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SHORT COMMUNICATIONS

TECHNOLOGY

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Physical Sciences

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Studies on Some Electrical Properties of Polycrystalline Calcium Fluoride at High Temperatures

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The electrical conductivity σ and the dielectric constant ε′ were measured at a frequency of 1 KHz on pressed pellet of CaF_2 in the temperature range 373-873°K. The pressure applied for preparing the sample varied from 3 to 5 tons/cm^2 on circular disk of 2.5 cm diameter and 0.45 cm thickness by hydraulic press. In the temperature range investigated the conductivity data exhibited to activate regions yielding activation energies of 0.532 ev and 0.42 ev. The region I called intrinsic region and region II is extrinsic region. The effect of temperature and the hydrostatic pressure on the real and imaginary part of the dielectric constant for this sample has been studied. The dielectric constant curve shows a slow increase of dielectric constant up to temperature 473°K and above this temperature, a fast increase in ε′, which may be attributed to lattice expansion and polarizability of constituent ions. At high temperature in the dielectric, two points of phase transition are attained. In the present study, it has been found that within the reported temperature range the dielectric constant and dielectric loss are predominately determined by the motion of the defects. The activation energy deduced from the dielectric studies is in good agreement with that obtained from the present electrical conductivity data.

Key words**:** Ionic conductivity, Dielectric constant, Phase, Activation energy.

Introduction

Currently there is considerable interest in materials with high ionic conductivity, solid electrolytes or fast ionic conductors (Perram 1983, Suresh 1973). The fluorite structure of alkaline earth metal fluorides exhibit super ionic behavior at high temperatures, and they have been attracting particular attention because they have important potential applications as electrolyte in high energy density batteries and other devices. The superionic conductors, because of their remarkable physical properties, are used in electrochemical devices (Vashista *et al* 1979)

Trnorcorn (1998) studied on mixing of isovalent cationic effect in multi components fluorite structured fluorides, the static permittivity and the diffusivity in concentrated solutions of alkaline earth fluorite which are rare earth flouride. With this, the influence of mixing the physical properties of super ionic conductor was also studied. The fast ionic conduction is found in $Ba_{0.7-x}SrLaF$, a single crystal of barium station lanthanum flouride.

There exists an essential characteristic of all super ionic conductors, due to which almost complete crystal disorder of one species of ions raises several important questions. First of all as the number of defects become equal to the number of ions, it becomes important to identify the vacancies and interstitials. There are no regular lattice sites in such conductors. This difficulty can be resolved by treating all the ions as interstitial with more than one available sites.

Thus electrical properties of the alkaline earth fluorides are of considerable interest. One of most basic form of the properties of solids is the static dielectric constant ε′ and the loss factor ε″. The value of dielectric constant plays a key role in the lattice dynamics of ionic crystal (Liang 1973), while considering the effect of electronic interaction on the transport of matter or charges in ionic crystals, a knowledge of dielectric constant is needed. The rate of increase of dielectric constant with temperature change dε′/dT is expected to be faster at high temperature in ionic crystals (Oberschmidt *et al* 1980). So, it was considered worth while to measure the dielectric properties of polycrystalline CaF_2 at high temperatures. From the application point of view it is easier to use well pressed powder in the form of pellets. Also to evaluate the electrical conductivity of well pressed powder as a function of temperature and to determine the transport parameters and compare their results with data those obtained by single crystal. (Oberschmidt *et al* 1980). The dielectric constant and its change with temperature is calculated from the measured sample capacitance C, by applying corrections for changes in sample diamensions. Due to the thermal expansion in an isotropic medium, Samara (1968) has given the following equation for the change in dielectric constant with temperature, at constant pressure:

$$I/\varepsilon_o (d\varepsilon/dT)_p = 1/C_o (dC/dT)_p - 1/3V_o (dV/dT)_p \text{ ..... } (1)$$

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Where ε_o, C and V_o are the values for dielectric constant, capacitance and volume at atmospheric pressure. At sufficiently high temperature, the loss factor is dominated by the conductivity of the sample which is given by equation (2):

$$\text{Tan}\,\delta = \varepsilon''/\varepsilon' = 4\pi\sigma/\varepsilon'' \quad \ldots\ldots (2)$$

Where ε'' is the imaginary part of the dielectric constant and ε' is the real part and σ is the conductivity of the sample and ω is the measuring frequency. The conductivity can also be written by equation (3):

$$\sigma = \sigma_o \exp(-E/2KT) \quad \ldots\ldots (3)$$

From this equation we can calculate the activation energy E at a particular temperature and σ is the DC conductivity and σ_o is a constant. Here we intend to study the ionic or electrical conductivity and the dielectric properties of pure and dispersed alkaline earth fluoride structured compound, CaF_2 at high temperature starting from 373°K, with a view to determine precisely the transport parameters and studying the defect mechanism for the flow of charge at these temperatures.

Dielectric constant is an important property in relation to the ionic conduction because the larger the value of dielectric constant of an ionic crystal, the lower energy formation for the lattice defect (Choudhry and Bichile 1986).

Experimental

Sources and preparation of sample. The powder sample of CaF_2 used in this work was obtained from M/s Koch Light Laboratories, France with specific purity of 99.99%. To obtain uniform particle size, the material were mortared in fine particles powder with a mesh size of 150 micron. The powder thus obtained were pressed and made in the form of pellet in a specially designed stainless steel die with plunger pressing with a hydraulic press which can read the pressure in Kbar or ton. For preparing such sample a pressure of 4 to 5 tons/cm^2 were applied. The density of pressed powder were obtained after pressing at different pressure. The typical diamension were 0.47 to 0.55cm thick. For measuring its electrical conductivity and the dielectric constant, the conducting electrodes were pasted on indium oxide paint and with a thin layer of graphite on them. Such electrodes were also used by Schoonman *et al* (1977). They obtained reproducible results on each thermal cycling.

Conductivity measurements. Following the ASTM standard 1983, the conductivity data were collected for the sample, kept in the holder has been designed and fabricated for this purpose. The AC conductivity or conductance G, were measured by Ogawa Seiki OSK 555 impedance bridge. It is a high resolution easy balancing bridge with three decades reading. It has built in variable frequency oscillator, IKHz tunable detector. It can give σ conductivity from 0.001μs - 120 S. Frequency dependent conductivity from 120Hz to 10KHz as well as the effect of applied voltage from 50mv to 1.5v A.C (Peak to Peak). The suitable frequency for measurement was 1KHz except at high temperature above 373°K where low frequency were selected. The measured resistance was independent of applied voltage.

Recording the temperature. A separate digital control unit was attached for recording the temperature. The internally heated system was driven by a thermocouple (Chromel-alumel thermocouple). The temperature can be controlled within an accuracy of ± 1%. This unit allowed the data to be taken at preset time interval as the sample was heated or cooled. The effect of heating rate was checked to see that the sample has sufficient time to equilibrate at rate of 10K/min sample was heated or cooled. The nominal heating rate was kept at 9K/min. During the cooling cycle, the a.c. conductivity was measured with an accuracy of ± 2%. This experiment was repeated three times for each sample at same temperature. Standard deviation for the three readings was taken to avoid the possible errors. Data obtained after several thermal cycling were found concordant and reproducible. The conductivity measurements were recorded during the cooling cycle. At high temperature > 700°K, it was observed that the sample was contaminated with oxygen of the atmosphere.

Measurement of dielectric parameters ε' and ε'' with temperatures. Capacitance and the dielectric loss measurements were made at a frequency of 1KHz employing transformer arm bridge OSK 555 impedance bridge which is equipped with three terminal cell (with connecting wires). Since the cell contains three terminal electrodes, the data were taken free from errors due to edges and the surface effects. The capacitance of the parallel plate is measured by depositing indium paint as electrodes on opposite sides on the specimen. The relative change in capacitance of the sample with temperature is equal to the relative change in permitivity, after applying the necessary edge corrections for the change in geometery of the sample. This instrument has a measuring accuracy of + 0.2 %. Shielded leads and specially designed sample holder is taken for the final results. The value of dielectric constant at room temperature were determined using standard geometrical technique in which the sample capacitance is calculated from the following formula:

$$C = \varepsilon' \varepsilon_o A / d \quad \ldots\ldots (4)$$

Where A is area of crossection, d is the thickness and ε_o is permitivity of free space.

From Equation (4) the dielectric constant ε' and its change with temperature is calculated from the measured sample capacitance C.

Table 1
Density of the sample at different applied pressure

S.No.	Hydrostatic pressure	Measured density gm/cm^3
1.	3.31 tons/inch^2	3.77
2.	3.85 tons/inch^2	3.88
3.	3.85 tons/inch^2	3.95
4.	4.4 tons/inch^2	3.91
5.	5.0 tons/inch^2	3.93
6.	5.4 tons/inch^2	3.93

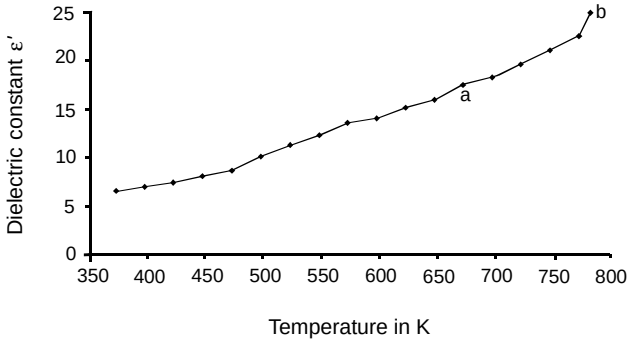

Fig 1. Variation of dielectric constant ε′ with temperature of pressed pellet of CaF_2.

Calculation of activation energy. From the equation given by Samara (1968), the activation energy can be determined from the slopes of the curve of Fig 3, at region I and II. It has also been calculated from equation (2) and (3) that the activation energy (E) is 0.532eV for region I and 0.423eV for region II, which is in accordance with the earlier studies. (Bone and Schoonman 1977). The CaF_2 compound has the following constants in the purest form at S.T.P:

CaF_2 Data

Mol. weight. 78.08, Refractive index =1.434,, . Density=3.18gm/cm^3, Melting point=1360˚C, Boiling point 2500˚C, Crystallographic phase = Cubic.

Results and Discussions

As shown in Fig 1, the dielectric constant of a CaF_2 pellet is 6.5 at 373˚K, and at an elevated temperature of 773˚K, it becomes 24.1. At temperature of 675˚K and 773˚K, the points a and b are the phase transition points from cubic to some other phase equilibrium. From 373˚K to a temperature of 473˚K, the dielectric constant is slowly increasing. At point b at a temperature of 800˚K the dielectric constant is increasing to a value of 25. 1 there is dε′ /dT. Table 1 gives the values of density of the pressed sample after applying different hydrostatic pressures.

It is observed that the density of pressed CaF_2 is not changing after increasing the applied pressure of more than 5 tons/inch^2.

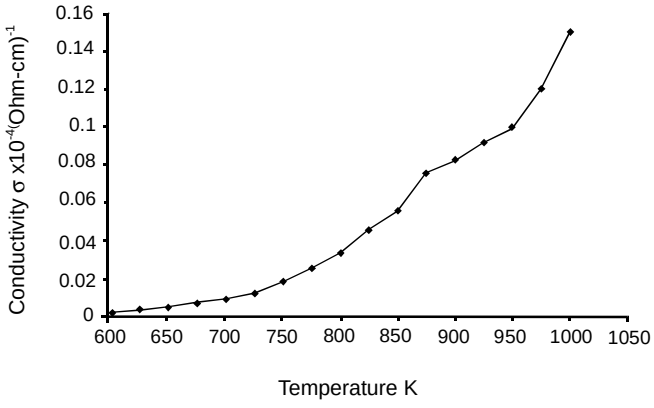

Fig 2. Variation of conductivity as a function of temperature.

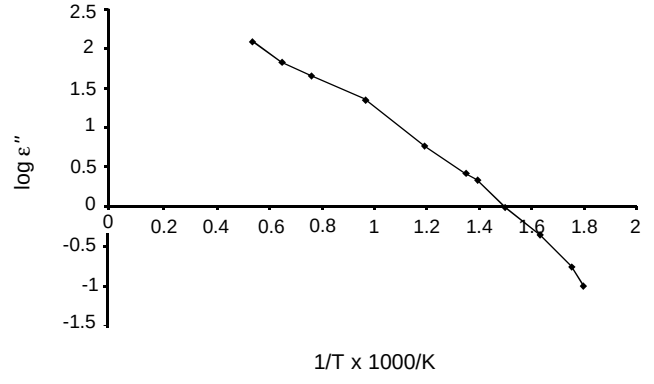

Fig 3. Plot of imaginary part of dielectric constant against inverse of temperature 1/T for CaF_2 (Pellet).

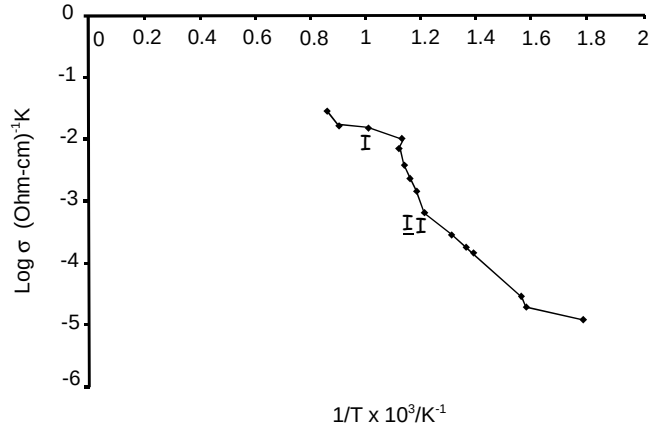

Fig 4. Curve between log σ vs 1/T for CaF_2 pellet 1/Tx 10^3/K^{-1}.

As shown in Fig 2, the ionic conductivity increases with temperature up to a temperature of 850˚K, a sharp increase in the conductivity from 0.077x10^{-4}/Ohm/cm to 0.975x10^{-4}/Ohm/cm.

As shown in the curve of Fig 4, the graph between log σ and inverse of temperature 1/T exhibits two activated regions of

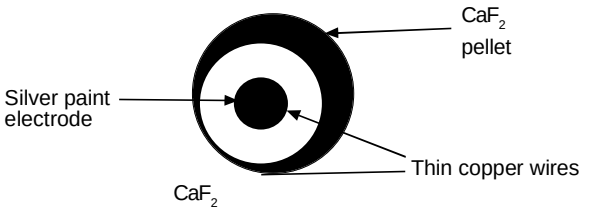

Fig 5. CaF_2 pressed pellet with silver electrodes used for the measurement of dielectric constant.

conductivity denoted by I and II. The data was fitted to a function by the analysis of method of least square. According to equation (3), the data is showing a transition from 830°K stage I to stage II. It is giving activation energy 0.532eV to 0.42eV respectively. This is the approximate method to calculate the energy required to create + ve ions vacancy or defect formation in ionic crystals.

Fig 3 illustrates log ε'' vs 1/T, for this material. It is seen that the temperature dependence loss as expected from equation 2 and 3. This graph shows a linear behavior, and by least square method of curve fitting, the data has been fitted to calculate the slope which is called the activation energy for the two regions in the curve. The density, dielectric constant and electrical conductivity were found to be depended on applied pressure. For pressure > 5tons /cm^2, the density and the related measured parameters do not remain constant and reproducible.

Conclusion

As we have seen the effect of applied pressure on the sample pellet of CaF_2 and its measured values of density, electrical conductivity and the static dielectric constant ε′ these values are not different from the earlier studies (Perram 1983).

Fig 1 shows the variation of dielectric constant ε′ with temperature of such CaF_2 pellet. It has been noticed that up to a temperature of 550°K, the dielectric constant ε′ increases slowly as expected from the ionic solids (Smyth 1955).

However, this increase becomes very much fast above 559°K. This fast increase in ε′ is due to lattice expansion, polarizability of the constituents ions. In addition to the polarization, another possible contribution to the static dielectric properties of a substance are the dipoles which are produced by the impurities and lattice defects. There are some factors which contribute to the temperature dependence of dielectric constant of a cubic crystal. For such a material, the temperature dependence of the dielectric constant at constant pressure is separated into volume dependent and volume independent contribution (Oberschmidt and Lazarus 1980).

For such a material, the microscopic Clausius Mossotti Formula holds, depending upon the polarizability of the ions.

$$\varepsilon' - 1 / \varepsilon' + 2 = 4 / 3\pi\alpha_m / V \quad \text{..... (5)}$$

Where α_m is the polarizability of microscopic, small sphere of volume C in vacuum (Bosman and Havings 1965). The following reasons are offered for the increase of ε at high temperatures.

i.) When the temperature rises, the number of polarizable particles per unit volume decreases as a result of volume expansion (Bosman and Havings 1965).

ii) Similarly as the temperature increases, the polarizability of a constant number of particles rises within an increase of available volume.

Ionic conductivity. As shown in Fig 2 which is in between ionic conductivity and temperature, the ionic conductivity is divided into two regions I and II that are analogous to the alkali halides. Region I identifies as extrinsic conduction region from 373°K to 573°K. Because the conduction occurs via extrinsic vacancies. The conductivity rises after heating the sample to high temperature and contaminate with the oxygen. The divalent oxygen ion replaces the monovalent fluorine atom and thus leaves one vacant anion site. Region II is identified as intrinsic region t in the range, 573-873°K and the doping experiment (Lian and Joshi 1975) shows that at this temperature the vacancies are more mobile than interstitials in CaF_2.

Frankel anion defects. As it is obvious from Fig 4, the transition from region I to region II results from a change of conduction from F ion by interstitial motion to ion vacancy motion (Kennedy *et al* 1973). The observed activation energy from stage I is 0.532eV in this stage and this energy is too small to be consistent with the formation and motion of defect with Frankel anion defect in the flourite structure. It has been explained by Schoonman *et al* (1973), that the formation energy for the Frankel defects required in CaF_2 is 2.2eV to 2.8eV.

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Pak. J. Sci. Ind. Res. 2004 ***47****(2) 96 - 98*

STUDIES OF REACTION MECHANISM AND PHYSICAL NATURE OF LIGHT-WEIGHT BASIC MAGNESIUM CARBONATE

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The reaction mechanism of light - weight magnesium carbonate has been studied and concluded that it is based upon formation of colloidal magnesium hydroxide and adsorption of HCO_3^- ions on magnesium hydroxide surface under optimum conditions. Precipitation, carried out in the presence of sodium bicarbonate over a concentration range of 1M to 0.01M, is a continuous function of the carbon dioxide remaining in the solution. Temperature between 70˚C and 80˚C, stirring speed of 900 rpm, gradual addition of magnesium sulfate solution and mass ratio of soda ash to sodium bicarbonate (3:2) were found to be the optimum conditions for obtaining light - weight magnesium carbonate.

Key words**:** Reaction mechanism, Light - weight magnesium carbonate, Effect of sodium bicarbonate.

Introduction

There have been extensive studies conducted on the preparation of light-weight magnesium carbonate with the aim to get optimum conditions. A number of researchers studied the preparation of basic magnesium carbonate. The following processes are the examples of the previous studies. Harold (1933) reported that light magnesium carbonate is precipitated by heating the solution containing magnesium bicarbonate in the presence of crystallizing agent such as gelatin, gum arabic, castor oil sulfonin, etc. Samuel (1936) studied the preparation of light magnesium carbonate using soda ash, sodium bicarbonate and magnesium salt solution by introducing steam into the mixture until temperature was 70 - 90˚C; the role of bicarbonate ions did not mention during the reaction. Morifuji *et al* (1991) added hydroxy carboxylic acid salts to magnesium carbonate to produce light-weight magnesium carbonate. Paul (1934) obtained light magnesium carbonate by adding giobesite (dolomite) to the solution of sodium bisulfate at room temperature instead of using epsom salt and soda ash. Bertrand (1932) converted heavy magnesium carbonate into light magnesium carbonate by mixing asbestos fibre to the slurry of heavy carbonate between 150˚F to 250˚F without using sodium bicarbonate; but he could not get the maximum degree of lightness. Okata (1995) and Mita *et al* (1994) also converted heavy magnesium carbonate into light magnesium carbonate by introducing steam directly into the solution to raise temperature to 180˚C and observed smaller changes of absorption and desorption of water.

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However, these studies are insufficient to explain the complete reaction mechanism for light - weight magnesium carbonate. Therefore, the present study was undertaken to evaluate the optimum conditions to get utmost degree of lightness and regulate other influencing factors for producing light - weight magnesium carbonate.

Experimental

Epsom salt, soda ash and sodium bicarbonate of technical grade were used throughout the experiments. De-ionized water with conductivity of $10^7\Omega$/cm was used in the preparation of all solutions. A flask (500 cm^3) with lid having two necks was used as reaction vessel. All experiments were conducted at concentration varying from 1M to 0.5M, at temperature between 70 - 80˚C and at a constant stirring speed of about 900 rpm. The central hole of the lid was used for the introduction of thermometer and second for the addition of the sample. A hot plate with controlled temperature and stirring speed was used throughout the studies. The solutions of magnesium sulfate , sodium carbonate and sodium bicarbonate used were prepared from IM-solution. In each experiment, 10.0g ± 0.05g of epsom salt solution was added in a thin stream to the reaction medium containing magnetically stirred solution of sodium carbonate (7.2g ± 0.05g) and sodium bicarbonate (4.8g ± 0.05g). Carbon dioxide gas was evolved and immediately estimated in the moist freshly precipitated basic carbonate, in order to avoid any loss of carbon dioxide from the precipitate which might arise through air-drying following precipitation. The dissolved carbon dioxide (both free and combined) in each experiments is directly estimated in a convenient volume

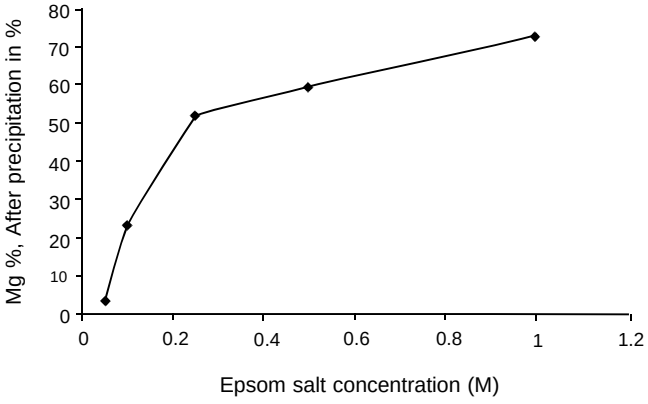

Fig 1. Effect of epsom salt concentration.

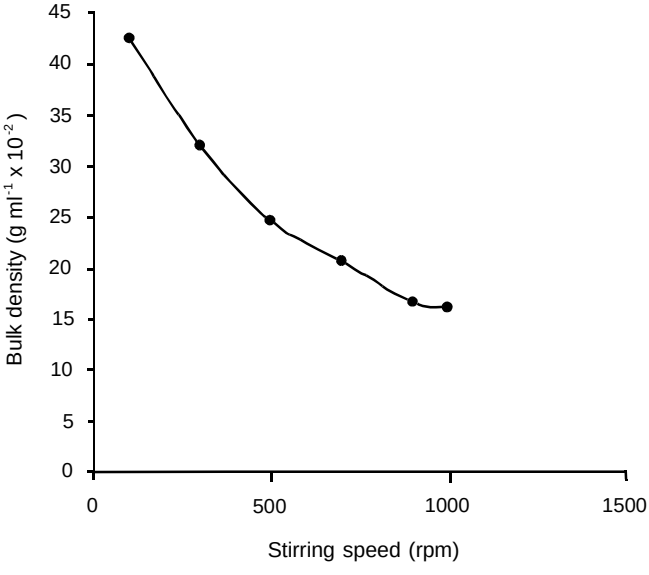

Fig 2. Effect of stirring speed on bulk density.

of solution, 10, 25 or 50 c.c. and bulk density of dried $MgCO_3$ in each experiment was also determined. After estimation of carbon dioxide gas, magnesium in solution was determined, by oxine method using hydroxyquinoline (Arthur 1964).

Results and Discussion

Effect of epsom salt concentration. Some experiments were conducted to study the effect of epsom salt concentration upon conversion rate by varying its concentration from 0.05M to 1M. The reaction medium contained the solutions of soda ash and sodium bicarbonate in the mass ratio 3:2 at temperature of 70°C. By addition of magnesium sulfate solution to reaction medium, carbon dioxide gas evolved and estimated in moist freshly precipitated basic carbonate. The results are shown in Fig 1. The results in the figure show that the conversion rate first increases sharply upto 52% during

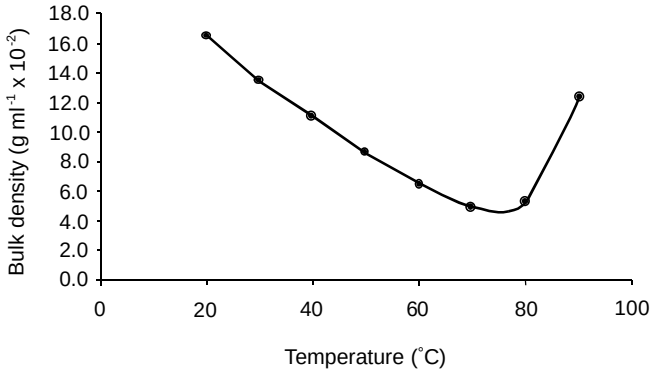

Fig 3. Effect of temperature on bulk density.

concentration change of 0.2M (i.e. 0.25 - 0.05M). Afterwards during further change of 0.2M (i.e. 0.25 - 0.45M) conversion rate was found to be very slow, which may be due to the formation of magnesium ions in the solution which are adsorbed on the surface of epsom salt. This caused the decrease in conversion rate of epsom salt to magnesium carbonate. From the above mentioned findings a logarithmic behaviour of con version rate is observed. Therefore, it may be concluded that conversion rate is enhanced by increasing concentration of epsom salt and degree of conversion rate is adversely effected by the increase in concentration of magnesium carbonate in the resulting mixture. Similar tendency was also reported by Jack (1939).

Effect of stirring speed. In order to examine the effect of stirring speed on degree of lightness, a number of experiments at different stirring speeds i.e. 100 - 1000 rpm were performed using the reaction medium containing 1M solution of epsom salt, soda ash and sodium bicarbonate in the mass ratio 3:2 at temperature of 70°C. The results described in Fig 2 show that bulk density is an inverse function of stirring speed. It is also observed from the results in the figure that bulk density decreases sharply during a change of stirring speed from 100 - 900 rpm, thereby it attains its least value at 900 rpm. Therefore, it may be concluded that the degree of lightness corresponding to slope in figure between stirring speed 900 rpm and 1000 rpm was independent. Hereafter, all experiments were conducted at 900 rpm.

*Effect of temperature.*Effect of temperature on bulk density was examined at temperatures ranging from 20°C to 90°C. The reaction bath contained 1M solutions of epsom salt, soda ash and sodium bicarbonate in the mass ratio 3:2. The values of bulk density obtained from different experiments were plotted against temperature in the Fig 3.

The results in Fig 3 shows that bulk density at temperature of 20°C was maximum, which goes on decreasing gradually, showing linear relation over whole temperature ranging from 20°C -

70˚C. The least bulk density was obtained at temperature of 75˚C. It is observed from the results that least bulk density was obtained due to the formation of colloidal magnesium hydroxide and subsequent adsorption of bicarbonate ions over the surface of magnesium hydroxide. At temperature beyond 75˚C decomposition of bicarbonate takes place resulting in the increase in bulk density again as appears in the figure. It can be concluded that the effect of temperature on bulk density may be interpreted in the ways that it not only helps in the formation of colloidal magnesium hydroxides but also in adsorption of HCO_3^- ions. This adsorption process of HCO_3^- phenomenon was in agreement with observation of earlier workers (Jack 1940).

a) $Mg(OH)_2 \xrightarrow[\text{adsorption}]{HCO_3} Mg\begin{matrix}OH\\HCO_3\end{matrix} + OH^-$

b) $MgCO_3^- + Mg\begin{matrix}OH\\HCO_3\end{matrix} \longrightarrow MgCO_3^- \quad Mg\begin{matrix}OH\\HCO_3\end{matrix}$

Conclusion

The reaction mechanism and physical nature of light weight magnesium carbonate was studied in the presence of sodium bicarbonate. The experimental results indicate that conversion rate is a logarithmic function of epsom salt concentration. From these studies, it is also concluded that temperature between 70˚C - 80˚C, stirring speed of 900 rpm and mass ratio of sodium carbonate to sodium bicarbonate (3:2) are the optimum conditions to get utmost degree of lightness of basic magnesium carbonate i.e. 0.05g/ml. Under optimum conditions employed, the reaction mechanism is based upon the formation of colloidal magnesium hydroxide and subsequent adsorption of HCO_3^- ions on magnesium hydroxide surface. A combination of these effects is supported by evidence.

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Pak. J. Sci. Ind. Res. 2004 ***47****(2) 99 - 103*

HIGH FIELD ^{13}C - NMR SPECTROSCOPIC ANALYSIS OF THE TRIACYLGLYCEROLS OF *ADENOPUS BREVIFLORUS* SEEDS OIL

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High resolution carbon - 13 NMR (gated decoupled) spectra of the carbonyl, saturated and olefinic carbons in *Adenopus breviflorus* seeds oil have been used for direct determination of the acyl composition and acyl positional distribution on the glycerol backbone. The spectra revealed the presence of saturated, oleic and linoleic fatty acids. Semi quantitative analysis using the integrals of the allylic carbons signals gave the percentage composition of the oil as saturated 25.00%, oleic 14.00% and linoleic 60.90%. These percentage compositions were confirmed by gas chromatography. The spectra further revealed that while the saturated fatty acids are distributed between the 1,3 (α) and 2 (β) glyceridic positions, oleic acid is attached only at the (α) glyceridic position while linoleic acid is attached mostly at the (β) glyceridic position.

***Key words*:** ^{13}C-NMR, *Adenopus breviflorus*, Linoleic fatty acids, Gas chromotography, Triacylglycerols.

Introduction

Most seed oils are composed of triacylglycerols which contain an array of fatty acids, saturated as well as unsaturated and distributed among the three positions of the glycerol backbone. In defining the acyl positional distribution between the α - (i.e. the 1 and 3 positions of the glycerol) and β - (i.e. the 2 position of glycerol), carbon - 13 NMR has been found most useful. There have been also some efforts in the past (Ng 1984; Gunstone 1993; Lie Ken Jie *et al* 1996), where ^{13}C - NMR was used to identify, confirm or evaluate the fatty acids composition of different seeds oil. These reports indicated that except for lack of differentiation of the saturated fatty acids, the ^{13}C - NMR technique provided the same information as the time consuming, conventional gas chromatographic technique for establishing fatty acid composition of oils and the tedious enzymatic hydrolysis for identifying the positional distribution of the oils acyl groups.

Adenopus breviflorus (Cucurbitaceae) grows in the wild in Savanah forest of Southern Nigeria. It has about 55-60% oil (Esuoso and Bayer 1998). Oderinde (1990) and Oshodi (1996) reported the fatty acids composition of the *Adenopus breviflorus* seeds oil. We have characterized the oil and indicated some possible uses of the seeds oil (Akintayo and Bayer 2002a). In an earlier investigation, we have tried to identify *Adenopus breviflorus* seeds oil by ^{1}H-NMR spectroscopy (Akintayo and Bayer 2002b). In continuation of our efforts on the systematic studies of the lesser known and under-utilised tropical seeds oils, the present effort aims at the ^{13}C-NMR spectroscopic analysis of *Adenopus breviflorus* seed oil to *(i)* confirm the presence of the reported fatty acids, *(ii)* identify and semi-quantiate the fatty acids and most importantly *(iii)* determine the fatty acids distribution on the glycerol backbone. The quantitative integrity of the NMR derived fatty acid composition is verified by gas chromatographic analysis of the oil.

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Experimental

Adenopus breviflorus (ADB) seeds were purchased from some markets in Ibadan, Akure and Ado-Ekiti in the south - western part of Nigeria. The seeds were screened, washed and dried in the oven (103°C) and the oils extracted with hexane for 20 h by Soxhlet method. The extracts were desolventised under reduced pressure in a rotavapour.

The ^{13}C - NMR of the samples dissolved in deuteriated chloroform were recorded on the BRUKER AMX -400(BRUKER Instruments, Inc. Karlsruhe, Germany) Fourier transforms spectrometer operating at 100.6MHz. The gated decoupling pulse sequence was used with the following parameters. Number of scans 512, acquisition time 1.3665sec, pulse width 10.3μsec, delay time 1.0 sec. Free induction decay (FID) was transformed and zero filled to 300K to give a digital resolution of 0.366Hz /point.

