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EVALUATION OF ANTIOXIDANT ACTIVITIES OF *BACILLUS CEREUS* AND *BACILLUS PUMILUS* METABOLITES

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Abstract: Soil samples were screened for bacteria with antibiotic production potential. The two isolates which showed very good antibacterial activity were identified as *Bacillus cereus* and *Bacillus pumilus*. The metabolites of the two bacteria were subjected to various solvent extractions and the extracts were tested for antioxidant activity by DPPH and ABTS radical scavenging assay. The metabolites of both bacteria exhibited significant antioxidant activity by exhibiting 97% and 88.5% radical scavenging by ABTS method for *B. pumilus* and *B. cereus* with IC_{50} values at 16.2±1.17µg/ml and 55.12±2.51µg/ml respectively. They showed less radical scavenging activity by DPPH assay. The two bacteria by their antioxidant activity promises to have bioactive molecules with therapeutic potential and other useful health benefits.

Keywords: *Bacillus cereus*, *Bacillus pumilus*, Antioxidants, antibacterial activity.

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INTRODUCTION

Oxidative stress occurs when abnormally high levels of reactive oxygen species (ROS) are generated and the available supply of the body's antioxidants is insufficient to handle and neutralize the free radicals [1,2]. Various physical, chemical and environmental stresses on cells may induce an overproduction of ROS [3].

Free radicals are highly unstable molecules that interact with other molecules in the body and cause considerable damage. It is a well known fact that free radicals can damage DNA, proteins, lipids and carbohydrates within the human tissues and can cause many diseases. These free radicals are responsible for various cellular anomalies like protein damage, inactivation of enzymes, alterations in DNA, lipid peroxidation which in turn can lead to pathological conditions. It can cause many diseases like cancer, rheumatoid arthritis, aging, diabetes, reperfusion injury, cardiovascular diseases, atherosclerosis and inflammatory diseases [4,5,6].

Antioxidants are chemical compounds that can protect the human body from free radicals by reducing or preventing oxidative damage. They retard the progress of many diseases [7,8]. Thus to maintain the normal cellular health it is important to have effective antioxidants that scavenges multiple types of free radicals so that it can be used in multiple diseases. Apart from human health antioxidants have larger applications in food industries. They can delay the deterioration and discoloration of foods due to radical mediated oxidation of fats and oils which is the major cause of food spoilage [9].

Many available bioactive antioxidants are derived from natural sources like plants and animals. They are isolated from rice bran, peanut kernels, cornmeal, frog skin, egg yolk proteins, casein and many other sources. The findings of synthetic antioxidants have resulted in replacement of natural ones by synthetic compounds. But, the usage of synthetics such as Butylhydroxyanisole (BHA), Butylhydroxytoluene (BHT), and tert-butylhydroxyquinone (TBHQ) are found to have carcinogenic effects [10,11]. Hence, the development and utilization of less harmful antioxidants with low cytotoxicity of natural origin are desirable and interest in finding natural antioxidants has increased in recent days mainly focused on plant compounds [12,13,14].

Microbial cells have a number of antioxidant defense mechanisms. They play a major role by removing or inactivating ROS and protect the biological system. They maintain the free radical levels that are not toxic to the cells. In the recent years, there has been an increased interest in the antioxidant effect of microorganisms and their role in health and diseases [15,16]. The studies have showed that a number of lactic acid bacteria, *Bacillus* species and many others bacteria exert antioxidant action[17,18,19,20,21]. Several yogurt starter cultures like *Lactobacillus bulgaricus, Lactobacillus casei* and many other *lactobacilli* have been reported to have several health benefits like hypoallergenic effects, control of gastrointestinal infections, stimulation of

immunological systems, anticarcinogenic effect, reduction of serum cholesterol and longevity. They have reported to enhance the bioavailability of calcium and other nutrients and improve lactose intolerance [22].

However, until now, the available literature has very little information regarding antioxidant activity on *B. cereus* and *B. pumilus* species. Therefore the present study was to examine the antioxidant potential of the above two bacteria by *in vitro* assays.

MATERIALS AND METHODS:

Collection of soil samples

Soil samples were collected from a hill station nearby Agumbe forest region, Shimoga district, Karnataka.

Isolation of antibiotic producing bacteria

The organisms present in the soil were screened for their antibiotic production potential by crowded plate technique [23]. (Nutrient agar was used in place of Tripticase soy agar). Twelve bacteria that showed promising activity were selected, isolated in pure form and preserved at 4°C. Among the twelve, the two bacteria KBAIA-0210 and JKNAB-0609 which showed very good antibacterial activity against all the target organisms were selected for identification and further screening for other pharmacological activities.

