and Ni(II) by di-2-ethylhexyl phosphoric acid D2EHPA-kerosene from aqueous fluoride medium, about 94% Al(III) and 2% Ni(II) were extracted with 0.3 M D2EHPA (pH 2.1 and temperature 30 ± 1 °C). Extraction of Ni(II) decreased with increasing extractant concentration. D2EHPA-kerosene-fluoride system showed better extraction of Al(III) with higher extractant concentration and aqueous pH and *vice versa* for the extraction of Ni(II). The maximum separation factor ($\beta \sim 1380$) was obtained for Al(III) at 20 °C and decreased to ($\beta \sim 732$) at 60 °C. The separation of Al(III) from Ni(II) was favoured at normal temperature. Extraction followed the order Al(III) $\gg \gg$ Ni(II). About 99% stripping of Al(III) was attained from the loaded 0.20 M D2EHPA. Much faster extraction of Al(III) compared to Ni(II) and preferential loading were shown by D2EHPA-kerosene in the presence of fluoride ion in the aqueous phase. Separation of Al(III) was the most outstanding from Ni-Al-F-complex solution.

Keywords: Al-Ni separation, D2EHPA-kerosene system, fluoride medium

Introduction

Liquid-liquid extraction is one of the most promising and effective extraction and separation technique in hydrometallurgy. Particularly phosphorus-based extractant, D2EHPA, (a commercial extractant) has proved to be of particular significance for its wide range of use in the extraction (from divalent to heptavalent) as well as separation of metal ions from different acid solutions (Nasr-Eddine Belkhouche *et al.*, 2005; Islam and Mostafa, 1995; Islam *et al.*, 1988; 1979). Leaching of the spent nickel catalyst from fertilizer factories produces aluminium and nickel containing solutions (Islam and Mostafa, 1993) and a treatment is necessary to separate the two metal ions. Previously, for this purpose, in our laboratory some separation studies had been attempted using various extractants and also with tolyl phosphate using NH_3 medium in the presence of fluoride ions (Islam and Mostafa, 1993) and using Cyanex 272 (Islam and Rahman, 2006). Cobalt-nickel separation using Cyanex 272, Cyanex 301 and Cyanex 302 has also been reported by some workers (Reddy and Sarma, 2001; Tait and Brian, 1993; Chou and Beckstead, 1990; Danesi *et al.*, 1984; Rickelton *et al.*, 1984). However, no report was found on the extractive separation of Al(III) and Ni(II) by D2EHPA from fluoride medium, though several reports are available on Ni(II) and Al(III) from acidic medium. Tributyl phosphate (TBP), di-*o*-tolyl phosphate (HDTP) and versatic acid-10 were attempted for extractive separation.

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TBP and versatic acid-10 were unable to extract Ni(II) from Ni-Al-F solution in NH_3 medium. Di-2-ethylhexyl phosphate extracted Ni(II) from Ni-Al-F complex solution, but phase separation was very difficult owing to emulsion formation in ammoniacal media. It was observed that the waste nickel catalyst from urea fertilizer factories is leached by HF acid more effectively than other leachants like H_2SO_4 , HCl etc. However, it was difficult to separate Al-Ni from such leach solution by conventional precipitation methods.

The efficacy of D2EHPA for Al-Ni separation in acidic fluoride medium was tried in this laboratory and is reported here.

Materials and Methods

Standard solution of Al(III) was prepared by dissolving exactly 17.582 g of analytically pure $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in a one litre volumetric flask and made up to the mark with 1% HNO_3 solution and standardized using spectrophotometric technique as indicated below. Another 1 litre standard solution of Ni (II) was prepared by dissolving exactly 8.956 g of analytically pure $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ in a 1 litre volumetric flask and made up to the mark with 0.1 N HCl solution and standardized. pH of the aqueous solutions was adjusted by using anhydrous Na_2CO_3 /dilute H_2SO_4 if needed.

The extractant D2EHPA, having 98% 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 D2EHPA (1 M) was prepared by weighing out exactly 658.02 g of D2EHPA in a 1 litre volumetric flask and diluting by distilled kerosene. Extractant solutions of different concentrations were prepared by proper dilution of the stock with distilled kerosene.

A definite aliquot (20 ml) of an aqueous phase was taken in a 125 ml reagent bottle and to it same aliquot (20 ml) of organic phase was added. The bottle was stoppered and shaken for a definite time period in a thermostatic water bath at 30 °C (except for temperature dependence). After attainment of equilibrium, phases were allowed to settle and disengaged. The aqueous phase equilibrium, pH and the metal ion concentrations were determined.

For the stripping study, 20 ml organic phase of 0.20 M D2EHPA-kerosene loaded with Al(III) and Ni(II) were taken in a 125 ml reagent bottle and to it, 20 ml of 6 N HCl was added as a stripping agent and shaken for 5 min to transfer metal content to the aqueous HCl phase. Two layers are formed and settled for 5 min. The aqueous HCl phase was separated and measured spectrophotometrically to obtain metal extracted in the organic phase. The metal content, Al(III) and Ni(II), of the aqueous phases were determined by subtracting the values of organic phase metal ion contents from the initial aqueous phase values. In the case of loading test, the organic phase was repeatedly contacted with fresh equal volumes of aqueous solutions until saturation of the organic phase with the metal was attained.

Al(III) ion concentration in the aqueous phase was estimated by Aluminon method (Scott, 1939) and Ni(II) ion concentration was determined by dimethyl-glyoxime colorimetric method (Vogel, 1989) using spectrophotometer ANA-75 (Tokyo Photoelectric Company). In all the cases, phase separation occurred readily, pH values of the aqueous solutions were measured by the digital pH meter (HD-700, Japan). Distribution ratio (D) of metal ion was calculated as the ratio of metal ion concentration in the organic phase to that in the aqueous phase at equilibrium.

Results and Discussion

Effect of phase- contact time. Figure 1 represents variation of distribution ratio on contact time. In both the cases of Al(III) and Ni(II), it was found that the extraction ratio increased continuously with the increase of phase contact time up to a period of 9 min and after that it remained unchanged with further increase of contact time. Extraction plots became horizontal after 10 min for both Al(III) and Ni(II) and on further contact for a longer time, extraction ratio did not increase. Extraction of Al(III) is about 80% and 85% at 5 and 10 min,

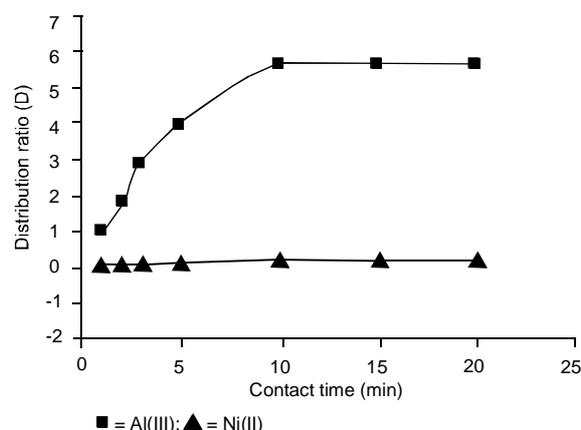


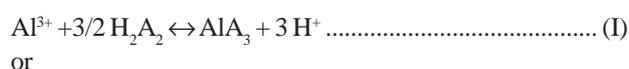
Fig. 1. Effect of distribution ratio of Al(III) and Ni(II) extraction by D2EHPA- F⁻-kerosene system on contact time.

Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; (SO₄⁻)_(ini) = 0.263 M; pH_(ini) = 2.10; D2EHPA = 0.10 M; phase ratio (O/A) = 1; F⁻ = 1.00 g/litre as NaF; temperature = 30±1 °C; diluent = kerosene.

respectively, for 0.10 M D2EHPA. On the contrary, extraction of Ni(II) was about 8% and 9% for the same duration. Therefore, it is concluded that equilibrium was attained within 10 min for both the metal ions. Equilibrium time was 9 and 2 min for Al(III) and Ni(II), respectively, by Cyanex 272 in kerosene systems (Islam and Rahman, 2006) and 30 and 10 min by HDTP in kerosene (Islam and Mostafa, 1995). It is concluded that extraction of Al(III) and Ni(II) from fluoride medium by analytical grade D2EHPA in kerosene system is much faster. Thus, in subsequent experiments, 10 min mixing was allowed to ensure equilibrium. Maximum separation factor β in this respect was found to be ~ 51 within 2 min.

Effect of equilibrium pH on the distribution of Al(III) and Ni(II) for extraction by D2EHPA- F⁻-kerosene system.

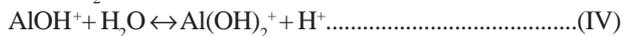
The result obtained, from the experiments at different equilibrium pH, is plotted as log D (distribution ratio) vs equilibrium pH. Fig. 2 shows that extraction of Al(III) ions increases with increase of aqueous phase equilibrium pH. However, extraction of Ni(II) remain unchanged with the increase of aqueous phase pH. In the case of Al(III), slopes of lines were calculated and the values are 0.50, 0.54, 0.54 and 0.50 for 0.05, 0.10, 0.20 and 0.30 M D2EHPA systems, respectively. But in the case of Ni(II) extraction, slope of the plots became zero for all the extractant concentrations investigated. Thus, extraction of Al(III) by D2EHPA did not give a pH dependence value of 3 as expected from the equilibrium reaction:



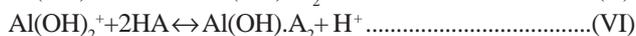
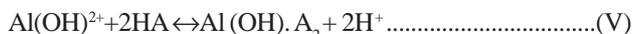
or



where: dimeric(H_2A_2) and monomeric(HA) extractant species were assumed. A very low aqueous pH dependence slope cannot be accounted for by simple ion exchange mechanism though a linear dependence on aqueous acidity is observed. The existence of hydrolyzed species of the form $Al(OH)^{2+}$ or $Al(OH)_2^+$ in the aqueous phase formed by the hydrolysis reaction may promote Al(III) extraction by the following reactions.



at the acidity of the aqueous phase, where complete hydrolysis to $Al(OH)_3$ is not supposed.



Where, HA is the monomer molecule of D2EHPA

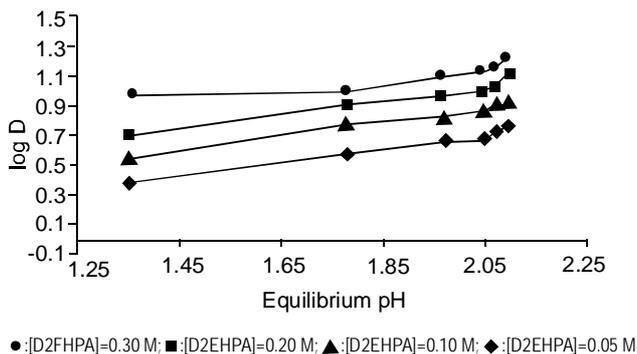


Fig. 2. Effect of distribution ratio of Al(III) on equilibrium pH for D2EHPA- F- kerosene system.

Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(SO_4^{=})_{(ini)} = 0.263$ M; $pH_{(ini)} = 1.40, 1.90, 2.40, 3.00, 3.70$ and 4.30 ; phase ratio (O/A) = 1; $F^- = 1.00$ g/litre as NaF; temperature = 30 ± 1 °C; diluent = kerosene.

Thus, less than unity slope may indicate the hydrolyzed Al species extraction. Almost zero slope for Ni(II) extraction in the presence of F^- is difficult to explain. So, Ni(II) extraction is pH independent in the range of the studied pH. F^- ion may catalyze the formation of hydrolytic species in the case of Al and retard Ni extraction(Fig. 3).

Effect of D2EHPA concentration on extraction of Al(III) and Ni(II). Figures. 4 and 5 represent variations in distribution ratio (D) of cations on the extractant concentration. Experiments were carried out at extractant concentrations of 0.05, 0.10, 0.20 and 0.30 M and at aqueous phase pH of 1.40, 1.90, 2.40, 3.00, 3.70 and 4.30. It was observed that the extraction of Al(III) increased with increasing extractant concentrations

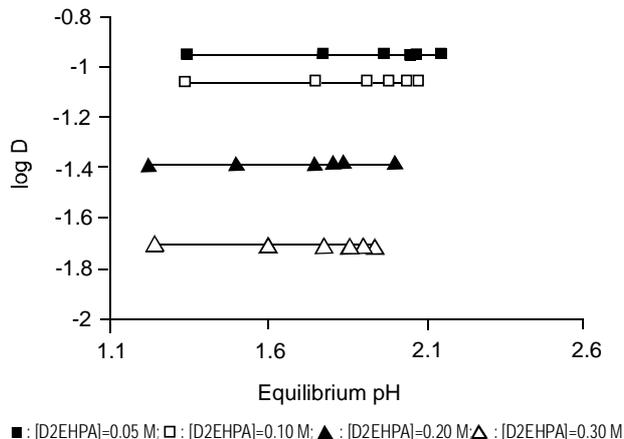


Fig. 3. Effect of distribution ratio of Ni(II) on equilibrium pH.

Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(SO_4^{=})_{(ini)} = 0.263$ M; $pH_{(ini)} = 1.40, 1.90, 2.40, 3.00, 3.70$ and 4.30 ; phase ratio (O/A) = 1; $F^- = 1.00$ g/litre as NaF; temperature = 30 ± 1 °C; diluent = kerosene.

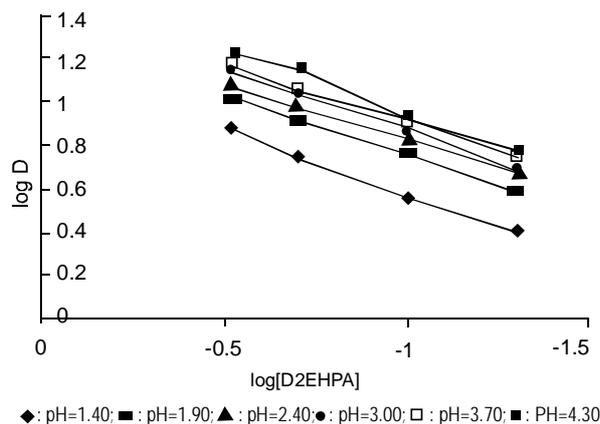


Fig. 4. Effect of distribution ratio of Al(III) on extractant concentration for D2EHPA - F-kerosene system.

Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(SO_4^{=})_{(ini)} = 0.263$ M; (D2EHPA) = 0.05,0.10,0.20 and 0.30 M; phase ratio (O/A) = 1; $F^- = 1.00$ g/litre as NaF; temperature = 30 ± 1 °C; diluent = kerosene.

in the organic phase, and the straight-line relationship was obtained for distribution ratio and extractant concentration. Slopes of the lines are 0.60, 0.60, 0.50, 0.60, 0.50 and 0.50 for pH values of 1.40, 1.90, 2.40, 3.00, 3.70 and 4.30 respectively. Here, linear relationship of the extraction ratio with the D2EHPA concentration indicated that with increasing extractant concentration, extraction of Al(III) improved. On the contrary, extraction of Ni(II) was extremely negligible and decreased with increasing extractant concentration and was independent of the pH of aqueous solutions. Since the extraction of Al(III) is significant and the extraction of Ni(II) is the same for

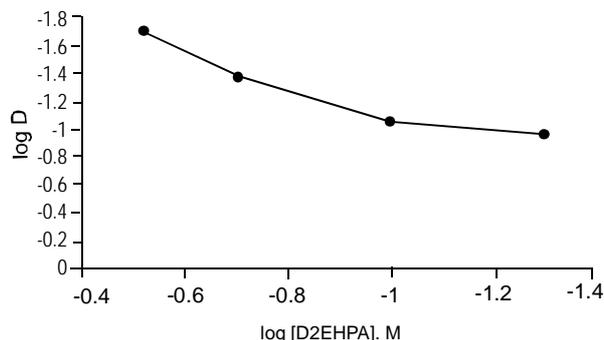


Fig. 5. Effect of distribution ratio of Ni(II) on extractant concentration for D2EHPA-F- kerosene system. Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(\text{SO}_4^-)_{(\text{ini})} = 0.263 \text{ M}$; D2EHPA = 0.05, 0.10, 0.20 and 0.30 M; $\text{pH}_{(\text{ini})} = 1.40, 1.90, 2.40, 3.00, 3.70$ and 4.30; phase ratio (O/A) = 1; $\text{F}^- = 1.00 \text{ g/litre}$ as NaF; temperature = $30 \pm 1 \text{ }^\circ\text{C}$; diluent = kerosene.

different pH values, a single curve was obtained due to very low extraction of Ni(II). It is assumed that with increasing extraction of Al(III), extraction of Ni(II) is hindered. Thus, Al(III) cation has higher affinity than Ni(II) towards D2EHPA in fluoride medium. Converting the distribution coefficient to percentage extraction shows that about 94 % Al(III) and 2% Ni(II) were extracted at the equilibrium pH of 2.1.

The slope of extractant dependence indicates that normal ion exchange mechanism for unhydrolyzed Al(III) is not followed. Rather hydrolyzed and polymerized species extraction is indicated by the extraction data. Here the dependence of Al(III) on extractant concentration indicates that 2:1 complexes are formed in the extraction process, as suggested before which may explain low pH and extractant dependence.

Effect of temperature on Al(III) and Ni(II) extraction by D2EHPA- F -kerosene system. Figure 6 shows variation of distribution ratio on temperature. In both the cases of metal ions, extraction ratio increased with increasing temperature. Slopes of the lines are 0.44 and 0.45 for Al(III) and Ni(II), respectively, at 0.20 M D2EHPA. Extraction reaction enthalpy changes (ΔH) has been calculated using Vant-Hoff equation. The calculated values are 8.50 and 8.70 kJ/mol for Al(III) and Ni(II), respectively. Positive enthalpy change suggests that the extraction process for both the ions is endothermic in nature. The distribution ratio/temperature relationship indicates that extraction reaction of the two is strongly influenced by temperature. Thus, the extraction using the system under study can be moderately increased by using a higher temperature.

Loading of D2EHPA by Al(III) and Ni(II). The loading capacity is defined as the amount of metal content in grams extracted per 100 g of an extractant. It is an important parameter for the

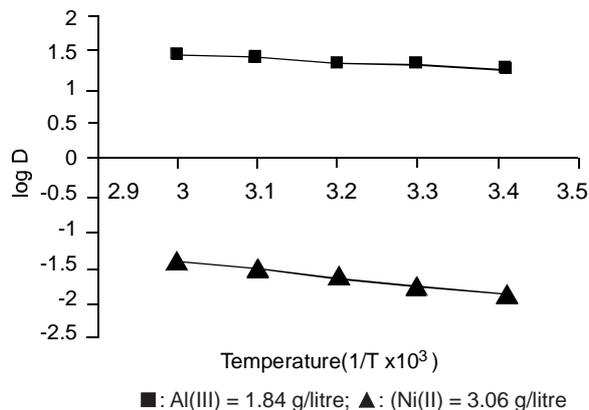


Fig. 6. Effect of distribution ratio of Al(III) and Ni(II) for D2EHPA- F -kerosene system on temperature. $(\text{SO}_4^-)_{(\text{ini})} = 0.263 \text{ M}$; $\text{pH}_{(\text{ini})} = 4.00$; D2EHPA = 0.20 M; phase ratio (O/A) = 1; $\text{F}^- = 2.45 \text{ g/litre}$ as NaF; diluent = kerosene.

study of extraction mechanism and also for the industrial evaluation of the extractant, as a high value of loading capacity is desirable for any particular extractant metal system for industrial applications.

The same organic phase (20 ml) was repeatedly contacted for 10 min at $30 \text{ }^\circ\text{C}$ with the fresh equal volumes of aqueous solutions containing fixed concentration of Al(III) and Ni(II) ions. For loading, 0.10 and 0.20 M [D2EHPA] were used. Aqueous pH 4.10 was used because of the instability of the metal ions in the aqueous phase at higher pH values. After equilibration, the phases were rapidly disengaged to avoid any mass transfer and the aqueous phases were analyzed for metal content. The amount of metal ions transferred into the organic phase for each contact was then determined by difference and the cumulative concentration of ions in the organic phase was estimated. The obtained results plotted as cumulative metal content vs contact number is given in Fig.7 and 8. In these figures, it is seen that the organic phase is saturated with Al(III) and Ni(II) within 4 to 5 contacts. Under the stated, experimental conditions, 2.50 g Al(III) and 0.035 g Ni(II) are extracted at pH 4.10 with 0.20 M D2EHPA giving loading capacity of 12.50 g Al(III) and 0.00175 g Ni(II) for 1 mole D2EHPA. For 0.10 M D2EHPA at the same pH, loading capacity was 12.8 g Al(III) and 0.0035 g Ni(II). Loading test data suggests the extracted species as $\text{Al}(\text{HA})_2$.

Separations studies: Effect of aqueous phase acidity and extractant concentration on Al-Ni separation factor (β). The separation factor (β) for Al(III) and Ni(II) is calculated for each extractant concentration 0.05, 0.10, 0.20 and 0.30 M D2EHPA, at initial aqueous pH values 1.40, 1.90, 2.40, 3.00, 3.70 and 4.30, respectively, and plotted as separation factor

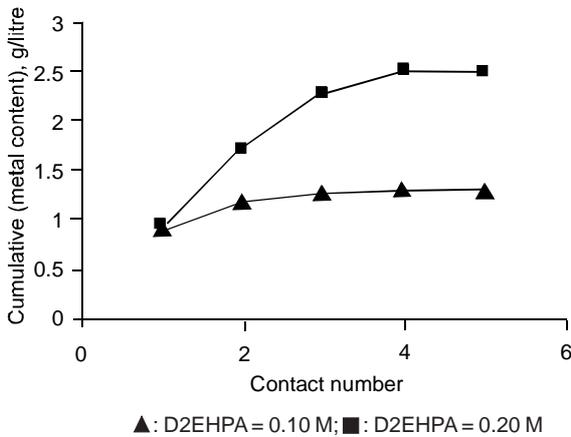


Fig. 7. Loading of Al(III) by D2EHPA from aqueous fluoride medium.
 Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(SO_4^{2-})_{(ini)} = 0.263$ M; $pH_{(ini)} = 4.10$; phase ratio (O/A) = 1; $F^- = 1.00$ g/litre as NaF; temperature = 30 ± 1 °C; diluent = kerosene.

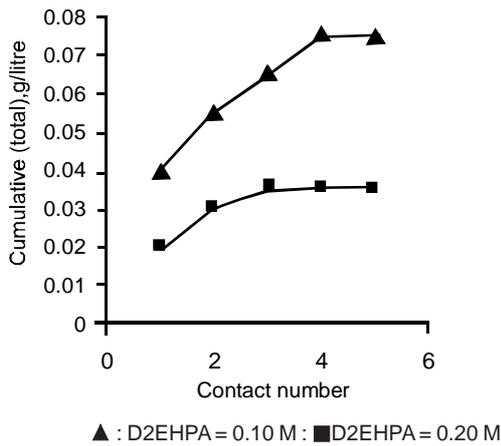


Fig. 8. Loading of Ni(II) by D2EHPA.
 Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(SO_4^{2-})_{(ini)} = 0.263$ M; $pH_{(ini)} = 4.10$; phase ratio (O/A) = 1; $F^- = 1.00$ g/litre as NaF; temperature = 30 ± 1 °C; diluent = kerosene.

(β) vs equilibrium pH in Fig. 9. It is seen from the plots that the separation factor increases with the decrease in aqueous phase acidities at all concentration of D2EHPA. Thus increase of pH favours the separation of Al(III) from Ni(II). The separation factor increases sharply with the increase of extractant concentration (Fig. 10). The increased extractant concentration decreases the Ni(II) concentration to a negligible value. Consequently, Al(III) can be almost completely removed from Ni(II) solution. Separation factor, β , values of more than ~650 were obtained at equilibrium pH 1.94 and 0.30 M extractant concentration, which may be due to the higher affinity of Al(III) for the extractant. It may be concluded that at high pH and higher extractant concentration, Al(III) and Ni(II) can be separated efficiently.

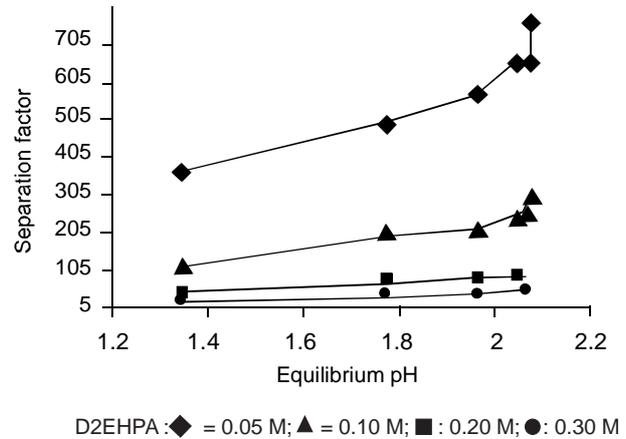


Fig. 9. Effect of aqueous phase acidity on separation factor (β).
 Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(SO_4^{2-})_{(ini)} = 0.263$ M; $pH_{(ini)} = 1.40, 1.90, 2.40, 3.00, 3.70$ and 4.30 for each respectively; phase ratio (O/A) = 1; $F^- = 1.00$ g/litre as NaF; temperature = 30 ± 1 °C; diluent = kerosene.

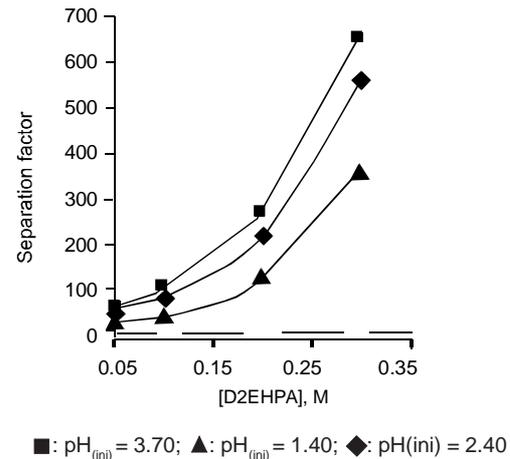


Fig. 10. Effect of extractant concentration on separation factor (β).
 Al(III) = 1.00 g/litre; Ni(II) = 0.50 g/litre; $(SO_4^{2-})_{(ini)} = 0.263$ M; D2EHPA = 0.05, 0.10, 0.20 and 0.30 M; phase ratio (O/A) = 1; $F^- = 1.00$ g/litre as NaF, temperature = 30 ± 1 °C; diluent = kerosene.

Effect of temperature of extraction on separation factor (β).
 The separation factor (β) is graphically represented as function of absolute temperature (Fig. 11). It is evident from that the separation factor decreases with the increase in temperature. However, extraction is still much higher for Al(III) than Ni(II). Maximum separation factor β (1380) was obtained for Al(III) at 20 °C and decreased to 732 at 60 °C temperature. Thus, it is concluded that the ordinary temperature favoured better separation of Al(III) from Ni(II) ions from D2EHPA-kerosene-fluoride medium.

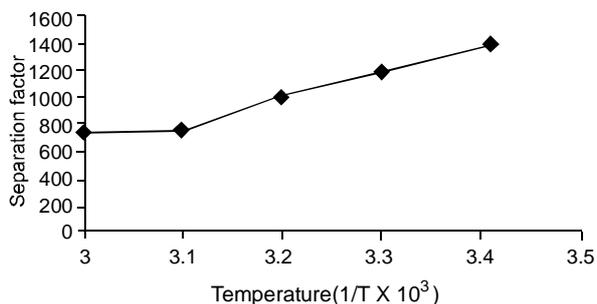


Fig. 11. Effect of temperature on separation factor (β). Al(III) = 1.84 g/litre; Ni(II) = 3.06 g/litre; $(\text{SO}_4^-)_{(\text{ini})} = 0.263$ M; $\text{pH}_{(\text{ini})} = 4.00$; D2EHPA = 0.20 M; phase ratio (O/A) = 1; F = 2.45 g/litre as NaF; diluent = kerosene.