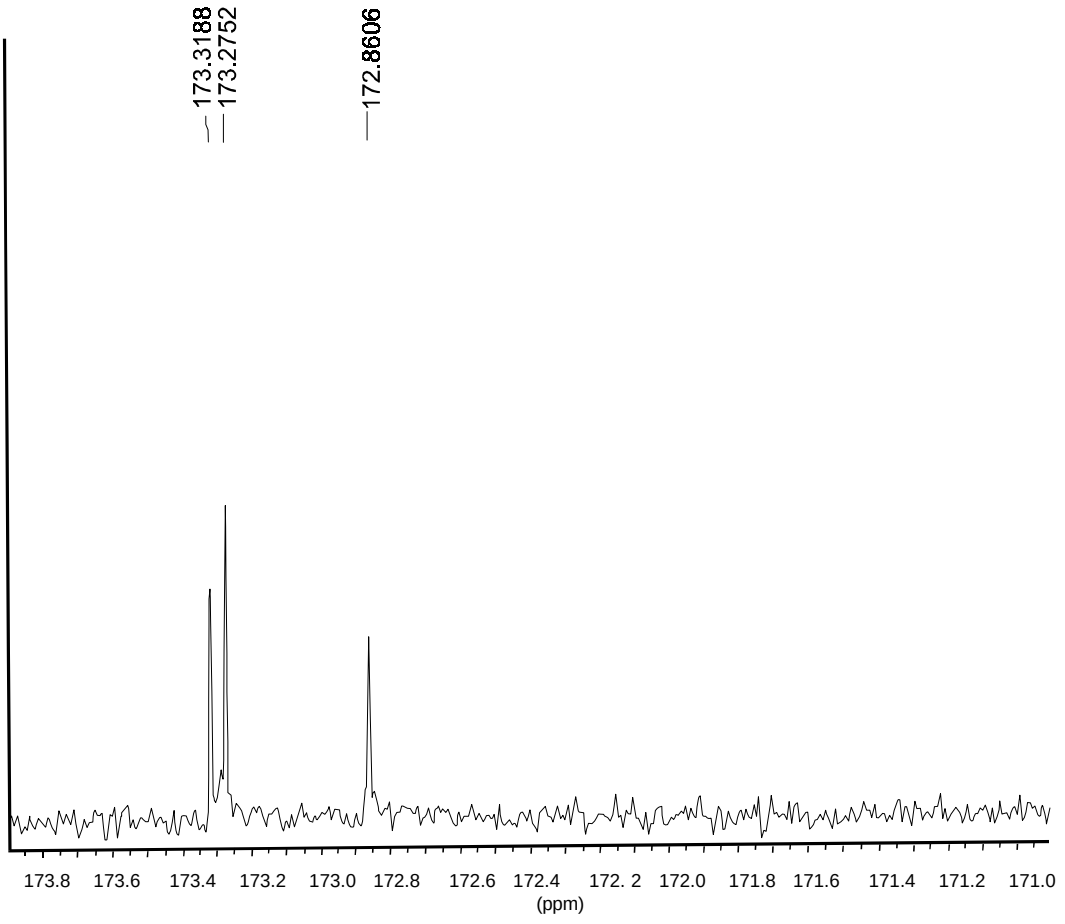

Fig 1. Proton-decoupled high resolution ^{13}C -NMR (100.6 MHz) of the carbonyl carbons of the triacylglycerols in *Adenopus breviflorus* seeds oil.

Fatty acid methyl esters (FAMES) of the oil was prepared as follows: Approximately 2mg crude seeds oil was transferred into a 5 - 10 ml glass vial and 1ml of diazomethane-ether solution added. The mixture was shaken thoroughly and allowed to stand for 1 min. Then 16µl of 3.33M CH_3ONa / CH_3OH solution was added, mixture shaken and allowed to stand for 10 min after which 10 µl acetic acid was added. The clear supernatant was used for Gas chromatographic analysis . 0.2 µl of the FAMES was injected into Hewlett-Packard 5890 GC (Hewlett - Packard Co, Palo Albo CA). The column was HP Ultra Performance coated with crosslinked 5% Phenol + 95% polysiloxane, 30 x 0.25nm, 0.2µ coating thickness. Temperature programming was as follows: Initial temperature,160˚C for 2 min, temperature increased at 2.5˚C / min up to 300˚C and maintained at this final temperature for 5 min. Injector and dectector temperature were 280˚C and 340˚C, respectively.

Results and Discussion

In this discussion we abbreviate saturated acyl groups as Sat., oleate [18:1 (9*Z*)] as O and linoleate [18:2 (9*Z*,12*Z*)] as L (where the first number in bracket denotes the number of carbon atoms in fatty acid chain, the second number denotes the number of double bonds, the other numbers denote the position of double bonds and *Z* stands for the *Z* configuration of the corresponding double bond). The structures of oleate and linoleate and the respective carbon numbers used throughout this discussion are as follows:

Oleate - $^{18}CH_3$, $^{17}CH_2$, $^{16}CH_2$, $^{15}CH_2$, $^{14}CH_2$, $^{13}CH_2$, $^{12}CH_2$, $^{11}CH_2$, $^{10}CH = {}^{9}CH$, $^{8}CH_2$, $^{7}CH_2$, $^{6}CH_2$, $^{5}CH_2$, $^{4}CH_2$, $^{3}CH_2$, $^{2}CH_2$, $^{1}CO\bar{O}$

Linoleate - $^{18}CH_3$, $^{17}CH_2$, $^{16}CH_2$, $^{15}CH_2$, $^{14}CH_2$, $^{13}CH = {}^{12}CH$, $^{11}CH_2$, $^{10}CH = {}^{9}CH$, $^{8}CH_2$, $^{7}CH_2$, $^{6}CH_2$, $^{5}CH_2$, $^{4}CH_2$, $^{3}CH_2$, $^{2}CH_2$, $^{1}CO\bar{O}$

where the superscripts stand for carbon numbers.

The high resolution ^{13}C - NMR spectrum of the carbonyl carbons of the triglycerides of ADB is presented in Fig 1 and it shows three signals at 173.3188 ppm, 173.2752 ppm and 172.8606 ppm. Referring to established data (Lie Ken Jie *et al* 1992; Lie Ken Jie and Cheng 1993; Lie Ken Jie and Lam 1995) two of the signals could be paired, 173.2752 / 172.8606 with a

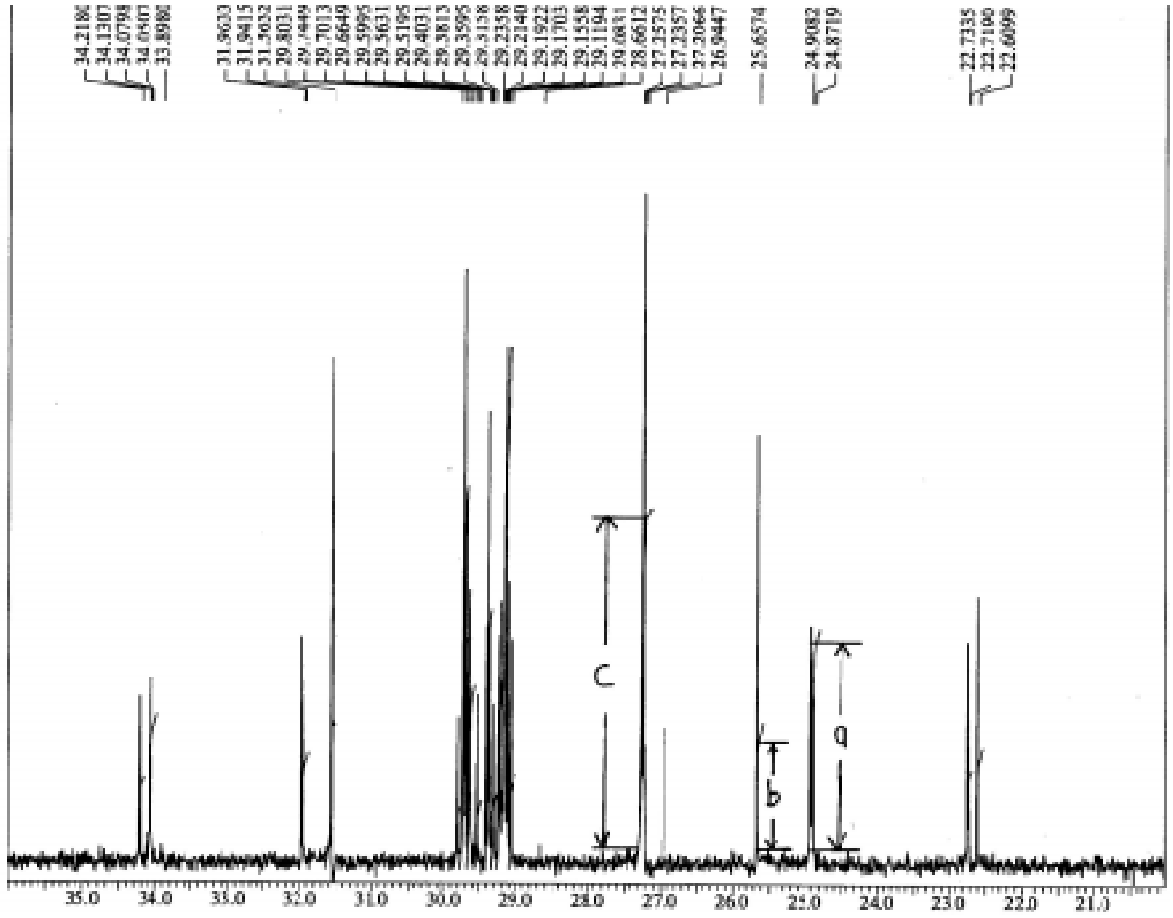

Fig 2. Proton-decoupled ^{13}C - NMR (100.6MHz) of the saturated carbons of the fatty acid chains in *Adenopus breviflorus* seeds oil. The integral value 'a' is for the peak at *ca* 24ppm,'b' is for the peak at *ca* 25 ppm and 'c' is for the peak at *ca* 27 ppm.

chemical shift difference of *ca* 0.415. The highest chemical shift in the spectrum 173.3188 ppm can be assigned to carbonyl carbon of Sat. in α position.

Ng (1983) has shown that C-1 of O and L attached to either of the 1,3 glyceridic carbons (i.e. at α position) occur at a slightly lower field to that of Sat. occupying the same position (O differs by 0.029 ± 0.002 ppm while L differs by 0.041 ± 0.002 ppm).

Rather than relying solely on chemical shift values, we have also made use of the difference values to ascertain the type of the ester and their positions on the glycerol backbone throughout this discussion. The higher value of the pair of signals, 173.3018 ppm differs from the 173.3188 ppm signal by *ca* 0.0043 ppm. Referring to Ng (1983), the pair of signals 173.2752 ppm/172.8606 ppm could, therefore, be assigned to L in α and β positions. Signals observed in the carbonyl region of this oil indicate the presence of Sat. and L. Earlier report by Ng (1983) has shown that resonances of saturated fatty acids were not resolved in the carbonyl region.

The ^{13}C - NMR signal profiles in the upfield region (20 - 36 ppm) of the ADB oil (Fig 2) were also found to be very characteristic and could be used for identification of the acyl groups and their positional distribution on glycerol backbone. There are two sub-regions in the spectra that are useful for these purposes (i) the C - 2 carbon shift region (*ca* 34 ppm) and (ii) the C - 3 (*ca* 24 ppm), allylic (25 - 27 ppm), C - 17 (*ca* 22 ppm) and C - 16 (*ca* 31ppm) carbon shift region.

C-2 carbon shift region (ca 34 ppm). Four signals 34.2180 ppm, 34.1307 ppm, 34.0798 ppm and 34.0507 ppm appear in this region. Two of the signals 34.2180 ppm/34.0507 ppm could be paired (shift difference of 0.167 ppm). These shifts are assigned to the C-2 carbon atoms of Sat. in the α and β positions. The 34.1307 ppm is assigned to L in β glyceridic position and the 34.0798 ppm assigned to O in α glyceridic position. These assignments were based on established data, (Lie Ken Jie *et al* 1992; Lie Ken Jie and Cheng 1993; Lie Ken Jie and Lam 1995).

C-3, allylic, C-17 and C-16 carbon shift region. The two signals in the C-3 region (*ca* 24 ppm) 24.9082 ppm and

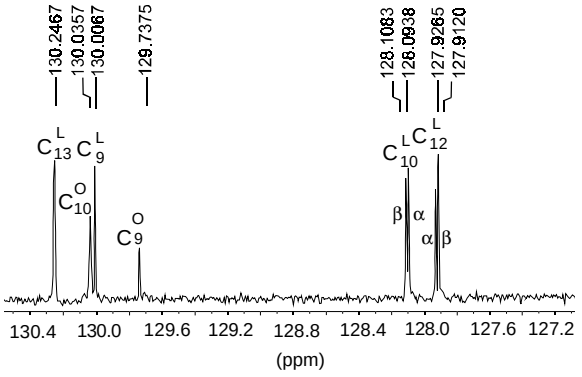

Fig 3. Proton-decoupled ^{13}C - NMR (100.6MHz) of the olefinic carbons of the triacylglycerols of *Adenopus breviflorus* seeds oil. In the assignment of the peaks, the superscripts of symbol C are defined as follows, O for oleic and L for linoleic. The subscripts of symbol C represents the specified carbon in the fatty acid chain.

Table 1
Fatty acid composition of *Adenopus breviflorus* seed oil

Fatty acids	a (%)	b (%)	c (%)	^{13}C NMR
Palmitic	10.10	10.10	10.84	*
Stearic	2.50	9.90	14.06	*
Oleic	24.56	19.40	13.84	14.10
Linoleic	62.86	60.70	61.26	60.90
Saturated	12.60	19.90	24.90	25.00
Unsaturated	87.42	80.10	75.10	75.00

a, % Fatty acid composition as reported by Oderinde (1990); b, % Fatty acid composition as reported by Oshodi (1996); c, % Fatty acid composition as obtained in the present effort by GC method; *, % Fatty acid composition reported together as total saturated.

24.8718 ppm can be paired having a chemical shift difference ($\Delta\delta$) of 0.036 ppm. Referring to established data, this pair of signals are assigned to C-3 of L distributed in the α and β glyceridic positions. No signal is found in the region *ca* 32 ppm, hence the presence of *trans* ethylenic system in the seeds oil can be ruled out.

Ten signals appear in the region (20 - 27 ppm). The signal at 27.2575 ppm is due to C-11 carbon atom of O, the 27.2356 ppm signal is due to C-14 carbon atom of L, the 27.2065 ppm is due to C-8 carbon atom of O and L and the 25.6573 ppm signal is due to C-11 of L. The relative intensities of the allylic methylene protons are distinct and the signals profile and intensity could serve as fingerprint for the identification of the oil.

Lie Ken Jie and Lam (1995) have observed a de-shielding order for the shifts of C-16 carbon nuclei as follows, Sat. (31.976 ppm) > O (31.954 ppm) > L (31.567 ppm). This trend was also

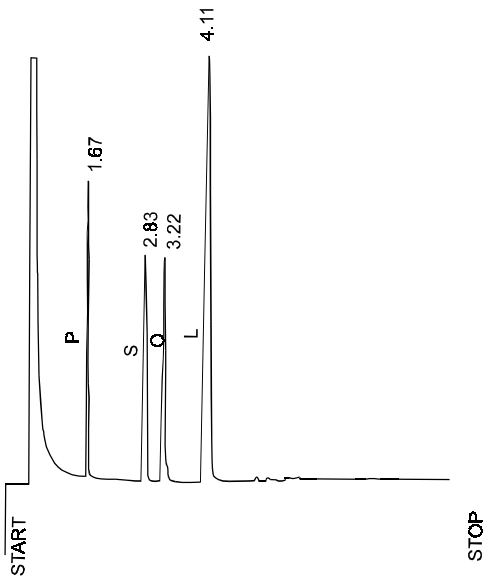

Fig 4. GC Chromatogram of *Adenopus breviflorus* seeds oil. The numbers are retention times. The symbols are: P for Palmitic acid, S for stearic acid O for oleic acid and L for linoleic acid.

observed by the same authors for C-17 carbon nuclei. The spectra of ADB also shows this de-shielding effect, so the signals at 31.9632 ppm, 31.9414 ppm and 31.5632 ppm are assigned to the shift of C-16 carbon nuclei of Sat., O and L respectively present in the ADB oil. In the same manner, the 22.7335 ppm, 22.7189 ppm and 22.6098 ppm are assigned to the shift of C-17 carbons of Sat., O and L respectively.

Another very characteristic region in the ^{13}C - NMR spectra of oils that defines the acyl composition and positional distribution on glycerol backbone is the olefinic carbon shift region. ^{13}C - NMR spectrum of ADB oil in this region is shown in Fig 3.

Ng (1983) had observed that the chemical shift between a pair of peaks become smaller for the olefinic carbon nearer to the methyl end of the fatty acid chain, i.e. in the O chain, magnitude of the peak separation is in the order C-9 > C-10 > C-12 > C-13. He also observed that in the O chain, the peak for C-9 attached at β glyceridic position appears at a lower field than that attached at the α -position and that the reverse order holds for C - 10. These high / low field alteration in peak position were also observed among the olefinic carbons of L chain. In general, in the O chain, $\Delta\delta$ between C -10 and C-9 α-positions is 0.30 ppm and that between their β- positions is 0.34 ppm. In the L chain, $\Delta\delta$ between C-13 and C - 9 α-positions is 0.20 ppm and $\Delta\delta$ between their α- positions is 0.34 ppm. In the L chain $\Delta\delta$ between C-13 and C-9 β -positions is 0.20 ppm and $\Delta\delta$ between their α-positions is 0.24 ppm while $\Delta\delta$ between C-10 and C- 12 β -positions is 0.17 ppm and their α positions is

0.19 ppm. Based on these difference values and other established data, the peaks in the olefinic regions are assigned as shown in Fig 3. The spectrum clearly shows the presence of O and L and absence of any triene ester. The intensity of the peaks show that L is more abundant than O in ADB oil. The sharpness of the C-9 and C-10 of O clearly indicate that they are single peaks. However, the chemical shift difference ($\Delta\delta$ = 0.30 ppm) points to the fact that O is attached only at the α glyceridic position. The chemical shift difference between the C-13 and C-9 of L ($\Delta\delta$ = 0.24 ppm) and the intensities of the pair of peaks observed for the C-10 and C-12 shows that L is mostly attached at the α glyceridic position. These results corroborates our observations from other regions of the spectra especially the C-3 carbon region which had indicated the distribution of L in the α and β glyceridic positions and the C-2 carbon shift region which had indicated presence of O in α position and L in mainly β position.

*Semi-quantitative analysis of the fatty acid composition.*The results discussed above revealed that ADB oil is composed mainly of Sat., O and L. For oils with non complex composition like this, the peaks at *ca* 24 ppm represents the total number of saturated, monoene and diene chain.The peaks at *ca* 25 ppm belongs to C-11 that is allylic to both double bonds of a *cis-cis* diene (linoleic) such that they represent the total number of diene chains and the peaks at *ca* 27 ppm belong to the two carbons allylic to *cis* double bond i.e. C-8, C-11 of O and C-8, C-14 of L, such that they represent twice the total number of monoene (O) and diene (L) chain (Ng and Ng 1984). The areas of these peaks, therefore permit quantitative analysis of Sat., O and L.

Integrals of these peaks are identified as a, b and c in Fig 2 and the percentage composition of the oil is calculated as:

Percentage of Sat. = [(a - 0.5c) / a] x 100
Percentage of O = [(0.5 c -b) / a] x100
Percentage of L = [b / a] x 100

For the ADB, a = 0.46, b = 0.28 and c = 0.69. The percentage of the acyl composition derived from the NMR spectra is presented in Table 1 along those side obtained by gas chromatography by Oshodi (1996) and Oderinde (1990) and also obtained by GC methods in the present effort. The GC chromatogram obtained in the present effort is presented in Fig 4. The NMR results confirm the GC results that L is the most abundant fatty acid in ADB oil. Our GC results compare very well with our NMR extrapolated results. However, results of other workers differ especially in their O and S contents. These variations may be due to geographical and environmental factors. Going by the agreement between our two results obtained by two independent methods, we can reasonably state that in ADB consumed in the South-western part of Nigeria, percentage saturated fatty acids is ca 25% and unsaturated fatty acids is ca 75% comprising of oleic(ca 14%) and linoleic(ca 61%) acids.

Acknowledgement

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Short Communication

Pak. J. Sci. Ind. Res. 2004 ***47****(2) 104 - 106*

ELECTROLYTIC OXIDATION OF TETRAHYDROCARBAZOLE

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Electro-oxidation of THC in a strong base, potassium methoxide in methanol, produces a series of dehyrodimers having interesting stereochemistry and ^{13}C dynamic N.M.R. properties (Bobbitt *et al* 1986a). Electro-oxidation of 1-carbomethoxy THC yields its 1- methoxy derivative in 44% yield (Bobbitt 1989). In aqueous acetonitrile containing $LiClO_4$, THC can be oxidized to a dehydrodimer in 60 - 90% yield. A two compartment cell at + 0.7 V vs. SCE having graphite anode is used (Bobbitt *et al* 1986b). Preparative anodic hydroxylation of 1- carbomethoxy THC and its 7- methoxy derivative have been reported (Rusling *et al* 1984). Related mechanistic investigations were also carried out (Rusling *et al* 1986). Characteristics of anodic normal pulse voltammograms for the monomeric indole alkaloids such as catharanthine, vindoline, THC, its N - methyl derivative, aniline, *p* - chloroaniline, and *m* - dimethylaminoanisidine, were described (Haque 1990).

The mechanisms of electrodimerization of THC and N- methyl THC were investigated using cyclic, linear sweep, normal pulse and reverse pulse voltammetry at carbon paste and platinum electrodes in 10% aqueous - acetonitrile (Kulkarni *et al* 1982). Adsorptive / extractive accumulation of THC at carbon paste electrode was used to improve the subsequent voltammetric determination with respect to the sensitivity and selectivity (Wang and Bonakdar 1985).

2,2,6,6 - Tetramethylpiperidine -1- oxonium tetrafluoroborate was shown to react with THC to give, in the presence of water, the 4 - keto derivative (Bobbitt *et al* 1988). In the absence of water, the oxidation of THC gave a mixture of dimers, probably derived from the Diels - Alder self condensation of 2,3-dihydro-1*H*-carbazole (Bobbitt *et al* 1990).

A saturated solution of solid sodium bicarbonate containing 0.2M lithium perchlorate in equivolume aqueous - acetonitrile was investigated as an electrolyte for the electrochemical oxidation of 2,3,4, 9-tetrahydro1*H*-carbazole, THC, to its 4a - 7′-dehydrodimer. THC was oxidized by a similar mechanism in the presence and absence of bicarbonate. A Bioanalytical Systems BAS-100 Electrochemistry System was used for voltammetry. The three-electrode cells used carbon paste working electrodes (A = 0.08 cm^2), a platinum counter electrode and, as reference a Ag / Ag+ (0.001 M acetonitrile solutions) was used. All working electrodes were disks surrounded by an outer layer of insulating material. All potentials are reported vs SCE. Acetonitrile (ACS reagent - grade or spectrograde), and THC (Aldrich) were used as received. Solutions were purged with purified nitrogen for 8 - 10 min prior to the beginning of an experiment carried out at an ambient temperature of the laboratory, 25 ± 2°C (Haque 1989).

For the electrochemical oxidation of THC, a saturated solution of solid $NaHCO_3$ containing 0.2M lithium perchlorate in equivolume aqueous - acetonitrile was investigated as an electrolyte, to determine whether THC is oxidized by a similar mechanism in the presence and absence of sodium bicarbonate. From voltammetric and preparative runs, it appears that THC is oxidized by a similar mechanism in the presence and absence of sodium bicarbonate. There is a substantial increase in the amount of dimeric product formed when sodium bicarbonate is present; this is probably due to the decreased acid catalyzed decomposition of dimeric product.

This is suggested by examination of the voltammograms obtained during the electrochemical oxidation of THC. Fig 1(a) shows cyclic voltammogram of 1mM THC at carbon paste electrode in an equivolume aqueous-acetonitrile containing 0.2M lithium perchlorate but no sodium bicarbonate. Two peaks appear in this voltammogram; first peak appears at a potential of 0.51 V with peak current 25.0 μ A. Fig 1(b) shows the voltammogram obtained for THC when sodium bicarbonate is added to the background electrolyte. A single peak appears in this voltammogram at a potential of 0.39V and 25.0μA peak current. Time single peak suggests that electrolysis of the dimer is suppressed in the presence of sodium bicarbonate. The cyclic voltammetric values of Ep for the first peak, for the electro-oxidation of tetrahydrocarbazole, shifted to more negative values as the concentration of the carbonate was increased, with dEp / dlog [bicarbonate] = -17.9 mV. Since neither a reliable acidity scale nor a suitable range of buffer is available in the equivolume aqueous - acetonitrile used, qualitative information can be obtained by the assumption that bicarbonate is acting as a proton acceptor. A value of -17. 9 mV / pH would be expected (Nadjo and Saveant 1973).

Fig. 2(a) shows large amplitude pulse voltammogram for THC at carbon paste electrode in equivolume aqueous-acetonitrile containing 0.2M lithium perchlorate. In this voltammogram

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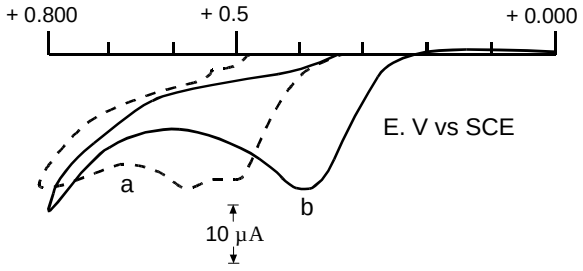

Fig 1. Cyclic voltammogram of 1 mM THC at carbon paste electrode in equivolume aqueous acetonitrile containing 0.2M lithium perchlorate **(a)** without (---) and **(b)** saturated with (—) sodium bicarbonate.

single wave appears with half-wave potential at 0.47V, and 53.0 µA limiting current. Fig. 2(b) shows large amplitude pulse voltammogram for THC when sodium bicarbonate was added to the previous solution Fig. 2(a).

This voltammogram also shows a single wave at $E_{1/2}$ 0.31 V, and 49.0µA limiting current. As revealed by cyclic voltammetry Fig. 1(a), the difference between peak potential for the formation of dimer (510 mV, Table 1) and peak potential for subsequent oxidation of dimer (570 mV, Table 1) is only 60 mV. This difference is too small to be manifest as two distinct plateaux in the large amplitude pulse voltammogram Fig. 2(a) in the present solvent mixture of equivolume aqueous acetonitrile. However, when the proportion of acetonitrile is increased to 90% in the electrolyte and 0.002 M tetra-*t*-butylammonium hydroxide is added in place of sodium bicarbonate, two separate $E_{1/2}$ values were reported earlier (Haque 1989) for the corresponding large amplitude pulse voltammogram.

Large amplitude pulse voltammetry (LAPV) gives only one anodic wave in the presence and absence of sodium bicarbonate. The height of this wave was nearly the same regardless of whether sodium bicarbonate was present or not. The wave height in LAPV is directly proportional to number of electrons transferred (Bond 1980) which did not increase in the presence of bicarbonate. Furthermore, there was no change in the height of the wave as compared with that in the electrolytic solution containing 90% acetonitrile and 10% water (Kulkarni *et al* 1982). LAPV shows there was no change in the number of electrons transferred Fig. 2(b) when bicarbonate is added to the electrolytic solution.

Fig. 3(a) shows differential pulse voltammogram for 1mM THC at carbon paste electrode in aqueous - acetonitrile containing 0.2M lithium perchlorate, when no sodium bicarbonate was added. This voltammogram shows three distinct summits. First summit appears at a potential of 0.43V and 15.3 µA summit current. The second and third electro - oxidation summits were not investigated further. Fig 3(b) shows differential pulse voltammogram of 1mM THC. This was obtained when

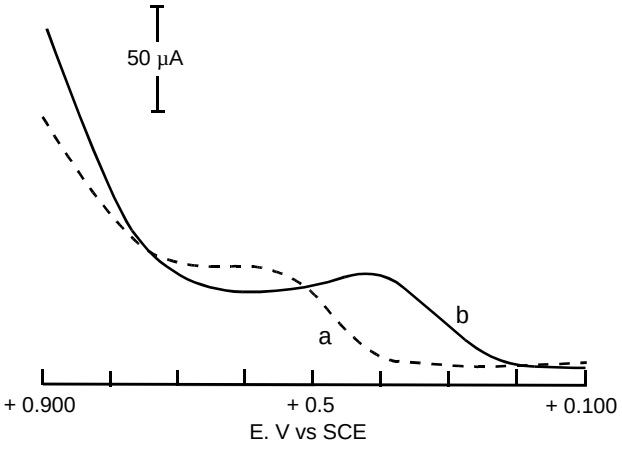

Fig 2. Large amplitude pulse voltammogram of 1 mM THC at carbon paste electrode in equivolume aqueous acetonitrile containing 0.2M lithium perchlorate **(a)** without (---) and **(b)** saturated with (—) sodium bicarbonate.

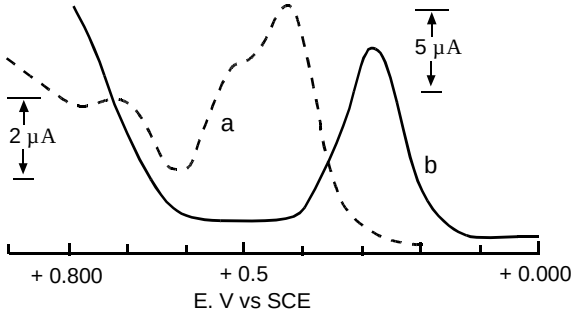

Fig 3. Differential pulse voltammogram of 1 mM THC at carbon paste electrode in equivolume aqueous acetonitrile containing 0.2M lithium perchlorate **(a)** without (---) and **(b)** saturated with (—) sodium bicarbonate.

solid sodium bicarbonate was added to the previous solution Fig 3(a). A single summit appears in this voltammogram at 0.28 V and 15.3 µA summit current, corresponding presumably to the first summit in Fig. 3(a). The differential pulse voltammetry suggests that eletrolysis of dimer is suppressed in the presence of sodium bicarbonate. There was no change in the identity (Bobbitt *et al* 1986a &b) of the dimeric product formed when sodium bicarbonate was added to the electrolyte solution, as confirmed by HPLC. Values of potentials, and currents for the three voltammetric techniques are summarized in Table 1.

Voltammetric and preparative runs indicate that THC is oxidized by a similar mechanism in the presence and absence of sodium bicarbonate. However, there is a substantial increase in the amount of dimeric product formed when bicarbonate is present. The yield of 4a-7′-dehyhdrodimer of THC in the presence of saturated sodium bicarbonate upon electrolysis in 0.2M $LiClO_4$ + 1 : 1 mixture of acetonitrile - water approached

Table 1

Anodic voltammetric characteristies of THC at carbon paste electrode in equivolume acetonitrile water containing 0.2M lithium perchlorate in the absence (A) and presence (B) of saturated sodium bicarbonate (1g 50ml)

Technique	Potential (mV) vs. SCE			Current (μA) I
	E_{1x}	E_{x2}	E_{x3}	
CV at 100mVs^{-1}				25.0
A	510	570	--	25.0
B	390	--	--	
DPV at 4mVs				15.3
A	430	510	720	15.3
B	280	--	--	
LAPV at 4mVs^{-1}A				
B	470	--	--	53.0
	310	--	--	49.0

nearly 25%. In the absence of bicarbonate, yield of the dimeric product was less than 16%. Sodium bicarbonate apparently suppresses the acid - catalyzed decomposition of the dimeric product.

Key words: Electrodehydrodimerization, Voltammetry, Tetrahydrocarbazole, Electrosynthesis.

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Biological Sciences

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Cultivation of Prawn in Polyculture with some Species of Indian and Chinese Major Carps

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The freshwater prawn *Macrobrachium rosenbergii* (de Man) was cultivated in polyculture with Indian and Chinese major carps for two successive years. The densities of prawn were 6000,8000 and 10,000 juveniles / ha with a constant fish density of 5,000 fish fingerlings / ha. The fish species were silver carp (35%), catla (15%), mrigal (20%) in the first year and in the second year silver carp, catla, rohu, mrigal, grass carp and black carp in the ratio of 30:15:34:5:15:1, respectively. Highest production of prawn and fish were 122 kg / ha and 4200 kg / ha / yr in the first year and 96 kg / ha and 3945 kg / ha / yr in the second year. The low production of prawn might be hampered by the low temperature. However, cultivation of prawn with Indian and Chinese major carps should be made in overwintering season and low prawn density should be maintained in polyculture system of *M. rosenbergii* with fish.

