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Physical Sciences


An Experimental Investigation of PAH Emissions from a Heavy Duty Diesel Engine Fuelled with Biodiesel and its Blend

Asad Naeem Shah a,b*, G. E. Yun-shan c, TAN Jian-wei a and Liu Zhi-hua a

aSchool of Mechanical and Vehicular Engineering, Beijing Institute of Technology, Beijing 100081, P.R China
bDepartment of Mechanical Engineering, University of Engineering and Technology, Lahore, Pakistan

dedicated to the memory of Dr. T. Y. Khan in recognition of his contributions to the field of science, technology, and engineering.

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Abstract. For the comparison of emissions of polycyclic aromatic hydrocarbons (PAHs) from diesel, biodiesel and its 20% blend with diesel, and their carcinogenic potencies, an experimental study has been conducted on a turbocharged, intercooled and direct injection diesel engine. Total PAHs (solid and gas) from diesel, B20 and B100 at low load were more than those at high loads. Total PAH emissions from the test fuels at the rated speed were more than those at maximum torque speed. Benzo[a]pyrene (BaP) brake specific emission of biodiesel is less than that of diesel. LMW-PAH emissions for the test fuels are all higher than those of MMW and HMW PAH. Biodiesel and B20 reduce both the total Benzo[a]pyrene equivalent concentration (BaPeq) and the total mean–PAHs as compared to commercial diesel fuel. BSFC of the engine increased but its brake power decreased in the cases of B20 and biodiesel.

Keywords: diesel engines, biodiesel, polycyclic aromatic hydrocarbons, carcinogenic potencies

Introduction

Diesel engines due to their high fuel efficiency, output power and fuel economy are widely used in heavy duty trucks, buses, generators, construction and agricultural machinery, in the face of the dwindling sources of conventional fossil fuels, their ever increasing demand and prices and stringent emission regulations. Among a number of alternative fuels like methanol, ethanol, LPG, LNG, CNG and vegetable oils, biodiesel consisting of alkyl monoesters of fatty acids from vegetable oils or animal fats can be used in unmodified diesel engines in pure or blended forms (Meher et al., 2006; Graboski and McCormick, 1998). It is a non-toxic, eco-friendly and biodegradable fuel (Lapinskiene et al., 2006). Use of biodiesel as an alternative fuel reduces the regulated air pollutants, including particulate matter, HC, CO and SO, (Labeckas and Slavinskas, 2006; Sinha and Agarwal, 2005; Usta, 2005; Senda et al., 2004; Turrio-Baldassarri et al., 2004; Monyem and Van Gerpen, 2001). Global use of biodiesel can curtail green house gas emissions as compared to mineral diesel (Gerpen, 2006; Carraretto et al., 2004; Tan et al., 2004; Peterson and Hustruid, 1998). It has higher cetane number, ultra-low sulphur concentration, higher flash point, high oxygen content and improved lubricating efficiency (Ebiura et al., 2005; Agarwal et al., 2003; Fukuda et al., 2001). Biodiesel has less adverse effect on human health as compared to diesel (Schroder et al., 1999) and mutagenicity of biodiesel particulate emissions is much lower than that of petroleum-based diesel fuel (McDonald et al., 1995). To encourage the use of biodiesel fuel, Austria, Germany and United States, Governments have announced tax benefits for the people (Raneses et al., 1999; Krawczyk, 1996).

Although biodiesel has widely been investigated as an alternative fuel in diesel engines for performance, regulated and somewhat unregulated emissions, however polycyclic aromatic hydrocarbons (PAHs) and their carcinogenic potencies still need to be addressed comprehensively. This study is an effort to determine PAHs and their corresponding carcinogenic potencies from the exhaust of a diesel engine alternately fuelled with biodiesel and its 20% blend with commercial diesel fuel.

PAHs are formed by incomplete combustion or high temperature pyrolytic process involving organic matter (Khalli et al., 1995). PAHs are semi volatile substances at atmospheric conditions and occur both in vapour-phase and as attached to particles depending on vapour pressure of each PAH component (Bashir et al., 2003). Lighter PAHs are in vapour phase, while those having four or more rings are found mainly adsorbed in particulate material (Park et al., 2002). Although lighter PAHs have weaker carcinogenic properties, they are the most abundant in the urban atmosphere and react with other pollutants to form more toxic derivatives (Ho et al., 2002). PAHs specially benzo (a) pyrene injure the respiratory and immune system, and is responsible for cell mutation and can cause skin and lung cancers (Grevenynghge et al., 2003; Yousef et al., 2002). PAHs are mainly contributed by the mobile sources like diesel and gasoline engines; the contribution of total PAHs from mobile sources to ambient air is 91.8% (Yang et al., 1999).

*Author for correspondence; E-mail: naeem_138@hotmail.com
Materials and Methods

Engine specifications. The engine under study is a turbo-charged, direct injection and intercooled, which runs on an electrical dynamometer (SCHENCK HT 350). The engine specifications are given in detail in Table 1. No modification or alteration has been made in the engine. The schematic diagram of the experimental set up is given in Fig. 1.

Table 1. Engine specifications

<table>
<thead>
<tr>
<th>Items</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cylinders</td>
<td>4</td>
</tr>
<tr>
<td>Bore (mm)</td>
<td>110</td>
</tr>
<tr>
<td>Stroke (mm)</td>
<td>125</td>
</tr>
<tr>
<td>Displacement (litre)</td>
<td>4.752</td>
</tr>
<tr>
<td>Compression ratio</td>
<td>16.8</td>
</tr>
<tr>
<td>Rated power (kW@ r/min)</td>
<td>117/2300</td>
</tr>
<tr>
<td>Maximum torque (N.m@ r/min)</td>
<td>580/1400</td>
</tr>
<tr>
<td>Nozzle hole diameter (mm)</td>
<td>0.23</td>
</tr>
<tr>
<td>Number of nozzle holes</td>
<td>6</td>
</tr>
</tbody>
</table>

Test fuels. Three fuels were used in this study namely D (commercial diesel), B100 (biodiesel), B20 (a blend of 20% biodiesel and 80% diesel). Biodiesel is provided by Zhenghe Bioenergy Co. Ltd., Hainan, China. Main properties of the test fuels are given in Table 2.

Sampling methodology and PAH analysis. An ejector-diluter (Dekati Ltd., Finland) was used to obtain the emission directly from the exhaust pipe by inserting a J-shaped stainless-steel sample probe into the exhaust pipe as shown in Fig. 1. The ejector-diluter consists of a set of filters, a dryer, a temperature controller, a pressurized air heater and two diluters. Dry, particle-free and pressurized air was introduced into the primary diluter and was heated up to the exhaust gaseous temperature. The diluted sample was then introduced into the secondary diluter for further dilution. The dilution ratio of the primary and secondary diluters was 8, so the overall dilution ratio of the instrument was about 64. Before sampling, the calibration was made by using two concentrations of CO₂ which were measured before and after the dilution instrument. The total residence time was about 0.1 seconds for the primary and the secondary diluters as delineated by Dekati Ltd.

Sampling of PAHs was performed by two distinct ways: one for the particulate phase and another for the vapour phase. Particulate phase PAHs were collected on a glass-fibre filter. Before sampling, filters were placed in an oven at about 450 °C for 8 h to avoid the possibility of presence of any organic compound. Cleaned filters were stored in a desiccator for 8 h to achieve moisture equilibrium and then were weighed. After the tests, filters were put again in a desiccator for 8 h to remove moisture, and were weighed to determine the net mass of the trapped particles. Gaseous phase PAHs were collected in a glass cartridge PUF/XAD-2/PUF, Sopelco USA, (Fig. 2), connected in series with particulate filters (Fig. 1). PUF is polyurethane foam of density 0.022 g/cm³, having outside diameter 113x83 mm, and with a nominal particle size of 80 μm.

Table 2. Properties of fuels

<table>
<thead>
<tr>
<th>Properties</th>
<th>B100</th>
<th>B20</th>
<th>D</th>
<th>Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (kg/m³)</td>
<td>886.4</td>
<td>845.1</td>
<td>834.8</td>
<td>SH/T 0604</td>
</tr>
<tr>
<td>Viscosity (mm²/s) at 20 °C</td>
<td>8.067</td>
<td>4.020</td>
<td>3.393</td>
<td>GB/T 265</td>
</tr>
<tr>
<td>Lower heating value (MJ/kg)</td>
<td>37.3</td>
<td>41.57</td>
<td>42.8</td>
<td>GB/T 384</td>
</tr>
<tr>
<td>Sulphur content (mg/l)</td>
<td>25</td>
<td>-</td>
<td>264</td>
<td>SH/T 0253-92</td>
</tr>
<tr>
<td>Cetane number</td>
<td>60.1</td>
<td>-</td>
<td>51.1</td>
<td>GB/T 386-91</td>
</tr>
<tr>
<td>Carbon content (%)</td>
<td>76.83</td>
<td>-</td>
<td>86.92</td>
<td>SH/T 0656-98</td>
</tr>
<tr>
<td>Hydrogen content (%)</td>
<td>11.91</td>
<td>-</td>
<td>13.08</td>
<td>SH/T 0656-98</td>
</tr>
<tr>
<td>Oxygen content (%)</td>
<td>11.33</td>
<td>-</td>
<td>0</td>
<td>Element analysis</td>
</tr>
</tbody>
</table>

Fig. 1. Experimental setup.

Fig. 2. PUF/XAD-2/PUF cartridges.
The collected sample was extracted in an ultrasonic extractor (for 30 min) for the solid-phase PAH emissions and in Soxhlet extractor (for 24 h) for gaseous phase PAHs, with 250 ml dichloromethane (DCM). The extracted liquid was collected in a flask of 500 ml, and was concentrated by a rotary evaporator (Kuderna-Danish evaporator) to 1.0 ml. The concentrated sample was cleaned by passing it through a silica gel column at a speed of 2 ~ 3 ml/min, which was installed to solid-phase extraction (SPE) device and was activated with 3 ml n-hexane. In order to wash the PAHs staying on the silica gel column, 15 ml mixture of n-hexane and DCM (volume ratio, 1:1) was added to the sample. At last PAHs-washing liquid was concentrated by evaporator (K-D) exactly to 1.0 ml, and was refrigerated until analysis.

For PM-phase PAHs extraction, fibre-glass filter was cut into several pieces which were put into a flask and then DCM was poured into the flask. The sample was extracted by ultrasonic agitation thrice (30 min each time), and was volatilized with water bath to 10 ml. The residues were filtered by core filter. Other aspects of the treatment procedure for the sample were identical to those described above.

After the sampling, the identification and quantification of PAHs were made by using a gas chromatograph (GC) Agilent 6890N and mass spectrometer (MS) Agilent 5795C according to the compendium method TO-13A (US/EPA, 1999). The specifications of GC/MS are given in Table 3.

<table>
<thead>
<tr>
<th>Table 3. GC/MS specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas chromatograph (GC)</td>
</tr>
<tr>
<td>Mass spectrometer (MS)</td>
</tr>
</tbody>
</table>

Compounds were identified from their mass spectral data, using U.S National Institute of Standards and Technology (NIST) library. After the identification, compounds were quantified by using the external standard methods to make the linear standard curves. The GC/MS was calibrated with a standard solution of 16 PAH compounds (EPA 610 PAHs Mixture, Supelco, Bellefonte, PA, USA). The standard samples, diluted 10, 50, 100, 500, 1000 and 2000 times, were injected into GC/MS as 1 µl liquid of each concentration with the help of micro-injector to get the standard ion flow graph, and hence the peak area of the 16 PAHs. The retention time, quantitative and reference ions, curve equations, R (correlation coefficient) and RSD (relative standard deviation) of different compounds are depicted in Table 4.

**PAH emissions and statistical analysis.** According to the number of rings, the PAH homologues have been grouped as under: Naphthalene (Nap) with 2-rings; acenaphthylene (AcPy), acenaphthene (Acp), fluorene (Flu), phenanthrene (PA) and anthracene (Ant) with 3-rings; fluoranthene (FL), pyrene (Pyr), benzo[a]anthracene (BaA) and chrysene (CHR) with 4-rings; benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP) and dibenzo [a,h]anthracene (DBA) with 5-rings; and, indeno[1,2,3-cd] pyrene (IND) and benzo[g,h,i]peryylene (BghiP) with 6-rings.

Total PAH concentration is the sum of the individual concentration of the 16 compounds. In order to analyze the PAH homologous distribution, the total PAHs have further been divided into three groups on the basis of their molecular weight, which are low molecular weight (LMW), medium molecular weight (MMW) and high molecular weight (HMW) PAHs. LMW-PAHs have two and three-ringed PAHs, MMW-PAHs have four-ringed PAHs and HMW-PAHs have five and six-ringed PAHs. In this way, first six PAHs are LMW-PAHs, next four are MMW-PAHs and last six are HMW-PAHs of the 16 compounds. Among these PAHs, several are known as...
human carcinogens. The carcinogenic potency of a certain PAH compound is, generally, evaluated on the basis of its Benzo[a]pyrene equivalent concentration (BaPeq) which is determined by the corresponding toxic equivalent factor (TEF) (Nisbet and LaGoy, 1992). For the assessment of carcinogenic potency of total PAHs, each individual BaPeq is added to get the total BaPeq of all the 16-PAHs.

**Engine conditions.** The experimental conditions of the engine, basing on speed-load characteristics, are given in the Table 5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Quantitative ion</th>
<th>Reference ion</th>
<th>Standard curve</th>
<th>Correlation coefficient</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nap</td>
<td>5.76</td>
<td>128</td>
<td>127, 129</td>
<td>$y = 4.19345e+006x$</td>
<td>0.9941</td>
<td>2.85</td>
</tr>
<tr>
<td>AcPy</td>
<td>8.33</td>
<td>152</td>
<td>153, 154</td>
<td>$y = 2.48049e+006x$</td>
<td>0.9927</td>
<td>6.26</td>
</tr>
<tr>
<td>Acp</td>
<td>8.70</td>
<td>154</td>
<td>153, 152</td>
<td>$y = 1.97297e+006x$</td>
<td>0.9993</td>
<td>5.73</td>
</tr>
<tr>
<td>Flu</td>
<td>9.97</td>
<td>166</td>
<td>167</td>
<td>$y = 1.87257e+006x$</td>
<td>0.9949</td>
<td>6.28</td>
</tr>
<tr>
<td>PA</td>
<td>12.92</td>
<td>178</td>
<td>176</td>
<td>$y = 2.13584e+006x$</td>
<td>0.9910</td>
<td>2.58</td>
</tr>
<tr>
<td>Ant</td>
<td>13.11</td>
<td>178</td>
<td>176</td>
<td>$y = 2.10768e+006x$</td>
<td>0.9927</td>
<td>6.39</td>
</tr>
<tr>
<td>FL</td>
<td>17.56</td>
<td>202</td>
<td>201</td>
<td>$y = 1.86261e+006x$</td>
<td>0.9944</td>
<td>1.64</td>
</tr>
<tr>
<td>Pyr</td>
<td>18.47</td>
<td>202</td>
<td>201</td>
<td>$y = 2.21573e+006x$</td>
<td>0.9924</td>
<td>3.33</td>
</tr>
<tr>
<td>BaA</td>
<td>23.93</td>
<td>228</td>
<td>226, 229</td>
<td>$y = 1.58855e+006x$</td>
<td>0.9910</td>
<td>5.99</td>
</tr>
<tr>
<td>CHR</td>
<td>24.08</td>
<td>228</td>
<td>226, 229</td>
<td>$y = 1.35516e+006x$</td>
<td>0.9920</td>
<td>6.66</td>
</tr>
<tr>
<td>BbF</td>
<td>28.59</td>
<td>252</td>
<td>250</td>
<td>$y = 1.21176e+006x$</td>
<td>0.9935</td>
<td>6.53</td>
</tr>
<tr>
<td>BkF</td>
<td>28.70</td>
<td>252</td>
<td>250</td>
<td>$y = 1.11217e+006x$</td>
<td>0.9949</td>
<td>7.39</td>
</tr>
<tr>
<td>BaP</td>
<td>29.84</td>
<td>252</td>
<td>250</td>
<td>$y = 1.03983e+006x$</td>
<td>0.9982</td>
<td>2.62</td>
</tr>
<tr>
<td>IND</td>
<td>35.19</td>
<td>276</td>
<td>274</td>
<td>$y = 849205x$</td>
<td>0.9955</td>
<td>2.99</td>
</tr>
<tr>
<td>DBA</td>
<td>35.52</td>
<td>278</td>
<td>276</td>
<td>$y = 380247x$</td>
<td>0.9973</td>
<td>11.34</td>
</tr>
<tr>
<td>BghiP</td>
<td>36.68</td>
<td>276</td>
<td>274</td>
<td>$y = 476422x$</td>
<td>0.9928</td>
<td>14.53</td>
</tr>
</tbody>
</table>

Table 4. PAHs with their equations of standardization curve, correlation coefficients and RSD

Table 5. Engine conditions

<table>
<thead>
<tr>
<th>Speed (r/min)</th>
<th>Load (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Engine condition 1</td>
<td>1400</td>
</tr>
<tr>
<td>Engine condition 2</td>
<td>2300</td>
</tr>
<tr>
<td>Engine condition 3</td>
<td>2300</td>
</tr>
</tbody>
</table>

**Results and Discussion**

**Brake specific emission of PAHs.** Brake specific emission (BSE) is defined as the PAH mass emitted per kilowatt power, developed in the engine in one hour. Table 6, 7 and 8 show BSE of PAHs both in PM and gas phases for engine conditions 1, 2 and 3, respectively. It is clear from Table 7 and 8 that total PAHs (solid and gas) from diesel, B20 and B100 at engine condition 2 are more than the corresponding PAHs at engine conditions 3. This finding is consistent with the previous studies that fuel PAHs survival is highest at low engine loads (Collier et al., 1995; Barbell et al., 1989; Williams et al., 1986). Low engine load gives rise to emissions by quenching of the flame front in the clearance between the piston top and the cylinder head near top dead center (Collier et al., 1995). Furthermore, at low load, oxidation rate of fuels may decrease due to increase in over-lean mixture area, which may result in incomplete combustion and hence may increase PAH emissions.