Identification of Bacteria

Morphological and biochemical tests were carried for the two bacteria KBAIA-0210 and JKNAB-0609. The lipid profile was also performed. The two bacteria were identified as *Bacillus cereus* and *Bacillus pumilus* respectively.

Solvent extraction and preparation of samples

The two bacteria *B. cereus* and *B. pumilus* were grown separately in large quantity in nutrient broth medium and incubated for three days at 35°C. The broth was centrifuged to separate the cells at 10,000 rpm for 20 minutes. The clear supernatant containing the metabolites was collected. The metabolites of both organisms were subjected to successive solvent extraction with petroleum ether, ethyl acetate and methanol (1:1) in a separating funnel. All the three solvent extracts were dried in separate plates. The *B. cereus* (BC) petroleum ether extract was labeled as sample BC-1, ethyl acetate extract as BC-2 and methanol extract as BC-3. Similarly the *B. pumilus* (BP) extracts were labeled as BP-1, BP-2 and BP-3 respectively.

IN VITRO ANTIOXIDANT STUDIES

Studies on Free Radical Scavenging Effects

In the present study, all the samples (BC-1, BC-2, BC-3, BP-1, BP-2 and BP-3) and the standard (Ascorbic acid) were tested for their *in vitro* antioxidant activity using two standard methods viz., 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical, and 2,2"-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS).The final concentration of the samples and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.812µg/ml. The absorbance was measured against the corresponding control solution. The DPPH prepared in methanol served as control. The percentage inhibition was calculated by using the following formula.

$$\text{Radical scavenging activity (\%)} = \frac{OD\ control - OD\ sample}{\text{OD control}} \text{x100}$$

DPPH scavenging assay

Preparation of sample solutions: 1mg of sample was dissolved in 1 ml of methanol separately to obtain a solution of 1mg/ml concentration. Solutions were serially diluted separately to obtain the lower concentrations.

Control: 1ml DPPH solution mixed with 1ml of methanol served as control.

Preparation of standard solution: 1mg of ascorbic acid was dissolved in 1 ml of methanol separately to obtain a solution of 1mg/ml concentration. Solutions were serially diluted separately to obtain the lower concentrations.

Procedure: The assay was carried out in small size test tubes. To 1ml DPPH solution, 1 ml of each of the test sample solutions was added separately. The tubes were incubated at 37°C for 30 min and the absorbance of each solution (Test standard and control) was measured at 540 nm using UV-spectrophotometer[24].

ABTS radical anion scavenging assay

Preparation of ABTS: ABTS (54.8 mg) dissolved in 50 ml of distilled water and potassium persulphate (17 mM, 0.3 ml) was added. The reaction mixture was left to stand at room temperature overnight in dark before use.

Preparation of sample solutions: 1mg of different sample solutions were dissolved in 1 ml of freshly distilled DMSO separately. These solutions were serially diluted with DMSO to obtain the lower dilutions.

Preparation of standard solution: 1mg of ascorbic acid was dissolved in 1 ml of freshly distilled DMSO separately to obtain a solution of 1mg/ml concentration. Solutions were serially diluted separately to obtain the lower concentrations.

Control: 1ml ABTS solution mixed with 1ml of DMSO served as control.

Procedure: To 0.2 ml of various concentrations of the samples standard and control, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution was added to make the final volume of 1.36 ml. Absorbance was measured spectrophotometrically after 20 min at 734 nm against the control[25].

RESULTS

DPPH radical scavenging assay

Bacillus cereus samples

The highest percentage of inhibition was observed in the sample BC-2 with 38.9% at 1000 µg/ml concentration. The samples BC-1 and BC-3 showed 19.56 and 14.19% inhibition respectively. However, at 62.5 µg/ml and concentrations lower from that BC-1 sample showed better activity than BC-2 sample. The comparative percentage of inhibition of BC samples is shown in fig 1.

Bacillus pumilus samples

The highest percentage of inhibition was observed in the sample BP-1 with 44.8% at 1000µg/ml concentration. The samples BP-2 and BP-3 showed 36.26 and 27.45 percentage inhibition respectively. The comparative percentage of inhibition of BP samples is shown in fig 2.

The IC_{50} value for all the samples (BC and BP) were above 1000µg/ml. For standard (Ascorbic acid) IC_{50} value was at 2.69 $\pm$0.05 µg/ml.