Key words: *Macrobrachium rosenbergii,* Polyculture, Production and culture system.

Indroduction

The freshwater prawn *Macrobrachium rosenbergii.* (de Man) has a very high potential for aquaculture in Bangladesh. The species has a number of advantages over many other crustaceans (Fujimura 1967, 1972 and 1974). It is a benthophagic omnivore, which makes it a good candidate in the polyculturing system (Parameswarn *et al* 1977). Polyculture of *M. rosenbergii* has been successfully investigated with Indian and Chinese carps in many countries of the world (Malecha *et al* 1981; Buck *et al* 1983; Wohlfarth *et al* 1985).

Prawns are the valuable cash crop and its polyculture with various species of fish has received considerable attention in temperature climates. Polyculturing system of *M. rosenbergii* with other fish species which are exclusively surface feeder and mid-water feeder produces more crops by utilizing the whole water body through improving the ecological stability or possibly through redistribution of food without hampering the growth rate of either the prawn or other fish species, (Tunsutapanich *et al* 1982). In Bangladesh, polyculture of *M. rosenbergii* with carp is a potential area of research at the prevailing context of the carp polyculture system. This is already an on-going practice in different places of Bangladesh (Shah 1991). Presently, the practice is that the farmers stock prawns and fishes without any scientific basis of stocking density, the level being very limited and extensive. There are thus enough scopes for improving the present practice through scientific culture and management. With this end in view, the study was undertaken to see how present system of carps polyculture can be improved through introducing prawn in the system.

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Materials and Methods

The study was conducted for two successive culture periods from December to September and from November to July and these two culture periods are called as first year and second year, respectively. In the first year treatments were carried out with or without prawn under some feeding and fertilization situation with two replications of each, having an area of 0.1 ha for each replications pond. The experimental design is given in Table 1.

In the second year there were five different treatments each with two replications. Out of the five treatments three treatments (T_1, T_2 and T_3) consisted of two different prawn densities with a constant density of fish, the treatments were tested with regard to two different feeds, viz., feed A and feed B under the same fertilized situation. The treatments T_4 and T_5 were adopted as the means to compare the growth of fish under the situation of fish with or without prawn under the same two types of feeding conditions. Hatchery produced seeds were used for the experiment. The experimental design is given in Table 2.

Results and Discussion

The production of fish and prawn as well as total production of each treatment for each year of trial are shown in Table 3. From the first year production, it was seen that out of two treatments, first treatment had given a production of 4,200 kg /

Table 1
First year experimental design

Treatment	Stocking densities and species combination		Feed and feeding rates		Fertilization
T_1	5000 Fish fingerlings / ha		Sesame oil cake	4%	Inorganic fertilizer
	6000 shrimp / ha		Rice bran	40%	(Urea plus TSP 1:1)
	Silver carp	35%	Fish meal	20%	@ 50 kg / ha / month
	Catla	15%	3% Body weight feed daily		
	Rohu	30%			
	Mrigal	20%			
	Prawn 600/pond				
T_2	5000 fish fingerlings / ha		-do-		-do-
	Silver carp	35%			
	Catla	15%			
	Rohu	30%			
	Mrigal	20%			

Table 2
Second year experimental design

Treatment	Stocking densities and species combination		Feed and feeding rates		Fertilization
T_1	Prawn 10,000 / ha		Fish meal	20%	Only inorganic
	Fish 5,000 / ha		Rice bran	50%	fertilizer (Urea & TSP
	Silver carp	30%	Oil cake	30%	1:1) @80 kg / ha
	Catla	15%	@ 3% body weight daily		
	Rohu	34%	(Feed A)		
	Mrigal	5%			
	Grass carp	15%			
	Black carp	1%			
T_2	Prawn	10,000 / ha	Fish meal	10%	Same
	Fish	5,000 / ha	Rice bran	15%	
	Fish species ratio same as T_1		Oil cake	45%	
			Feeding rate same as T_1		
			(Feed B)		
T_3	Prawn	8,000 / ha	(Feed A)		Same
	Fish	5,000 / ha			
	Fish species ratio same as T_1				
T_4	Prawn	Nil	(Feed A)		Same
	Fish	5,000 / ha			
	Fish species ratio same as T_1				
T_5	Prawn	Nil	(Feed B)		Same
	Fish	5,000 / ha			
	Fish species ratio same as T_1				

Table 3

Details of stocking average final attained by each species and the total production of fish and prawn under polyculture of *M. rosenbergii* with fish

Treatment	Details of stocking		Initial weight (g)	No. fish harvest	% survival	Final weight (g)	Contribution to the production (kg)	Total production (kg / ha / yr)
	Species	No.of fish stocked						
T_1	S.Carp	175	24.0	170	97	1358	231	Fish: 4200
(First year)	Catla	75	21.0	72	96	525	38	Prawn: 122
	Rohu	150	13.0	138	92	701	97	
	Mrigal	100	18.0	90	90	607	54	
	Prawn	600	2.5	112	18.6	109	12.20	
T_2	S. Carp	175	24.0	148	84	1291.9	191.20	
	Catla	75	21.0	63	84	497	31	Fish: 3672
	Rohu	150	13.0	125	83	637	79	
	Mrigal	100	18.0	82	82	805	66	
	Prawn	Nil						
T_1	S.Carp	150	23	143	95	1215	173.75	
Feed A	Catla	75	37	62	82	475	29.45	Fish: 3645
	Rohu	170	12	144	84	550.8	79.30	
	Mrigal	25	11	25	100	804	20	Prawn: 75
	G. Carp	75	2.5	70	94	246	52	
Second	B. Carp	5	43	5	100	2080	10	
year	Prawn	1000	5.6	157	15.7	48	7.50	
T_2	S.Carp	150	23	131	87	1233.7	161.61	
Feed B	Catla	75	37	55	73	468	25.74	Fish: 3125
	Rohu	170	12	117	69	565	66.10	
	Mrigal	25	11	25	100	705	17.62	Prawn: 59
	G. Carp	75	2.5	55	73	585	32	
	B. Carp	5	43	5	100	1900	9.50	
	Prawn	1000	5.6	132	13.2	45	5.90	
T_3	S. Carp	150	23	143	95	1315	188.01	
Feed A	Catla	75	37	69	79	535	36.92	Fish: 3945
	Rohu	170	12	138	81	678	93.56	
	Mrigal	25	11	24	96	455	10.92	Prawn: 96
	G. Carp	75	2.5	61	81	890	54	
	B. Carp	5	43	5	100	2200	11	
	Prawn	800	5.6	192	14	50	9.60	
T_4	S.Carp	150	23	141	80	1318	185.84	
Feed A	Catla	75	37	65	60	520	33.80	Fish : 3796
	Rohu	170	12	147	57	476	69.97	
	Mrigal	25	11	25	100	909	23	
	G. Carp	75	2.5	51	68	1000	51	
	B. Carp	5	43	5	100	3200	16	
	Prawn	Nil						
T_5	S. Carp	150	23	138	78	1312	181	
Feed B	Catla	75	37	65	60	495	32.18	Fish : 3565
	Rohu	170	12	144	70	438	63.11	
	Mrigal	25	11	25	100	820	20.50	
	G. Carp	75	2.5	43	57	1191	51	
	B. Carp	5	43	5	100	1750	8.70	
	Prawn	Nil						

S. carp = Silver carp; B. carp = Black carp; G. carp = Grass carp.

Table 4

Treatment wise average values and the range of physico-chemical and biological parameters under polyculture of *M.rosenbergii* with fishes in the two successive years of trial

Year	Treatments	Temperature °C	Water transparency (cm)	Dissolved O_2 (ppm)	pH	Hardness (ppm)	Plankton (org./l)	
							Zooplankton	Phytoplankton
Ist year	T_1	18.67 - 29.17	35.5 - 63.4	3.35 - 5.45	7.0 - 8.0	128 - 135	7151 - 10830	13875 - 33850
	T_2	18.67 - 29.17	41.18 - 65.25	2.25 - 5.50	7.3 - 8.5	95 - 130	6983 - 8500	10158 - 30155
2nd year	T_1	9.5 - 34.5	15.4 - 43.0	1.0 - 8.5	6.7 - 9.0	85 - 135	830 - 7600	5430 - 32000
	T_2	9.5 - 34.5	23.0 - 64.0	1.5 - 7.5	6.5 - 8.5	98 - 138	780 - 8300	4380 - 28000
	T_3	9.5 - 34.5	14.0 - 74.0	1.5 - 9.0	6.5 - 9.0	74 - 175	800 - 7500	5080 - 30200
	T_4	9.5 - 34.5	12.0 - 61.0	2.0 - 7.0	7.0 - 8.5	98 - 135	650 - 9500	5220 - 35000
	T_5	9.5 - 34.5	13.5 - 59.0	1.9 - 6.5	6.5 - 8.5	75 - 140	850 - 9320	6430 - 38000

ha / yr of fish plus 122 kg / ha / crop of prawn in polyculture at a stocking density of 6,000 juveniles/ha and 5,000 fingerlings of carps / ha and in the second treatment fish production obtained from ponds without prawn was 3,672 kg / ha / yr which is lower than the production obtained from the first treatment. This low production of fish in the second treatment could be explicable on the basis of the fishes, effected by the argulosis disease which might hampered the total fish production. As compare to the growth of mrigal in the first trial, mrigal showed better performance in these ponds, where prawn was nil. This year trial indicates that the ecological niche of the aquaculture system utilized by the prawn with carps polyculture ponds are not overlapped with niches of other carps, except mrigal. Similar results have also been reported by Tunsutapanich *et al* (1982); Rouse and Stieckney (1982).

In the second year trial out of the three treatments (T_1, T_2 and T_3), the treatment (T_3) had produced the higher production of fish 3945 kg / ha / yr plus 96 kg / ha / crop of prawn where prawn were stocked at 8,000 juveniles / ha and the Feed A was used, having 25.34% protein level. The other two treatments T_1 and T_2 each has produced 3,645 kg / ha / yr of fish plus 75 kg / ha / crop of prawn and 3,125 kg / ha / yr of fish plus 59 kg / ha / crop of prawn, respectively where prawn density was 10,000 juveniles / ha in each treatment and the Feed A, Feed B, respectively were used as supplementary feed. In feed B having 24.5% protein level. Other two treatments T_4 and T_5 each has produced 3,795 kg / ha / yr and 3,565 kg / ha / yr of only fish where prawn was nil and the supplementary feed A and B, respectively were applied. These two treatments were adopted as the means to determine the growth of fishes effected by situation of with or without prawn under two types of feeding conditions. The results of these two treatments T_4 and T_5 indicating that with or without prawn in polyculturing of *M. rosenbergii* with carp no significant loss is caused in total biomass. Similar results have also been cited by Buck *et al* (1983); Cohen and Raianan (1983) and Ahmed *et al* (1996). From Table 3 it was seen that the average growth of individual fish was below marketable size which was the most plausible reason for the high density situation effected by the shallowness of the ponds; the effective depth (1.5m) of ponds was quite low in consideration of the density of fish stocked and due to their higher ratios circumstances in the surface (45%) and midwater region (49%).

The prawn production of 122 kg / ha / crop, 96 kg / ha / crop and the lowest production of 59 kg / ha / crop under the stocking density of 6,000 / ha, 8000 / ha and 10,000 / ha, respectively in the two sucessive years, with a constant density of 5,000 fish / ha; it is seen that the production of *M. rosenbergii* was higher in ponds with lower stocking densities of prawn. Smith *et al* (1978); Willis and Berrigan (1977) and Huner *et al* (1980) recommended that low density culture was feasible where growing season for prawn was 5-6 months. The survival rate of prawn ranged from 13-24% and fish 69-100%. In this study it was also found that prawn survival increased with decreased stocking density. It has been reported by some researcher (Sandifer and Smith 1975; Willis and Berrigan 1977; Brody *et al* 1980). The low survival of prawn could be explicable on the basis of some probable factors such as temperature, stocking density etc.

Considering the shallowness of the ponds that resulted in to the reduced space, food and shelter. The result of the physico-chemical parameters (Table 4) particularly temperature, dissolved oxygen, transparency and pH data explain the low survival rate. During the culture period dissolved oxygen level ranged from 1-8.5 ppm. This confirms more generally, the low level of dissolved oxygen at early in the morning the prawns were observed to move very slowly along the shoreline of the pond. Humayun *et al* (1986) reported that low dissolve oxygen content of water was the most important cause for heavy mortility of prawn. Cohen and Raianan (1983) re-

ported that to get an optimal prawn production, dissolved oxygen level should always be maintained above 4 ppm. On the other hand, culture cycle of the species has been erroneous; the prawn suffer from cold condition very much and as such their culture cycle should not be through cold season. Wohlfarth *et al* (1985) terminated their experiment on polyculture of prawn with fish at the beginning of November, not to expose the prawns to low water temperature, which might be lethal for prawns. During second year culture period of this experiment the atmospheric temperature dropped down to 5.6°C and the water temperature at 7.00 am. was 9.5°C (Table 2); which might be most plausible for heavy mortality of prawn. Sang and Fujimura (1977) cited that *M. rosenbergii* adopts minimum 15°C to maximum 35°C temperature and the maximum growth rate occur near at 31°C. However, the temperature data (Table 2) in the present study were 9.5-34.5°C in the second year trial but in the first year trial the temperature data were in the range of 18-33°C. It can also be noted that low survival of prawn might be plausibly the reason of predation by piscivorous animals such as fox which were available in the research area, as the species is more vulnerable to predation during molting stages.

From the present study, it can be suggested for the next, culture of prawn should be made at overwinter season and the another observation in the polyculture of prawn with six months rearing but at the same time fish can not attain marketable size. It would be advisable that fishes will be stocked at least four months before; than the stocking of prawn. Therefore, further studies are needed to develop the methods and techniques for polyculture of *M. rosenbergii* with fishes.

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Pak. J. Sci. Ind. Res. 2004 ***47****(2) 112 - 117*

ARTIFICIAL GROUND FREEZING METHOD FOR SHAFT CONSTRUCTION IN MADDHAPARA HARDROCK MINE, BANGLADESH: MINIMIZATION OF ITS COST

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The Korea South-South Cooperation Corporation (NAMNAM) used Artificial Ground Freezing (AGF) during 160 m depth shafts (cage and skip) construction in the Maddhapara Hardrock Mining Project (MHMP). The freezing design calculation for AGF operation showed that freezing wall thickness was satisfactory for both kaolin and sand layer at existing vertical ground pressure. But after AGF operation freezing status revealed that the ice wall thickness in skip shaft attained as per design, but in cage shaft the achieved thickness was more than the expected due to deviation from original design for freezing hole by NAMNAM i.e., drilling of 31 freezing holes instead of 32 for cage shaft. The ice-wall bonding had affected the whole rock mass of the inner diameter of cage shaft that became hard like rock, but this effect was not so intensive for the skip shaft towards the inner portion and did not create any severe problem. As a result the cage shaft was excavated with explosive (drilling blasting) involving additional time (3 months) and cost (US$1,51,866), which NAMNAM could avoid by sinking an additional 160 m deep freezing hole during cage shaft construction with a cost of US$18,045 and thus saving a total of US$1,33,820 for the whole operation in MHMP.

***Key words*:** Artificial Ground Freezing, Maddhapara Hardrock Mine, Cost effectiveness.

Introduction

Freezing is reliable, safe and cost - effective approach for the development of mine and civil engineering construction within the water bearing zone for the protection of water leakage from aquifer, especially to counter the tendency of seepage and collapse of the side wall (Harris 1995). Artificial Ground Freezing (AGF) method was used for the first time in 1862 for a mineshaft construction in Swansea in South Wales and is widely used till today for ground freezing without affecting the water table or the quality of ground-water.

In 1974 - 1976, the Geological Survey of Bangladesh (GSB), based on the results of geophysical prospecting, located dome-shaped body of Archean Basement of the Indian Platform of granodioritic composition in the Maddhapara area at the shallowest depth of 128 m from the surface. The Korea South-South Cooperation Corporation (NAMNAM) of the Democratic People's Republic of Korea came into contract with the Petrobangla (Bangladesh Oil, Gas and Mineral Corporation) in 1994 for the development of underground hardrock mine in Maddhapara. Then, Maddhapara Hardrock Mining Project (MHMP) came into existence with annual production target of 1.65 million ton of rock. It is the first experience of hardrock mining and the second major mining project in Bangladesh (Fig 1).

The Basement Complex has two units; fresh and weathered. The fresh rock forms the fissure artesian aquifer (NAMNAM 2000). The weathered portion is divided into weathered layer in the lower part, and impervious kaolin layer in the upper part. Tertiary Dupi Tila Formation, Tura Formation and Permian Gondwana Group lie above the Basement and form porous aquifer with 26˚C temperature at the top and 31˚C at the bottom, and hold non-pressure gravitational water that infiltrate into it. Impervious Madhupur Clay overlies the porous aquifer.

Keeping in mind hydrogeological and engineering properties of the formations, AGF method was used for sinking cage and skip shaft, which is the first experience of this kind in Bangladesh. The period of construction as per schedule for the skip and cage shafts was 3 months. The construction of skip shaft was completed as per schedule, but additional 3 months were needed for the cage shaft construction involving additional cost of manpower, pneumatic air, explosive per shift per hole and power, which was not considered in the project proposal. So, the study on the AGF for shaft construction in MHMP is carried out to authenticate the financial involvement in shaft construction after practical experience.

Experimental

Shaft sinking by AGF method in MHMP. Two types of vertical shafts were constructed; cage shaft for transporting class locomotive and mine car for man and material lifting, and skip shaft for lifting 1.65 million tons of rock a year. Polish double-step compression of refrigerant plant was used for

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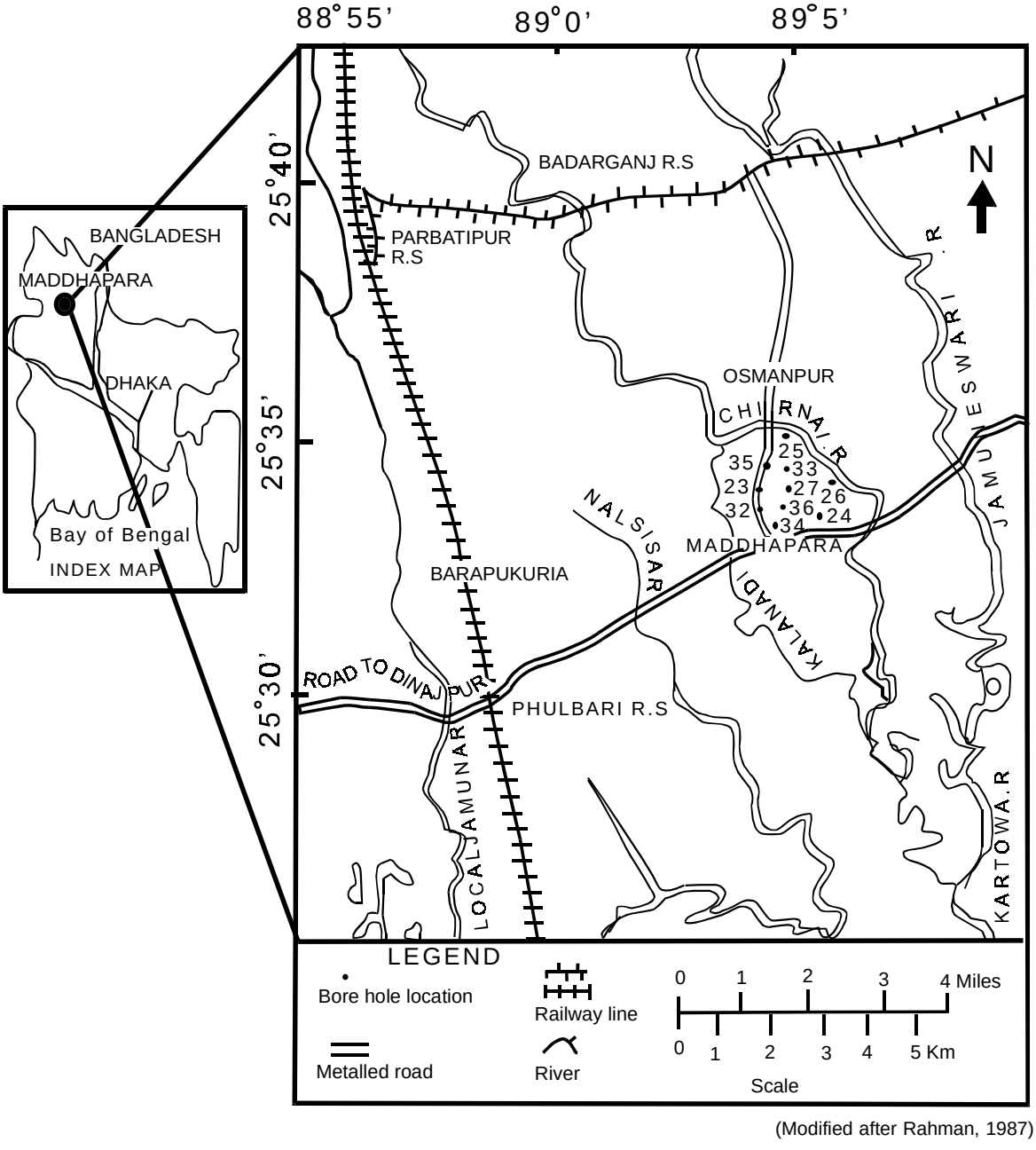

Fig 1. Location map of the study area.

AGF method in MHMP. At first, boreholes were drilled with 0.8-2.0 m interval along the perimeter of shafts and then freezing pipes were put into the holes. Water solution mixed with $CaCl_2$ was supplied into the freezing pipes by pump. As a result of continuous circulation of the chilled salt water into the freezing pipes, water within the rock mass was frozen. The ice column was formed gradually around each borehole and after a certain period a united ice wall was formed and the sinking of shaft began. The longitudinal section of freezing boreholes is shown in Fig 2.

Drilling of freezing boreholes for cage and skip shaft. Shaft construction design in MHMP was divided into; the freezing section for aquifer zone and non-freezing section for fresh rock at the bottom of mine. The total depth of freezing borehole in the freezing section of aquifer zone was 154 m (Juche 1998). The initial sinking diameter and finished diameter of shafts were 6.8 and 5.0 m, respectively. The number and dia-meter of freezing hole were 32.0 and 11.5 m, respectively.

According to the project proposal of NAMNAM for ground freezing operation before the construction of shaft, a total of

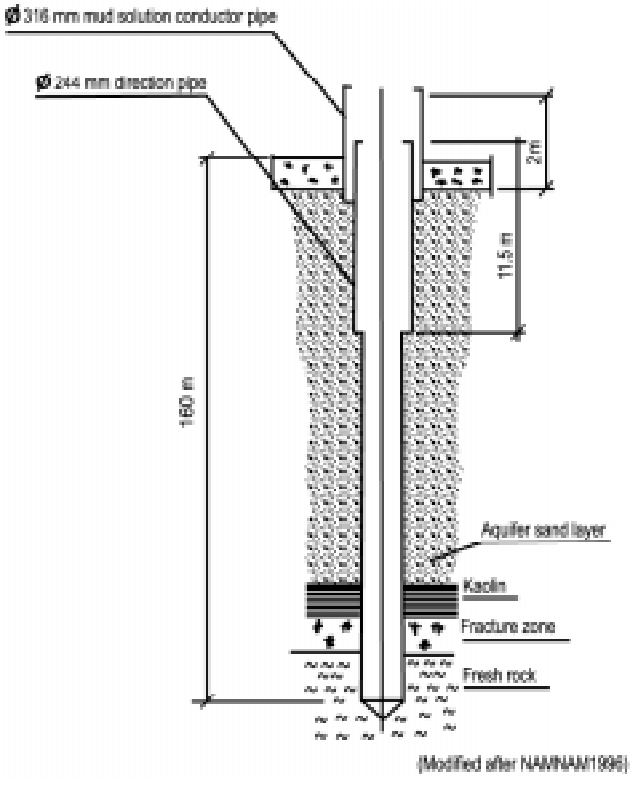

Fig 2. Longitudinal section of freezing borehole in MHMP.

75 freezing and temperature measuring boreholes were planned to drill. But they drilled 74 freezing, center, supplementary and temperature measuring boreholes of which 63 were freezing boreholes (32 for skip shaft and 31 for cage shaft) from October 28, 1995 to October 21, 1996 (NAMNAM 1999). The arrangements of freezing borehole for cage and skip shafts are shown in Fig 3 and 4, respectively.

Calculated values for freezing design before AGF operation in the present study showed that the thickness of freezing wall was satisfactory for both kaolin and sand layers and their values were 2.83 and 1.88 m, respectively at existing vertical ground pressure, but during AGF operation, the thickness were achieved as 3 and 2 m, respectively.

Technology of mass freezing. Freezing work in MHM included two steps; (i) Active freezing work, it started to activate the freezing wall first. The difference of temperature of inlet and outlet brine at the initial time of active freezing work was 3 - 5˚C in normal. The difference of temperature of outlet and inlet brine for the first 10-15 days of freezing was about – 3.5˚C and gradually it reached to 2 - 3˚C at the end of active freezing and it reached to below 1˚C in the passive freezing period. Temperatures were recorded from temperature measuring borehole at a depth of 10 m interval once in a day during active freezing period.

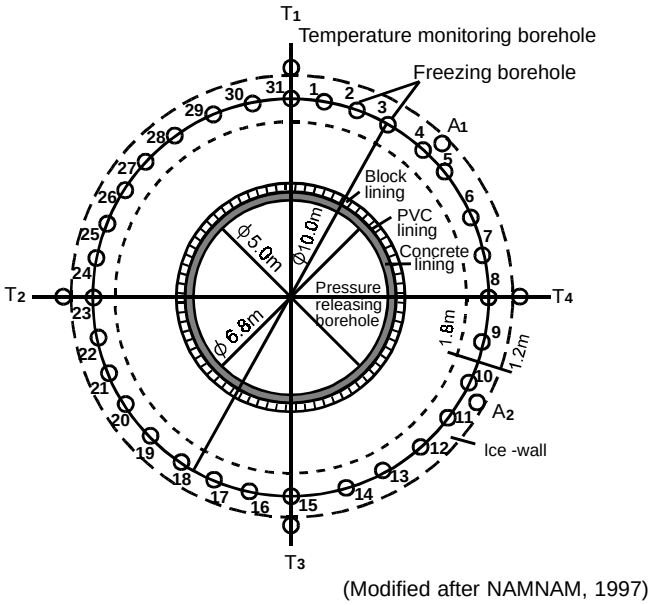

Fig 3. Arrangement of freezing borehole for cage shaft sinking.

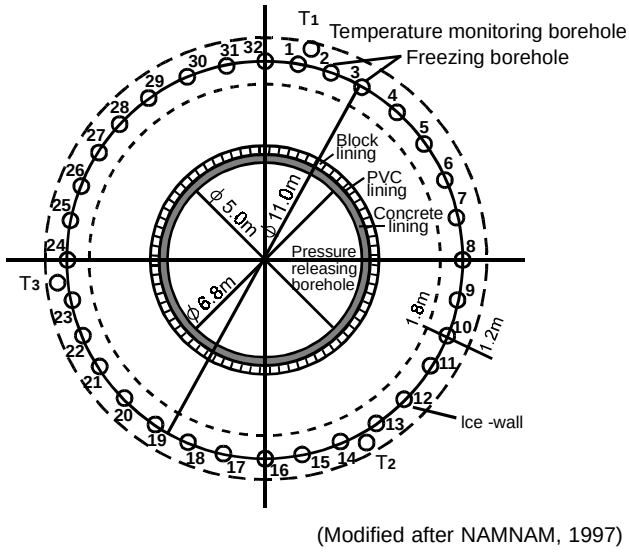

Fig 4. Arrangement of freezing borehole for skip shaft sinking.

(ii) Passive freezing work; Passive freezing starts to maintain the required thickness of ice wall just after completion of active freezing and is continued up to total construction.

Observations in course of stratum freezing. After AGF operation and before the construction of shaft, the temperature of stratum freezing and ice wall thickness at 10 m interval from three temperature monitoring holes arranged at different distances (0.8, 1.0, 1.25 and 1.5 m) at inner and outer circle of the freezing ring. It was measured in MHMP by NAMNAM (NAMNAM 1996). These data are used in the present study. The temperature data are plotted graphically with respect to depth for the months of November 1996 to December 1997 and

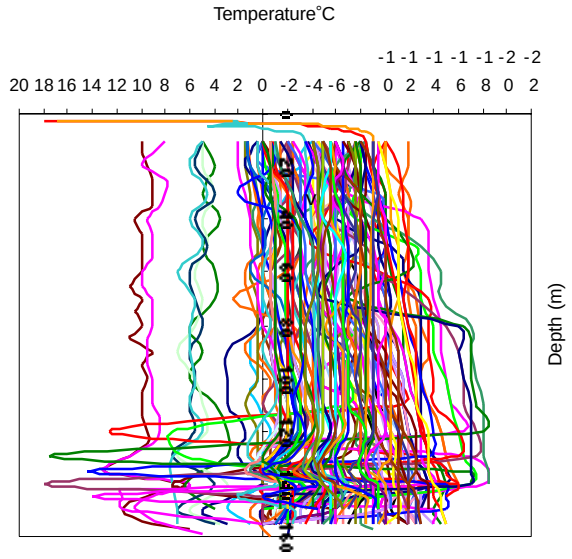

Fig 5. Freezing status of cage shaft.

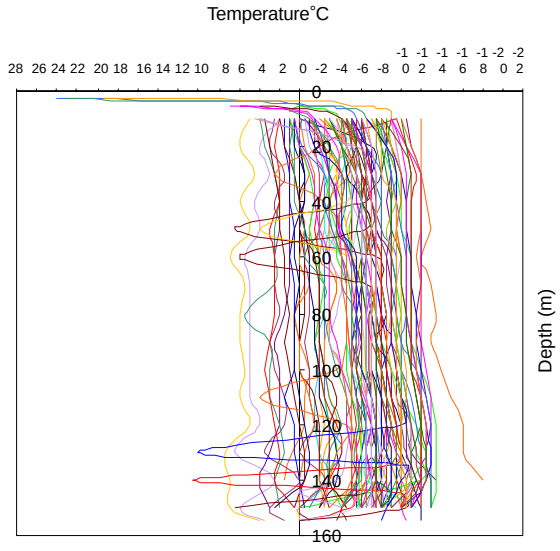

Fig 6. Freezing status of skip shaft.

the freezing status of the stratum freezing for the shafts are shown in Fig 5 and 6.

It reveals that the thickness of ice wall in skip shaft was achieved as per design but in cage shaft the ice wall attained thickness more than the original design due to wrong freezing planning i.e., drilling of 32 freezing holes was done ins-tead of 31 during cage shaft construction by NAMNAM. The ice-wall thickness in clay layer (up to 7 m depth) did not form well-satisfactory required ice-wall thickness due to presence of capillary water or soil moisture but with increasing depth, on the other hand the ice-wall is formed more satisfactorily in porous sandy aquifer layer.

The ice wall bonding (overlapping) at 10, 50, 130, 140, 150 and 160 m depths of cage and skip shafts are constructed in the present study and their representatives are shown in Fig 7a - f. It is observed that the ice wall bonding has affected the whole rock mass of the inner diameter of cage shaft but for skip shaft, this effect is not so intensive towards the inner portion. The effect of ice wall bonding between depth intervals of 10 - 40 m in the cage shaft is not so intensive but that of between 40 - 50 m depth interval is intensive. This effect became severe below 100 m depth and continues up to 160 m, where ice-wall bonding ingress up to the center of the cage shaft. But the effect of ice wall bonding in case of skip shaft construction through porous aquifer, weathered hardrock and fissure artesian aquifer did not create any severe problem.

Results and Discussion

The periods of skip and cage shaft construction as per schedule were 3 months. But additional 3 months for the cage shaft construction were needed due to wrong freezing planning i.e., deviation from original design for freezing hole and as a result freezing was done so intensively that the total area of the freezing ring as well as the cage shaft excavated area become hard like rock and was excavated with the help of explosive (drilling blasting) involving the additional cost of followings; (a) man - power, (b) consumption of pneumatic air (compressor of 800 kwa / h capacity), (c) consumption of explosive per shift per hole, and (d) power consumption.

(a) *Manpower*. Total cost of additional manpower needed for cage shaft construction is given in Table 1.