It is elucidated from the Tables 6 and 8 that total PAHs for the fuels at rated speed are more than the corresponding PAHs at maximum torque speed. Since engine speed can affect the swirl characteristics, injection timing and combustion temperature of the engine (Collier et al., 1995; Rao et al., 1993; Gomes and Yates, 1992), so, the possible reason for increased PAHs at higher speed may be the increase in turbulence in the combustion chamber of the engine at higher speed, which may

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Quantitative ion</th>
<th>Reference ion</th>
<th>Standard curve</th>
<th>Correlation coefficient</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nap</td>
<td>6.603</td>
<td>123.198</td>
<td>189.03</td>
<td>188.028</td>
<td>8.575</td>
<td>143.606</td>
</tr>
<tr>
<td>AcPy</td>
<td>0.749</td>
<td>1.559</td>
<td>3.869</td>
<td>0.251</td>
<td>3.406</td>
<td>2.505</td>
</tr>
<tr>
<td>Acp</td>
<td>0.562</td>
<td>0.778</td>
<td>1.781</td>
<td>0.139</td>
<td>2.950</td>
<td></td>
</tr>
<tr>
<td>Flu</td>
<td>1.617</td>
<td>1.946</td>
<td>7.096</td>
<td>0.581</td>
<td>13.627</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>2.436</td>
<td>17.393</td>
<td>4.171</td>
<td>4.546</td>
<td>18.152</td>
<td></td>
</tr>
<tr>
<td>Ant</td>
<td>3.243</td>
<td>1.253</td>
<td>1.487</td>
<td>0.543</td>
<td>1.153</td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>3.688</td>
<td>0.556</td>
<td>2.361</td>
<td>0.390</td>
<td>1.416</td>
<td></td>
</tr>
<tr>
<td>Pyr</td>
<td>6.095</td>
<td>0.680</td>
<td>3.531</td>
<td>1.990</td>
<td>1.737</td>
<td>5.158</td>
</tr>
<tr>
<td>BaA</td>
<td>1.651</td>
<td>1.848</td>
<td>0.488</td>
<td>0.243</td>
<td>0.694</td>
<td></td>
</tr>
<tr>
<td>CHR</td>
<td>1.228</td>
<td>0.544</td>
<td>1.603</td>
<td>2.933</td>
<td>0.348</td>
<td></td>
</tr>
<tr>
<td>BbF</td>
<td>0.401</td>
<td>0.356</td>
<td>0.077</td>
<td>0.031</td>
<td>0.133</td>
<td></td>
</tr>
<tr>
<td>BkF</td>
<td>0.254</td>
<td>0.293</td>
<td>0.351</td>
<td>0.027</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td>BaP</td>
<td>0.566</td>
<td>0.363</td>
<td>0.157</td>
<td>0.014</td>
<td>0.225</td>
<td></td>
</tr>
<tr>
<td>IND</td>
<td>0.160</td>
<td>0.046</td>
<td>0.290</td>
<td>0.001</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>DBA</td>
<td>1.883</td>
<td>0.638</td>
<td>0.133</td>
<td>0.009</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>BghiP</td>
<td>1.685</td>
<td>0.624</td>
<td>0.464</td>
<td>0.014</td>
<td>0.049</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. PAH brake specific emission at engine condition 1 ($\mu g/kW\cdot h$)
increase the heat losses to the combustion chamber walls, and hence may decrease the combustion temperature. The low temperature in the combustion chamber decreases the oxidation rate and hence increases the PAH emissions.

From the Table 6, 7 and 8, it is obvious that after the naphthalene, phenanthrene is prominent in PAH emissions from the test fuels. The BSE of phenanthrene from diesel, B20 and B100 are 9.2%, 53.1% and 18.1% more, respectively, at low load (condition 2) as compared to high load (condition 3). This is in good agreement with the previous finding that percentage recovery of phenanthrene, in case of diesel fuel, is more at low load as compared to that at high load (Collier et al., 1995).

The BSE of total PAHs (sum of BSE of all PAHs at the three conditions) for B100, B20 and diesel is 1325.740, 1224.974 and 1516.488 μg/kWh, respectively which reveals that biodiesel and its blends reduce the PAH emissions. This is consistent with other results (Lin et al., 2006a, Cardone et al., 2002).

Benzo[a]pyrene (BaP), the most carcinogenic compound, shows less BSE in case of B100 as compared to diesel for all the engine conditions. Although B20 shows less BSE in terms of Benzo[a]pyrene at engine conditions 1 and 3, its BaP emission improves surprisingly at engine condition 2 compared to diesel fuel.

Total LMW, MMW and HMW-PAHs. Fig. 4 shows the comparison of total lower, medium and higher molecular weight PAHs. ΣLMW-PAHs show higher BSE as compared to ΣMMW-PAHs and ΣHMW-PAHs, while the least BSE is depicted for ΣHMW-PAHs at the three engine conditions for all the test fuels. For diesel fuel, ΣLMW-PAHs is 87.1%, 94.5% and 90.2% of total PAH emissions at engine conditions 1, 2 and 3, respectively. Similarly, ΣLMW-PAHs is 94.5%, 80.1% and 80.4% of the total PAH emissions from B20 at conditions 1, 2 and 3, respectively, and 90.4%, 90.8% and 91% of the total PAHs from B100 at the engine conditions 1, 2 and 3, respectively. This finding is in good agreement with the previous studies that two and three ring PAHs contribute a large fraction of the total PAH emissions (Lin et al., 2006b, c).

### Table 7. PAH brake specific emission at engine condition 2 (μg/kWh)

<table>
<thead>
<tr>
<th>Compound</th>
<th>D (μg/kWh)</th>
<th>B20 (μg/kWh)</th>
<th>B100 (μg/kWh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM-PAHs</td>
<td>Gas-PAHs</td>
<td>PM-PAHs</td>
<td>Gas-PAHs</td>
</tr>
<tr>
<td>Nap</td>
<td>27.015</td>
<td>638.157</td>
<td>28.292</td>
</tr>
<tr>
<td>AcPy</td>
<td>0.896</td>
<td>3.459</td>
<td>0.767</td>
</tr>
<tr>
<td>Acp</td>
<td>0.773</td>
<td>1.351</td>
<td>0.576</td>
</tr>
<tr>
<td>Flu</td>
<td>3.494</td>
<td>5.295</td>
<td>3.377</td>
</tr>
<tr>
<td>PA</td>
<td>15.293</td>
<td>42.726</td>
<td>35.746</td>
</tr>
<tr>
<td>Ant</td>
<td>2.364</td>
<td>1.046</td>
<td>1.616</td>
</tr>
<tr>
<td>FL</td>
<td>6.672</td>
<td>1.304</td>
<td>5.100</td>
</tr>
<tr>
<td>Pyr</td>
<td>14.219</td>
<td>1.579</td>
<td>11.067</td>
</tr>
<tr>
<td>BaA</td>
<td>0.786</td>
<td>1.955</td>
<td>6.011</td>
</tr>
<tr>
<td>CHR</td>
<td>2.091</td>
<td>1.434</td>
<td>11.686</td>
</tr>
<tr>
<td>DBF</td>
<td>0.754</td>
<td>0.387</td>
<td>21.138</td>
</tr>
<tr>
<td>BKF</td>
<td>0.197</td>
<td>0.204</td>
<td>13.420</td>
</tr>
<tr>
<td>BaP</td>
<td>2.854</td>
<td>0.998</td>
<td>4.475</td>
</tr>
<tr>
<td>INd</td>
<td>0.838</td>
<td>0.028</td>
<td>4.507</td>
</tr>
<tr>
<td>BaA</td>
<td>1.712</td>
<td>0.327</td>
<td>1.426</td>
</tr>
<tr>
<td>BghiP</td>
<td>4.022</td>
<td>0.823</td>
<td>3.382</td>
</tr>
<tr>
<td>Total</td>
<td>785.049</td>
<td>681.162</td>
<td>547.200</td>
</tr>
</tbody>
</table>

### Table 8. PAH brake specific emission at engine condition 3 (μg/kWh)

<table>
<thead>
<tr>
<th>Compound</th>
<th>D (μg/kWh)</th>
<th>B20 (μg/kWh)</th>
<th>B100 (μg/kWh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM-PAHs</td>
<td>Gas-PAHs</td>
<td>PM-PAHs</td>
<td>Gas-PAHs</td>
</tr>
<tr>
<td>Nap</td>
<td>31.130</td>
<td>390.330</td>
<td>2.521</td>
</tr>
<tr>
<td>AcPy</td>
<td>0.691</td>
<td>2.345</td>
<td>0.377</td>
</tr>
<tr>
<td>Acp</td>
<td>0.428</td>
<td>1.709</td>
<td>0.146</td>
</tr>
<tr>
<td>Flu</td>
<td>2.138</td>
<td>10.015</td>
<td>1.189</td>
</tr>
<tr>
<td>PA</td>
<td>12.220</td>
<td>40.466</td>
<td>20.736</td>
</tr>
<tr>
<td>Ant</td>
<td>1.353</td>
<td>2.758</td>
<td>1.992</td>
</tr>
<tr>
<td>FL</td>
<td>6.613</td>
<td>3.342</td>
<td>6.335</td>
</tr>
<tr>
<td>Pyr</td>
<td>14.365</td>
<td>8.702</td>
<td>14.707</td>
</tr>
<tr>
<td>BaA</td>
<td>2.670</td>
<td>0.986</td>
<td>0.529</td>
</tr>
<tr>
<td>CHR</td>
<td>1.995</td>
<td>2.561</td>
<td>3.584</td>
</tr>
<tr>
<td>DBF</td>
<td>0.202</td>
<td>1.289</td>
<td>0.203</td>
</tr>
<tr>
<td>BKF</td>
<td>1.266</td>
<td>0.143</td>
<td>1.134</td>
</tr>
<tr>
<td>BaP</td>
<td>1.297</td>
<td>0.884</td>
<td>1.797</td>
</tr>
<tr>
<td>INd</td>
<td>0.939</td>
<td>0.021</td>
<td>1.341</td>
</tr>
<tr>
<td>BaA</td>
<td>2.729</td>
<td>0.439</td>
<td>1.064</td>
</tr>
<tr>
<td>BghiP</td>
<td>3.424</td>
<td>0.016</td>
<td>1.789</td>
</tr>
<tr>
<td>Total</td>
<td>549.464</td>
<td>307.780</td>
<td>560.539</td>
</tr>
</tbody>
</table>
the 16-PAHs was calculated by the following formula:

\[ \text{BaPeq} = \sum (M_i \cdot \text{TEF}_i) \]  

(1)

Where \( M \) is mean BSE in (\( \mu g/\text{kW} \cdot \text{h} \)); \( i = 1 \sim 16 \)

It is clear from the Table 9 that total BaPeq and total mean BSE (sum of mean BSEs) for the three fuels follow the order 

\( B_{100} < B_{20} < D \). The above results reveal that use of biodiesel and its 20% blend decreases both the total- BaPeq and the total mean- PAH emissions. This finding is consistent with the data quoted in other literature (Lin et al., 2006a, b).

**Brake specific fuel consumption and brake power.** Fig. 5 shows comparison of the brake specific fuel consumption (BSFC) of the test fuels at different engine conditions. The increase in BSFC of the engine in the cases of B20 and B100 is 6.4% and 13.9%; 2.5% and 13.8%, and 1.2% and 13.2%, respectively at conditions 1, 2 and 3, respectively.

Fig. 6 depicts the effect of biodiesel and its blend on brake power of the engine at the three different conditions. The percentage decrease in power of B20 and B100 compared to diesel is of the order of 3.3% and 0%; 9.8% and 7.5%, and 12% and 11.7%, at engine conditions 1, 2 and 3, respectively.

The possible reasons for higher BSFC but lower power, in the cases of B20 and B100, are lower heating value and more
density of biodiesel as compared to those of diesel. Furthermore, at the same degree of crank angle, more mass is injected in case of biodiesel due to its earlier injection start with raised pressure and rate, as compared to diesel.

**Conclusion**

The current study aims at the comparative assessment of PAHs in terms of brake specific emissions and their corresponding carcinogenic potencies from a heavy duty diesel engine alternatively fuelled with biodiesel and its 20% blend with diesel. The main findings are as follows:

- BSE of total PAHs of diesel, B20 and B100 is more at low load than that at high load.
- BSE of total PAHs emitted from diesel, B20 and B100 is more at rated speed compared to that at maximum torque speed.
- Naphthalene and phenanthrene emissions for the test fuels are all dominant to other PAHs and the phenanthrene BSE for diesel, B20 and B100 is 9.2%, 53.1% and 18.1% more, respectively, at low load as compared to high load.
- The BSE of total PAHs for B100, B20 and diesel follows the order B20 < B100 < D.
- Benzo[a]pyrene (BaP)- BSE is less for biodiesel as compared to diesel for all the engine conditions.
- LMW-PAH emissions for the test fuels are all higher than those of MMW and HMW PAH, but HMW-PAHs are least among the three major categories of PAHs for all the engine conditions.
- Total BaP eq for the three test fuels follows the order as B100 < B20 < D.
- BSFC of the engine increases following the decrease in its brake power in the cases of biodiesel and B20 for all the engine conditions used in this study except condition 2 where diesel and B100 show almost the same brake power.

**Acknowledgement**

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**References**


**Introduction**

Recycling of secondary raw materials is an important part of the industries in developing countries like Pakistan. Silver is a valuable natural resource of finite supply; it is used as a component of various products. The discharged silver remains a pollutant of concern due to its aquatic toxicity and is subject to control by both hazardous waste and water quality regulatory programmes. Wastewashings containing silver at a concentration greater than 5 mg/l are regulated as hazardous waste (Paul, 1997) and cannot be discharged without first being rendered non-hazardous. Silver has monetary value as a recovered commodity as well. Harper and Siegel (2003) studied two different types of processes, electrolytic plating units alone and electrolytic plating units in combination with metallic replacement backup units and using them, they described the recovery of silver, down to concentrations of less than 5 mg/l.

Major source of recoverable silver is photo processing activity wherein the metal appears in different forms depending on the type of process. The photographic processing industry has four options in silver recovery i.e., electrolytic plating, metallic replacement, ion exchange and chemical precipitation. Chemical precipitation and recovery of silver by gravimetric methods is an effective technology. The precipitation process converts soluble metal compounds into relatively insoluble sulphide compounds through the addition of precipitating agents, such as sodium sulphide (Na₂S). Over a broad pH range, sulphides (S²⁻, HS⁻) are extremely reactive with heavy metal ions. Sulphide precipitation can be operated over a wide range of pH, typically from pH 2 to 12. Metal-sulphide precipitates are less amphoteric than the corresponding metal-hydroxides and, therefore, less likely to resolubilize because of changes in pH.

Extraction of silver from industrial wash waters of film-printing plants has been the subject of interest of many investigators. Syed et al. (2002) obtained a high yield of silver on heating the films with oxalic acid solution to boiling temperature to separate the inorganic components from the polymer substrate. Lanzano et al. (2006) studied one year anthropogenic stocks and flows of silver as it progresses from extraction to final disposal. They concluded that in total 62% of all discarded silver is recycled and 38% is sent to landfills. Rivera et al. (2007) studied silver precipitation in elemental form in the S₂O₃²⁻ - S₂O₄²⁻ system and recorded recovery of more than 99%. Arsalan and Sayiner (2008) followed the process of thiosulphate leaching of precious metals and recovered 99.57% Au and 95.87% Ag at optimized conditions. Zhouxiang et al. (2008) reported total silver recovery from the spent fixing bath and waste X-ray films as 98.0% and 95.8%, respectively.

In the present investigation, X-ray washings, were treated with activated carbon under certain conditions prior to its precipitation as silver sulphide in a bid to separate the inorganic compounds from gelatin and polymer compounds to gain the maximum recovery of silver. Possibilities of application of the developed procedure for processing of secondary raw materials containing silver, at large scale, were also explored.

**Materials and Methods**

The present study was performed to study a process at large scale that was earlier made at the laboratory scale.

**Laboratory scale experiment.** In the laboratory, at first, X-ray washings with no activated carbon yielded 77.57% recovery at 25 °C. X-ray washings were then stirred with activated carbon, supplied by Merck, at 20 and 50 °C; pH
was kept constant at $5.4 \pm 0.1$ and recorded with Corning pH-meter. The highest recovery of silver was obtained from washings treated with activated carbon at 50 °C (Table 1). At each temperature the experiment was repeated in triplicate to optimize the parameters and the average value was reported. Analytical grade reagents were used for the optimization of parameters. Concentration of silver was determined by Zeeman 8000 atomic absorption spectrophotometer.

**Recovery of silver at large scale.**

**Sampling.** X-ray washings, 24 litres (9 l from Khyber Teaching Hospital and 15 l from Popular X-rays, a private organization) were collected in polyethylene bottles, prior washed, cleaned with dilute nitric acid and then rinsed with deionised water. Washings were poured in a large clean tub and were mixed thoroughly with a thick glass rod. Silver, 7 g/l, was detected in the aggregated solutions by atomic absorption spectrophotometer as well as by the standard method of chemical analysis.

