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Antimicrobial activity of thermotolerant bacterial isolate from coal mine spoil

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The study deals with isolation of bacteria from fresh coal mine spoil and their ability to produce antimicrobial compound. In detection of antagonistic effect, one of the four bacterial isolates, strain-I exhibited maximum inhibitory effect against Escherichia coli and was selected for further study. Our results indicated, antimicrobial activity against both Gram negative (E. coli) as well as Gram positive (Staphylococcus aureus) pathogens and exhibited greater zone of inhibition against E. coli at 40 to 50°C where as against S. aureus at 40°C at the 24th hour of incubation. However, with increase in time of incubation, the zone of inhibition showed a declining trend. The antimicrobial activity of strain-I was the highest between pH 5 to 7. Culture of the isolate with glucose as a substrate resulted relatively larger zone of inhibition against E. coli at 48th hour of incubation.

Key words: Strain-I, coal mine spoil, antimicrobial compound, antagonistic effect, zone of inhibition.

INTRODUCTION

Freshly led overburden dump of coal mine spoil due to nutrient deficiency, high internal temperature and low pH represents an extreme habitat (Juwarkar et al., 2004; Machulla et al., 2005; Gogoi et al., 2007; Daniels et al., 2009). However, in spite of such extreme profile, the coal mine spoil was reported not to be microbiologically sterile and on culture harbours bacterial colony forming units (Darland et al., 1970; Belly and Brock, 1974; Norris and Owen, 1992; Johnson, 2003; Sethy and Behera, 2009). Bacterial strain isolated from unusual extreme habitat were often reported to produce antimicrobial compound (Khalil, 2002; Mendo et al., 2004; Walczak and Donderski, 2004; Khalil et al., 2006; Bushra et al., 2007; Awais et al., 2008; Muhammad et al., 2009). It is expected that such strain will be used for bioprospecting ability for industrial production of different secondary metabolites including antibiotics, proteins, unusual enzymes, etc (Costa et al., 1989).

In the present study, we isolated and characterized bacterial strains from a freshly dumped coal mine spoil for production of secondary antimicrobial metabolite.

MATERIALS AND METHODS

For the present study, samples were collected from the fresh coal mine overburden dumps of Basundhara coal fields, located between 22°03'32" and 22°04'11" North Lat. and 83°42'18" and 83°44'08" East Long in the Sundergarh district of western part of Orissa. The spoil of the overburden due to its pyrite composition maintains a temperature of 65-75°C and the pH of the spoil varies from 4-5. Since the overburden dump was freshly lead the overburden was without any vegetation cover. The samples (n=10) were collected randomly from different locations of the overburden from a depth of 0-20 cm depth, following the aseptic procedure. The samples after their collection were subjected to mixing, resulting a composite sample which were appropriately kept in a sterilized polythene packet and were brought to the laboratory for further analysis.

Isolation of bacterial strain

Strains were isolated by dilution plate count technique (Parkinson et al., 1971; Ward and Cockson, 1972). For the homogenization, 1 g of sample was mixed in 100 ml of double distilled water for 30 min in a shaking incubator with 120 rpm at 50°C. After incubation culture was serially diluted. 100 µl of diluted suspensions were spread on nutrient agar plates. The plates were incubated for 24 to 48 h at 50°C. Discrete colonies were observed after 24-48 h of incubation. Colonies were differentiated on the basis of their

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lates were incubated for 24 h.
2. After incubation, the pure cultures were streaked against the growth line of standard test organisms on the surface of the synthetic culture medium, using separate petriplates for each test organism and incubated for 24 h. The inhibitions of the isolate against growth line of the standard test organism were measured in terms of zone of inhibition (in mm) were observed and measured.

Detection of antagonistic effect by cross streak method

Detection of antimicrobial activity was performed with cross-streak method, in which the 3 types of standard test organisms were streaked on a side of the petriplate containing nutrient agar medium, using separate petriplates for each test organism and plates were incubated for 24 h. After incubation, the pure cultures of the strains were streaked against the growth line of standard test organism and again the plates were incubated for 24 h. The inhibitions of the isolate against growth line of the standard test organism were measured in terms of zone of inhibitory distance.