Stripping of Al(III) and Ni(II) from loaded organic phase. In industrial applications, the back extraction of loaded organic phase is as important as the forward extraction. The metal loaded organic phase was stripped with aqueous HCl. The data obtained from the experiment is plotted (Fig. 12) as stripping duration (min) vs percentage of stripped metal ions. It was observed that about 87.18% and 70% of Al(III) and Ni(II) ions, respectively, were stripped from the organic phase within 5 min, about 96.8% and 90%, within 10 min by 6 N HCl and about 99% and 95%, within 15 min. However, with prolonged time for stripping, extraction percentage did not increase, which indicates that the back extraction equilibrium is attained within 15 min stripping of the loaded organic phase. However, the selective stripping of these metals is not pos-

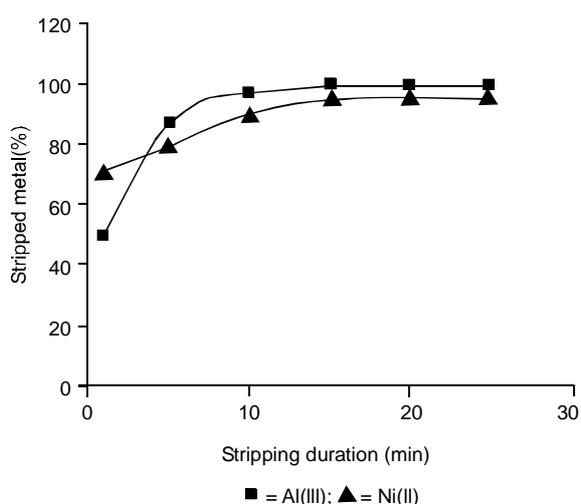


Fig. 12. Effect of time on stripping of loaded organic phase. $\text{Al(III)}_{\text{org}} = 0.78$ g/litre; $\text{Ni(II)}_{\text{org}} = 0.02$ g/litre; stripping agent = 6 N HCl; D2EHPA = 0.20 M; phase ratio (O/A) = 1; F = 1.0 g/litre as NaF; temperature = 30 ± 1 °C; diluent = kerosene.

sible. Separation is obtained only in the forward extraction of the aqueous phase.

Conclusion

D2EHPA is an exceptionally effective extractant for the extraction and separation of Al(III) and Ni(II) from an aqueous fluoride medium containing Al(III) and Ni(II). The equilibrium is reached within 10 min for Al(III) and Ni(II). The maximum separation factor ($\beta \sim 1380$) is obtained for Al(III) with respect to Ni(II) at 20 °C and decreased to ($\beta \sim 732$) at 60 °C temperature. D2EHPA loading capacity is 12.50 g Al(III) and 0.00175 g Ni(II)/mole D2EHPA. The data indicates that simple ion exchange mechanism is not followed for the extraction of Al(III) from fluoride medium. Extraction involves the hydrolyzed species of Al(III) as the acid dependence values are only ~ 0.50 . Extraction of Ni(II) is negligible in the fluoride medium. From the temperature dependence data, the extraction reaction enthalpy change (ΔH) value suggests that the extraction process is endothermic in nature and ΔH values are found to be 8.50 and 8.70 kJ/mol, respectively, for Al(III) and Ni(II) by D2EHPA.

Therefore, the extractant D2EHPA has good prospect for use in the treatment of leach liquors, from the waste nickel catalyst which may be obtained from HF leaching of the waste nickel catalyst from the reforming process of natural gas in the fertilizer plants.

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Studies on the Lipolytic Enzymes of *Sesamum indicum* Seed Powder

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Abstract. Optimum conditions for the hydrolysis of simple triglycerides and phosphoglycerides for the activity of the lipolytic enzymes (lipase and phospholipase) extracted from the defatted seeds of *Sesamum indicum* were established for use in laboratory and industry. The enzymes showed optimum activity at 40 °C and pH 7 in aqueous media. N-heptane was found to be the most satisfactory solvent for maximum activities. The activity of lipase extracted from germinated seeds increased with the stage of seed development, but was reverse for the phospholipase activity.

Keywords: lipase/phospholipase activity, solvent media, triglycerides, lipolytic enzymes

Introduction

Sesamum indicum DC. oil is reckoned equal to olive oil in medicinal properties, especially in the treatment of ulcers, psoriasis, prurigo, leucoderma and wounds (Nadkarni, 1982).

Earlier studies on sesame lipids are available (Javed *et al.*, 2000; Toro-Vazquez *et al.*, 2000; Yashida *et al.*, 1995; Kamal and Appelqvist, 1994) but research concerning its lipolytic enzymes (lipase and phospholipase) has so far not been reported. Enzymes play an important role in *in vivo* synthesis as well as metabolism of a number of organic compounds in the animal and plant kingdom.

In the present studies, enzymes from mature and germinated seeds of sesame were extracted and the enzymatic activity of lipase and phospholipase was investigated at different temperature, pH, aqueous media and organic solvents. The objective was to establish optimum conditions for the hydrolysis of simple triglycerides and phospho-glycerides by lipase and phospholipase, respectively, therefore, these conditions can be applied both in the laboratory and industry. Such investigations were also made earlier on wheat grains, castor bean, oat grains and corn (Banu and Serban, 1970; Berner and Hammond, 1970; Ory, 1969; Ferrigan and Geddes, 1958). Similar studies on *Cassia* sp., *Nicotiana rustica*, *Zea mays*, *Carum capticum*, *Citrullus* sp., of local origin were carried out at PCSIR Laboratories (Waheed *et al.*, 2002; Javed *et al.*, 1999; Ahmad *et al.*, 1993; Aman and Akhtar, 1991; Zaka *et al.*, 1989). The present work on sesame is thus an extension of the earlier studies.

Materials and Methods

Extraction of lipase and phospholipase. Dried seeds of sesame collected from local market, were ground to a fine powder and defatted in a soxhlet extractor with diethyl ether. The defatted seed powder (50 g) was suspended in 200 ml citrate buffer (citric acid 0.1 M and disodium hydrogen phosphate 0.2 M) of pH 7 in 500 ml conical flask and was shaken at 200 rpm for one h at 40 °C, using a Gallenkamp orbital shaker. The supernatant containing enzymes was obtained by centrifugation for 15 min at 12,000 rpm. The extract was diluted to 200 ml with citrate buffer and utilized to study enzyme activities under different condition (Waheed *et al.*, 2002).

Preparation of substrate. Olive oil (Italian origin) was purchased from local market and its triglycerides were separated and purified by thin layer chromatography. Triglycerides (1 g) were emulsified by blending with 10% gum acacia solution (aqueous media) to determine lipase activity, whereas, 10% egg lecithin (BDH, England) emulsion was used as substrate for the phospholipase activity (Kausar and Akhtar, 1979). Hydrolysis of the two substrates by enzymes (lipase and phospholipase) extracted from mature seeds under different parameters is described below.

Effect of pH. The enzyme extract was shaken for one h at 40 °C and 200 rpm in the presence of substrates (triglycerides or lecithin emulsion) separately with citrate buffer (pH 7) and calcium chloride (0.1 M). The released fatty acids after extraction with 5 ml hexane: chloroform (1:1, v/v), were treated with 2.5 ml of Cu- TEA reagent in a test tube, shaken for 5 min and then centrifuged. The upper layer (3 ml) was reacted with 0.5 ml of 0.1% sodium diethyldithiocarbamate which resulted in golden yellow colour, whose absorbance (A) at a fixed wave length (440 nm) was recorded on a spectrophotometer

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(Beckman, model 24, England) against a blank prepared by boiled enzyme powder. A linear standard curve was drawn between the concentration, 80 µg/litre - 800 µg/litre of palmitic acid against the absorbance (A 0.300 - A 0.500) at fixed wavelength (440 nm). The standard curve was used to calculate µ equivalent of fatty acids released per g/h. The activity of lipase or phospholipase was calculated according to Guven *et al.* (1979) as follows.

$$\text{Lipase/phospholipase activity } (\mu\text{U}) = \frac{\text{concentration of fatty acid}}{1000} \times 8$$

Experiments were conducted with citrate buffer solutions to observe the effect of pH (3.0-8.0) on hydrolysis of substrates.

Effect of temperature. Experiments to study the hydrolysis of substrate were conducted by changing the incubation temperature from 20 °C to 70 °C at 10 °C intervals under the same conditions as mentioned above.

Effect of solvents. Defatted seed powder (1 g) was placed in a 50 ml stoppered conical flask containing 50 µ litre water and 5 ml liquid triglyceride: solvent (1:9) to observe the effect of various organic solvent on lipase activity. Lecithin solvent (1:9) was used to study the effect of solvents on phospholipase activity. The above mixtures were shaken for 2 h at 40 °C (Blain *et al.*, 1976). The mixture was cooled to room temperature and an additional 3 ml of solvent was added and thoroughly mixed. The rest of the experiment was conducted as indicated in the the effect of pH.

Lipase and phospholipase activities in germinated seeds. Seeds of sesame were germinated in an incubator at 30 °C ± 1 °C (Javed *et al.*, 1999). Seedlings with roots at root lengths of 5, 10, 15, 20, 25 and 30 mm were dried and crushed separately. The lipase and phospholipase, extracted (see section i) from above various root lengths, were assayed on substrates (triglycerides and lecithin) with buffer solution of pH 7 and an incubation temperature of 40 °C. The fatty acids

released were measured from the standard curve and enzymatic activity was calculated.

Results and Discussion

Enzyme systems play an important role in the synthesis and breakup of a number of organic compounds in animals and plants. The present study is concerned with the lipase and phospholipase enzymes of sesame seed which are involved in the degradation of lipids. These enzymes hydrolyse triglycerides and phosphoglycerides, respectively, and the liberated fatty acids serve as indicator of their activity. The defatted material (meal) of resting and germinated seeds of sesame after treatment of citrate buffer under specific conditions was centrifuged to get lipase and phospholipase enzymes for studying their activities under different parameters.

Fatty acids develop golden yellow colour on treatment with cupric nitrate, triethanolamine and diethyldithiocarbamate solution. In the present study, the absorbance of golden yellow colour was measured by spectrophotometer at 440 nm. Concentration of fatty acids is directly proportional to the development of colour showing the activity of the particular enzyme. The concentration on the basis of absorption was determined with the help of a standard curve drawn between the concentrations of palmitic acid against the absorbance at the same wavelength as mentioned above. The activity of lipase and phospholipase was calculated by Guven's method.

The lipase and phospholipase activities were determined under different conditions of pH, temperatures and solvents. The conditions of pH and temperature, which gave better activity of lipase and phospholipase to mature seeds in aqueous media, were also applied to germinated seeds.

The lipase and phospholipase activities of defatted seeds in the pH range of 5.0 to 8.0 were studied by carrying the experiment for 1h (Table 1). Data showed that the activity of lipase in neutral media (pH 7) was maximum (2.53 µU). In case of phospholipase, maximum activity (2.42 µU) was also obtained at pH 7. Optimum pH 7 is also reported for these

Table 1. Lipase and phospholipase activity of mature seeds at different pH

pH	Lipase			Phospholipase		
	Absorption(A) at 440 nm	Conc. of F.A. (µ equiv./g/h)*	Activity (µ U)**	Absorption (A) at 440 nm	Conc. of F.A. (µ equiv./g/h)*	Activity (µ U)**
3	0.324	103	0.82	0.321	90	0.72
4	0.333	142	1.12	0.328	122	0.98
5	0.350	218	1.74	0.348	212	1.70
6	0.368	298	2.38	0.364	282	2.25
7	0.372	316	2.53	0.369	303	2.42
8	0.358	253	2.02	0.354	238	1.89

* = taken from the standard curve (Guyen *et al.*, 1979); ** = calculated on dry matter basis (Guyen *et al.*, 1979)

enzymes in other seeds such as groundnut, coconut, maize, wheat and almond (Akhtar *et al.*, 1975). It was observed that pH 7 played a vital role for the best activity in both lipase and phospholipase. Other studies were carried out by adjusting the reaction media to pH 7 and varying the reaction temperature and by changing the solvent in the media.

The activities of lipase and phospholipase in the defatted mature seeds were determined under various temperature conditions i.e., 20 °C -70 °C at pH 7 for 1 h. The optimum activity of lipase and phospholipase (2.49 μ U, 2.46 μ U, respectively) was found to be at 40 °C for both the enzymes (Table 2). The activity decreased when the temperature was increased or decreased from 40 °C. These observations show that these enzymes are more active at 40 °C and are in accor-

dance with the studies of Kenaf seed lipase (Kausar and Akhtar, 1979) showing its maximum activity at 40 °C.

A set of experiments was also conducted at pH 7 and 40 °C in which different organic solvent suspensions were used in the media to determine the most appropriate solvent for hydrolysis of triglycerides and lecithin substrates by lipase and phospholipase of mature seeds. The *n*-heptane proved to be the best solvent for optimum enzymatic activity for both enzymes as compared to cyclohexane, di-isopropyl ether and cyclo-hexanol. The observed order of activity was *n*-heptane > cyclohexane > di-isopropyl ether > cyclohexanol, values being 2.32 > 1.79 > 0.90 > 0.61 μ U for lipase and phospholipase 2.16 > 1.70 > 0.75 > 0.48 μ U, respectively (Table 3); it was due to straight chain structure and non polar nature of *n*-heptane. This pattern of

Table 2. Lipase and phospholipase activity of mature seeds at different temperatures

Temp. °C	Lipase			Phospholipase		
	Absorption(A) at 440 nm	Conc. of F.A. (μ equiv./g/h)*	Activity (μ U)**	Absorption (A) at 440 nm	Conc. of F.A. (μ equiv./g/h)*	Activity (μ U)**
20	0.352	228	1.82	0.350	218	1.74
30	0.363	276	2.21	0.360	262	2.10
40	0.371	311	2.49	0.370	308	2.46
50	0.355	240	1.92	0.352	228	1.82
60	0.326	112	0.90	0.323	98	0.78
70	0.318	77	0.61	0.317	72	0.57

* = taken from the standard curve (Güven *et al.*, 1979); ** = calculated on dry matter basis (Güven *et al.*, 1979)

Table 3. Lipase and phospholipase activity of mature seeds in the presence of different solvents

Solvent	Lipase			Phospholipase		
	Absorption(A) at 440 nm	Conc. of F.A. (μ equiv./g/h)*	Activity (μ U)**	Absorption (A) at 440 nm	Conc. of F.A. (μ equiv./g/h)*	Activity (μ U)**
<i>n</i> -heptane	0.366	298	2.32	0.362	270	2.16
Cyclohexane	0.351	224	1.79	0.348	212	1.70
Di-isopropyl ether	0.326	112	0.90	0.322	94	0.75
Cyclohexanol	0.318	77	0.61	0.314	60	0.48

* = taken from the standard curve (Güven *et al.*, 1979); ** = calculated on dry matter basis (Güven *et al.*, 1979)

Table 4. Lipase and phospholipase activity of germinated seeds of different root lengths

Root length (mm)	Lipase			Phospholipase		
	Absorption(A) at 440 nm	Conc. of F.A. (μ equiv./g/h)*	Activity (μ U)**	Absorption (A) at 440 nm	Conc. of F.A. (μ equiv./g/h)*	Activity (μ U)**
5	0.375	328	2.62	0.369	303	2.42
10	0.386	380	3.04	0.358	253	2.02
15	0.396	422	3.37	0.347	205	1.64
20	0.405	464	3.71	0.342	181	1.45
25	0.414	502	4.01	0.332	139	1.11
30	0.418	520	4.16	0.326	112	0.90

* = taken from the standard curve (Güven *et al.*, 1979); ** = calculated on dry matter basis (Güven *et al.*, 1979)

activity was also observed by Waheed *et al.* (2002) for *Nicotiana rustica*.

The parameters of temperature (40 °C) and pH (7) which showed better activity for the enzymes from mature seeds were also applied to germinated seeds at root lengths of 5 to 30 mm (Table 4). The activity of lipase, carried out in aqueous media was found to be directly proportional to the increase in root length of germinated seeds. The maximum activity of lipolytic enzyme is 4.16 µU at root length of 30 mm. In contrast the activity of phospholipase was inversely proportional to the root length of germinated seeds. The best activity of phospholipase was 2.42 µU at a root length of 5 mm; similar patterns were observed in other studies (Ahmad *et al.*, 1993; Aman and Akhtar, 1991) on *Zea mays* and *Carum capticum*.

Conclusion

Lipase and phospholipase of mature and germinated seeds of *Sesamum indicum* exhibit optimum activities at pH 7 and 40 °C in aqueous media. In case of organic solvents, *n*-heptane showed better activities for both the enzymes at pH 7 and 40 °C. The lipase activity is maximum at 30 mm root length, but phospholipase activity was minimum at 30 mm root length. It is concluded that multiple factors are involved for the lipase and phospholipase activity of mature and germinated seeds. The optimum conditions evaluated for the activities of these enzymes can be utilized in the industry to resolve technical processing problems and to reduce the cost and processing time for sesame and other seeds.

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An Ecofriendly Synthesis of 4-Thiazolidinone Derivative Using Tributylammonium Bromide Under Microwave Irradiation

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Abstract. A series of new compounds 5-benzylidene-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone were synthesized by adopting environment friendly microwave irradiation methodology, their structures and *in vitro* antibacterial and antifungal activities are reported. The synthesized compounds exhibited different levels of antibacterial activities. Three compounds showed broad spectrum antibacterial activity.

Keywords: microwave irradiation, tributylammonium bromide, 4-thiazolidinone, environment friendly, antibacterial compounds

Introduction

Thiazolidinone is a useful precursor for a variety of heterocyclic products including drugs, dyes, herbicides, sulfur drugs, chemical reaction accelerators, flavouring substances and is associated with broad spectrum of biological activities including antibacterial, antifungal, tuberculostatic, anthelmintic, antitumor, anticonvulsant, diuretic, insecticidal and pesticidal properties (Singh *et al.*, 1981).

The derivatives of 4-thiazolidinone moiety have been synthesized by condensation of aromatic aldehyde and piperidinium benzoate in refluxing toluene (Kasmi-Mir *et al.*, 2006). Such methods involve long reaction time, require large quantities of organic solvents and generally yield unsatisfactorily. Microwave radiation has been employed for the formation of different products under simple operational conditions (Algul *et al.*, 2008).

Phase transfer catalysts (PTC) are environmentally benign and are used for reactions in which tetraalkylammonium cations are preferred in heterogeneous two-phase system.

Solvents like carbon tetrachloride, pyridine, dimethyl sulphoxide, dimethyl sulphate, toluene, 1,4-dioxane are commonly used as reaction media and for purification purposes. The common adverse effects of these solvents may include redness, itching and rashes on skin, swelling of face, troubled breathing, shortness of breath, nasal congestion, headache, vomiting, severe upper abdominal pain, back pain, and possible allergic reaction to material if inhaled, ingested

or even contacted. Particularly adverse effects like loss of appetite, insomnia, fatigue, depression, delirium, fever, frequent urination, and loss of coordination or judgment are caused by pyridine, while confusion, drowsiness, diarrhoea are due to carbon tetrachloride (Ballell *et al.*, 2004; Rao *et al.*, 2004; Merck Index, 1996).

In the present investigations, reaction of 3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone with different aromatic aldehyde in the presence of tributylammonium bromide (TBAB) as phase transfer catalyst in an aqueous medium was carried out under microwave irradiation and *in vitro* activity of the newly synthesized compounds was evaluated focusing on qualitative as well as quantitative analysis. This reaction requires only 6-8 min, is environmentally benign with low energy consumption and easy workup.

Materials and Methods

Melting points determined on digital melting point apparatus (Gallenkamp, England) were uncorrected. IR spectra were recorded on a Shimadzu FTIR-8400 instrument as KBr discs and only noteworthy absorption levels (cm^{-1}) were listed. $^1\text{H-NMR}$ spectra were recorded on a Bruker AC-300 MHz using TMS as the internal standard and represented in chemical shift as δ ppm downfield from TMS. Elemental analysis was carried out using a Perkin Elmer CHNS analyzer and mass spectra were recorded on a Juel D-300 spectrometer. Zone of inhibition was calculated on digital automatic zone reader (AZ-II, SUPICO, Korea). All solvents and reagents were purchased from Fluka, Merck, Sigma-Aldrich and used without purification.

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The reaction mixture was irradiated with microwave of low power (200 W). The temperature of the reaction mixture was monitored by temperature reader. Temperature of the reaction mixture continuously increased and the reaction was completed in 6-8 min using microwave oven (Model No.MV 32/8-O, SUPICO, Korea). The reactions were monitored by TLC using *n*-hexane: ethylacetate (3:7, v/v) as developing solvent, and target compounds were isolated in high yield. This novel energy-saving procedure was found to be useful for the efficient preparation of several compounds. The crude product was purified by column chromatography on silica gel to ensure purity.

Schematic presentation of magnetron is given in Fig. 1.

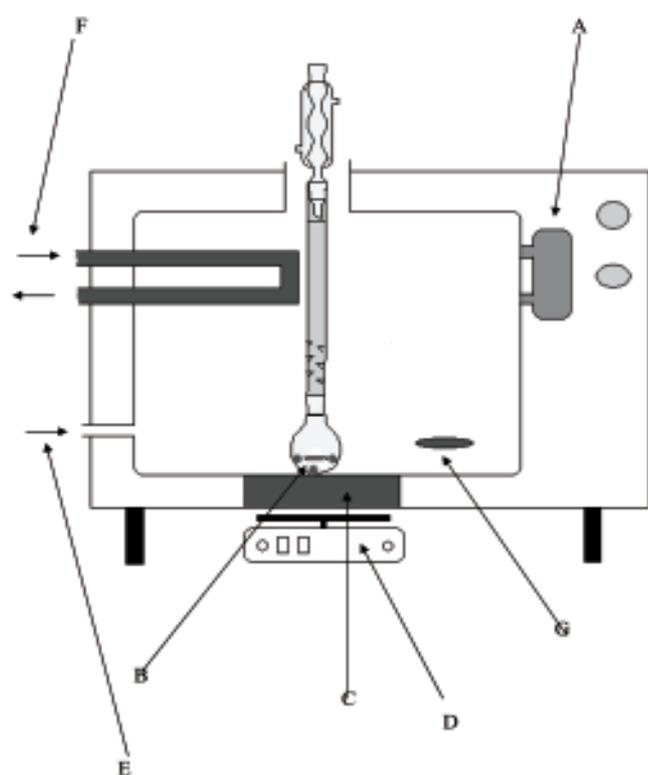


Fig. 1. Schematic diagram of magnetron.

- A. Magnetron
- B. Reaction mixture with the EDL and a magnetic stir bar
- C. Aluminum plate
- D. Magnetic stirrer
- E. Pyrometer
- F. Circulating water in a glass tube
- G. Dummy load inside the oven cavity

Series (1a-j). Synthesis of 5-benzylidene-3-(4-methylphenyl)-2-(phenyl-imino)-4-thiazolidinone. Equimolar (5 mmol) quantity of compound 3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone (1.4118 g) was treated with different aromatic aldehydes in the presence of phase transfer catalyst (1.6 mmol tetrabutylammonium bromide) and 20 ml water. The mixture

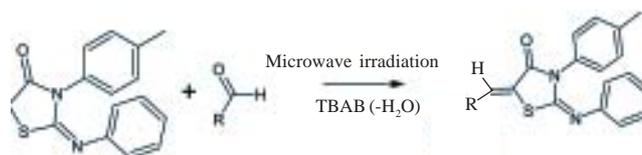
was irradiated in microwave oven at 200 watts at 110 °C for 6-8 min and then cooled to room temperature. The prepared compounds were recrystallized from ethanol to get target compounds.

Antimicrobial activity test. The test was performed according to the disk diffusion method (United States Pharmacopoeia, 2004). All compounds were screened for their antimicrobial activity against a variety of bacterial strains, such as *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Aspergillus niger* at 20 µg/ml concentration. Apparatus were sterilized by using autoclave for 15 min at 121 °C (Fazzini, Model F-31, Italy). Agar plates were surface inoculated uniformly using the broth culture of the tested microorganisms. The potency of synthesized compound was inserted in a hole made by the porcelain cylinders under laminar flow hood. The impregnated disks were placed on the medium suitably spaced apart and then transferred to an incubator at 37 °C for 72 h for bacteria, and at 28 °C for fungi (Naeem *et al.*, 2008). Inhibition zones caused by various compounds on the microorganisms were examined. The results of the preliminary screening are listed in Table 3.

Results and Discussion

A series of ten compounds **1(a-j)**, 5-benzylidene-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone were synthesized using phase transfer catalyst (TBAB) under microwave irradiation (synthetic scheme).

