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Study on Antioxidant activity and strain development of *Bacillus subtilis* (MTCC No.10619)

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The present study focused on the antioxidant activity for the *Bacillus subtilis* which showed potential activity and strain improvement of *B. subtilis* through random mutagenesis to obtain mutant having high antibacterial activity. *B. 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. They were tested for the presence of enzymatic and non-enzymatic antioxidant activity.

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

Introduction

Antioxidant compounds in food play an important role as a healthprotecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables and bacteria. Bacteria sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from bacterial sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants.

Bacitracin ($C_{66}H_{103}N_{17}O_{16}S$) is a branched cyclic dodecyclpeptide produced by *Bacillis subtilis* and some strains of *Bacillus licheniformis* (Ishihara, 2002). It is most commonly used in complex with zinc that seems to

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stabilize the antibiotic complex (Ikai, 1992). 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 (Paleckova, 1981). Vegetative cells of *B. subtilis* were exposed with N-methyl-N'-nitro-N-nitrosoguanidine by which the antibiotic activity was drastically increased (Lukin, 1986). The marine bacteria are capable of producing various metabolites. *B. subtilis,* in particular has 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 (Skropeta, 2008). The antibacterial compounds isolated from marine bacterial species are inhibitory to terrestrial bacteria and also many bacterial strains, which are considerable ecological significance (Saz, 1963). 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 *B. subtilis* and have 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 *B. subtilis* was increased. In searches for bioactive antibiotics, *B. 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 (Kelecom, 2002). Also deep-ocean sediments hold great promise as a source of genetically novel microorganisms producing structurally unique secondary metabolites (Fenical, 2006). In this quest for new bio-diversity, the exploration of marine microorganisms, particularly marine bacteria has proved to be a rich source of secondary metabolites that display antibacterial properties (Burgess, 1991; Baam, 1996). Therefore, the present study was undertaken to isolate the antagonistic *B. subtilis* from the soil sample collected from Bay of Bengal and check its antibiotic production efficiency by mutation methods.

Material and mtehods

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

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 by preparing in saline water by making serial dilutions by plate method (Warcup, 1950). Nutrient agar 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 (Schillinger, 1989) in NA plates. A modified cross-streak method (Balagurunathan, 1992) was used for antimicrobial activity. Single streak of *B. subtilis* was made on surface of the modified nutrient agar and incubated at 37^0C. After observing a good ribbon-like growth of the *B. subtilis* on the plates, the overnight bacterial strains, such as *B. subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Proteus vulgaris*, *Candida albigans*, *Aspergillus niger* were streaked at right angles to the original streak of *B. subtilis* and incubated at 37^0C and the incubation distance was measured after 24-48 h. A control plate was also maintained without inoculating the *B. subtilis*, to assess the normal growth of the bacteria.

The *B. subtilis* isolate was inoculated in nutrient broth and kept in shaker under 250 rpm at 37^0C 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 (Michael, 1989).

Effect of Mutation on Antimicrobial activity

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

UV Irradiation

For UV irradiation, method of (Parekh, 2000) was adopted. *B. 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 37^0C 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 37^0C. 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 (Philips, 1960).

NTG treatment

For chemical mutagenesis using NTG (N-methyl-N-nitro-N-nitroso guanidine), MNNG, and HNO_2 was follwed the method of Delic (1970). *B. 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 37^0C 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 37^0C for 30 minutes. After incubation, 1ml of chemical treated culture transferred in to 9ml of nutrient agar medium and the tubes with culture of antibiotic production were incubated for 24 h. on a shaker at 37^0C. 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 (Muthurayar, 2006).

Total antioxidant activity

Chemicals: 0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate.

Procedure: The total anti-oxidant capacity of the samples was evaluated by the method of prieto et.al. An aliquot of each sample extract (0.05 ml) was mixed with 0.5 ml of reagent in 1.5 ml eppendorf tube. The tubes were capped and boiled in a boiling water bath at 95^oC for 90 min and cooled. The absorbance was measured at 695 nm against blank in carpary spectrophotometer. Simultaneously, a blank was setup using buffer instead of sample and treated in same manner as test. The anti-oxidant capacity was expressed as micro moles of ascorbic acid equivalents of anti-oxidant capacity (AEAC).

Assay of enzymes involved in anti-oxidant activity

Assay of catalase

Chemicals: 0.1M phosphate buffer, pH 7.5: **a.** 1.39 gm of monobasic sodium phosphate was dissolved in 100 ml of water, **b.** 2.68 gm of dibasic sodium phosphate was dissolved in 100 ml of water. 16.0 ml of **a**, 84.0 ml of **b** was mixed and made upto 200 ml with water, 0.9% Hydrogen peroxide (v/v): 0.9 ml of 30% Hydrogen peroxide made upto 100 ml with water, 2 N Sulphuric acid: 11.11 ml of concentrated Sulphuric acid made up to 100 ml with water and 0.1M Potassium permanganate: 1.58 gm of $KMnO_4$ was dissolved in 100ml of water.