**Treatment of sample.** To the washings in three different clean plastic tubs, each containing 8 l of washings, HNO$_3$ was added to bring the pH down to 5.4±0.1. Granular activated carbon, at 6 g/l, was added to the washings to increase the removal efficiency and tubs were placed in the incubator at 50 °C. Washings were stirred, from time to time, with a thick glass rod. After 6 h, washings were taken out and filtered through the suction pump. The filtrates were collected in large bottles.

**Precipitation.** Treated washings, 4 l, were taken at a time in a tub and made alkaline with 92 ml of 10N NaOH. The alkaline washings were then transferred to a steel bowl and heated to 60 °C. Dissolved 160 g commercial grade sodium sulphide was added to each 4 l of washings while still hot. A substantial excess of sodium sulphide was preferred in order to ensure complete precipitation of silver as silver sulphide. The precipitate was massed for 2-3 h and then kept over night to cool down. As silver sulphide is a colloidal precipitate, it is extremely difficult to filter it. Therefore, excess water was decanted and the precipitate was oven dried.

**Metallic silver.** The precipitate was processed at pilot plant. A large graphite crucible was used for the gravimetric analysis. Silver was recovered from the black precipitate by smelting at 970 °C utilizing commercial grade reagents for smelting. Natural gas was used as the source of heat.

The produced silver was again smelted in a furnace using analytical grade reagents.

**Characterization of the produced silver.** Specific gravity of the produced silver was determined using density meter (Mettler Toledo model XS 203 S) as well as manually.

**Used activated carbon.** The organic substances and gelatin laden activated carbon was air dried and stored in a bottle, for treatment in a separate process to displace the organic substances and gelatin from it to render the carbon fit for reuse.

**Results and Discussion**

The parameters of the process, investigated for their influence on the recovery of silver, were temperature, pH, concentration of activated carbon and its stirring time.

On the first smelting 168 g metallic silver was obtained from the molten mass and on the second smelting, 158 g shining metallic silver was obtained. The density of the metal was 10.60 g/cm$^3$ and thus the purity of the product was 99.62%.

From 24 l of washings, 158 g silver were recovered at pilot scale which shows 94.04% recovery. Compared to the work carried out at laboratory scale (Table 1), the result achieved at the pilot scale was less by 0.50%; this amount of metal might have been lost during handling at large scale.

The activated carbon without absorbing the silver ions, sorbs organic substances, particularly gelatin from the solution and retains mechanical impurities, which makes the medium clear. It eventually results into the release of more silver ions from the washings and increases the recovery of silver. It could be concluded that through this process, silver recovery increases rapidly after treatment with activated carbon at high temperature whereas at lower temperatures and without treatment, silver recovery efficiency is insignificant.

The study shows technological justification of using activated carbon as a purifier for the removal of gelatin and organic compounds from X-ray washings, with high silver extraction efficiency and low reagent consumption. However, during the precipitation of silver as silver sulphide, H$_2$S gas is evolved, which is considered a broad-spectrum poison. At 0.0047 ppm H$_2$S gas has rotten egg smell. At

**Table 1.** Treatment of X-ray washings with activated carbon; dependence of temperature (lab. results)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Recovery of silver with no activated carbon (%)</th>
<th>Recovery of silver after treatment with activated carbon (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>77.57</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>82.57</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>94.54</td>
</tr>
</tbody>
</table>

conditions: X-ray washings, 500 ml; pH, 5.4±0.1; activated carbon, 4 g/l; stirring time, 6 h.
150-250 ppm the olfactory nerve is paralyzed after a few inhalations of the gas and the sense of smell disappears, giving signals of danger. Higher concentrations of 700-800 ppm of H₂S tend to be fatal. In addition to this, some caustic chemicals have also been used with the risk of forming potential toxic fumes. This disadvantage was overcome by performing the precipitation experiment in the fuming chamber and sometimes in the very open air.

References
Comparative Study of Quality Changes in Okra
*Abelmoschus esculentus* (L) Moench Stored at Different Relative Humidities

F. O. Adetuyi*, A. U. Osagieb and A. T. Adekunle*

*a*Food Science and Technology Department, Rufus Giwa Polytechnic, Owo, Nigeria

*b*Biochemistry Department, University of Benin, Benin City, Nigeria

*c*Crop Science Department, University of Benin, Benin City, Nigeria

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**Abstract.** Okra (*Abelmoschus esculentus* L) pods were stored at the relative humidity of 90% and 100% for up to 10 days. The moisture content, crude fibre, and protein, fat, viscosity, hydrolysable and condensed tannin, total phenol, vitamin C and reducing power of the okra were determined on alternate days. Okra pods stored at 100% RH experienced the least percentage loss in all the determined parameters though the loss of antinutrient was lower at this relative humidity.

**Keywords:** relative humidity, antioxidant, okra, storage conditions

**Introduction**

Okra (*Abelmoschus esculentus* (L) Moench) is a tall annual dicotyledonous plant widely grown as vegetable crop in the tropics and subtropics and also in the warmer temperate areas (Kochhar, 1986). It has many different cultivars, varying in many respects. (Tindall, 1986). Okra is traditionally grown in African regions, the most important production regions being Ghana, Burkinafaso and Nigeria (De Lannoy, 2001).

Fresh and green tender fruits of okra are used as vegetable, whereas its mucilage has medicinal applications. Okra has industrial applications as well and is used in confectionary (Siemonsma and Kouame, 2004; Shalau, 2002; Kochhar, 1986).

Okra is a powerhouse of valuable nutrients. Nearly half of its fibre is soluble and half is insoluble which helps to keep the intestinal tract healthy (Shalau, 2002). The fibres from stem and mature pods have a number of uses in papermaking, in textile and other industries. (Siemonsma and Kouame, 2004; Kochhar, 1986). In Nigeria, fresh okra is preferred to dried okra by the majority of the people and as such its consumption is highest in the rainy season when production is at its highest. The sites of okra production are always very far from the market, therefore, post-harvest deterioration of fresh okra results in loss of produce due to poor storage and transportation from the outlying villages to the city markets.

Information regarding optimum environmental conditions for extending post harvest life of okra is not available. Due to the transportation, to long distance, optimum relative humidity might be needed for storage life extension. The aim of this research work is to investigate the quality changes in Akure indigenous okra when stored at the relative humidities (RH) of 90% and 100%. The qualities assessed are nutrients, antinutrients, antioxidants and viscosity.

**Materials and Methods**

Okra plant (*Abelmoschus esculentus* L Moench) used in this study was of indigenous origin in Akure, Nigeria. Plants were grown on a fallow land of 5 years, measuring 14 m×14 m, located in a farm in Ifon in Ose local Government of Ondo State, Nigeria. The experiment was laid out in randomized complete block design (RCBD). Each experimental unit was planted on the side of ridges. Spacing was 0.9 m between and 0.45 m within rows. Three seeds were planted per hole and thinned to one per stand, two weeks after planting (WAP), giving a plant population of about 24,690 plants/hectare. Each potential okra pod was tagged on the day, the flower dropped and pods free of apparent mechanical injuries, insect damage or diseases were harvested using knife on the 8th day after the flower had dropped.

Pods were randomly divided into two lots and stored at 90% and 100% relative humidities at the temperature of 10 °C ± 2 °C for 10 days. The temperature of storage rooms were controlled using a single point thermostat. Relative humidity inside the storage room was manually measured daily using wet bulb/dry bulb hygrometer.
Okra pods were analyzed five times during the storage period for moisture content, crude fibre, crude protein, fat, viscosity, hydrolysable and condensed tannin, phytate, total phenol, ascorbic acid and reducing power. All determinations were done in triplicate.

Nutrient composition, (moisture, fat and crude fibre) of the fresh and stored okra were determined using standard AOAC (1990) method, the protein content was determined using micro-Kjeldhal method (Nx6.25).

The method of Wang and Hwang (1993) was used for the determination of hydrolysable tannin (HT) and condensed tannin (CT). For hydrolysable tannin (HT), phenolic extract (0.5 ml) was diluted with 2 ml distilled water in 10 ml flask. Folin-ciocalteau phenol reagent (1 ml) was added and shaken vigorously. Five ml of 20% Na₂CO₃ was pipetted into the mixture and made up to the mark with distilled water and again shaken vigorously. It was allowed to stand for 20 min for colour development. Absorbance of the sample, standard and the blank were read on spectronic 21D spectrophotometer at a wavelength of 735 nm. For condensed tannin (CT), phenolic extract (0.1 ml) was pipetted into a 30 ml test tube and covered with aluminium foil. Three ml of 4% vanillin (w/v) in methanol was added and the tube was shaken vigorously. Concentrated HCl, 1.5 ml, was added and the tube was shaken again. It was allowed to stand for 20 min for colour development. Absorbance of the sample, standard and the blank were read on spectronic 21D spectrophotometer at a wavelength of 500 nm. Viscosity was measured using Ostwald viscometer as described by AOAC (1990).

For determination of Vitamin C content (AOAC, 1990), 5 g of the sample was extracted with 100 ml H₂O. Twenty five ml of 20% glacial acetic acid was added to 10 ml of the extracted sample and titrated against standardized 2.6 dichloroindophenol (0.05 g/100 ml) solution.

Total phenol was determined by mixing 0.2 ml phenolic extract (0.2 g of okra extracted with 20 ml of 70% acetone) with 0.8 ml Folin-ciocalteau reagent and 2 ml of 7.5% sodium carbonate. The mixture was diluted with 7 ml distilled water and absorbance was measured at 765 nm after 2 h. The result was calculated as gallic acid equivalent (Iqbal et al., 2004).

Reducing power of okra was determined by assessing the ability of okra to reduce FeCl₃ solution as described by Pulido et al. (2000); briefly, 2.5 ml of okra aliquot (0.5 g of okra homogenized in 20 ml methanol) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1 g/100 ml potassium ferrocyanide. The mixture was incubated at 50 °C for 20 min and thereafter 2.5 ml of 10 ml/100 ml trichloroacetic acid was added. The mixture was subsequently centrifuged at 650 rpm for 10 min. Five ml of the supernatant was mixed with equal volume of water and 1 ml of 0.1 g/100 ml ferric chloride. Absorbance was later measured at 700 nm; higher absorbance indicates higher reducing power.

Statistical analysis. Data collected was subjected to the analysis of variance (SAS, 2002). Mean separation was done, where, there was significant differences using Duncan multiple range test procedure as described in the SAS soft ware; significance was accepted at P>0.05.

Results and Discussion

Physiological activities continue in all plant crops following the harvest. These processes involve changes in the chemical composition and physical characteristics of the plant material and can influence its quality as food, whether it is consumed fresh or used as raw material for subsequent processing operations (Rhodes, 1980).

Storage life of okra pods at the two relative humidities (RH) was determined after 10 days. Okra pods stored at 90% RH and 100% RH differed from the freshly harvested okra pod in the intensity of determined parameters.

The proximate content of okra pods at the two relative humidities are shown in Table 1. At the end of the storage period the proximate contents of pods differed from those of freshly harvested ones and were reduced in storage.

Moisture. The moisture content of pods stored at 90% RH reduced from 89.025% to 86.66% and at 100% RH reduced from 89.02% to 87.02%. Reduction in moisture content could be the result of respiration in the stored pods which releases water to the atmosphere and also due to dehydration of the pods as a result of environmental conditions. There was a significant difference (P>0.05) between the moisture content of pods stored at 90% RH and 100% RH. The pods at 100% RH recorded the least percentage loss of 2.25%. A compromise of about 95% RH is usually used to minimize both water loss and storage rots (Rhodes, 1980). A very high relative humidity (95-100%) is needed to retard dehydration, pod toughening and loss of freshness (Cantwell and Suslow, 2005).

Crude fibre. At the end of the storage period, crude fibre reduced from 10.93% to 8.83% at 90% RH and from 10.93% to 9.57% at 100% RH; the reduction in crude fibre might be the result of conversion of cellulose to carbohydrates, used during respiration; however at every stage of the storage, there was no significant difference (P>0.05) between the crude fibre of the pod at the two relative humidities but pods at 100% RH recorded the percentage loss of 12.44% against 19.21% at 90% RH. Decrease in the crude fibre observed in this study
does not conform to the findings of Oti and Mgbolu (1987) who reported increase in crude fibre of the two varieties of Nigerian ginger during storage. Amusa et al. (2002) also reported increase in crude fiber of the breadfruit with increase in the storage time.

**Crude protein:** Crude protein of the pods reduced with the increase in the storage period, from 15.17% to 12.22 at 90% RH and from 15.17% to 12.34 at 100% RH; this could be attributed to the breaking down of proteins after harvest and recycling of the component amino acid. This result conforms with the findings of Omueti and Adepoju (1988) about okra. Agbor-Egbe and Rickard (1990) also reported decrease in crude protein of aroid stored for 14 days. A significant difference (P>0.05) between the crude protein at the two relative humidities was noted at every stage of the storage period, percentage loss in crude protein being the least (18.72%) at 100% RH.

**Fat.** Fat content of the okra pod at the two relative humidities decreased as the storage period increased; at 90% RH it reduced from 9.97% to 7.38%, while at 100%, from 9.97% to 7.25%. This could be the result of recycling of the carbon stored as triacylglycerols into lipids through the action of the enzyme lipase. This result agrees with the findings of Amusa et al. (2002), regarding the bread fruit but contrary to the report of Udoessien and Ifon (1984) about the increase in fat content of the flesh of stored pepper fruit. There was a significant difference (P>0.05) between the fat content at the two relative humidities with 90% RH having the least loss of 25.98%.

**Tannin.** The tannin content of okra pod reduced with the storage period (Fig. 1) which could be attributed to the action of polyphenol oxidase enzyme.

The hydrolysable tannin (HT) is more important nutritionally because it can be readily hydrolyzed into carbohydrate and phenols (Osagie, 1998; Bullard et al., 1981). The hydrolysable tannin reduced from 0.480% to 0.268% at 90% RH and from 0.480% to 0.280% at 100% RH. There was no significant difference (P>0.05) at every stage of storage of the okra pod at the two relative humidities, but 90% RH recorded the high-

---

### Table 1. Nutrient composition of Akure okra (%)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Day</th>
<th>90% RH*</th>
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<td>2</td>
<td>88.45±0.044</td>
<td>88.40±0.044</td>
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<td>4</td>
<td>87.73±0.044</td>
<td>88.24±0.051</td>
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<td>6</td>
<td>87.44±0.053</td>
<td>87.92±0.044</td>
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<td>87.56±0.053</td>
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<td>10</td>
<td>86.66±0.044</td>
<td>87.02±0.035</td>
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<tr>
<td>Loss (%)</td>
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<td>2.25</td>
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<tr>
<td>Crude fiber</td>
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<td>10.93±0.062</td>
<td>10.93±0.062</td>
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<tr>
<td></td>
<td>2</td>
<td>10.33±0.026</td>
<td>10.40±0.026</td>
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<td>4</td>
<td>10.18±0.010</td>
<td>10.28±0.026</td>
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<td>12.22±0.026</td>
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<tr>
<td>Loss (%)</td>
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<td>20.41</td>
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<td>8.99±0.017</td>
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<td>7.78±0.017</td>
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<td>Loss (%)</td>
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<td>25.98</td>
<td>27.28</td>
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</tbody>
</table>

* = values represent means of triplicate tests; values with the same alphabet in the same row are not significantly different (p> 0.05).

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Fig. 1. Condensed tannin and hydrolysable tannin. (A) Content (%); (B) Loss (%).
est percentage loss (44.09%) at the end of the storage period which makes much of the HT not available. Condensed tannins (CT) are complex flavanol polymers which cannot be hydrolyzed to simple components; they have limited solubility and extractability and hence may have little nutritional significance (Osagie 1998; Mehansho et al., 1987). Condensed tannin reduced from 0.630% to 0.484% at 90%RH and from 0.630% to 0.501%, at 100%RH with the former having the highest percentage loss of 23.06% at the end of the storage period, making much of the CT unavailable. Fleck (1988) reported decrease in tannin level during storage of four acorn species.

Viscosity. The viscosity reduced during storage at the two relative humidities Fig. 2. At 90% RH, the viscosity of Akure okra pod reduced from 63.27 cp to 51.38 cp and at 100% RH, from 63.27 cp to 51.26 cp. The highest percentage loss of 18.95% was recorded at 100% relative humidity and there was a significant difference (P>0.05) at every stage of the storage at the two relative humidities. This could be attributed to the loss of moisture in the okra through metabolic activities.

Total phenol. Total phenol reduced as the storage period increased (Table 2). This conforms with the findings of Ose et al. (1997) who reported decrease in total phenol content of water convolvulus leaves during the storage. Lim et al. (2006) reported decrease in total phenol content of guava. Total phenol of okra pods at 90%RH reduced from 0.098 to 0.063 mg/g gallic acid equivalent (GAEg) while 100% RH from 0.098 to 0.071 mg/g GAE; this could be attributed to the oxidation of phenols by phenolase to quinones (Kays, 1991). There was a significant difference (P>0.05) at every level of storage of the pods at the two relative humidities. The relative humidity of 100% recorded the lowest percentage loss of total phenol (27.54%), which means it conserves more of this antioxidant and makes them available.

Reducing power. Reducing power of okra followed the trend of the total phenol and reduced during storage (Table 2). At 90% RH, it reduced from 1.16 to 0.65 and at 100% RH, from 1.16 to 0.71 at absorbance of 700 nm. However, there was no significant difference (P>0.05) at every stage of storage at the two relative humidities. The least percentage loss of 38.79% was recorded at the relative humidity of 100% RH.