Synthetic scheme



Compounds	R	Compounds	R
1a	-C ₆ H ₅	1f	4-CH ₃ C ₆ H ₄
1b	4-ClC ₆ H ₄	1g	2-ClC ₆ H ₄
1c	4-N(CH ₃) ₂ C ₆ H ₄	1h	4-NH ₃ C ₆ H ₄
1d	4-OCH ₃ H ₄	1i	3-OH-5-OHC ₆ H ₃
1e	3-OCH ₃ -4-OHC ₆	1j	4-OHC ₆ H ₄

Structures of synthesized compounds were elucidated by spectral data mentioned in Table 1 and Table 2. Previously, under microwave irradiation, a reaction of 3-phenyl, 4-thiazolidinone with aromatic aldehyde was conducted without TBAB using water as solvent, for 8 min but target compounds were not obtained, because 3-phenyl,4-thiazolidinone with

Table 1. Characterization of synthesized compounds by FTIR, NMR, and MS spectrometer

Compounds	Yield % physical state	M.p. °C	IR (KBr, cm ⁻¹)	¹ H-NMR (CDCl ₃) δ	Mass Molecular ion peak <i>m/z</i>
1a [5-benzylidene-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	88, off white powder, reaction time 6 min	208	3460 (–N=thiazolidinone), 3040-3010 (Ar-C-), 1724 (C=O, thiazolidinone)	8.01 (s, 1H, CH=C ₅) 7.58-7.24 (m, 15H, <i>J</i> =8.4 Hz, aromatic proton), 3.20 (t, 3H of CH ₃)	370.467, Calc. 370.202
1b [5-(4-chlorobenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	96, yellow crystalline powder, reaction time 6 min	224	3440 (–N=thiazolidinone), 3044-3010 (Ar-C-), 1716 (C=O, thiazolidinone), 660 (-Cl)	8.36 (s, 1H, CH=C ₅) 7.48-7.30 (m, 10H aromatic proton), 7.18 (d, 2H, <i>J</i> =7 Hz), 7.10 (d, 2H, <i>J</i> =6.4 Hz), 3.16 (t, 3H of CH ₃)	404.912 Calc. 404.891
1c [5-[4-(dimethylamino)phenyl]-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	86, yellow powder, reaction time 8 min	210	3414 (–N=thiazolidinone), 3040-3010 (Ar-C-), 1740 (C=O, thiazolidinone)	8.35 (s, 1H, CH=C ₅) 7.45-7.34 (m, 10H aromatic proton), 7.22 (d, 2H.), 7.10 (d, 2H), 3.28 (s, 6H, (CH ₃) ₂), 2.46 (t, 3H of CH ₃)	413.535 Calc. 413.52
1d [5-(4-methoxybenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	73, light yellow powder reaction time 8 min	206	3430 (–N=thiazolidinone), 3024-3002 (Ar-C-), 1740 (C=O, thiazolidinone).	8.24 (s, 1H, CH=C ₅) 7.46-7.30 (m, 10H aromatic proton), 7.18 (d, 2H.), 7.06 (d, 2H), 3.48 (s, 3H, CH ₃ O), 3.02 (t, 3H of CH ₃)	400.493, Calc. 400.48
1e [5-(4-hydroxy-3-methoxybenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	77, off white powder, reaction time 8 min	236	3380 (–N=thiazolidinone), 3200(-OH), 3030-3010 (Ar-C-), 1738 (C=O, thiazolidinone).	10.05 (s, 1H, OH) , 8.14 (s, 1H, CH=C ₅) 7.54-7.28 (m, 10H aromatic proton), 7.12 (d, 2H, aromatic), 6.98 (s, 1H, aromatic), 3.68 (s, 3H, CH ₃ O), 2.88 (t, 3H of CH ₃)	416.492 Calc. 416.48
1f [5-(4-methylbenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	69, yellow powder, reaction time 8 min	245	3450 (–N=thiazolidinone), 3040-3010 (Ar-C-), 1730 (C=O, thiazolidinone).	8.34 (s, 1H, CH=C ₅) 7.44-7.26 (m, 10H aromatic proton), 7.22 (d, 2H, aromatic), 7.14 (d, 2H, aromatic), 3.32 (s, 3H, CH ₃), 2.68 (t, 3H of CH ₃)	384.493, Calc. 384.47
1g [5-(2-chlorobenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	78, white crystalline powder, reaction time 6 min	218	3440 (–N=thiazolidinone), 3024 (Ar-C-), 1728 (C=O, thiazolidinone)	8.58 (s, 1H, CH=C ₅) , 7.48-7.34 (m, 10H aromatic proton), 7.24 (m, 4H, aromatic), 3.18 (t, 3H of CH ₃)	393.04, Calc. 393.89
1h [5-(4-aminobenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	92, off white crystalline powder, reaction time 6 min	225	3445 (–N=thiazolidinone), 3020 (Ar-C-), 1730 (C=O, thiazolidinone)	9.78 (d, 2H, NH ₂), 8.46 (s, 1H, CH=C ₅), 7.38-7.22 (m, 10H aromatic proton), 7.10 (d, 2H, aromatic), 6.90 (d, 2H, aromatic), 3.21 (t, 3H of CH ₃)	385.481, Calc. 385.458
1i [5-(3,5-dihydroxybenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	44, white crystalline powder, reaction time 8 min	240	445 (–N=thiazolidinone), 3025 (Ar-C-), 1715 (C=O, thiazolidinone)	10.02 (s, 1H, OH) , 9.094 (s, 1H, OH), 8.20 (s, 1H, CH=C ₅), 7.54-7.32 (m, 10H aromatic proton), 7.24 (d, 2H, aromatic), 7.16 (s, 1H, aromatic), 3.22 (t, 3H of CH ₃)	402.466, Calc. 402.391
1j [5-(4-hydroxybenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone]	90, white crystalline powder, reaction time 8 min	216	3440 (–N=thiazolidinone), 3015 (Ar-C-), 1720 (C=O, thiazolidinone)	9.94 (s, 1H, OH) , 8.22 (s, 1H, CH=C ₅), 7.66-7.34 (m, 10H aromatic proton), 7.28 (d, 2H, aromatic), 3.18 (t, 3H of CH ₃)	386.466 Calc. 385.886

Table 2. Elemental analysis data of synthesized compounds (CHNS analyzer)

Compounds	Chemical formula	Elemental analysis %							
		Calc.		Analysis		Calc.		Analysis	
		value	value	value	value	value	value	value	value
		C	C	H	H	N	N	S	S
1a	C ₂₃ H ₁₈ N ₂ O ₂ S	74.55	74.57	4.92	4.90	7.55	7.56	8.68	8.66
1b	C ₂₃ H ₁₇ ClN ₂ O ₂ S	68.16	68.22	4.26	4.23	6.94	6.92	7.90	7.92
1c	C ₂₅ H ₂₃ N ₃ O ₂ S	72.60	72.61	5.60	5.61	10.17	10.16	7.77	7.75
1d	C ₂₄ H ₂₀ N ₂ O ₂ S	71.99	71.98	5.12	5.03	6.98	6.99	8.00	8.01
1e	C ₂₄ H ₂₀ N ₂ O ₃ S	69.20	69.21	4.49	4.48	6.71	6.73	7.68	7.70
1f	C ₂₄ H ₂₀ N ₂ O ₂ S	74.96	74.97	5.22	5.24	7.28	7.29	8.33	8.34
1g	C ₂₃ H ₁₇ ClN ₂ O ₂ S	68.20	68.22	4.25	4.23	6.95	6.92	7.91	7.92
1h	C ₂₃ H ₁₉ N ₃ O ₂ S	71.67	71.66	4.96	4.97	10.88	10.90	8.33	8.32
1i	C ₂₃ H ₁₈ N ₂ O ₃ S	68.66	68.64	4.50	4.51	6.96	6.96	7.96	7.97
1j	C ₂₃ H ₁₈ N ₂ O ₂ S	71.50	71.48	4.70	4.69	7.24	7.25	8.29	8.30

aromatic aldehyde were non-miscible mixture of oil and water. Then phase transfer catalysts [tetraethylammonium bromide (TBAB), triethylbenzyl-ammonium chloride (TEBAC), polyethyleneglycol (PEG)] were tried (Shi *et al.*, 2005) and found that TBAB was best in molar ratio 1.5: 5: 5 (TBAB, 3-phenyl, 4-thiazolidinone and aromatic aldehyde), respectively. Increase in the quantity of TBAB had no effect on yield and time. The compounds **1b**, **1h**, and **1j** gave better yields of 92%, 90% and 93%, respectively.

Tetraalkylammonium cations are preferred in heterogeneous two-phase system, one phase containing reacting base used to generate organic anions and the second phase containing organic reactant. (Alexander *et al.*, 2004; Anjaiah *et al.*, 2004; Appleby *et al.*, 1986). The (C₂H₅)₄N⁺Br⁻ serves both as a phase-transfer catalyst and a base because the reactants would exist as a non-miscible mixture of oil and water in the absence of (C₂H₅)₄N⁺Br⁻ and 3-CH₂ of 4-thiazolidinone cannot remove the alkali effect. The enolate ions would not be formed in the reaction that explains the reaction does not take place in the presence of polyethylene glycol, or in the absence of TBAB. Microwave irradiation was used to accelerate the rates of the reactions (Chiappe and Pieraccini, 2005).

The synthesized compounds were subjected to bacterial analysis that was presented in Table 3, which exhibited different activities depending on the nature and position of the substituent on thiazolidinone ring. The compounds **1c**, **1e** and **1h** showed the best activity against *E. coli*, *B. subtilis*, *S. aureus*, compounds **1d** and **1i** were observed slightly active against *E. coli*, *B. subtilis*, *S. aureus* and compounds **1b** and **1g** were active against *Proteus vulgaris* and *Aspergillus niger*. Ciprofloxacin and Gentamycin were used as reference antibacterial compounds and results were shown in Table 3.

Table 3. Results of antimicrobial activity of the compounds **1a-j**

Compounds	Zones of inhibition (mm)				
	Antibacterial activity			Antifungal activity	
	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. vulgaris</i>	<i>A. niger</i>
1a	+	+	+	-	-
1b	+	-	+	++	++
1c	+++	++++	++++	-	-
1d	++	++	++	-	-
1e	+++	++++	++++	+	++
1f	+	+	+	+	-
1g	+	+	+	++	++
1h	+++	++++	++++	-	-
1i	+	+	+	-	-
1j	-	+	+	+	+
Ciprofloxacin	+++	++++	++++	-	-
Gentamycin	+++	++++	++++	-	-

Highly active = ++++ (inhibition zone > 20 mm); highly active = +++ (inhibition zone 15-20 mm); active = ++ (inhibition zone 10-15 mm); slightly active = + (inhibition zone 5-10 mm); inactive = - (inhibition zone < 5mm)

The data given in Table 3, revealed compounds **1c**, **1e** and **1h** to be highly active against *B. subtilis*, *S. aureus* and compounds **1a**, **1f**, **1g** and **1i** were observed to be slightly active against *B. subtilis*, *S. aureus* and *E. coli*. Compound **1d** was active against *P. vulgaris* and **1e**, **1j** were active to fungi *A. niger*.

Conclusion

One pot synthesis carried out using nontoxic solvent, phase transfer catalyst under microwave irradiation was a simple, time saving, economically feasible, environment friendly and efficient method for the preparation of 5-benzylidene-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone compounds. The synthesized compounds yielded very promising results

when compared with Ciprofloxacin and Gentamycin as reference antibiotic (fourth generation). The compounds **1c**, 5-[4-(dimethylamino)phenyl]-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone. **1e**, 5-(4-hydroxy-3-methoxybenzylidene)-3-(4-methylphenyl)-2-(phenylimino)-4-thiazolidinone and **1h** [5-(4-aminobenzylidene)-3-(4-methylphenyl)-2-(Phenylimino)-4-thiazolidinone] can be used as broad spectrum antibiotics after carrying out toxicological studies; since bacteria are developing resistance against the existing antibiotics, there is always need for developing new antibacterial compounds.

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Salicylic Acid Induced Physiological and Biochemical Changes in Wheat Under Drought Stress Conditions

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Abstract. Experiment for finding the effect of pre-soaking of wheat seeds varieties, viz Wafaq-2001 and Punjab-96, in salicylic acid (SA) solution on the drought tolerance of wheat, revealed increase in the total biomass and grain yield per plant as well as in spikes per plant, 100 seed weight, proline, total soluble sugars, membrane stability index (MSI), superoxide dismutase (SOD) and ascorbate peroxidase (APOX) activity in both the tested varieties. The yield increase in drought tolerant variety Wafaq-2001 was more as compared to drought sensitive Punjab-96. Results signify the role of SA in regulating the drought response of wheat and that SA could be seed primed and used as a potential growth regulator under drought stress conditions.

Keywords: wheat, salicylic acid, drought resistance

Introduction

Wheat (*Triticum aestivum* L.) is the most important food crop of the world including Pakistan and ranks first among all the cereals. In Pakistan, it occupies around 8.6 million hectares with annual production of 22.0 million tones (Economic Survey, 2007). Wheat yields of the country are much lower as compared to many other countries of the world due to abiotic stresses particularly drought, salinity and high temperature (Sial *et al.*, 2005; Khan, 2003; Reynolds *et al.*, 2001).

To overcome the consumption pressure of ever increasing population, efforts are concentrating on improving wheat-yield by developing new varieties with desirable genetic make up. Although selection and breeding is the ultimate way to produce stress tolerant crop plants, exogenous application of osmoprotectants, growth promoters and antioxidant compounds to plants has been considered a short-term solution for alleviating the adverse effects of different stresses on plants during the last decade (Arfan *et al.*, 2007; Raza *et al.*, 2006; Iqbal and Ashraf, 2005).

Various physiological and biochemical effects of salicylic acid on plant systems have been documented in response to environmental stresses (Raskin, 1992). These include effects on membrane permeability, SOD activity, chlorophyll, relative water contents etc. (Agarwal *et al.*, 2005). It is also an important molecule for modulating plant responses to stress (Senaratna *et al.*, 2000). Any compound can be applied exogenously either as a pre-sowing seed treatment, as a foliar spray or through

the rooting medium (Ashraf and Foolad, 2007; Iqbal and Ashraf, 2005) but pre-sowing seed treatment is easy, time saving and economical for the farmers to mitigate the adverse effects of drought stress.

The hypothesis of present research study was to analyze the effects of salicylic acid seed pre-treatment in ameliorating the adverse effects of drought stress in wheat.

Materials and Methods

Seeds of two wheat varieties viz Wafaq-2001 and Punjab-96, obtained from wheat programme, National Agriculture Research Centre (NARC) Islamabad, Pakistan, were treated in aerated aqueous solution of salicylic acid (10^{-4} M) for 12 h and control (no SA) in black painted flasks. A separate set of plants was maintained which served as well watered. After hormone treatment, seeds were washed with distilled water and sown in pots filled with soil.

Drought stress was imposed at three developmental stages viz tillering (48 DAS), preanthesis (80 DAS) and mid-milky stage (128 DAS) by withholding irrigation for about 5-7 days till the signs of temporary wilting/leaf rolling started. At this stage, samples of flag leaf were collected and analyzed for proline, soluble sugar, superoxide dismutase (SOD), ascorbate peroxidase (APOX) activity and membrane stability index (MSI). After sampling, pots were regularly irrigated. Proline content of flag leaf was determined by the method of Bates *et al.* (1973). Total soluble sugars were measured as described by Pattanaik and Mohapta (1988).

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For SOD activity measurement, leaves (0.5 g) were homogenized in 50 mM sodium phosphate buffer containing 1% polyvinyl pyrrolidone (PVP). The homogenate was centrifuged for 15 min at 4 °C and the supernatant was used for assay of the activities of APOX and SOD. The activity of SOD was assayed by monitoring its ability to inhibit the photochemical reduction of NBT (Beauchamp and Fridovich, 1971). One unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of absorbance reading to 50% in comparison with tubes lacking enzyme.

APOX was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm (Nakano and Asada, 1981). Reaction mixture (3 ml) contained, 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H₂O₂ and 0.1 ml enzyme. The reaction was started with the addition of 0.1 mM hydrogen peroxide. Decrease in absorbance for a period of 30 sec was measured at 290 nm. Activity was expressed by calculating the decrease in ascorbic acid content by comparing with a standard curve drawn with known concentrations of ascorbic acid.

The MSI was determined according to Sairam *et al.* (2002). Leaf samples (0.1 g each) were cut into discs of uniform size and placed in 10 ml of double-distilled water in two sets. One set was kept at 40 °C for 30 min and its conductivity was (C₁) recorded using conductivity meter. The second set was kept in a boiling water bath (100 °C) for 15 min and its conductivity was also recorded (C₂). The MSI was calculated as:

$$MSI = [1 - (C_1/C_2)] \times 100$$

At maturity, agronomic data were recorded for biomass, spikes, grain yield per plant and 100 seed weight. Data were analyzed using a completely randomized design three factor factorial split plot arrangement, where, factor A=hormone, factor B=varieties and factor C=growth stages. Factor C is split plot on factor A and B. The treatment means were compared by LSD test at 0.01 and 0.05 probability levels (Steel and Torrie, 1984).

Results and Discussion

The analysis of data revealed significant differences between the well watered, salicylic acid pretreated seeds and the control. However, there were non-significant differences among the varieties for biomass accumulation (Fig. 1). In both wheat varieties, significant ($p < 0.001$) increase in biomass was observed as compared to the control. SA treatment enhanced biomass production by 38.84% as compared to the control.

Further significant differences were found among the treatments for grain yield per plant (Fig. 2), 100 seed weight (Fig. 3)

and proline content (Fig. 4). The highest grain yield per plant was recorded in pretreated Wafaq-2001; the percent increase in grain yield alone by SA seed pretreatment was 26.17% as compared to control. Similarly, there was 29.24% increase in yield of pretreated seeds in case of (HXV). In Punjab-96 this increase was 21.45%.

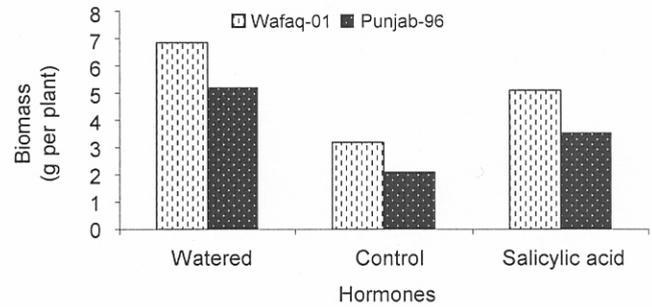


Fig. 1. Effect of salicylic acid seed pretreatment on the biomass of wheat under watered and water stressed (control and salicylic acid) conditions.

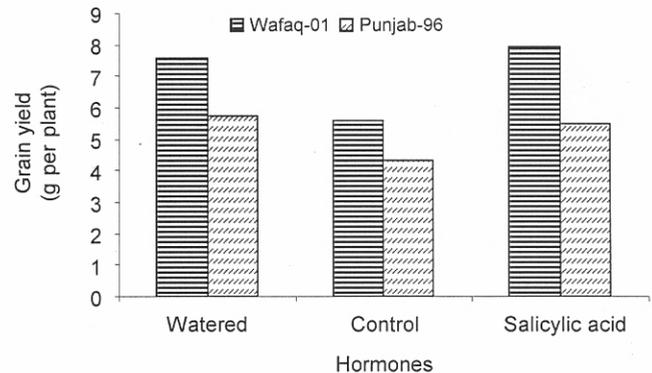


Fig. 2. Effect of salicylic acid seed pretreatment on the grain yield of wheat under watered and water stressed (control and salicylic acid) conditions.

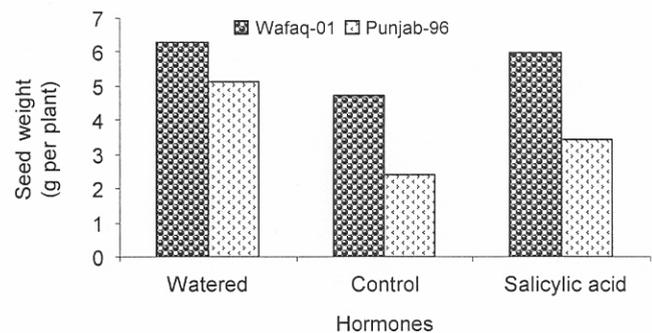


Fig. 3. Effect of salicylic acid seed pretreatment on 100-seed weight of wheat under watered and water stressed (control and salicylic acid) conditions.

The highest 100 seed weight (23.9%) increase was observed in pretreated Wafaq-2001. However, in case of HxV, this increase was 20.8% in case of Wafaq-2001 and 29.61% in case of Punjab-96.

Wafaq-2001 accumulated highest proline content, whereas, proline content was non-significant under HxV interaction. There was 40.4% increase in proline as compared to the control.

The data showed significant differences among the treatments for total soluble sugars accumulation (Fig. 5), membrane stability index, superoxide dismutase and ascorbate peroxidase activities and also between the two varieties, by SA seed pretreatment. Wafaq-2001 accumulated the highest total soluble sugar as well as displayed high membrane solubility index (Fig. 6), high superoxide dismutase activity (Fig. 7) and high ascorbate peroxide activity (Fig. 8).

In Wafaq-2001, there was 29.23% increase in total soluble sugar accumulation as compared to the control; however, in case of HxV, total soluble sugars increased 36.7% in Wafaq-2001 and 19.68% in Punjab-96.

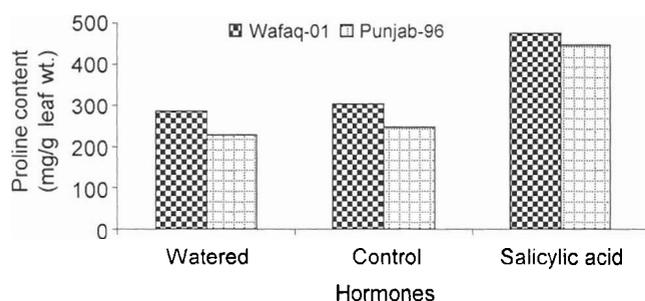


Fig. 4. Effect of salicylic acid seed pretreatment on the proline concentration in wheat leaves of wheat under watered and water stressed (control and salicylic acid) conditions.

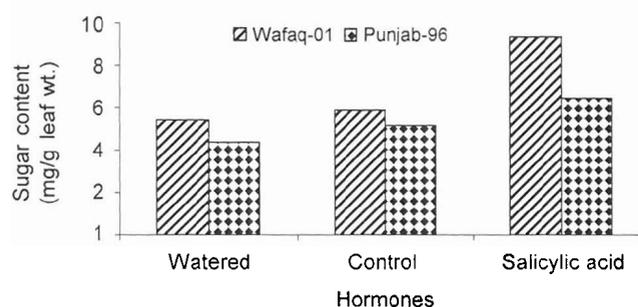


Fig. 5. Effect of salicylic acid seed pretreatment on the soluble sugar content in wheat leaves under watered and water stressed (control and salicylic acid) conditions.

There was 20.07% improvement in membrane stability index, highest being at mid milky growth stage followed by preanthesis and tillering.

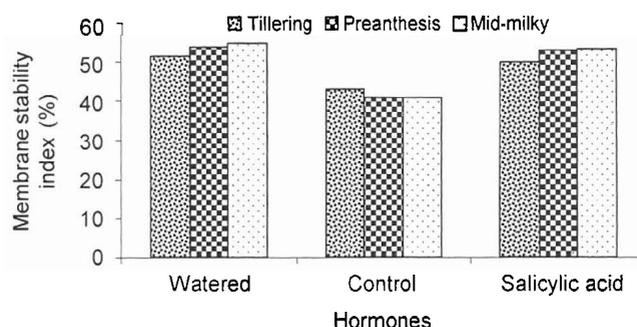


Fig. 6. Effect of salicylic acid seed pretreatment on the membrane stability index of wheat under watered and water stressed (control and salicylic acid) conditions.

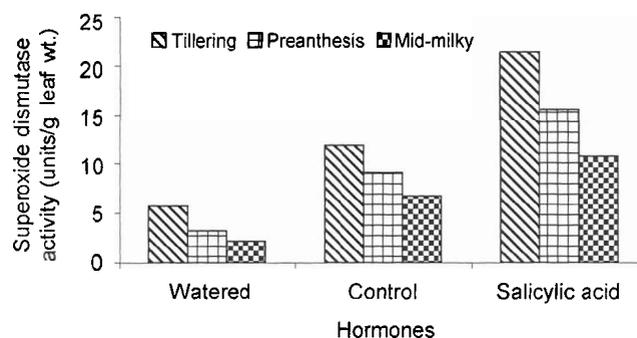


Fig. 7. Effect of salicylic acid seed pretreatment on the superoxide dismutase activity in wheat at different growth stages under watered and water stressed (control and salicylic acid) conditions.

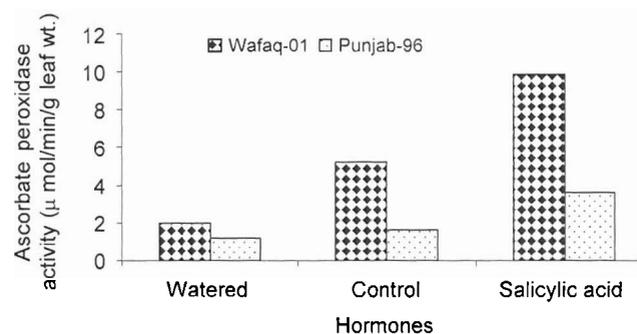


Fig. 8. Effect of salicylic acid seed pretreatment on the ascorbate peroxidase activity of wheat under watered and water stressed (control and salicylic acid) conditions.

There was 40.5% and 49.3% increase in SOD and APOX activities, respectively, by SA seed pre-treatment alone. In case of HxV, the increase was 40.2% and 46.6%, respectively, in Wafaq-2001 and 43.8% and 55.5%, respectively, in Punjab-96 by SA seed pretreatment.

SA seed pretreatment significantly affected plant growth properties i.e., biomass per plant, grain yield per plant, 100 seed weight, proline, total soluble sugars, activity of SOD and APOX enzymes. Drought stress caused a significant reduction in the growth of two wheat varieties. However, application of salicylic acid seed pretreatment counteracted the adverse effects of low water availability on the growth of the varieties.

From the results it can be concluded that beneficial effect of SA application depends on type of cultivar. Bezrukova *et al.* (2004) also reported improvement in growth of wheat by SA application. In the present study increase in grain yield and 100-grain weight of Wafaq-2001 was mainly due to increase in grain size and number with SA application. This is in agreement with findings of Grieve *et al.* (1992), who inferred that the beneficial effects of SA on grain may have been due to translocation of more photo-assimilates to grains during grain filling, thereby increasing the grain yield per plant. Zhou *et al.* (1999) also reported 9% increase in grain weight of maize plants seed-soaked with SA. The second possible mechanism of SA induced yield enhancement might be an increase in the number of spikelets and number of grains, because SA has the capacity to both directly and indirectly regulate the yield (Agarwal *et al.*, 2005).

SA treatment increased proline content more in Wafaq-2001 than Punjab-96. Proline indirectly causes increase in metabolic activation by providing osmoregulation for the plants under stress (El-Tayeb, 2005; Shakirova *et al.*, 2003; Pesserakli and Huber, 1987) and also found increase in proline content with SA treatment under salinity and drought.

SOD activity increased significantly, by SA seed pretreatment (Fig. 4). The highest increase in SOD activity was observed in Wafaq-2001 by SA seed pretreatment as compared to control. SA seed pre-treatment increased the activity of ascorbate peroxidase. The highest increase in ascorbate peroxidase was observed in Wafaq-2001 followed by Punjab-96 (Fig. 5). This might be due to drought tolerant character of Wafaq-2001. Salicylic acid-induced-increase in SOD, CAT and GR activities has also been reported by Agarwal *et al.* (2005), Clark *et al.* (2002) and Molina *et al.* (2002). The higher MSI in drought tolerant Wafaq-2001 suggests a better protection from the oxidative damage. The better protection in Wafaq-2001 seems to result from the high CAT and APOX activity. These results

are in agreement with the results of Bor *et al.* (2003) and Sairam *et al.* (2002) who found a correlation between increased antioxidant enzymes activities and decreased lipid peroxidation in salt drought tolerant wheat and wild beet, *Beta maritima*, respectively, under salt stress.

Conclusion

From the results it was inferred that SA seed treatment caused an increase in biomass and grain yield in both the two wheat varieties. The increase in yield was more pronounced in the tolerant variety Wafaq-2001 as compared to drought sensitive Punjab-96. The SA pre-soaking increased total biomass, proline, total soluble sugars, membrane stability index, activity of superoxide dismutase and ascorbate peroxidase in both the tested varieties. Results signify the role of SA in regulating the drought response of wheat and suggest that SA could be seed primed and used as a potential growth regulator under drought stress conditions.

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Micronutrient (Zn) Role in Stimulating Root Nodules and Yield of Chickpea

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Abstract. In the study of the effect of zinc (Zn) on root nodule stimulation and yield of chickpea under the climatic condition of D.I. Khan, Pakistan, significant effect of application of varying levels of zinc was noted. Plant height was significantly more as compared to the control. The plants receiving 7.5 kg/ha Zn had the highest weight of nodules/plant and produced maximum grain yield of 1185 kg/ha.

Keywords: *Cicer arietinum* L.; zinc application, nodule formation, grain yield, Pakistan

Introduction

In Pakistan, soils are generally poor in micronutrients. Zinc deficiency has particularly been reported in rainfed areas causing substantial yield losses (Rashid and Rafique, 1996). Zinc deficiency restricts RNA development which in turn inhibits protein synthesis and results in stunted growth. Previous research findings revealed that Zn application improved the nitrogen metabolism, yield and nitrogen fixation in legume crops (Shukla and Yadar, 1982). Similarly, Khanzada and Ahmad (1989) indicated that the application of iron (Fe) and zinc (Zn) significantly increased the grain yield of soybean due to improvement in the yield components and nitrogen metabolism of crop. Muhammad *et al.* (1999) reported that the application of Zn @ 5 kg/ha significantly increased the grain yield of rapeseed but 10 kg/ha of Zn showed depressing effect. In contrast, Sherazi *et al.* (2001) reported that the level of 10 kg/ha Zn significantly increased cotton yield as against other applications in cotton zone of Punjab, Pakistan. Nathan *et al.* (2005) indicated that Zn fertilization increased the paddy yield by 12 to 18% compared to the unfertilized flooded rice. Hussain and Yasin (2004) concluded that the application of 5 kg/ha zinc increased the wheat grain yield by 16% over the control.