ABTS radical scavenging assay

Bacillus cereus samples

The highest percentage of inhibition for *Bacillus cereus* was recorded in the sample BC-1 with 88.5% at 1000µg/ml concentration. The samples BC-3 and BC-2 showed 87.21 and 78.31% inhibition respectively. The comparative percentages of inhibitions are shown in fig 3.

The IC_{50} value obtained for BC-2 was least at 55.12±2.51µg /ml followed by the samples BC-3 and BC-1 with 59.3±2.21 and 118.23±2.02 µg/ml respectively. For standard IC_{50} was at 11.25±0.49 µg/ml.

***Bacillus pumilus* samples**

The highest percentage of inhibition for *Bacillus pumilus* was recorded in the sample BP-1 with 97% at 1mg/ml concentration. The samples BP-2 and BP-3 showed 95.62 and 82.03% inhibition respectively. The comparative percentages of inhibition are shown in fig 4.

The IC_{50} value obtained for BP-1 was least at 16.2±1.17µg/ml. The samples BP-2 and BP-3 showed 48.8±3.32 and 80.5±2.37 µg/ml respectively.

The percentage inhibition values obtained against all the samples by both methods are shown in table 1. The IC_{50} values obtained for all the samples by both methods are shown in table 2.

DISCUSSION

In the present work the two bacteria *B. cereus* and *B. pumilus* isolated from soil samples were tested for their antioxidant activities by *in vitro* methods. They were originally isolated for their antibiotic production potential. The results obtained showed both the bacteria exhibiting very good antioxidant activity by ABTS method with 97% inhibition for *B. pumilus* and 88.5% inhibition for *B. cereus* metabolites. The antioxidant activity exhibited by DPPH method was low with a maximum of 44.8% inhibition for *B. pumilus* and 39.8% inhibition for *B. cereus* metabolite respectively. The IC_{50} values obtained at 16.2±1.17µg/ml for BP-1 and 55.12±2.51µg /ml for BC-2 by ABTS method in comparison with 11.25±0.49 µg/ml obtained for standard shows that they are potential antioxidants.

Though there are several reports on bacteria producing active antioxidants, most of them are reported from *lactobacilli* group of bacteria. Several commercial yogurt starter cultures like *Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus acidophilus* and *Lactobacillus rhamnosus* have shown different levels of antioxidant activity by hydroxyl radical scavenging, ferrous iron chelating, linoleic acid peroxidation inhibition and superoxide dismutase (SOD) activity[26,27,28]. Some of the other *lactobacillus* species reported with similar properties are *Lactobacillus brevis, Lactobacillus fermentum* and *Lactobacillus jonsonii*[29,30,31]. Lactic acid bacteria are also reported to synthesize antioxidant peptides during fermentation which prevents oxidative stresses associated with numerous degenerative diseases like aging, cancer and atherosclerosis [9, 32].

Other than lactic acid bacteria reports are available on *Bacillus* species, especially on *Bacillus subtilis.* Many strains of *Bacillus subtilis* exerted antioxidant activity on fermented foods acting

as natural preservatives [33, 34, 35]. A strain of *Bacillus subtilis* with a biosurfectant production potential has also exhibited antioxidant activity [36]. The *Bacillus subtilis* strain with antibiotic production potential exhibiting antioxidant activity[34]. The antioxidant activity in a different *Bacillus* species, *Bacillus simplex* were reported [37].

Regarding antioxidant activity of *B. cereus* and *B. pumilus* very little information is available. Though antioxidant activity was reported on *B. cereus*, it was on the determination of prolongation of the period required for initiation of rancidity as measured by changes in peroxide value [38]. Hence, as per the available literature and to the best of our knowledge, this may be the first report on antioxidant activity on *B. cereus* and *B. pumilus* strains by DPPH and ABTS radical scavenging assay.

CONCLUSION

The two isolates *B. cereus* and *B. pumilus* bacteria isolated from soil have proved to be potential antioxidants. Experiments by other methods are necessary to obtain more evidence on their antioxidant potential. Further experiments are also required to understand the exact mechanisms responsible for their antioxidant activity. The study has to be carried further to purify the active compounds in order to expatiate on the exact chemical structure.

The available report on bacterial metabolites with antioxidant activity has also exhibited other health benefits such as immunomodulants, anticarcinogenic effects, reduction of serum cholesterol, antiaging and hypoallergenic effects. Hence, the metabolites of the two bacteria can be analyzed for the potential compounds responsible for any of these health benefits. Since, the usages of synthetic antioxidants such as BHA, BHT and TBHQ are found to have toxic and carcinogenic effects, the development and utilization of harmless antioxidants of natural origin are desirable and this work will provide bases for future study in this area.