Vitamin C. Vitamin C contributes to the antioxidant properties of vegetables by protecting the erythrocyte membrane, maintaining the blood vessel flexibility and improving the blood circulation in the arteries as well as facilitating the absorption of iron in the body (Oboh, 2005). Vitamin C content (Table 2) reduced during storage at the two relative humidities. Evensen (1983) reported loss of vitamin C in the musk melon during storage. Vitamin C in Okra pods reduced from 48.73 mg/100 g to 16.52 mg/100 g at 90% RH and from 48.73 mg/100 g to 18.92 mg/100 g at 100% RH, which could be attributed to the conversion of ascorbic acid to dehydro ascorbic acid in stored produce by the enzyme, ascorbate oxidase. At every stage of storage, there was a significant difference (P>0.05) between the vitamin C content of the stored

Table 2. Total phenol, reducing power and vitamin C content of ‘Akure’ okra

<table>
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<th>Parameters</th>
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<th>90% RH*</th>
<th>100% RH*</th>
</tr>
</thead>
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<td>Total phenol (mg/GAEg)</td>
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<td>0.098±0.003a</td>
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<td>0.088±0.003a</td>
<td>0.089±0.003a</td>
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<td>4</td>
<td>0.082±0.003ab</td>
<td>0.085±0.003a</td>
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<td>0.076±0.003b</td>
<td>0.080±0.003a</td>
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<td>0.078±0.008a</td>
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<td>10</td>
<td>0.063±0.003b</td>
<td>0.071±0.003a</td>
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<tr>
<td>Loss (%)</td>
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<td>27.54</td>
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<td>1.16±0.052a</td>
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<td>0.82±0.026a</td>
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<td>0.80±0.026a</td>
<td>0.81±0.026a</td>
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<td>0.76±0.026a</td>
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<td>0.73±0.026a</td>
<td>0.75±0.026a</td>
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<tr>
<td></td>
<td>10</td>
<td>0.65±0.026bc</td>
<td>0.71±0.026a</td>
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<tr>
<td>Loss (%)</td>
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<td>43.96</td>
<td>38.79</td>
</tr>
<tr>
<td>Vitamin C (mg/100 g)</td>
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<td>48.73±0.029a</td>
<td>48.73±0.029a</td>
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<td>2</td>
<td>21.86±0.012a</td>
<td>22.84±0.026a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20.59±0.032a</td>
<td>20.46±0.015a</td>
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<td>6</td>
<td>19.78±0.044a</td>
<td>20.01±0.040a</td>
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<tr>
<td></td>
<td>8</td>
<td>16.64±0.040a</td>
<td>19.75±0.023a</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>16.52±0.023a</td>
<td>18.92±0.038a</td>
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<tr>
<td>Loss (%)</td>
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<td>64.42</td>
<td>59.62</td>
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</tbody>
</table>

* = values represent means of triplicate tests; values with the same alphabet along the same row are not significantly different (p> 0.05).
pod at the two relative humidities with 100% RH having the lowest percentage loss (61.17%).

Conclusion

It is concluded from the observations presented that though in a few of the parameters observed, there was no significant difference (P>0.05) in “Akure” okra pods stored at 90% RH and 100% RH but the pods stored at 100% recorded more acceptable percentage loss. Thus for storage of okra, 100% RH at the temperature of 10 ºC ± 2 ºC is recommended.

References


Cantwell, M., Suslow, T. 1997-2005. Recommendations for Maintaining Postharvest Quality of Okra, Department of Plant Science, University of California, Davis, California, USA.


Introduction
During the last three decades, Gulf’s agricultural production has dramatically increased. (Talukder and Kaakeh, 2006; Thacker et al., 2000) which is associated with different factors including the increased use of agrochemicals, especially pesticides for crop protection (Tivy, 1991). Since 1960, the value of pesticide imports to Oman has increased more than 10 fold (FAO, 2003). Around 835 pesticides have been registered in the UAE, and among them, the insecticides have the greatest share (49.8%) followed by fungicides (22%). The average amount of pesticides used in the UAE has been 9.86 l or kg per hectare per year (Kaakeh et al., 2004). This increased use of different pesticides in Gulf countries is a cause for serious concerns. Hazardous effects of pesticide residues in plants, soil, water, food, environment, humans and domestic animals and on beneficial organisms including pollinating honeybees, predators, parasites, fishes, birds and other wild animals are very serious issues world wide (Perry et al., 1998; Edwards, 1987). Pesticides may also cause resistance and resurgence problems in target pest populations, resulting in use of their higher doses at greater frequencies in the farms (Talukder and Kaakeh, 2006).

The use of pesticides in vegetable production in Oman is a regular practice. In Northern Oman all agricultural farms use different types of pesticides for the protection of crops (Kaakeh et al., 2007; Thacker et al., 2000); 95% of the farms use insecticides, 60%, fungicides and 20%, herbicides. Regular use of pesticides in crop production might be associated with pesticide residue problems. Three out of 11 selected pesticides, widely used in the UAE, have the potential to leach to groundwater due to their solubility that exceeds the US EPA threshold values (Kaakeh et al., 2004). Therefore, over the years, contamination of soil and ground water with the pesticides has been a major concern. The present investigation was designed to determine pesticide residue levels in agricultural soils of Omani and UAE vegetable farms, so as to estimate the extent of soil pollution caused by the use of pesticides under local environmental conditions. It may consequently aid in developing better management strategies.

Materials and Methods
Sampling techniques. In Oman, twenty farms located at different sites were selected from the Al-Batinah region, from which soil samples were collected randomly from two locations in each farm at two different depths (0-15 cm and 15-30 cm). In the UAE, twenty farms representing four agricultural regions of Al-Ain (five farms per region) were selected for the present study and soil samples were collected in a similar way. Soil samples were separately sieved to exclude foreign materials and the samples were stored in previously marked plastic bags which were transferred to Central Laboratory Unit, UAE University for analysis. The types of pesticides applied in all of these surveyed farms were also recorded.

Analyses of pesticide residues in soil samples. Analytical techniques. Analytical techniques used for the soil analysis at the laboratory involved extraction, identification, confirmation (wherever possible) and quantitation of pesticides. The studied pesticides included propamocarb, diazinon, pirimiphos-methyl, malathion, chlorpyrifos, phenthoate, triazophos, deltamethrin, cypermethrin, dimethoate and metalaxyl. Dimethoate and metalaxyl in the extract were separated and
carbamates were analysed by HPLC on XTerra MS C-18 column using photodiode array detector. All other pesticides were analyzed using GC-ECD for organochlorine and GC-NPD for nitrogen and phosphorous compounds. Confirmation was made by GC-MS.

**Sample preparation.** 10 ± 0.5 g of each soil sample was blended with 10 ± 0.5 g of anhydrous sodium sulfate and placed in an extraction thimble and put inside the Soxhlet equipment. Approximately 80 ml of the extraction solvent (1: 1 methylene chloride: acetone) was added, and the sample was extracted for 2 h (with boiling for 1 h and rinsing for 1 h) at 150 °C. Each sample was carefully evaporated after extraction. The evaporation was carried out using a rotary evaporator till the volume of the extract was reduced to around 2 ml. Then 5 ml methanol was added and the solution was again concentrated. The process was repeated twice; and finally the extract was concentrated to about 1 ml. The extract was collected in round flask and exchanged to hexane solvent and quantitatively made up to 2 ml, transferred to a 2 ml GC auto sampler vial; 2 µl of the extract was sequentially injected using both injectors, one connected to the NPD and then to the ECD with 0.5 min delay.

**GC conditions.** A Varian 3800 GC-ECD-NPD was used for current analyses with two different types of columns ( CP-Sil 8, 30 m* 0.32 mm ID, df = 1 connected to NPD and CP-Sil 19 CB 30 m* 0.25 mm ID, df= 0.25 connected to ECD). Helium (1.5 ml/ min) was used as the carrier gas for both the columns. The injector temperature was maintained at 240 °C, splitless for both injectors and detector temperature at 300 °C for both ECD and NPD. The temperature programme was: initial temperature at 80 °C, held 1 min, 80 °C to 160 °C at 8 °C/min, held for 2 mins, followed by 160 °C to 180 °C at 2 °C/min, held for 7 mins, 180 °C to 200 °C at 2 °C/min, held for 2 min and 200 °C to 260 °C at 5 °C/min, held for 20 min. The level of detection was upto 0.01 ppm.

**HPLC.** The HPLC equipment used for the current analyses was a gradient solvent delivery system (Waters, Alliance 2695 separation module with column oven or equiv.), equipped with XTerra MS C18 (150 mm x 4.6 mm ID, 5 µm) column and Waters 2996 PDA Detector. The isocratic programme for LC pump was with flow of 1.0 ml/min, temperature 25 °C, 65% A (water) and 35% B (CH3CN). The photo-diode-array detector was monitored at 215 nm. The limits of detection were 0.25 µg/g and 0.10 µg/g for dimethoate and metalaxyl, respectively.

**Expression of results.** The amount of pesticide residues (µg/g), detected in each sample from each farm, was calculated according to the following equation:

\[
\text{Pesticide residue (µg/g)} = \frac{\text{area of sample x conc. of std (µg/ml) x vol. made up to (ml)}}{\text{area of std. x wt. of sample (g)}}
\]

**Results and Discussion**

Among the tested pesticides, cypermethrin was the most frequently detected in Omani farm soil samples followed by chlorpyrifos and to a lesser extent, phenthoate, triazophos, and deltamethrin (Table 1). The highest level of cypermethrin was detected as 0.48 mg/kg in the soil samples from farm 7, followed by 0.36 mg/kg in farm 9. The levels of phenthoate, triazophos, and deltamethrin in the soil were within the range of 0.01-0.03 mg/kg. The chlorpyrifos and malathion were detected in some of the Omani farm soils with different concentration levels. As for example, 1.01 mg/kg level of chlorpyrifos was detected in farm 6, followed by 0.79 mg/kg in farm 16. Malathion was observed in the soil samples of farm 3 only. Propamocarb, diazinon, pirimiphos-methyl, dimethoate and metalaxyl were not detected in any Omani soil samples.

In the UAE soil samples, different pesticides were detected in different regions (Table 2). In the central region, the most frequently detected pesticide was chlorpyrifos (in all farms), followed by cypermethrin and deltamethrin, while malathion and propamocarb were detected only once or twice. The level of chlorpyrifos reached up to 0.37 mg/kg in some of the farms (farm C1) while the level of other detected pesticides did not exceed 0.08 mg/kg. Diazinon, pirimiphos-methyl, phenthoate, triazophos, dimethoate and metalaxyl were not detected in the central region soil samples. In the eastern region, the most frequently detected pesticides were chlorpyrifos and cypermethrin followed by phenthoate and deltamethrin. The least frequently detected pesticides were propamocarb and diazinon. The level of cypermethrin was generally high (0.1-0.8 mg/kg). The highest level of cypermethrin was detected in soil samples from farm E1 (0.86 mg/kg). On the other hand, the highest level of chlorpyrifos (1.46 mg/kg) was detected in farm E2. However, several pesticides including pirimiphos-methyl, malathion, triazophos, dimethoate, and metalaxyl were not detected in the eastern region soil samples. In the western region, low concentrations of deltamethrin (0.02-0.19 mg/kg) and chlorpyrifos (0.01-0.04 mg/kg) were detected at relatively low levels; cypermethrin was detected in a few samples only, whereas propamocarb, diazinon, pirimiphosmethyl, malathion, phenthoate, triazophos, dimethoate, and metalaxyl were not detected in this region. In the northern region, the most frequently detected pesticides were deltamethrin (0.02-0.19 mg/kg) and cypermethrin (0.01-0.12 mg/kg) followed by
Table 1. Pesticide residues in selected soil samples from Al-Batinah region of Sultanate of Oman

<table>
<thead>
<tr>
<th>Region</th>
<th>Farms</th>
<th>Sample</th>
<th>Depth (cm)</th>
<th>HPLC Method</th>
<th>Results (mg/kg)</th>
<th>GC Method</th>
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</thead>
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<td></td>
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<td></td>
<td></td>
<td>Metalaxyl</td>
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|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
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| 13 | 1   | -   | -   | 0.02| -   | -   | -   | -   | -   | 0.05| -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
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| 2  | 1   | -   | -   | -   | -   | 0.01| 0.03| -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
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| 14 | 1   | -   | -   | 0.01| -   | -   | -   | -   | 0.01| -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
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| 2  | 1   | -   | -   | 0.04| 0.01| -   | 0.03| -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|    | 2   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| 15 | 1   | -   | -   | -   | -   | -   | -   | -   | -   | -   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   |
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| 2  | 1   | -   | -   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   | *   |
|    | 2   | -   | -   | 0.02| -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| 16 | 1   | -   | -   | 0.04| 0.01| -   | 0.23| -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
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| 2  | 1   | -   | -   | 0.10| -   | -   | 0.79| -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
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|    | 2   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |

- = pesticide residues not detected (level of detection is 0.01 ppm); * = soil sample was not available for analysis; depth 1= 0-15 cm; depth 2= 15-30 cm
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Table 2

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Continued...
life is around 13 weeks. It might be the reason of presence of higher amount of cypermethrin in different soil samples. The highest level of chlorpyrifos found was 1.01 mg/kg in Oman and 1.46 mg/kg in UAE, but generally its average levels were below 0.1 mg/kg. Depending on the type of soil, microbial metabolism of chlorpyrifos may have a long half-life; soil temperature, organic content and acidity levels play important roles in its degradation (US EPA, 1984).

Chapman et al. (1981) reported that persistence of cypermethrin in soil was related to organic matter, clay content, microbial activity and anaerobic conditions. In the present study, in Oman soil samples, organic matter in the range of 0.201-6.048% and in UAE farm soil samples, in the range of 0.201-5.342% was recorded. It is possible that the higher levels of pesticides observed in the soil samples were associated with higher soil organic matter content. Redondo et al. (1997) calculated the degradation half-lives of chlorpyrifos as 10 days, and found that the distribution through the soil profile shows that the pesticide concentrations were always highest in the upper layer (0-5.0 cm) of soil. This report was in agreement with our current results, where we also observed that the pesticide residue levels in top-soil (0-15 cm) were higher than at lower depths (15-30 cm). Putnam et al. (2003) reported that chlorpyrifos residues could be detected in cranberry fruits at harvest, after 62 day post-chlorpyrifos application. Gyldenkaerne et al. (2000) reported that the deltamethrin and cypermethrin have almost the same physical and chemical properties. Gana et al. (2005) warned that although pyrethroids are known for their immobility in soil, however, surface erosion and runoff may ultimately lead to significant off-site pesticide movement to surface streams over a sufficiently long time scale due to their long persistence. However, as Oman and UAE both are desert countries with average rainfall of 20-100 mm/year in Oman and 65 mm/year in UAE and commercial vegetable production is fully dependent on irrigation, this type of off-site movement is irrelevant in both the countries. In Gulf countries like Oman and UAE, the use of pesticides for crop protection is increasing, but systematic investigations on the presence/persistence of pesticide residues in agricultural soils are not available. Therefore, the current study will be helpful to create awareness in both the countries.

Conclusion

Detected pesticides in Oman and UAE soil varied in their levels in different agricultural farms and regions. In Omani vegetable farms, pesticide residue of cypermethrin was most frequently detected followed by chlorpyrifos. In vegetable farms of all the four regions of UAE, chlorpyrifos, cypermethrin and deltamethrin were detected in soil samples, but, phenthoate was detected only in the eastern and northern regions. The knowledge gained from the current investigation on pesticide residues in vegetable farm soils of Oman and UAE will aid in future management for pesticide application and handling. The current results might help in the selection, use and application of different pesticides that may lead to a reduction in pesticide residues in agricultural soils in both the countries. In addition, this outcome will help in the future pesticide management strategies in the Sultanate of Oman and the United Arab Emirates.

Acknowledgement

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References


Prevalence and Pathology of Helminth Infections in Pigs


*Department of Parasitology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
bDepartment of Agricultural Statistics, Faculty of Agricultural Economics and Rural Sociology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
cDepartment of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
c/o Nurul Islam, 18/I/1, Kristopur, Mymensingh-2200, Bangladesh

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*Author for correspondence; E-mail: islam_ausrf@yahoo.com

Abstract. In 30 viscera of local slaughtered pigs from different areas of Tangail and Mymensingh districts of Bangladesh, six species of helminths were identified; 2 of them were trematodes namely Fasciolopsis buski (36.70%) and Gastrodiscoides hominis (26.70%) and 4 species were nematodes namely Ascaris suum (60%), Metastrongylus elongatus (53.33%), Stephanurus dentatus (10%) and Physocephalus sexalatus (56.71%). Three nematode species, viz. M. elongatus extracted from lung, S. dentatus from peri-renal fat and P. sexalatus from stomach, were recorded for the first time in pigs in Bangladesh. No gross lesion was observed in pigs affected with M. elongatus. In A. suum infection, intestinal wall was infiltrated with plasma cells, lymphocytes and eosinophils. In M. elongatus infection, lymphocytes and macrophages mainly eosinophilic infiltration was observed in the parenchyma of lung. Age exerted a significant (p<0.05) influence on the development of the helminths, P. sexalatus, F. buski, A. suum and G. hominis.

Keywords: helminth infection, pigs, Bangladesh

Introduction

In Bangladesh, the pig rearing is limited to the minority people. Pig population in Bangladesh is estimated as 8 millions (Rahman, 2004). Some of the pig parasites have public health significance (Talbot, 1972). It is reported that most of the pig parasites are the causes of great economic loss in terms of poor growth and weight loss (Johnson et al., 1972). Internal parasites devitalize pigs by robbing them of essential nutrients and injuring vital organs. Pigs heavily parasitized are more susceptible to diseases such as scour and pneumonia (Soulsby, 1982). The resulting diseases and unthriftiness are a major cause of economic loss.