Due to high nitrogen concentration in the tissues, pulses contribute considerably towards soil fertility. The soil enriching property of mashbean is of greater value especially to our country, where soils are deficient in organic matter, nitrogen and other soil fertility parameters (Ofori and Stern, 1987). Ibrahim *et al.* (1987) reported that soybean can fix 60-168 kg of atmospheric nitrogen and add to the soil per year. Pal and Sheshu (2001) reported that the transfer of residual N from nodulating soybean, lablab bean, green gram and black gram to the succeeding maize crop was of the order of 18.4-20.9, 19.5-29.9, 12.0-13.7 and 9.3-10.3 kg/ha, respectively.

Chickpea (*Cicer arietinum* L.) pulse group is widely grown, both in rainfed and irrigated regions of Pakistan. During 2003-04, chickpea was grown on an area of 982.3 thousand hectares with a production of 611.1 thousand tons (Qureshi, 2004). Being leguminous in nature, it not only demands less fertilizer but also improves soil fertility by virtue of fixing free atmospheric nitrogen through the root nodules. Being the rich source of protein (17-23%), it is called poor man's meat.

In consideration of the acute shortage of proteins in cereal based diets of mankind and livestock, in general, and in Pakistan, in particular, study on the effect of Zn on the production of chickpea was undertaken in the climatic conditions of D.I. Khan, Pakistan.

Materials and Methods

The field study was conducted to determine the response of Zn manuring on chickpea at Arid Zone Research Institute, PARC, D.I.Khan, Pakistan during Rabi season of 2004-05. The treatments comprised of five Zn levels (0, 2.5, 5.0, 7.5 and 10 kg/ha) applied as zinc sulphate ($ZnSO_4$) at sowing time with a basal dose of 20-50 kg NP/ha to the field. The experiment was laid out in a randomized complete block design (RCB) with three replications. A plot size of 5 x 1.8 m with six (6) rows per plot was maintained, keeping row to row distance of 30 cm. Soil samples collected from experimental site were air dried, grinded, sieved and mixed thoroughly for making accurate physical and chemical analysis (Table 1).

An improved variety of chickpea "NIFA-88" was sown in a well prepared seed bed using seed rate of 70 kg/ha. Recommended cultural practices and plant protection measures were adopted till harvest of the crop. The agro-meteorological data recorded during the growing period of crop is depicted in Fig. 1.

At complete flowering and pod formation stage, ten (10) plants from each treatment were uprooted at random. Roots of

Table 1. Physicochemical soil properties of the experimental field

Parameter	Value
pH	8.20
E _{Ce} (ds/m)	2.75
Organic matter (%)	0.76
Total nitrogen (%)	0.37
Olsen P(mg/kg)	6.91
DTPA-Zn (mg/kg)	0.52
Clay (%)	51.0
Silt (%)	37.5
Sand (%)	11.5
Textural class	clayey

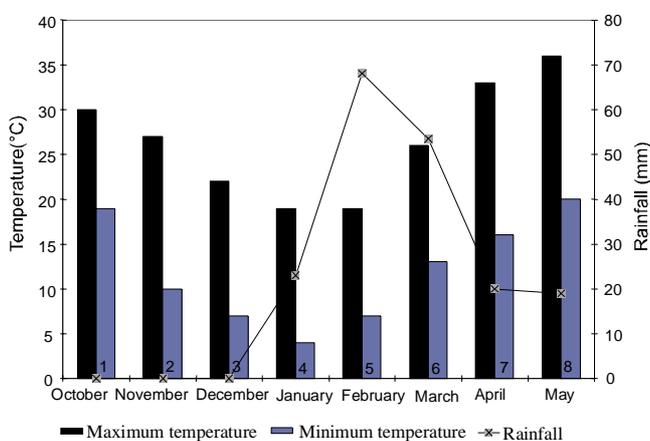


Fig. 1. Mean monthly rainfall and temperature during 2004-2005.

individual plant were carefully washed in buckets to collect and count nodules of each plant separately. Then the nodules of 10 plants were mixed and averaged for the number of nodules/plant of each treatment. The nodules of each treatment were weighed on electrical balance and computed to record the weight of nodules per plant. The crop was harvested at physiological maturity and the sun-dried samples were threshed manually to record grain yield data. On weighing, grain moisture content was 12%. The data recorded on different parameters of plant growth and development was subjected to statistical analysis of variance. The LSD at 5 percent level of probability was used for comparison of the treatment means (Steel *et al.*, 1997).

Results and Discussion

Plant height. The height of plant is an important growth character directly linked with the productive potential of the plant in terms of fodder and grain yield. The recorded data (Table 2) showed that all the levels of Zn significantly

produced taller plants relative to the plants grown as control. A maximum of 111.7 cm plant height was recorded in the plots fertilized with 10 kg/ha Zn, while the minimum plant height of 105.1 cm was recorded for the control. Analysis of variance for plant height of chickpea as affected by different treatments of Zn also indicated a significant difference (P=0.05) between different means of Zn treatments. The maximum plant height produced with 10 kg/ha Zn was statistically at par with the plant heights produced by the application of 5.0 and 7.5 kg/ha Zn. This increase in the plant height might be attributed to the effect of Zn on vegetative growth of chickpea particularly plant height. Similar increase in plant height of wheat by 3.2%, effective tillers by 11.6% and per grain panicle by 11% over control was reported by Jana *et al.* (2005).

Table 2. Effect of different levels of zinc on growth parameters and yield of chickpea

Zn levels (kg/ha)	Plant height (cm)	No. of nodules/plant	Nodule weight/plant (mg)	1000 seed weight (g)	Grain yield (kg/ha)	Increase over control (%)
0	105.1 ^c	14.3 ^d	338.0 ^d	160.3 ^d	821 ^c	-
2.5	109.0 ^b	16.0 ^{bc}	393.0 ^c	165.6 ^c	953 ^d	16
5.0	111.2 ^a	17.0 ^{bc}	435.0 ^b	170.3 ^b	1989 ^c	32
7.5	111.5 ^a	17.3 ^a	459.3 ^a	173.3 ^a	1185 ^a	44
10.0	111.7 ^a	17.3 ^a	455.0 ^a	173.1 ^a	1142 ^b	39
LSD	1.009	0.901	13.39	1.595	22.85	-

Means followed by same letter do not differ significantly

Number of nodules on roots. The data pertaining to the number of nodules per plant of chickpea (Table 2) indicated that the application of Zn, significantly increased the development of nodules on the roots of the plants. The data evinced that all the levels of zinc produced significantly more number of nodules per plant relative to the control. The number of nodules per plant linearly increased from 14.3 to 17.3 with increasing level of Zn but beyond the level of 7.5 kg/ha of Zn evinced declining trend in the development of nodules on the roots. The difference between the number of nodules per plant due to the treatment of 5.0, 7.5 and 10.0 kg/ha Zn was non-significant, whereas, the lowest number of 14.3 nodules per plant was produced by the control. These results suggested that the application of Zn might have stimulated the native rhizobia population to increase nodule production on the root system of chickpea.

Weight of nodules. The data (Table 2) indicated that the treatments of Zn also affected the weight of nodules per plant of chickpea. The maximum weight of 459.3 mg of nodules was

obtained by the treatment of 7.5 kg/ha Zn, whereas, minimum weight of 338 mg was recorded in the control. The difference between the treatments of different levels of zinc was also significant except for application of 7.5 and 10 kg/ha Zn levels. Low levels of zinc gave low weight of nodules/plant as compared to higher levels. This confirmed the synergistic effect of Zn in the development of nodules which ultimately improved the efficiency of atmospheric nitrogen fixation by legumes as reported by Shukla and Yadar (1982) that the application of phosphorus and zinc improved the metabolic activities relating to nitrogen fixation and grain yield of chickpea.

Grain weight. The magnitude of grain development is indicated by the 1000 grain weight of crop. The data in this respect, depicted in Table 2, indicate that 1000 grain weight of chickpea was significantly affected by the application of Zn. Different levels of Zn significantly increased 1000 grain weight relative to control. Maximum of 173.3 g of 1000 grain weight was obtained by the treatment of 7.5 kg/ha Zn followed by 173.1 g obtained by that of 10 kg/ha showing non-significant difference between the two. Minimum 1000 grains weight of 160.3 g was recorded in the control. The data revealed that 1000 grain weight showed positive relationship with plant height and number of nodule per plant of the crop. This increase in 1000 grain weight of chickpea due to Zn application might be attributed to vigorous vegetative growth which ultimately produced well developed seeds. These results are in harmony with the findings of Ammanullah and Hatam (2000), who observed positive relationship of 1000 seed weight of soybean with plant height and other parameters.

Grain yield. Data concerning grain yield of chickpea is presented in Table 2. Statistical analysis of the data showed that different levels of Zn had significant ($P=0.05$) effect on grain yield of chickpea. It can be inferred from the data that maximum grain yield of 1185 kg/ha was produced by Zn treatment at the rate of 7.5 kg/ha followed by the yield of 1142 kg/ha obtained by application of 10 kg/ha Zn; the minimum grain yield of 821 kg/ha was produced by control. These results are in line with the findings of Hussain *et al.* (2007), Iqbal *et al.* (2002) and Khanzada and Ahmad (1989), who observed similar increase in grain yield of different crops with application of micronutrients. The data further revealed that the difference between the treatment means of different levels of Zn was also significant. The percentage increase in grain yield of chickpea with different levels of Zn ranged from 16 to 44% over control. The maximum increase was recorded by the treatment of 7.5 kg/ha Zn as compared to the low and the high level of Zn. Beyond this level, grain yield of chickpea declined with higher level of treatment. These results are in conformity

with the work of Muhammad *et al.* (1999) who observed similar decrease in yield of rapeseed with 10 kg/ha of Zn as compared to the lower level. Similarly, Sharma *et al.* (2000) reported that wheat responded only to 5 kg Zn/ha and resulted in 13.62 and 6.14% higher grain yield as compared to the control and application of 10 kg Zn/ha, respectively.

Conclusion

The findings of this study suggest that soils, poor in micro-nutrients may be supplied with the respective micronutrient fertilizer, so as to overcome the deficiency. The data showed considerable increase in the development of root nodules that might improve the soil fertility through the symbiotic nitrogen fixation. Increase in grain yield of chickpea with zinc (Zn) supply also suggests application of micro-nutrients for improvement of yield and nitrogen fixing capability of the leguminous crops.

However, for sustainable productivity, further research is recommended for studying the multiple effects of micro-nutrients with reference to the mobilization/stimulation of the root system and the yield of pulses.

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Status of Plant Available Sulphur and Its Relationship to Other Soil Characteristics in Pothwar Soils

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Abstract. Assessment of the amount of plant available sulphur (SO₄-S) in soils of Pothwar, Pakistan revealed the average S contents in the soil to range from 5.7 to 21.7 µg/g. Five out of fifteen soil series were deficient (< 10 µg S/g) in SO₄-S with a range of 5.0 to 9.0 µg/g, six were deficient only at upper (0-15 cm) soil depth while, the remaining four had satisfactory level (10-30 µg S/g) at both the soil depths. Sulphur exhibited significant positive correlation with clay (r = 0.77**), EC_e (r = 0.77**), organic C (r = 0.82**), total N (r = 0.88**) and extractable P (r = 0.72**) contents in soil. Correlation coefficients of SO₄-S with sand (r = -0.41), soil pH (r = -0.49) and CaCO₃ (r = -0.60*) contents were negative. Organic C and N had the most pronounced effects (R² > 65) on S availability in soil.

Keywords: soil sulphur, soil characteristics, Pothwar

Introduction

The Pothwar Plateau (latitude 32° 10 to 34° 9 N and longitude 71° 10 to 73° 55 E) constitutes an important part of the rainfed agricultural area of Pakistan. It consists mainly of Rawalpindi, Chakwal, Attock and Jhelum districts of northern Punjab, Pakistan. The annual rainfall varies from 500 to 1000 mm, out of which almost 70 percent is received in the form of heavy summer rains.

The main crops of Pothwar are wheat, millet, pulses, rapeseed and groundnut. The area contributes significantly, to agricultural and livestock production of Pakistan. Farmers of Pothwar region practice low inputs in agriculture because of high costs, uncertainty of rainfall and unawareness of modern technology. The use of fertilizers in this area is 3 to 4 times less in comparison to that of irrigated regions (Ahmed and Rashid, 2003; Ali *et al.*, 2002). Almost all soils of Pothwar are deficient in nitrogen and phosphorus while potassium is adequate for plant growth except in sandy soils.

Sulphur is the fourth major plant nutrient and in conjunction with NPK, plays a vital role in cell wall formation, protein synthesis and enzyme reactions as well as in seed formation and oil synthesis in oilseed crops. The deficiency of S has been reported by Rashid *et al.* (1995) and Ahmad *et al.* (1994) in the Pothwar area. However, the information regarding the sulfur status of Pothwar soils and its response in crops is not adequate (Ahmad *et al.*, 1994).

Rapeseed and mustard are important oilseed crops of the Pothwar. They require comparatively greater amount of S for

proper growth and higher yields than cereals. It has been estimated that 60 kg of sulphur is removed in producing one ton of rapeseed (Ahmed and Rashid, 2003). Therefore, to get better yields of oilseeds in this area, the knowledge of sulphur status and S supplying capacity of soil is imperative.

Sulphur in Pothwar soils is mostly inherited from parent material (inorganic) or added through rainwater because it is speculated that the amount of S generated from organic sources in Pothwar soils cannot be appreciable due to relatively low organic matter in soil and lack of recycling of crop residues. Rich source of S from canal and tubewell water is not available in this rainfed area. The present study was carried out to assess plant available S (SO₄-S) in prominent soil series of Pothwar region of Pakistan and to study its relationship with other important soil characteristics.

Materials and Methods

Soils belonging to 15 dominant series in the Pothwar tract of the Punjab province of Pakistan were collected from their relevant locations, earmarked by the Soil Survey of Pakistan (Soil Survey of Pakistan, 1971; 1967). Four soil series (Qutbal, Missa, Rajar and Basal) from Attock district, five soil series (Talagang, Balkassar, Chakwal, Satwal and Therpal) from Chakwal district and six soil series (Guliana, Kahuta, Rawalpindi, Rawal, Tirnaul and Qazian) from Rawalpindi district were collected. These soils were classified as (1) *Typic Ustochrepts*: Missa, Basal, Talagang, Rawalpindi, Rawal, Tirnaul, (2) *Typic Haplustalfs*: Balkassar, Chakwal, Therpal, Kahuta and Gulianan, (3) *Typic Ustorthents*: Qutbal, Missa and Rajar, (4) *Typic Torripsamments*: Qazian and (5) *Typic*

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Chromusterts: Satwal (Table 1). These soils fall into two climatic zones, high and medium rainfall, of the Pothwar region. Soil sampling was carried out during the months of September and October, 2004. At the time of sampling, most of the sampling fields were kept fallow in order to conserve moisture for the winter crops. The moisture content in most of the soils was below the field capacity as sampling was carried out during the dry period following the summer rains. Information about the sampling area, soil series, soil type and parent material is given in Table 1. Quadruplicate soil samples were taken from two depths, i.e., 0-15 and 15-30 cm using a soil auger, transferred into polyethylene bags and brought to the laboratory. The field moist samples were spread over the polyethylene sheets separately, hand-picked to remove stones, homogenized and stored in plastic containers until analysis. The mean annual temperature in the experimental area was 24.5 °C and the mean annual precipitation was 750 mm. However, precipitation is unevenly distributed over the year, i.e., approximately 60% in the monsoonal months of July and August.

A small portion of each sample was taken, air-dried, ground, mixed thoroughly and analysed for different soil properties. The samples were analysed for texture, EC_e, pH, soil organic C, total N and extractable P by methods described by Page *et al.* (1982); 0.15 % CaCl₂ extractable SO₄-S by turbidimetric method (Verma *et al.*, 1977). The data for SO₄-S were classified into four categories, deficient (< 10 µg/g), satisfactory (11-30 µg/g), adequate (31-100 µg/g), and excessive (> 100 µg/g) as described by Ahmad *et al.* (1994).

The relationships between plant available S content and different soil properties were analysed by simple linear correlation and regression analysis using MS Office (Excel) package-2003 and Stat View 5.0 (SAS Inst. Inc.).

Results and Discussion

Plant available sulphur (SO₄-S) status of Pothwar soils. The data presented in Table 2 and Fig. 1 revealed that Satwal and Chakwal soils had significantly higher plant available S (SO₄-S) contents, though both were statistically at par with each other, followed by Talagang soils. Five out of fifteen soils i.e., Missa, Rajar, Rawal, Therpal and Qazian were deficient (<10 µg S/g), having 5.0 to 9.0 µg S/g at both soil depths; six soils, i.e., Basal, Qutbal, Guliana, Rawalpindi and Tirnaul, were deficient only at upper depth (0-15 cm), while the remaining four i.e., Balkassar, Chakwal, Satwal and Talagang had satisfactory level of sulphur (10-30 µg S/g) at both the soil depths.

Overall, the average SO₄-S content in soils under study was 11.7 µg/g (Table 3). The minimum mean SO₄-S (5.7 µg/g) was recorded at Qazian (*Typic Torripsammets*) and maximum (21.7 µg/g) at Satwal (*Typic Chromusterts*). Though the average values of SO₄-S were in the satisfactory range but all the soils of Attock district and most of the Rawalpindi district were deficient in sulphur, while most of the soils belonging to Chakwal area, except Therpal, had sufficient S content.

The present results of study are supported by Ahmad *et al.* (1994) and Saleem and Davide (1987) who attributed low S in soils of rainfed areas of Pakistan to leaching losses and no

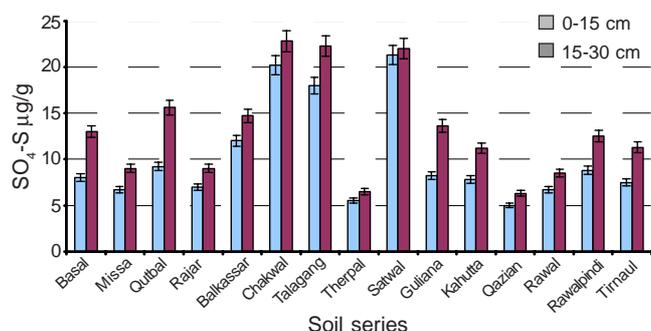
Table 1. Parent material, climate and cropping pattern and texture of soils of Pothwar region

Soil series	Area	Climate	Parent material	Textural class	Soil classification
Basal	Attock	Sub-humid to semi arid	Loess	Silt loam	<i>Ustochrepts</i>
Missa	Attock	Sub-humid	Loess	Silt loam	<i>Ustochrepts</i>
Qutbal	Attock	Sub-humid	Loess	Loam	<i>Ustorthents</i>
Rajar	Attock	Sub-humid	Loess	Loam	<i>Ustorthents</i>
Balkassar	Chakwal	Sub-humid	Residuum (sandstone)	Loam	<i>Haplustalfs</i>
Chakwal	Chakwal	Sub-humid	Loess	Loam	<i>Haplustalfs</i>
Talagang	Chakwal	Sub-hum to semi arid	Alluvium	Loam	<i>Ustochrepts</i>
Therpal	Chakwal	Sub-humid	Alluvium	Sandy loam	<i>Haplustalfs</i>
Satwal	Chakwal	Sub-humid	Loess	Sandy clay loam	<i>Chromusterts</i>
Guliana	Rawalpindi	Humid to sub-humid	Loess plain	Loam	<i>Haplustalfs</i>
Kahutta	Rawalpindi	Humid	Residuum (sandstone)	Sandy loam	<i>Haplustalfs</i>
Qazian	Rawalpindi	Humid to sub-humid	Tertiary (sandstone)	Loamy sand	<i>Torripsammets</i>
Rawal	Rawalpindi	Humid	Mountain Outwash	Sandy loam	<i>Ustochrepts</i>
Rawalpindi	Rawalpindi	Sub-humid	Loess	Loam	<i>Ustochrepts</i>
Tirnaul	Rawalpindi	Humid	Residuum, colluvial(shale)	Silt loam	<i>Ustochrepts</i>

Table 2. Plant available S (SO₄-S) in Pothwar soils

Soil series	Depth		Mean
	0-15 cm	15-30 cm	
Basal	8.3	13.0	10.7 ^e
Missa	6.2	9.6	7.9 ^{gh}
Qutbal	9.2	15.6	12.4 ^d
Rajar	7.0	9.0	8.0 ^g
Balkassar	10.5	16.3	13.4 ^c
Chakwal	20.2	22.8	21.5 ^a
Talagang	18.0	22.3	20.2 ^b
Therpal	5.5	6.5	6.0 ⁱ
Satwal	21.3	22.0	21.7 ^a
Guliana	8.7	13.2	11.0 ^e
Kahutta	7.8	11.2	9.5 ^f
Qazian	5.0	6.3	5.7 ^j
Rawal	6.7	8.5	7.6 ^h
Rawalpindi	8.8	12.5	10.7 ^e
Tirnaul	7.5	11.3	9.4 ^f
Mean	10.0 ^b	13.30 ^a	11.7
ANOVA		LSD	SE
Series		0.307	0.108
Depth		-	-
SD		0.435	0.153
CV (± %)	2.28		

Means followed by the same letter (s) are not significantly different (P<0.05; DMR test)

**Fig. 1.** Plant available sulphur (SO₄-S) content in soils of Pothwar plateau, Punjab, Pakistan.

addition of S by the canal/ tubewell waters. The crop production Pothwar area depends on rainfall, therefore, the soils did not receive any S from canal/ tubewell waters, except whatever, little is present in rainwater as compared to irrigated areas where, canal and tubewell water add considerable amount of S to soils (Bhatti, 1980). The soils of this area remain fallow during heavy summer rains (monsoon) in order to conserve moisture for subsequent crop that might also increase leaching losses of SO₄ to deeper depths with rapidly percolating

water; many lysimeter studies had shown that more leaching of SO₄ occurred in fallow than cropped soils and it was minimum during growing period of crop (Shepherd and Bennett, 1998; Kirchmann *et al.*, 1996).

Lower S content in the upper depth as compared to the lower depth in all the soils might be due to eluviations or leaching of SO₄ from the upper part of the soil to lower depths particularly in the well drained soils under high rainfall conditions (Rawalpindi area). Havlin *et al.* (2004) advocated less than 10% of the total S in surface soil.

Relationships between plant available S (SO₄-S) and soil properties. The simple linear correlation and regression analysis highlighting the pattern of association among SO₄-S and other soil characteristics are presented in Table 3 and 4, Fig. 2 and 8 are discussed as under:

The particle size analysis and textural classes of the soil under study, presented in Table 4, showed that soils were generally medium to coarse textured. Sandy loam and silt loam were the dominant textural classes with relatively high amounts of sand and silt. Soil SO₄-S exhibited positive correlation with silt ($r = 0.001$) and clay ($r = 0.77^{**}$) and negative correlation ($r = -0.41$) with sand particles of the soils under study (Fig. 2 and 3). These results are supported by Tiwari and Sakal (2002). The highly significant and strong correlation ($r = 0.77^{**}$) between SO₄-S and clay, indicated that amount of clay contributes to the soil S because it might adsorb SO₄ strongly, as compared to silt and sand fractions. Solomon *et al.* (2001) reported 1.5-2.0 times greater amount of inorganic S in the clay as compared to silt separates in soils. SO₄ adsorbed to clay is subsequently slowly released to soil solution thus preventing SO₄ leaching losses from the surface soils (Scherer, 2001), while, high sand contents in soil might encourage leaching losses of SO₄ due to their low adsorption capacity.

Strong positively correlation ($r = 0.77^{**}$) between SO₄-S and EC_e indicated that S in soil increased as EC_e increased. The fact is that EC_e is related to the concentration of soluble cation and anions present in soil; so, increase in anions like SO₄ increases EC_e of the soil. Similar relationships were also reported by Ghosh and Agrawal (2005) and Tiwari and Sakal (2002) in Indian soils. Sulphur exhibited negative correlation ($r = -0.49$) with soil pH; it decreased as pH increased (Fig. 4). These results are supported by the studies of Bandyopadhyay and Chattopadhyay (2000). The alkaline (pH 8.0-8.5) and calcareous nature of Pothwar soils might encourage the adsorption of cations on exchange sites and as a result, anions like SO₄ are subsequently released into the soil solution where, it might be leached with downward percolating water in well drained soils of the area.

Table 3. Physicochemical characteristics of soils of Pothwar*

Soil series	Sand (%)	Silt (%)	Clay (%)	EC _e dS/m	pH	CaCO ₃ (g/100 g)	SOC (g/100 g)	TN (g/100 g)	EP (µg/g)	SO ₄ -S (µg/g)	C:S ratio	N:S ratio
Basal	41.5	47.0	11.5	0.38	8.0	7.50	2.12	0.25	4.15	10.7	197.7	22.9
Missa	37.5	54.0	8.5	0.42	8.2	13.15	2.43	0.26	4.75	7.9	307.0	32.9
Qutbal	45.5	41.0	13.5	0.35	7.9	5.20	2.88	0.28	5.15	12.4	231.9	22.2
Rajar	52.0	41.0	7.0	0.36	7.9	11.35	2.23	0.24	4.55	8.0	278.1	29.4
Balkassar	40.5	39.0	20.5	0.58	7.6	3.40	3.72	0.34	6.15	13.4	278.0	25.4
Chakwal	31.5	42.0	26.5	0.53	7.7	3.60	4.05	0.38	7.40	21.5	188.4	17.7
Talagang	41.0	36.5	22.5	0.63	7.6	3.25	3.35	0.39	5.10	20.2	165.8	19.1
Therpal	69.0	21.0	10.0	0.40	7.7	7.45	2.23	0.23	3.40	6.0	370.8	37.5
Satwal	52.0	28.0	20.0	0.49	7.8	5.25	3.86	0.38	5.30	21.7	177.6	17.3
Guliana	39.5	42.5	18.0	0.36	7.9	9.55	3.26	0.33	5.15	11.0	298.6	29.8
Kahutta	47.0	34.5	18.5	0.39	8.1	5.25	3.05	0.32	5.40	9.5	321.1	33.7
Qazian	63.5	24.0	12.5	0.29	7.9	5.60	1.94	0.22	3.95	5.7	340.4	37.7
Rawal	45.0	39.5	15.5	0.34	7.7	8.25	2.90	0.27	4.50	7.6	381.6	35.5
Rawalpindi	43.0	38.0	19.0	0.41	7.9	7.30	3.20	0.33	5.00	10.7	299.1	30.4
Tirnaul	32.0	51.0	17.0	0.33	7.9	10.7	2.95	0.30	4.35	9.4	313.8	31.9
Mean	45.37	38.6	16.0	0.42	7.9	7.1	2.94	0.3	5	11.7	276.7	28.2
SD	10.38	9.09	5.49	0.1	0.2	3	0.65	0.06	1	5.3	69.3	7.0
CV	0.23	0.24	0.34	0.23	2.2	0.4	0.22	0.19	0.2	0.5	17.9	1.8
Min	31.5	21.0	7.0	0.29	7.6	3.3	1.94	0.22	3.4	5.7	165.8	17.3
Max	69.0	54.0	26.5	0.63	8.2	13.2	4.05	0.39	7.4	21.7	381.6	37.7

*=average of two soil depths 0-15 and 15-30 cm; SOC = soil organic carbon; C:S = carbon:sulphur ratio; TN = total nitrogen; N:S = nitrogen : sulphur ratio; EP = extractable-P

Table 4. Relationship between soil characteristics (x) and SO₄-S contents (y) in different soil types of Pothwar (n=15)

Soil characteristics	r	Regression equations	R ²
Sand	- 0.41	y = 21.20 - 0.20x	0.17
Silt	0.00	y = 11.70 + 0.00x	0.00
Clay	0.77**	y = - 0.30 + 0.75x	0.60
E C _e	0.77**	y = - 5.90 + 42.46x	0.59
pH	- 0.49	y = 131.42 - 15.28x	0.25
CaCO ₃	- 0.60*	y = 19.20 - 1.06x	0.36
Organic C	0.82**	y = - 7.80 + 6.63x	0.67
Total N	0.88**	y = - 12.65 + 81.65x	0.77
Extractable P	0.72**	y = 8.21 - 4.02 x	0.52

r=simple linear correlation coefficient; R²=multiple reg. coefficient of determination; *=significant at P=0.05 (> 0.52); **=highly significant at P=0.01(> 0.64)

The soils of Pothwar plateau inherited CaCO₃ contents from their parent materials because most soils have originated from fine grained loess material containing calcite. The significant negative correlation ($r = -0.60^*$) of S with CaCO₃ indicated that SO₄ in calcareous soil (Fig. 5) may form insoluble compounds with CaCO₃ and become less available. These results are in line with those of Havlin *et al.* (2004) and Trivedi *et al.* (2000).