Procedure: The catalase activity is determined by titrimetric method based on the procedure described by Radha Krishna and Sarma. To 0.5 ml of sample extract, 2.5ml of 0.9% H_2O_2 at incubated at 28^oC for 3 min. the reaction was then arrested by adding 5 ml of 2N H_2SO_4 and the residual H_2O_2 was titrated with 0.1 n $KMnO_4$ solution. Blank was set up using buffer without extract. Unit of catalase activity was expressed as ml of 0.1N $KMnO_4$ equivalents of hydrogen peroxide decomposed per minute per µg protein.

Assay of non–enzymatic parameters of antioxidant activity:

Estimation of ascorbic acid

Chemicals: 4% oxalic acid**:** dissolve 4 gm of oxalic acid in 100 ml distilled water, Dye solution: dissolve 26 mg of 2,6 dichloro phenol indophenol and 21 mg of sodium bicarbonate in 100 ml of volumetric flask, Stock standard solution: dissolve 100 mg of ascorbic acid in 100 ml of 4% oxalic acid solution and Working standard: dilute 10 ml of stock solution in 100 ml with 4% oxalic acid solution.

Procedure: By Sadasivan and Theymoli Balsubramian method. A stock solution of 100 µg of Ascorbic acid was dissolved in 100 ml of 4% oxalic acid solution. For working standard 10 ml of the stock solution was diluted to 100ml with 4% oxalic acid. For control 5 ml, of working standard solution was taken in to a 100 ml conical flask. Then 1 ml of acetic acid was added and titrated against the dye until the appearance of pink color which persists for few minutes. For blank, 5 ml of distill water and for test 5 ml of sample extract are taken and procedure is repeated.

Amount of Ascorbic acid (mg) = test – blank/ control – blank x 8.

Results

A total of 178 strains were isolated from sponges of Bay of Bengal, Visakhapatnam. 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* and *Aspergillus niger*. Out of these the bacterial isolates *Bacillus subtilis* showed higher antagonistic activity against all the pathogens tested. Among the bacterial strains, *B. subtilis* was effective antagonistic strains and it used for further studies of UV and NTG treatments.

Screening of antimicrobial activity

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

Table 1. Antimicrobial activity of *Bacillus subtilis*

Test organisms	***Bacillus subtilis* (mm)**
Pseudomonas aeruginosa (MTCC 424)	22±0.577
Escherichia coli (MTCC 443)	20±0.577
Proteus vulgaris (MTCC 1771)	16±0.577
Staphylococcus aureus (MTCC 96)	18±0.577
Bacillus subtilis (MTCC 441)	13±0.000
Bacillus cereus (MTCC 430)	22±0.577
Candida albicans (MTCC 227)	16±0.333
Aspergillus niger (MTCC 1344)	12±0.577

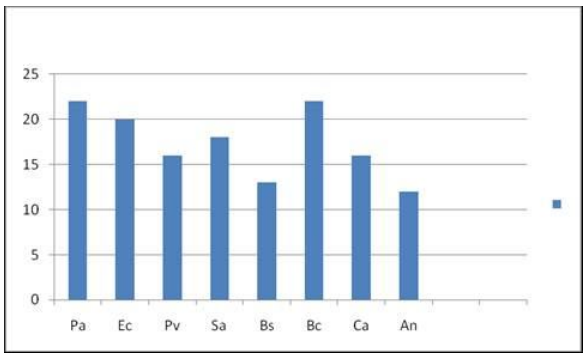

Fig 1. Antimicrobial activity of *Bacillus subtilis*:
Pa- *Pseudomonas aeruginosa,* Ec- *Escherichia coli,* Pv- *Proteus vulgaris,* Sa- *Staphylococcus aureus*, Bs- *Bacillus subtilis,* Bc- *Bacillus cereus,* Ca- *Candida albicans,* An- *Aspergillus niger*

Effect of mutation on antimicrobial ativity

The strain *Bacillus subtilis* that showed antimicrobial activity against pathogens tested 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. The strain exposed to UV radiation showed variation in zone of inhibition against all the ten pathogens tested with the 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* (+1 mm), *Staphylococcus aureus* (+4 mm), *Pseudomonas aeruginosa* (+2 mm) and *Escherichia coli* (+2 mm). The decreased inhibition zone was observed against *Aspergillus niger* (-1 mm) and *Candida albicans* (-2 mm). There was no change against *Bacillus cereus* and *Proteus vulgaris*.

The chemically mutated strain showed variation in the inhibition zone with all the ten tested bacterial and fungal pathogens than that of non-mutated strain. As compared to the control and chemically 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 albigans* (-1 mm).