Bangladesh due to its conducive geo-physical condition and tropical climate is considered as haven of parasites (Salkeld et al., 2008). Situation is further aggravated by orthodox husbandry methods such as rearing pigs in clay, feeding the pig herd in dirty places etc. As such pigs remain susceptible to both ecto- and endoparasites. Basak (1988) has documented 5 species of parasites from visceral samples of pigs from some districts of Bangladesh. Moreover, Shaikh and Huq (1984) recorded Ascaris suum, Trichurus suis, Fasciolopsis buski and Ancylostoma duodenale. But unfortunately no attention has been paid to study the pathology of parasites in pigs in Bangladesh. The present study was aimed at determining the prevalence of helminth infections and pathological lesions produced by them.

Materials and Methods

For the study, a total of 30 viscera of slaughtered pigs were examined for helminths during the period from July, 2005 to May, 2006. Visceral examination, parasite identification and preservation were conducted in the laboratory at the Department of Parasitology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, whereas, pathological study was performed in the Department of Pathology, Bangladesh Agricultural University, Mymensingh.

Post-mortem examination, parasite collection and identification. Viscera of 30 slaughtered pigs were collected from different places of Mymensingh and Tangail districts. In Mymensingh district the pigs are reared by lower cast Hindus and in Tangail district, non-Muslim ethnic people are engaged in the practice. These were the only two areas from where the visceral samples could be collected and brought to the laboratory within five to six hours and so it was possible to examine all the samples on the day of collection. Viscera were collected from local markets where the pigs are slaughtered. After collection the viscera were brought to the laboratory. Each part of the gastrointestinal tract was opened through long axis by giving longitudinal incision with scissors in separate clean buckets. The contents of the respective part were
made clear by repeated washing and sedimentation. The mesentery were cut into small pieces with the help of a sharp pair of scissors and kept in a jar containing sufficient amount of normal saline. After 20 min, the supernatant was decanted and the sediment was examined. Lungs, kidney with perirenal fat, liver, mesentery and spleen were also separated carefully and examined for the detection of lesions if any produced by parasites. These organs were cut into small pieces and kept in two separate glass jars with normal saline. Then the pieces were squeezed gently and removed from the jar. After several washings, the supernatant was poured off carefully and the sediment was examined for presence of parasites. Lungs were collected from all slaughtered pigs and were examined grossly for lung worms after opening the bronchial passages with a pair of scissors. The collected parasites were carefully washed in saline to remove mucus and other waste materials. Diaphragms and intercostal muscles were examined for the larvae of *Trichinella spiralis* and *Cysticercus cellulose* by artificial digestion method.

**Artificial digestion method.** Selected piece of muscle was minced in a household mincer. Minced tissues 10 g were then stirred in 100 ml peptic digestion fluid at 37 °C. This was incubated at 37 °C for 4 to 6 h. Then the content was poured through a wire mesh screen with an aperture of 0.25 mm, held over another one with an aperture of 0.75 mm. This was then washed well in running tap water. Then the content was allowed to settle and the sediment was examined for *Trichinella spiralis* and *Cysticercus cellulose* (Rahman *et al.*, 1996).

Nematodes were preserved in 70% glycerine alcohol and trematodes, in 10% formalin.

**Trematode identification.** Trematodes were identified according to the keys and description given by Soulsby (1982) by preparing permanent slide following the methods, described by Cable (1957).

**Nematode identification.** Nematodes were identified by preparing temporary slides adding one drop of lactophenol (Cable, 1957) according to the keys and description given by Soulsby (1982), Anderson (1992) and Yorke and Meplestone (1962).

**Study for pathological lesions.** Lesions containing tissues or organs were collected and preserved in 10% buffer neutral formalin. Fixed tissue sections were processed, paraffin embedded, sectioned and were routinely stained with haematoxylin and eosin (H & E) as per standard procedure (Luna, 1968).

**Statistical analysis.** The multivariable logistic regression models were fitted to determine whether, age and sex significantly influenced the helminth infections (Hosmer and Lemeshow, 1989).

**Results and Discussion**

To study the prevalence of helminth parasites and pathological lesions produced by them in pigs, 30 viscera were examined. Of them 27 (90%) viscera were found to be infected with one or more species of helminths. During visceral examination only 6 species of parasites were recovered, among them were 2 species of trematodes such as *F. buski* (Fig. 1) and 4 species of nematodes namely *A. suum* (Fig. 2), *M. elongatus*, *P. sexalatus* and *S. dentatus* and *G. hominis* (Table 1). This finding is almost similar to the finding of Davidson and Taffs (1965) who reported 95% helminth infections in pigs. However, the present finding was much higher than the results reported by Rajkhowa *et al.* (2003) in India who recorded 63.31% gastrointestinal

![Fig. 1. Fasciolopsis buski, Lankester, 1975.](image1)

![Fig. 2. Ascaris suum, Goeze, 1782.](image2)
helminth infection in pig. These parasites were also recorded in pigs by Carstensen et al. (2002), Saikh and Huq (1984), Varma (1957) and Lapage (1962) in different countries. The nematodes, *P. sexalatus* (Nematoda: Spiruroidea, Fig. 3, 4), *M. elongates* (Nematoda: Stongyloidea, Fig. 5, 6), and *S. dentatus* (Nematoda: Stongyloidea, Fig. 7, 8), were recorded for the first time in lung, stomach and perirenal fat of local pigs, respectively, in Bangladesh.

Prevalence of helminth parasites was the highest in case of *A. suum* (60%) followed by *M. elongatus* (53.33%), *T. suis* (56.71%), etc. Overall mean parasitic burden was 33.22±3.30 (Table 2).

**Effect of age and sex on parasitism.** In this study, the result of multivariable logistic regression model lead to the conclusion that age was a significant (P<0.05) risk factor for developing each of the helminths, *P. sexalatus*, *F. buski*, *A. suum* and *G. hominis*. But sex exerted no significant (P> 0.05) influence on the development of these helminths. The positive sign of the coefficients of age indicates that the risk of being infected by the helminths, *P. sexalatus*, *F. buski* and

<table>
<thead>
<tr>
<th>Class or order of parasites</th>
<th>Name of parasite</th>
<th>Location in the host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trematoda</td>
<td><em>Fasciolopsis buski</em>, Lankester, 1875 (Fig. 1)</td>
<td>Small intestine</td>
</tr>
<tr>
<td></td>
<td><em>Gastrodiscoides hominis</em>, Lewis and McConnel, 1913</td>
<td>Caecum and colon</td>
</tr>
<tr>
<td>Nematoda</td>
<td><em>Ascaris suum</em>, Goeze, 1782 (Fig. 2)</td>
<td>Small intestine</td>
</tr>
<tr>
<td></td>
<td><em>Physocephalus sexalatus</em>, Molin, 1860 (Fig. 3, 4)</td>
<td>Stomach</td>
</tr>
<tr>
<td></td>
<td><em>Metastrongylus elongatus</em>, Gmelin, 1790 (Fig. 5, 6)</td>
<td>Bronchi and bronchioles of lung</td>
</tr>
<tr>
<td></td>
<td><em>Stephanurus dentatus</em>, Diesing, 1839 (Fig. 7, 8)</td>
<td>Perirenal fat</td>
</tr>
</tbody>
</table>

**Table 1.** Helminth parasites of pigs with their location in the host

Fig. 4. Posterior portion of *Physocephalus sexalatus* 4x.

Fig. 5. Anterior portion of *Metastrongylus elongatus* 4x.

Fig. 6. Posterior portion of male *Metastrongylus elongatus* 4x.
by a pig increases with the increase in age. This result could not be compared and contrasted due to the lack of relevant literature. Probably inverse age resistant phenomenon is associated with more prevalence of these parasites in aged animals. Besides, life cycle of these parasites are indirect (Lapage, 1962). Dung beetle acts as intermediate host in the life cycle of *P. sexalatus* and aquatic snail in case of *F. buski* and *G. hominis*. Adult pigs are allowed to scavenge in the fields and marshy areas. So adult pigs have more chance of getting infection. On the other hand, the negative sign of the age coefficient of the 3rd model implies that the risk of the pig being infected by *A. suum* decreases as age increases (Table 3). This finding is in full agreement with the findings of Lapage (1962) and Soulsby (1982). Perhaps, immunity increases with the increase in age.

### Table 2. Prevalence and mean burden of helminths in pigs

<table>
<thead>
<tr>
<th>Name of helminth</th>
<th>Animals affected (%)</th>
<th>Parasitic load</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=30</td>
<td>Range</td>
<td>Mean ± SE</td>
<td></td>
</tr>
<tr>
<td><em>A. suum</em></td>
<td>18 (60%)</td>
<td>7-50</td>
<td>21.89±3.14</td>
<td></td>
</tr>
<tr>
<td><em>M. elongatus</em></td>
<td>16 (53.33%)</td>
<td>10-30</td>
<td>17.31±1.63</td>
<td></td>
</tr>
<tr>
<td><em>T. suis</em></td>
<td>17 (56.71%)</td>
<td>1-4</td>
<td>2.12±0.21</td>
<td></td>
</tr>
<tr>
<td><em>F. buski</em></td>
<td>11 (36.70%)</td>
<td>1-5</td>
<td>2.73±0.45</td>
<td></td>
</tr>
<tr>
<td><em>G. hominis</em></td>
<td>8 (26.70%)</td>
<td>8-20</td>
<td>12.25±1.46</td>
<td></td>
</tr>
<tr>
<td><em>S. dentatus</em></td>
<td>3 (10%)</td>
<td>17-25</td>
<td>20.67±2.33</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>90%</td>
<td>1-71</td>
<td>33.22±3.30</td>
<td></td>
</tr>
</tbody>
</table>

Study of pathological lesions. No gross lesions were detected but parasite *M. elongatus* was present in the bronchi and bronchioles. The lung showed inflammatory exudates in the lumen of the bronchus. During the histopathological study, infiltration of lymphocytes, macrophages mainly eosinophils in the parenchyma of the lung was observed (Fig. 9). There was an increased cellularity in the alveolar wall of the affected

**Fig. 7.** Anterior portion of *Stephanurus dentatus* 4x.

**Fig. 8.** Posterior portion of male *Stephanurus dentatus* 4x.

**Fig. 9.** Lung: Note the infiltration of lymphocytes, macrophages and mainly eosinophils in parenchyma.
lung. These findings conform to the findings of Vercruysse et al. (1986), Anthony and Lewis (1961) and Soulsby (1965). *M. elongatus* is incapable of lacerating the lung tissue (Porter, 1936). Probably for this reason, no gross lesion was produced.

No gross lesions were observed in case of *A. suum* infection. The wall of the intestine was infiltrated by plasma cells, lymphocytes and eosinophils in the mucosa and sub mucosa. This tissue reaction occurred due to parasitic infestations. The lumen consisted of necrotic debris (Fig. 10). These observations were supported by the observations of Soulsby (1965). Mouth parts of *A. suum* are adapted for the feeding of epithelial cells of intestine. In fact they feed on epithelial cells of the intestine. But there is no evidence that they actively suck blood (Soulsby, 1965). For that reason, probably, they usually do not produce deep wounds in the mucosa of the intestine.

![Fig. 10. Intestine: Note the Infiltration of eosinophils in the wall of intestine due to *A. suum* infection.](image)

During the present study, the prevalence and pathology of parasites in pig which were reared in two different management systems were studied. But it was not possible to determine the effect of parasites on the production performance of pig in terms of growth rate, meat production, furrowing etc. With the use of available anthelmintics in our country, such as the combination of levamisole and triclabendazole or tetramisole and oxyclozanide at 4 months interval, this parasitism can be controlled. This parasitism in pigs can be easily detected by examination of faecal samples provided by any government veterinary hospital. But due to lack of awareness most of the pig rearers are not involved in deworming practices regularly. So, all pig rearers should deworm their pigs at an regular interval to keep their pigs healthy and to keep away the risk of zoonotic parasitic diseases.

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Oxford, UK.


Concentration of Electrolyte Reserves of the Juvenile African Catfish *Clarias gariepinus* (Burchell, 1822) Exposed to Sublethal Concentrations of Portland Cement Powder in Solution

Mohammed Kabir Adamu* and O. Arimoro Francis
Department of Animal and Environmental Biology, Delta State University, Abraka, Nigeria

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Abstract. The study investigated the effect of sublethal concentrations (39.10, 19.55, 9.87 and 0.00 mg/l) of Portland cement powder in solution on the electrolyte reserves (sodium, potassium, calcium, chloride and inorganic phosphorus) in the serum, liver and kidney of the juvenile African catfish *Clarias gariepinus* after a 15 day exposure period. The basic function of the determined electrolyte reserves in the body lies in controlling fluid distribution, intra and extra cellular acidobasic equilibrium, maintaining osmotic pressure of body fluid and normal neuro-muscular irritability. The result revealed significant (P<0.05) changes in serum sodium, potassium, calcium and chloride and insignificant (P>0.05) changes in inorganic phosphorus. Sodium, calcium, chloride and inorganic phosphorus and potassium were significantly (P<0.05) and insignificantly (P>0.05) different in liver and kidney, respectively. Ipso-facto, the effector organs viz: liver and kidney of teleost species – *Clarias gariepinus* which are primarily responsible for regulating water and ionic movement between external and internal milieu of fishes are susceptible to deleterious effects of Portland cement powder thus sublethal concentration (39.10 mg/l) of Portland cement powder in solution after a 15 day exposure has been most toxic and debilitating to the test fish.

Keywords: Portland cement, electrolyte reserves, serum, liver, kidney, *Clarias gariepinus*.

Introduction

Though involved in the developmental structure of any country in the modern world but cement industry generates dust/aerosol during its operations. Portland cement also known as hydraulic cement is composed of tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrate and gypsum with trace constituents like potassium oxide, sodium oxide, chromium compound and nickel compound (Mindess and Young, 1981). Use of these important ingredients of concrete and mortar as well, cannot be avoided in building construction. Cement and most of its constituents have been found to be toxic to living organisms in the environment (Adamu and Audu, 2008; Adamu and Kori-Siakpere, 2008; Akinola et al., 2008; Khattak et al., 2005, Fatima et al., 2001; Hansen, 1998).

The development and severity of the injury caused by the cement dust on plants or animals depend besides the concentration of the pollutant, also on the duration of exposure to the pollutant, the species and its stage of development as well as the environmental factors conducive to the accumulation of the cement dust in the organism, which makes it susceptible or resistant to injury (Heather, 2003).

According to Musa and Omorogie (1999), fish are intimately associated with an aqueous environment and physical and chemical changes in the environment are rapidly reflected as measurable physiological changes in the fish.

Electrolyte reserves are the ions balance that change within certain limits depending on metabolic activities caused by some environmental factors such as pollution. In fresh water fishes, blood and electrolyte concentrations are regulated by interacting processes, such as absorption of electrolyte from the surrounding medium through active mechanism mainly at the gills and through selective re-absorption of electrolytes from urine. Any alteration in one or more of these processes results in a change in the plasma electrolyte composition. These ions play vital role in several body functions. The monovalent ions, sodium, potassium and chloride are involved in neuromuscular excitability, acid base balance and osmotic pressure (Verma et al., 1981), whereas divalent ions such as calcium and magnesium facilitate neuromuscular excitability, enzymatic reaction and retention of membrane permeability. Inorganic phosphate acts as a major cytoplasmic buffer and is the basis of energy exchange (Aurbach et al., 1985).

Despite the fact that cement production leads to the formation of aerosols which invariably reach the aquatic system, no detailed study has been reported on its sublethal effect on concentration of the electrolyte reserves of fish. Therefore, the present study seeks to determine the sublethal effect of Portland cement powder in solution on the concentration of
the electrolyte reserves of the juvenile African catfish, *Clarias gariepinus*. *Clarias gariepinus* occupies a position within a food chain leading to man. It is widely available and abundant and thus amenable to laboratory testing. It is genetically stable and thus a uniform population can be tested. Further it can be produced artificially.

**Materials and Methods**

**Experimental fish.** Juveniles of the African catfish *Clarias gariepinus* (Burchell, 1822) of the same brood-stock and mixed sex were obtained from Asaba Fish Farm in Igbide, Delta State, Nigeria. The mean length and weight of fish were 16.35±0.23 cm and 28.07±0.45 g, respectively. The fish were transported in an oxygenated bag to the laboratory (Animal and Environmental Biology Laboratory, Delta State University, Abraka, Nigeria) and placed in a large plastic aquarium of 60 l capacity with well aerated borehole water to acclimatize for ten (10) days during which they were fed with commercial feed (Duranteed feed) to avoid starvation. After acclimatization, the fish were transferred to the experimental plastic aquaria, ten (10) fish per 40 l aquarium in replicate thus twenty (20) fish per concentration.

**Preliminary investigation.** The following concentrations of Portland cement powder in solution (Dangote Portland cement powder, Nigeria) were used for the range finding test: 156.40, 78.20, 39.10, 19.55, 9.78, 4.89 and 2.45 mg/l. The tests were conducted on five (5) *Clarias gariepinus* juveniles per concentration of Portland cement powder in solution with replications, including the control with 0% Portland cement powder in solution. Based on the result of the range finding test, the definitive concentrations of 39.10, 19.55, 9.78 and 0.00 (control) mg/l of Portland cement powder in solution were prepared and replicated for the experimentation.