Strong positive correlation ($r = 0.82^{**}$) between SO₄-S and organic C exhibited the importance of organic C to S availability in soil (Fig. 6). However, the low (<5.0 mg/g) organic C content (Table 2) with little addition of farmyard manure (FYM) and no recycling of crop residues in the soils under study indicated that the amount of S generated from this source cannot be appreciable. These results support other findings (Hedge and Murthy, 2005; Srinivasarao *et al.*, 2004; Trivedi *et al.*, 2000; Ahmad *et al.*, 1994). Since S is an integral constituent of soil organic matter, the enrichment with S generally determines the S supplying capacity of soils. In the fifteen soil series analysed, C:S ratio showed much variations (Table 2)

and ranged from 165 to 381; the lowest was observed in Talagang while, the highest was recoded in Rawal soil.

The C:S ratio (with respect to the C levels) tend to be more variable; it has been reported in the range of 143:1 in alkaline soils of Oregon, USA (White, 2005); 92:1 in Eastern Australia (Havlin *et al.*, 2004) and 100:1.2 in Alfisols of India (Sharma and Jaggi, 2001). However, the C:S ratio of 100:1 was described as a reasonable representative by Brady and Weil (2002). The rather wide C:S ratios in all soils under study suggested that these soils contained less proportion of both organic C and S contents.

Sulphur exhibited highly significant positive correlation with N ($r = 0.88^{**}$) and P ($r = 0.72^{**}$) in soils under study. Sulphur

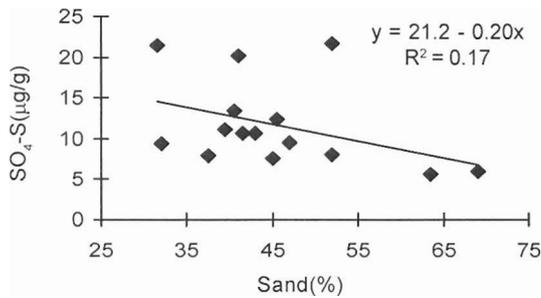


Fig. 2. Correlation between SO_4 -S and sand fraction.

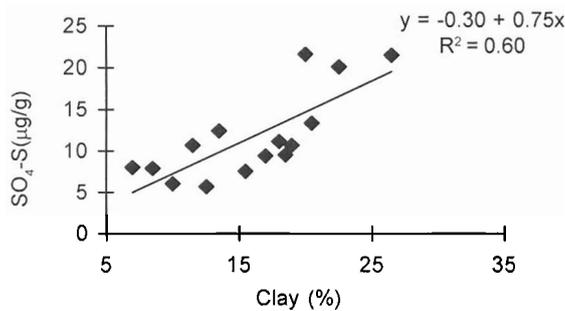


Fig. 3. Correlation between SO_4 -S and clay fraction.

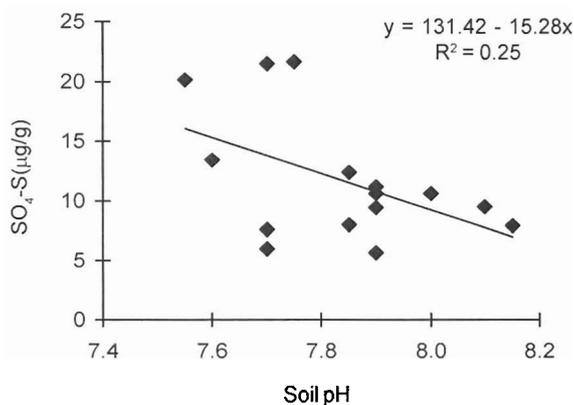


Fig. 4. Correlation between SO_4 -S and pH.

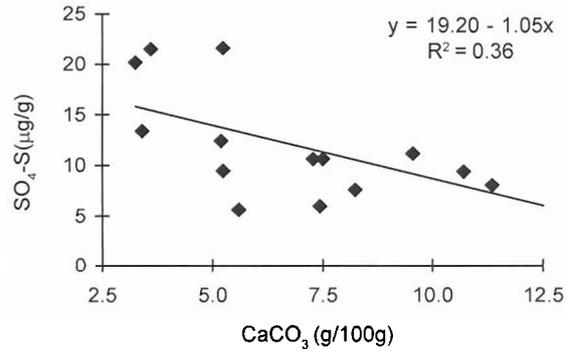


Fig. 5. Correlation between SO_4 -S and $CaCO_3$.

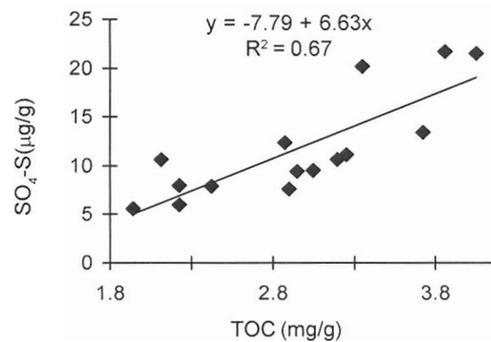


Fig. 6. Correlation between SO_4 -S and total organic C.

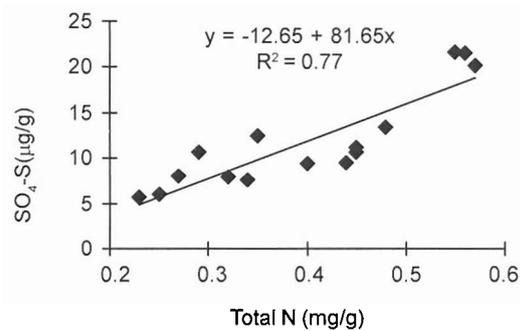


Fig. 7. Correlation between SO_4 -S and total N.

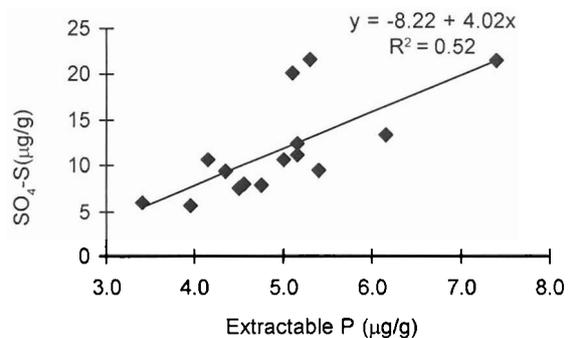


Fig. 8. Correlation between SO_4 -S and extractable P.

in soil decreased as NP concentration decreased (Fig. 7 and 8). It may be due to coarse texture and calcareous nature of Pothwar soils, which caused low availability of these plant nutrients. The high N:S ratios (Table 3) in the soils might be due to more use of N fertilizers like urea in the soils. Farmers of Pothwar region practice low agricultural input because of uncertainty of rainfall; N alone accounted 90 percent of the total consumption of all the three major nutrients (NPK) in Rawalpindi area during 2004-05 (NFDC, 2005). Application of high rates of N fertilizer on marginally S deficient soils can result in faster depletion of S from the soil, with little or no increase in the yield (Malhi and Gill, 2002). Maximum rapeseed yield responses to N and S were observed only when the availability of N and S was in approximate balance (7:1).

Sulphur and P both form insoluble compounds in the presence of CaCO_3 in the soil and get converted into insoluble fractions in alkaline calcareous soils at higher soil pH. Similar results were reported by earlier workers (Ghosh and Agrawal, 2005; Kaistha *et al.*, 2002) in case of Indian soils.

Overall influence of soil characteristics on $\text{SO}_4\text{-S}$ availability are presented through regression analysis and equations (Table 4) while, the corresponding relations are presented in Fig. 2-8. It indicated that effect of all the parameters on soil S was not similar ($P < 0.05$). Some soil characteristics had more influence on plant available S than others. Higher coefficient of determination values ($R^2 > 50$) for clay content, EC_e , organic C, N and P depicted close association of these characteristics with $\text{SO}_4\text{-S}$, while, sand content, soil pH and CaCO_3 had less impact ($R^2 < 50$) on soil S content. Higher association of clay content ($R^2 = 59$) to soil S as compared to sand ($R^2 = 17$) indicated presence of clay to be more important for adsorption and availability of S than other soil fractions. However, among all soil characteristics, organic C and N had the most pronounced effect ($R^2 > 65$) on S availability which emphasized the importance of organic C to plant nutrients availability particularly S. Therefore, to get better yields of crops particularly oilseed in Pothwar soil, management of organic C is very important through crop residue incorporation and FYM addition.

Conclusion

The results revealed that majority of soils in Pothwar were deficient in plant available sulphur. All the soils of Attock district and most of the Rawalpindi district were found to be deficient while, most soils of Chakwal area had sufficient plant available S content. The problem was found very prominent in areas with light textured soils, low organic matters and under medium to high rainfall conditions, which might encourage leaching losses of $\text{SO}_4\text{-S}$ to lower soil depths. A significant,

positive relationship between organic C content and $\text{SO}_4\text{-S}$ contents suggested that organic matter content in spite of being low, may have contributed to the increase in S availability in soil.

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A Weak Current Amperometric Technique in Physiological and Bioelectromagnetic Measurements

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Abstract. A technique for measuring ultra-low electric currents from living cells, using electrodes, biosensors or magnetic detectors is reported, based on the design of a sensitive, ultra-low-noise trans-impedance amplifier. This technique offers a low-noise, low current measurement capability down on the order of 2×10^{-14} amperes, with specifications such as input leakage current of less than 1×10^{-15} amperes and a dynamic range of $30-100 \times 10^{-14}$ amperes. Maximum bandwidth of roughly 10KHz was observed, while working in the specified dynamic range. This set of specifications is quite satisfactory and desirable for many low-frequency applications in bioelectromagnetism and bio-amperometry. The technique finds numerous applications in studying intrinsic cellular fields and induced currents originated in cells under physiological conditions. A few applications envisaged for its possible utility include bio-sensing amperometry, general studies in bioelectromagnetism and ion transport studies in plasma membrane and mitochondrial inner membrane, by incorporation of the amplifier with suitable micro-electrodes or nano-scale electrical, magnetic or optical sensors.

Keywords: intrinsic currents, biosensors, bio-amperometry, bioelectromagnetic measurement

Introduction

A large number of important measurement applications in experimental physiology and biophysics, such as living cell bioelectromagnetism, weak current amperometry and bio-sensing with the help of embedded nanotechnology, require extremely sensitive and ultra-low-noise current measurement techniques, which can measure tiny currents at the levels of pico-amperes to femto-amperes. These measurements are typically made with micro-electrodes, quantum devices and detectors, and low-temperature cryogenic superconducting quantum interference device (SQUID) detectors, or SQUID-based nanotubes (Cleuziou *et al.*, 2006). Various electrophysiological studies with ion channels (Hamill *et al.*, 1981) or investigations on intrinsic currents (Axmacher and Miles, 2004) or weak electrical field processes within living cells (Bullock, 1997) also require sensitive instrumentation and amplification at the level of femto-amperes to pico-amperes input current. Pico-ampere amplifiers are widely available and can be easily fabricated on an electronics workbench in a physiological laboratory. Unfortunately, techniques to measure ultra-low-current (such as on the level of femto-amperes) cellular signals with least noise susceptibility are neither easy to

develop nor widely available in market, in view of the special design and fabrication considerations required for their development. The main impediments faced in such designs are leakage of tiny input bias currents through the amplifier circuitry and the inherent large noise associated with measurement of ultra-low currents. With the help of special design and fabrication measures, one can venture down to an order of about 2-10 femto-amperes (with considerable noise reduction), while keeping the leakage current to a minimum extent, not affecting the measurement in a significant way. Going beyond that domain becomes an impervious task for in-house development, as at first, there are no general-purpose operational amplifiers available in market which can sense lower bias currents beyond that range, and secondly, various terrestrial and celestial effects (such as ambient electromagnetic fields in the vicinity and cosmic ray-induced electron shower events) induce tiny currents in the front-end section of the amplifier, creating unavoidable current leakages.

Industrial-grade commercial pico- and femto-ampere measurement instruments are available in market, but unfortunately they are an expensive modality and beyond the range of budgets of small biophysics and physiology laboratories,

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especially in colleges with limited budgets. Buying a commercial product off-the-shelf also precludes the experimenters from making alteration or customization in the device, in view of their specific applications. A small-scale biophysical or physiological research laboratory has to thus rely on in-house designs.

There are some public-domain designs available for low-level biological signal voltage and current amplifiers, as published extensively in literature, such as instrumentation amplifiers and pico-ampere electrometers for physiological applications, but there are a number of problems with these designs. First of all, most of the designs are for voltage mode meters. Secondly, if there are few current amplifier designs published, they are limited to nano-amperes or pico-amperes range, not presenting a design which can venture down to femto-amperes. These design ideas also pose limitations in terms of keeping the signal integrity conserved. Most importantly, these designs do not present special considerations and techniques entailed to measure ultra-low-level signals, while limiting the leakage currents and inherent noise which are coupled to small-current measurements. Thus, availability of a mere design, such as by an amplifier chip's manufacturer specifications sheet, is not sufficient to implement it in practical application.

In a biophysical study by authors to investigate the possibility and manifestations of intrinsic electric currents and endogenous electromagnetic fields in living cell systems, it was undertaken to design and fabricate a sensitive current to voltage converting amplifier which could successfully and efficiently present a practical design of an ultra-low-current and ultra-low-noise trans-impedance amplifier.

The design of this technique and its constituent amplifier differs from other conventional techniques and amplifiers in a number of ways. First of all, it presents a design paradigm which can venture down to a few tens of femto-amperes, while minimizing the leakage current and noise, with the help of its careful choice of components and special considerations followed during the fabrication of the device. Secondly, instead of just showing a way to measure the induced field currents from applied potentials, it presents a technique for low-current measurement from the living cells, which attempts to measure low-frequency intrinsic electric fields and induced femto-ampere currents, produced as a result of intrinsic effects inherent within the cell plasma membrane. This is done in an electromagnetically shielded environment and in the absence of applied fields. Third, the amplifier and measurement technique can be developed in a small laboratory with a very limited budget, costing even less than the cost of an average digital multimeter (DMM).

This technique offers an enhanced accuracy in cell amperometry or bioelectromagnetism studies by the virtue of an improvement in its meticulous design and development. Design of the amplifier is made on a special glass fiber board instead of a conventional printed circuit board (PCB) and components (including the amplifier integrated circuits) are mounted on teflon stand-offs using special considerations, as summarized in the next section. Noise susceptibility and current leakage paths are kept to a very minimum. Usage is made of some of the most precision components available in market, conserving the precision and signal integrity. The operational amplifier device used in this design, National LMP7721, has a few excellent specifications, especially the large error rejection capabilities due to the use of giga-ohm value feedback resistances, incorporated in the design. The constructed amplifier prototype is enclosed in a shielded miniature aluminum box and mounted on the micro-manipulator arm on the microscope stage, and the input of the amplifier is directly connected to the cell electrode via a short 90 μ wire, without using any cable or connector. This eliminates the noise and signal losses in the cable communicating the signal from cells to the amplifier and to the data acquisition stage. Thus, adoption of special measures and extensive trials have resulted into a design which has lower current measurement range and higher accuracy and precision in terms of measurement and signal integrity, as compared to other designs described in the literature.

Materials and Methods

Amplifier design. The design of the amplifier comprises of two stages and is built around a recently-introduced precision operational amplifier IC (integrated circuit), national semiconductors LMP7721. The design reported in this paper is based on meticulous modification of a basic design as per the manufacturer's specifications (National Semiconductors, 2008). This device is an ultra-low-noise, ultra-low input bias current, operational amplifier, manufactured with metal oxide silicon (MOS) technology input stage. It has one of the lowest input bias current operations available in the market, guaranteed by manufacturer after extensive testing at around 3×10^{-15} A. Moreover, it offers superior noise performance, tested by manufacturer at $10\text{fA-Hz}^{-1/2}$ @ 1KHz and $7\text{nV-Hz}^{-1/2}$ @ 1KHz input-referred current and voltage noise, respectively, and a total harmonic distortion (THD) of 0.003% @ 1KHz, as claimed by the manufacturer. Its one of the most important features is curtailing of the magnitude of error produced when used with a large-value resistance, such as giga-ohms. It suppresses the large error by a factor of about 10^3 to 10^6 , a great advantage in femto-ampere level current measurement using a high-value feedback resistor. This is a major advantage of

this device, which became the reason for our choosing its utility in this application, in addition to its low bias current and low noise spectral density. In addition, a theoretical (open-loop) gain bandwidth product (GBP) of 17MHz and average slow rate of around 10.0V/ μ s, as claimed in the manufacturer's specifications for LMP7721 in its data sheet (National Semiconductors, 2008) are appropriate for application in electrophysiology and general biophysics (although, it should be noted that, practically, the theoretical GBP described above is neither achievable in an ultra-low current amplifier design nor it is applicable in the design presented here).

Design of the amplifier is illustrated in the circuit diagram in Fig. 1. The first stage comprises a unity gain current to voltage amplifier with a zero-resistance front-end, followed by the second stage, with approximate gain of twenty inverting amplifier. The input signal is presented *via* a 50 G Ω Input Resistor to the inverting input of U1, LMP7721 device. This Resistor is only used for testing purposes to measure the minimum current readable by the amplifier. Once the amplifier is tested, this resistor is removed, enabling a direct zero-resistance connection between the input terminal and the amplifier. A precision resistor of 50 G Ω is connected as the Feedback Resistor to provide roughly 10¹⁰ current/voltage

transfer function. However, the voltage gain of the amplifier remains unity.

Output from the current/voltage pre-amplifier is passed on to a second amplification stage, comprising of the U2 (LMP7721) *via* C3, which removes the DC voltage presented at the output of U1 from the input current. U2 has a feedback resistor, R3, which can be of any value close to around 1 M Ω (an optimal value for our application was found to be 997 K Ω), however, a resistance higher than 1.1 M Ω was found to be unsatisfactory. This stage yields a voltage gain of roughly x10 to x40 for the second amplifier (depending on the value of R3). Capacitor C5 prevents coupling of the amplifier to mains noise and also acts as a pseudo-cut-off for the high frequency content of input signal. A successive power supply filtering scheme is adopted for the amplifier power rails by means of numerous 10 nF and 100 nF capacitors at the power rail employed in the circuit. In addition, a notch filter design can also be devised at this stage for elimination of mains and high-frequency noise components. However, we did not implement it in order to conserve the original signal, as entailed in our application.

Construction. After the design, a number of prototypes of the amplifier were fabricated to achieve the optimal performance

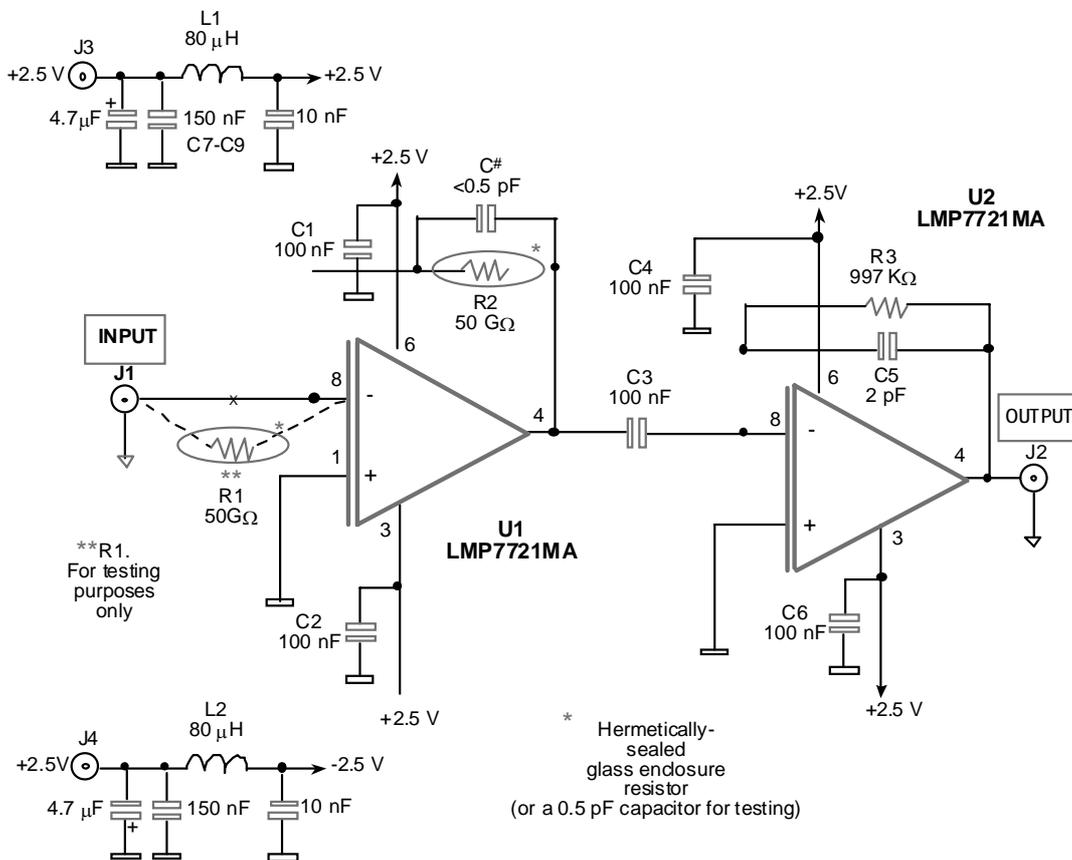


Fig. 1. A schematic diagram of the amplifier circuit.

and operation on the order of 5 to 100 femto-amperes. The device was tested with various time-domain and frequency-domain (spectrum analysis) methods used in a usual electronic workbench setup. The input current was calculated using voltage method, by utilizing Ohm's law. Alternatively, a lock-in amplifier could also be employed for this purpose. A sinusoidal low-voltage AC signal from agilent waveform generator 33220 A (Agilent, Santa Clara, CA) at various frequencies and amplitudes was applied to the amplifier's input *via* the 50 G Ω resistor. Response was recorded on Stanford SR-760 spectrum analyzer (Stanford Instruments, Stanford, CA). For instance, spectrum analyzer showed a signal of 1.000 KHz at 1.2 mV corresponding to a 1KHz, 0.1 mV input signal at the front-end of amplifier, demonstrating a total voltage gain of 12 from both stages (U1 and U2). Using Ohm's law, this input voltage, on the order of \sim 0.1 mV across the 50 G Ω resistor, yielded an input current of around 2 fA, which seemed to be the lowest current recorded by means of this amplifier. However, there was a substantial degradation of its performance in terms of noise. After extensive trials and recalculation of component values and replacement by clean components, the lowest level recorded was about 20 to 30 fA, with a substantial reduction in the noise and leakage current. We take the average of this range, 25 fA, as the lowest current measured. The leakage current, after various trials and improvements on the construction of initial two prototypes, was recorded at about 1 fA. This leakage current is extremely small and impossible to be eliminated in any realistic practical design, owing to minute leakage pathways and cosmic ray shower-induced discharges. This seems to be a reasonable specification and ceiling of amplifier's capabilities, and sufficient for the measurement capabilities of the amplifier in the envisaged applications.

Significant measures were taken to minimize the current leakage and noise from the amplifier. These included, fabrication of the amplifier on a glass polyester printed circuit board (PCB), use of hermetically-sealed vacuum glass enclosure resistors (Micro-ohm Corp., Duarte, CA), suspension of the I.C. in air and contact *via* 120 μ Au-plated Cu wires enclosed in Teflon stand-offs, inverting input pin of I.C. (pin #2) and the 50 G Ω front-end resistor suspended in air with no connections to PCB and shielded with a grounded copper mesh, a grounded tight metal enclosure mounted a few centimeters from the cells sample holder, and power supply provision by batteries. A significant reduction in mains noise amplitude was observed by operation of the device in a (sufficiently) electromagnetically shielded faraday cage.

For optimal amplifier operation, it is recommended to use a regulated \pm 5 or 6 volts supply (even with the battery power).

This power supply, based on LM7805 and LM7905 series regulators, was added to the prototype in last stages of testing, following observation of minor fluctuations in battery power (especially after prolonged burn-in hours). In addition, care must be taken not to exceed the Op-Amp's quite stringent input voltage and current limits (National Semiconductor, 2008).

For operation in the required femto-ampere dynamic range, it is extremely essential that the unit is clean and free of any deposition or contamination, especially the surface on the Op-Amp package, the Giga-ohm feedback resistor and the PCB contacts. Even traces of microscopic dust on fingertips during manual work can affect the leakage through the I.C. or these resistors. After fabrication, unit was washed with a solution of diluted ethyl alcohol (C₂H₅OH), wiped dry with high-pressure clean air and then treated in an ultrasonic bath, so as to eliminate any possible residue from the fabrication stages.

Response was also recorded and analyzed on a computer by means of a data acquisition (DAQ) system by IOtech (IOtech Corp., Cleveland, OH), using customized fast fourier transform (FFT)-based spectrum analysis routines written in national instruments labview 8.2 software (National Instruments Corp., Austin, TX).

Application in bioelectromagnetism and cellular amperometry. Amplifier design was used in the application of a bioelectromagnetism and amperometry technique devised by us to detect minute intrinsic cellular currents induced by underlying physiological processes and applied electric fields within and around the cell plasma membrane, using a budding yeast (*Saccharomyces cerevisiae*) cell model. An overview of the experiment is illustrated in Fig. 2, which is in essence a microelectrode current measurement technique. The amplifier prototype was mounted on a micro-manipulator device (Nikon, Kyoto) on an inverted microscope stage, with its input connected to a 99.9% 75 μ gold wire (Chemtel Chemicals Corp., NJ) electrode immersed in a sample of cells contained in a mini-petri dish.

