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ASSESSMENT OF ANTIOXIDANTS AND ANTIMICROBIAL EFFICACY OF BIOACTIVE METABOLITES ISOLATED FROM *PSEUDOMONAS MONTEILII*

Thampy Aditya Sreekumaran and S. Umamaheswari*

Microbial Biotechnology Laboratory, Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli-627012, Tamil Nadu, India.

***Corresponding Author: S. Umamaheswari**
Microbial Biotechnology Laboratory, Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli-627012, Tamil Nadu, India.

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ABSTRACT
The aim of the work deals with assessment of the antioxidants and antimicrobial activity of crude bacterial culture *Pseudomonas monteilii* collected from the Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu. Hydroxyl radical scavenging assay is based on the qualification of the degradation product of 2 deoxy ribose by condensation with TBA. The radical scavenging activity of different extracts was determined by using DPPH assay. The decrease in the absorption of the DPPH solution after the addition of the sample was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference. 1, 1-diphenyl-2-picryl hydrazyl is a stable free radical with pink color which turns yellow when scavenged. Antioxidants react with DPPH and reduce it to DPPH-H and as a consequence the absorbance decreases indicating the scavenging potential of the antioxidant compounds in terms of hydrogen donating ability. The antibacterial analysis of the crude extract of *Pseudomonas monteilii* was evaluated and was found to exhibit activity against both Gram-positive (*Bacillus cereus*) and Gram-negative (*Escherichia coli*) bacteria while the antifungal efficiency of the crude sample against the isolated fungus from the homemade pickle was investigated with their minimum inhibitory concentration. Hence the bioactive metabolites isolated from *Pseudomonas monteilii* proved to be potentially effective and can be used as biopreservatives such as bacteriocin that can preserve food stuffs avoiding health hazards of chemically antimicrobial agent applications.

KEYWORDS: Bioactive Metabolites, *Pseudomonas monteilii*, Hydroxyl Radical, DPPH, Biopreservatives, Bacteriocin.

INTRODUCTION
Apart from the commonly known antibiotics, there are other enormous ranges of bioactive secondary metabolites with many other biological activities. This makes them economically important for related industries. These are mainly synthesized with the aim of either providing protection to the microbes from any other biological stimulus or harm including plants, insects, humans, even other microorganisms or regulating many biochemical pathways of higher organisms. Some of the important substances, apart from antibiotics are other medicinals, toxins, biopesticides and animal and plant growth factors.[1] These important classes of highly valuable compounds play a wide range of roles as drugs, agrochemicals, biofuels and food additives. However, these compounds are usually produced in very low amounts under typical laboratory conditions in the species from which they originate. The secondary metabolites isolated from microbes exhibits either antimicrobial which includes-antibacterial, antifungal, antiprotozoal action, antitumor or antiviral activities earlier known as antibiotics. The genus *Pseudomonas* is the most heterogeneous and environmentally significant known bacterial group and includes mobile Gram-negative aerobic rods extended in all nature and characterized by its high metabolic versatility given by a complex enzymatic system. *Pseudomonas* species tend to be predominant among the bacteria associated with plants rhizosphere. Several species of this genus exhibiting biocontrol and bioremediation activities in some species of leguminous plants have been studied.[2,3,4,5]

Bacterial volatile organic compounds (VOCs) can have direct antagonistic effects against other bacteria. Many species of *Pseudomonas* and *Bacillus* that are used as biocontrol agents against plant pathogens have been reported to produce VOCs with antibacterial activity.[6,7,8,9,10] In addition to exerting antagonistic effects towards other bacteria, VOCs can also modify the behavior of other bacteria and modulate their resistance to antibiotics. Bacterial volatiles such as ammonia,

trimethylamine, hydrogen sulfide, nitric oxide, and 2-amino-acetophenone can alter biofilm formation or dispersal or affect motility of bacteria[11] and.[6] All microbial species produce a unique reproducible profile of VOCs under specific conditions.[12] These antagonists include bacteria such as *Pseudomonas* sp., *Streptomyces* sp. and *Bacillus* sp.[13] and.[14] Strains of *Pseudomonas* sp. have been reported to produce several VOCs such asbenzothiazole, cyclohexanol, n-decanal, dimethyl trisulfide, 2-etyhl-1-hexanol and nonanal.

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals or their actions (15). Free radicals have been implicated in the etiology of several major human ailments including cancer, cardiovascular diseases, neural disorders, diabetes and arthritis[16,17,18] In recent years, there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance. Therefore, a greater attention has been paid to antimicrobial activity screening and evaluating methods. Microorganisms especially fungi not only cause the food spoilage but also have the ability to produce secondary metabolites like mycotoxins that cause serious health issues in humans. Three main stages of microbial contamination routes include the field (water, soil, and air), raw materials (crops, meats, and milk) and food processing and manufacturing levels. Various methods and technologies can be used to control the contamination at each stage (19). Natural bio preservatives from animal and plant origin are considered as alternatives to chemical preservatives because of the good hygienic quality, safety and extension of shelf life of food products.