Figure 1. The % inhibition of oxidation of *Bacillus cereus* metabolites by DPPH method

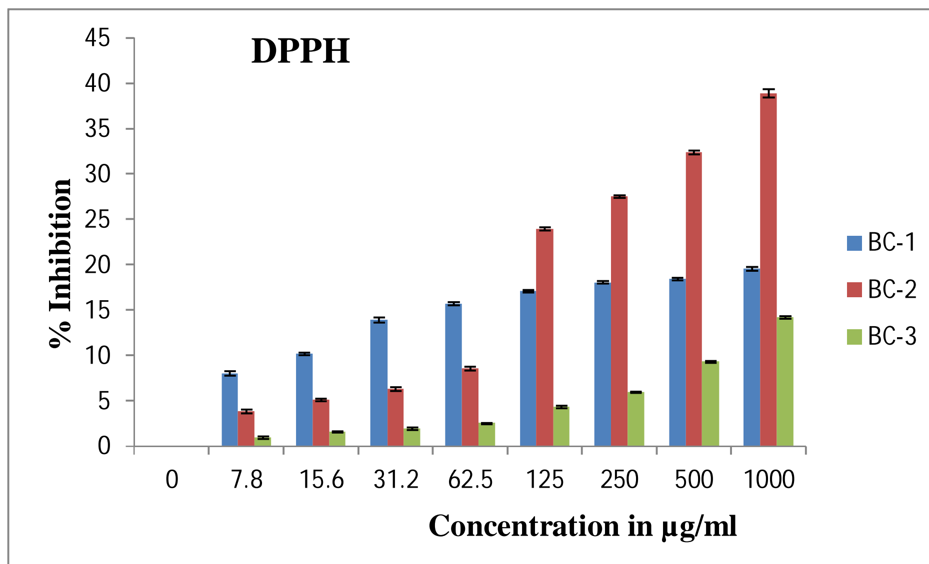


Figure 2. The % inhibition of oxidation of *Bacillus pumilus* metabolites by DPPH method

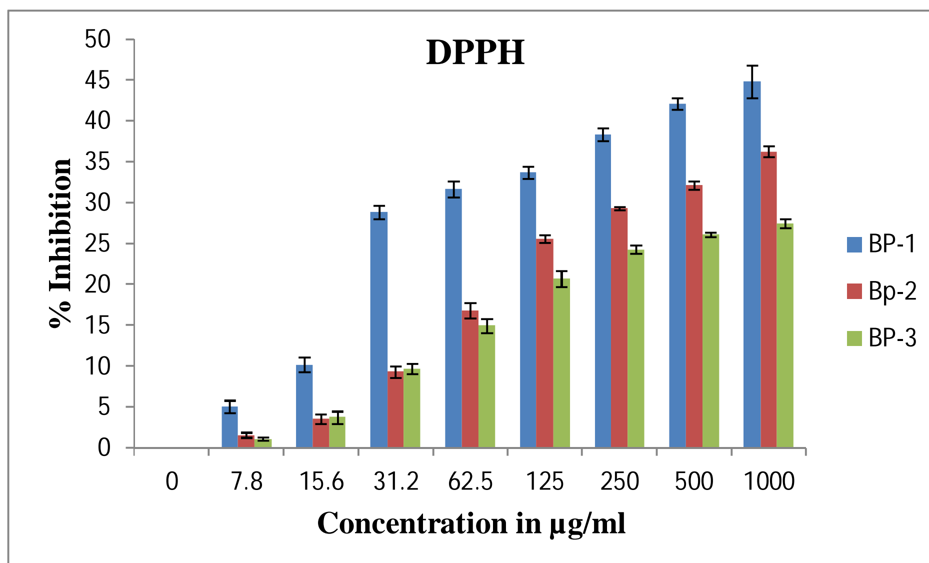


Figure 3. The % inhibition of oxidation of *Bacillus cereus* metabolites by ABTS method