A strain of wild-type *Saccharomyces cerevisiae* S288C (ATCC 26108), provided by Widger Labs at the Department of Biology and Biochemistry, the University of Houston, was preserved at 4 °C in an autoclaved YPD agar medium. Cells were grown at a temperature of 29 °C with agitation (160 rpm) in YPD (1% yeast extract, 2% peptone and 2% dextrose). Detailed materials and methods of growing and preparation of the cells and preparation of YPD medium are well-known (Wright and Philipsen, 1991). The main buffer used in the study was phosphate buffered saline (PBS) (Roche Corp., Indianapolis, IN). A solution was prepared with deionized water

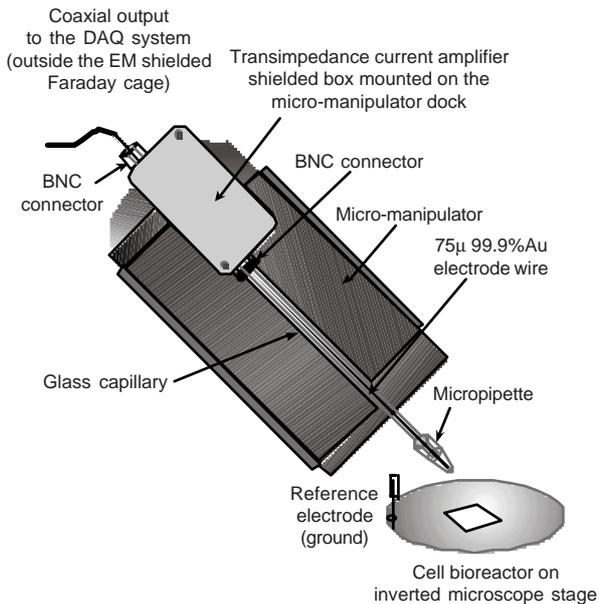


Fig. 2. An overview of the experiment to measure intrinsic electric fields as produced in cultured yeast cells as a result of various physiological processes.

passed through a milliQ system (Millipore, Billerica, MA), the resistivity of which was measured at 17.8 mV-cm.

It was assured that the experiments were conducted in aerobic conditions, by means of a static aeration through an external air supply through the sides of the reactor cell vessels. Oxygen concentration in the reactor was monitored with digimed oxygen concentration monitor (Digimed, Tampa, FL). Acidity changes due to electrical fields were measured with a standard pH meter (Cole-Palmer, Vernon Hills, IL); however, no significant changes in pH were observed. The experiments were carried out at room temperature, maintained at 20.5 °C.

For intrinsic field studies, experiments involved no external electrical fields application to the cells. Signal was picked up in a shielded environment from a micropipette making contact with the cell plasma membrane. However, for measuring the external field-induced response and in the application of dielectric spectroscopy (Miller *et al.*, 2005), time-dependent electric fields of varying frequencies from 100 Hz to 10 KHz were used, by obtaining an external AC signal from the waveform generator. An external field source electrode was immersed in the cells sample holder, in the form of a three-probe mode, common ground electrode (Woodward and Kell, 1991), or four-probe mode, individual grounds (Miller *et al.*, 2005), creating a uniform electric field in the sample holder. Magnitude of the applied voltage was varied between 0.5 V_{p-p} to 3.0 V_{p-p}. Fig. 3 illustrates a block schematic of the three-probe method,

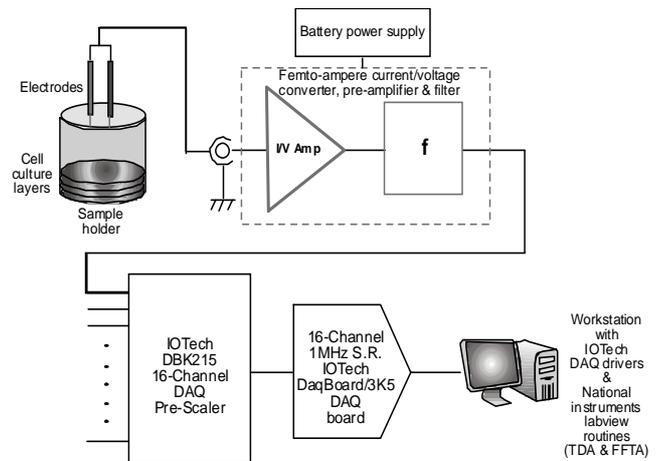


Fig. 3. A block schematic of the three-probe (two input/output electrodes and the third common ground) dielectric spectroscopy experiment to measure induced currents produced in cultured yeast cells as a result of applied time-varying electric fields. TDA stands for Time-Domain Analysis and FFTA for Fast Fourier Transform Analysis (spectrum analysis).

designed around two main electrodes, one sensing electrode and the other applied field electrode, and one common ground.

Results and Discussion

Response was quite satisfactory as expected from the manufacturer specifications for LMP7721. Minuscule currents at the level of a few tens of femto-amperes could be measured with low noise content in our experiments. The design of the chip indeed demonstrated a conspicuous suppression in noise even in the presence of a large value resistor, yielding an RMS voltage noise at the level of a few hundred microvolts (around 250 μV on an average), unlike hundreds of millivolts using any other operational amplifier. The noise voltage and current spectral densities of the amplifier, on the order of about 10nV-Hz^{-1/2} (±2n V/Hz^{1/2}) and 35fA-Hz^{-1/2} (±5 fA/Hz^{1/2}), respectively, at 1.0 KHz. limit, seemed to comply well with the manufacturer-tested LMP7721 specifications of 10 fA-Hz^{-1/2} @ 1 KHz and 7n V-Hz^{-1/2} @ 1 KHz input-referred current and voltage noise, respectively, as reported by the manufacturer in the device data sheet. There is room for improvement and meeting manufacturer's lowest limits by improving the finesse of prototype's fabrication. There were some noise harmonics seen with floating inputs, however their amplitudes reduced conspicuously in the presence of an input signal or connection to the electrodes, as seen in the Fig. 4, which depicts recording of a 3 fA event at the input terminals (the least input bias current limit allowed by the LMP7721 device) corresponding

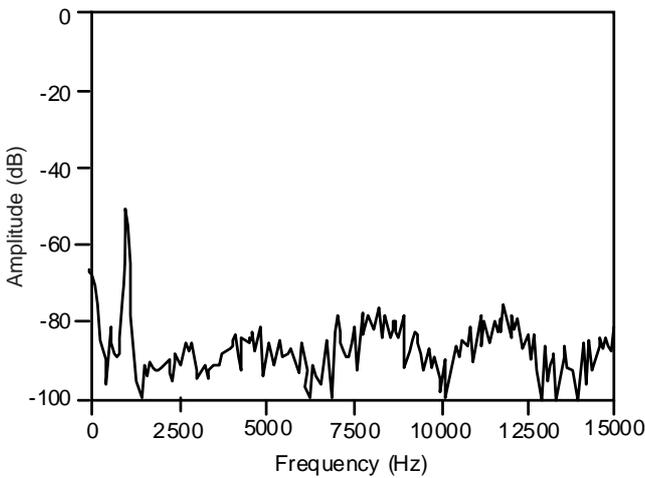


Fig. 4. Power plot of an amplified 1KHz at 0.14 mV AC test signal event as recorded by the amplifier (translating to a 3 fA input current recording).

to a 0.14 mV, 1 kHz input signal. But at this level, there was a large noise component in the signal. After extensive trials, a reasonable performance, in terms of the least noise, was obtained at an input current level of average 25 fA, which we claim as the lowest current limit of measured input current, using this amplifier.

The bandwidth of amplifier seemed to be satisfactory till 9.8 KHz before a substantial signal-to-noise ratio degradation is observed. After further development of the amplifier, this limitation on frequency range could be improved.

The transfer function or the trans-impedance gain of the front-end amplifier is calculated using the relationship of output voltage to input current in an inverting amplifier, as follows:

$$V_{out} = -I_{in} \times R_f \dots\dots\dots(1)$$

With an input current of 30 fA and a feedback resistor value of 50 GΩ, as used in this design, a value of 1.5 mV output voltage is determined. This seems to be the maximum magnitude of the obtained voltage, with low noise susceptibility, corresponding to a low-current on the order of few tens of femto-amperes which can be measured with the front-end stage. After signal conditioning through the second-stage amplifier, this is amplified about ten-fold and one can obtain few tens of milli-volts from the constructed prototype while measuring an ultra-low-current. Typically, the range of obtained voltage amplified from a cellular intrinsic signal is 0-20 mV, corresponding to minuscule currents on the order of 1-35 fA being generated within the cell plasma membrane.

The transfer ratio for the two-stage amplifier with 15 mV measured output and 30 fA input current was determined to be

0.05 x 10¹² rho (or 0.05 pico-rho) using the relationship for transfer ratio:

$$k = \frac{V_{out}}{I_{in}} \dots\dots\dots(2)$$

This transfer ratio, expressed in terms of the dimensions of resistivity, is found to be quite high as expected, and complies to the transfer function of the amplifier. The magnitude of this ratio, in the dimensions of resistivity, is slightly less than the resistivity of rubber and glass (Serway, 1998).

This implies that, if the minimum possible detected current, distinguishable from noise, or the minimum detected signal (MDS), of this amplifier is around 30 fA, the input electronic transduction capacity of the amplifier and this technique is to transduce an electronic pulse of approximately 18,720 electrons/second (from the fact that 1 fA current involves transport of 6242 electrons/second) with the help of a suitable input sensor.

A number of three successive prototypes were built, beginning with the use of another low bias current amplifier (LMC662) and a set of input and feedback resistors of 10 GΩ and utilizing various configurations up to 100 GΩ, before a successful operation could be achieved at the input range of around 5 fA-25 fA with 50 GΩ, using LMP7721. A use of resistors above this optimal value of 50 GΩ, such as 100 GΩ, was found to be ineffective in decreasing the input current while keeping the noise to a minimum value. It was found that large value resistors (higher than 50 GΩ) are inherent with large noise susceptibility and low SNR, as described earlier. The best value measured is thus determined at a range of 20-30 fA, with minimum possible leakage current and high SNR.

After testing of the amplifier with spectrum analysis, it was used in a micro-electrode-based biological experiment, incorporating a bioreactor and patch-clamp design, using gold and gold-plated-tungsten (Au-W) micro-electrodes. Aim of this experiment was to investigate the form and manifestations of tiny currents and intrinsic noise produced by metabolic pathways or physiological processes in living cells and organelles primarily in cultured wild-type *Saccharomyces cerevisiae* (brewer’s yeast) cells *in vitro*. Cell membranes have been known to be associated with membrane noise (Verveen and DeFelice, 1981) and their intrinsic ability to amplify external electric fields. The membrane noise is beyond the thermal or white noise and in fact some form of stochastic cellular activity, which has been termed to carry a valuable signature of underlying physiological processes, a possibility cited by Bullock in his detailed treatise (Bullock, 1997) in the realm of neurophysiology. The technique revealed presence of a simi-

lar intrinsic noise from respiring and active yeast cells. Amplitudes of aerobic living cells were found to be conspicuously higher (about ~70%) than dead or anaerobic cells or the phosphate buffered saline (PBS) medium alone without cells. We measured currents ranging from 20 fA to 55 fA from the active, respiring yeast cell membranes, induced as a result of a number of processes, without any applied or ambient electric fields. The source and underlying mechanisms of these currents, which are referred as intrinsic noise, may be ion channel transport across the membrane or some correlations with the metabolic states of the cells. These possibilities are currently being investigated and a few of our reports in this context are in the process of publication. Fig. 5 illustrates the sample intrinsic electric response (amplified hundred times in this figure) from the plasma membrane of yeast cell while in its active aerobic-respiring state and under physiological conditions, as measured with this technique, and in the absence of any ambient or applied external electromagnetic fields. Fig. 6 depicts a dielectric response (most likely from the cell plasma membrane in view of very low frequency), from an active and aerobically respiring yeast cell, in response to an applied AC field of frequency 1.2 KHz @ 1.0 V_{p-p}, using a three-probe configuration. The effects of medium in which cells were suspended have been subtracted from the amplitude displayed.

One of the final amplifier prototypes, as used in testing, is illustrated with the help of a photograph in Fig. 7, as assembled in the Biophysics Research Laboratory at the Texas Center for Superconductivity at University of Houston (TcSUH). Some of the special considerations entailed for manufacturing such amplifiers can be appreciated from the photograph.

While testing of the amplifier, it was revealed that amplifier is not only very sensitive to surrounding electrical and magnetic fields, but also to external vibrations present in environ-

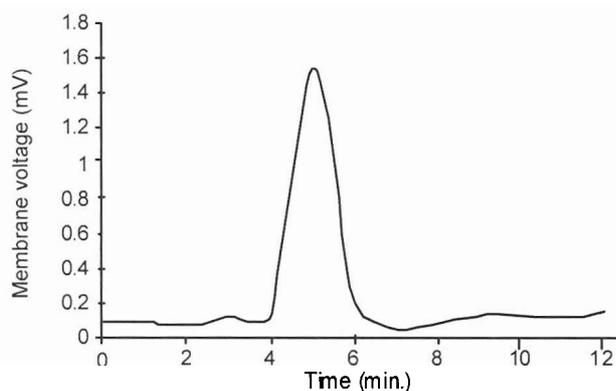


Fig. 5. A sample intrinsic cellular signal as measured *in vitro* from cell membrane of an aerobically-respiring yeast cell (x100).

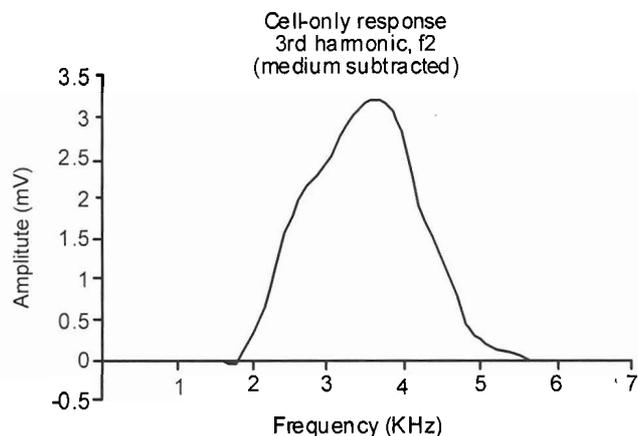


Fig. 6. A snapshot of an induced AC signal as measured from respiring yeast cells in response to an applied AC field, using three-probe method.

ment, such as acoustic vibrations from the surroundings. Electromagnetic and acoustic shielding presented themselves as mandatory requirement for the reliable measurements using this device.

It is difficult to make an accurate claim for the most minimum possible current level measured by the amplifier, in view of the fact that every operational amplifier is susceptible to parasitic capacitances, which affect the measurement of input current using the Ohm's law-based voltage method (by measuring the voltage across the 50 GΩ resistor, as done in this study). However, the operational amplifier integrated circuit employed in this design, national semiconductor LMP7721, guarantees a tested ultra-low-level femto-ampere operation at very low noise voltage and current densities, as reported in the abstract. Nevertheless, we have done earnest efforts in meeting the lowest possible limit of measured current, while keeping signal integrity conserved. It can be surmised that if contin-

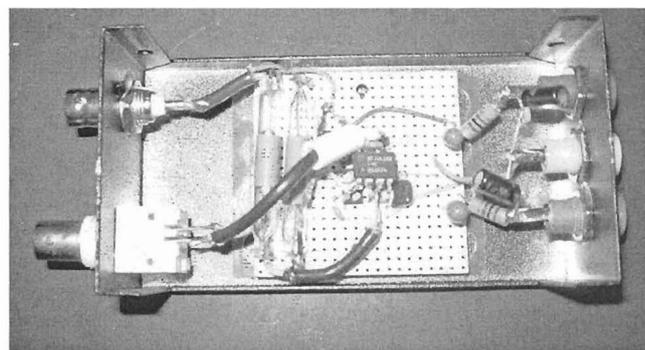


Fig. 7. A photograph of amplifier prototype, where the fiberglass PCB, the amplifier chip and high-resistance vacuum-sealed resistors, can be clearly seen.

ued improvements are made in the proper selection of components and careful assembling of the amplifier, as described in this report, it could possibly result into an amplifier capable of measuring a lowest possible current of 1 to 3 fA. Nevertheless, in our study we could make measurements with currents at the scale of around 25-30 fA at a bandwidth of 10 KHz, with satisfactory noise suppression. This is our claimed minimum input level of measurement, which is a reasonable lower limit and sufficient for a broad spectrum of applications in biophysics and electrophysiology.

Although, theoretically the claimed bandwidth for LMP7721 is quite high, as highlighted earlier, but our analysis of the amplifier and testing revealed the experimental bandwidth limited to around 10 KHz. This constrains the amplifier's application to its use limited to low-frequency signals regime, such as in general cell electrophysiology, low-frequency biological amperometry, electroencephalography (EEG) and electrocardiography (ECG) etc. Modifying the design of the first stage front-end amplifier can increase the bandwidth window manifold, but would adversely affect the minimum current measurement capabilities and noise performance. A trade-off would be required in the two parameters, depending on the problem at hand.

Conclusion

Design and development of a very sensitive, low-noise and low-cost amplifier have been carried out, as reported in this paper, which is found to work at an input current dynamic range of $\sim 2 \times 10^{-14}$ to 1×10^{-13} amperes, with a lowest measured current limit of around 25 femto-amperes with low-noise content, yielding voltage and current noise spectral densities on the order of about $10 \text{ nV-Hz}^{-1/2}$ and $35 \text{ fA-Hz}^{-1/2}$, respectively, at 1.0 KHz. Initial testing was done with workbench time-domain and spectrum analysis methods. The amplifier was incorporated in an application study by investigating its response to very low current sources, such as membrane currents and intrinsic noise in cultured living cells, as well as in recordings of minute changes in the harmonic response of cells to applied sinusoidal electrical fields. On the basis of this, a technique was developed to measure ultra-low currents within the living cell plasma membranes.

Another application of this amplifier and its based technique lies in the experimental measurement of mass-transport-diffusion current (Taylor and Schultz, 1996) in biosensors or bioelectronic electrodes which work on the principle of amperometric transduction of biological processes. By use of a suitable biosensor, such as a carbon electrode, or an embedded field effect transistor (FET) in a constant-potential configuration, minuscule fluctuations in current may be detected

which are the direct measure of the rate of electron transfer reaction in the diffusion layer (the region of solution in bioreactor in which the sensor/electrode is immersed). The technique can be utilized in measuring the electron transport current, which has a mathematical value as expressed by Equation 3.

$$I = \frac{nFADC}{\delta} \dots\dots\dots(3)$$

Where F is the Faraday's Constant, A the area of electrode, δ the thickness of diffusion layer, C concentration, D the diffusion coefficient and n the number of transferred electrons.

Knowing the current, one can easily deduce the diffusion coefficient in a biological experiment. With the incorporation of this amplifier design, transduction of a weak stream of mass-transport-diffusion electrons from a biosensor can be detected, which would otherwise be difficult with conventional amperometry techniques. This area needs investigation. An experiment in this direction is currently being considered by us for carrying out.

The design has great potential in its application in many areas in general biological and physiological measurement. It is earnestly hoped that this design will stimulate further efforts in this direction which could bring forth improved designs of similar ultra-low-level-current, ultra-low-noise and low-cost amplifiers, advancing the field of measurement science and technology in biosensors, bioelectronics, electrophysiology and quantum computing applications. By offering a measurement technique to measure non-thermal noise stochastic signals, which may be rich in knowledge pertaining to underlying physiological processes, as suggested by Bullock (1997) in the case of neurophysiology, this technique has great potential to be further investigated.

Acknowledgement

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Heterologous Expression of *Chaetomium thermophilum* Xylanase 11-A (CtX 11-A) Gene

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Abstract. *Chaetomium* has a potential source of xylanase and cellulase enzymes, both of which are required in the treatment of fibre in the poultry feed. The titre of the enzymes needs to be enhanced by using recombinant DNA technology for fulfilling the requirement of the industries. Efforts are made to construct prokaryotic and eukaryotic expression cassettes that can be cloned under specific strong promoters i.e., T7 and AOX1, respectively, and the enhancer elements to get the maximum gene expression. In the present study BL21 *E. coli* and GS115 *Pichia pastoris* strains are used as model organisms to express the CtX 11-A gene in the presence of 1 mM IPTG and 100% methanol upto final concentration of 0.5. In case of BL21 expression, the maximum xylanase activity was observed after 1.5 h in the presence of 1% xylose, which was 2.302 U/ml and after 7 h in the presence of 0.5% lactose, was 1.708 U/ml. However, in *Pichia pastoris* the maximum production of xylanase was 2.904 and 0.006 U/ml as compared to control 0.484 and 0.06 U/ml, respectively.

Keywords: thermophilic fungi, *Chaetomium thermophilum* xylanase A (CtXA), cloning and gene expression, *Escherichia coli*, *Pichia pastoris*

Introduction

Among the most abundant hemicelluloses in plant cell wall polysaccharides, xylanase has many important applications in various industries such as foods, chemicals, paper, fuels etc., (Boettner *et al.*, 2002; Christov and Prior, 1996; Alam *et al.*, 1994; Coughtan and Hazlewood, 1993; Wong and Saddler, 1992).

Xylanases are classified into two distinct families, F/10 and G/11, of the glycoside hydrolases (Henrissat and Bairoch, 1993). A number of xylanase-producing fungi and bacteria have been isolated from a variety of sources, which have a close relation with the characterization of the produced xylanases. Sinha *et al.* (2004) isolated an extremely thermostable xylanase from a thermophilic eubacterium. Collins *et al.* (2002) isolated a cold active xylanase from the antarctic bacterium *Pseudoalteromonas haloplanktis*. *Bacillus* sp., strain AR-009, an alkaliphile from an alkaline soda lake, produced two alkaliphilic xylanases (Emami *et al.*, 2002). In addition, an acidophilic xylanase was isolated from *Penicillium* sp. 40, which was screened from an acidic soil (Kimura *et al.*, 2000).

Production of thermostable cellulases and xylanases from thermophilic fungi is an important industrial source for

hemi-cellulases such as glucanases, xylanases, galactanases, mannanases, galactomanases and pentosanases. *C. thermophilum* is a thermophilic filamentous fungus that produces thermostable xylanase (Latif *et al.*, 1995) It is frequently found in soil, air and plant debris and produces endoxylanase, Xyn11-A. Enhanced enzyme production can be achieved by isolation, characterization, cloning and expression of the genes under specific strong promoters and enhancer elements.

In recent years, several industrial yeasts have been developed as recombinant host systems for the commercial production of heterologous proteins. These organisms combine ease of genetic manipulation with the ability to perform many eukaryotic post-translational modifications (Lin Cereghino *et al.*, 2002). One of the most commonly used systems is the methylotrophic yeast *Pichia pastoris*, in which expression is driven by one of the strongest known regulated promoters, the alcohol oxidase I (AOX1) promoter, which is induced by methanol and repressed by other carbon sources such as glucose, glycerol, and ethanol (Lin Cereghino and Cregg, 2000). Another important feature of this system is its ability to achieve extremely high cell densities, enabling efficient protein production and secretion (Pickford and O'Leary, 2004; Sreekrishna *et al.*, 1997).

At the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan, there is a large collection of thermophilic fungi (Latif *et al.*, 1994), which have

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potential applications in the poultry feed industry and paper and pulp industry. Wong and Saddler (1992) demonstrated that *C. thermophilum* shows large amounts of extra cellular cellulase and xylanase activity when grown on cellulosic or lignocellulosic substrates as carbon sources. In the present studies efforts were made to isolate xylanase (Xyn 11-A) gene from *C. thermophilum* strain NIBGE-1 and clone the xylanase gene in prokaryotic and yeast model systems. In this context, the expression model systems BL21 (Novogen) and *P. pastoris* is selected (Invitrogen, USA). *P. pastoris* has many advantages of higher eukaryotic expression systems such as protein processing, protein folding and post-translational modification. It is faster, easier and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture and generally gives higher expression levels.

Materials and Methods

Prokaryotic expression system. Isolation and amplification of Xyn 11-A gene. Xylanase gene (860 bps) was isolated from *Chaetomium thermophilum* NIBGE-1 strain by designing a set of primers Xyn 11-A (F) 5'-GGC GATAGC TAG CAT GGT CAA CTT CTC AAC TCTC -3' (34 mers) and Xyn 11-A (R) 5'-GGAAGG GCC CGC ACT GCATGC TTG TTAGC -3' based on the reported sequence from Gene bank nucleotide database accession no. AJ508931. This fragment was cloned into T/A cloning vector pTZ57R (MBI Fermentas) and the cloned was sequenced from Microsynth GmbH, Switzerland. The sequence was submitted to Gene bank and assigned accession No. AY366479. Sequencing and characterization of gene information revealed a 35 bp intron with two exons in the isolated gene fragment. The gene sequence AY366479 was compared with other xylanase genes from other organisms using online software. Intron was removed by amplifying the insert along with vector backbone except intron by using primers P₃ 5'-AGA CTC GAG TCG AAC CCC GGT ATC GAC -3' 27 mers and P₄ 5'-CTG CTC GAG GCG CTG GAAATG TTT TGT TGG -3' 30 mers. The desired gene was named as Xyn 11-A, and the resultant construct was named as pSWXyn11-A(a) as indicated in Fig. 1.

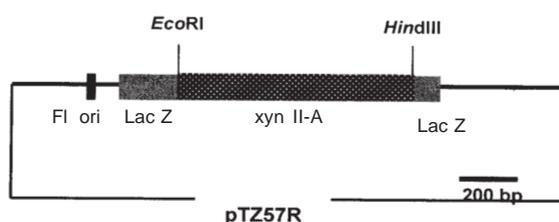


Fig. 1. Recombinant clone pSWXyn11-A(a) with restriction sites.

Cloning and Expression of Xyn 11-A in BL21 *E. coli* strain. Xyn 11-A gene, (810 bps), from pSWXyn11-A(a) and pET32a (+) was restricted with *EcoRI* and *Hind III*, respectively. The eluted fragments were ligated for overnight at 16 °C and transformed into 10b *E. coli* strain through heat shock at 42 °C. The transformants were selected on Laurie bertini (LB) medium supplemented with 100 mg/ml ampicillin. The resultant construct pSWXyn11-A(b) was confirmed with the combination of different restriction enzymes *EcoRI*, *Hind III*, *Xho I* and *Xba I*, respectively. The recombinant clone pSWXyn11-A(b) as indicated in Fig. 2, was transformed into heat shock BL21 competent cells and the transformants were verified through PCR analysis. The colony was cultured into broth LB medium supplemented with 100 mg/ml ampicillin and incubated at 37 °C for overnight. Next day, 2 ml of overnight culture was transferred to 50 ml (100 mg/ml) ampicillin LB broth medium and again incubated for 3 h at 37 °C. After three hours of growth, 70 µl of 1 mM IPTG inducer was added and the cells were again incubated at 37 °C for 3 h. Samples were collected every 30 min, cells were pelleted down and given the freeze thaw treatment. Cell lysate was dissolved in 1 ml citrate buffer and sonicated for complete lysis. The lysate was centrifuged at 13,500 rpm for 10 min and supernatant was used for further analysis.

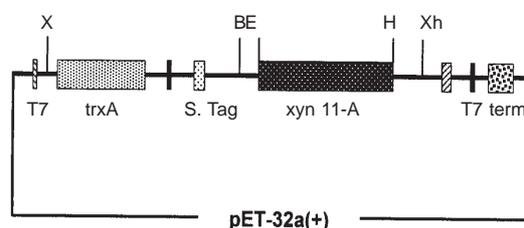


Fig. 2. Recombinant clone pSWXyn11-A(b) with restriction sites. E: *Eco RI*; H: *Hind III*; B: *BamHI*; X: *XbaI*; Xh: *XhoI*; T7: T7 promoter; T7 term: T7 terminator.

