

Strain improvement for the production of antioxidant activity from *Bacillus sp.* by induced stress

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Abstract

In the present study, water samples were collected from different beaches in Chennai such as Marina beach, Elliott beach, VGP Golden beach and Kovalam beach to evaluate the best source for antioxidants. The bacteria were isolated on Starch Casein Agar media and screened for the antimicrobial activity. Among 11 isolates, 5 isolates showed antimicrobial potential which were further evaluated for the DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity. Among 5 isolates, one sample showed significant DPPH scavenging activity with half minimal inhibitory concentration of (IC₅₀)344.754µg/mL. The maximum antioxidant production was observed at pH 7 and at temperature of 37°C with an IC₅₀ of 188.66µg/mL and 293.76µg/mL respectively. The potent antioxidant producing strain was subjected for mutagenesis. In physical mutagenesis, the organism exposed for UV light for 25 minutes showed maximum antioxidant production with an IC₅₀ of 133.55µg/mL. This mutant strain was then subjected for chemical mutagenesis with the addition of different concentrations of Ethidium bromide such as 10µL, 20µL, 30µL, 40µL and 50µL. The mutant strain obtained with the addition of 20µL Ethidium bromide (EtBr) showed significant antioxidant activity with an IC₅₀ of 325.4µg/mL. The sample was purified by solvent extraction method and was evaluated for antioxidant production. The analyte was subjected to HPLC (High Performance Liquid Chromatography) analysis to avince the presence of antioxidants. The ethyl acetate extract showed the total phenolic content of 0.892 mg GAE/g of dry extract. It also showed the total flavonoid content of 0.522mg RE/g of dry extract.

Keywords: Antioxidants, DPPH scavenging activity, HPLC, Mutagenesis

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INTRODUCTION

The microbial extracts have served as a valuable source of diverse molecules in many drug discovery efforts and led to the isolation of several important drugs (Santas *et al.*, 2008). They produce the most bioactive compounds that are potentially useful for human in pharmaceutical industry sector, for instance as antimicrobial, antifungal, antioxidant, anticancer and anti-inflammatory agents (Yoghiapiscessa *et al.*, 2016).

Antioxidants are the free radical scavengers (Takao *et al.*, 1994). Antioxidant compounds have

an important role in improving human health and can decrease the risk of multiple chronic degenerative diseases, for instance gastrointestinal infection, cancer, Alzheimer's disease, cataract and coronary heart disease (Yoghiapiscessa *et al.*, 2016).

Antioxidant properties have been reported from grains, cereals, medicinal herbs, plants, eggs, meat, legumes and nuts (Pereira *et al.*, 2015). The study of microbial antioxidants came into focus in the early 1980s, although various studies have established a relationship between antioxidants

and microorganisms (Gupta et al., 2013). However, studies on microorganisms with respect to antioxidant and free radical scavenging activities are very limited (Saravana Kumar et al., 2014)

Several works have been already reported on the antioxidant activity of bacteria. For instance, The probiotic bacteria *Streptococcus thermophilus* has been shown to aid malnutrition due to short-term fasting and reduce the associated intestinal disorders in animals. *S. thermophilus* is also known to have powerful antioxidant activity, protecting the body from aging, stress, sugar, antibiotics and other toxins (Gupta et al., 2013).

Further, the organisms like *Bacillus simplex*, *Bacillus natto*, *Bacillus cereus*, *Lactobacillus dextraniscum*, *Micrococcus freudenreichii*, *Sarcina lutea*, *Bacillus stearothermophilus*, *Bacillus longum*, *Lactobacillus acidophilus*, *Thermothrix* species have also shown considerable antioxidant activity (Varinder et al., 2013).

In the present study, the research focus was to screen bacteria found in water bodies producing bioactive compounds and to determine potential bacterial isolates through the antimicrobial and antioxidant activities of the bacterial extracts.

MATERIALS AND METHODS

Collection of samples: The samples used in this study were collected from different beaches in Chennai such as Marina beach, Elliott beach, VGP Golden beach and Kovalam beach. The samples were collected using sterilised plastic bags and were then taken to the laboratory for further analysis.

Isolation and identification of organism: A known volume (1mL) of each water sample was added to 9mL of deionized water. Then the serial dilution method was carried out to obtain different concentrations ranging from 10^{-1} to 10^{-5} , after which 500 μ L of the sample from the last dilution was aseptically drawn and poured onto the sterile petriplates. The Starch Casein Agar media (Starch-10g, Casein-1g, NaCl-37g, Agar-20g, Distilled water-1000mL) was poured on the inoculum and allowed to solidify. The petriplates were incubated for 24hours at 37°C. After the incubation, the colonies formed on the petriplates were subcultured on SCA slants. The isolated strain was identified to the genus level based on their morphological characters such as colour, shape, size, texture etc., of the bacterial colonies and biochemical characteristics such as IMViC tests, carbohydrate fermentation, nitrate reduction tests etc.