**Experimental procedure.** Forty (40) litres of aquaria were maintained throughout the exposure period. Ten (10) juveniles were placed in each 40 litres plastic aquarium with one replication. Well-aerated borehole water was used during acclimatization and exposure periods. Fish were fed with commercial feed during acclimatization period of 8.00, 14.00 and 18.00 h. In order to monitor the level of the toxicant and dissolved oxygen, the effect of evaporation and ammonia concentrations during experimentation, the toxicant was changed daily. The exposure period lasted for 15 days during which some water quality parameters were monitored at 5 days intervals using the method described by APHA (1998). The experiment was conducted at the mean water temperature of 25±2.00 °C, dissolved oxygen of 7.22±0.04 mg/l, pH and total alkalinity of 6.94±0.23 and 10.08±0.32 mg/l, respectively. A total of twelve (12) fish from each concentration were sacrificed for electrolyte reserve analyses. Blood was collected from the sacrificed fish by the method described by Kori-Siakpere (1998) and placed in lithium heparin test tubes. Serum was obtained from the blood using a Pasteur pipette after centrifuging with Centurion 1000 series (centrifuge model 1020D.E) at 3,000 rpm for 5 mins (Ogbu and Okechukwu, 2001). Thereafter, liver and kidney were obtained from the fish, homogenized separately using laboratory mortar and pestle and extractants was prepared by adding 2 ml of 10% sucrose solution (Mahobia, 1987). The preparations were then centrifuged separately at 3,000 rpm for 20 mins. Supernatant was transferred to a 2 ml microcentrifuged tube for the determination of electrolyte reserve concentration immediately. These measurements were determined in accordance to the procedure of Cromatest kit (Cromatest Linear Chemicals, Barcelona, Spain). Thereafter, the absorbance (A) was recorded using a spectrophotometer (UV-7504 spectrophotometer, Surgi Friend Medicals, England).

**Data analysis.** The data was first analyzed using a single factor (concentration) ANOVA after which individual means were compared using Tukey HSD multi-sample correction / test. Control values obtained at the beginning and the end of the 15 day exposure were not significantly different and were therefore combined as one control. In all cases, differences were considered statistically significant at P<0.05. Statistical analysis was carried out using NCSS statistical programme. All the data is presented as mean ± standard error.

**Results and Discussion**

Results relating to the levels of sodium (Fig. 1), potassium (Fig. 2), calcium (Fig. 3), chloride (Fig. 4) and inorganic phosphorus (Fig. 5) in the serum, liver and kidney of African catfish (*Clarias gariepinus*) after 15 days exposure to sublethal concentrations of portland cement powder in solution are presented as follows.

**Sodium.** It was observed that sodium levels in serum and liver significantly (P<0.05) decreased during 15 days exposure period as compared to the control, while sodium level in kidney increased significantly (P<0.05). Concentration of Portland cement powder in solution, 39.10 mg/l, caused the reported significant difference. Statistical analysis revealed that sodium concentration was the most significant in the liver, less in the kidney and least in the serum of the test fish after 15 days exposure.

**Potassium.** The level of serum potassium in the fish, after 15 days exposure, revealed a significant (P<0.05) decrease when compared to the control. However, the level of potassium in the liver and kidney exhibited insignificant (P>0.05) decrease. Concentration of Portland cement powder in solution, 39.10 mg/l, caused the reported significant difference.
Fig. 1. Mean and standard error values of sodium of the exposed *Clarias gariepinus*.

Statistical analysis revealed that the potassium concentration was the most significant in the serum, less in the kidney and least in the liver of the test fish.

**Calcium.** The level of calcium in serum and liver of the exposed fish insignificantly (P>0.05) decreased while the levels of the liver and kidney calcium increased significantly (P<0.05) when compared to the control. Concentration of Portland cement, 39.10 mg/l, caused the significant difference, whereas statistically, calcium concentration was the most significant in the liver, less in the kidney and the least in the serum.

**Chloride.** The level of serum chloride insignificantly (P>0.05) increased, while the levels of the liver and kidney chloride significantly (P<0.05) increased and decreased, respectively. Concentration of Portland cement, 39.10 mg/l, caused the significant difference. Statistically, the chloride concentration was the most significant in the liver, less in the kidney and the least in the serum.
Inorganic phosphorus. The concentration of inorganic phosphorus in the liver insignificantly (P>0.05) decreased, while in serum and kidney, significantly (P<0.05) increased and decreased, respectively. Portland cement concentration, 39.10 mg/l, caused the significant difference. Statistical analysis revealed the inorganic phosphorus concentration to be the most significant in the kidney, less in the serum and least in the liver of the test fish.

The result of water quality parameters used in the study were within the optimal range, required by *Clarias gariepinus* as reported by Viveen et al., (1985). Oleru (1984) reported that traces of copper and chromium in cement dust played an important role in disturbing various metabolic processes. Akinola et al. (2008) reported high levels of calcium, silicon, zinc, aluminum and iron in the rats exposed to cement dust in Sagamu as compared to the unexposed rats. Chronic exposure to Portland cement powder in solution has been reported to cause reduction in TLC, PCV, TEC, Hgb and ESR in fish (*Oreochromis niloticus*) (Adamu and Audu, 2008). Adamu and Kori-Siakpere (2008) reported significant alterations in nitrogenous waste products and tissue aminotransferases in catfish *Clarias gariepinus* exposed to sublethal concentrations of Portland cement powder in solution.

Sodium is the chief regulator of osmotic pressure of the body fluid. It initiates and maintains the rate of heart beat and excites involuntary muscles and nerves. Mazon et al. (2002) reported, significant decrease in the concentration of plasma sodium in *P. scrofa*, exposed to lethal and sublethal concentrations of copper. In addition, Pelgrom et al. (1995) observed marked decrease in plasma Na⁺ concentrations in *Oreochromis mossambicus* exposed to copper concentrations for 6 days. Stouthart et al. (1995) reported reduced total sodium concentration in larvae of *Cyprinus carpio* exposed to chromium.

In the present study a significant decrease was observed in serum sodium concentration in *Clarias gariepinus* exposed to the highest concentration of Portland cement powder in solution after 15 days. This decrease might reflect sodium influx rate which may be due to ion loss as a result of complete failure of osmoregulatory processes. The mechanism of osmoregulatory disruption by metals normally involves inhibition of Na⁺/K⁺ ATPase enzymes in gills and perhaps in the gut, as well. Therefore, Portland cement powder in solution might inhibit gill Na⁺/K⁺ ATPase in *Clarias gariepinus* causing disruption of sodium regulation. The increase in kidney sodium concentration may reflect decreased urinary excretion of sodium due to renal tubular dysfunction or reduced intestinal absorption which might be the cause of the observed decline and increase in sodium concentration in serum and kidney, respectively.

Potassium is the main intracellular cation involved in several physiological functions viz, nerve and muscle function, acid-base balance and osmotic pressure. Swarnlata (1995) reported an increase in the concentration of potassium in blood and spleen and a decrease in kidney, liver, muscle and brain of *Clarias batrachus* after 15 days of treatment with carbaryl. Similar findings were observed in the present study. The decreased concentration of potassium observed in the test fish may be attributed to cell damage in the gills and kidneys as revealed by the histopathological examination of *Oreochromis niloticus* exposed to Portland cement powder in solution (Adamu et al., 2008). Disturbed potassium regulation might be due to an impaired active reabsorption of potassium in renal tubules.

Calcium is of great importance in blood coagulation and as regulator of permeability of cell membrane to water and inorganic ions. It also contributes to the maintenance of the membrane potential as well as the development of action potential in muscles and nerves. Hypocalcemia was recorded in the serum of the test fish *Clarias gariepinus*. Koyama and Itazawa (1977) showed a relationship between renal damage, hypocalcemia and skeletal deformities in cadmium-treated carp. Seemingly the persistent hypocalcemia observed may be due to defective intestinal calcium absorption or to impaired calcium reabsorption in the renal tubules accompanied by neuro-muscular hyperexcitability and cramp conditions. Similarly, the hypocalcemia observed may be attributed to
diffusional losses caused by increased permeability of gill epithelium to water and ions (Adamu et al., 2008).

Chloride ions along with sodium and potassium play an important role in neuromuscular excitability, acid-base balance and osmotic pressure of the body. There was an insignificant increase in serum chloride concentration and significant increase and decrease in liver and kidney chloride, respectively. It was however, noted that as the concentration of sodium in the serum and liver decreased there was a corresponding increase in chloride concentration. The inability of the kidney to perform the function of re-absorption due to damage may have been responsible for the decrease and increase in concentrations of chloride recorded in kidney and liver, respectively.

The inorganic phosphate acts as a major cytoplasmic buffer and is the basis of energy exchange. A non-significant decrease in serum phosphorus reported in this study may have resulted from decreased oxidative metabolism and lowered ATP production due to the ability of the Portland cement powder to inhibit the enzymes involved in electron transport chain, affecting the phosphorylating capacity of mitochondria. The decrease in the concentration of inorganic phosphorus may be linked to redistribution of electrolytes between intracellular and extracellular compartments and/or impairment of renal function.

**Conclusion**

The study shows that the liver and the kidney, which are primarily responsible for regulating water and ionic movement between external and internal milieu of fishes, are susceptible to deleterious effects of Portland cement powder in teleost species. The result further confirms that Portland cement powder in solution as high as 39.10 mg/l is pathogenic and toxic to the test fish.

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A List of Damselflies (Zygoptera: Odonata) Recorded from Azad Jammu and Kashmir, Pakistan

Ahmed Zia**, Muhammad Naeem**, Muhammad Ather Rafi** and Soaib Ali Hassan**

**National Insect Museum, NARC-Islamabad, Pakistan
**Pir Meher Ali Shah Arid Agriculture University, Rawalpindi, Pakistan

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Abstract. In the intensive survey of the valley of Kashmir for updating the record of damselflies inhabiting the region, a total of 15 genera and 31 species of damselflies were collected during the summer season of three consecutive years (2005-2007) which are reported.

Keywords: damselflies, Zygoptera, Odonata, Azad Jammu and Kashmir, Pakistan

Introduction

Damselflies are predatory both in larval and adult stages. The larvae are voracious feeders and prey upon aquatic insects e.g., mosquito larvae and aquatic larvae of other species (Sahayaraj, 2004). Adults normally feed on small flying insects such as gnats (Meyer, 2005), mosquitoes and midges during flight (Pedigo, 2002). The presence of odonates is taken as an indicator of ecosystem health for both aquatic and terrestrial ecosystems (Watson et al., 1982). Adult damselflies feed on the insect pests of crops as well, especially of rice (Yousuf et al., 1998; Yasumatsu et al., 1975) and cotton (Yousuf et al., 1995; Yunus et al., 1980).

Odonates have been the focus of extensive research in many countries. They belong to one of the few insect orders that have been intensively studied in the tropics (Woodward, 2001). They have been documented on all continents except Antarctica and are usually concentrated in warmer, tropical habitats (Boyd, 2005). According to Sahayaraj (2004), approximately 5600 named species of Odonata have been described, so far, all over the world.

In the past, Fraser (1933-36) and Laidlaw (1915) reported Odonata from the subcontinent. Kanth (1985) studied the Odonata of Azad Jammu and Kashmir, but his survey was not comprehensive. Yousuf et al. (2000a; 2000b), Luqman (1995), Khaliq et al. (1995; 1990), Khaliq and Siddique (1995), listed odonates from different districts of Azad Jammu and Kashmir. However, this area has high topographic diversity and requires further surveys to reveal the existing zygopterous fauna of the region.

The distribution of damselflies is not well known in Azad Jammu and Kashmir. The valley is bestowed with diverse habitats and abundant streams and springs. The objective of the present study is to prepare a comprehensive and updated record of the damselflies (Zygoptera) of Azad Jammu and Kashmir.

Materials and Methods

Twenty-eight different sites (four sites per district), as follows, were selected to collect adult damselflies from the valley of Azad Jammu and Kashmir (Fig. 1), concentrating on the habitat requirements of damselflies.

District Poonch: Rawalakot (L1), Banjosa (L2), Hajira (L3), Abbasspur (L4).

District Sudhnoti: Tarar Khal (L5), Palanadri (L6), Goraha (L7), Azad Pattan (L8).

District Muzaffarabad: Chikar (L9), Chakothi (L10), Patika (L11), Muzafarabad (L12).

District Bagh: Arja (L13), Bagh (14), Bajri (15), Harighal (L16).

District Mirpur: Mangla (L17), Dudial (L18), Palak (L19), Azad Pur (L20).

District Kotli: Sensah (L21), Sarsawa (L22), Dongi(L23), Kotli city (L24).

District Bhimber: Samahni (L 25), Barnala (L26), Kodala (L27), Bhimber city (L28).

Adult damselflies were collected during the summer season of three consecutive years, 2005-2007 (Table 1). Specimens were collected using aerial nets, sweep nets and dip nets when catching over water. The specimens were killed in cyanide bottles and transferred to paper bags for transportation to the laboratory. In the laboratory they were softened and rehydrated.
localities such as: *Rhinocypha hilarye* (F.), *Aciagrion hisopa* (S.), *Agriocnemis dabeau* (F.) and *Agriocnemis splendidissima*, which were collected from a single locality only. Followed by these were *Lestes viridulus* (Ramb.), *Pseudagrion spencei* (F.), *Coeliccia renifera* (S.), *Ellatoneura nigerrima* (L.) and *Bayadera longicauda* (F.) which were collected from two localities. More field surveys as well as systematic research is needed to reveal additional species and gain a more complete understanding of their distribution.

**Acknowledgement**

We are thankful to Higher Education Commission (HEC) for providing us sufficient funds to carry out these surveys and to collect damselflies (*Zygoptera: Odonata*) from the valley of Azad Jammu & Kashmir. We are also grateful for the services provided by the staff of the National Insect Museum, NARC, Islamabad in the identification of specimens. We extend our thanks to Mr. Muhammad Irshad (Consultant, National Insect Museum, Islamabad, Pakistan) for the critical reading of this manuscript.

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Response Surface Methodology for the Optimization of Alpha Amylase Production by *Serratia marcescens* SB08

C. K. Venil* and P. Lakshmanaperumalsamy

*Division of Environmental Microbiology, Department of Environmental Sciences, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India

13/262 MGR Nagar, Podanur, Coimbatore, Tamil Nadu, India

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**Abstract.** In this work, central composite design combining with response surface methodology was successfully employed to optimize medium composition for the production of alpha amylase by *Serratia marcescens* SB08 in submerged fermentation. The process parameters that influence the enzyme production were identified using Plackett-Burman design. Among the various factors screened, inoculum concentration, pH, NaCl and CaCl₂ were found to be most significant. The optimum level of pH was 5.0, inoculum concentration 3%, NaCl 0.30 g/l and CaCl₂ 0.13 g/l. The actual enzyme yield before and after optimization was 56.43 U/ml and 87.23 U/ml, respectively. Thus, it is advisable to the microbial industry sponsors to apply such profitable bioprocess to maintain high yield for mass production of α-amylase.

**Keywords:** alpha amylase; fermentation; *Serratia marcescens*; Plackett-Burman design; central composite design

**Introduction**

Alpha amylase catalyses hydrolysis of α-D-(1,4) glycosidic linkages in starch components and related carbohydrates. It is a key enzyme in the production of starch derivatives and also can be used in desizing fabrics, baking industry, pharmaceuticals and detergents. The growth and enzyme production of the organism are strongly influenced by medium composition thus optimization of media components and cultural parameters are the primary tasks in a biological process (Djekrif-Dakhmouche *et al.*, 2006). The main strategy used is media engineering for which the operating conditions parameters are optimized by changing one parameter at a time and keeping the others at a constant level (Liu and Tzeng, 1998). Optimization studies do not take into consideration effects of interaction among different variables as any process can be influenced by several variables (Silva and Roberto, 2001). Limitations of single factor optimization can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process (Elíbol, 2004). Single variable optimization methods are not only tedious, but also can lead to misinterpretation of results, especially because the effect of interaction between different factors are overlooked (Wenster-Botz, 2000). Response surface methodology may be summarized as a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables.

Statistical methods are applied for optimization of alpha amylase (Kunamneni *et al.*, 2005; Ahuja *et al.*, 2004; Francis *et al.*, 2002; Dey *et al.*, 2001). No defined medium has been established for the optimum production of alpha amylase by different microbial sources. Each organism has its own special conditions for maximum enzyme production. A statistical approach has been employed in the present study for which a Plackett-Burman design is used for identifying significant variables influencing alpha amylase production by *Serratia marcescens* SB08 (GenBank Accession Number: AB061685). The levels of significant variables were further optimized using response surface methodology.

**Materials and Methods**

**Microorganism and culture maintenance.** Potent strains of bacteria were isolated from the gut of sulphur butterfly (*Kricogonia lyside*). The sulphur butterfly was washed with 70% ethanol and with sterile distilled water several times to eliminate surface bacteria. All dissections were performed under sterile conditions. After disrupting the walls, the contents of the stomach were collected in sterile eppendorf tube, containing phosphate-buffered saline; contents were serially diluted, spread onto the surface of nutrient agar plates and incubated for 48 h at 30 °C in order to record total colony forming units (CFU/ml). The *Serratia marcescens* SB08 isolated from the gut of sulphur butterfly was maintained at 4°C on nutrient agar slants and subcultured every 2 to 4 weeks.

**Amylase production.** Five ml starch broth was inoculated with 1 ml of inoculum and was incubated at 30 °C for 18 h. This 5 ml...
of 18 h old culture was then transferred to 95 ml of sterile starch broth medium and was incubated for 30 °C for 24 h.
After incubation, the crude enzyme was obtained by centrifugation of the culture broth at 10,000 rpm for 10 min and this Cell Free Filtrate (CFF) was stored at -20 °C.

**Alpha-amylase assay.** Amylase production was assayed in terms of amylase activity exhibited by the culture supernatant. The reaction mixture containing 0.1 ml of crude enzyme and 1.0 ml (1.0%) solution of soluble starch in 50 mM phosphate buffer (pH 7.5) was incubated at 50 °C for 5 min. The reaction was stopped by addition of 1.0 ml of 1 N NaOH. The level of amylase activity was determined by measuring the reducing sugar released from soluble starch (Nelson, 1944). One unit of amylase activity was determined by measuring the amount of enzyme which liberates 1 μmol of reducing sugar as glucose per min under the conditions of assay. The experiment was performed in triplicate.