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

S.No.	Pathogens	Diameter of Inhibition zone *Bacillus subtilis* (mm) (UV)	Diameter of Inhibition zone *Bacillus subtilis*(mm) (NTG)
1.	*Pseudomonas aeruginosa* (MTCC 424)	24±0.577	24±0.333
2.	*Escherichia coli* (MTCC 443)	22±0.577	22±0.333
3.	*Poteus vulgaris* (MTCC 1771)	16±0.333	17±0.000
4.	*Staphylococcus aureus* (MTCC 96)	22±0.577	21±0.666
5.	*Bacillus subtilis* (MTCC 441)	14±0.666	14±0.577
6.	*Bacillus cereus* (MTCC 430)	22±0.333	23±0.666
7.	*Candida albicans* (MTCC 227)	14±0.333	15±0.333
8.	*Aspergillus niger* (MTCC 1344)	11±0.000	08±0.577

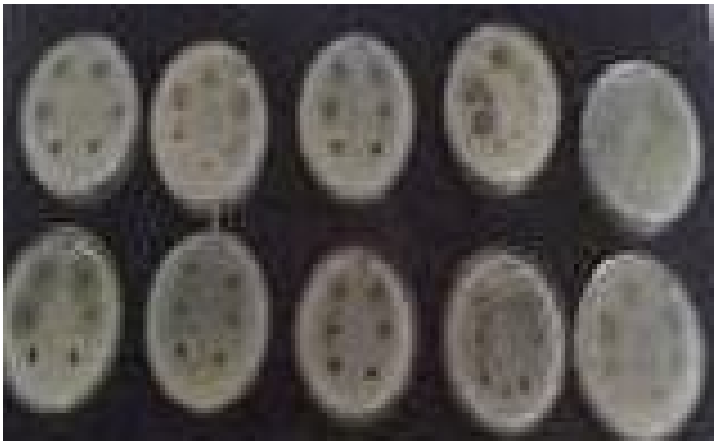

Fig 2. Strain improvement results of *Bacillus subtilis* against pathogens

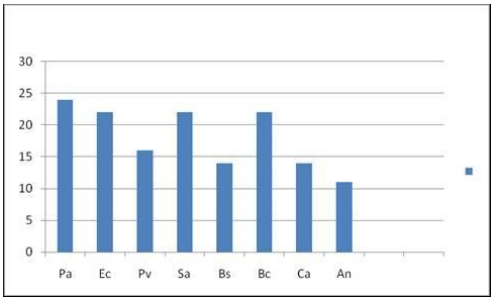

Fig 3. Antimicrobial activity of Bacillus subtilis after exposing to UV

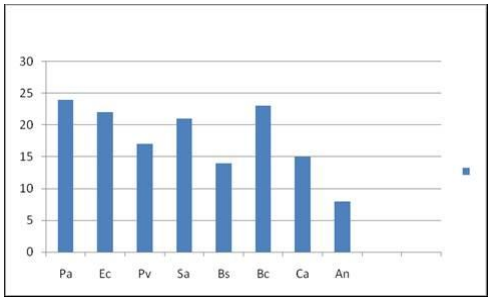

Fig 4. Antimicrobial activity of *Bacillus subtilis* after exposing to NTG

Pa- *Pseudomonas aeruginosa,* Ec- *Escherichia coli,* Pv- *Proteus vulgaris,* Sa- *Staphylococcus aureus*, Bs- *Bacillus subtilis,* Bc- *Bacillus cereus,* Ca- *Candida albicans,* An- *Aspergillus niger*

Enzymatic parameters of antioxidant activity

Catalase activity: Catalase promotes wound healing through the degradation of hydrogen peroxide to oxygen and water. This specific action protects cell structure during the primary inflammatory reaction of wound healing.

Non-enzymatic parameters of antioxidant activity: The concentration of non-enzymatic parameters of antioxidant activity was also determined.

Ascorbic acid Activity:The highest activity of ascorbic acid was observed (37.16 units/mg protein)

Total Antioxidant Activity: The total anti-oxidant activity of different extracts was shown in figure. The total anti-oxidant activity ranges from 1.58µm to 2.74µm of ascorbic acid equivalents.

Discussion

From the results of preliminary investigations, it can be concluded that the crude bacterial extracts tested are good resources for the presence of bioactive compounds. After the analysis of all the antioxidant parameters tested have highest anti-oxidant activity.In view of the presence of antioxidant activity and other bioactive nature of putative compounds, further studies for isolating the compound/(s) can be undertaken. However for exploring potential use and therapeutic application further studies on chemical characterization and purification of compounds of interest can be carried out.

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 (Hung, 2008). Several other reports have shown the induction of novel bioactive compounds by UV, X- and γ-radiation in various models, including bacteria and plants (Vilenskaya, 1972; Karentz, 2001). A plethora of theories advocate that marine bacteria produce secondary metabolites to protect themselves against the harmful effects of UV radiation (Motta, 2004). As was demonstrated earlier, representatives of the genus *Bacillus* are capable of producing several antibiotic substances (Rosenfeld, 1947). Therefore, amongst various mutagens causing multiplicity of mutations (Hung, 2008), UV and nitrous acid were also employed.

In the present study, after mutation, the strain *Bacillus subtilis* exhibited more against all pathogens namely *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.* But there was no increase or decrease in the activity against *Bacillus cereus* and *Proteus vulgaris* and there was decrease in the activity against *Aspergillus niger* and *Candida albigans* (UV treated). There was an increase in the antibacterial activity against all the bacterial test pathogens namely *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus cereus* and *Proteus vulgaris*. There was decrease in the activity of *Aspergillus niger* and *Candida albigans* (NTG 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 (Parekh, 1964).

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