SDS-PAGE analysis. The supernatant fluid from cell lysate of selected cultures of *E. coli* were separated by SDS-PAGE on 10 % and visualized by coomassie blue staining.

Western blot analysis. Protein, 15 µg, from both transformed and non-transformed *E. coli* cells were run on 15 % SDS-PAGE gels and transferred to nitrocellulose paper. The polyclonal antibodies, raised against fusion part of the protein, conjugated to alkaline phosphatase detected bands that bound antibodies.

Inoculum preparation for induction of xylanase gene by using different carbon substrates. Different soluble carbon substrates were used at 0.5% and 1% level to induce the

xylanase gene expression in Dubose Salt (DS) media. These carbon sources were xylose, glucose, cellubiose and lactose. Colonies were picked and cultured in the test tubes containing 3 ml DS broth media with 100 mg/ml ampicillin (antibiotic) and xylose, glucose, cellubiose and lactose at 0.5% and 1% were used as carbon sources. Next day 1 ml culture was transferred to 2 ml DS broth media with 100 mg/ml ampicillin, and grown for 3 h on a shaker at 37 °C. After 3 h growth, 4.7 ml of (1m M) IPTG was added to act as inducer. Cultures were further grown on a shaker at 37 °C for one and a half hour and for 7 h. Medium containing growth was centrifuged at 13,500 rpm for 5 min. The xylanase activity from *E. coli* was assayed against oat spelt xylan method as described by Tuncer *et al.* (1999). Two ml of above reaction mixture containing 1 ml of cell lysate, 0.5 ml of 1 % oat spelt xylan and 0.5 ml of citrate phosphate buffer (pH 6) was incubated at 40 °C for 2 h at 60 rpm. The reducing sugar was determined by dinitrosalicylic acid (DNS) procedure.

***Pichia pastoris* (yeast expression system). Cloning and transformation of xylanase gene into *E. coli* TOP10F' strain.** The intron-less 810 bp fragment of xylanase gene from pSWXyn11-A(b) and pPIC3.5K *P. pastoris* vector were digested with *Eco*RI and *Not*I restriction enzymes and placed at 37 °C for 1 h. Digestion was run on 0.5 % agarose gel and the targeted fragments were eluted from the gel by using extraction Kit (MBI, Fermentas). Eluted fragments were ligated and transformed into heat shocked competent cells of *E. coli* TOP10F' strain (Li *et al.*, 2005). Colonies were selected randomly from overnight grown *E. coli* in LB agar medium containing ampicillin (100 mg/ml). Plasmid isolation was done by miniprep method (MBI, Fermentas). The resultant recombinant clone was confirmed through combination of different restriction enzymes i.e., *Eco*RI, *Not*I and *Kpn*I.

Transformation and screening of resultant recombinant clone into *Pichia pastoris*. The resultant recombinant vector pSWXyn11-A(c) (Fig. 3) was linearized with *Not*I restriction enzyme, transferred to the cuvette and electric shock was given at 2.0 kvolts for integration in the genomic DNA of *P. pastoris*. Then immediately 1ml of 1M sorbitol was added and cuvettes were placed on shaker at 30 °C. After 2 h the medium was spread on the 0.75 mg/ml concentration of geneticin YPD agar medium plates for the selection of transformants. These plates were incubated at 30 °C until colonies appeared. Colonies were picked from 0.75 mg/ml geneticin plate and cultured into YPD liquid medium without antibiotic and incubated at 30 °C till the time that cell density reached up to ($OD_{600} = 1.0$). The transformants were confirmed through PCR analysis by using set of primers P3 and P4. The

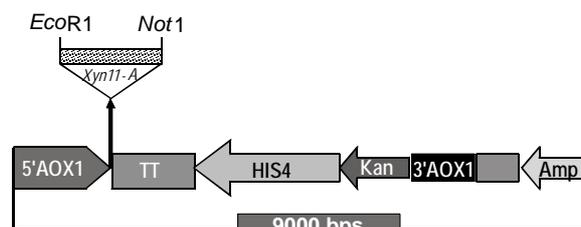


Fig. 3. Recombinant clone pSWXyn11-A(c) with restriction sites.

sample was prepared for PCR reaction under denaturation at 94 °C for 5 min, annealing at 60 °C for 1 min, extension to 72 °C for 1 min and 35 cycles, as 10 µ litre of *P. pastoris* culture into 1.5 ml microcentrifuge tube, 5 µ litre zymolyase enzyme was added and incubated at 30 °C for 10 min. The sample was frozen at -70 °C for 10 min.

Optimization of *P. pastoris* growth for xylanase assay and SDS-PAGE analysis. *P. pastoris* transformants having pSWXyn11-A(c) were picked from 0.75 mg/ml geneticin YPD agar media plates along with non-transformant GS115 as control and cultured into YPD broth media without antibiotic at 30 °C ($OD_{600} = 1.0$). Cells were harvested by centrifugation at 3000 rpm for 5 min at room temperature. Supernatant was discarded and cell pellets were resuspended into 25 ml minimal glycerol medium (MGM) and 0.02% 10 X dextrose in a 100 ml flask. Cultures were placed at 28-30 °C in a shaking incubator (150-200 rpm) until growth reached the log phase. Once the cells are in log phase, they can be induced for xylanase expression. One ml culture was taken before each induction of 100 % methanol to a final concentration of 0.5% in 25 ml MG medium. Induced culture was collected at different time intervals i.e. 24, 48, 72, 96 and 120 h. One ml of culture was transferred to 1.5 microcentrifuge tubes. These samples were used to analyze expression levels and determine the optimal time from post-induction to harvest. Cells were centrifuged at 13,500 rpm at room temperature for 2-3 min. For intracellular and secreted expression, both supernatant and pellet were stored at -70 °C until ready for protein assay.

Preparation of samples for xylanase assay and SDS-PAGE. The activity of xylanase was determined by the method described by Tuncer *et al.* (1999) against oat spelt xylan. The sample was prepared for both SDS-PAGE and xylanase assay as thawed cell pellets and quickly placed on ice. Pellets, were dissolved in 1 ml distilled water and 100 µ litre breaking buffer and an equal volume of acid washed glass beads (size 0.5mm) was added, vortexed for 30 sec., incubated on ice for 30 sec (repeated for several times) than centrifuged at 13,500 rpm for 10 min. Clear supernatant was transferred to a fresh 1.5 ml microcentrifuge tube, and 50 µ litre SDS-PAGE loading dye

was added for SDS-PAGE analysis and boiled for 10 min at 100 °C in a dry bath; 10-20 µ litre sample per well was loaded into SDS-PAGE gel whereas other used for xylanase assay and the rest were stored at -20 °C for use in future. Electrophoresis was performed using a discontinuous buffer system, for the analysis and separation of proteins. Developer solution was added to enhance and bands were visualized during silver staining of SDS-PAGE of xylanase Xyn11-A protein in *P. pastoris*.

Results and Discussion

Results in prokaryotic expression system. Confirmation of cloning of Xylanase into pET vector. The resultant recombinant clone pSWXyn11-A(a) was confirmed through digestion as shown in Fig. 1. After digestion, the xylanase gene of approx. 810 bp was cloned in the pET expression vector, and finally the recombinant clone pSWXyn11-A(b) was transformed into *E. coli* strain BL21 for bacterial expression. To confirm the cloning of intron-less Xyn11-A gene in pSWXyn11-A(b), *EcoRI* and *HindIII* restriction enzymes were used. Digestion with *HindIII* and *EcoRI* produced approx., 900 bp fragment. Similarly, digestion with *HindIII* and *XhoI* which produced a correct sized fragment of approx. 600 bp. Confirmation of pSW Xyn 11-A(b) was also made with *HindIII* and *XbaI* restriction enzymes which produced approx., 800 and 600 bp fragments.

Confirmation of transformants BL21 having pSWXyn 11-A(b). After confirmation of recombinant clone pSWXyn11-A(b) through combination of different restriction enzymes, pSWXyn11-A(b) and pET 32a(+) vector was transformed into *E. coli* BL21 strain by heat shock transformation method. The target gene from pSWXyn11-A(b) was confirmed through PCR analysis by using set of Xyn 11-A specific primers P₁ and P₂. The amplification of 810 bp fragment confirmed the transformation of recombinant vector in *E. coli* strain BL21.

Estimation of the xylanase gene expression in the form of fusion protein was carried out using xylanase assay. The xylanase gene expression in *E. coli* strain BL21 was induced by IPTG (1mM) inducer in LB broth media as indicated in Table 1 and shown in Fig. 4. The activity of xylanase by DNS method was obtained in U/ml. The maximum activity of xylanase gene U/ml was obtained after incubation of 2 h at 40 °C. The maximum and minimum production of xylanase was 4.62 and 3.99 U/ml, respectively, as compared to the control (4.19 and 3.91 U/ml, respectively).

Effect of different carbon sources on xylanase production.

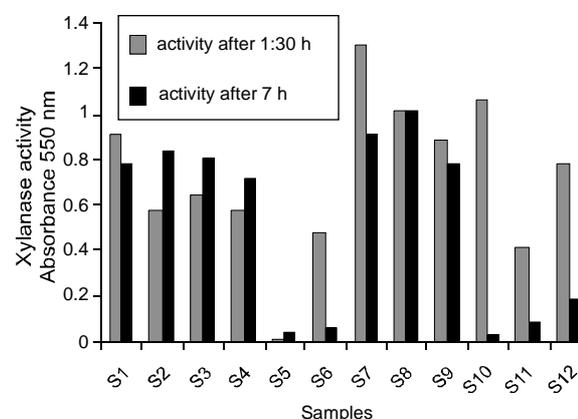
Effect of different carbon sources was observed on the xylanase gene expression, which was carried out through

xylanase assay. The xylanase activity in *E. coli* BL21 strain in DS liquid media containing ampicillin (100 mg/ml) and different carbon sources i.e., xylose, glucose, cellobiose and lactose, was carried out after 1.5 and 7 h, (Table 2, Fig. 4). Maximum activity of xylanase (U/ml) in the presence of carbon sources was obtained after incubation for 10 min at 40 °C. The maximum xylanase activity after 1.5 h, observed in the presence of 1% xylose, was 2.302 U/ml whereas minimum activity, observed in

Table 1. Xylanase activity in cell lysate of *E. coli* (BL21) harboring the pET expression vector with intron-less xylanase fragment.

Time of induction*	Enzyme activity U/ml **		
	Sample 1	Sample 2	Sample 3 (control)
At zero h	3.19	3.74	3.44
After 30 min	4.62	3.40	3.81
After 60 min	4.03	3.80	4.19
After 90 min	3.95	3.99	3.92
After 120 min	4.19	3.94	3.75

* = induction with IPTG (1 mM); ** = HI sample 1 and 2: pSWXyn11-A(b); sample 3: pET 32a(+)



- S 1: (0.5% xylose + DS media + xylanase gene)
- S 2: (0.5% glucose + DS media + xylanase gene)
- S 3: (0.5% cellobiose + DS media + xylanase gene)
- S 4: (0.5% lactose + DS media + xylanase gene)
- S 5: (without carbon source + DS media + pET)
- S 6: (without carbon source + DS media + xylanase gene)
- S 7: (1% xylose + DS media + xylanase gene)
- S 8: (1% glucose + DS media + xylanase gene)
- S 9: (1% cellobiose + DS media + xylanase gene)
- S 10: (1% lactose + DS media + xylanase gene)
- S 11: (LB media + pET)
- S 12: (LB media + xylanase gene).

Fig. 4. Xylanase activity in cell lysate of *E. coli* (BL21) in the presence of different carbon sources

Table 2. Xylanase activity in cell lysate of *E. coli* (BL21) in the presence of different carbon sources.

<i>E. coli</i> strain transformed with vector	Medium used	Carbon source	Enzyme activity U/ml with vector	
			After one and half h	After 7 h
pSSZ810 (b)	DS*	0.5 % xylose	1.375	0.575
pSSZ810 (b)	DS	0.5 % glucose	-0.454	1.139
pSSZ810 (b)	DS	0.5 % cellubiose	0.515	1.539
pSSZ810 (b)	DS	0.5 % lactose	0.860	1.708
PET 32a (+) control	DS	without carbon source	-0.303	0.139
pSSZ810 (b)	DS	without carbon source	2.787	0.236
pSSZ810 (b)	DS	1 % xylose	2.302	0.133
pSSZ810 (b)	DS	1 % glucose	1.363	1.369
pSSZ810 (b)	DS	1 % cellubiose	-0.136	-1.424
pSSZ810 (b)	DS	1 % lactose	0.412	-5.853
PET 32a (+) control	LB**	without carbon source	2.411	0.488
pSSZ810 (b)	LB	without carbon source	2.696	-0.983

* = first media; ** = second media

the presence of 1 % cellobiose, was -0.136 U/ml. On the other hand the maximum activity of xylanase after 7 h, observed in the presence of 0.5 % lactose, was 1.708 U/ml, whereas, minimum activity, in the presence of 1 % lactose, was -5.853 U/ml.

SDS-PAGE analysis for xylanase protein induced by IPTG inducers. The production of recombinant xylanase by *E. coli* BL21 was indicated by the appearance of a 43 kDa protein on SDS-PAGE. The amount of recombinant protein increased with different induction times as indicated in Fig. 5.

Western blotting of recombinant xylanase. Western blot analysis was carried out by using polyclonal antibodies raised against fusion protein.

Results of *Pichia pastoris* (yeast expression system). **Confirmation of recombinant clone pSWXyn11-A(c) through restriction analysis and transformation into GS115 *Pichia pastoris* strain.** Xylanase gene was digested with *EcoRI* and *NotI* enzymes and cloned at the same sites in pPIC3.5K. Upon digestion with *EcoRI* and *NotI*, recombinant clone pSWXyn11-A(c) produced (approx.) 810 bp fragment along with vector backbone of pPIC3.5 K of 9000 bp. pSSZ810 recombinant clone has two *KpnI* restriction sites, an internal and the other *KpnI* site is located in the vector backbone. Therefore, upon digestion with *KpnI*, it produced two fragments of sizes 500 and 300 bp, respectively.

The purified and linearized fragment of pSWXyn11-A(c) with *NotI* was transformed into *P. pastoris* strain GS115 through electroporation for integration into genomic DNA of *P. pastoris*. Concentration of geneticin was optimized for the selection of

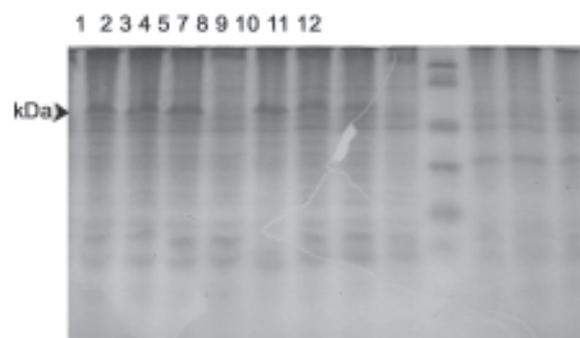


Fig. 5. SDS-PAGE analysis of total protein isolated from bacterial strain BL21 transformed with pSWXyn11-A(b) and PET 32a(+). Lane 1-8: pSWXyn11-A(b) transformed BL21. Lanes 10-12: PET 32a(+) transformed BL21. From right to left in lane 12, 8 and 4: protein induced after zero minute, Lane 11, 7 and 3: protein induced after 30 minutes, Lane 10, 6 and 2: protein induced after 60 minutes, Lane 5 and 1: protein induced after 90 minutes. 9: represents prestained protein marker #SM0441 118 k Da (MBI Fermentas).

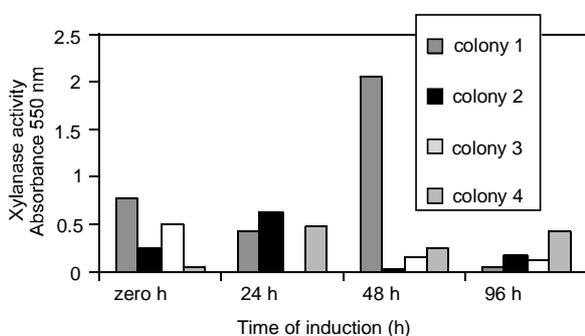
transformants from 30, 50, 70, 90, 110, and 130 µg/ml. Suitable concentration of geneticin (antibiotic) for selection of transformants was found to be 0.75 mg/ml. The transformants were grown on YPD agar media plates containing 0.75 mg/ml geneticin. The integration of linearized fragment pSWXyn11-A(c) having xylanase gene in the genome of *P. pastoris* was confirmed through PCR amplification by using xylanase specific primers.

Xylanase assay of pSWXyn11-A(c) in GS115 *Pichia pastoris* strain. Production of xylanase protein by recombinant *P. pastoris* GS115 strain was induced by 100 % methanol up to a final concentration of 0.5 % in minimal glycerol media (MGM) broth as indicated in Table 3. The maximum and the minimum production of xylanase was 2.04 and 0.006 U/ml as compared to the control, 0.484 and 0.06 U/ml, respectively. Li *et al.* (1993) determined the expression of Mmr-1 protein in *P. pastoris* by induction under the concentration of 0.5% methanol. The level of this recombinant protein was about 50 mg/ml which is greater than our expressed protein after 48 h of induction (Fig.6).

Table 3. Xylanase activity in *Pichia pastoris* GS115 strain

Time of induction*	Xylanase activity (U/ml)**			
	Sample 1	Sample 2	Sample 3	Sample 4
At zero h	0.769	0.242	0.5	0.06
After 24 h	0.424	0.632	0.006	0.484
After 48 h	2.04	0.014	0.151	0.242
After 96 h	0.06	0.175	0.133	0.436

* = 100 % methanol inducer upto 0.5% final concentration; ** = sample 1-3: transformants having pSSZ810(c) xylanase gene; sample 4: non-transformant *Pichia pastoris* GS115 strain



Colony 1: having pSWXyn11-A(c) at zero, 24, 48 and 96 h

Colony 2: having pSWXyn11-A(c) at zero, 24, 48 and 96 h

Colony 3: having pSWXyn11-A(c) at zero, 24, 48 and 96 h

Colony 4: without pPIC3.5k at zero, 34, 48 and 96 h

Fig.6. Xylanase production by *Pichia pastoris* strain GS115 at different time period

SDS-PAGE (silver staining) of pSWXyn11-A(c) in GS115 *P. pastoris* strain. The intensity of desirable protein (approx.) 28 kDa increased with increase in time after 100 % methanol up to a final concentration of 0.5 % induction. The maximum activity of pSWXyn11-A(c) having xylanase protein was observed after 96 h of induction period whereas no band was observed in case of non-transformant *P. pastoris* GS115.

Conclusion

Hyperactivity of this xylanase versus those from other sources may be due to the presence of glycine rich C-terminal region which is absent in other fungi. However, for determination of its exact function further investigations are required. The maximum xylanase activity was observed in the presence of 100% methanol inducer and, after incubation for 2 h at 50 °C, is lower as compared to 2 mg/ml, observed in the previous studies (Faber *et al.*, 1995)

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Review

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Lobsters from Northern Arabian Sea (Pakistan Coast)

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Abstract. Pakistan is situated between latitude 24° and 37° North and longitude 62° and 75° East, on the eastern part of the Arabian Sea with a coastline of about 900 km. Lobsters are found both on its northwest (Balochistan) and southeast coasts (Sindh). Important fishing grounds of lobsters are located along Ormara, Pasni Gwader and Jiwani besides Karachi coast. To date, 16 species of lobsters have been recorded from Pakistan. The only predominant commercial species is *Panulirus polyphagus*, which constitutes 83% of the catch. The paper provides an identification key for northern Arabian Sea lobsters and outlines the available information on the biology, fisheries and management of the lobster species from Pakistan. Most of the studies have been focused on *P. polyphagus*.

Keywords: Arabian Sea lobsters, *P. polyphagus*, Arabian Sea, lobsters

Introduction

Pakistan is situated between latitude 24° and 37° north and longitude 62° and 75° east on the eastern part of the Arabian Sea with a coastline of about 900 km. It is divided into two ecological zones: Balochistan coast on the northwest and Sindh coast on the southeast. The Balochistan coast is about 630 km long and the Sindh coast is about 270 km long. Along the Balochistan coast, the continental shelf is 10-20 km wide; the bottom is mostly rocky except at Sonmiani Bay. The Sindh coast is characterized by innumerable creeks; the continental shelf extends to about 30-100 km; the bottom of the shelf is muddy and muddy-cum-sandy. The river Indus discharges in this area and forms the Indus Delta, which is one of the largest deltas in the world. The Balochistan coast is endowed with stable hydrological conditions characterized by high temperature and salinity owing to little fresh water drainage.

Species diversity. A sound knowledge of biodiversity and biology of commercial species forms the basis for a sustainable management. To date, 16 species of lobsters have been recorded from Pakistan (Kazmi, 2004, 2003; Holthuis, 1991; Williams, 1986). These species belong to eight genera and two families. The family Palinuridae is represented by two genera *Panulirus* and *Puerulus*. Genus *Panulirus* has five species and two sub species recorded from Pakistan coast: *Panulirus versicolor*, *P. homarus megasculpta*, *P. homarus rubellus*, *P. ornatus*, *P. polyphagus* and *P. penicillatus*; *Puerulus* has two species: *Puerulus angulatus* and *P. sewelli*. The family Scyllaridae comprises of six genera and nine species viz. *Thenus orientalis*, *T. pariindicus*, *T. indicus*, *Scyllarides*

tridacnophaga, *S. haani*, *Scammarctus batei*, *Eduarctus martensii*, *Acantharctus ornatus* and *Parribacus antarcticus*.

History of Taxonomy of Pakistani Lobsters. The history of carcinological studies in Pakistan reveals that the lobster fauna of Pakistan reported in the beginning belonged to only one Palinurid genus *Panulirus* and its three species and one Scyllarid genus *Thenus*, till then monotypic (Ahsanullah, 1965; Tirmizi and Ahsanullah, 1965; Government of Pakistan, 1960; Hussain, 1958). Additions of several genera and species have been made through reports as World Lists (Burton and Davie, 2007; Holthuis, 2002, 1991, 1984; Williams, 1986) and new records made by local scientists (Kazmi, 2003, 1995; Moazzam *et al.*, 2003; Fatima, 2000; Tirmizi and Kazmi, 1983).

In recent years several nomenclatural changes have also been made world wide. The scyllarine genus *Thenus* was represented only by *Thenus orientalis* until recently; now Burton and Davie (2007) have revised the taxonomy on the basis of DNA studies and recognized four species within the genus (Burton and Davie, 2007). They established three new species, one of these, *T. parindicus*, is also included in the material from Pakistan; another species *T. indicus* was separated from the old *T. orientalis* as non-type material from Karachi. The record of *T. orientalis* from Pakistan is given with some doubts by Burton and Davie (2007). Prof. Dr. L. B. Holthuis revised the Indo-Pacific species of the scyllarine genera in 2002 and separated several genera from the species formally placed in the genus *Scyllarus* Fabricius. Three such species which were reported from Pakistani waters were also transferred to the new genera created by Holthuis. The material examined for new genus *Acantharctus* included

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material collected from Pakistan (then British India) in 1930. More works on taxonomic status also altered the species composition occurring in the northern Arabian Sea up to 10°N. Some old records were found to be misidentified, for example *Jasus lalandi* reported by Prasad and Tampi (1968) is actually *Parribacus antarcticus* (Holthuis, 1985). Pollock *et al.* (2000) have given the distribution of the species *Jasus lalandi* in the south east Atlantic region, so *J. lalandi* is no more included in the faunal list from Arabian Sea. Similarly the case of genus *Parribacus* (*P. antarcticus*), reported by Williams (1986) from the northern Arabian Sea (including Pakistani waters), has to be treated with some reservation since it was not included by Holthuis (1991) in the world list of lobsters from Pakistan or either Arabian Sea. Another problematic genus is *Palinustus*. What is believed to be *P. waguensis* has been reported under the name of *P. mossambicus* from the Arabian Sea. The taxonomic status of *P. waguensis vis-à-vis P. mossambicus* is far from clear and closer study of the complex is still highly desirable (Holthuis, 1991).

The nominotypical form of *Panulirus homarus* (*P.h.homarus*) is found throughout the range of species but *P. h. megasculpta* is known only from the northern Arabian Sea and *P. h. rubellus* inhabits S. E. Africa and S. E. Madagascar (Holthuis, 1991). These subspecies were not distinguished in the study by Tirmizi and Ahsanullah (1965). Kazmi (1995) reported a specimen of *P. homarus* collected from Churna Island, Makran Coast (25° 5' E, 67° 5' N). Interestingly, this specimen appeared to be more similar to *P. h. rubellus* than to the local subspecies *P. h. megasculpta*. The colour pattern differed from all the known forms and some minor morphological differences were also noticed in the antennal peduncle, antennular plate, and the abdominal pleura. There is a prominent spine at the inner anterolateral angle of the basal antennal segment (Fig. 1a). The antennular plate has two unequal pairs of principal spines, two tufts of course plumose setae arranged in double row with three sharp spinules (Fig. 1b). The scallops of the abdominal grooves are deep, large and interrupted; the armature on the posterior margin of the abdominal pleurae is strongly developed (Fig. 1c). The live specimen (MRC Catalogue No. Rapt. 1) was dark brown with the anterior portion of the carapace brick red mid-dorsally and the abdomen was light orange. It is probable that two mentioned forms may have intermingled and a hybrid is emerging in our area since hybrids are common in areas where subspecies overlap (Pollock, 1993).

An earlier unpublished key prepared for the northern Arabian Sea up to 10° N, under the Pakistan Science Foundation Research Project (1988-89) titled "Guide to the Malacostraca of

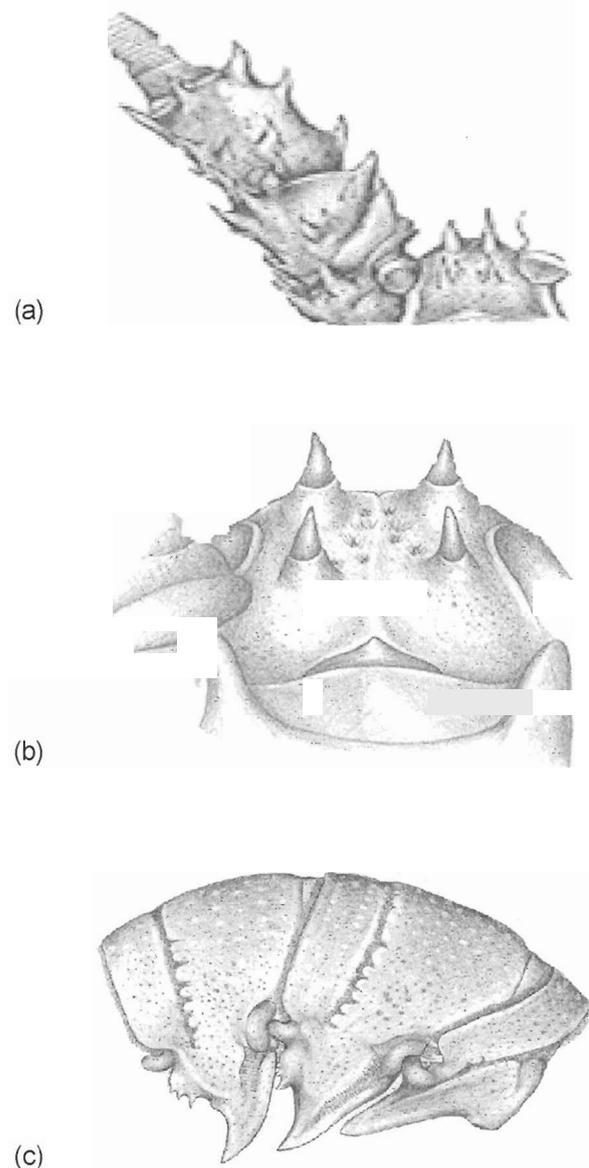


Fig. 1a-c. (a) The prominent spine at the inner anterolateral angle of the basal antennal segment. (b) The antennular plate showing two unequal pairs of principal spines, two tufts of plumose setae in double row with three sharp spinules. (c) The strong armature on the posterior margin of the abdominal pleurae.

the Arabian Sea" by Tirmizi and Kazmi (1989) included fewer taxa. This key has been updated and included in this publication. To date two nephropine genera, *Nephropsis* and *Acanthocharis*, one polychelid genus *Polycheles*, one ibacine genus *Parribacus*, one arctidine genus *Scyllarides*, sole thenine genus *Thenus*, five scyllarine genera *Scammarctus*,

Bathyarctus, *Eduarctus*, *Acantharctus* and *Biarctus* and two palinurid genera *Panulirus* and *Puerulus* are reported from the Arabian Sea. Out of these *Parribacus*, *Thenus*, *Scyllarides*, *Scammarctus*, *Eduarctus*, *Acantharctus*, *Panulirus* and *Puerulus* extend to north in the Arabia Sea (24-37 °N) in Pakistani coastal waters.