(b) *Comsumption of pneumatic air*. Jumbo drill machines pneumatic air compressor run in each shift for 6 h per day with two electric compressor of total capacity of 1600 kwa / h. Therefore, the total amount of electricity consumption per day was 9600 kwa / h (6 h × 1600 kwa / h). So, the total cost of electricity for drilling blasting operation during excavation stood US$ 960 (9600 kwa / h × US$ 0.1) considering per unit cost of electricity for industrial purpose in Bangladesh is US$ 0.1. The construction of shaft below 40 m from the surface that was for 120 m (160 - 40) m, compressed air was circulated in 3 shifts in a day with an efficiency of 1.8 m per blasting, involved the total time 22.22{120 ÷ (1.8 × 3)}. So, that the cost of consumption of pneumatic air was US$ 21, 331.2 (US$ 960 × 22.22).

(c) *Consumption of explosives per shift in a hole*. Each blasting had an efficiency of 1.8 m of excavation in the shaft, so excavation of 120 m of freezing part of the shaft needed 66.66 times (120 m / 1.8) of blasting. Each operation consumes 100 kg of explosive and 100pcs of detonators were consumed for

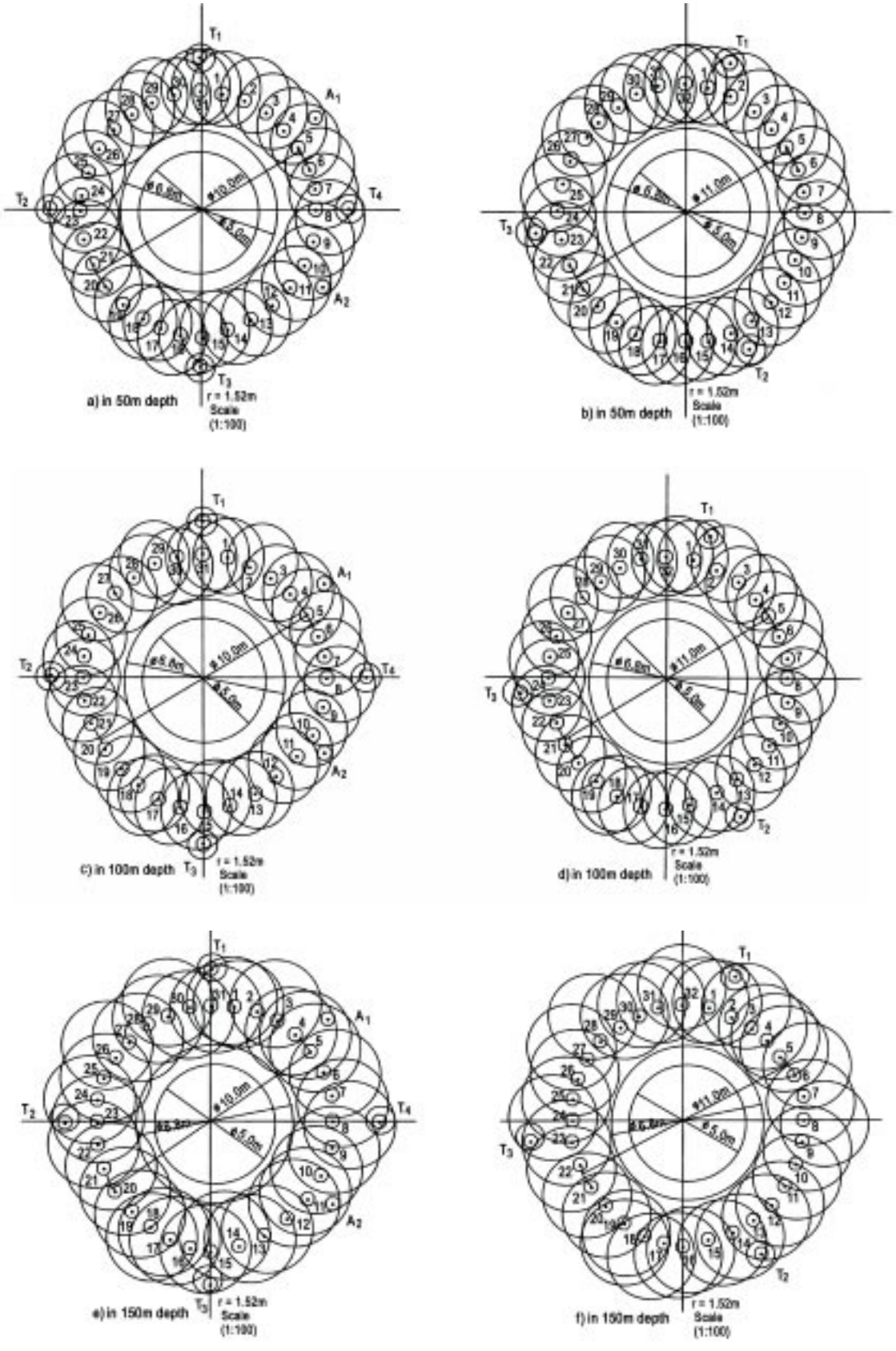

Fig 7. Representatives of ice wall bonding (overlapping) at different depths (a, c, e) for cage shaft and (b, d, f) for skip shaft.

Table 1
Total cost of additional manpower

Personnel expertise	Unit	Per unit salary (US$)	Total salary (US$)
Engineer	1	1,500	1,500
Explosive expert	1	1,500	1,500
Shot firer	2	900	1,800
Explosive loader	4	900	3,600
Mucking labour	10	700	7,000
Signal man	2	700	1,400
		Total for 1 month	= US$ 16,800
		Therefore additional cost for 3 months	= US$ 16,800×3
			= US$ 50,400

blasting operation, so that the blasting explosive involve the cost of US$ 50,000 (66.66 × 100kg = 6666.66 × US$ 7.5) and that of the detonator was US$ 1335 (66.66 × 100pcs = 6666 × US$ 0.2). The total cost of explosive and detonator for blasting operation during shaft construction was US$51,335(US$50,000 + US$ 1,335).

(d) *Electricity consumption for freezing plant*. Each freezing plant was consisted of one compressor with a capacity of 800 kwa / h and four freezing plant units were running uninterruptedly for 3 months. So, the involvement of excess cost was estimated as follow:

800 kwa / h × 4 units × 3 months or 90 days × US$ 0.1 = US$ 28,800.

Finally, the total cost for additional 3 months needed for cage shaft construction due to involvement of excess manpower, consumption of pneumatic air, explosive per shift per hole and electricity for freezing plant was US$ 1,51,866.2, which NAMNAM could avoid by sinking another single 160 m deep freezing boreholes in addition to 31 freezing borehole during the construction of cage shaft, with a cost of US$ 18,045 and thus saves excess time (3 months) and additional cost (US$ 1,33,820) for mining operation in the Maddhapara Hardrock Mining Project.

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Pak. J. Sci. Ind. Res. 2004 ***47****(2) 118 - 120*

NATURAL PROTEIN FORTIFICATION OF CASSAVA (*MANIHOT ESCULENTA*, CRANTZ) PRODUCTS (FLOUR & GARI) USING BAKER'S YEAST SOLID MEDIA FERMENTATION

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In an attempt to enhance the nutritional quality of cassava products (flour & gari), Baker's yeast was used in the fermentation (solid media) of cassava pulp. The mash obtained was subsequently processed into flour and gari (the forms in which cassava products are popularly consumed in Nigeria) and analyzed. The protein (flour, 10.90%; gari, 6.30%) and fat (flour, 4.50%; gari, 3.00%) contents of the products were high. Conversely, the tannin (flour, 0.2%; gari, 0.1%) and cyanide (flour, 9.5mg / kg; gari, 9.1mg / kg) contents were low, though, the cassava flour had higher protein, fat, tannin and cyanide contents than gari. The results indicated that Baker's yeast, a cheap and non-pathogenic saprophyte, could be used in enhancing the nutritional potentials of cassava products by increasing nutrients (protein and fat) and decreasing antinutrient contents (tannin and cyanide). However, nutrient increase was higher in cassava flour while the antinutrient decrease was higher in gari.

Key words: Baker's yeast, Protein, Fat, Tannin, Cyanide, Cassava products.

Introduction

Cassava is often considered an inferior food because the tuber is low in protein, essential minerals and vitamins (Onwueme 1978; Aletor 1993). However, in many cassava-growing areas, its use as food helps to alleviate problems of hunger and thus, its importance in terms of food security in these areas cannot be over emphasized (Aletor 1993). The processes for upgrading the protein value of cassava using solid substrate fermentation have been developed in some countries such as Canada where *Aspergillus fumigatus* has been used (Read and Gregory 1975) and Burundi (Vlavonou 1988) and Nigeria (Akindahunsi *et al* 1999a) where *Rhizopus oryzae* was used in enriching cassava product with protein. This study is a continuation of our study on nutrient enrichment and detoxification of cassava products using cheap, non-pathogenic and saprophytic fungus, *Saccharomyces cerevisae*.

Materials and Methods

Cassava tubers were collected from the Research farm of the Federal University of Technology, Akure, Nigeria. The chemicals used, sodium hydroxide, sulphuric acid, potassium dihydrogen phosphate, citric acid and magnesium sulphate pentahydrate were the products of Eagle Scientific Limited, Nottingham, England, while the urea, tannic acid, ferric chloride and ammonium thiocyanate and petroleum ether (40-60°C) were the products of BDH Chemicals Limited, Poole, England. In addition, glass distilled water was also used.

Cassava tubers were peeled, crushed, and pressed using hydraulic press. The pressed pulp was later subjected to fermentation (Vlavonou 1988). Pure strain of Baker's yeast was sub-cultured and inoculated (1:100) into 1kg of the mash (cassava pulp) as the starter culture and 730ml nutrient solution containing urea (80g), $MgSO_4$. $2H_2O$ (7g), KH_2PO_4 (13g) and citric acid (20g) and then allowed to ferment for 3 days. The product obtained was subsequently, processed into flour and gari. The gari was produced by pressing the fermented pulp by using a locally fabricated mechanical press and then fried in a hot metal dish to gari (Adewusi *et al* 1999).

Sample analysis. The proximate composition (ash, fat, crude fibre and carbohydrate) of the micro-fungi fermented cassava products was evaluated using the standard AOAC (1984) method and the protein content was determined using the Micro-Kjeldhal method (N x 6.25). The tannin content was determined using Makkar *et al* method (1993) while the cyanide content was determined using the method of De Bruijn (197I). The Na, Zn, Ca, Mg, K and Fe contents were determined on aliquots of the solutions of the ash by established Flame Atomic Absorption Spectrophotometric procedures using a Perkin-Elmer Atomic Absorption Spectrophotometer (Model 372) (Perkin-Elmer 1982).

Analysis of data. The data were analysed by students t-test (Zar 1984).

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Results and Discussion

The results of the proximate analysis revealed that the protein contents of the Baker's yeast fermented cassava products were high (flour 10.90 ± 0.1; gari 6.30 ± 0.2). This unusual high protein content could be attributed to the ability of the Baker's yeast to secrete some extracellular enzymes (protein) into the cassava mash during their metabolic activities on the cassava mash and fermentation of the cassava by the fungi. The multiplication of the fungi in the cassava in the form of single cell proteins could also provide explanation for the increase in the protein content of fermented cassava products (Akindahunsi *et al* 1999a; Okafor 1998). However, the protein content of the flour is significantly ($P > 0.05$) higher than that of the gari. This could be attributed to the method of preparation of each of the products. During the processing of gari which entails pressing, sieving and frying of the fermented cassava, some of the protein may have leached off while pressing and burnt off while frying (Akindahunsi *etal* 1999a). It is documented that pre-processing, processing and post-processing methods of preparation of cassava products determine the quality of the products (Akindahunsi *et al* 1999a). The protein content of the product as shown in Table 1, compared favourably with the protein content of *Rhizopus oryzae* fermented cassava products (Akindahunsi *et al* 1999a).

The reason for the unusual high fat content of the cassava products could not be categorically stated. However, there could be possible transformation of carbohydrate to fat (Lehninger 1987) while Akindumila and Glatz (1998) reported that certain fungi can produce microbial oil during the course of fermentation. The decrease in carbohydrate could be attributed to the possible transformation of some of the carbohydrate which the organism possibly use as its carbon source to some other metabolites such as protein or fat (Lehninger 1987).

The mineral contents (Zn, Mg, Fe, Ca, Na and K) of the Baker's yeast fermented cassava products (Table 2) were considerably low when compared to other food crops such as fruit, mushroom, yam tubers and vegetables (Akindahunsi and Oboh 1998, 1999b; Ola and Oboh 2001). However, the gari had a significantly higher ($P > 0.05$) Fe, Mg, Ca and Na contents than the cassava flour. This could be the result of the fact, that some of the metals in the frying pan used may have leached into gari (Akindahunsi *et al* 1999a).

Table 1
Proximate composition* of Baker's yeast fermented cassava products (flour and gari)

Sample	Flour	Gari
Protein	10.90 ± 0.1	6.30 ± 0.2
Fat	4.50 ± 0.2	3.00 ± 0.2
Crude fibre	3.20 ± 0.1	4.30 ± 0.4
Carbohydrate	77.90 ± 0.3	84.50 ± 0.3
Ash	3.50 ± 0.1	1.90 ± 0.0

*Values refer to mean ±SD (dry weight) of three replicates.

Table 2
Mineral composition* of Baker's yeast fermented cassava products (gari and flour)

Sample	Flour	Gari
Zn	4.90 ± 0.2	4.8 ± 0.1
Mg	32.40 ± 0.2	34.1 ± 0.3
Fe	2.20 ± 0.1	2.8 ± 0.1
Ca	11.00 ± 0.1	13.8 ± 0.1
Na	29.60 ± 0.2	30.8 ± 0.1
K	38.40 ± 0.3	36.9 ± 0.1

*Values refer to mean ± SD (dry weight) of three replicates.

Table 3
Tannin (%) and cyanide (mg / kg) contents* of Baker's yeast fermented cassava products (gari and flour)

Sample	Flour	Gari
Cyanide	9.5 ± 0.2	9.1 ± 0.2
Tannin	0.2 ± 0.0	0.1 ± 0.0

*Values refer to mean ± SD (dry weight) of three replicates.

The levels of antinutrients (cyanide and tannin) are shown in Table 3. Tannins affect nutritive value of food products by forming a complex with protein (both substrate and enzyme) thereby inhibiting digestion and absorption. They also bind Fe, making it unavailable and recent evidence suggests that condensed tannins may cleave DNA in the presence of copper ions. It also imparts a dull colour to the processed products, which affects their market value. The tannin contents of the Baker's yeast fermented cassava products flour (0.2 ± 0.0), gari (0.1± 0.0) were very low when compared with the usual tannin content of cassava products (0.4 - 0.5%) (Hahn 1992). It is worth noting that the tannin content of the flour was significantly ($P > 0.05$) higher than that of the gari, which indicates that the processes of garrification could also decrease the tannin content of cassava products. The tannin levels compared favourably with the 0.2% tannin content reported by Akindahunsi *et al* (1999a) for *Rhizopus oryzae* fermented cassava products. The products could also be considered to be safe with regard to tannin poisoning since

the levels reported in this study are far below the critical value of 0.7- 0.9% (Aletor 1993).

The levels of the residual cyanide present in both the cassava flour (9.5 ± 0.2) and gari (9.1 ± 0.2) were very low when compared with the usual cyanide content of cassava products in Nigeria (gari, 19.0mg / kg; fufu, 25mg / kg) and the cyanide content of *Rhizopus oryzae* fermented cassava products (flour, 17.2mg / kg; gari, 13.5mg / kg). This shows that Baker's yeast is capable of utilizing cyanogenic glucosides and the breakdown of the products and explains why it is one of the natural flora involved in cassava fermentation during gari processing (Oke 1968; Akindahunsi *et al* 1999a). The cyanide levels are far below the detrimental level of 30 mg / kg (Akinrele *et al* 1962). These products could therefore be considered safe with regard to cyanide poisoning. Thus from this study, it could be concluded that Baker's yeast, a cheap, non-pathogenic and saprophyte anaerobe, would efficiently increase the protein content of cassava products and reduce the level of tannin and cyanide.

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ISOLATION, CHARACTERIZATION AND STUDY OF MICROBIAL ACTIVITIES OF THE BRAIN LIPID AND CHEMICAL ANALYSIS OF THE BRAIN OF BAGHDA CHINGRI (*Penaeus monodon*) OF THE BAY OF BENGAL

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The brain lipid of Baghda chingri (*Penaeus monodon*) was extracted and characterized with respect to various physical and chemical constants and compared with those of standard oils and fats. Thin layer chromatographic (TLC) and gas liquid chromatographic (GLC) investigation of the lipid showed the presence of myristic, palmitic, stearic, oleic, linoleic, arachidic and some other unidentified fatty acids. The microbial activities of the lipid were investigated. Nitrogen, phosphorus, potassium and calcium contents of the total brain containing the lipid were determined.

Key words: Lipid, Chingri, Polyunsaturatted fatty acid (PUFA), TLC, GLC.

Introduction

Bangladesh earns substantial amount of foreign exchange by exporting shrimps prawns and lobster to many countries of the world including USA, UK and Japan. Processing of these is associated withe expulsion of head and brain. Literature survey (Endinkeau and Kiew 1993; Gutierrez and Da Silva 1993; Heyden 1994; van Schacky *et al* 1999, Harper and Jacobson 2001; Bucher *et al* 2002; Holub 2002) shows that fish lipids including brain lipids of shrimps and prawns contain pharmaceutically important and physiologically active ω-3 and ω-6 unsaturated fatty acids, which play an effective role in reducing cardiovascular problems. This phenomenon has attracted investigators to analyze fish lipids of both marine and fresh water origins for polyunsaturated fatty acids (PUFA). Lovern (1953) has found that PUFA are present in high ratios in lipids of marine fish of which acids of ω-3 configuration are predominant Bang and Dyerberg (1975) studied the dietary habits of the Eskimos, based on fish oils containing higher proportion of PUF As dominated by ω-3 fatty acids. Both ω-3 and ω-6 fatty PUFAs have been found to inhibit the biosynthesis of cholesterol in liver (Murray *et al* 1990). These essential fatty acids constitute integral part of nervous tissues in the brain as complex lipid. The exportable marine species from Bangladesh, Baghda chingri (*Penaeus monodon*) is remarkable from demand point of view. The present investigation is concerned with the isolation of the brain lipid of Baghda chingri with a view to find out the PUFAs presence in it and studying its physico-chemical and microbial characteristics including the chemical analysis of brain.

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Experimental

The lipid was extracted from the brain of Baghda chingri by Bligh and Dyer method (Gurr and James 1977) using chloroform: methanol (2:1, v/v). The extract thus obtained was dried, free of solvent first by rotary evaporation and finally by blowing a slow stream of nitrogen gas. The yield was 25%. The refractive index, moisture, crude fat, crude fibre and ash contents of the lipid were determined by standard methods (Ranganna 1991). Saponification value, saponification equivlent value, acid value and percentage of free fatty acid (as oleic), iodine value, acetyl value (Griffin 1972), peroxide value (Morris 1965), thiocyanogen value, Reichert - Meissl value and Polenske value (Ranganna 1991), Henher value, Elaiden test result (Das 1989) and the quantity of unsaponifiable matter (Williams 1966) of the lipid were determined by standard methods.

The fatty acid mixture was prepared from the lipid sample, which was then converted into corresponding methyl esters by proper treatment with methanolic solution of sulphuric acid and purified (Loury 1966 and 1967; Mangold and Kammereck 1961). Thin layer chromatographic separation was made using thin layer of silica gel as the stationary phase and petroleum ether: ether (80:20, v/v) as the mobile phase. The separation of methyl esters depending on R_f values was visualized by spraying with a 0.2% ethanolic solution of 2,7-dichlorofluo-

rescein air drying and inspection under UV - light. Mixture of standard methyl esters of fatty acids was used for comparison and the fatty acid composition of the lipid was identified from R_f values. The methyl esters prepared as before were also analysed by GLC. (Mangold and Kammereck 1961; Loury 1966 and 1967). A portion of the sample was injected into one end of the column of the GLC equipment (PYE-UNICAM PU 4500, Phillips) using a flame ionization detector and a chart recorder. A column (internal diameter 2mm, length 1.5 meter) was filled with 10% diethyl glycol succinate (DEGS) on 100 - 200 (British Std. Sieve) mesh. The injector temperature was 230°C and the detector temperature was 250°C. The temperature of the column was programmed initially at 100°C for 1 min, then allowed to rise to 225°C at a rate of 4°C / min. Nitrogen gas was used as the carrier gas at a flow rate of 11.3 ml / min. Standard methyl esters of caprylic, nonanoic, capric, undecanoic, lauric, myristic, palmitic, stearic, oleic, arachidic and behenic acids (Sigma Chemical Company, USA) were used for identification of the peaks. The fatty acids present in the lipid under investigation were thus identified by comparison of relative retention time and peak position. The percentage of the acids was computer estimated from the GLC peaks.

A portion of the lipid sample was screened for its anti-bacterial activity by disc diffusion method (Bauer *et al* 1966) against four human pathogenic bacteria, viz. *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi and Escherichia coli.* Another portion was screened for its anti - fungal activity by poisoned food technique (Grover and Moore 1962) against three phyto pathogenic fungi, viz. *Macrophomina phaseolina, Alternaria alternata* and *Curvularia lunata*. Nutrient Agar and Potato Dextrose Agar were used as basal medium for anti-bacterial and anti-fungal activity tests respectively. Dimethyl formamide (DMF) was used as a solvent for preparation of lipid solution of desired concentration (1%).

The brain was first sun dried with occasional stirring, and then vacuum oven dried at 40°C. The dried materials were ground in an electric grinder into 60 mesh powder and digested according to the Modified Kjeldahl Method. Percentage of N, P, K and Ca were estimated by standard procedure (Ranganna 1991).

Results and Discussion

The physical and chemical characteristics of the brain lipid investigation may help to evaluate its suitability for a given purpose. Some of the physical and chemical constants are shown in Table 1, for comparison with of standard commercial oils. The refractive index of the lipid was found to be 1. 4736, a quite high value that is an indication of moderate amount of unsaturation in the fatty acid components. The moisture, ash, crude fat and crude fibre contents of the lipid were found to

Table 1

Physical and chemical constants of the brain lipid of Baghda chingri and some related fats and oils (Williams 1966; Lange 1987; Das 1989)

Name of the sample	S.V.	S.E.V	A.V.	F.F.A (%)	I.V.	T .V.	Acetyl Value	U.S.M. (%)	R.M.V.	P.V.	H.V.	R.I.
Olive oil	190-195	287-295	0.6-1.5	0.25-0.60	80-88	75-83	10.04	0.5-1.2	0.6-1.5	0.5	0.6	1.4657 - 1.4667
Sunflower oil	190-194	287-295	0.6-2.4	0.15-0.45	125-140	78.4-81.3	---	0.3-0.9	0.5	---	---	1.4659 - 1.4721
Cotton seed oil	192-198	283-292	1.0-5.0	0.4-0.9	103-111	61-69	0.7-12.2	0.8-1.8	0.95	---	94.2	1.4743 - 1.48
Linseed oil	189-195	287-296	4.0	0.5-0.75	175-200	---	---	1.0-1.5	---	---	94.8	1.479 - 1.480
Soybean oil	190-195	287-295	1.27-1.54	0.35-0.85	129-137	77-85	---	0.7-1.6	0.5-2.5	0.2-1.0	---	1.4723 - 1.4756
Coconut oil	255-260	210-250	2.5-10.0	---	8.2-9.6	6.1-70	---	0.15-0.7	7.0-8.0	15-17	82	1.4530
Palm-kerneloil	248	220-250	----	---	15-18	---	---	---	28	---	94.2	---
Sardine oil	189.8-193.8	---	2.2-21.7	---	138.8-	---	---	---	---	---	---	---
Whale oil	184-200	---	0.3-51.4	---	126.9	---	---	---	---	---	---	---
Brain lipid of Baghda chingri	229.25	244.71	1.11	0.56	95.83	43.63	10.58	0.566	1.04	0.796	95.32	1.4736 at 28°C

S.V, Saponification value; S.E.V, Saponification equivalent value; A.V, Acid value; F.F.A, Free fatty acid; I.V, Iodine value; T.V, Thiocyanogen value; U.S.M, Unsaponifiable matter; R.M.V, Reichert - Meissl value; P.V, Polenske value; H.V, Henher value, R. I, Refractive Index.

be 2.102%, 0.93%, 1.85% and 1.34%, respectively. The comparatively high saponification value and saponification equivalent value, 229.25 and 244.71, respectively, indicate the presence of higher proportion of high molecular weight fatty acid components. The acid value and percentage of free fatty acid (as oleic) were found to be 1.11 and 0.56, respectively. The low values of these characteristics are an indication of the suitability of the lipid for edible purpose. The iodine value of 95.83 indicates that the lipid contains moderate proportion of unsaturated fatty acid components and is of semidrying type, also confirmed by the Elaiden test. The peroxide value of 194.95 and thiocyanogen value of 43.63 also indicates moderate content of unsaturated fatty acid components. The acetyl value of 10.58 is an indication of low content of free hydroxyl groups in the lipid. The low value of unsaponifiable matter 0.566% (w/w) indicates that the lipid contains a small amount of unsaponifiable sterols, tocopherols, vitamins A and D, hydrocarbons and so on. The Reissert-Meissl and Polenske value, 1.04 and 0.796, respectively are an indication of low content of both volatile water soluble and volatile water insoluble but alcohol soluble fatty acid components in the lipid sample. The higher Henher value of 95.32% is an indication of high percentage of water insoluble nonvolatile fatty acid components present in the lipid.

A study of the effect of storage time showed an increase of acid value and peroxide value and a decrease of R-M value, thiocyanogen value and iodine value with time. That means, the quality of the lipid deteriorates with storage time.

Thin layer chromatographic (TLC) analysis showed the presence of myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), arachidic acid ($C_{20:0}$), arachidonic acid ($C_{20:1}$) and erucic acid ($C_{22:1}$), well-separated in petroleum ether: ether (80:20) solvent system. The chromatogram showed several other spots, which could not be identified due to non - availability of suitable standards in the laboratory. These unidentified acids may be some PUFAs such as eicosapentaenoic acid ($C_{20:5}$), docosahexaenoic acid ($C_{22:6}$) etc. which are available in marine plants, phytoplankton, zooplankton, algae etc. (Beare 1962; Dyerberg 1986) on which the Baghda chingri lives on.

Qualitative and quantitative information about myristic acid, palmitic acid, stearic acid, oleic acid and arachidic acid present in the lipid has been obtained from GLC (Table 2). These acids comprised of about 33% of the total acids present in the lipid as calculated from the area of the peaks in the chromatograph. The chromatograph showed several other peaks, which could not be identified due to non - availability of suitable standards in the laboratory. However, TLC analysis in a different laboratory (conducted by the same authors) showed the presence of linoleic, linolenic, arachidonic, erucic acids in addition to the acids identified and quantified by GLC.

It is evident from Table 3 that the lipid sample has positive activity against all the test pathogenic bacteria. Maximum inhibition was found in the case of *Staphylococus aureus* (28 mm) and minimum in *Bacillus subtilis* (12 mm), while oil soaked paper discs were used. *Staphylococcus aureus* and *Salmonella typhi* were found sensitive towards the lipid sample at 0.05 ml / disc and 0.1 ml / disc while *Bacillus subtilis* and *Escherichia coli* showed very low or no inhibitory activity. It is evident from Table 4 that the mycelial growth of all test fungi was stimulated by the lipid sample. It is hoped that this work employing the lipid sample as chemical test will help the development of pesticides and medicines for human diseases.

The brain of Baghda chingri was found to contain a good amount of nitrogen (3.54) as well as protein (Proteineous nitrogen), which is well-balanced in respect of essential amino acids. The percentage of phosphorus, 0.5506 indicates that phospholipid may be presented in the lipid, which was extracted from the brain. The percentage of potassium was found to be 1.123. Calcium content of brain, 0.914% may help in the formation of rigid bone structure of the community children in their growing age who eat these Baghda chingri (Table 5).

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Table 2

Fatty acids as obtained by GLC analysis of methylated brain lipid of Baghda chingri

Peak number of acids identified by GLC	Name of the fatty acids	Inference	Retention time, RT	Area	Relative area, %
1	Myristic acid	$C_{14:0}$	16.52	8722	3.748
2	Palmitic acid	$C_{16:0}$	21.83	31280	13.443
3	Stearic acid	$C_{18:0}$	26.07	18483	7.943
4	Oleic acid	$C_{18:1}$	26.70	16291	7.001
5	Arachidic acid	$C_{20:0}$	31.21	1572	0.676

Table 3
Anti-bacteria screening data of the brain lipid of Baghda chingri

Name of the bacteria	Diameter of inibition zone in mm		
	Oil soaked disc	0.05 ml / disc	0.1 ml / disc
Staphylococcus aureus	28	23	25
Bacillus subtilis	12	-	-
Salmonella typhi	26	20	22
Escherichia coli	13	-	-

'--' means no inhibition

Table 4
Anti-fungal screening data of the brain lipid of Baghda chingri

Name of the fungi	% Inhibition after 5 days
Macrophomina phaseolina	- 2.09
Curvularia huna ta	- 20.229
Alternaria alternata	- 1.1730

-ve sign indicates the stimulation of test fungi

Table 5
Percentage of nutrient elements in the brain of Baghda Chingri

Name of the sample	N	P	K	Ca
Brain of Baghda Chingri	3.54	0.5506	1.123	0.914

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Response of a Cotton Cultivar to Sulphur Fertilization

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Field experiments on sulphur fertilization in cotton cultivar CIM-109 were carried out at Central Cotton Research Institute Multan, Pakistan, in silty loam soils. The treatments consisted of four sulphur doses (0, 7, 14, 28 kg ha^{-1}) and two sulphur sources (gypsum and ammonium sulphate). Sulphur fertilization showed significant increase in seed cotton yield, boll number and boll weight. The addition of 7 kg S ha^{-1} seemed sufficient to overcome deficiency in silty loam soils for optimum cotton production. There were no differences in seed cotton yield due to sulphur sources. The petiole sulphate-sulphur concentration increased with increasing doses of sulphur fertilizer. The concentration of SO_4–S > 2000 ppm seemed sufficient for normal cotton growth and optimum seed cotton yield.

***Key words*:** Sulphur fertilization, Fruit production, Petiole NO_3–N, Petiole SO_4–S, Fibre quality, Seed cotton yield.

Indroduction

Sulphur deficiency is increasing due to the adoptation of high yielding varieties, intensive cultivation and use of high analysis sulphur free fertilizer throughout the world. The increased occurrence of sulphur deficiency is attributed to the exhaustive cultivation of oilseed crops. In general, soils in the Punjab have enough sulphur to cater for plant needs, but 25% of cultivated area has SO_4–S less than 10 mg kg^{-1} of soil, which is critical threshold for most crops (Ahmad *et al* 1992).

Total sulphur requirements of cotton may approach the level of phosphorus. Cotton absorbed 12-15 kg ha^{-1} of sulphur and for adequate nutrition 0.2% SO_4–S was desired in cotton petioles and leaves during mid-season (Mitchell and Baker 1997). Cotton requires a continuous supply of external sulphur for normal growth. Proteolysis hardly occurs during sulphur starvation of cotton plant (Mengel and Kirkby 1978). Experiments conducted in Malawi (Mathews 1972) and elsewhere (Makhdum *et al* 2001) showed significant increase in seed cotton yield due to sulphur fertilization. A sulphur dose of 22 kg ha^{-1} gave higher seed cotton yield than control. Cotton growers in South Carolina invariably add 11 kg ha^{-1} of sulphur to obtain maximum cotton yield (Messick 1992).

Fertilizers which contain sulphur as a combining ingredient are being replaced with high analysis fertilizer that are low in sulphur. Crop intensity has increased and determined cotton cultivars which develop nutrient demands more rapidly are common in culture. Taking into account these factors sulphur deficiency is likely to occur in context of prevailing cropping systems. Experiments, therefore, were conducted to test cotton response to sulphur fertilization on its growth yield and fibre qualities.

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Materials and Methods

Experiments were conducted on silty loam soils for two seasons 1998-2000 at Central Cotton Research Institute, Multan, Pakistan. Soil samples were collected before sowing from the plough layer of experimental sites and analysis carried out as per methods described by Klute (1986) and Page *et al* (1982). These soils have pH of 8.37, organic matter 0.4% $NaHCO_3$ extracted phosphorus 14 mg kg^{-1} of soil and NH_4OAc extracted potassium 220 mg kg^{-1} of soil.

Sulphur from soil was extracted using 0.001M $CaCl_2 \cdot 2H_2O$ solution as an extraction agent. Plant leaf samples were washed with distilled water, dried at 70°C, ground and digested in acid mixture (HNO_3 and $HClO_4$). Analyses were conducted by the turbidimetric method with $BaCl_2.2H_2O$; readings were taken using a spectrophotometer at 470 mm (Verma *et al* 1977). Sulphate values in experimental plots ranged from 11 to 13 mg kg^{-1} of soil at the time of planting cotton.