Preparation of cell free supernatant: Sterile Starch Casein broth (Starch-10g, Casein-1g, NaCl-37g, Distilled water-1000mL) was inoculated with the obtained pure cultures of the isolates and incubated at 37°C for 24 hours. The cell free supernatant was obtained by centrifugation of this

culture at 10000rpm for 10 minutes at 4°C.

Estimation of antioxidant activity by DPPH assay: The free Radical scavenging activity using 1,1- diphenyl-2-picryl-hydrazyl (DPPH) reagent was determined according to the method given by Blois (Marsden., 1958). The cell free supernatant with different concentrations ranging from 10 μ L to 50 μ L were pipetted into different test tubes. 3mL of freshly prepared DPPH solution was added and incubated in dark chamber for 15 minutes. Absorbance was recorded at 517 nm and compared with the control. The antioxidant activity was given as percentage of DPPH scavenging and the IC50 value was calculated (Akter et al., 2010)

Effect of pH on the production of antioxidant compound: To determine optimal pH, the organism was cultivated in a conical flask containing 50mL of Starch Casein broth with different pH ranging from 4-9. The pH of the medium was adjusted using 1N HCl and 0.2M phosphate buffer. The culture was incubated in a rotary shaker for 24 hours at 37°C. After incubation, the cell free filtrate was used for DPPH assay.

Effect of temperature on the production of antioxidant compound: In order to determine the optimum temperature for the production of antioxidant compound, the Starch Casein broth was prepared in different conical flasks and was adjusted to pH 7. 1mL of the bacterial culture was inoculated and incubated at different temperatures such as 4°C, 37°C, 50°C and 60°C in a rotary shaker. After incubation, DPPH assay was performed as described earlier.

Effect of induced stress on the production of antioxidant compound

Effect of UV irradiation: Sterilized Starch Casein Agar media was poured into 5-6 petriplates and allowed to solidify. 100 μ L of the culture was spread uniformly. The plates were exposed to UV light for different intervals of time such as 5, 10, 15, 20 and 25 minutes and incubated for 24 hours at 37°C. After incubation, the colonies formed were sub-cultured on SCA slants. These cultures were inoculated in Starch Casein broth and incubated at 37°C for 24 hours. The cell free supernatant was prepared by centrifugation and the DPPH assay was performed.

Effect of chemical stress: 1mL of sterilised Starch Casein Broth was taken in 5 different vials and inoculated with the bacterial culture and different concentration of Ethidium Bromide ranging from 10-50mL was added to each vial. Incubate at 37°C for 24 hours. After incubation, the broth is centrifuged at 10,000 rpm for 10 minutes and the DPPH assay was performed using the supernatant.

Purification

Production of pure culture: Sterile 250mL Starch Casein broth with pH (7) was inoculated with the bacterial culture and incubated in shaker

incubator for 24 hours. The cell free supernatant was prepared using this broth and the DPPH assay was performed.

Solvent extraction by ethyl acetate: In this method, equal volume of the supernatant and ethyl acetate were added in a separating funnel, mixed well and allowed to settle down for overnight. Formation of two immiscible layers observed i.e., organic layer and aqueous layer. The organic layer was taken and kept for drying in a water bath at 30-40°C for overnight. The dried powder (10mg) was dissolved in 1mL of DMSO solution. Taking this solution, DPPH assay was performed.

High performance liquid chromatography (HPLC): Methanol and water was used in the ratio 7:3 to prepare mobile phase and was degassed. The crude sample and purified sample obtained was membrane filtered using nitrocellulose membrane. Further, 20µL of the sample was injected into the injector and the results were recorded.

RESULTS AND DISCUSSION

Isolation of the bacteria: Marine water bodies are the potential sources for isolation of novel bacteria yielding new products and are recognized as source of novel antibiotic and anticancer agents. Thus, bacteria represent an important group of microbes found in environment. In this study, 14 aerobic bacterial colonies were isolated on the basal media from 4 marine water bodies in Chennai. The macroscopic appearance of the

isolates showed white smooth translucent colonies on the media.