The key presented here has been fabricated using different sources mainly from Holthuis (1991). The first reports of the species from the area are referenced. For the higher taxa the key by Martin and Davis (2001) has been followed. Further Ah Yong and O'Meally (2004) and Dixon *et al.* (2003) revised reptantian classification and changed the position of Scyllaridae and Palinuridae and put them in the infraorder Achelata.

Key to the northern Arabian Sea lobsters.

- 1a. First three pairs of pereopods with true chelae, first pair the largest and most robust ----- 2
- 1b. Third pereopod never with a true chela, in most groups chelae also absent from first and second pereopods --- 3
- 2a. Fourth pereopod and usually also the fifth, without true chelae. Carapace cylindrical not flattened ----- Infraorder Astacidea, Superfamily Nephropoidea, Family Nephropidae - Deep sea true lobsters and lobsterettes ----- 4
- 2b. All pereopods, or at least the first four, with true chelae. Carapace flattened ----- Infraorder Palinuridea, Superfamily Eryonoidea, Family Polychelidae -----
A single genus and single species: *Polycheles andamanicus*
(Ramadan, 1938).
- 3a. Antennal flagellum reduced to a single broad and flat segment, similar to the other antennal segments -----
Infraorder Palinuridea, Superfamily Palinuroidea, Family Scyllaridae ---- Slipper lobsters ----- 9
- 3b. Antennal flagellum long, multi-articulate, flexible, whip-like or more rigid. Epistome short, far shorter than 1/3 of the carapace. Eyes not placed on an elevation of the cephalon. Carapace with numerous strong and less strong spines and two frontal horns over the eyes. Rostrum absent or reduced to a single spine. Legs 2 to 4 (usually also 1) without chelae or subchelae ----- Infraorder Palinuridea, Superfamily Palinuroidea, Family Palinuridae-Spiny lobsters or Langoustes ----- 19
- 4a. Rostrum laterally compressed for the larger part of its length, with dorsal and ventral, but no lateral teeth. Carapace with branchiostegal spine. Body entirely covered by numerous closely placed and sharply pointed spinules. Lateral margin of the telson with 6 to 12 spines -----
----- Subfamily Neophoberinae -----
- 4b. Rostrum dorsoventrally depressed with lateral (and sometimes ventral) but without dorsal teeth; sometimes without any teeth. Carapace without a branchiostegal spine. Body never uniformly covered with spinules, although granules may be present all over or spinules may be placed on the carapace. The lateral margin of the telson with at the most three lateral spines, which if present, are usually small and irregular.
Scaphocerite absent. Carapace without postorbital spine. Abdominal sternites unarmed in both sexes. No podobranch on second maxilliped ---- Subfamily Thymopinae. Antennal scale absent. Pincers of first pair of legs with soft pubescence. Body not uniformly spinulose. Abdomen without a distinct median ridge ----- single genus: *Nephropsis* ----- 5
- 5a. Rostrum without lateral teeth. A strong post-supraorbital spine present behind the supraorbital spine. Abdominal somites III to VI with a median dorsal carina. Anterior margin of pleura of abdominal somite II without spines. Telson without medio-dorsal spine - Gladiator lobsterette-----
----- *N. ensirostris*
(Macpherson, 1990).
- 5b. Rostrum with lateral teeth. Other characters mentioned under 5a present or absent ----- 6
- 6a. Rostrum with one pair of lateral teeth (one tooth on either margin). Anterior margin of pleuron of second abdominal somite without a spine, although the pleuron itself may end in a sharp, spine-like tip ----- 7
- 6b. Rostrum with two pairs of lateral teeth. Anterior margin of second abdominal somite with or without spine ----- 8
- 7a. Abdominal somites without any trace of a mid-dorsal carina. No post supraorbital spine on carapace. The distance between the supraorbital spines and the gastric tubercle is less than half the distance between the gastric tubercle and the cervical groove. Exopod of uropod with a diaeresis ----- Indian Ocean lobsterette ----- *N. stewarti*
(Macpherson, 1990).
- 7b. Median dorsal carinae on abdominal somites III to VI, but not second ----- Ridge-back lobsterette -----
----- *N. carpenteri*
(Holthuis, 1991).
- 8a. Abdomen with a dorsomedian carina on the somites II to VI. Exopod of uropod with a diaeresis. Rostrum with two pairs of lateral teeth in the basal part. The supraorbital spine is followed by a post supraorbital spine. Anterior

A single genus and single species: Prickly deep-sea lobster ----- *Acanthacaris tenuimana*
(Holthuis, 1984; Alcock and Anderson, 1894).

- margin of pleura of abdominal somite II with one or two spines in the basal half. Telson without mediodorsal spine in the basal part.
Median groove of rostrum reaching distinctly beyond anterior pair of lateral rostral teeth. Distance between supraorbital spine and gastric tubercle is half the distance between gastric tubercle and postcervical groove ----- Grooved lobsterette ----- *N. sulcata* (Holthuis, 1991).
- 8b. Abdomen without mediodorsal carina. Exopod of uropod without diaeresis ----- Red and white lobsterette -----
----- *N. suhmi*.
(Macpherson, 1990; Ramadan, 1938).
- 9a. Exopod of all maxillipeds with a multiarticulate flagellum. ----- 10
- 9b. Exopod of third and first maxilliped without a flagellum; the flagellum of the second maxilliped transformed to a single laminate segment ----- 11
- 10a. Carapace strongly depressed, with a deep cervical incision in the lateral margin. Mandible with a simple two-segmented palp ----- Sculptured mitten lobster -----
----- Subfamily Ibacinae
A single genus and single species: *Parribacus antarcticus* (Williams, 1986).
- 10b. Carapace rather highly vaulted, with a small, shallow cervical incision, which may be lacking altogether. Mandible with three-segmented palp ----- Subfamily Arctidinae.
A single genus: *Scyllarides* ----- 19
- 11a. Orbits on the anterolateral angle of the carapace. Body strongly depressed. Lateral margin of the carapace with only the cervical incision. No teeth on the lateral margin of the carapace, apart from the anterolateral and postcervical. Fifth leg of female without a chela -----
----- Subfamily Theninae.
A sole genus *Thenus* and single species: Flat-head lobster ----- 12
- 11b. Orbits on the anterior margin of the carapace, some distance from the anterolateral angle. Body not depressed, but rather high and vaulted. Lateral margin of the carapace with both cervical and postcervical incisions, neither of which is very deep. Lateral margin of the carapace with numerous teeth or squamiform tubercles. Fifth leg of female with a chela ----- Subfamily Scyllarinae ----- 14
- 12a. Spotting on pereopods absent. Outer face of propodus of second leg having upper most longitudinal grooves with setae reduced to short thin line near base, or completely lacking. Merus of third maxilliped with out a small spine proximally on inner ventral margin; inner margin of ischium distally dentate, but smoother and slightly molariform proximally ----- *T. parindicus*.
(Burton and Davie, 2007).
- 12b. Spotting on pereopods either present or absent. Outer face of propodus of second leg having upper most longitudinal grooves bearing obvious setae over at least proximal half. Merus of third maxilliped with a small spine proximally on inner ventral margin; inner margin of ischium prominently dentate along entire length ----- 13
- 13a. Numerous small spots or blotches present on some or all segment of pereopods ----- *T. orientalis* (Ahsanullah, 1965).
- 13b. Small spotting on pereopods absent, ventral face of some segments may be darkly blotched. Inner face of merus of one or more pereopods lacking a large purple to black pigmented blotch ----- *T. indicus* (Burton and Davie, 2007).
- 14a. Posterior half of the dorsal surface of abdominal somites II to IV with a rather wide transverse groove over the middle, sometimes with tubercles on either side, but without an arborescent pattern of narrow grooves. Abdominal somites with a distinct median longitudinal carina sharply set off from the rest of the dorsal surface ----- 15
- 14b. Posterior half of the dorsal surface of abdominal somites II to IV with an arborescent pattern of a narrow central transverse groove with side grooves, that often are branched ----- 16
- 15a. Anterior part of thoracic sternum gutter-like sunken and directed down, its anterior margin tapering anteriorly and ending in a median point, which is placed lower than the anterolateral angles of the sternum. Fourth and fifth legs unusually long and slender, the fifth reaching the base of the antenna. Dactylus of third to fifth legs with a double dorsal fringe of setae. Fourth antennal segment without additional carina *Scammarctus*
A single species: Soft locust lobster ----- *S. batei* (Holthuis, 1991, as *Scyllarus batei*).
- 15b. Anterior margin of thoracic sternum V- or U- shapedly incised, truncate or convex, but in a horizontal plane, top not sunken. Fourth and fifth legs not remarkably slender and without a double dorsal fringe of setae on the dactylus.
Anterior margin of thoracic sternum truncate or convex, sometimes with a median tubercle but with median incision; this margin situated on about the same level as the anterolateral teeth of the rostrum. Propodii of first four

- legs often with ventral setae ----- *Bathyarctus* A single species: *B. rubens*
(Holthuis 1991; George, 1969, as *Scyllarus rubens*).
- 16a. Fourth segment of antenna with an additional carina or a row of tubercles outside the main oblique carina. Abdominal somites II to IV with a median dorsal carina, that of somite III usually highest. Rostral tooth absent or small. Fourth antennal segment with an additional row of tubercles ----- *Eduarctus* ----- 17
- 16b. Fourth segment of antenna with a single oblique carina over its full length; no additional carina or rows of tubercles. Abdominal somites usually without a median carina ----- 18
- 17a. The posterior margin of the smooth anterior half of abdominal somites II to IV crenulated ----- *E. aesopius*
(Holthuis, 1991 as *Scyllarus aesopius*).
- 17b. The posterior margin of the smooth anterior half of abdominal somites II to IV is straight, non crenulated ----- Striated locust lobster ----- *E. martensii*
(Holthuis, 1991, as *Scyllarus martensii*).
- 18a. Last segment of thoracic sternum with a sharp median thorn ----- *Acantharctus* ----- A single species: *A. ornatus*
(Ramadan, 1938, as *Scyllarus arctus* var. *paradoxus*).
- 18b. Last segment of thoracic sternum at most with a central tubercle.
Anterior margin of thoracic sternum deeply V - shapedly incised, without additional tubercles, pregastric tooth absent ----- *Biarctus* ----- A single species: *B. sordidus*
(Chhapgar and Deshmukh, 1964, as *Scyllarus sordidus*).
- 19a. Red spot on smooth part of somite 1 small and irregular; somites III-IV distinctly humped - Humpbacked locust lobster or Aeosop slipper lobster ----- *S. haanii*
(Moazzam *et al.*, 2003; Williams, 1986).
- 19b. Smooth part of somite 1 without spot, or more often with 3 or more red or reddish spots of varying size and intensity ----- 20
- 20a. Somite 1 with median spot diffuse, not surrounded by ring of yellow; pale red blotches on body surface, marbled with brown or grey; side plates of somite II toothed on margin ----- Locust lobster or Blunt slipper lobster -----
----- *S. squamosus*
(Holthuis, 1991).
- 20b. Somite 1 with spots placed widely apart and nearly equal in size; yellowish brown - Clam - killer slipper lobster -----
----- *S. tridacnophaga*
(Tirmizi and Kazmi, 1983).
- 21a. Flagella of antennules long, whip-like, longer than peduncle of antennules ----- *Panulirus* ----- 24
- 21b. Flagella of antennules short, shorter than last segment of antennular peduncle ----- 22
- 22a. Frontal horns truncated with anterior margin crenulated; first segment of antennular peduncle reaching beyond antennal peduncle ----- *Palinustus* ----- A single species: *P. mossambicus*
(Kurian, 1967).
- 22b. Frontal horns tapering to a sharp point; first segment of antennular peduncle not over-reaching antennal peduncle. Antennular plate distinct, a stridulating organ present. Carapace with a median ridge behind the cervical groove, often with spines or tubercles, but without submedian rows ----- *Puerulus* ----- 23
- 23a. Median keel of carapace with 8 small teeth (5 postcervical, 3 intestinal). Surface of carapace covered with scattered granules, large tubercles present on ridges, 2 spines behind supraorbital spines ----- Arabian whip lobster -----
----- *P. sewelli*
(Ramadan, 1938).
- 23b. Median keel of carapace with 5 teeth (3 postcervical, 2 intestinal); body spines distinct and sharp; 3 spines behind supraorbital spines; fifth pereopod of male not chelate ----- Banded whip lobster ----- *P. angulatus*
(Moazzam *et al.*, 2003).
- 23c. Median keel of carapace with 5 to 7 teeth (3 postcervical, 2 to 4 intestinal); fifth leg of male chelate - Red whip lobster ----- *P. carinatus*
(Moazzam *et al.*, 2003).
- 24a. Abdominal somites with a distinct transverse groove which may be interrupted in the middle. Third maxilliped with or without exopod ----- 25
- 24b. Abdominal somites smooth, without transverse groove. Third maxilliped without exopod ----- 26
- 25a. Anterior margin of transverse groove of abdominal somites crenulated. Groove itself either complete or interrupted in the middle. Antennules plate with 4 equal large, well separated spines, arranged in a square with additional very small spinules scattered in between. Exopod of third maxilliped absent. Colour: body dark green or reddish brown, finely spotted with white. No distinct bands of light colour on the abdomen. A light anterior spot at the base of the abdomen pleura. Antennules banded. Legs rather uniform in colour, sometimes with faint, longitudinal streaks ----- Scalloped spiny lobster -----
----- *P. homarus*.
(Tirmizi and Ahsanullah, 1965).

25b. Transverse groove of abdominal somites with straight margins, not crenulated.

Antennular plate with 4 strong spines, which are fused at their bases, forming a single bunch of 4 diverging points; the anterior pair shorter than the posterior. Exopod of third maxilliped present, with flagellum. Transverse grooves over the abdominal somites usually uninterrupted. Colour: body greenish or reddish, ranging from yellowish green through brown green to blue-black or dark reddish brown; speckled on carapace and abdomen with tiny whitish spots. No transverse colour bands on abdomen, but two rather large whitish spots on somite I. Antennules not banded. Legs with wider or narrower longitudinal yellowish lines or streaks on a dark (greenish or reddish) background ----- Pronghorn spiny lobster ----- *P. penicillatus*

(Tirmizi and Ahsanullah, 1965).

26a. Abdominal somites 1 to 6 with a distinct uninterrupted white transverse band along the posterior margin ----- 27

26b. Abdomen without distinct transverse bands on all somites, sometimes there is a line of pale spots there, or a narrow line is present on somites I to III, but on somites IV and V, this is replaced by a row of spots. Antennular plate with 4 spines.

Abdominal somites smooth and naked. Colour of abdomen brownish or greenish grey with at most minute indistinct speckles. The usual large eyespot in the anterior half near the base of the pleura is accompanied by an oblique pale streak placed somewhat middle of the eyespot. The pleura have white tips, sometimes this white colour extends slightly up the anterior and posterior margins. Carapace with a peculiar and very characteristic marbling of pale lines near the bases of the frontal horns. Legs not streaked, but with very sharply defined irregular dark spots of a bluish or brownish colour, which often form incomplete rings around the various segments. Antennal flagella distinctly ringed ----- Ornate spiny lobster ----- *P. ornatus*

(Fatima, 2000; Holthuis, 1991; Chhappgar and Deshmukh, 1964; Government of Pakistan, 1960).

27a. Antennular plate with 2 strong spines; surface of abdominal somites naked and smooth Colour: abdominal somites II to V with a white transverse band along the posterior margin which, however, is not set off by dark bands. Colour of body and abdomen usually greyish green without spots. Tailfan of a rather uniform colour. Legs irregularly spotted, not distinctly streaked ----- Mud spiny lobster ----- *P. polyphagus*

(Tirmizi and Ahsanullah, 1965).

27b. Antennular plate with 4 strong spines arranged in a quadrangle. The whitish transverse bands along the posterior margin of the abdominal somites very distinct because they have a dark band in front and just behind them - Painted spiny lobster ----- *P. versicolor*
(Tirmizi and Kazmi, 1983).

Lobster fisheries. The main fishing grounds for lobsters along the coast of Pakistan are shown in Fig. 2. Best lobsters in terms of size and weight are caught along Karachi coast though the quantity is low; other important fishing grounds are located along Balochistan coast (Ormara, Pasni, Gwader and Jiwani). The spiny lobsters, after being caught, are mostly kept alive in seawater cages in shallow submerged areas of the coast and at processing units till they are either locally marketed or exported to other countries. The average size of spiny lobsters is 106 mm carapace length (Tirmizi and Bashir, 1975). Out of 16 recorded species, *P. polyphagus* was the only species exploited commercially; 83% of the samples studied consisted of *P. polyphagus*, whereas 16% comprised of *P. homarus*, and 1% included *Thenus orientalis* and other species. The magnitude of lobster fisheries is low despite considerable species diversity. Total lobster catch during 1993 to 2005 is presented in Fig. 3 (Marine Fisheries Department, 2002) alongwith the total export and catch of lobsters separately from two maritime provinces *viz* Sindh and Balochistan. The lobster catch ranged between 615 to 1077 metric ton during 1993-2004; the maximum catch was observed in 1999. Lobster catches were high from Balochistan during 1993 to 1998 comprising of 62-73% of the total catch, whereas from 1999 onwards more than 50 percent (57-73%) of the total catch was obtained from Sindh coast; increase in lobster catch from Sindh coast was due to the intensification of catch effort along Sindh coast. Lobster fishing is conducted almost round the year but the best catches were observed by the end of August to end of September, whereas, minimum or almost nil catches were observed in June, July and early August due to the strong southwest monsoon which limits fishing activities. Export of lobsters ranged between 66 to 288 metric tones (Table 1) and the earnings ranged between Rs.6.3 to 75.8 million (1US\$ = Rs.80).

Lobsters are exported in both the live and the frozen forms (whole, meat, tails) to USA, Japan, Saudi Arabia, Oman, Italy, The Netherlands, Hong Kong, Singapore, Belgium, France, Kuwait and Maldives (Marine Fisheries Department, 2002).

Biological studies. Present knowledge on lobster biology is based exclusively on a project report conducted by Marine

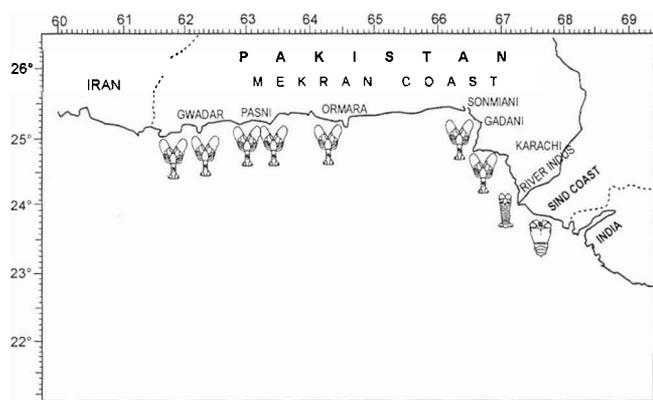


Fig. 2. The main fishing grounds for lobsters along Pakistan coast.

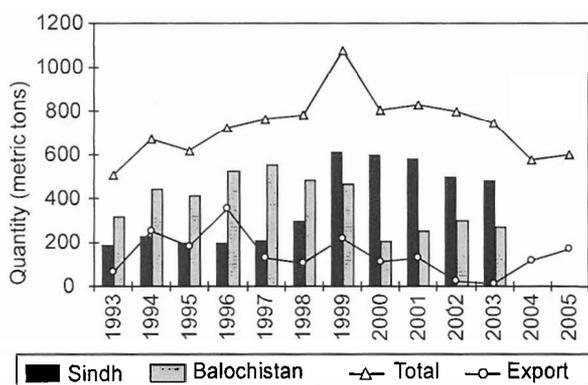


Fig. 3. Lobster catch during 1993 to 2004.

Table 1. Catch and export data of lobsters

Frozen meat, tail (met. tons)	Value (Rs. in million)	Export		Total quantity (met. tons)	Total value (Rs. in million)
		Live (met. tons)	Value (Rs. in million)		
066	12.976	0	0	066	12.976
175	49.819	80	14573	255	64.392
152	37.331	33	5733	185	43.064
288	75.785	64	13534	352	89.319
129	48.583	03	121	132	48.704
079	29.624	25	8489	104	38.113
116	42.227	101	42.779	217	85.006
079	45.361	31	17.218	110	62.579
103	71.921	25	16.915	128	88.836
013	10.110	10	6.938	23	17.048
009	6.265	5	2607	14	8.872
013	5790	102	37317	115	86215
033	14522	136	47472	169	61994

Fisheries Department under a grant by Pakistan Agriculture Research Council (PARC, 1980). The study comprised of the information about the lobster species, habitat, fishing grounds, abundance, partial larval development, larval morphology, breeding season, fecundity and bionomics. The studies revealed that *P. polyphagus* (maximum total length: 37 cm) prefers muddy-cum-sandy bottom, close to the Indus River mouth at depths between 5-90 meters, generally at 40-50 meter depth, while *P. homarus* (maximum total length: 32 cm) normally inhabits rocky bottom, which are more common on northwest (Balochistan) coast. *P. ornatus* (maximum total length: 50 cm) was found in shallow coastal waters up to 10 meter depth mostly on muddy bottom including river Indus mouth, occasionally found in sandy areas. *P. versicolor* (maximum total length: 32 cm) is found between 1 to 15 meter depth, in clear waters on rocky substrates (Paradise point, Buleji), this species tends to hide in rock crevices and corals; juveniles are found in low salinity areas. *P. penicillatus* (maximum total length: 45 cm) inhabits clear water rocky substrates; it also hides under boulders and in crevices. *Puerulus swelli* is a rare species found in offshore deep waters (maximum total length: 5 cm). *Thenus orientalis* is found mostly from muddy areas along Indus deltaic areas (Fig. 2) in shallow waters of up to 100 meter depth; it is rarely found on Balochistan (Makran) coast (PARC, 1983).

Studies have been focused on *P. polyphagus* because of its abundance whereas little work has been carried on *P. homarus*. The size distribution of fished stock was between 6.5 cm to 24.5 cm tail length in *P. polyphagus*, whereas, for *P. homarus*, size range was 7.2-20 cm tail length. The sex ratio slightly varied both in *P. polyphagus* and *P. homarus* from typical 1:1 ratio and found to be 1.1:1 and 1.5:1, respectively, during the four year study period from 1976-1979. *Phyllosoma* larvae of *Panulirus* were described from I-IX stage. Stomach contents of *P. polyphagus* revealed the presence of shell fragments of foraminiferans, bivalves, gastropods, juvenile crabs, crustacean remains (antennules, carapace, chelae, appendages, and gill rakers), fish eggs, fish scales, bones, vertebrae, sand grains and pebbles.

Hussain and Amjad (1980) worked on the breeding and fecundity of *P. polyphagus*. According to this study, berried females of *P. polyphagus* were found during the period October to May; the peak of breeding season was found during March-April. The eggs were oval in shape, average length of recently released eggs was 0.5023 mm, whereas, the breadth was 0.4255 mm. Fecundity was estimated as 83,960 to 790880 eggs (average = 306,700 ± 58,000) from 30 berried

females ranging in tail length from 139 mm to 211 mm and weighing between 130 to 360 g tail weights (measured from anterior tip of the first abdominal terga to the tip of telson). Fecundity has a positive relationship with both tail length and tail weight of the lobster. Larval stages of lobsters collected from plankton from near shore and offshore samples were also studied and described by Ghory *et al.* (2005). Nutritional value and macro- and micro-nutrients of *Panulirus* sp. have been studied by Nisa *et al.* (1995).

Management. During the past decade, a number of international conventions included new obligations for management activities regulating ocean uses. The conventions (and codes) make explicit reference to protection of ecosystem features. The overarching convention in this respect is the Convention on Biological Diversity (CBD). Other international legal instruments include the UN Fish Stock Agreement and the FAO Code of Conduct. In response to these international agreements, national legislation and policies are required to incorporate ecosystem considerations more explicitly within national ocean management regimes. Pakistan is a signatory to the above cited conventions and has taken some important measures into consideration for the management of lobster fisheries in Pakistan:

1. Minimum catchable size of lobsters. Minimum legal size for catch is <15.0 cm, i.e. lobsters of less than 15.0 cm are not allowed to be caught and when captured should be immediately released back into the sea alive and shall not be landed or marketed.

2. Protection of berried females. No berried female should be caught and when captured should be released immediately into the sea alive

3. Protection of soft shelled lobsters. Protection of soft shelled or newly moulted lobster is considered necessary because moulting precedes mating (reproductive cycle).

Fisheries management in Pakistan is generally carried out through licensing, indicating exploitable stocks, designating the environmental friendly fishing gears and methods and by enforcing restrictions with regard to the closed season and closed areas. Pakistan also adopted guide lines to the code of conduct for responsible fisheries laid down by FAO. There are various regulations formulated within the country for management of fishing in Exclusive Fishing Zone (EEZ of the country) (Regulation of Fishing Act, 1975, amended 1993; the Sindh Fisheries Ordinance, 1980; the Balochistan Sea Fisheries Act No. IX, 1971). Co-operation among fishermen, scientists, and government agencies is important for imple-

mentation, which at present, is lacking. There is a need for effective management programmes for sustainable utilization of lobster resources. Research on population dynamics, effects of dwindling habitats on various populations and stocks assessment are urgently needed. In addition, legal implementation of fishing regulations, creation of awareness among fishers on the negative impact of over-fishing and marketing of egg-bearing lobsters and juveniles are the most important aspects, which have not been given due consideration. There is a need of adopting less destructive methods like use of lobster traps and to educate the fishermen to release egg-bearing lobsters. Pakistan has no commercial fishing fleet to explore the deep sea resources; the rights for deep sea fishing have been given to various foreign countries through licensing. Deep sea fishing zone and catch, both are not inspected or documented by any agency. It may be inferred that deep sea forms of Pakistani waters are still unexploited.

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