Cotton cultivar CIM-109 was planted in early June at a spacing of 75 cm between rows and 30 cm between plants. The layout of experiment was randomized complete block design with four repeats. The area of each plot was 105 m^2. Sulphur was applied in the form of gypsum and its doses consisted o 0, 7, 14 and 28 kg S ha^{-1}, all broadcasted and incorporated in the soil at the time of seed bed preparation. An additional treatment of ammonium sulphate was included for gypsum comparison and its dose consisted of 28 kg S ha^{-1}. All experimental units received nitrogen and phosphorus. Nitrogen was

Table 1
Effect of sulphur fertilization on development of plant shape (mean of 2 seasons)

Treatments		Plant height (cm)	Number of nodes on main stem	Inter-nodal length (cm)
Sulphur (kg ha^{-1})	Source			
00	--	102	35	2.94
07	Gypsum	107	36	3.01
14	-do-	109	36	3.04
28	-do-	111	36	3.09
28	Ammonium	114	37	3.14
LSD ($p < 0.05$)		4.26*	1.11**	0.16^{ns}

ns, non significant at 0.05 levels; **, highly significant at 0.01 level.

Table 2
Effect of sulphur fertilization on fruit production (mean of 2 seasons)

Treatments		Fruiting positions/ m^2	Intact fruit/ m^2	Fruit shedding (%)
Sulphur (kg ha^{-1})	Source			
00	--	516	121	76.30
07	Gypsum	534	133	75.30
14	-do-	535	133	75.10
28	-do-	540	134	75.00
28	Ammonium	539	133	75.10
LSD ($p < 0.05$)		20.75*	7.09**	1.25^{ns}

ns, non significant at 0.05 levels; **, highly significant at 0.01 level.

applied in the form of urea at the rate of 150 kg N ha^{-1} and phosphorus in the form of diammonium phosphate at the rate of 50 kg P_2O_5 ha^{-1}. Crop production practices were the standard ones of the cotton growing area.

Five consecutive plants in each plot were harvested at maturity and measurements were taken on plant height, fruiting positions and intact fruit. Seed cotton yield and its components were determined by harvesting whole plot and calculations made on area basis. Data obtained were subjected to statistical analysis as per methods described by Gomez and Gomez (1984).

Results and Discussion

Plant height is a good measure of overall growth and development. Field application of sulphur showed significant increase in cotton plant height and its node number (Table 1). Internodal length also increased, but to a non-significant level. These data demonstrate that role of sulphur seemed to be more pronounced in terms of node differentiation than that of elongation of internodal lengths in cotton plant.

Cotton plant requires continuous supply of sulphur for its normal growth. Sulphur deficiency causes accumulation of nitrates and organic nitrogen compounds in cotton plant (Mengel and Kirkby 1978). The slow utilization of nitrogen compounds is likely to reduce plant size in sulphur deficient soils. Sulphur deficient cotton plants, in general, were short and showed abnormal growth and development. Mitchell *et al* (1992) stated that plants deprived of sulphur showed some visible effects which resemble those caused by nitrogen deficiency. The plants show marked decrease in growth and canopy size besides being abnormal in appearance.

Fruiting positions provide indication of potential yield whereas, intact fruits represent actual yield. Data provided in Table 2 indicate significant differences in fruiting positions and intact fruit due to sulphur fertilization. However, there were no differences in fruit shedding. Sulphur fertilization increased plant

Table 3
Effect of sulphur fertilization on seed cotton yield and other characters (mean of 2 seasons)

Treatments		Seed cotton yield (kg ha^{-1})	Number of bolls per plant	Boll weight (g)	Lint (%)
Sulphur (kg ha^{-1})	Source				
00	--	2095	22	2.57	35.50
07	Gypsum	2242	24	2.60	35.50
14	-do-	2255	24	2.62	35.60
28	-do-	2255	24	2.62	35.60
28	Ammonium sulphate	2257	24	2.63	35.60
LSD ($p < 0.05$)		31.37**	0.51**	0.03**	0.48^{ns}

ns, non significant at 0.05 levels; **, highly significant at 0.01 level.

Table 4
Effect of sulphur fertilization on fibre characteristics (mean of 2 seasons)

Treatments		Fibre length (mm)	Fibre uniformity ratio (%)	Fibre fineness (µg inch^{-1})	Fibre strength (000 lbs inch^{-2})
Sulphur (kg ha^{-1})	Source				
00	--	25.60	46.20	4.60	92.50
07	Gypsum	25.80	46.40	4.50	93.10
14	-do-	25.50	46.40	4.60	93.60
28	-do-	25.70	46.20	4.50	93.10
28	Ammonium sulphate	25.80	46.70	4.50	93.70
LSD ($p < 0.05$)		0.45^{ns}	0.88^{ns}	0.23^{ns}	1.70^{ns}

ns, non significant at 0.05 levels.

growth and this led to more fruiting positions and intact fruit. Sulphur fertilization per season did not significantly reduce fruit shedding. Cotton plant has in built capacity to maintain vegetative-reproductive balance called relative fruitfulness (Guinn 1998). Cotton plant due to nutrient deficiency tends to curtail vegetative expansion and thus reduces overall plant size and boll load. This phenomenon is fully exhibited from data on plant size and fruit load obtained in control and sulphur receiving treatments.

The benefit of vigorous plant growth and higher number of intact fruit was reflected in seed cotton yield. Sulphur fertilization caused significant increase in seed cotton yield, boll number and boll weight (Table 3).

Increase in yield occurred with the first level of sulphur application (7 kg ha^{-1}) and no further increase occurred at the second and third sulphur level. Increase in yield was similar irrespective of fertilizer sources viz gypsum and ammonium sulphate. Results obtained in this experiment fully demonstrate sulphur need for cotton crop in soils having SO_4–S in the range of 11-18 mg kg^{-1} of soils. More increase in yield due to sulphur fertilization has been obtained in several cotton growing areas of the world (Matthews 1972; Mascagni *et al* 1991; Tandon 1995; Makhdum *et al* 2001).

Lint samples analyzed for quality showed a little variation due to sulphur fertilization (Table 4).

The reason being that genetic and climatic factors exert so much influence on fibre quality that a little direct effect from sulphur can be elucidated (Mullins 1996; Makhdum *et al* 2001).

Sulphate-sulphur concentration in leaf petioles collected during mid-season increased with sulphur addition in soil (Table 5). The control plots showed a level of 1355 ppm SO_4–S, which increased to 3089 ppm with increased sulphur fertilization.

Table 5
Effect of sulphur fertilization on SO_4–S concentration in leaf petioles

Treatments		Leaf petiole		
Sulphur (kg ha^{-1})	Source	NO_3–N ppm	SO_4–S ppm	NO_3–N/ SO_4–S
00	--	15582	1355	11.5
07	Gypsum	12700	2045	6.20
14	-do-	128520	2567	5.00
28	-do-	12550	3089	4.00
28	Ammonium sulphate	12570	3120	4.00

The concentration of 1355 ppm SO_4–S in petioles of control plot confirmed sulphur deficiency, since 2000 ppm value is considered as a critical level in cotton leaves (Mascagni *et al* 1991). Furthermore, the ratio of NO_3–N to SO_4–S was wide engough to indicate sulphur deficiency problem. A ratio of 10 or more is thought to represent sulphur deficiency (Mitchell and Baker 1998).

Conclusions

Cotton yield increased due to application of sulphur at the rate of 7 kg ha^{-1} in soils having alfkaline pH and low organic matter. The critical level of SO_4–S was 11 mg kg^{-1} of soil for the purpose of soil fertilization. Plant analysis indicated 2045 ppm SO_4–S in cotton leaf tissues for harvesting good yield.

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ANTIULCER EFFECT OF *ARTEMISIA ABSINTHIUM* L. IN RATS

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The extracts of *Artemisia absinthium* induced a significant decrease in volume of gastric juice, acid output and peptic activity but no effect was determined on mucin activity in acetylsalicylic acid (ASA) ulcerated rats. Moreover, they decreased the ulcer index significantly. Phytochemical analysis indicated the presence of saponins and glycosidic sugars in the extract.

Key words**:** *Artemisia absinthium*, Antiulcer activity, Saponins.

Introduction

Plants and plant products have served mankind as medicines since ancient times. The use of herbal medicines and herbal products constitute a large portion of consumers choice and continue to rise in popularity all over the world (Sanyal *et al* 1964, 1965, 1971, 1982; Elliot and Heward 1976; Al Habbal *et al* 1984; Blum 1985; Geol *et al* 1985a, b & c, 1986; Lorincz 1994; Shalita 1995; Priest 1995; Rowe 1998). *Artemisia absinthium* L., a member of the Asteraceae family, has long been used in traditional system of medicine in Pakistan and it is reported to possess well-marked antipyretic and tonic properties (Ikram *et al* 1987). Its oil is produced commercially and used as a tonic. It has stimulating effect on the digestive organs (Manjunath 1948). Keeping in view, the folkloric use of plant species in the indigenous system of herbal medicines, different semi pure extract were tested for their antiulcer effects on acetylsalicylic acid (ASA) induced ulcers in rats. In addition the effects on volume of gastric juice, acid output, peptic activity and mucin activity were also studied to evaluate the action of these extracts.

Materials and Methods

Artemisia absinthium L. was collected in July, identified and authenticated from the herbarium specimens of Peshawar Laboratories. Voucher specimens were preserved and catalogued in the said herbarium. The plant material was shade dried, powdered and stored carefully. All the solvents and chemicals used were of analytical grade. Acetylsalicylic acid was purchased from the local market. For TLC, precoated silica gel, G 60, F 254 plates (0.2mm thick, Merck) were used.

Animals. Sprague-Dawley albino rats of either sex, weighing 200 ± 4g and housed under standard conditions, were used.

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Preparation of extracts and fractionation. The air dried powdered plant material (2.0 kg) was cold percolated with 95% ethanol (three times). The combined alcoholic extracts were concentrated under reduced pressure. The crude extract thus obtained was defatted with hexane. The defatted material was then extracted successively with chloroform and carbon tetrachloride. The fraction finally obtained (in 3.75% yield) was dissolved in methanol and then passed through a charcoal - celite column to remove the coloring matter. Thin layer chromatography (TLC) of the mixture was carried out in the solvent systems ethylacetate and formic acid (1.0:0.1), chloroform, methanol and water mixture in the ratios of (65:35:10; 70:30:10 and 70:30:5) which showed the presence of six components. For visualizing the spots, the detection reagents used were $SbC1_3$ in $CHC1_3$ (15%) and anisaldehyde (0.5 ml) +EtOH (9 ml)+ H_2SO_4 (0.5 ml), followed by heating at 60 -70°C for 5 min. The crude saponin mixture was separated into five purified / semipurified fractions by chromatography on silica gel, eluting with chloroform and chloroform - methanol mixtures. Solvents were removed *in vacuo* and each of the eluent was examined for its antistress activity.

Phytochemical screening. Tests for the presence of alkaloids, glycosidic sugars, free sugars, anthraquinones and saponins in each eluent were carried out by reported standard methods (Siddiqui and Ali 1997).

ASA - induced gastric ulceration. This assay was conducted in accordance with the modified method of Geol *et al* (1985). The animals were divided into three test groups, each group contained six animals, while the untreated control group contained 10 animals. The control (ulcerated) group was given oral dose of aqueous suspension of ASA (200 mg / kg) in 1% carboxymethylcellulose (CMC). Aqueous extracts of *Artemisia absinthium* were administered orally at the dose of 5 mg / kg 3 h prior and 3 h after ASA treatment for three days. The

Table 1

Effect of different fractions (Fraction 1-5) of *Artemisia absinthium* L. on volume gastric juice secreted, acid output and peptic activity of gastric juice in untreated control and stressulcerous rats (20 h cold stressing)

S.No.	Treatment	Number	Volume of gastric juice (ml)	Acid output (mmol / h)	Peptic activity velocity constant / ml of the gastric juice / min
1.	Control Propylene Glycol 5 mg / kg i / p	10	21.00 ± 3.50	0.90 ± 0.03	0.080 ± 0.002
2.	Fraction 1 5 mg / kg in PG i / p	6	15.00* ± 3.00	0.55** ± 0.16	0.076 ± 0.004
3.	Fraction 2 5 mg / kg in PG i / p	6	13.00* ± 2.00	0.60* ± 2.00	0.040** ± 0.004
4.	Fraction 3 5 mg / kg in PG i / p	6	14.00* ± 3.00	0.70 ± 0.01	0.060 ± 0.004
5.	Fraction 4 5 mg / kg in PG i / p	6	17.50 ± 0.78	0.89 ± 0.02	0.094 ± 0.007
6.	Fraction 5 5 mg / kg in PG i / p	6	15.80 ± 1.42	0.90 ± 0.02	0.120 ± 0.030

Levels of significance compared to control *P< 0.05; **P< 0.005; PG = Propylene Glycol; i/p, intraperitoneally.

effects on healthy rats were also evaluated with 1% CMC (10 ml / kg) only. The animals were operated on the fourth day in accordance with the method of Shay *et al* (1945). The feed was withheld 18 h prior to surgery. The pylorus was ligated and gastric juice was collected for a period of 4 h. The animals were then killed and stomach was removed by clam-ping the oesophagus. The gastric juice was collected and centrifuged (5000 rpm, 5 min). The supernatant liquid was col-lected in a graduated cylinder and volume calculated (ml / 100 g b.w.). The stomachs were then inflated with 1.00% formalin (10 ml; 10 min). The average number of ulcers per stomach were recorded and percent inhibition of ulcer formation cal-culated (Okaba *et al* 1978).

Acid output. Acid output was determined by titration of volume gastric juice secreted with 0.01 N NaOH using phenolphthalein as an indicator (Oser 1965).

Peptic activity. Peptic activity was measured by the modified method of Rigges and Stadie (1933) with some modifications, as 50 ml of distilled water was used instead of 10 ml. Mercuric chloride was used as a preservative in place of thiomersalate.

Mucin activity. The ratio of total carbohydrates to total protein was calculated as an index of mucin activity. Fucose was determined by the method of Dische and Shettles (1948). Protein bound hexose was quantified by slight modification of Lusting and Langer procedure (1931). Hexosamine was estimated in accordance with the procedure of Elson and Morgan (1933). Gastric juice was heated with hydrochloric acid in boiling water bath (3 N; 16 h). Sialic acid was measured by the method of Ayala *et al* (1951). Whereas, total protein was determined in accordance with the procedure of Winzler *et al* (1948).

Statistical analysis. Significance of the values obtained was evaluated by Student's t-test.

Results and Discussion

The phytochemical studies were undertaken to evaluate the antiulcerogenic effects of purified / semipurified extracts of *A. absinthium* L. Phytochemical analysis showed the absence of alkaloids and anthraquinones but indicated the presence of glycosidic sugars and saponins, which showed the characteristic saponic properties in aqueous alcoholic extracts such as foaming, toxicity towards fish and hemolytic activity. The Liebermann-Burchard test was also used as a color test which is adopted in the Japanese Pharmacopea as identification method for crude drugs containing saponins e.g. Platycodi Radix, *Anemarrhenae rhizoma*. (Sinsaku *et al* 1981). The ASA induced ulcerated group treated with different fractions of *Artemisia absinthium* L. has shown a significant decrease in the volumes of gastric juice, decrease in acid output and peptic activity (Table 1). Peptic activity was measured according

Table 2

Effect of different fractions (Fraction 1-5) of *Artemisia absinthium* L. on percent of ulcer incidence, Average No. of ulcer per stomach, ulcer index and percent inhibition of ulcer formation in stress ulcerated rats (20 h cold stressing at 23°C)

S.No.	Treatment	Number	Ulcer present	Absent	Ulcer incidence (%)	Average No. of ulcer per stomach	Ulcer index	Inhibition (%)
1.	Control Propylene Glycol 5 mg / kg i / p	10	10	0	100	7.00 ± 0.05	18.00 ± 6.00	-
2.	Fraction 1 5 mg / kg in PG i / p	6	5	1	83	3.00** ± 1.00	8.00* ± 4.00	65
3.	Fraction 2 5 mg / kg in PG i / p	6	5	1	83	5.00 ± 0.77	10.00* ± 4.00	44
4.	Fraction 3 5 mg / kg in PG i / p	6	5	1	83	5.50 ± 0.90	12.00 ± 5.00	33
5.	Fraction 4 5 mg / kg in PG i / p	6	5	1	83	2.60 ± 0.76	15.26 ± 2.60	11
6.	Fraction 5 5 mg / kg in PG i / p	6	5	1	83	3.50 ± 1.00	13.00 ± 4.00	27

Levels of significance compared to control *P< 0.05; **P< 0.005 vs ASA Control, Studen's t-test.

Table 3

Effect of *Artemisia absinthium* L. extracts on mucin activity of gastric juice in untreated control and ASA treated, pylorus ligated rats

S.No.	Treatment (g / kg x 3 days P.O.)	N	Carbohydrates (µg / ml)					Total Protein (µg / ml)	Total Carbohydrates / Total Protein
			Total hexoses	Hexosamine	Fucose	Sialic acid	Total		
1.	Control	10	1312 ± 104	520 ± 72	180 ± 95	159 ± 20	2236 ± 193	2485 ± 188	0.90 ± 0.06
2.	Fraction 1 (2.0 b.i.d.)	8	1298 ± 132	250* ± 2.00	285* ± 21	158 ± 42	2399 ± 26	6000* ± 900	0.41* ± 0.075
3.	ASA (0.2 o.i.d.)	8	813* ± 69	438 ± 52.00	29 ± 5.0	164 ± 15	1443 ± 93	2000 ± 470	0.75 ± 0.090
4.	Fraction 2 (2.0 b.i.d)	6	900 ± 30	250 ± 12.00	200** ± 50	170 ± 80	1520 ± 80	4000* ± 430	0.38 ± 0.020

Results are means ± S.E. *P<0.05 vs untreated control **P<0.001 vs ASA control: Student's t-test; o.i.d = once daily; b.i.d = twice daily.

to the method of Rigges and Stadie (1933) with some modifications, as 50 ml of distilled water was used instead of 10 ml. Mercuric chloride was used as a preservative in place of thiomersalate. Significant effects have been observed in ulcer activity. The 65.00% reduction in ulcer index with fraction-I and 44.00% with fraction - II has been observed (Table 2). A qualitative change in the contents of carbohydrates (hexose and fucose) has been observed (Table 3). The extracts caused a decrease in acid and pepsin output and a qualitative change in hexose and fucose contents of carbohydrates in ASA-ulcerated rats, although the drug did not exert a quantitative change in the dissolved mucin contents of the gastric juice. The effects on swimming performance of rats have been observed (Table 4). Significant increase in the activity has been

Table 4
Effect of *Artemisia absinthium* L. fractions on swimming performance of rats

S.No.	Group	Drug treatment	N	Mean duration of swimming (Seconds ± S.E.)
1.	Control	Propylene Glycol 5 mg / kg	6	270 ± 11
2.	Fraction 1	5 mg / kg in PG i/p	5	363* ± 68
3.	Fraction 2	5 mg / kg in PG i / p	6	257 ± 23
4.	Fraction 3	5 mg / kg in PG i / p	6	255 ± 15
5.	Fraction 4	5 mg / kg in PG i / p	6	270 ± 36
6.	Fraction 5	5 mg / kg in PG i / p	5	240 ± 12

Note: *Levels of significance compared to control P<0.05 P.G. = Propylene Glycol, i/p = intraperitoneally.

observed in all fractions, especially with fraction - I and fraction - IV as compared to the controlled conditions.

LD_{50} values were not recorded as the extracts had no lethal effects upto 10 mg / kg and mortality has not been observed in the experimental animals. Therefore, it is concluded that different crude extracts of *Artemisia absinthium* have displayed significant antiulcer effects, decrease in volume of gastric juice and acid output. Injurious or toxic effects were not detected.

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AN ECO - FRIENDLY APPROACH FOR THE MANAGEMENT OF NEMATODES ASSOCIATED WITH CHILLI

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This investigation focuses on the effects of three organic amendments namely Fertinemakil and sugarcane bagasse alone or in combination on the population density of three nematode species and yield of chilli. For the purpose of comparison, a chemical nematicide carbofuran was also used. Population densities of *Helicotylenchus indicus*, *Tylenchorhynchus curvus* and *Meloidogyne* spp. (J2) were markedly reduced by the treatments. Yield of chilli was increased significantly over the control by all treatments except sugarcane bagasse alone. However, highest yield was obtained in carbofuran treatment.

***Key words*:** Nematodes, Fertinemakil, Sugarcane bagasse, Control, Chilli, *Capsicum annuum*.

Introduction

Chilli (*Capsicum annuum* L.) a cash crop grown in all the four provinces of Pakistan is affected by a number of diseases caused by fungi, bacteria, virus and nematodes.

Yield loss can be substantial in fields heavily infested with nematodes and in warmer region damage can be amplified by secondary pathogens.

Nematicides that are being used for the control of plant parasitic nematodes are costly and can cause environmental pollution. Organic amendments such as plant materials are safe to use and also effective against plant nematodes (Akhtar and Yadav 1990; Gupta and Kumar 1997; Ramakrishnan *et al* 1997; Khan *et al* 2001; Shaukat and Siddiqui 2001). Therefore, efforts are being made to study different organic amendments for the control of nematodes associated with various crops. Recently, Khan *et al* (2001) reported the efficacy of three organic amendments including pigeon manure, poultry manure and saw dust on the populations of *Tylenchorhynchus curvus*, *Helicotylenchus indicus* and *Meloidogyne* spp.

Khan and Shaukat (2001) studied effect of castor, neem and mustard oil - cakes on nematodes associated with chilli namely *Helicotylenchus indicus*, *Pratylenchus thornei* and *Meloidogyne* spp. Population densities of the nematodes were effected by the organic amendments to varied extent. Comparatively, castor oil - cake caused greatest reduction in nematode population. The present investigation deals with the nematicidal effect of Fertinemakil (a pesticide containing neem cake and a fungicide produced by PCSIR Laboratories Complex, Karachi), sugarcane bagasse alone or in combination for the control of nematodes associated with chilli. For comparison a systematic nematicide Carbofuran was used.

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Materials and Methods

The experiment was conducted in a field of Crop Diseases Research Institute, PARC, University of Karachi, Karachi.

This experiment was conducted using a randomized complete block design with four replications. The microplot size was 1 m^2. The initial populations of *Helicotylenchus indicus*, *Tylenchorhynchus curvus* and *Meloidogyne* spp. (J2) larvae were 140, 76 and 18/100 ml soil, respectively. These three species constituted 90.0% of the total nematode population. The soil was tilled three times within a month so as to facilitate the release of any residual effect prior to treatments. Six weeks old seedlings were transplanted in the third week of March 2001 in microplots. The treatments applied in this study were: Fertinemakil 800 kg/ha, sugarcane bagasse 800 kg/ha, Carbofuran 10 kg/ha, Fertinemakil + sugarcane bagasse and control. All treatments were applied in accordance with the recommendations of Plant Protection, Pakistan. The harvest of chilli was done from June to September 2001. Initial nematode population was determined one week before treatment and final at the time of last harvest in accordance with Cobb's (1918) method using a composite root and soil sample from each microplot.

Table 1
Effect of nematicidal treatments on dynamics of *Helicotylenchus indicus*, *Tylenchorhynchus curvus* and *Meloidogyne* spp. associated with chilli

Treatment	Nematodes / 100 ml soil			% of nematodes with respect to initial population density		
	H. indicus	*T. curvus*	*Meloidogyne* spp.	*H. indicus*	*T. curvus*	*Meloidogyne* spp.
Fertinemakil (FE)	36.5	18.0	9.0	26.1	23.7	50.0
Sugarcane bagasse (SB)	99.5	56.7	11.2	71.7	74.6	62.5
Fertinemakil + SB	12.2	24.0	4.7	8.7	31.6	26.1
Carbofuran	10.0	26.5	2.2	7.1	34.9	12.5
Control	99.0	45.5	20.0	70.7	59.9	111.1
Initial population density	140.0	76.0	18.0	-	-	-
LDS P = 0.01	V 7.3	V 6.6	V 5.4	V 6.2	V 6.7	V 9.2
P = 0.05	V 10.2	V 9.3	V 7.6	V 8.7	V 9.4	V 10.8

The data were analysed using two - way analysis of variance (ANOVA) followed by least significant difference (LSD) and Duncan's multiple range test (Zar 1994). Some specimens of *Helicotylenchus indicus*, *Tylenchorhynchus curvus* and *Meloidogyne* spp. (J2) second stage juveniles isolated from this study have been deposited in the Nematode Laboratory, USDA, Beltsville, Maryland, USA.

Results and Discussion

Yield was significantly ($p < 0.001$) increased over the non-treated plots by all treatments except sugarcane bagasse when applied alone. Highest yield of chilli was obtained in plots treated with carbofuran treatment (Fig 1). Fertinemakil in combination with sugarcane bagasse also gave considerably higher yield.

Final population densities of the nematodes were also significantly affected by treatments. The population density of *Helicotylenchus indicus* was drastically reduced by Carbofuran and Fertinemakil in combination with sugarcane bagasse (Table 1).

The population density of *T. curvus* was also greatly reduced by Fertinemakil and Carbofuran.

Meloidogyne spp. second stage juveniles density was decreased by Carbofuran followed by Fertinemakil + sugarcane bagasse and Fertinemakil. The reduction of population density of nematodes by using application of Fertinemakil was similar to that observed on nematodes associated with onion and rice (Khan *et al* 2000a & b). Organic amendment, sugarcane bagasse was suggested for nematode control as it has been effective in controlling nematode population (Khan *et al* 1997; Khan and Shaukat 2000), thereby, improving yield and at the same time its availability is easy. This effective control in population could be due to biochemical composition of nematicidal products released by Fertinemakil in soil.

From the present study it can be inferred that Fertinemakil along with soil amendments not only controls nematode population but also improves the yield.

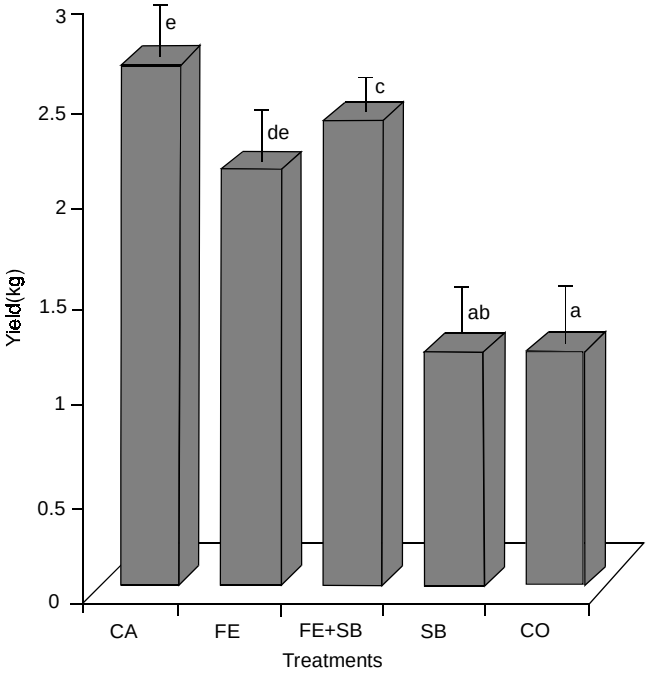

Fig 1. Yield of chilli in different nematicidal treatments (CA = Carbofuran, FE = Fertinemakil, SB = Sugarcane bagasse, CO = Control). Error bars indicate + 1 standard error of the means. Treatments and control not sharing a common letter on the bars are significantly different at P = 0.05 (Duncan's multiple range test).

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Pak. J. Sci. Ind. Res. 2004 ***47*** *(2) 138 - 141*

MICROBIAL SIDE - CHAIN DEGRADATION OF PROGESTERONE II. APPLICATION OF DIFFERENT TECHNIQUES FOR PROGESTERONE CONVERSION BY *FUSARIUM DIMERIUM*

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A local isolate of *Fusarium dimerium,* firstly reported to be able to degrade progesterone side chain to give certain C-19 androgenes derivatives, proved to perform this important conversion more efficiently adopting the cell immobilization technique. Thus, relatively more amounts of Androst - 4 - ene - 3, 17 - dione (AD), Testololactone (TL) (major products) as well as, Testosterone (T), Androsta - 1,4 - diene 3, 17 - dione (ADD) (minor products) were recorded after 72 h using 2% Ca - alginate immobilized fungal cells. Relatively lower bioconversion rates were achieved when the bioconversion process was carried out in top - laboratory fermentor using free cells of the promising fungus.

Key words: *Fusarium dimerium,* Progesterone, Bioconversion.

Introduction

Microbial side - chain degradation of steroids has become an important transformation process for the production of 17 - ketosteroid intermediates, such as androsta - 1, 4 - diene - 3, 17 -dione (ADD) and androst - 4 - ene - 3, 17 dione (AD) (Imada *et al* 1981). Much attention has been focussed upon the transformation of steroids by immobilized microbial cell (Atrat 1982; Kolot 1982; Koshcheyenko *et al* 1983; Rehm and Omar 1993; Gemeiner *et al* 1994; Manosroi *et al* 1999).

Immobilization of whole microbial cells has gained considerable interest, mainly due to the possible industrial applications.

The utilization of immobilized cells as industrial catalysts could also be advantageous when compared to traditional fermentation procedures for several reasons:

1) Immobilized microorganisms permit easy separation of the products.
2) They are reusable and suitable for continuous or repeated batch operation allowing better process control.
3) Immobilized cells and in particular, entrapped ones are less susceptible to microbial attack.
4) Pollution effects e.g. the problem of disposal of spent cell mass, are less pronounced with immobilized cells.

Due to these facts the present investigation was mainly designed to evaluate the efficiency of applying the immobilization techniques in the bioconversion of progesterone into C - 19 derivatives by *F. dimerium.* Moreover, the utilization of the top - lab fermentor in the bioconversion process was also carried out.

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Materials and Methods

Microorganism and culture conditions. Fusarium dimerium used as an experimental organism in this study was obtained from the centre of Plant Pathology Department, National Research Centre (NRC), Cairo, Egypt. The fungus was maintained on a medium composed of (g / l) glucose, 40; peptone, 1; yeast extract, 1 $MgSO_4.7H_2O$, 1; KH_2PO_4, 0.7; L - asparagine, 0.70; agar, 20 (Kinawy 1974). The same composition (except agar) was used as a liquid fermentation medium.

Immobilization technique. F. dimerium cells were entrapped in 2% Ca alginate as described by El - Diwany *et al* (1992), then standard weight of 2% Ca - alginate pellets was suspended in 50 ml 0.05 M tris HCI buffer (pH7) containing 0.7% NaCI and 0.02% Tween 80, in a 250 ml Erlenmeyer flasks. Progesterone (5 mg / 50 ml buffer) was then added as ethanolic solution and the flasks were agitated on a reciprocal shaker (150 rpm) at 30°C for certain time.

Utilization of free fungal cells. The bench top chemostat C_{32} "Bioflo" standard 2 litres vessel was used. One litre of the fermentation medium (Adham *et al* 2002) was sterilized, then inoculated with 20 ml of the standard fungal suspension. The fermentation was conducted at 30°C, agitation speed 200 r/min, aeration rate 1 / v / v / mm, initial pH 7, and exhaust gas escaping was applied under gauge pressure 17 cm water column for 72 h, progesterone was then added as solution in 96% ethanol

at a concentration of 100 µg / ml. The fermentation was continued for 24 h unless otherwise stated.

Transformation product assay. At the end of the transformation period, the culture filtrate was extracted with chloroform (2 volumes), washed with distilled water, then dried over anhydrous sodium sulphate. The combined extracts were evaporated under vacuum to give a semi-solid residue (test material). The different transformation products in the test material were separated and determined by two methods:

A - THIN - LAYER CHROMATOGRAPHY (TLC). It was performed on silica gel G plates (Sallam *et al* 1969), for identification and resolution of the test material, the following solvent systems proved to be suitable:

I - Cyclohexan: acetone: chloroform (15:5:2 v / v / v).
II - Cyclohexane: chloroform: isopropanol (10:5:2 v / v / v).
III - Benzene: ethyl acetate: acetone (4:1:1 v / v / v).

Two different colour reagents were used for the identification of the different steroids present. (i) Libermann - Burchard reagent. (ii) Chlorosulphonic acid: acetic acid (3:1 v / v).

The experimental results revealed the presence of more than one steroid compounds in the transformation medium. The distance between these were sufficient to enable them to be separated and eluted with chloroform. The extract was filtered and evaporated to dryness in a test tube in a water bath. For the determination of the different steroid products, 8 ml aliquot of chromogen reagent (45 ml conc. H_2SO_4 + 55 ml absolute ethanol) were added to the test material, heated in boiling water bath for 15 min, and cooled, the absorbance was measured photometrically at specific λ_{max} for each product in the tested material.

