Strain Improvement of Selected Strain *Bacillus subtilis* (MTCC No.10619) for Enhanced Production of Antimicrobial Metabolites

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**ABSTRACT**

A total of 178 strains (bacteria, fungi, yeast, actinomycetes) were isolated from sponges, Bay of Bengal and their antagonistic activities were tested against eight pathogenic bacteria *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 96, *Pseudomonas aeruginosa* MTCC 424, *Escherichia coli* MTCC 443, *Bacillus cereus* MTCC 430, *Proteus vulgaris* MTCC 1771, *Candida albicans* MTCC 227, *Aspergillus niger* MTCC 1344. Among these the potent bacteria *Bacillus subtilis* showed high antibacterial activity. The present study is focused on the improvement of *Bacillus subtilis* through random mutagenesis to obtain mutant having high antibacterial activity. The Bacillus subtilis was subjected for mutation study by using physical (UV radiation) and chemical (NTG) mutation methods. After the mutations, antibacterial activity of the strain was increased and different at different time intervals.

**Key words:** *Bacillus subtilis*, mutation, Antibacterial activity, strain improvement.

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**INTRODUCTION**

Bacitracin (C_{66}H_{103}N_{17}O_{16}S) is a branched cyclic dodecylpeptide produced by *Bacillus subtilis* and some strains of *Bacillus licheniformis* [1]. It is most commonly used in complex with zinc that seems to stabilize the antibiotic complex [2]. Bacterial cells were treated with 0.5M Ethyl Methane Sulphonate (EMS) for 3 h and cultured in a medium containing soybean meal, sucrose and mineral salts [3]. Vegetative cells of *Bacillus subtilis* were exposed with N-methyl-N’-nitro-N-nitrosoguanidine by which the antibiotic activity was drastically increased [4]. The marine bacteria are responsible in producing various metabolites. *Bacillus subtilis* in particular have given us a number of useful compounds of various chemical structures, so called secondary metabolites, including antibiotics. As a result of the increasing prevalence of antibiotics-resistant pathogens and the pharmacological limitations of antibiotics, there is an exigency for new
antimicrobial substances. The development of methods for sampling, identification and successful culture of deep-sea microorganisms has uncovered a new resource for drug discovery [5]. The antibacterial compounds isolated from marine bacterial species are inhibitory to terrestrial bacteria and also many bacterial strains, which are considerable ecological significance [6]. The result of extensive screening have been the discovery of about 4000 antibiotic substances from bacteria and fungi, many of which have found applications in medicine, most of them are produced by *Bacillus subtilis* shows a moderate antagonistic activity against human pathogens. These strains are mutated by using standard Physical (UV) and Chemical (NTG) mutation methods. After mutations, antibacterial activity of the strain *Bacillus subtilis* was increased. In searches for bioactive antibiotics, *Bacillus subtilis* strain has been isolated from various marine samples. Studies showed that distribution of antibacterial metabolites is high in sediment living marine bacteria as compared to other sources [7]. Also deep-ocean sediments hold great promise as a source of genetically novel microorganisms producing structurally unique secondary metabolites [8]. In this quest for new bio-diversity, the exploration of marine microorganism’s particularly marine bacteria has proved to be a rich source of secondary metabolites that display antibacterial properties [9, 10]. Therefore, the present study was undertaken to isolate the antagonistic *Bacillus subtilis* from the soil sample collected from Bay of Bengal and check its antibiotic production efficiency by mutation methods.

**MATERIALS AND METHODS**

The marine sample were collected from different places of Bay of Bengal, the samples was brought to the laboratory in asceptic condition. *Bacillus subtilis* was isolated by spread plate technique on nutrient agar medium (purchased from high media).

**Screening of Bacillus subtilis isolate for Antimicrobial activities:**

Identification of antibiotic producing species was carried out by studying morphological, cultural, physiological and biochemical characters on the basis of Bergey’s Manual of Determinative Bacteriology. The sponges were collected by SCUBA diving at depths of 3–20 m in the Bay of Bengal near Visakhapatnam coast (GPS: 24°21.432 N; 28°72.725 E) of Andhra Pradesh, INDIA. Different strains were isolated from the marine sponges prepared in saline water by making serial dilutions by plate method [11]. NA plates were prepared and inoculated with isolates by a single streak of inoculum in the center of the petridish. The antimicrobial activity was determined by agar well method [12] in NA plates. A modified cross-streak method [13] was used for antimicrobial activity. Single streak of *Bacillus subtilis* was made on surface of the modified nutrient agar and incubated at 37°C. After observing a good ribbon-like growth of the *Bacillus subtilis* on the plates, the overnight bacterial strains, such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Proteus vulgaris*, *Candida albicans*, *Aspergillus niger*, were streaked at right angles to the original streak of *Bacillus subtilis* and incubated at 37°C and the incubation distance was measured after 24-48 h. A control plate was also maintained without inoculating the *Bacillus subtilis*, to assess the normal growth of the bacteria.