DPPH radical scavenging activity: Besides antimicrobial activity, marine bacteria also develop unique metabolic and physiological capabilities, which enable them to survive in extreme habitats and to produce compounds that might not be produced by their terrestrial counterparts. Marine bacteria were isolated and screened for their antioxidant producing capacities. Since there is no specific method to evaluate the antioxidant activity of a compound/extract, due to different antioxidant mechanisms, the antioxidant activity was evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. DPPH is a stable free radical, accepts protons from the antioxidant source and shows a decreasing absorption. In the present study, the bacteria that showed antimicrobial potential were evaluated for the DPPH scavenging assay and the DPPH activity was determined in percentage of inhibition.

It was witnessed that the bacteria in sample 2 showed significant level of DPPH radical scavenging activity than other samples. The bacteria in sample 2 showed an IC₅₀ of 336.64/mL that is comparable with the IC₅₀ value of standard gallic acid (10.8±0.02 ug/mL). These results are extremely interesting, especially when compared with the work of Kalirajan and co-workers who evaluated the DPPH radical scavenging activity of bacteria associated with a marine sponge and obtained an IC₅₀ of 857.49 µg/mL, which is 2.5 times less

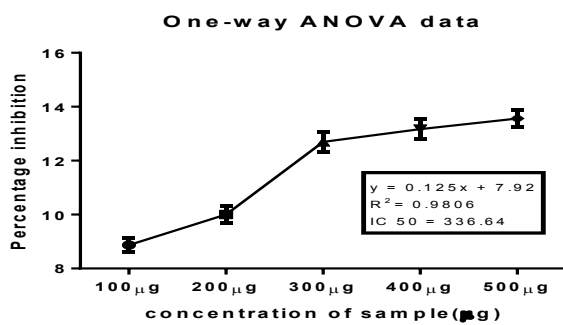


Fig. 1. Percentage inhibition shown by the bacteria at different concentrations.

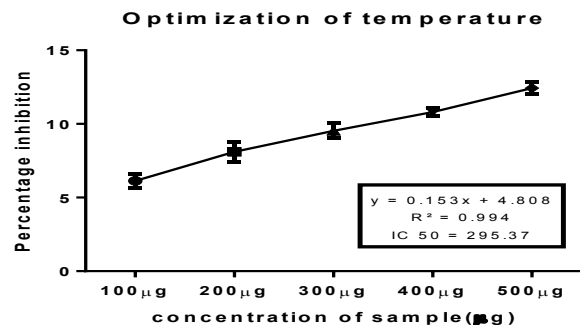


Fig. 3. Percentage inhibition shown by the bacteria at room temperature.

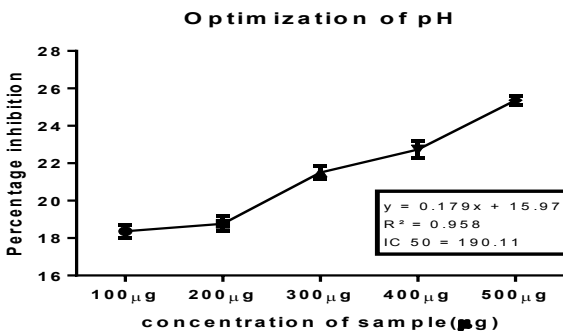


Fig. 2. Percentage inhibition shown by the bacteria at pH 7.

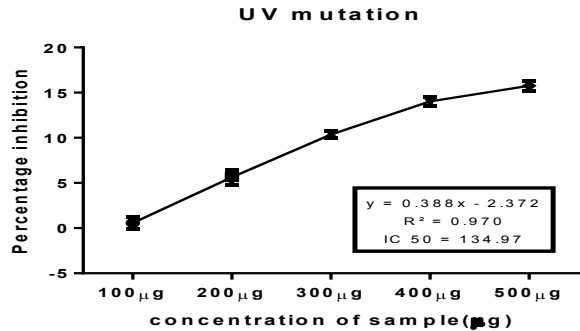


Fig. 4. Percentage inhibition shown by the bacteria after UV mutation.

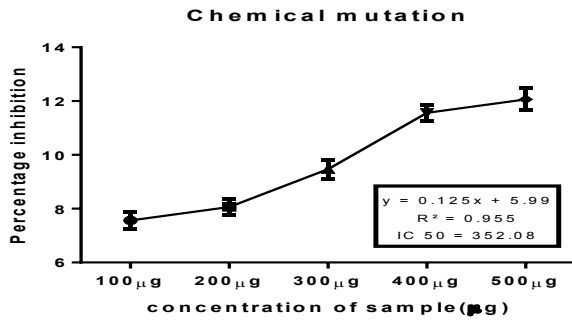


Fig. 5. Percentage inhibition shown by the bacteria after chemical mutation.