B - ISOLATION BY COLUMN CHROMATOGRAPHY: The collected extract (test material) was dissolved in the minimum volume of n - hexane and then fractionated on silica gel S column. The following sequence of solvents were used: n - hexane, n - hexane: benzene (1 : 1, v / v), benzene containing different concentrations of chloroform, chloroform containing different concentrations of methanol and finally methanol.

Results and Discussion

Bioconversion of progesterone by immobilized cells of F. dimerium. In a previous communication (Adham *et al* 2002) thirty seven different fungal cultures were tested for their ability to degrade the side - chain of progesterone. The data shows that some tested organisms metabolized progesterone in different degrees. Also *F. dimerium; F. oxysporum* No. 153; *F. oxysporum* No. 152; *F. moniliforme* were the most active progesterone side - chain degrading fungi and were, thereafter, subjected to quantitative studies. The obtained data shows that the tested organisms metabolized progesterone, however, *F. dimerium* was the most active and highest yields of both testosterone and androstadienedione were obtained by this species.

To our knowledge, side - chain degradation with *F. dimerium* have not yet been reported. In literature also it is the most active with the formation of considerable yield of testosterone (Adham *et al* 2002). Due to the above reasons, we choose this organism for elucidating the most favourable conditions for it to perform the side - chain degradation of progesterone. The physiological aspects of the bioconversion process were also evaluated (Adham *et al* 2002). In the preceding work, the bioconversion processes were routing carried out by the shaked free fungal cultures. Therefore, in the present study the biotransformation of progesterone with the immobilized cells of the tested fungus was investigated. The transformation capacity of the immobilized cells of *F. dimerium* seems to be relatively low at the early stage of the transformation process (about 40% of the steroid substrate remained unchanged), thereafter, the bioconversion rates were markedly enhanced as indicated by the consumption of the total amount of the charged progesterone (Fig 1). Androstenedione (AD) was traced as the major product during the different phases of the transformation period.Δ^1 - dehydroderivative; namely androstadiendione (ADD) was only detected in minor amounts indicating a weak dehydrogenase activity. On the other hand, testolactone seems to be the second major product which was produced with increasing amounts particularly during the early phase of the transformation process. Similarly, testosterone was produced in good yields after 96 h. This may reflect the remarkable lactonization activity catalyzing the formation of testololactone.

Bioconversion of progesterone by free fungus cells in a stirred tank fermentor. Transformation in a stirred tank fermentor using different aeration rate was carried out. The results (Table 1) showed that the same pattern of trans-

Table 1
Effect of aeration on transformation of progesterone by *F. dimerium* using Bioflo fermentor

Aeration Vvm	Residual P%	T %	AD %	ADD %	TL %	Missing steroids %
0.5	Traces	23.9	25.6	8.2	20.5	21.8
1.0	Traces	25.8	32.3	7.5	20.7	13.7
0.5	Traces	22.6	32.2	7.0	20.0	18.2

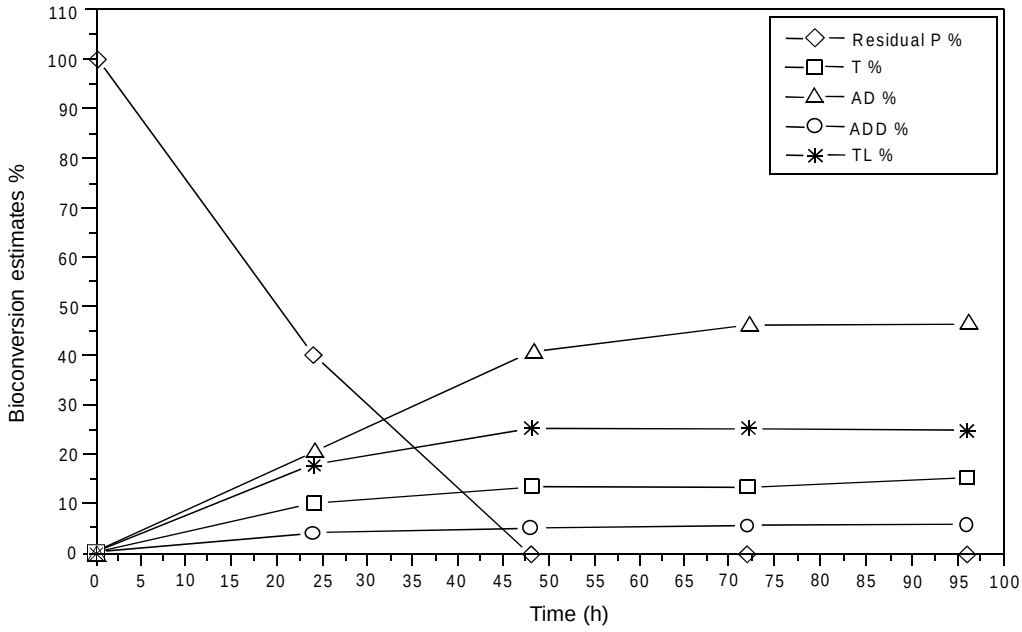

Fig 1. Bioconversion of progesterone by shaked immobilized cells of *F. dimerium* entrapped in 2% calcium alginate.

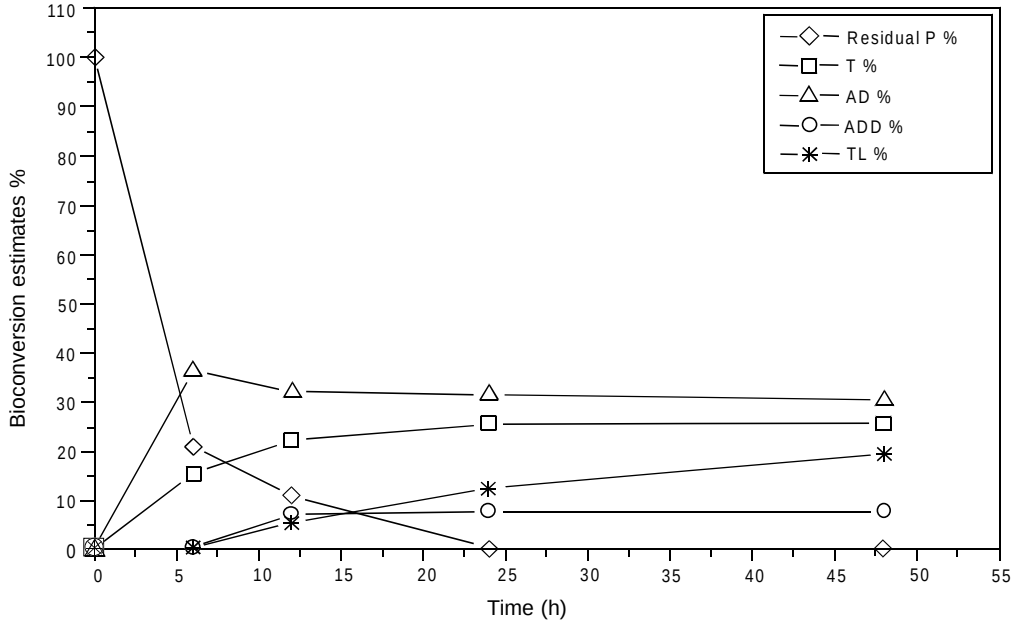

Fig 2. Bioconversion of progesterone by *F. dimerium* using Bioflo fermentor.

formation was observed regardless of the aeration rate applied. Thus, the charged progesterone concentration was almost converted mainly to AD and TL as major products and ADD in relatively minor levels. However, the adjustment of the aeration rate at 1 v / v afforded relatively higher bioconversion yields. In subsquent experiment, the bioconversion activities were traced at different time intervals while the aeration rate was adjusted at 1 v / v level. The results revealed that 80% of the progesterone substrate was successfully converted mainly into AD and after the first 6 h (Fig 2). The following phase of the bioconversion course (mainly 6 - 12 h) exhibited further progesterone conversion in favour of AD and T production with the concomitant production of ADD and TL in relatively lower yields. As the bioconversion process prolonged to 24 and 48 h, the charged progesterone was completely converted with the formation of AD and T as major products and the detection of TL in elevating yields.

Table 2
Properties of the transformation products of progesterone bioconversion by *F. dimerium*

Isolated compounds	Eluted solvents	Solvent of crystallization	Crystal properties
ADD	Benzene : Chloroform 70 : 30	Chloroform: Methanol	m.p. 140 - 142 ˚C UV λ_{max} 244 nm
AD	Benzene : Chloroform 1 : 1	Chloroform : Methanol	m.p. 174 - 176 ˚C UV λ_{max} 239 nm
T	Chloroform	Chloroform : Methanol	m.p. 153 - 158 ˚C UV λ_{max} 240 nm
TL	Chloroform : Methanol 1 : 1	Methanol	m.p. 207 - 209 ˚C UV λ_{max} 242 nm

Assessment of the progesterone side-chain derivatives produced by F. dimerium. It seems appropriate to verify the identity of the different products formed as a result of progesterone bioconversion with the tested fungus, since the identity so far has been based solely on the TLC separation of each compound. Therefore, the column chromatographic resolution of the transformation mixtures was performed. The fractions containing the same product (as judged by TLC analysis) were collected, then evaporated through vacuum and crystallization, from suitable solvent. Identification of each compound was made by determination of m.p. and UV absorption spectra (Table 2).

The aforementioned investigations clearly proved that the tested fungus *(F. dimerium)* can transform progesterone (C_{21}-steroid) into the same product, namely AD, T as major products in addition to TL and ADD as minor derivatives. This transformation pattern existed upon using the immobilized or the free cell conversion techniques. However, relatively higher transformation outputs were recorded with the immobilized fungal cells, whereby, the progesterone substrate was completely converted after 72 - 96 h into AD, TL, T and ADD at 48, 26, 16.5 and 7 %, respectively.

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Pak. J. Sci. Ind. Res. 2004 ***47*** *(2) 142 - 145*

CONSTITUENTS OF *PRUNUS ARMENIACA*

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Phytochemical screening of the non-alcoholic extract of *Prunus armeniaca* has revealed the presence of a triterpenoid belonging to Ursane / Oleanane series and a steroid alongwith its glucoside for the first time from this source. Structures were confirmed by spectroscopic methods, using IR, ^{1}H-NMR,^{13}C-NMR and Mass spectra.

Keywords: *Prunus armeniaca,* Fruits, Steroidal glycosides and a triterpenoid.

Introduction

Prunus armeniaca is a member of family *Rosaceae* and *Genus Prunus*. It is commonly known as Apricot (English) and Zardalu in urdu. Apricot is normally found in areas of higher altitudes. The fruit is very popular, besides being a table fruit apricot is also employed in making jams and nector. In Pakistan, it is cultivated in the inner valleys of Baluchistan and Kashmir from the plains to 12,000m (Baquar 1989). *Prunus* species are reported to have antipyretic and leucodermatic activity in the treatment of leprosy. Apricot kernel oil closely resembles almond oil and employed as an adulterant or substitute for it. It is also used in medicine for earache and in variety of ailments (Chopra 1956, Gupta 1969). The kernel is used as an expectorant and a remedy for dry throat, laryngitis, lung diseases and abscesses. It is regarded as bechic, depurative, sedative for the respiratory centre, tonic and antispasmodic, a remedy for severe colds and bronchial asthma. In Indo China a special preparation of the fruit is chewed but not swallowed to protect the bronchial tubes from cold during winter (Lily 1980).

Triterpenoids/steroids are the compounds of wide occurance and structural diversity, which have always attracted attention, and their pharmacological activities. Keeping in view the biological / pharmacological importance, present studies were undertaken on this plant to carry out the isolation and structural studies of such compounds. Plant aqueous ethanolic extract also showed antibacterial activity, which is under process.

Materials and Methods

Plant material (2kg) fruits were purchased from local market, Karachi and verified by Botany Department, University of Karachi. The fruits were percolated with methanol at room temperature for 15 days (3x5 extractions), methanolic extract was concentrated and the residue obtained upon concentration was treated with n-hexane whereupon a white gummy solid separates out leaving behind n-hexane extract (**1**). Methanol soluble filtrate was further extracted with ethyl acetate and butanol saturated with H_2O. Ethyl acetate extract was evaporated and labelled as extract (**2**).

The compound isolated from non-alcoholic (hexane) fraction of the fruit was identified as α-amyrin acetate. Two other compounds were obtained from ethyl acetate fraction (non-alcoholic) and were identified as Stigmasterol and Stigmasterol glucoside.

Results and Discussion

The separation of compounds was achieved by column chromatography followed by preparative TLC and fractional crystallization. The compound generally belonged to Oleanane/ Ursane series (Fourneau and Hocquemiller 1996) and sterol derivatives (Jamshed and Fazal-ur-Rehman 1991) reported first time from this source.

Compound 1: It was eluted with pet ether-benzene 70:30, a white waxy solid gave Leibermann's burchard test positive. On re-crystallization with chloroform-methanol gave a white crystalline compound melting at 219-222°C, showing molecular ion peak, M^+ at m/z 468 ($C_{32}H_{52}O_2$) and a base peak m/e at 218.The base peak at 218 indicated the presence of α/β amyrin type of compound and arise due to retro Diels-Alder fragmentation. The fragment at m / z 203 and 189 arises from 218 fragment due to the loss of (M-CH_3) and (-CH_2CH_3). Its IR spectrum showed absorption at 2910, 2870 cm^{-1}due to CH stretching and 1735 (C=O), 1245 cm^{-1} (C-O), a sharp singlet at 1735 and 1245 cm^{-1} due to C-O single bond confirming

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Table 1
^{13}C-NMR ($CDC1_3$,75.43 MHz) Data of Compound (**1**)

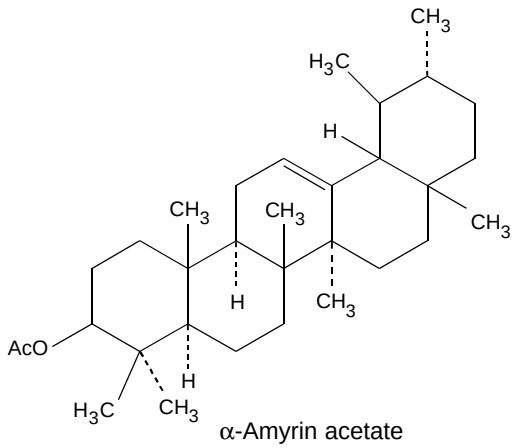
S No.	Multiplicity (DEPT)	^{13}C-NMR (δ)	S No	Multiplicity (DEPT)	^{13}C-NMR (δ)
1	CH_2	38.4	17	C	33.8
2	CH_2	23.6	18	CH	59.0
3	CH	80.7	19	CH	39.7
4	C	37.6	20	CH	39.7
5	CH	55.3	21	CH_2	31.3
6	CH_2	18.3	22	CH_2	41.5
7	CH_2	32.8	23	CH_3	28.1
8	C	40.1	24	CH_3	16.8
9	CH	47.6	25	CH_3	15.7
10	C	36.8	26	CH_3	16.8
11	CH_2	17.5	27	CH_3	23.2
12	CH	124.0	28	CH_3	28.1
13	C	139.0	29	CH_3	23.2
14	C	42.0	30	CH_3	21.4
15	CH_2	28.7		$COCH_3$-3	170.4
16	CH_2	26.7		$COCH_3$-3	21.2

Table 2
^{13}C-NMR ($CDC1_3$, 75.43 MHz) Data of Compound (**2**)

S No.	Multiplicity (DEPT)	^{13}C-NMR (δ)	S No.	Multiplicity (DEPT)	^{13}C-NMR (δ)
1	CH_2	37.2	16	CH_2	26.1
2	CH_2	28.2	17	CH	56.0
3	CH	71.8	18	CH_3	12.1
4	CH_2	40.3	19	CH_3	19.4
5	C	140.8	20	CH	36.1
6	CH	121.7	21	CH_3	19.0
7	CH_2	31.7	22	CH	138.3
8	CH	31.9	23	CH	129.3
9	CH	51.2	24	CH	50.2
10	C	36.5	25	CH	29.2
11	CH_2	21.1	26	CH_3	21.2
12	CH_2	39.7	27	CH_3	21.0
13	C	42.2	28	CH_2	23.1
14	CH	56.9	29	CH_3	12.2
15	CH_2	24.4	–	--	--

the ester grouping. Peak at m/e 408 (M^+-60) also indicated the presence of CH_3COO^- group. Presence of a sharp singlet at δ 2.01 ppm (3H,s -$COCH_3$) in (^{1}H- NMR) also provide the evidence. The NMR spectrum also showed eight methyl singlets from δ 0.78-1.16 and a distorted triplet at δ 5.02 due to proton at C-12 because of olefinic double bond and a broad singlet at δ 4.72 accounted for C-3 β-hydrogen, bearing OA_c group. The two other signals appeared at δ 170.4 and δ 21.2 in ^{13}C- NMR indicated the presence of carbonyl carbon and methyl carbon of acetate group (Table 1). On the basis of spectral studies and data that is available in literature the compound was identified as α-amyrin acetate. (Ahmed 2001)

Compound 2: It was obtained from ethyl acetate extract by repeated column chromatography using silica gel. The compound was eluted by chloroform-methanol with increasing percentage of methanol (10%, 2.5%, 50%, & 7.5%). The fractions obtained from 5% methanol were all similar to each other, showing a very prominent single spot on TLC when developed with spraying reagents. For further purification, the compound was re-crystallized with methanol-chloroform, and repeated re-crystallization gave white pure solid UV active compound (m.p=168°C), with intense coloured spot on TLC when sprayed with ceric sulphate and Leibermann's burchard reagents.



α-Amyrin acetate

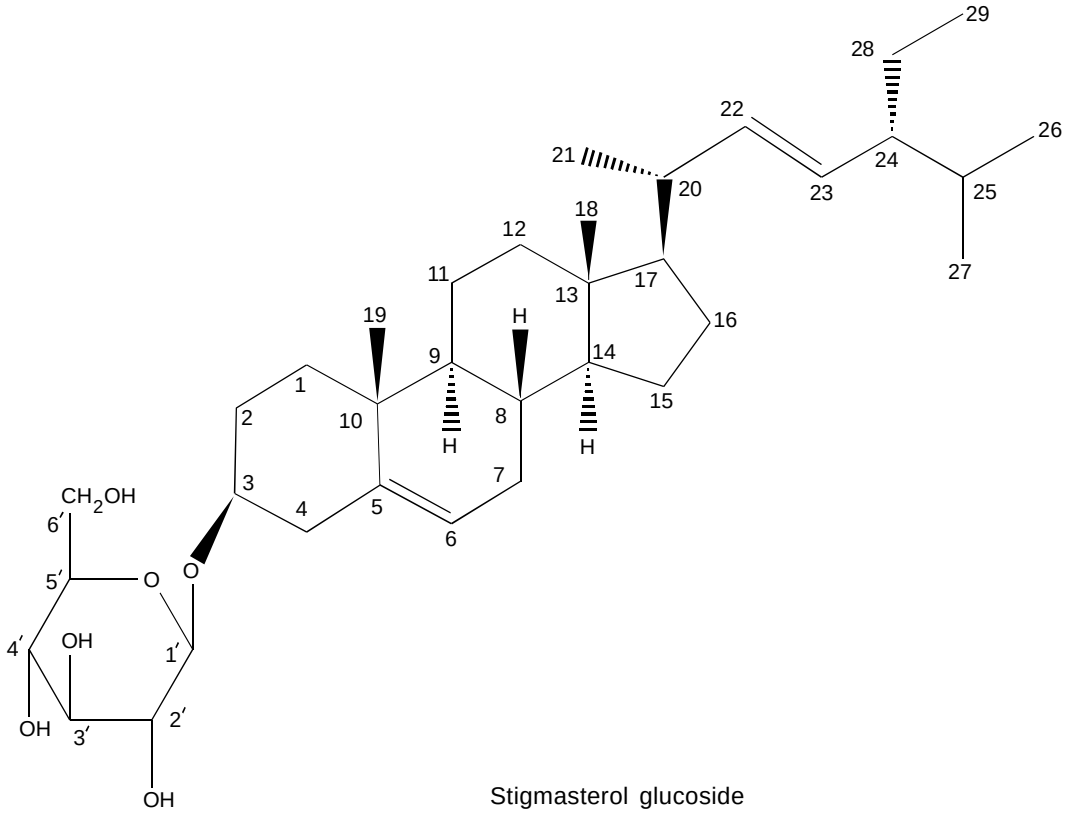
EI mass showed the molecular ion peak M^+at m/z 412.3809 ($C_{29}H_{48}O$) and base peak at 203, other peaks in mass spectra appeared at 369, 301, 273 (M-139), 220 and 164. IR absorption exhibited υ_{max} at 3412 cm^{-1} (OH-br), 2900 (CH-str), 1680 due to C=C bond, and 1041 (C-O-C). The ^{13}C-NMR showed 29 carbon signals indicating six methyl, nine methylene, eleven methine and three quaternary carbons (Table2). The six methyls appeared in the ^{1}H-NMR spectrum at δ 0.69 (s,H-18), 0.97 (s, H-19),1.00 (d, *J* = 6.4 Hz, H-21), 0.81(d, *J* = 6.1 Hz, H-26), 0.76(d, *J* = 6.0 Hz, H-27) and 0.78 (t, *J* = 7.5Hz, H-29). The olefinic signals, each of one proton resonated at δ 4.97 (dd, *J* = 15.4, 8.5 Hz, H-23), 5.11 (dd, *J* = 15.5, 8.5 Hz, H-22) 5.31 (br.s, H-6) and their associated carbons resonated at δ129.3(C-23), 138.3 (C-22) and 121.8 (C-6), respectively, which indicated the two double bonds in the molecule. The methine carbon resonated in the ^{13}C-NMR spectrum at δ 71.8 (C-3) and in the ^{1}H-NMR spectrum at δ 3.46 (m, H-3) revealed that hydroxyl group was attached to C-3.

Table 3
^{13}C-NMR ($CDC1_3$+ CD_3OD, 75.43 MHz) Data of Compound (**3**)

S No.	Multiplicity (DEPT)	^{13}C-NMR (δ)	S No.	Multiplicity (DEPT)	^{13}C-NMR (δ)
1	CH_2	39.2	19	CH_3	19.3
2	CH_2	29.3	20	CH	36.7
3	CH	79.6	21	CH_3	19.1
4	CH_2	40.0	22	CH	138.8
5	C	141.1	23	CH	129.9
6	CH	122.4	24	CH	46.6
7	CH_2	32.4	25	CH	29.8
8	CH	32.5	26	CH_3	20.0
9	CH	51.9	27	CH_3	19.6
10	C	37.3	28	CH_2	23.6
11	CH_2	21.6	29	CH_3	12.4
12	CH_2	40.3	1′	CH	101.7
13	C	42.8	2′	CH	71.0
14	CH	57.5	3′	CH	76.6
15	CH_2	25.8	4′	CH	74.3
16	CH_2	26.8	5′	CH	77.2
17	CH	56.7	6′	CH_2	62.5
18	CH_2	12.2	–	--	--

On the basis of above spectral data, TLC and comparison with authentic sample the compound was identified as stigmasterol. (Funes 1978)

Compound 3: It was isolated from ethyl acetate soluble part of methanolic extract. The molecular mass of (**3**) was confirmed as 574 ($C_{35}H_{58}O_6$) with the help of peak observed in the negative FAB mass spectrum at m/z 573 [M-H]. Other fragments in mass appeared at m/z 432 (M-162 glucose), 369 (M-43 $CH(CH_3)_2$, 273 (M-139), loss of side chain. Other fragments at 203/205 the base peak of steroidal skeleton. Its IR showed intense absorption band at 2853, 2921 cm^{-1} (CH stretch), a strong doublet at 1696 and 1649 (two C = C) bonds and also strong bands at 1461, 1025 cm^{-1}. The ^{13}C-NMR spectrum showed the presence of 35 signals, which were resolved as six methyl, ten methylene, sixteen methine and three quaternary carbons. Six-anomeric carbon in ^{13}C-NMR spectra showed resonance absorption between δ 60 and 71 (Table 3), a signal at β 3.18 due to proton (C-3) and a methine carbon at 101.7 indicated the presence of sugar moiety in the molecule. The compound was subjected to hydrolysis. The sugar confirmed was β-D glucose through magnitude of coupling constant of anomeric carbon at δ 4.35(J = 7.7 Hz) and co-TLC of hydrolyzed product with the authentic sugar sample. The values were found very similar to that previously reported in the literature (Zlatanov 1998). The sapogenine was found to be



Stigmasterol glucoside

stigmasterol.

The ^{1}H-NMR spectrum showed three olefinic signals at δ 4.92 (dd, *J* = 15.2, 8.2 Hz, H-23) 5.09 (dd, *J* = 15.2, 8.4 Hz, H-22) and 5.31 (br.s, H-6). The six methyl confirmed by the ^{13}C-NMR spectrum appeared in the ^{1}H-NMR spectrum at δ 0.69 (s, H-18), 1.00 (s, H-19), 0.88 (d, *J* = 6.2 Hz, H-21) 0.82 (d, *J* = 6.3 Hz, H-26), 0.76 (d, *J* = 6.4Hz, H-27) and 0.77 (t, *J* = 7.0 Hz, H-29). Compound **3** was identified as 3-O-β-D-glucopyranosyl-stigmasterol and was confirmed by matching the spectral data with that of reported in literature.

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EFFECT OF SELECTED FOOD ADDITIVES ON PHYTIC ACID CONTENT OF SOYBEAN DURING SOAKING

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The effect of food additives of sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) was studied on the hydrolysis of phytic acid in soybean, soaked in water and in solutions of pH4 and 6 at 30°C for 6 and 12 h. Four varieties of soybean V1 (B1-080/36), V2 (AGS-62), V3 (G1-0031) and V4 (EPPS) were selected. The amount of phytic acid in V1, V2, V3, and V4 of soybean was 12 mg, 11 mg, 13 mg and 12.5 mg/g respectively. Soaking of soybean flour for 6 and 12 h in water and in pH4 and 6 solutions have significantly reduced ($P < 0.05$) the levels of phytic acid. The effect of pH6 solution was most effective, while the effect of water and pH4 was similar in lowering the phytic acid in soybean. The presence of mixed food additives (1% NaCl + 2% $NaHCO_3$) in water, and pH4 and 6 solutions, reduced the level of phytic acid in soybean to 38%, 52% and 56% for 6 h, and 48%, 55% and 68% for 12 h in respective solutions. It was observed that soaking of soybean flour in pH 6 solution in the presence of NaCl and $NaHCO_3$ phytase enzyme of the flour is well activated to hydrolyse phytic acid. These results suggest that soaking with sodium chloride and sodium bicarbonate can reduce the phytic acid in soybean flour. This treatment can improve the nutritional value of the soyabean flour, which is used in various food products of dairy and confectionery etc.

Key words: Food additives, Phytic acid, Soyabean, Soaking.

Introduction

Legumes are the important source of dietary protein for the large segment of the world population, especially in areas where use of animal protein in human diet is limited due to its non-availability, or cultural and religious constraints. In addition to protein, legumes also provide energy, minerals, and some vitamins. The quality of protein depends on their amino acids composition and legumes are generally rich in lysine, threonine, valine, leucine and isoleucine. This pattern of amino acids in legumes increases their importance as a supplement for cereal diets, which are usually deficient in lysine (Mtenga and Sugiyamma 1974). In plants, number of chemical compounds are synthesized which can adversely affect the quality of plant products used as human and animal food. One of the most important of such anti-nutritional factors is the phytic acid, which occurs in cereals, legumes and oilseeds. The presence of phytic acid decreases the bioavailability of many essential minerals and protein (Maga 1982).

Soybean is a leguminous crop extensively grown for the production of edible oils and proteins. The soybean belongs to the family Leguminoseae, subfamily Papilionoideae, and the genus *Glycine.* Soybean seeds vary in color from yellow, green or brown to black, and are either solid colored or bi-colored. They are spherical, elongated, or oval in shape. Soybean originated in China, where records of it go back to 2838 B.C. and it was spread to other parts of the world very late. The present commercial soybean cultivars were introduced in Pakistan in early of the year 1960 from the USA for experimental purposes. Cultivation of soybean on a commercial scale in the province of NWFP was reported in the years of 1970 - 1971, in Sind province in 1975 - 1976, and in Punjab province in 1983. In the province of Balochistan, it is still in the experimental stage. The total production of soybean in Pakistan is 7228 tones and in NWFP 6410 tones (Agriculture Statistics of Pakistan, 1994 - 1995).

To improve the nutritional quality of soybean by elimination the anti-nutritional factors, the influence of food additives of sodium biocarbonate and sodium chloride in soaking was studied on phytic acid in soybean flour soaked in water and various pH solutions at 30°C for different times.

*Chemistry of phytic acid.*The utilization of protein in human body depends on the quality of protein that is the presence of essential amino acids, digestibility and anti-nutritional factors including phytate. Phytic acid is the normal constituent of cereals and leguminous seeds and usually occurs in the form of phytate. This represents a complex class of naturally occurring compounds that can significantly influ-

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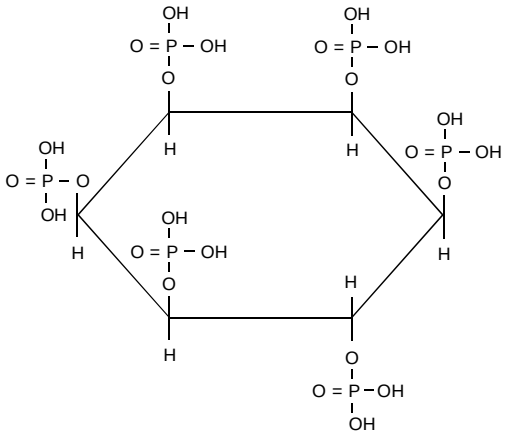

Fig. 1 Structure of phytic acid ($C_6H_{24}0_{27}P_6$) suggested by Neuberg (1908).

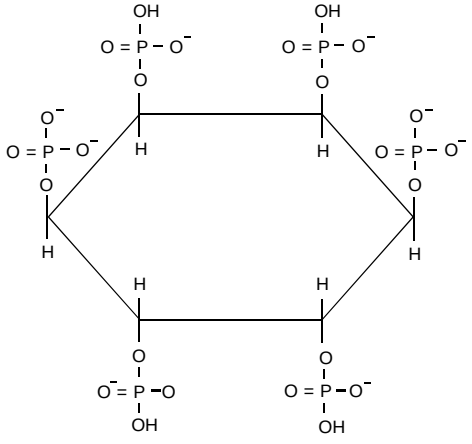

Fig. 2 Structure of phytic acid ($C_6H_{18}O_{24}P_6$) proposed by Anderson (1914).

ence the functional and nutritional properties of foods. Phytic acid is commonly called myo-inositol hexaphosphoric acid or 1, 2, 3, 4, 5, 6-hexakis (dihydrogen phosphate) myo-inositol. Phytic acid can interact with various minerals make insoluble complexes thus decreasing the bio-availability of many essential minerals such as calcium, phosphorous, magnesium, iron and zinc and also protein in human diet (Maga 1982). The interaction of phytic acid with protein is depend on the pH of the medium. At pH level below the isoelectric point of protein, phytate binds directly to protein action and at pH above the IP it binds to the protein through an alkaline earth metal (Cheryan 1980).

In legumes, a level of phytic acid is approximately up to 5% by weight. In soybean, 70 - 80% of the phosphorous is present in the form of phytic acid, the phosphoric acid ester of inositol. The interaction between phytic acid, mineral and/or protein appears to be primary factor responsible for its adverse nutritional effects in high phytate diets. The mechanism for this interaction can be explained by the structure of phytic acid. Neuberg (1908) proposed phytic acid chemical formula $C_6H_{24}O_{27}P_6$ having three P-O-P linkages between pairs of adjacent phosphates (Fig 1). Anderson (1914) proposed phytic acid chemical formula $C_6H_{18}P_6O_{24}$. (Fig 2). Gosselin and Coughlan (1953) studied calcium-phytic acid interaction using an ion-exchange equilibrium technique and concluded that there is P-O-P within the phytic acid molecules, in accordance with Neuberg structure. Fischer and Kurten (1932) also came to similar conclusion.

Materials and Methods

(1) Sample collection. For this research project, the following approved varieties of soybean of the year 1996, were collected from the Malakandher Farm, of NWFP Agricultural University, Peshawar:

(a) V1 (B1 - 080/36) (b) V2 (AGS - 62)
(c) V3 (GL - 0031) (d) V4 (EPPS)

The study was carried out in the Laboratory of the department of Food Science and Technology, NWFP Agricultural University, Peshawar.