**Optimization of process parameters. Screening of important nutrient components using Plackett-Burman design.** This study was performed using Plackett-Burman design for screening medium components with respect to their main effects but not their interaction effects (Placket and Burman, 1946) on enzyme production by *Serratia marcescens* SB08. The medium components were screened for eleven variables at two levels, maximum (+) and minimum (-). According to the Plackett-Burman design, the number of positive sign (+) is equal to (N+1)/2 and the number of negative sign (-) is equal to (N-1)/2 in a row. A column should contain equal number of positive and negative signs. The first row contains (N+1)/2 positive signs and (N-1)/2 negative signs and the choice of placing the signs is arbitrary. The next (N-1) rows are generated by shifting cyclically one place (N-1) times and the last row contains all negative signs. The experimental design and levels of each variable is shown in Table 1. The medium was formulated according to the design and the flask culture experiments for each enzyme were assayed as described earlier. Response was calculated as the rate of enzyme production, expressed as U/ml. All experiments were performed in triplicate and the average of the rates of enzyme production was considered as the response.

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

\[
E = (\Sigma M_+ - \Sigma M_-)/N
\]

where:

E is the effect of tested variable, M_+ and M_- are responses (enzyme activities) of trials at which the parameter was at its higher and lower levels, respectively, and N is the number of experiments carried out.

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

\[
t = E_{oi} / SE
\]

where:

E_{oi} is the effect of the tested variable. The variables with higher confidence level were considered to influence the response or output variable.

**Optimization of concentrations of the selected medium components using response surface methodology.** Response surface methodology is an empirical statistical modelling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously (Rao et al., 2000). The screened medium components affecting enzyme production were optimized using central composite design (CCD) (Box and Hunter, 1957; Box and Wilson, 1951).

According to this design, total number of treatment combinations is \(2^k + 2k + n_0\), where ‘k’ is the number of independent variables and n0, the number of repetitions of the experiment at the centre point. For statistical calculation, the variable \(X_i\) has been coded as \(x_i\), according to the following transformation:

\[
x_i = \frac{X_i - X_o}{6X}
\]

where \(x_i\) is dimensionless coded value of the variable \(X_i\), \(X_o\) is the value of the \(X_i\) at the centre point, and \(6X\) is the step change. A 2^k-factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components.

Behaviour of the system was explained by the following quadratic equation:

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

where:

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

**Validation of the experimental model.** The statistical model was validated with respect to alpha amylase under the conditions predicted by the model in shake flask conditions. Samples were withdrawn at the desired intervals and alpha amylase assay was determined as described above.
Results and Discussion

Plackett-Burman design. The influence of eleven medium factors namely pH, temperature, agitation, inoculum concentration, incubation time, sucrose, peptone, KH$_2$PO$_4$, yeast extract, NaCl and CaCl$_2$ in the production of alpha amylase was investigated in 12 runs using Plackett-Burman design. Table 1 represents the Plackett-Burman design for 11 selected variables and the corresponding response for alpha amylase production in 12 runs. Variations ranging from 24.35-79.07 U/ml in the production of alpha amylase in the 12 trials were observed by Plackett-Burman design.

Alpha amylases can be produced by submerged fermentation (Aguilar et al., 2000; Egas et al., 1998; Bose and Das, 1996). Alpha amylase production by Serratia marcescens SB08 was subjected to response surface methodology and pH, inoculum concentration, NaCl and CaCl$_2$ were found to be the positive factors. These factors have a positive effect on the production of alpha amylase. These findings support other investigations of the same enzyme belonging to other microbial species (whether fungal or bacterial) (Narang and Satyanarayana, 2001; Ilori et al., 1997).

Supplementation of metal ions has been reported to provide good growth and also influences higher alpha amylase production (Sivaramakrishnan et al., 2006). Most of the alpha amylases are metalloenzymes and in most of the cases, Ca$^{2+}$ ions are required for maintaining the spatial conformation of the enzyme, thus playing an important role in enzyme stability. Both calcium and manganese are necessary for the alpha amylase biosynthesis (McTigue et al., 1994). Ca$^{2+}$ ions are important for thermal stability of the enzyme activity, (Egas et al., 1998; Bose and Das, 1996).

Statistical analysis of the Plackett-Burman design demonstrates that the model F-value of 0.79 is significant. P-value < 0.05 indicates that model terms are significant. The model’s goodness of fit was checked by determination of coefficient (R$^2$). In this case, the value of R$^2$ (0.85) closer to 1 denotes better correlation between the observed and the predicted responses. Coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. Lower reliability of the experiment is usually indicated by high value of CV. In the present case, a low CV (4.55) denotes that the experiments performed are highly reliable (Table 2).

Regression analysis was performed on the results and first order polynomial equation was derived representing alpha amylase production as a function of the independent variables:

\[
\text{Amylase} = 47.00 + 5.10A + 8.58D + 1.92K + 1.33L
\]

Magnitude of the effects indicates the level of significance of the variable on amylase production. Consequently, based on the results of the present experiment, statistically significant variables i.e., inoculum concentration, pH, NaCl and CaCl$_2$ with positive effects were further investigated with central composite design to find the optimal range of these variables. The optimized medium composition by Plackett-Burman design was as follows (g/l): soluble starch, 20.0; yeast extract, 4.0; peptone, 10.0; MgSO$_4$.7H$_2$O, 0.5; NaCl, 0.5; CaCl$_2$, 0.2.

<table>
<thead>
<tr>
<th>Run</th>
<th>Factor A: pH</th>
<th>Factor B: Temp. (°C)</th>
<th>Factor C: Agit. (rpm)</th>
<th>Factor D: Inocul. conc. (%)</th>
<th>Factor E: Incub. time (h)</th>
<th>Factor F: Sucrose (g/l)</th>
<th>Factor G: Peptone (g/l)</th>
<th>Factor H: KH$_2$PO$_4$ extract (g/l)</th>
<th>Factor J: Yeast (g/l)</th>
<th>Factor K: NaCl (g/l)</th>
<th>Factor L: CaCl$_2$ (g/l)</th>
<th>ÿα-amylase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>40</td>
<td>200</td>
<td>1</td>
<td>96</td>
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<td>200</td>
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<td>1</td>
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<td>0.5</td>
<td>0.2</td>
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<td>1</td>
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<td>0.05</td>
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<td>0.1</td>
<td>0.05</td>
<td>43.91</td>
</tr>
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</table>
Central composite design. This is a very useful tool to determine the optimal level of medium factors and their interaction. Based on Plackett-Burman design, inoculum concentration, pH, NaCl and CaCl₂ were selected for further optimization using response surface methodology. To examine the combined effect of these factors on alpha amylase production, a central composite design (CCD) was employed within a range of -2 to +2 in relation to production of alpha amylase. The results obtained from CCD are given in Table 3. These were fitted to a second order polynomial equation to explain the dependence of alpha amylase production on the medium components:

\[ Y = 66.50 + 15.83A + 4.00B + 2.67C + 0.33D - 3.38AB - 4.38AC + 3.63AD - 1.63BC + 4.13BD + 1.63CD - 4.48A^2 - 9.48B^2 - 3.48C^2 - 3.73D^2 \]

where:

- \( Y \) is the predicted response (alpha amylase production),
- \( A, B, C \) and \( D \) are the coded values of \( \text{pH}, \text{inoculum concentration,} \text{NaCl} \) and \( \text{CaCl}_2 \), respectively.

The analysis of variance of the quadratic regression model suggest that the model is very significant as is evident from the Fisher’s F-test (Table 4). The model’s goodness of fit was checked by determination coefficient (\( R^2 \)). In this case, the value of \( R^2 (0.894) \) (multiple correlation coefficient) closer to 1 denotes better correlation between the observed and the predicted responses. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (5.45) denotes that the experiments performed are highly reliable. The P-value denotes the significance of the coefficients and is also important in understanding the pattern of the mutual interactions between the variables.

The fitted response and contour plotted for the above regression model is shown in Fig. 1. 3D response surface curves were plotted to understand the interactions of medium components.

### Table 2. Analysis of variance for \( \alpha \)-amylase production by *Serratia marcescens* SB08

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of square</th>
<th>Degree of Freedom</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
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<td>315.1508</td>
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</tr>
<tr>
<td>A-pH</td>
<td>312.220083</td>
<td>1</td>
<td>312.222</td>
<td>0.785091826</td>
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</tr>
<tr>
<td>D-Inoculum</td>
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<td>1</td>
<td>882.8821</td>
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<tr>
<td>K-NaCl</td>
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<tr>
<td>L-CaCl₂</td>
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<td>Cor Total</td>
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</tr>
</tbody>
</table>

CV = 4.55; \( R^2 = 0.85 \)

### Table 3. Experimental plan for optimization of alpha amylase production using central composite design

<table>
<thead>
<tr>
<th>Run</th>
<th>pH</th>
<th>Inoc. conc. (%)</th>
<th>NaCl (g/l)</th>
<th>CaCl₂ (g/l)</th>
<th>Alpha amylase (U/ml)</th>
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<td>3</td>
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<td>0.125</td>
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<tr>
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<td>3</td>
<td>0.3</td>
<td>0.125</td>
<td>44.01</td>
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<td>0.125</td>
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<td>0.2</td>
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<tr>
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<td>0.3</td>
<td>0.125</td>
<td>52.01</td>
</tr>
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</table>

### Table 4. ANOVA for the experimental results of the central composite design (quadratic model)

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<tr>
<th>Source</th>
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<th>Degree of Freedom</th>
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<th>F-value</th>
<th>P-value</th>
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<td>6016.667</td>
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<td>&lt; 0.0001</td>
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<td>B-Inoculum</td>
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<td>384</td>
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<tr>
<td>C-NaCl</td>
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CV = 5.45; \( R^2 = 0.894 \)
**Fig. 1(a-f).** Response surface and contour plot of the combined effect of factors: (a) inoculum concentration, pH; (b) NaCl, pH; (c) CaCl₂, pH; (d) NaCl, inoculum concentration; (e) CaCl₂, inoculum concentration; (f) CaCl₂, NaCl, on the production of alpha amylase by *Serratia marcescens* SB08.
components and their effect on enzyme production. Graphs highlight the roles played by various factors and also to emphasize the roles played by the physical constraints. From the central point of the contour plot the optimal process parameters were identified.

Fig. 1a shows the response surface plot obtained as function of pH vs inoculum concentration, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at pH 5.5 and inoculum concentration of 3%.

Fig. 1b shows the response surface plot obtained as a function of pH vs NaCl, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at pH 5.35 and NaCl, 0.32 g/l.

Fig. 1c shows the response surface plot obtained as a function of pH vs CaCl2, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at pH 5.23 and CaCl2, 0.13 g/l.

Fig. 1d shows the response surface plot obtained as a function of inoculum concentration vs NaCl, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at inoculum concentration of 3.27% and NaCl, 0.43 g/l.

Fig. 1e shows the response surface plot obtained as a function of inoculum concentration vs NaCl, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at inoculum concentration of 3.83% and CaCl2, 0.16 g/l.

Fig. 1f shows the response surface plot obtained as function of NaCl vs CaCl2, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at NaCl, 0.38 g/l and CaCl2, 0.15 g/l.

**Validation of the model.** The experiment was conducted for 12 runs, evaluated statistically and found significant. Optimum values of the tested variables are pH 5.0, inoculum concentration 3%, NaCl, 0.30 g/l and CaCl2, 0.13 g/l. Maximum experimental response for alpha amylase production was 87.23 U/ml, whereas the predicted value was 80.25 U/ml indicating a strong agreement between experimental and predicted values, thus proving the validity of the model.

Alpha amylase production was sustainable (87.23 U/ml) in Ehrlenmeyer flask and in 2 litre fermentor, suggesting feasibility of scale up of alpha amylase production. There was a slight decline in enzyme production in 2 litre fermentor (86.92 U/ml), which could be due to the reduction in dissolved oxygen (Uma Maheshwar Rao and Satyanarayana, 2003).

**Conclusion**

It is important to discover new bacterial strains that produce enzymes with novel properties of industrial interest. In the present study, production of α amylase under submerged fermentation was obtained by optimizing medium components by RSM. Application of response surface methodology, represented by central composite design, to optimize the selected factors for maximum production, is an efficient method that tests the effect of factor interaction. Besides, it converts the bioprocess factor correlations into a mathematical model that predicts where the optimum is likely to be located. It is worthwhile to advise the microbial industry sponsors to apply such experimental design to maintain high efficiency and profit bioprocess. In the present investigation, the optimized medium succeeded to increase α amylase yield. The production level in basal medium was 56.43 U/ml and increased after applying RSM to 87.23 U/ml.

**References**


Beneficiation of Malakand Graphite Ore

K. R. Kazmi*, M. Arif Bhatti, M. Shafique Anwar and Ansar Mehmood
Material Science Research Centre, PCSIR Laboratories Complex, Ferozepur Road, Lahore-54600, Pakistan

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Natural graphite is a form of carbon, found as crystals of high graphitic content or in amorphous form of lower purity. (Brady, 2004; Liu and Yuyan, 2003; Kalyoncu, 2001; Gordon, 1995). Due to its conductivity, high thermal stability and lubricating properties, graphite is used in various industries according to the grade. (Anwar et al., 2006; Kalyoncu, 2001; Gordon, 1995).

At present, Pakistan imports all its requirements of foundry grade graphite containing 75-85% graphitic carbon (Anwar et al., 2006; Kalyoncu, 2001; Crossley and Peter, 2000; Qureshi et al., 1967). In the Malakand area of North Western Frontier Province (NWFP) of Pakistan, an extensive deposit of low grade graphite ore, containing 10-17% graphitic carbon, has been found which can be exploited on commercial scale.

This paper deals with the beneficiation study of the said graphite deposit using froth flotation technique. Chemical analysis was carried out by conventional gravimetric and volumetric methods. Characteristics of the ore were defined by petrography, X-ray diffraction and ore microscopy. Flotation feeds of the ore were prepared by subjecting the ore to crushing and wet grinding at 1:1 solid/liquid ratio. Flotation tests were carried out in a Denver D-12 flotation machine. The steps of beneficiation of the ore are given in the flowsheet. Optimum flotation parameters and metallurgical balance are presented in Table 1 and 2.

The grade (graphitic carbon=16.94%) of the ore (Table 3) is sufficient to exploit the ore on commercial scale (Hand, 1997; Hussain et al., 1967). The ore was found as fine grained graphitemica schist. The gangue mostly comprised of clay with hydrated oxides of iron and predominant quantities of silica and mica. The ore contains crystalline graphite (Table 4) so flotation can be considered a proper route for its beneficiation (Kalyoncu, 2001).

The ore can be upgraded up to 38.5% at rougher flotation stage @ 91.56% recovery at a feed size of 84.81%. Regrinding of the rougher concentrate before three cleaning operations and re-circulating the cleaner tailings to the rougher flotation feed ensured a final concentrate grade of 84.05% @ 72.09% recovery.

The grade of the rougher concentrate improves tangibly while the recovery increases slightly as the amount of fine material increases in the flotation pulp. Although the increase in the recovery is not very significant with the decrease in the percentage of gangue, the increase in grade is sufficient to meet the specifications of the foundry grade graphite.

### Flowsheet: Beneficiation of Malakand graphite ore.

- **Ore**
  - Crushing and grinding
  - Flotation feed
  - Rougher flotation → Rougher tailings
  - Rougher concentrate
  - Reregrinding
  - I Cleaner tailings ← I Cleaner flotation  
    - I Cleaner concentrate
  - II Cleaner tailings ← II Cleaner flotation  
    - II Cleaner concentrate
  - III Cleaner tailings ← III Cleaner flotation  
    - III Cleaner concentrate

*Author for correspondence; E-mail: kamranrazakazmi@hotmail.com*
particle size, it is quite reasonable at 91.56%. Through grinding for 6 min, 84.81% of the ground material passes 73 μm screen (Table 5); further increase in grind time does not affect the size distribution of ground mill product. The maximum liberation of graphite grains in Malakand ore was found to be 73 μm. Maximum purity was achieved at a pH of 7.5. A neutral pH or a more alkaline pH adversely affects the optimum conditions.

The pulp density variation has a significant effect on the grade and the recovery of graphite. As the pulp density is reduced from 40 to 20% solids, the grade of the concentrate improves and vice versa. At pulp density of 33% solid, the grade of the rougher concentrate is better than 40% solid, while there is no big difference in the recovery. The better result at 33% solid is due to fine grained nature of Malakand ore while for flaky...
produced was quite suitable for the production of graphite based products.

Acknowledgement
The authors are highly grateful to Mr. Sadiq Naeem, Mr. Wasim Anwar and Mr. Mohabbat Khan for their assistance in the course of this work.

References

<table>
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<th>Table 5. Size distribution of ground mill product</th>
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Sesquiterpenes: The Potent Antioxidants

Abdul Latif Khan*, Hamayun Khan**, Javid Hussain*
Mohammad Adnan³, Iqbal Hussain³, Taous Khanc and Abdur Rahman Khan⁴

¹Department of Chemistry, Kohat University of Science & Technology, Kohat-26000, Pakistan
²Department of Botany, Kohat University of Science & Technology, Kohat-26000, Pakistan
³Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad, NWFP, Pakistan
⁴Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad, NWFP, Pakistan

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Abstract. Sesquiterpenes (STs) are 15 carbon terpenoids, having significant phytomedicinal, phytotoxic and agrochemical potentials. They are found to be present in the essential/edible oils and have also been reported from numerous plant species. In this review article, the antioxidant potentials of STs isolated from various plants have been presented. As the antioxidants prevent, protect or reduce the damaging/aging of the cell, therefore, they are of prime importance. This review will provide the literature on the antioxidative potency of STs probably for the first time, which could be used as scientific data base for the researchers working in this field.