Antimicrobial production medium

For the production of antimicrobial activity, 1% inoculums (24 h old bacterial culture) was aseptically transferred to synthetic medium (Bushra et al., 2007; Awais et al., 2008) with the following composition (g/l) L-glutamic acid-5.0, KH₂PO₄ -0.5, K₂HPO₄-0.5, MgSO₄.7H₂O-0.2, MnSO₄.2H₂O-0.01, NaCl-0.01, FeSO₄.7H₂O-0.01, CuSO₄.7H₂O-0.1,CaCl₂.2H₂O-0.015, pH 7.

Antimicrobial assay

The agar well diffusion method was used to test the isolates for the production of antimicrobial activity (Sen et al., 1995). Fresh culture (24 h) of Escherichia coli, Staphylococcus aureus and Streptococcus mutans were used as the test microorganisms. Lawning the standard test organisms on the surface of the respective nutrient agar plates and wells were made using a sterile borer (7 mm). 100 µl cell free supernatant was added to each well respective agar plates and were incubated at 37°C for 24 h, after which diameter of zone of inhibition (in mm) was observed and measured.

Effect of temperature, pH and glucose concentration

The production of antimicrobial activity was studied at various temperatures by incubating the synthetic culture medium at 35 to 60°C. Growth of strain was carried out in 250 ml Erlenmeyer flask containing 50 ml synthetic medium and incubated at respective temperature in a rotary shaker at 150 rpm for 120 h. At every 24 h the cell free supernatant was obtained by centrifugation (10,000 rpm for 15 min) and assayed for antimicrobial activity in terms of zone of inhibition.

Similarly, the effect of pH was determined by incubation of isolated bacteria at different pH values (3.0 to 9.0). The pH was adjusted with 1N H₂SO₄ and 1N NaOH. Further, the effect of glucose at different concentrations (1 to 6%) was studied. As described previously, the cell free supernatant was obtained by centrifugation and assayed at different pH and glucose concentration.

RESULTS

Dilution plate count technique resulted 8 × 10⁶ CFU of bacteria on the nutrient agar plates after an incubation period of 48 h at 50°C. The detailed analysis of the developed bacterial colonies indicated four types of bacteria with different colony morphology and biochemical attributes (Table 1). However, all isolates

Table 1. Colony morphology and biochemical attributes of four isolated bacterial isolates.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Strain-I</th>
<th>Strain -II</th>
<th>Strain -III</th>
<th>Strain -IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>White slimy to dry brown</td>
<td>White and dull</td>
<td>White and shining</td>
<td>White and mucoid</td>
</tr>
<tr>
<td>Colony shape</td>
<td>Irregular</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>3-4</td>
<td>1-2</td>
<td>0.5-2</td>
<td>1-2</td>
</tr>
<tr>
<td>Margin</td>
<td>Undulate</td>
<td>Entire</td>
<td>Entire</td>
<td>Undulate</td>
</tr>
<tr>
<td>Elevation</td>
<td>Raised</td>
<td>Raised</td>
<td>Raised</td>
<td>Flat</td>
</tr>
<tr>
<td>Gram stain</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Spore formation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amylase activity</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cellulase activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pectin degradation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂S production</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vp</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>A (+) &amp; G(-)</td>
<td>A (+) &amp; G (-)</td>
<td>A (+) &amp; G (-)</td>
<td>A (+) &amp; G(-)</td>
</tr>
<tr>
<td>Lactose</td>
<td>A (+) &amp; G(-)</td>
<td>A (+) &amp; G(-)</td>
<td>A (+) &amp; G(-)</td>
<td>A (-) &amp; G(-)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>A (+) &amp; G(-)</td>
<td>A (+) &amp; G(-)</td>
<td>A (+) &amp; G(-)</td>
<td>A (+) &amp; G(-)</td>
</tr>
</tbody>
</table>

(+ positive; (-) negative; (A) Acid; (G) Gas.)
were Gram negative nonsporulating bacilli and showed motility. Two of the strains (strain-III and IV) were amylase positive. Except strain-IV, all the isolates were capable of utilizing citrate. Strain-I and II were marked to be methyl red positive where as none of the isolates were VP positive. All the strains except strain-IV produced acid, but no gas during the fermentation of glucose, lactose and sucrose.