(2) Sample preparation. The sample was cleaned off from all the impurities and sorted out for uniform shape and size. The seeds were ground by using a mesh of 40mm sieve. The ground and sieved samples were packed in plastic bags and stored at room temperature for subsequent chemical analysis.

(3) Protein analysis. Duplicate sample (1.5g) was placed in a digestion flask, 5g of digestion mixture and 30 ml of concentrated H_2SO_4 were added. The digestion flasks were then transferred to digestion assembly and the temperature adjusted to 100˚C which was increased gradually to 400˚C. The digestion was continued until the solution in the digestion flask was cleared and all organic matter oxidized. The digestion was completed within 2 h, after which the flasks were cooled for one h.

Distillation of the digest was performed by Micro-Kjeldahl apparatus using 5 ml from digestion flask and 5 ml NaOH and distilled water added. The sample was distilled and distillate was collected in a conical flask containing 5 ml of 2% boric acid solution with mixed indicator of methyl red. The distillation was completed in 5 minutes. The pinkish color was changed during distillation. Distillate collected in conical flask was titrated against standard HCl (0.01 N) solution. End point was noted when pink color appeared. Milliliters (ml) of standard HCl solution used were noted and percent crude protein was calculated as follows:

$$\% \text{ protein} = \frac{(S\text{-}B) \times 0.014 \times D \times 100 \times 6.26}{\text{Weight of sample x V}}$$

S = Volume of standard acid used for sample titration.
B = Volume of standard acid used for blank titration.
N = Normality of the acid used.
D = Sample dilution after digestion.
V = Volume of the digest taken for distillation after dilution.
0.014 is the equivalent weight of nitrogen and 6.25 is the general nitrogen to protein conversion factor.

(4) Food additives, and soaking of the soybean sample. The ground sample of soybean (approx.10 g) was soaked in 100 ml beaker in water and solutions of pH 4 and 6, with and without food additives of sodium chloride (1%) and sodium bicarbonate (2%) separately and mixed of both at 30°C for 6 and 12 hours.

(5) Phytic acid determination. Method developed by Huag and Lantzch (1983) was used for the determination of phytic acid. This method is based on phytic acid precipitation with an acid iron-III solution of known iron contents.

Preparation of solution: (i) HCl (2N). HCl (167.4 ml) was dissolved in distilled water to make a final volume of 1 litre.*(ii) Ferric solution.* Ammonium iron-III-sulphate [$NH_4(SO_4)_2$.12 H_2O] (2 g) was dissolved in 100 ml of 2N HCl and the volume was made to 1 liter with distill water in volumetric flask. *(iii) 2,2 Bipyridine solution.* 2,2, bipyridine (5 g) dissolved in 5 ml of thioglycollic acid ($HSCH_2COOH$) and then the volume were made up to 500ml in volumetric flask. *(iv) Phytic acid solution.* Phytic acid (Purity 98%) 0.15 g was dissolved in water and the volume was made to 100 ml in volumetric flask.

(6) Statistical analysis. The data were analyzed statistically by the procedure of analysis of variance by using Randomized Complete Block Design (RCBD) with split plot design. The mean were separated by applying the Least Significant Differences (LSD) test. This test is recommended by Snedecor and Cochran (1967).

Results and Discussion

(1) Analysis of soybean for protein and phytic acid. Four varieties of soybean were analyzed for the protein and phytic acid contents. The name of these varieties are V1 (B1 -080/36), V2 (AGS - 62), V3 GL - 0031 and V4 EPPS. Table 1 shows the protein and phytic acid contents analyzed in these varieties of soybean. The data revealed that the protein content in V1, V2, V3 and V4 varieties is 39.8%, 37.3%, 41.0% and 40.4%, respectively. To analyzed phytic acid the standard calibration curve was prepared from standard phytic acid according to the method of Haug and Lantzch (1983) (Fig 1). The amount of phytic acid content in V1, V2, V3 and V4 varieties of soybean is 12 mg, 11 mg, 13 mg and 12.50 mg/g, respectively (Table 1).

Table 1
Protein and phytic acid contents of whole soybean flour

Variety	% Protein	Phytic acid (mg/g)
(V1) B1-080/36	39.8%	12.00
(V2) AGS-62	37.3%	11.00
(V3) GL-0031	41.0%	13.00
(V4) EPPS	40.4%	12.50

It was reported that soybean is composed of three major components, the hull, cotyledon and hypocotyl, in which protein is approximately 8%, 90% and 2% respectively. Typical composition of soybean was found to be, protein 42% fat 20%, total carbohydrate 35%, ash 5.0% and crude fiber 5.5%. One third of soybean is carbohydrates, which included various polysaccharides and sucrose. The balance of the materials present in soybeans was described as ash which included many minerals (FAO 1977). Rham and Jost (1979) reported the nutrient composition of soybean as moisture 7.6%, protein 50.0%, fat 0.9%, phytate 1.5%, Ca 0.24%, Mg 0.32%, Na 0.01%, K 0.05%, P 0.7% and ash 4.2%. Andrew and Winton (1965) described that soybean seed contained 5.85 - 19.27% moisture, 26.25 - 40.22% protein, 12.27 - 19.0% fat, 3.07 - 5.40% ash, 2.45 - 6.13% crude fiber and 26.17 - 32.84% nitrogen free extract. Rehman and Nawaz (1975) determined the protein and oil contents of various varieties of soybean and concluded that the protein content was lowest 37.75% in Bragg and highest 43.73% in Lee variety. The mean protein content of the varieties was 40.35% over 500 lines of soybean seed and reported that the protein and oil contents ranged from 30 to 46% and 12 to 24% respectively. Cartter and Hopper (1942) had earlier reported the average composition of 10 common varieties of soybean, which contain 42.78% protein, 19.83% fat, 4.99% ash, 5.52% crude fiber and 7.97% sugar. Ranjana *et al* (1988) and Clark and Proctor (1994) reported 54.93, 46.84 and 50.20% protein and 6.15, 7.65 and 5.35% ash in soybean flour.

Lolas *et al* (1976) reported that the phytic acid content of 15 soybean varieties ranged from 1.00 to 1.47% dry weight which represented between 51.4 and 57.1% of the total phosphorous. They also evaluated phytic acid levels in 19 oat varieties in a range of 0.84-1.01% based on dry weight and proposed that phosphorous measurement could estimate the amount of phytic acid content. He also found a phytic acid 0.62-1.35% dry weight in whole kernels of wheat, whereas, the bran portion had phytic acid levels ranging from 4.59 to 5.52%, demonstrating that foods containing added wheat bran could have unexpectedly high levels. Chen and Pan (1977) reported that soybean and two varieties of pea seed (Dwarf and Early Alaska)

contained 2.48, 1.13 and 1.86 mg/g phytate respectively. After germination for 5 days and extracted three times each with 5 ml of 0.5 N HCl for 40 minutes, the phytate decreased to 1.94, 0.59 and 1.20 mg/g respectively.

Ganesh Kumar *et al* (1978) reported that green gram, cowpea, and chickpea contain phytic acid 0.65, 0.43 and 0.28%, respectively. The extraction pattern of phytin-P in aqueous for uncooked legumes were 0.124, 0.090 and 0.056%, respectively. The maximum extraction of phytin-P by hydrochloric acid (0.5 N) media for uncooked legumes was 0.185, 0.123 and 0.078% respectively and for the cooked legumes was 0.150, 0.090 and 0.078%, respectively. Cooking resulted in the decrease of both water and acid extractable phytin-P, but the loss of acid extractable was much less then water extractable ones.Gad *et al* (1982) found that phytic acid content in Broadbean seed is 274.9 mg/100g, peas 222.7 mg/100g, fenugreek 190.2 mg/100g, chickpea 184.5 mg/100g, lentil 149.7 mg/100g, and lupine 91.9 mg/100g respectively. They further reported that beans contained total phosphorus 518.2 mg/100g, peas 345.2 mg/100g, lentil 357.5 mg/100g and lupine 340.1 mg/100g respectively.

(2) The influence of food additives, pH, temperature and times on phytic acid during soaking of soybean. The effect of various factors such as (i) Food Additives (ii) pH (iii) Temperature and (iv) Times were studied on the hydrolysis of phytic acid content in soaking of four varieties of soybean. The research was carried out in the model system, in which, the ground sample of soybean was soaked in tap water and in solution of pH 4 and pH 6 at 30˚C for 6 and 12 h; (a) with no food additives, (b) with 1% sodium chloride, (c) with 2% sodium bicarbonate and (d) with 1% sodium chloride + 2% sodium bicarbonate mixture.

The results in Table 2 shows the effect of soaking on phytic acid content in soybean in tap water, pH4, pH6 solutions (with no food additives) at 30˚C for 6 and 12 h, and the analysis of variance is given in Appendix-I. Soaking for 6 h at 30˚C, in V1 of soybean the phytic acid was reduced from its original level (12 mg/g) in water, pH 4 and pH 6 solutions to 11.54 mg, 11.46 mg and 10.50 mg/g and in V2 (11 mg/g) to 10.64 mg, 10.41 mg and 9.73 mg/g, respectively. Similarly in V3, the phytic acid (13 mg/g) was reduced to 12.65 mg, 12 mg and 11.36 mg/g and in V4 (12.50 mg/g) to 11.85 mg, 11.58 mg and 10.86 mg/g, respectively. Soaking the soybean for 12 hours at 30˚C, in tap water, pH 4 and pH 6 solutions, the phytic acid in V1 was reduced to 11.28 mg, 10.88 mg and 9.88 mg/g, and in V2 to 10.54 mg, 9.96 mg and 9.48 mg/g respectively. In V3 the phytic acid reduced to 11.93 mg, 11.64 mg and 10.56 mg/g and in V4 11.46 mg, 11.30 mg and 10.36 mg/g respectively. Among these varieties, the highest reduction in the level of phytic acid was found at pH 6, 12 h soaking. Statistically, soaking the soybean all four varieties in tap water, pH 4 and pH 6 solutions for both times 6 and 12 h, phytic acid was significantly reduced ($P<0.05$).

Table 2

Retention of phytic acid in soybean flour (mg/g) after soaking at 30˚C for different times with no food additives. Before soaking phytic acid level in soybean was in V1 (12 mg/g), V2 (11 mg/g), V3 (13 mg/g) and V4 (12.5 mg/g)

Variety	Time(h)	Phytic acid (mg/g) Soybean flour			Mean
		Water	pH4	pH6	
V1	6	11.54F	11.46G	10.50Q	11.17C
	12	11.28J	10.88K	9.88U	10.68F
V2	6	10.64N	10.41R	9.73V	10.26G
	12	10.54P	9.96T	9.08W	9.86H
V3	6	12.65A	12.00B	11.36H	12.00A
	12	11.93C	11.64D	10.56O	11.38B
V4	6	11.85M	11.58E	10.86L	11.10D
	12	11.46G	11.30I	10.36S	11.04E
Mean		11.486A	11.154B	10.291C	

Figures showing the same letter(s) are statistically not different from one another (LSD Test).

Table 3

Retention of phytic acid in soybean flour (mg/g) after soaking at 30˚C for different times with 1% sodium chloride (NaCl). Before soaking phytic acid level in soybean was in V1 (12mg/g), V2 (11 mg/g), V3 (13 mg/g) and V4 (12.5 mg/g)

Variety	Time(h)	Phytic acid (mg/g) Soybean flour			Mean
		Water	pH4	pH6	
V1	6	10.04H	10.68K	10.08P	10.60D
	12	10.84J	10.30N	9.43T	10.52F
V2	6	10.24O	9.72S	9.34U	9.76G
	12	9.92Q	9.40T	8.64V	9.32H
V3	6	12.00A	11.51C	10.86I	11.46A
	12	11.66B	11.23F	10.30N	11.06B
V4	6	11.45D	11.10G	10.62L	11.06C
	12	11.26E	10.68K	9.80R	10.58E
Mean		11.051A	10.578B	9.883C	

Figures showing the same letter(s) are statistically not different from one another (LSD Test).

The results in Table 3 show the effect of soaking on phytic acid content in soybean in tap water, pH 4 and pH 6 solutions in the presence of 1% sodium chloride at 30˚C for 6 and 12 h. The analysis of variance is given in Appendix-II. Soaking for 6 h, in V1 of soybean, the phytic acid was reduced from its original level (12 mg/g) in water, pH 4 and pH 6 solutions to

11.04 mg, 10.68 mg and 10.08 mg/g and in V2 (11 mg/g) to 10.24 mg, 9.72 mg and 9.34 mg/g, respectively. Similarly in V3, the phytic acid (13 mg/g) was reduced to 12 mg, 11.51 mg and 10.86 mg/g and in V4 (12.50 mg/g) to 11.45 mg, 11.10 mg and 10.62 mg/g, respectively. Soaking the soybean for 12 hours at 30˚C, in tap water, pH 4 and pH 6 solutions the phytic acid in V1 was reduced to 10.84 mg, 10.30 mg and 9.43 mg/g and in V2 to 9.92 mg, 9.40 mg and 8.64 mg/g respectively. In V3, the phytic acid reduced to 11.66 mg, 11.23 mg and 10.30 mg/g and in V4 11.26 mg, 10.68 mg and 9.80 mg/g respectively. Statistically, soaking all the four varieties of soybean in tap water, pH 4 and pH6 solutions in the presence of 1% sodium chloride for both times 6 and 12 h at 30˚C, phytic acid significantly reduced ($P < 0.05$). The effect of other processing on phytic acid content in various food has been reviewed by other workers.

Lyer *et al* (1980) found that when Pinto, Great Northern and Red kidneybeans were soaked in distilled water for 18 h at room temperature the phytate content of beans was appreciably reduced to 52.7, 69.6 and 51.7% respectively. However, they noticed a somewhat lessen phytate hydrolysis when the beans were soaked in a mixed solution (2.5% sodium chloride + 1.5% sodium bicarbonate + 9.5% sodium carbonate + 1.0% sodium tripolyphosphate) at pH7 and room temperature of 21˚C.

Prattley *et al* (1982) reported that in commercial concentrate of soya isolates free phytic acid concentration was obtained at pH 5. On neutralization of the isolates, the formation of phytate-protein complexes increased. Under alkaline conditions, divalent cations (e.g. Ca, Mg, and Zn) interact with phytic acid and mediate in protein-phytate interaction. Under acid conditions the protein form an insoluble complex with phytic acid. Calcium produce different effect at higher pH (>6). Soluble protein-calcium phytic acid complexes were formed which were less stable to heat and dissociation above pH 10. Since this interaction occurred only in the presence of calcium, a salt linkage is implicated in which divalent cations bind to the phytic acid in the form of a complex. Prattley *et al* (1982) therefore, proposed that either addition of divalent cations at low pH could effectively remove phytate from soya products by ultra filtration.

Chompreeda and Fields (1984) have reported that autoclaving the soybean meal at 121˚C for 30 min reduced phytate content by 17.5%. The corn meal which containing 220.3 mg/100g phytate phosphorous, after fermentation at 32˚C for 4 h was decreased by 77.7%, whereas the same fermentation in meal mixture (90% corn-10% soybean), (85% corn-15% soybean) and (80% corn-20% soybean), decreased the phytate phosphorus by 44.4%, 50.2% and 35.5% respectively.

Serriano *et al* (1985) reported that phytic acid in rape seed flour was reduced at pH 5.15 with subsequent dialysis or by phytase treatment. The effect of phytate reduction on the rate and extent of protein and amino acid digestibilities were determined using *in vitro* pepsin-pancreatin proteolysis method. Phytic acid reduction (51%) increased the release of many amino acids. Further reductions of phytic acid (89%) not enhance this process. It was suggested that further removal of phytate did not improve the digestibility of protein.

Table 4

Retention of phytic acid in soybean flour (mg/g) after soaking at 30˚C for different times with 2% sodium bicarbonate ($NaHCO_3$). Before soaking phytic acid level in soybean was in V1 (12mg/g), V2 (11 mg/g), V3 (13 mg/g) and V4 (12.5 mg/g)

Variety	Time(h)	Phytic acid (mg/g) Soybean flour			Mean
		Water	PH 4	pH 6	
V1	6	8.45C	7.28H	6.67M	7.47C
	12	6.66N	6.30P	5.65V	6.20G
V2	6	7.68E	6.68L	6.12R	6.83D
	12	5.96T	5.58W	5.10X	5.55H
V3	6	9.18A	7.92D	7.27I	8.12A
	12	7.35G	6.58O	6.07S	6.68E
V4	6	8.86B	7.56F	6.90J	7.78B
	12	6.80K	6.18Q	5.84U	6.27F
Mean		7.617A	6.760B	6.203C	

Figures showing the same letter(s) are statistically not different from one another (LSD Test).

Table 5

Retention of phytic acid in soybean flour (mg/g) after soaking at 30˚C for different times with 1% sodium chloride (NaCl) + 2% sodium bicarbonate ($NaHCO_3$). Before soaking phytic acid level in soybean was in V1 (12 mg/g), V2 (11 mg/g), V3 (13 mg/g) and V4 (12.5 mg/g)

Variety	Time(h)	Phytic acid (mg/g) soybean flour			Mean
		Water	pH 4	pH 6	
V1	6	7.48	5.72	5.28	6.16B
	12	6.20	5.48	4.12	5.27CD
V2	6	6.80	5.33	4.39	5.51C
	12	5.81	5.05	3.60	5.15D
V3	6	7.92	6.21	5.70	6.61A
	12	6.71	5.82	4.28	5.60C
V4	6	7.80	6.08	5.56	6.48AB
	12	6.65	5.57	4.22	5.48CD
Mean		6.921A	5.657B	4.643C	

Figures showing the same letter(s) are statistically not different from one another (LSD Test).

Table 6
Effect of food additives on average percent reduction of phytic acid of four varieties of soybean soaked in water and solutions of pH4 and pH 6 for 6 h

Food additive used	Phytic acid (% reduction)		
	Water	pH 4	pH 6
No additive	8	6	12
1% NaCl	8	11	16
2% $NaHCO_3$	40	40	45
1% NaCl + 2% $NaHCO_3$	38	52	56

Table 7
Effect of food additives on average percent reduction of phytic acid of four varieties of soybean soaked in water and solutions of pH 4 and pH 6 for 12 h

Food additive used	Phytic acid (% reduction)		
	Water	pH 4	pH 6
No additive	7	10	18
1% NaCl	10	14	21
2% $NaHCO_3$	45	50	53
1% NaCl + 2% $NaHCO_3$	48	55	68

The effect of soaking on phytic acid content in soybean in tap water, pH 4 and pH 6 solutions in the presence of 2% $NaHCO_3$ at 30˚C for 6 and 12 h is shown in Table 4 and analysis of variance is given in Appendix-III. Soaking for 6 h, in V1 of soybean, the phytic acid was reduced from its original level (12 mg/g) in water, pH 4 and pH 6 solutions to 8.45 mg, 7.28 mg and 6.67 mg/g, and in V2 (11 mg/g) to 7.68 mg, 6.68 mg and 6.12 mg/g, respectively. Similarly in V3, the phytic acid (13 mg/g) was reduced to 9.18 mg, 7.92 mg and 7.27 mg/g and in V4 (12.50 mg/g) to 8.86 mg, 7.56 mg and 6.90 mg/g respectively. Soaking the soybean for 12 hours at 30˚C, in tap water, pH 4 and pH 6 solutions the phytic acid in V1 was reduced to 6.66 mg, 6.30 mg and 5.65 mg/g and in V2 phytic acid reduced to 5.96 mg, 5.58 mg and 5.10 mg/g respectively. In V3, the phytic acid reduced to 7.35 mg, 6.58 mg and 6.07 mg/g and in V4 phytic acid reduced to 6.80 mg, 6.18 mg and 5.84 mg/g respectively. In this treatment the effect of pH6 for 12 h was most effective in lowering the phytic acid in soybean. Statistically, the results show that soaking in tap water, pH4 and pH6 solutions in the presence of 2% $NaHCO_3$ for both times 6 and 12 h significantly reduced ($P < 0.05$) the level of phytic acid in all four varieties of soybean.

The results in Table 5 show the effect of soaking on phytic acid content in soybean in tap water, pH 4 and pH 6 solutions in the presence of 1% sodium chloride (NaCl) + 2% sodium bicarbonate ($NaHCO_3$) at 30˚C for 6 and 12 h. The analysis of variance is given in Appendix-IV. Soaking for 6 hours, in V1 of soybean the phytic acid was reduced from its original level (12 mg/g) in water, pH4 and pH6 solutions to 7.48 mg, 5.72 mg and 5.28 mg/g and in V2 (11 mg/g) to 6.80 mg, 5.33 mg and 4.39 mg/g, respectively. Similarly in V3 the phytic acid (13 mg/g) was reduced to 7.92 mg, 6.21 mg and 5.70 mg/g and in V4 (12.50 mg/g) to 7.80 mg, 6.08 mg and 5.56 mg/g, respectively. Soaking soybean for 12 h at 30˚C in tap water, pH 4 and pH 6 solutions the phytic acid in V1 was reduced to 6.20 mg, 5.48 mg and 4.12 mg/g and in V2 to 5.81 mg, 5.05 mg and 3.60 mg/g, respectively. In V3, the phytic acid reduced to 6.71 mg, 5.82 mg and 4.28 mg/g and in V4 to 6.65 mg, 5.57 mg and 4.22 mg/g respectively (Table 6 & 7).

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Pak. J. Sci. Ind. Res. 2004 ***47*** *(2) 153 - 156*

LEAF MODIFICATIONS TO QUANTIFY YIELD, EARLINESS AND FIBRE TRAITS IN *GOSSYPIUM HIRSUTUM* L.

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Ten BC_4 : F_2 back-cross derived near-isolines developed from ten different cross combinations, differing in only leaf shape i.e. Normal, Okra, Sub-okra and Super-okra were compared for quantifying their yield, earliness and fibre traits. Sub-okra leaf cotton (L^{u_2}) was observed as an appropriate replacement for the normal leaf to improve the traits. Sub-okra types in all the combinations were superior for most of the traits. On an average over the populations, Sub-okra gave 19.7% higher yield, 1.5% earliness, 4.7% longer fibre and 2.1% more uniform fibre than the normal leaf isolines. Nevertheless, Sub-okra ginned and gave equally better fibre strength with the normal leaf. Yield, earliness, longer and uniform fibre superiority of Sub-okra leaf cotton over the normal leaf coupled with established insect resistance of modified leaves suggested that the potentiality of mutant leaves be exploited in future breeding programmes.

Key words: Leaf modifications, Fibre and earliness characters, *Gossypium hirsutum* L.

Introduction

Very little effort has yet been put to breed cotton for more open canopy types. However, in the recent past, emphasis are being diverted to breed cotton with modified leaf shape BH - 41 is the only Okra leaf type bred and released in Pakistan. Advantages of using open-canopy (Okra, Sub-okra and Super-okra) cotton are numerous as reported by several workers. Jones (1982) summarized their earliness and pest resistance, whereas, Landivar *et al* (1983), using model studies, characterized that under favourable moisture conditions, leaf shapes other than the normal might produce higher yields. Wells *et al* (1986) reported that Sub-okra leaf canopy photosynthesis was 7.0% greater than that of normal leaf near-isolines and is one of the causes for increased yields associated with Sub-okra leaf trait. Meredith (1984), using F_3 bulk hybrid populations of Okra (L^{o_2}), Sub-okra (L^{u_2}), and Super-okra (L^{s_2}) leaves, observed a significant lint yield increase of 4.8% in Sub-okra over normal leaf cotton. It was Burton (1966) who suggested using isolines to compare mutants with the normal leaf. After that suggestion, Meredith (1984) compared the yield of eight BC_4: F_3 Sub-okra leaf (L^{u_2}) lines with the normal leaf (l_2) cotton plants and reported that Sub-okra (L^{u_2}) cotton gave significantly higher yield (3.0%) than normal leaf. From his studies, it was conclude that the use of Sub-okra to replace normal leaf cotton offers a potentiality in yield increase. Recently, comparative studies were conducted by El-Zik and Thaxton (1993) at Texas A&M, USA, who besides yield, compared earliness and fibre traits of Okra cotton with the normal leaf. Comparing with the normal leaf, they reported that Okra leaf produced less or equal lint yield, earlier in maturity, less or equal in lint %, also produced longer, stronger and equally uniform and fine fiber. It appears that there is a room for comparing all the leaf types simultaneously and observe their potentiality over the normal leaf. The present study was carried out to evaluate yield, earliness and fibre differences of genetically similar (BC_4 : F_2) isolines of each Okra, Sub-okra, Super-okra and normal leaves produced by the backcross breeding method (Burton 1966).

Materials and Methods

Ten different populations, of which six segregated into Okra, Sub-okra, Super-okra and normal leaves, whereas, four segregated into Okra, Sub-okra and normal but not in Super-okra leaf types. The strains with mutant leaf genes were considered as donor parents of their respective recipient normal leaf types. Four back crosses were made for each of the ten cross combinations. In F_1 and subsequent generations, each leaf type was back crossed with their respective original normal leaf parents. In this way, four back crosses and one self (BC_4 : F_2) were made, thus all the leaf types had become near-isolines of their corresponding normal leaf parents except retaining mutant genes in Okra, Sub-okra and Super-okra populations. Since, six crosses segregated into four types (Normal, Okra, Sub-okra and Super-okra) and four segregated into three leaf types (Normal, Okra and Sub-okra), thus in total, 36 BC_4 : F_2 populations were produced. All the 36 populations were replicated four times in a split plots with randomized, complete block design arrangement, treating populations as main plots and leaf types as sub-plots. The trial was carriedout at Cotton Research Institute, Sakrand during crop

Table 1
Mean yield, lint % and earliness of various near-isogenic cotton differing in leaf shape

Population	Seed cotton yield (g)					Lint (%)					Earliness (% of bolls picked at 140 DAP)				
	Normal	Okra	Sub okra	Super okra +	Av.	Normal	Okra	Sub okra	Super okra +	Av.	Normal	Okra	Sub okra	Super okra +	Av.
Rode okra x CRIS - 52	72.1	63.1	73.9	41.0	62.5	36.5	35.0	36.0	34.1	35.4	65.5	83.7	75.4	84.3	75.6
Rode okra x 9L - 34 - ICCC	68.3	62.8	75.1	54.2	65.1	35.6	34.0	35.4	33.8	34.7	72.8	91.6	89.8	91.8	86.5
Super okra x CRIS - 9	75.8	71.0	85.0	40.3	68.0	36.7	34.9	36.8	34.0	35.6	80.7	96.5	89.2	92.3	89.7
LA_2 x 9L - 34 -ICCC	47.5	45.3	72.1	-	55.5	34.8	33.1	34.6	-	34.2	71.7	93.6	85.4	-	83.6
BH - 41 x CRIS - 21	77.8	74.8	83.5	-	78.7	37.8	35.2	37.8	-	36.9	61.0	73.0	70.0	-	68.0
BH - 41 x NIAB - 78	93.4	77.3	115.2	-	95.3	34.0	33.2	34.1	-	33.8	67.2	74.3	77.8	-	73.7
BH - 41 x CRIS - 9	94.3	74.9	101.3	-	90.2	35.2	34.1	35.6	-	35.0	63.0	71.2	76.4	-	70.2
Okra, T. Jam x CRIS - 127	91.9	62.4	130.5	45.8	82.6	36.6	34.2	36.4	33.9	35.7	81.1	84.7	85.1	99.4	87.6
Rode okra x CRIS - 129	68.8	44.1	107.2	54.3	69.3	36.8	34.8	36.6	34.0	35.5	65.7	85.8	84.8	90.7	81.7
Super okra x CRIS - 52	49.5	47.3	75.8	34.1	51.7	36.6	34.2	36.1	34.1	35.3	78.9	95.8	94.7	98.7	92.0
Experimental mean	73.9	62.8	92.0	45.0	71.9	36.1	34.3	35.9	34.0	35.2	69.9	85.0	82.9	92.9	80.9

LSD (0.05); For main plots, Seed cotton yield; 17.7, Lint %; 1.6, Earliness; 16.0. LSD (0.05); For sub-plots, Seed cotton yield; 13.2, Lint %; 1.5, Earliness; 12.2. DAP = Days after planting + Some averages excluded Super-okra populations.

Table 2
Mean fibre length, uniformity ratio and fibre strength of various near isogenic cotton differing in leaf shape

Population	Fibre length (mm)					Uniformity ratio					Fibre strength lbs/sq inch				
	Normal	Okra	Sub okra	Super okra +	Av.	Normal	Okra	Sub okra	Super okra +	Av.	Normal	Okra	Sub okra	Super okra +	Av.
Rode okra x CRIS - 52	26.6	26.8	27.0	26.0	26.6	44.7	44.8	46.3	44.9	45.2	97.5	98.5	98.1	98.2	98.1
Rode okra x 9L - 34 - ICCC	27.0	27.9	28.7	26.1	27.4	45.2	45.7	46.0	43.1	45.0	95.1	96.3	95.8	96.5	95.9
Super okra x CRIS - 9	25.1	24.9	26.7	24.8	25.4	45.8	44.9	46.4	44.8	45.5	97.8	98.1	98.0	98.6	98.1
LA_2 x 9L - 34 -ICCC	25.9	26.9	27.8	-	26.9	45.8	41.8	47.0	-	44.9	95.7	96.8	95.9	-	96.1
BH - 41 x CRIS - 21	27.2	27.2	27.8	-	27.4	46.5	45.7	47.9	-	46.7	98.1	99.8	98.3	-	98.7
BH - 41 x NIAB - 78	26.5	27.3	28.6	-	27.5	45.7	45.6	49.3	-	46.9	98.0	98.4	98.2	-	98.2
BH - 41 x CRIS - 9	26.5	27.5	28.5	-	27.5	46.4	46.0	46.5	-	46.3	97.3	97.9	97.8	-	97.7
Okra, T. Jam x CRIS - 127	26.3	26.8	27.1	27.0	26.8	47.2	48.2	47.4	46.0	47.2	98.8	99.1	98.9	99.2	99.0
Rode okra x CRIS - 129	25.7	25.4	26.7	24.5	25.6	45.8	46.0	46.3	45.7	46.0	98.7	99.0	98.8	99.1	98.9
Super okra x CRIS - 52	26.3	26.0	27.5	27.1	26.7	48.0	46.3	48.3	46.2	47.2	97.8	98.0	98.9	98.1	98.2
Experimental mean	26.3	26.4	27.6	25.9	26.8	46.1	45.5	47.1	45.1	46.1	97.5	98.2	97.9	98.3	97.9

LSD (0.05); For main plots, Fibre length; 1.8, Uniformity ratio; 1.9, Fibre strength; 2.1. LSD (0.05); For sub-plots, Fibre length; 1.1, Uniformity ratio; 0.8, Fibre strength; 1.3. DAP; Days after planting + Some averages excluded Super-okra populations.

year 2000. The plot size was 45′ x 15′. The distance between rows and plants were kept at 2.5′ and 9.0″, respectively. For recording the data, 15 random plants of specified leaf types from each genotype in a replication were tagged and treated as index plants. Earliness was recorded as number of open bolls divided by the total bolls obtained after 140 days of planting calculated in %. The yield was recorded in g per plant and lint in % calculated as the proportion of seed and lint per plant. Fibre length was measured in millimeter, fibre uniformity as the ratio of 25.0 and 50.0% span length and fibre strength in lbs/sq inch.

Results and Discussion

Ten different cross combinations with four different leaf morphologies were compared for six important traits of cotton and the results summarized are presented in Table 1, 2 and 3. For yield per plant, the genotypes differed significantly and the combination BH - 41 x NIAB - 78, on average over populations, gave maximum yield of 95.3 g. Among the leaf types, averaged over the populations (populations and genotypes hereafter will be used inter changeably) Sub-okra types gave higher yields (92.0) followed by normal leaf (73.9 g), however,

Super-okra leaf ranked poor (45.0 g). These results suggested that Sub-okra populations produced 19.7% more yield than the normal leaf. There was no genotype x leaf shape interaction for any trait because all the leaf shapes behaved similarly within the genotypes. The yield superiority of Sub-okra over normal leaf was also supported by Meredith and Randy (1987) and Meredith *et al* (1996). A high yielding variety, Siokra, with Okra leaves, for the first time was introduced commercially into Australia (Thomson 1985). Jones *et al* (1978) and Soomro *et al* (1998) also observed that Okra leaf plants out yielded normal leaf cultivars by 5.0 and 4.4% respectively. The genotypes have ginned differently and the highest lint% (36.9) was obtained by BH - 41 x CRIS - 121 and the lowest (33.8%) was obtained by BH - 41 x NIAB - 78. For leaf type, averaged over genotypes, Sub-okra ginned similar to normal and better than the Okra and Sub-okra leaves (Tables 1 and 3). El-Zik and Thaxton (1993) also reported non-significant difference in lint percentage between Sub-okra and normal leaf genotypes. The percent of bolls opened after 140 days of planting averaged over population varied significantly (Table 1) where population Super-okra x CRIS - 9 opened maximum number of bolls (89.7%). Among the leaf types averaged over populations, all the mutant leaf populations were earlier than the normal leaf, nonetheless Super-okra which had comparatively more open canopy than other leaf shapes was earliest of the all (92.9%).