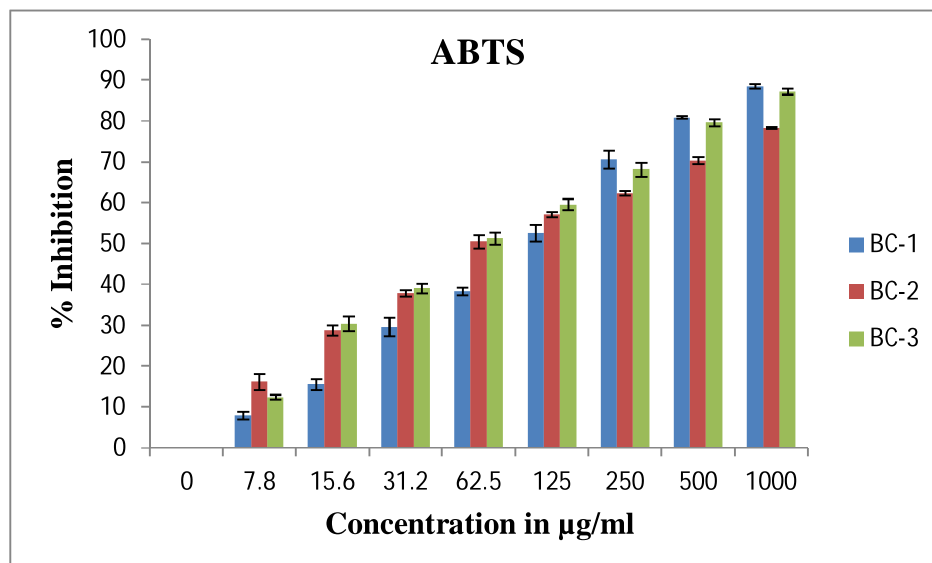
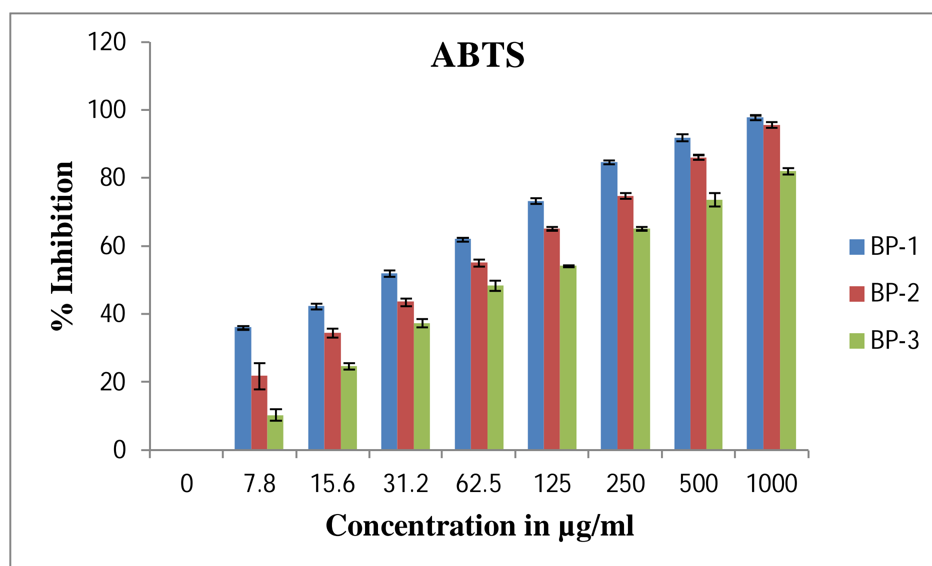


Figure 4. The % inhibition of oxidation of *Bacillus pumilus* metabolites by ABTS method



BC – *Bacillus cereus*; BC-1 Petroleum ether extract; BC-2 Ethylacetate extract; BC-3 Methanol extract

BP – *Bacillus pumilus*; BP-1 Petroleum ether extract; BP-2 Ethylacetate extract; BP-3 Methanol extract

Table 1: The % inhibition values of BC and BP samples by ABTS and DPPH methods

Description	% Inhibition	
	$ABTS^{\bullet-}$ Scavenging	DPPH Scavenging
Bacillus Cereus		
BC1	88.5	19.56
BC2	78.31	38.9
BC3	87.21	14.19
Bacillus Pumilus		
BP1	97	44.8
BP2	95.62	36.26
BP3	82.03	27.45

Table 2: Comparison of the IC_{50} values of various extracts of BC and BP metabolites against various free radicals.

Description	IC_{50} values ± SD. (µg/ml)* by methods	
	ABTS$^{\bullet-}$ Scavenging	DPPH Scavenging
Bacillus Cereus		
BC1	118.23 ± 2.02	>1000
BC2	55.12 ± 2.51	>1000
BC3	59.3 ± 2.21	>1000
Bacillus Pumilus		
BP1	16.2 ± 1.17	>1000
BP2	48.8 ± 3.32	>1000
BP3	80.5 ± 2.37	>1000
STANDARD		
Ascorbic acid	**11.25 ± 0.49**	**2.69 ± 0.05**

******Average of three determinations, values is mean* ± SD

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