The *Bacillus subtilis* isolate was inoculated in nutrient broth and kept in shaker under 250 rpm at 37°C for 24 h. The culture was separated from the cells by centrifugation at 5000 rpm and the supernatant was used for testing the antimicrobial efficiency. The pathogens were subjected to
pour plate method and the supernatant was tested by Agar-well method and incubated at room temperature for 24 hours. The diameter of the inhibition zone for each strain was recorded [14].

**Effect of Mutation on Antimicrobial activity:**

The strains which showed efficient antimicrobial activity were further selected to study the effect of mutation on their antibiotic production.

**UV Irradiation:**

For UV irradiation, method of [15] was adopted. The *Bacillus subtilis* was cultured in the tubes containing 9 ml nutrient broth. The tubes were inoculated with one loopful of the strain and incubated in a rotator shaker at 37°C for 24 h. After incubation, the tubes were removed from the shaker and 3 ml of each culture was exposed to UV irradiation at a distance of 30 cm for 180 sec. One ml of the exposed cultures was transferred to 9ml of the nutrient broth and the tubes were incubated for 24 h. on a shaker at 37°C. After incubation, the tubes were removed from the shaker and the broth was centrifuged at 2000 rpm for 20 min and the supernatant was used to examine the post mutation effect on the strain for their antimicrobial activity [16].

**NTG Treatment:**

For chemical mutagenesis using NTG, MNNG, and HHO₂ method of [17] was followed. The *Bacillus subtilis* was cultured in the tubes containing 9 ml nutrient broth. The tubes were inoculated with one loopful of the strain and incubated in a rotator shaker at 37°C for 24 h. The culture broth was centrifuged at 3000 rpm for 10 min and the pellets were collected. The pellets were suspended with 2ml of Tris buffer (pH 7.2) in the test tubes and 50µg/ml of NTG (N-methyl-N-nitro-N-nitroso guanidine) was added to the test tubes. Then the test tubes were incubated at 37°C for 30 minutes. After incubation, 1ml of chemical treated culture transferred into 9ml of nutrient agar medium and the tubes with culture of antibiotic production were incubated for 24 h. on a shaker at 37°C. The tubes were removed from the shaker and the broth was centrifuged at 2000 rpm for 20 min. The supernatant was used to examine the post mutational effect on the strains for antimicrobial activity [18].

**RESULTS**

A total of 178 strains were isolated from Bay Of Bengal Sponges, Visakhapatnam and the isolated strains were tested for their antagonistic activity against pathogens, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Proteus vulgaris*, *Candida albicans*, *Aspergillus niger*. Out of these the bacterial isolate *Bacillus subtilis* showed higher antagonistic activity against all the tested pathogens. Among the bacterial strains, only *Bacillus subtilis* has effective antagonistic strains was selected to irradiate with UV and treated with NTG.

**Screening of Antimicrobial activity:**

In the antimicrobial activity of the *Bacillus subtilis* with the eight pathogens and the highest activity is shown for the *Pseudomonas aeruginosa* (22mm) and *Bacillus cereus* (22mm). The other pathogens which exhibit antimicrobial activity are *Bacillus subtilis* (13mm), *Staphylococcus aureus* (18mm), *Escherichia coli* (20mm), *Proteus vulgaris* (16mm), *Candida albicans* (16mm), *Aspergillus niger* (12mm).
Table 1: Antimicrobial activity of Bacillus subtilis

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test organisms</th>
<th>Bacillus subtilis (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Pseudomonas aeruginosa (MTCC 424)</td>
<td>22</td>
</tr>
<tr>
<td>2.</td>
<td>Escherichia coli (MTCC 443)</td>
<td>20</td>
</tr>
<tr>
<td>3.</td>
<td>Proteus vulgaris (MTCC 1771)</td>
<td>16</td>
</tr>
<tr>
<td>4.</td>
<td>Staphylococcus aureus (MTCC 96)</td>
<td>18</td>
</tr>
<tr>
<td>5.</td>
<td>Bacillus subtilis (MTCC 441)</td>
<td>13</td>
</tr>
<tr>
<td>6.</td>
<td>Bacillus cereus (MTCC 430)</td>
<td>22</td>
</tr>
<tr>
<td>7.</td>
<td>Candida albicans (MTCC 227)</td>
<td>16</td>
</tr>
<tr>
<td>8.</td>
<td>Aspergillus niger (MTCC 1344)</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig 1: Antimicrobial activity of Bacillus subtilis:

Effect of mutation on antimicrobial activity:
The strain Bacillus subtilis showed antimicrobial activity against tested pathogen was treated with physical and chemical mutagens to study the effect of mutation on their antimicrobial activity. Mutated strain was checked for their antimicrobial activity against pathogens.