The open canopy of mutant leaves probably has contributed more towards light interception into the plant canopy Table 1. Jones (1982) observed that mature Okra leaf plants have about 40.0% less foliage than normal leaf, thus permit 70.0% more sun light to penetrate the canopy. Meredith *et al* (1996) suggested that earliness is indicated by yield at first harvest and also observed that Sub - okra isolines yielded significantly higher than normal leaf at first harvest, however, at second harvest the yield differences were not different between the leaf types. Genotypes varied significantly for fibre length where population BH - 41 x NIAB - 78 recorded longer fibre (27.5 mm). The leaf types averaged over genotypes also differed significantly and Sub-okra populations averged longer fibre (27.6) mm) than the normal and other mutant leaf populations Table 2. The Super-okra however, produced rather smaller fibre (25.9 mm) probably due to less food reserve and smaller leaf lobbing consequently have affected the fibre to grow longer. El-Zik and Thaxton (1993) recorded similar results where Okra leaf produced longer fibre than the normal leaf. The populations differed significantly in fibre uniformity where Super-okra x CRIS -52 and Okra, T. Jam x CRIS - 127, were at par with each other but both populations gave equally more uniform fibre than other populations Table 2. Among the leaf types Sub-okra populations averaged significantly more uniform fibre (27.1%) than the normal and other mutant types. However, the lowest uniformity ratio was recorded in Super-okra populations (45.1%). Again this could be attributed to less food reserve in Super-okra leaves, consequently retarded the fibre growth and eventually uniformity ratio. Contrary to our findings, El-Zik and Thaxton (1993) observed no significant difference between the normal and mutant leaves for fibre uniformity. The populations produced significantly variable fibre strength and the cross BH - 41 x CRIS - 121 expressed the maximum strength of 98.7 lbs/sq inch (Table 2), however, among the leaf types, no significant difference was recorded.

Table 3
Comparison of mutant leaves with the normal leaf for various traits

Trait	Leaf types		
	Okra	Sub-okra	Super-okra
Seed cotton yield	=	<	>
Earliness	>	>	>
Lint%	<	=	<
Fiber length	<	>	<
Uniformity ratio	<	>	<
Fiber strength	>	=	>

=; Declared non-significant when the difference was smaller than the LSD (0.05) value, >; Declared significantly higher when the difference was equal or higher than the LSD (0.05) value, <; Declared significant lower when the difference was equal or less than the LSD (0.05) value.

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Short Communication

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STUDY OF SOME KINETIC PARAMETERS FOR CITRIC ACID BIOSYNTHESIS BY *ASPERGILLUS NIGER* MUTANT NG - 110 USING SHAKE FLASK TECHNIQUE

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Citric acid can be produced from various microorganisms such as bacteria, filamentous fungi and yeast by applying various fermentation techniques. Because of its high solubility, palatability and low toxicity, citric acid has now become one of the most commonly used acids. Approximately, 75% of this compound is used as food acidulate and 12% in pharmaceutical industry (Haq *et al* 2001). Various fungi have been evaluated for citric acid production but best one for abundant citric acid production is *Aspergillus niger* (Maddox and Brooks 1998). The present study is concerned with the effect of pH and various concentrations of $K_4Fe(CN)_6$ and K_2HPO_4 on citric acid bio-production and their kinetic analysis.

Organism and inoculum preparation. The mutant strain of *Aspergillus niger* NG - 110 has been screened for citric acid accumulation from various available cultures in Biotechnology Research Centre of Government College University, Lahore, Pakistan developed by the treatment of ultraviolet irradiation (1.6×10^2 j / m^2 / S) for different time intervals (5 - 45 min). The culture was maintained on sterilized potato dextrose agar medium (Diced potato 200 g / l, Dextrose 20 g / l and Agar 15 g / l), pH 4.5 and stored at 4°C in the refrigerator. Conidial inoculum was used in the present study. Conidia from 3 - 5 days old slant culture were used for inoculation. The conidial suspension was prepared in sterilized 0.005% Monoxal O.T. (Dioctyle ester of sodium sulfosuccinic acid). One ml of the suspension contained 1.5×10^7 conidia. The count was made on a haemocytometer slide bridge under microscope.

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Fermentation technique. Submerged fermentation technique in 250 ml Erlenmeyer flasks was employed to investigate the optimum conditions for maximal production of citric acid. Twenty-five ml of clarified cane molasses with 15% sugar level (initial pH 6.0) was taken in each of the flasks. After sterilization, the flasks were cooled at room temperature and inoculated with 1.0 ml of conidial suspension. The flasks were then incubated at a rotary incubator shaker (Gallenkamp PLC, UK) at 30°C for 7 days. The shaking speed was kept at 200 rpm. After incubation, fermented broth was filtered through pre-weighed Whatman filter paper No.44 to remove the fungal mycelia and filtrate was used for the estimation of citric acid and residual sugar contents.

Analytical techniques. The filtrate was analysed for the estimation of residual sugar gravimetrically by DNS method (Tasun *et al* 1970) and citric acid anhydrous was estimated spectrophotometrically using pyridine-acetic anhydride method as reported by Marrier and Boulet (1958) whereas, for the calculation of dry cell mass, mycelia were thoroughly washed with tap water and dried at 105°C for two hours (Haq and Daud 1995).

Any increase or decrease in the pH greatly reduced citric acid biosynthesis. It might be due to that at lower pH the ferrocyanide was more toxic for the growth of mycelium in molasses medium. This has been reported by Pessoa *et al* (1984) whereas, a higher pH leads to the accumulation of oxalic acid. Fermentation medium with initial pH 6.0 resulted in maximum citric acid production (65.20 ± 0.2 g / l). Any increase or decrease in the phosphate quantity reduced citric acid production due to improper growth of mould mycelia. A high concentration of phosphate in the fermentation medium promotes more growth and less acid production (Khan *et al* 1970).The sugar consumption and mycelial dry weight were 93.50 ± 2.0 and 16.00 ± 0.3 gl / l, respectively. The percentage yield of citric acid on the basis of sugar fermented was 69.73%. Figure 1 shows the comparison of specific growth rate of NG - 110 (µg / h) for citric acid production.

Potassium ferrocyanide concentration. Effect of addition of different concentration of potassium ferrocyanide (50 - 300 ppm) on citric acid production by *Aspergillus niger* NG - 110 from molasses was investigated in shake flask. The fermentation medium containing 200 ppm potassium ferrocyanide showed the maximum citric acid production (69.3 ± 0.8 g / l). The sugar consumption and mycelial dry weight were 83.5 $\pm$ 4.0 and 25.3 ± 0.4g / l, respectively. The percentage yield of citric acid on the basis of sugar consumed in the medium containing 200 ppm $K_4Fe(CN)_6$ was 80.99%. A decrease in citric acid production was observed, when the concentration of potassium ferrocyanide was increased or decreased from

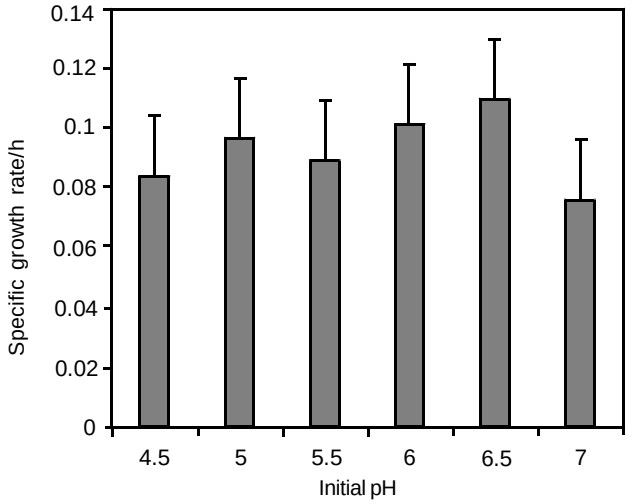

Fig 1. Comparison of specific growth rate for citric acid production at various initial pH.

Kinetic parameter: Specific growth rate, $\mu(h^{-1})$ = g cell mass produced / l / h, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p < 0.05$.

200 ppm. Product and growth yield coefficients as kinetic parameters were also studied for citric acid at potassium ferricyanide concentration of 200 ppm (Fig 2).

Dipotassium hydrogen phosphate concentration. The effect of addition of different concentrations of K_2HPO_4 ranging from 0.15% - 0.30% w/v, on citric acid fermentation by *Aspergillus niger* NG - 110 in shake flask was studied. The addition of K_2HPO_4 in the fermentation medium resulted in maximum citric acid production (81.21± 0.2 g / l) of K_2HPO_4. Sugar consumption and mycelial dry weight were 92.20 ± 3.5 and 20.40 ± 0.2 g / l, respectively. The percentage yield of citric acid on the basis of sugar used was 88.11%. The mould growth was in the form of small round pellets, observed in the fermented broth. Product and growth yield coefficients as kinetic parameters were also studied for citric acid production using different concentrations of dipotassium hydrogen phosphate (Fig 3). The values for Yp/s and Yp/x (g / g) at 2 g / l K_2HPO_4 were found to be significant.

The kinetic parameters such as growth yield coefficients (Y p/s and Y p/x in g / g), were also undertaken. The mutant strain of *Aspergillus niger* NG - 110 showed improved values for Y p/s and Y p/x. Similar kind of work has also been reported by Pirt (1975). Maximum growth in terms of specific growth rate (μ / h) was only marginally different during growth of mutant *A. niger* NG - 110 on 150 g / l carbohydrates in molasses at 30°C (than 32°C or 165 g / l sugar). Therefore, when the culture was monitored for Y p/s and Y p/x, there was a significant enhancement in these variables at optimal nutritional conditions.

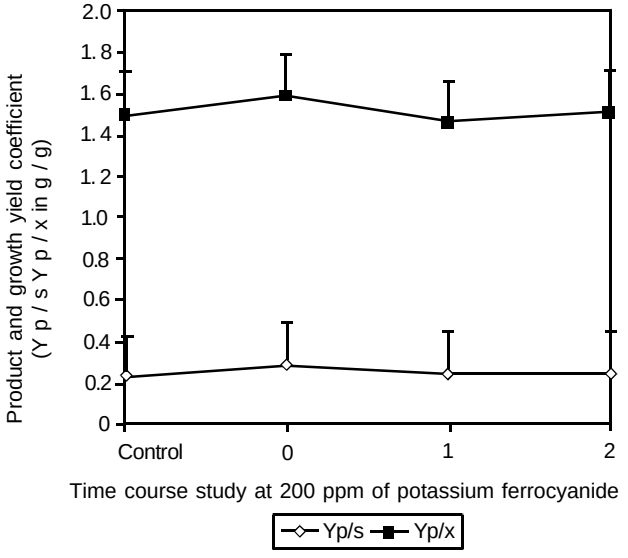

Fig 2. Comparison of product and growth yield coefficients for various time intervals at potassium ferrocyanide concentration of 200 ppmn.

Kinetic parameter: Y p/s = g citric acid produced/g substrate consumed; Y p/x = g citric acid produced/g cells formed; Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p < 0.05$.

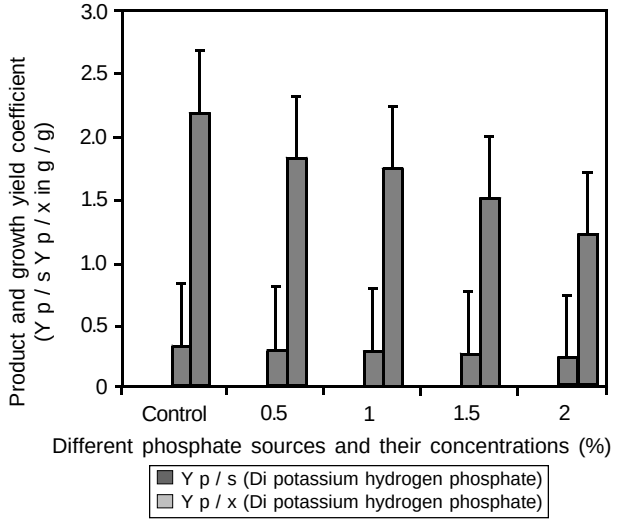

Fig 3. Comparison of product and growth yield coefficients for citric acid production.

Kinetic parameter: Y p/s = g citric acid produced/g substrate consumed; Y p/x = g citric acid produced/g cells formed; Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p < 0.05$.

This indicated that the mutant strain used in the current studies is a faster growing organism and has the ability to overproduce citric acid without additional replacements. The study is directly substantiated with the findings of Rajoka *et al* (1998). Maximum values for Y p/s, were several folds improved

over the previous workers (Pirt 1975; Roehr 1998; Kamal *et al* 1999).

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Short Communication

Pak. J. Sci. Ind. Res. 2004 ***47****(2) 160 - 162*

PHYTOCHEMICAL ANALYSES AND ANTIMICROBIAL ACTIVITIES OF THE LEAF AND STEM BARK EXTRACTS OF *GARCINIA KOLA* - HERKEL (FAMILY GULTIFERAE)

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Preliminary phytochemical analyses carried out on the leaves and stem bark extracts of *Garcinia kola* revealed the presence of glycosides, saponins, tannins and flavonoids. Alkaloids were present only in the leaf extract. Thin layer chromatography (TLC) on silica gel using different solvent systems showed the alkaloids to be both of salt and basic forms. Different concentrations of methanolic extracts (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) were tested on cultures of bacteria and fungi to determine their sensitivity and minimum inhibitory concentrations (MIC). Antibiotic gentamycin (1mg/ml) was used as the standard drug. The stem bark and leaf extracts of *Garcinia kola* had very high inhibitory activities, only on *Staphylococcus aureus* with MIC of 100µg / ml and 150µg/ml, respectively. The stem bark and leaves of this plant can be used for the treatment of diseases caused by *Staphylococcus aureus.*

The use of plants as medicines is due to the presence of secondary metabolites in form of alkaloids, saponins, glycosides, anthraquinones and volatile oils which may be present in the roots, barks, stems, fruits, flowers leaves and seeds of plants has promoted their use as medicines, (Gill 1992). *Garcinia kola* commonly known as bitter kola. It is a plant indigenous to the West and Central African sub - regions. It is easily recognised, by its fine, hairy flowers and large fruits. It has numerous pharmacological effects. Fresh fruit of *Garcinia kola* is used as a food; hot water extracts of the dried fruits are used in treating arthritis, liver disease, and cough; while the dried fruit is used as an antiseptic for cuts, sore throats and laryngitis, (Iwu and Igboko 1982). The hot water extract is also used in treating asthma, dysmennorhea, gastroenteritis, diarrhoea, hepatitis, abdominal colic and as a general antidote (Elujoba 1995).

The bark of *Garcinia kola* is used for fever cough, as an antihelmintic and for respiratory disease while the hot water extract has anti-oxidant activity (Ebana *et al* 1991). The alkaloid fractions of the bark has spasmolytic activity, while the ethanolic extracts and glycoside mixtures have antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Streptococci* and antifungal activity against *Aspergillus spp.* (Braide 1989; Madunyi 1995).

This study investigated the phytochemical constituents of the stem bark and leaves of *Garcinia kola* and evaluated the antimicrobial activities of their extracts on some human isolates from a reference hospital in Benin City, Nigeria.

Plant material. The fresh leaves of *Garcinia kola* were collected from Okodobor village, near Benin City, while the stem bark was collected from Iyowa village close to Okada town, Benin City, Nigeria. They were identified by, Alhaji Abubakar-a taxonomist. The leaves were dried in an oven at a temperature of 40-50˚C and ground into fine powder with the aid of a grinding machine. The bark was chopped into bits and pieces and also dried in the oven at 40-50˚C for two weeks. It was then ground into powder. Both the stem bark and leaf powders were stored in clean dry containers at 5˚C until needed.

Preparation of extracts. *Leaves*: One hundred gram of powdered *Garcinia kola* leaves were extracted with one litre of methanol using the Soxhlet extractor and then concentrated with the aid of the rotary evaporator. Twenty-two gram of the methanolic extract was dissolved in 22 ml of distilled water (w/v).

Stem bark: Two hundred gram of powdered stem bark of sample was put into 800 ml of water and macerated for two days with occasional stirring. It was then filtered and the filtrate concentrated over a water bath. Approximately, 4.85g of stem bark extract was obtained.

Phytochemical analysis: Extracts were subjected to phytochemical analysis to detect glycosides, flavonoids, tannins, saponins and alkaloids using the standard analytical procedures (Shoppe 1964; Harborne 1973; Trease and Evans 1983). Thin layer chromatography (TLC) using silica gel as adsorbent and different solvent systems and as well as Dragendorff's spray reagent were used to the alkaloidal spots.

Antimicrobial activity: Agar diffusion assay technique of Tramer and Fowler was used. Autoclaved nutrient agar medium was immediately place in a 55˚C water bath. When cooled, 18ml aliquots of the medium were aseptically poured

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Table 1
Phytochemical analyses of the stem bark and leaf extracts of *Garcinia kola*

Compound	Leaf Extract	Stem Bark Extract
Glycosides	Present	Present
Flavonoids	"	"
Tanins	"	"
Saponins	Absent	Absent
Alkaloids	Present	Absent

into round, plastic petri dishes and allowed to solidify and cool on room temperature. Then, excess moisture on the surface of the plates was evaporated in an incubator (30˚C) for 24 h. Six wells were punched in the solidified medium using a sterile glass tube (6 mm diameter). The disks of agar were removed from the plate using a vacuum device. Crude extracts of the samples were diluted to the desired concentrations and were immediately added into the wells in the agar plates, in triplicates and allowed to solidify. A soft agar overlay consisting of 10^4 CFU/ml of the appropriate test culture and 5ml of molten nutrient agar was poured over the surface of the plates. Plates were then incubated for 24 h at 37˚C, and the diameters of the inhibition zones were measured using a vernier caliper. Mean diameters of triplicate inhibition zones were calculated. Standard drug was gentamycin antibiotics (1mg/ml), which was already in solution. The tests organisms used were clinical isolates obtained from a reference hospital in Benin City, Nigeria and included; *Staphylococcus aureus, Klebsiella pneumonia Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger and Geotrichum species.*

The impregnated paper disc method described Casal 1979 and Chung *et al* 1990 was also used to determine the antimicrobial activities of the two extracts. The minimum inhibitory concentrations (MIC) of both the stem bark and leaf extracts were also determined by using varying concentrations of the extracts and testing using the method already described.

Phytochemical tests carried out on the leaves of *Garcinia kola* revealed the presence of glycosides, saponins, flavonoids, alkaloids and tannins. Thin layer chromatography (TLC) on silica gel using different solvent systems (methanol, water and ammonia, Benzene/Methanol showed the alkaloid to be both of salt and basic forms.

The stem bark also revealed the presence of glycosides, flavanoids and tannins. Alkaloids were absent (Table 1). Antimicrobial properties of plants have been linked to the presence of glycosides, saponins, tannins, terpenes alkaloids, unsaturated terpenes and steroids, especially tannins (Leven *et al* 1979; Hashem *et al* 1980). All test organisms except *Staphylococcus aureus* showed no activity. Table 2 also shows the antibacterial activity of the leaf and stem bark extracts.

Plants are made up of primary and secondary metabolites. These metabolites in most cases have medicinal values, (Iwu and Anyanwu 1982). Results from the phytochemical analyses of the stem bark and leaves of *Garcinia kola* have revealed the presence of flavonoids, tannins and glycosides. Saponins and alkaloids were detected only from the stem bark and the alkaloids were present in both the basic and salt forms.

The microbiological evaluations of the stem bark extract showed that the aqueous extract only had inhibitory activity against *Staphylococcus aureus* at all concentrations used, while there was no activity on the other test organisms. This showed the extract to be highly potent but very limited in its spectrum of activity. The MIC of the aqueous extract of the stem bark was found to be 100mg/ml on *Staphylococcus aureus.*

For the leaf extract, the result only showed inhibitory activity on *Staphylococcus aureus* using methanolic solvents. The minimum inhibitory concentration of the extract on *Staphylococcus aureus* was 150mg/ml. *Garcinia kola* is well used in traditional medicine for various ailments and diseases and can be very readily used in the treatment of diseases caused by *Staphylococcus aureus*.

Table 2
Zones of Inhibition of *Garcinia kola* leaf and stem bark extracts after 24hr on *Staphylococcus aureus.*

Concentration of Extract (Leaves) (mg/ml)	Zone of Inhibition (mm)	Concentration of Extract (Stem bark) (mg/ml)	Zone of Inhibition (mm)
100	-	100	10.1
150	12	150	10.2
200	19	200	11.8
Gentamycin (1mg/ml)	32	Gentamycin (1mg/ml)	20.1

- indicating no activity

***Key words*:** Phytochemical, Antimicrobial activity, Leaf, Stem bark, *Garcinia kola*.

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Pak. J. Sci. Ind. Res. 2004 ***47****(2) 163 - 165*

Studies on Sodium Sulphide Prepared from Sodium Sulphate

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The aim of this investigation was to prepare purified sodium sulphide which is utilized for multiple purposes. The reaction between commercial sodium sulphate and coal gives sodium sulphide and various products. The effect of reaction temperature and maximum composition were studied to establish the optimum conditions for maxium yield. The reaction is found suitable for large scale production of sodium sulphide from commercial sodium sulphate.

Key words**:** Commercial sodium sulphate, Coal, Sodium sulphide.

Indroduction

The alkali and alkaline earth sulphides are colorless, whereas, the heavy metal sulphides are usually deep colored. Sodium sulphide has attained a very important position in the chemical industry (Meraw-Hill 1987; Lide 1996). It is used as a reducing agent and to dissolve cellulose ester in the manufacture of pigments and also used in drugs and drug intermediate industries. (Huheey *et al* 1993). It is main ingredient in dying of textiles. Sulfur dyes are applied from dye bath containing sodium sulfide which gives good to moderate light fastness and good fastness at low cost and rapid processing and also as a solvent for water insoluble dyes. Sodium sulphide plays a very important role of a chemical compound used in liming and in manufacturing of lubricating oil and production of heavy water used for atomic power plants (Greenwood and Earnshaw 1997). In the production of pulp and paper, it is main component to maintain the sodium balance of the mill, the oxygen stage normally uses the oxidized white liquor and sodium sulphide has been oxidized to thiosulphate. In rubber industry, its main area of application is of vulcanizing agent in processing. It is also used in tanneries for dehairing because sulphide of sodium provides stronger alkalinity but less sulphidity than hydrosulphides.

In froth flotation, it is usually used to separate one solid from another, for solid-liquid separation, as in dissolved air flotation and for liquid-liquid separation as in foam fractionation and in flotation of sulphide ores. (Huang and Ling 2001). The mining industry uses sodium sulphide to form (insoluble) metal sulphide of copper, lead and molybdenum. The same raction is used to remove heavy metals like copper cadmium, mercury, lead from wastes water in many industries because of toxicity their concentration must be reduced to very low levels. (Krik Othmer 1983; Cotton *et al* 1999).

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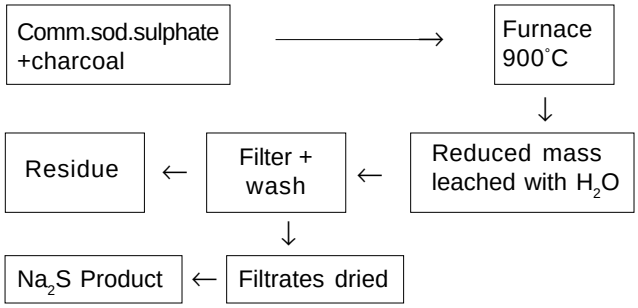
In kraft wood pulping process, the sulphide is used as cooking liquor. Other uses include the preparation of lubrication of oil and the production of polysulphide elastomer and plastics (Krik Othmer 1983).



Flow diagram of sodium sulphide

Materials and Methods

General procedure. Sodium sulphide was prepared by mixing known quantity of commercial sodium sulphate with known quantity of coal and heating in furnace at known temperature. The reaction product was crushed into course fragments and leached with water, filtered and the clear solution was evaporated to dryness. The light yellowish gray color dehydrated sodium sulphide was formed. The reaction involved in Na_2S production is as follow (Brady and Clauser 1986).

$$Na_2SO_4 + 2C = Na_2S + 2CO$$

The yield of sodium sulphide (Na_2S) was calculated by taking a known weight from the dried Na_2S product and titrating it again sodium sulphate and I_2 using starch as an indicator (Vogel 1987).

Results and Discussion

The present work demonstrates the optimum conditions for the preparation of sodium sulphide from commercial sodium

sulphate by varying the temperature, time period of heating and the ratio of commercial sodium sulphate and charcoal. The discussion follows:

a) The effect of temperature on maximum recovery of sodium sulphide was noted by raising the temperature from 650˚C to 1000˚C, the maximum yield have been obtained as shown in Table 1and Fig 1 at 900˚C and further increase in temperature is not important.

b) The effect of charcoal on maximum recovery of sodium sulphide from commercial sodium sulphate was studied by varying proportions of charcoal used for maximum recovery of sodium sulphide. The maximum yield obtained was shown

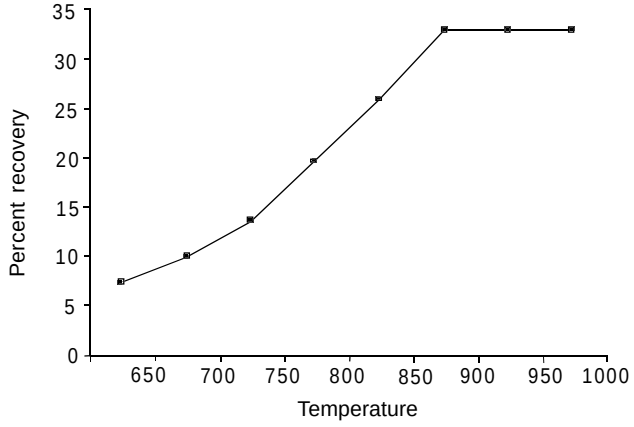


Fig 1. Effect of temperature on maximum recovery of sodium sulphide.

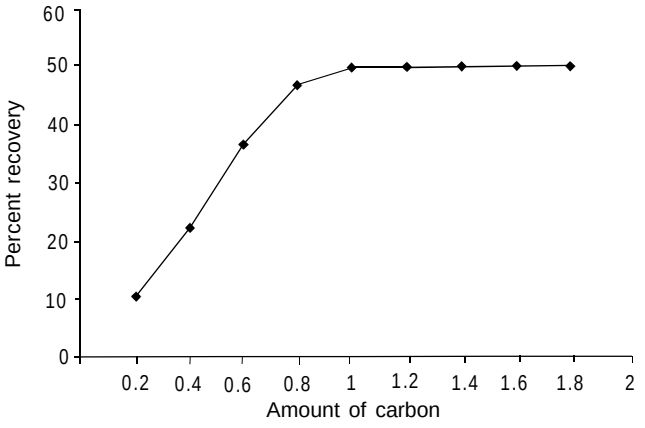


Fig 2. Effect of carbon on maximum recovery of sodium sulphide.

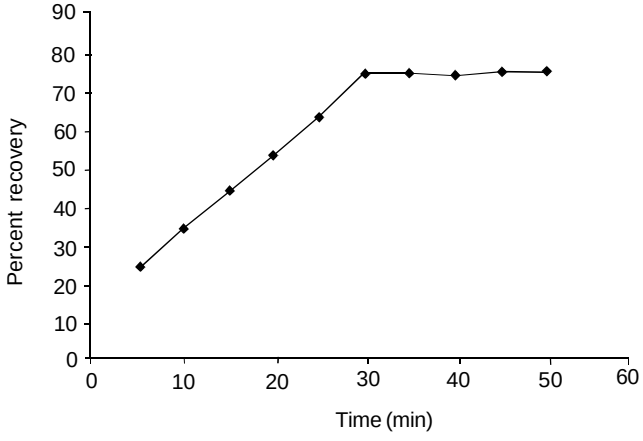


Fig 3. Effect of time on maximum recovery of sodium sulphide.

Table 1
Effect of temperature on maximum recovery of sodium sulphide

Sodium sulphate + Charcoal (g)	Temperature (˚C)	Recovery of sodium sulphide (%)
1:1	650	7.40
1:1	700	9.90
1:1	750	13.50
1:1	800	19.60
1:1	850	25.90
1:1	900	32.90
1:1	950	32.88
1:1	1000	32.89

Table 2
Effect of charcoal on recovery of sodium sulphide

Sodium sulphate (g)	Charcoal (g)	Ratio	Sodium sulphide (%)
2.0	0.20	1:0.1	10.00
2.0	0.40	1:0.2	22.00
2.0	0.60	1:0.3	36.10
2.0	0.80	1:0.4	46.20
2.0	1.00	1:0.5	49.10
2.0	1.20	1:0.6	49.08
2.0	1.40	1:0.7	49.09
2.0	1.60	1:0.8	49.10
2.0	1.80	1:0.9	47.05

Table 3
Effect of time on maximum recovery of sodium sulphide

Sodium sulphate + Charcoal	Time (min)	Recovery (%)
2:1	05	25.50
2:1	10	36.20
2:1	15	46.30
2:1	20	56.50
2:1	25	66.70
2:1	30	78.88
2:1	35	78.72
2:1	40	77.95
2:1	45	78.80

in Table 2 and Fig 2, in the ratio of 1:0.5 is more appropriate for maximum yield.

c) The effect of time from 5 - 40 min at a temperature 900°C was observed to get maximum extraction. The time was noted 30 min for the maximum recovery of sodium sulphide as shown in Table 3 and Fig 3. Further increase in time doesn't raise more yield.

Conclusion

Sodium sulphide of metallurgical grade was obtained by the reaction of commercial sodium sulphate with charcoal (ratio 1:0.5) at a temperature of 900°C. The reaction was completed in 30 min. The maximum recovery by 79.1% was found. The commercial sodium sulphate appears to be amenable to processing for the extraction of metallurgical sodium sulphide, as per experimental procedure given above.

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Erratum

The address of co-author was overlooked in the paper published in Vol. 47, January – February, 2004, Page 50. The correct address of co-author Y.M. Khanif is Department of Land Management, Faculty of Agriculture, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia. Also in Table 2, page 52, Grain yield (t ha^{-1}) was erroneously printed. This may be read as N Content (%) in grain.

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EXAMPLES

Journal Articles

In Bibliography:

Reid R W, Watson J A 1995 Reaction of lodgepole pine to attack by blue stain fungi. *Can J Bot* **45** (2 Part 1) 45-50.

Weenen H, Nkunya M H H, Bray D H, Mwasumbi L B, Kinabo L S, Kilimali V A E B 1990 Antimalarial activity of Tanzanian plants. *Plant Medic* **56**(4) 368-370.

Solhein H 1992a The early stages of fungal invasion in Norway spruce. *Can J Bot* **70** (4 Ser A) 112-119.

Solhein H 1992b Fungal succession in sapwood. *Eur J Pathol* **22** 143-158.

In Text:

(Reid and Watson 1995)

(Solhein 1992a)

(Solhein 1992a & b)

(Weenen *et al* 1990; Solhein 1992a; Reid and Watson 1995)

Chapters of Books

Demarchi DA, Marsh RD, Harcombe AP, Lea EC 1990 The environment. In: *The Birds of British Columbia*, eds Cambell R W & McNall M C E. Royal British Columbia Museum, Victoria, B C USA, Vol 2, 2nd ed, pp 366-425.

Books

Bennet O C, Myers J E 1974 *Momentum, Heat and Mass Transfer*. McGraw Hill, New York, USA, pp 540-562.

Thesis

Hu C 1989 A comparative study of peanut peroxidase isozyme. Ph.D thesis, Department of Plant Sciences, University of Western Ontario, London, Ontario, Canada.

Papers of Conferences/Symposia/Seminars

Jhonson S R, Knapp A K 1994 The role of *fire in Spartina pectinator* dominated tallgrass prairie wetlands. In: *Proceedings on 19th Tall Timbers Fire Ecology Conference*, Tallahassee, Fl, USA, November 3-6, 1993.

Technical Reports

Tiller FM, Leu WF 1984 *Solid Liquid Separation for Liquefied Coal Industries*. Final Report for RP - 1411-1. EPRI, AP-3599, Electric Power Research Institute, Palo Atto, CA, USA.

Patents

Verschuur E 1978 *Agglomerating Coal Slurry Particles*. US Patent 4126426.

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