Figure 1 illustrates the cross streak effect of the isolates against *E. coli*, *S. aureus* and *S. mutans*. As shown in the figure, the strains when cross streaked against *E. coli*, exhibited maximum inhibitory distance in comparison to *S. aureus*, whereas no antagonistic effect was found against *S. mutans*. Out of the two bacterial isolates, the one showing the maximum antagonistic distance (strain-I) was selected for further study.

Production of antimicrobial compound by the bacterial isolate was evaluated by measuring the diameter of zone of inhibition against *E. coli* and *S. aureus* at regular interval of 24 h of incubation with respect to different temperature regime (Tables 2 and 3). The zone of inhibition was relatively greater with respect to 24th hour of incubation at 40, 45 and 50°C. With increase in time of incubation the zone of inhibition showed a declining trend at all the temperature except 45 and 60°C at 48th hour of incubation. Against *S. aureus* the bacterial isolate exhibited zone of inhibition up to 55°C. However, the zone of inhibition was observed to be maximum at 40°C.

Antimicrobial activity of bacterial isolate against *E. coli* was evaluated at different pH (Figure 2). The zone of inhibition was observed at all pH tested in this study except pH 3. However zone of inhibition was relatively greater between pH 5 and 7. The effect of the glucose concentration on antimicrobial activity of bacterial isolate against *E. coli* was studied (Figure 3). The results indicated that the zone of inhibition exhibited maximum during 48th hour of incubation. At 24th as well as 72nd hour of incubation the zone of inhibition was relatively smaller. In general increase in glucose concentration did not exhibit any impact on the zone of inhibition.

**DISCUSSION**

Production of antimicrobial compounds by bacteria and fungi is considered to be one of the strategies for their survival through which they can eliminate competition and maintain their niche (Talaro and Talaro, 1996;
Table 3. Antimicrobial activity of bacterial isolate at different temperature against *S. aureus*.

<table>
<thead>
<tr>
<th>Time in hour</th>
<th>Zone of inhibition in mm at different temperature °C (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35</td>
</tr>
<tr>
<td>24</td>
<td>14±1</td>
</tr>
<tr>
<td>48</td>
<td>9±0.5</td>
</tr>
</tbody>
</table>

Figure 2. Antimicrobial activity of bacterial isolate at different pH values against *E. coli*.

Figure 3. Antimicrobial activity of bacterial isolate at different glucose concentration against *E. coli*. 
Jensen and Wright, 1997; Motta et al., 2004; Awais et al., 2010). The screening of antibacterial spectrum of the thermostolerant bacterial strain isolated from different habitats, has been reported by several studies (Khalil, 2002; Mendo et al., 2004; Walczak and Donderski, 2004; Khalil et al., 2006; Bushra et al., 2007; Awais et al., 2008; Muhammad et al., 2009). Isolated bacterial strain from extreme unusual habitat, as noted in the study did exhibit antimicrobial activity against some common pathogens. Exhibition of the antimicrobial activity at 55°C is an indication about thermostolerance nature of the isolate as well as its antimicrobial compound. Such report about the antimicrobial activity by the bacterial isolate from an extreme habitat at higher temperature, throw light on the possible bioprospectibility of the bacterial isolate for industrial application (Johnson, 2003; Ren et al., 2010).

Relatively higher zone of inhibition during 48th hour of incubation indicates that the synthesized antimicrobial compound is secondary metabolites. There have been studies that usually bacillus species produce in pH 7-8 (Muaz et al., 2007). However in this study being non-sporulating bacilli could exhibit antimicrobial activity at lower pH. Thus the isolate has the ability to regulate the biosynthesis of secondary metabolites at lower pH. As per the Yousaf (1997) among the sugars, glucose supports the maximum yield of antimicrobial activity. On the basis of such report effect of glucose concentration was studied. However our study did not reveal any positive impact on antimicrobial activity.

In the present study the isolated thermostolerant bacteria synthesized an antimicrobial compound that can withstand a temperature up to 60°C and showed activity against Gram positive as well as Gram negative bacteria. The study indicates the possible synthesis of broad spectrum antibiotic by the bacterial isolate. There is need to further study isolation, purification and characterization of the antimicrobial metabolite.

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