Keywords: sesquiterpenes, sesquiterpene lactones, antioxidant, plant extracts, biologically active compounds

Review

Sesquiterpenes. Sesquiterpenes (STs) are 15 carbon terpenoids comprising of mainly two types, oxygenated sesquiterpenes and hydrocarbon sesquiterpenes. The oxygenated forms occur bearing functional groups such as alcohols, ketones, aldehydes, acids or lactones in nature. Due to their small molecular weight, they are important volatile organic parts of especially the essential oils, which are of great medicinal potential. Besides their presence in essential oils, they are also major constituents of various medicinal and economically important plants (Merfort, 2002).

STs are one of the largest biogenetically homogenous groups of natural products known. More than 11,000 entries of sesquiterpenes have been reported so far. These sesquiterpenes have been divided into almost 24 different kinds (Schmidt, 2006). However, germacranolides, eudesmane, farnesane, elemane, guaiane, and chamigrane are well known besides some esters or lactone group-linked sesquiterpenes. Almost 5000 sesquiterpene lactones have been reported. Among them 60 to 65% have been reported to be present as essential/edible oils (Schmidt, 2006; Fraga, 2000).

Various STs have been found to be biologically active against cell proliferation, abnormal cell growth (cancer), inflammation, bearing antibacterial, antifungal, antispasmodic, cytotoxic, antimalarial, hepatoprotective, insecticidal, allelopathic, enzyme inhibitory, antilipase effects as well as many other diseases and problems (Khan et al., 2008; Liu et al., 2008; Macias et al., 2007; Pan et al., 2007; Miguel et al., 2005; Ding et al., 2005; Rafi et al., 2005; Sharma et al., 2005; Yun et al., 2002a,b).

Antioxidants. Antioxidants are classically defined as molecules present in concentrations lower than the biomolecules and may prevent, protect or reduce the extension of oxidative damage, such as glutation peroxidase, catalase and superoxide-dismutase. Other antioxidants, such as ascorbic acid (vitamin C) and tocopherol (vitamin E) are non-enzymatic antioxidants (Hussain et al., 2008; Khan et al., 2007, 2006, 2005; Foyer and Noctor, 2005; Bolwell and Wojtaszek, 1997; Harborne, 1993). Thus, there is a delicate balance between the generation and destruction of oxidants, which may be beneficial or deleterious to the organism (Hussain et al., 2008; Khan et al., 2007, 2006, 2005; Maffei et al., 2007; Foyer and Noctor, 2005; Novelli, 2005). Oxidation products from lipids and cholesterol are thought to be the contributing factor to the cause of various diseases, including cancer, atherosclerosis and some age-related diseases (Chun et al., 2007; Jatoi et al., 2007; Pandhair and Sekhon, 2006; Ho et al., 2003; Andersson et al., 1996; Chan, 1987; Scott, 1985). Lipid oxidation in food affects its nutritional quality which results in rancid flavour, one of the main consequences. Also loss of vitamins, polyunsaturated fatty acids and other essential compounds can occur during the process (Khan et al., 2008; Manzoor et al., 2007; Pandhair and Sekhon, 2006; Anwar and Bhanger, 2003; Andersson et al., 1996; Chan, 1987; Eriksson, 1982).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase catalyses the reduction of molecular oxygen to super-
oxide anion (\(O^2-\)) and the burst respiratory is paralleled by a higher consumption of oxygen (Krol \textit{et al.}, 1995). \(O^2-\) is the precursor of other reactive oxygen intermediates, including hydroxyl radical (\(OH^*\)), hypochlorite (\(OCl^-\)) and hydrogen peroxide (\(H_2O_2\)). Oxidants produced by phagocytes may destroy important biomolecules as well as phagocytosed microorganisms, and are also involved in the tissue injury associated with inflammatory diseases (Hussain \textit{et al.}, 2008; Khan \textit{et al.}, 2008, 2007, 2006, 2005; Sforcin, 2007; Pandhair and Sekhon, 2006; Moonis \textit{et al.}, 1992).

Mitochondria are important intracellular sources of reactive oxygen species (ROS). During the oxidative phosphorylation process, mitochondria reduce \(O_2\) to \(H_2O\) \textit{via} the respiratory chain. ROS are continuously produced by plants under different stress conditions and in different cellular compartments (Navrot \textit{et al.}, 2007; Foyer and Noctor, 2003). Both the chemical identity of a given ROS and the intracellular site of its production seem to affect the specificity of its biological activity, further increasing the complexity of ROS signalling within plants (Laloi \textit{et al.}, 2004). In several systems, various signalling pathways, particularly those involving, mitogen-activated protein kinase (MAPK)s, are modulated by ROS (Pitzschke and Hirt, 2006; Neill \textit{et al.}, 2004). Oxidative stress, resulting from the generation of ROS, such as superoxide (\(O^2-\)) hydrogen peroxide (\(H_2O_2\)) and hydroxyl radicals (\(HO^*\)), is a common phenomenon (Maffei \textit{et al.}, 2007). In absence of stress and under physiological conditions, the level of ROS is maintained low by the activity of antioxidative systems, which include secondary plant metabolites and scavenging enzymes (Pandhair and Sekhon, 2006; Foyer and Noctor, 2005). Both biotic and abiotic factors induce changes in the ROS equilibrium and trigger cascades of signals eventually leading to increased ROS production and/or decreased antioxidant and scavenging activities (Khan \textit{et al.}, 2008; Asada, 2006; Apel and Hirt, 2004; Hancock \textit{et al.}, 2002).

Antioxidants are commonly used to increase the shelf life of lipids and lipid-containing products. Many \textit{in vitro} studies indicate that phenolic compounds like flavonoids, coumarines, phenolic acid, lignans, hydroxycinnamates and stilbenes can have substantial antioxidant activity (Khan \textit{et al.}, 2008; Kim \textit{et al.}, 2002; Duthie and Crosier, 2000; Park \textit{et al.}, 2000). A large number of plants have been screened as a source of new additives for the food and pharmaceutical markets, which can provide a supplement to cope with oxidative stress (Hussain \textit{et al.}, 2008; Khan \textit{et al.}, 2008, 2007, 2006, 2005; Rosa \textit{et al.}, 2007; Pandhair and Sekhon, 2006; Laloi \textit{et al.}, 2004; Kim \textit{et al.}, 2002; Park \textit{et al.}, 2000; Shahidi, 1997).

The redox state of the cell has been shown to be involved in cell cycle regulation and cell death/survival (Dong-Yun \textit{et al.}, 2003). Glutathione (GSH) is the main intracellular antioxidant and plays an important role in these processes. GSH depletion leads to cell death, and increase in GSH inhibits cell proliferation (Menon \textit{et al.}, 2003).

**Antioxidant sesquiterpenes from plants.** Various sesquiterpenes belonging to different sub-classes have been isolated from different plant species. Since we have excluded the sesquiterpenes reported from essential/edible oils, therefore, information relating to only the isolated compounds have been compiled and presented here for future research. Details of sesquiterpenes reported from various plant species is given in Table 1.

Sesquiterpenoids \textbf{1} (7-hydroxy-3,4-dihydrocadalin) and \textbf{2} (7-hydroxydacadalin) have been isolated from \textit{Heterotheca insuloides} (Haraguchi \textit{et al.}, 1997), which showed potent 1,1-diphenyl-2-pircylhydrazyl (DPPH) radical scavenging activity and almost 80% inhibition was observed at 10 \(\mu\)g/ml concentration. Structurally these sesquiterpenes, especially compound \textbf{2}, contain \(\beta\)-napthol (3a) and \(p\)-cymene (3b) moieties. These structurally related compounds 3a and b showed almost no effect on linoleic acid autoxidation up to a concentration of 30 \(\mu\)g/ml (Haraguchi \textit{et al.}, 1997).

Feruloylpospesmic acids A and B, scorzoneric acid and scorzonerin (4) were purified from the crude extract of the aerial parts of the Mongolian medicinal plants, \textit{Scorzonera divaricata} and \textit{S. pseudodivaricata} (Tsevegsuren \textit{et al.}, 2007). These were detected in the DPPH active fractions. The potential of compound against oxidative stress, was determined using naturally occurring antioxidant, chlorogenic acid as control. Feruloylpospesmic acids A and B gave IC\(_{50}\) values of 36.36 and 34.24 \(\mu\)mol/ml, respectively, while chlorogenic acid had an IC\(_{50}\) value of 67.92 \(\mu\)mol/ml. However, the sesquiterpene, scorzonerin (4) did not show higher values compared to others. Scorzonerin (4) is a matricarin-based sesquiterpene lactone that carries an esterified dihydrocoumaric acid moiety, which in turn is glycosidically bound to glucose (Tsevegsuren \textit{et al.}, 2007).
<table>
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<td>5-Hydroxy-6,9-epoxyguaiane (not analysed for AO)</td>
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<td>Euphorbiaceae</td>
<td>Roots, dichloromethane extract</td>
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<td>Leaf extract</td>
<td>LC, VP, HPLC, NMR and spectroscopic techniques</td>
<td>Amakura et al., 2002</td>
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<td>Methylarzanol, sesquiterpene alcohol rosifoliol</td>
<td><em>Helichrysum italicum</em> ssp. <em>microphyllum</em></td>
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<td>Leaves and flowers, acetone extract</td>
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<td>Rosa et al., 2007</td>
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<td>Parthenolide</td>
<td><em>Tanacetum parthenium</em></td>
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<td>Nakamura et al., 2004</td>
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<td>Kim et al., 2006</td>
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<td>Fukaneufurochromone A, B, C, D, E and F</td>
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<td>Motai and Kitanaka, 2005</td>
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<td>1,2,3,4-Tetrahydro-1a,28,7-trihydroxy-1-6-dimethyl-4P-isopropynaphthalene-1-O-p-D-glucoside</td>
<td>Cotton seeds (Gossypium hirsutum)</td>
<td>Malvaceae</td>
<td>Methanolic extract</td>
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<td>Ortho-naphthaquinones, davidanones A, B and C</td>
<td><em>Ulmus davidiana</em></td>
<td>Ulmaceae</td>
<td>Methanol extract</td>
<td>-</td>
<td>Kim et al., 1996</td>
</tr>
<tr>
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<td>Magnoliaceae</td>
<td>Leaves</td>
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<td>Umemura et al., 2008</td>
</tr>
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<td>Parthenolide</td>
<td><em>Tanacetum parthenium</em></td>
<td>Asteraceae</td>
<td>HT22 cells</td>
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<td>Herrera et al., 2005</td>
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</table>
A bioassay guided separation from the methanolic extract of the fruiting bodies of *Stereum ostrea* led to the exploration of a new sesquiterpene, methoxylaricinolic acid (5), along with the known compound, laricinolic acid (6). In a panel for antioxidant effect, however, these compounds exhibited marginal inhibitory activity with an IC₅₀ of 50 mg/ml (vitamin E, 1 mg/ml) against lipid peroxidation in rat liver microsomes evaluated by the thiobarbituric acid method (Kim et al., 2006).

Sesquiterpenes like hirsutenols A, B and C (Yun et al., 2002a), D, E and F (Yoo et al., 2006) and sterins A, B (Yun et al., 2002b) and C (Yoo et al., 2005) were isolated from the culture broth of *Stereum hirsutum* and were found to be good antioxidants. Two new sesquiterpenes, godotol A (7) and godotol B (8), were isolated from *Pluchea arabica* (Fatope et al., 2004). DPPH free radical scavenging activity tests were performed on extracts and compounds 7 and 8. These compounds lack antioxidant activity, inhibiting DPPH radicals at less than 10%, with BST, LC₅₀ value of 290 μg/ml for 7 and 540 μg/ml for 8, respectively (Fatope et al., 2004).

The sesquiterpene, cacalol was isolated from *Cacalia delphiniifolia* and characterized by MS and NMR spectroscopy. Cacalol showed potent antioxidant activities of IC₅₀ of 40 nM (Shindo et al., 2004). Three new sesquiterpenes orthonaphthoquinones, davidianones A (13), B (14) and C (15), together with four known compounds, namely, mansonones E, F, H and I, were isolated from the 80% aqueous methanolic extract of root bark of *Ulmus davidiana* (Kim et al., 1996). The sesquiterpene, cacalol was isolated from *Cacalia delphiniifolia* and characterized by MS and NMR spectroscopy. Cacalol showed potent antioxidant activities of IC₅₀ of 40 nM (Shindo et al., 2004). Three new sesquiterpenes orthonaphthoquinones, davidianones A (13), B (14) and C (15), together with four known compounds, namely, mansonones E, F, H and I, were isolated from the 80% aqueous methanolic extract of root bark of *Ulmus davidiana* (Kim et al., 1996). The

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antioxidative activities of compounds were evaluated by a thiobarbituric acid method using rat liver microsomes. The result shows that compounds 13 and 15 were active. The IC₅₀ values of compound 13, 14, and 15 were 0.12, 6.90 and 0.80 μg/ml, respectively, compared with α-tocopherol (IC₅₀ 0.10 μg/ml) (Kim et al., 1996).

Two new sesquiterpenes, 1S*, 4R*, 5S*, 6R*, 7S*, 10S*-1(5), 6(7)-diepoxy-4-guaiol (16) and 1S*, 4S*, 5S*, 10R*-4,10-guianediol (17) have been isolated from the ethyl acetate soluble portion of the soft coral Sinularia sp. and their stereostructures were determined by spectroscopic methods and X-rays single crystal analysis. Both compounds showed antioxidant and cytotoxic activities (Zhang et al., 2006).

Zerumbone (ZER) (18), is a sesquiterpene occurring in tropical ginger Zingiber zerumbet Smith (Nakamura et al., 2004). ZER induced nuclear localization of the transcription factor Nrf2 that binds to antioxidant response element (ARE) of the phase II enzyme genes, suggesting that ZER is a potential activator of the Nrf2/ARE-dependent detoxification pathway. In order to protect against excessive ROS, aerobic organisms have developed a number of cellular defences composed of non-enzymatic and enzymatic components. The results preliminarily confirmed that ZER did not show any scavenging effect against the stable free radical DPPH (Nakamura et al., 2004).

Polygodial (23) is a sesquiterpene, which exhibits a strong affinity for sulfhydryl groups with which it interacts by the Michael-type reaction; it can thus inactivate alcohol dehydrogenase, a typical thiol enzyme and thereby interfere with an enzymatic reaction essential for plasma membrane function. On the other hand, the widespread disruptive effects of polygodial against mitochondria and some other organelles prompted us to consider the involvement in its yeastcidal activity of a chemical reaction which can directly attack the phospholipid bilayers (Machida et al., 1999).

ROS including hydrogen peroxide, superoxide anions, and hydroxyl radicals, are highly toxic oxidants. These oxidants cause lipid peroxidation and can induce disruption of plasma membrane phospholipid bilayers, when overproduced or not suitably eliminated. Mitochondria are equipped with Mn-superoxide dismutase and a redox cycle involving GSH and GSH peroxidase (Foyer and Noctor, 2005; Machida et al., 1999; Bolwell and Wojtaszek, 1997; Harborne, 1993). In the

Arzanol, a pyrone-phloroglucinol etherodimer and helipyrone, a dimeric pyrone [rosifoliol (19), 10-hydroxytremetone (20), acetoxytremetone (21) and acetoxyhydroxytremetone (22)], isolated from Helichrysum italicum ssp. microphyllum, showed antioxidant activity (Rosa et al., 2007). They could protect linoleic acid against free radical attack in assays of autoxidation and EDTA-mediated oxidation. Methylarzanol, as well as the sesquiterpene alcohol rosifoliol, showed a decreased, but still significant, protective effect against linoleic acid oxidation. Arzanol and helipyrone were also tested in an assay of thermal (140 °C) autoxidation of cholesterol, where arzanol showed significant antioxidant activity (Rosa et al., 2007).
mitochondrial matrix which lacks catalyse, GSH is the only defence available to cope with the potential toxic effects of hydrogen peroxide, produced endogenously in the electron transport chain. Mammalian cells with markedly depleted mitochondrial GSH were more sensitive to oxidative stress imposed by mitochondrial generation of ROS than those lacking cytosolic GSH. The effects of polyglydial on the glutathione content and ROS generation of the yeast cells (Saccharomyces cerevisiae) were further examined in the isolated mitochondrial suspension (Machida et al., 1999).

**Conclusion and Recommendations.** The present review attempts to summarize the outline of the existing knowledge of STs with special emphasis on their antioxidant activities. In conclusion, STs isolated from various plant species have significant antioxidant potentials and most of them are isolated from the plant family, Compositae, which is among the largest and ecologically most diverse plant families. Although there are several reports available on the STs bioactivities, however, only very few systematic studies on structure-activity relationships have been carried out. Detailed studies of this kind, however, would be highly desirable with respect to several aspects of medicinal/pharmaceutical, agrochemical and ecological interest, as most of the plant species, containing STs, have been used in traditional medicines for many centuries and continue to be utilized also in modern phytotherapy (Hussain et al., 2008; Khan et al., 2008, 2007, 2006, 2005). Therapeutic use of STs as pure chemicals, in spite of their broad utilization in the form of plant or crude extracts is restricted to very few examples. This is due to the lack of knowledge about establishment of structural relationship and its requirements for selectivity to a desired biological activity. It may, however, be conceived, that STs could play a valuable role as starting point for developing new therapeutic agents, if more information, especially in the form of quantitative structure-activity relationship (QSAR), existed.

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