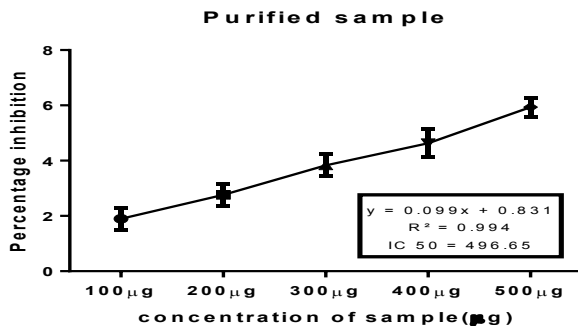


Fig. 6. Percentage inhibition shown by the bacteria after purification.

potent than that obtained for the bacteria in our work (Kalirajan et al., 2013).

Genus level Identification of potential isolate:

The isolate recovered from the water sample appeared as a smooth, translucent white colony. It was identified as Gram positive rods from Gram staining. From the biochemical tests, the bacterium was identified as Bacillus species since it showed positive result for carbohydrate fermentation, catalase, starch hydrolysis and methyl red tests.

Effect of pH on the production of antioxidant compound: An important factor significantly affecting the production of antioxidant was the initial pH. The production of antioxidant was carried out at different pH at 4, 5, 6, 7, 8, and 9. The table below shows the effect of medium pH on production

of antioxidant compound by the isolate.

The Maximum antioxidant production is observed at pH 7 which showed an IC50 value of 190.11µg/mL. The results are in the accordance with the work in the case of antioxidant production by *Pseudomonas fluorescens* at pH 7 (Raza et al., 2014) and also with the results of Chowdhury in the case of *Bacillus megaterium* (Sougata et al., 2011).

Effect of temperature on the production of antioxidant compound:

Temperature influences all the physiological activities in living cell and is one of the important environmental factors to control the growth, microbial activities, normal functioning of the cell. The production of antioxidant at different temperature viz., 4°C, 37°C, 50°C and 60°C, was examined by keeping the other conditions constant. The table below shows the effect of temperature on the production of antioxidant.

The Maximum antioxidant production was observed at room temperature i.e., 37°C which showed an IC50 value of 295.37µg/mL. Mata et al (2008) have reported a maximum EPS production at 32°C in *Alteromonadaceae* sp., a halophilic bacterium which is likely in agreement with the present study. Also there are reports of maximum antioxidant production at temperatures ranging from 28 to 37°C (Raza et al., 2014; Sougata et al., 2011; Varinder et al., 2013).

Effect of induced stress on the production of antioxidant compound

Effect of UV irradiation: Ultraviolet (UV) light exerts its mutagenic effect by exciting electrons in molecules. The excitation of electrons in DNA molecules often results in the formation of extra bonds between adjacent pyrimidines in DNA (Suribabu et al., 2014). The UV light is the best studied mutagenic agent in prokaryotic organisms (Meenu et al., 2000). In the current study, the mutant strain which was subjected to UV treatment for 25 minutes showed maximum antioxidant production.

The mutant strain showed an IC50 of 134.97µg/mL which confirms that the mutant strain is more

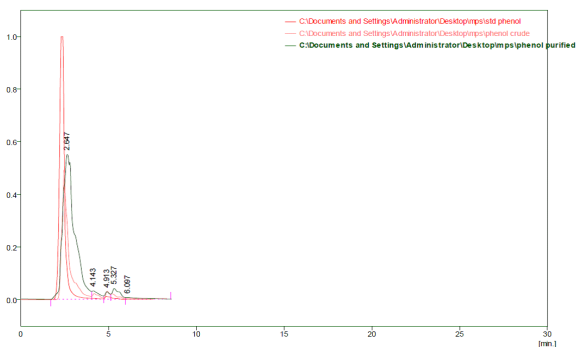


Fig. 7. HPLC chromatogram for the analysis of phenols extracted from bacteria by solvent extraction method.

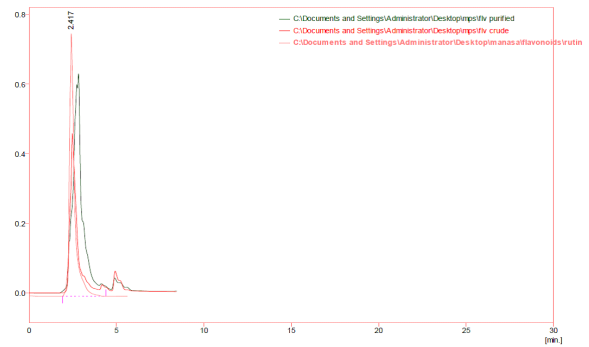


Fig. 8. HPLC chromatogram for the analysis of flavonoids extracted from bacteria by solvent extraction method.

potent than the parental strain. Here, mutations were found to have enhanced the antioxidant production ability by 2.5 fold than the parental strain. Thus, in the present study, mutations were found to have contributed significantly in strain improvement with respect to the production of antioxidants.