Strain exposed to UV radiation showed variation in zone of inhibition against all the ten tested pathogens non UV radiation exposed strains. As compared to the control and UV mutated strain at 180 sec an increased trend in the inhibition zone against Bacillus subtilis (+1mm), Staphylococcus aureus (+4 mm), Pseudomonas aeruginosa (+2mm) and Escherichia coli (+2mm). The decreased inhibition zone was observed against Aspergillus niger (-1mm) and Candida albicans (-2mm). There was no change against Bacillus cereus and Proteus vulgaris.

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Table 2: Antimicrobial activity of *Bacillus subtilis* after exposing to UV and NTG

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test organisms</th>
<th>Bacillus subtilis (mm) (UV)</th>
<th>Bacillus subtilis (mm) (NTG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Pseudomonas aeruginosa</em> (MTCC 424)</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>2.</td>
<td><em>Escherichia coli</em> (MTCC 443)</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>3.</td>
<td><em>Proteus vulgaris</em> (MTCC 1771)</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>4.</td>
<td><em>Staphylococcus aureus</em> (MTCC 96)</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>5.</td>
<td><em>Bacillus subtilis</em> (MTCC 441)</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>6.</td>
<td><em>Bacillus cereus</em> (MTCC 430)</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>7.</td>
<td><em>Candida albicans</em> (MTCC 227)</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>8.</td>
<td><em>Aspergillus niger</em> (MTCC 1344)</td>
<td>11</td>
<td>08</td>
</tr>
</tbody>
</table>

Fig 1: Strain improvement results of *Bacillus subtilis* against pathogens

Fig 2: Antimicrobial activity of *Bacillus subtilis* after exposing to UV

The chemical mutated strain showed variation in the inhibition zone in all the ten tested bacterial and fungal pathogens than the non-mutated strain. As compared to the control and chemical mutated strain *Bacillus subtilis* at 15 min showed an increase in the inhibition zone against *Bacillus subtilis* (+1 mm), *Staphylococcus aureus* (+3 mm), *Pseudomonas aeruginosa* (+2 mm) and *Escherichia coli* (+2 mm) *Bacillus cereus* (+1 mm) and *Proteus vulgaris* (+1 mm). There was decrease in the inhibition zone against *Aspergillus niger* (-4 mm) and *Candida albicans* (-1 mm).

Fig 3: Antimicrobial activity of *Bacillus subtilis* after exposing to NTG

![Graph showing antimicrobial activity of Bacillus subtilis after exposing to NTG](image)

**Pa**: *Pseudomonas aeruginosa*, **Ec**: *Escherichia coli*, **Py**: *Proteus vulgaris*, **Sa**: *Staphylococcus aureus*, **Bs**: *Bacillus subtilis*, **Be**: *Bacillus cereus*, **Ca**: *Candida albicans*, **An**: *Aspergillus niger*

**DISCUSSION**

Classical strain improvement for years, has allowed for the selection of strains, which are probably altered in the gene regulation and have increased ability to over produce secondary metabolites. This empirical approach has a long history of success, best exemplified by the improvements achieved for pencillin production [19]. Several other workers have shown the induction of novel bioactive compounds by UV, X- and γ-radiation in various models, including bacteria and plants [20, 21]. A plethora of theories advocate that marine bacteria produce the secondary metabolites to protect themselves against the harmful effects of UV radiation [22]. As was demonstrated earlier, representatives of the genus *Bacillus* are capable of producing several antibiotic substances [23]. Therefore, amongst various mutagens causing multiplicity of mutations [19], UV and nitrous acid were employed in addition to MNNG.

In the present study, after mutation, the strain *Bacillus subtilis* increased the antibacterial activity against all tested pathogens *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. But there is no increase or decrease in the activity of *Bacillus cereus* and *Proteus vulgaris* and there is decrease in the activity of *Aspergillus niger* and *Candida albicans* (UV treated). There was an increase in the antibacterial activity against all the bacterial test pathogens *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus cereus* and *Proteus vulgaris*. There was decrease in the activity of *Aspergillus niger* and *Candida albicans* (NTG treated).
treated). Due to mutation partial activity gene of this strain which is responsible for the production of antibiotics could have been activated. Similar results regarding mutagenesis were reported by [24, 25].

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REFERENCES