Effect of Chemical stress: Chemical mutagens are stronger mutagenic agents which cause permanent changes in DNA sequence by bringing about transitions from G: C → A: T (Miller., 1972). Ethidium bromide (EtBr) acts as a mutagen because it intercalates double stranded DNA and deforms the DNA (Joyce *et al.*, 1975). Different concentrations of Ethidium bromide was added to the UV mutant strain and was assayed for antioxidant production.

The maximum antioxidant production was observed with the addition of 20 μ L of Ethidium Bromide. The observed IC₅₀ value was 352.08 μ g/mL which showed slight increase in the antioxidant activity than the parent strain. Thus, in the present study, it can be concluded that the mutations play a very important role in the strain improvement of the bacteria in order to obtain a high yield of the metabolite.

Purification

Solvent extraction by ethyl acetate: The method used in the separation of antioxidants becomes essential for the accurate quantification and determination of antioxidant capacity (Santas *et al.*, 2008). Several extraction conditions are reported in the literature, however there is no single extraction method which may be considered standard (Cacace and Mazza, 2003). The chemical nature of phenolic compounds, the extraction method employed, the sample particle size, storage time and conditions, as well as the presence of interfering substances affect the efficiency of the extraction methods (Joana *et al.*, 2014). In the present study, ethyl acetate was used as an extraction solvent and the extracted solvent was used for the DPPH assay. The percentage of inhibition is shown graphically.

The purified extract showed an IC₅₀ of 496.65 μ g/mL. The solvent extraction using ethyl acetate led to the recovery of a potent antioxidant compound. Remya and Vijaykumar have reported the antimicrobial potential of compound obtained from ethyl acetate extract of *Streptomyces* strain RM42 (Mohan *et al.*, 2008). Similarly, the extraction of antibiotics has been carried out from *Streptomyces* by using various solvents including ethyl acetate and methanol.

High performance liquid chromatography (HPLC): High performance liquid chromatography constitutes a crucial, reliable technique for the characterization of phenolic compounds due to its versatility, precision and relatively low cost (Irene *et al.*, 2002). Moreover, HPLC techniques offer a

unique chance to analyze simultaneously all components of interest together with their possible derivatives or degradation products (Sakakibara *et al.*, 2003). The introduction of reversed-phase (RP) columns has considerably enhanced HPLC separation of different classes of phenolic compounds and RP-C18 columns are almost exclusively employed (Sai *et al.*, 2012). In this study, the analyte was subjected to HPLC analysis to avince for the presence of antioxidants. The results obtained were expressed in terms of mg/g of dry extract. The ethyl acetate extract showed the total phenolic content of 0.892 mg GAE/g of dry extract. It also showed the total flavonoid content of 0.522mg RE/g of dry extract. A number of works on Analysis of antioxidant compounds in plants using HPLC have been already reported. Very less works have been reported on the antioxidant compounds present in the bacteria (Shoib *et al.*, 2015). Thus, HPLC analysis of the ethyl acetate extracts of this sample led to the identification of phenolic compounds and flavonoids in bacteria and our results confirmed the presence of compounds to be responsible for showing antimicrobial and antioxidant activities. The results obtained here is in accordance with the results of the work reported on extraction and identification of antibacterial secondary metabolites from marine *Streptomyces sp. VITBRK2* (Benita *et al.*, 2014). This study thus confirms the potential of the bacteria as a valuable source of bioactive compounds and could be potential alternatives for synthetic antimicrobial drugs and antioxidants.

Conclusion

The marine bacterial isolates from different water sources of Chennai, India exhibited antioxidant activity with an IC₅₀ of 344.75 μ g/mL which revealed a potential source of novel antioxidant compounds. The organism was found to be *Bacillus sp.* by performing gram staining and various biochemical tests. Strain improvement of the bacteria was carried out by using physical and chemical mutagens such as UV light and Ethidium Bromide. The bacteria showed IC₅₀ of 133.55 μ g/mL with UV light which was 2.5 fold higher than the wild parent strain. Also, the antioxidant activity increased slightly with the chemical mutagen with an IC₅₀ of 325.4 μ g/mL. Further, a simple and reliable extraction technique, and a validated HPLC method were developed for the simultaneous separation and quantification of antioxidants from the bacteria.

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