MATERIALS AND METHODS

Collection of *Pseudomonas monteilii*

Bacterial culture, *Pseudomonas monteilii* was collected from glycerol stock preserved in Microbial Biotechnology Laboratory, Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu. Subculturing of *Pseudomanas monteilii* culture was done using specific media named Pseudomonas Isolation Agar (PIA) and the culture was also preserved in glycerol stocks at -20^{0}C and used for further analysis.

Partial purification of *Pseudomonas monteilii*

About 1000ml of broth was prepared by filtering the Pseudomonas Isolation Agar (PIA) to which 20ml of purified glycerol was added and sterilized at 121^{0}C for 15 minutes. After sterilization the media was allowed to cool completely and later 10ml of the freshly prepared *Pseudomonas monteilii* culture was added to it and was kept in an orbital rotary shaker at 37^{0}C for 48 hours for further multiplication so as to produce the *Pseudomonas* culture in large mass. After 48 hours, the culture was taken out of shaker and kept inside the refrigerator. Ammonium sulphate with a saturation of 60% was used for precipitating the *Pseudomonas* culture. For every 100ml of the culture, a concentration of 36.6gm of Ammonium sulphate salt was added to the *Pseudomonas* culture broth and agitated using magnetic stirrer until the culture was precipitated and the appearance of foam at the top of the culture. The precipitation was carried out in a cool temperature (20^{0}C).

Now the precipitated crude *Pseudomonas* culture was centrifuged at 15,000 rpm for 15 minutes at 4^{0}C using high speed cooling centrifuge. After centrifugation the supernatant was discarded and the pellet which is considered to be the protein was weighed and then stored into a sterile glass bottle. Each time the pellet was separated after centrifugation, 1 ml of PBS (Phosphate Buffer Saline) buffer was added to it and was stored into 20^{0}C refrigerator and the crude protein extract was isolated and purified. The centrifuged sample was then dialyzed using dialysis membrane to remove the excess salt from the crude protein extract.

Antioxidants Activity

The antioxidants activity was carried out using 2 radial assays

1). Hydroxyl Radical Scavenging Activity

Different concentration of extracts 12.5-200µL from the stock solution was mixed with 500µl reaction mixture (2 deoxy 2 ribose (2.8mM), $FeCl_3$ (100µm), EDTA (100µm), H_2O_2 (1.0mM), ascorbic acid (100µm) in KH_2PO_4 – KOH buffer (20mM, pH 7.4) and made up to a final volume of 1 ml. A control without the test compound, but an equivalent amount of distilled water was taken. After 1 hour of incubation at 37°C, 1ml of 2.8% TCA was added to which 1ml of 1% aqueous TBA was added, the mixture was incubated at 90°C for 15 minutes to develop the colour. After cooling the absorbance was measured at 532nm against an appropriate blank solution (21).

Calculation

% inhibition = (control-test)/control x 100.

Table 1: Gallic Acid Standard.

Concentration (µg/ml)	Absorbance	Percentage of Inhibition (%)
Control	0.562	0
125	0.521	7.29
250	0.473	15.83
500	0.448	20.28
1000	0.247	56.04
2000	0.208	62.98

2). DPPH Radical Scavenging Assay

The radical scavenging activity of different extracts was determined by using DPPH assay (22). The decrease in the absorption of the DPPH solution after the addition of the sample was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference. Different volumes of extracts 1.25µL-20µL from stock solution were made up to a final volume of 20µl with DMSO and

1.48ml DPPH (0.1mM) solution was added. A control without the test compound, but an equivalent amount of distilled water was taken. The reaction mixture was incubated in a dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

Calculation

% inhibition = (control-test)/control x 100.

Table 2: Ascorbic Acid Standard.

Concentration (µg/ml)	Absorbance	Percentage of Inhibition (%)
Control	1.7983	0
12.5	1.4044	21.90
25	1.0782	40.04
50	0.7121	60.40
100	0.2921	83.75
200	0.0692	96.15

Preparation of Homemade Pickle

The homemade pickle was prepared without adding any preservatives. It was stored in a small plastic container and used for further purposes.

Isolation of Fungus from Homemade Pickle

The fungus grown in the homemade pickle was isolated using sterile cotton buds and inoculated into PDA (Potato Dextrose Agar) broth and incubated for 36-48 hours for multiple growth of the unknown fungus. After 48 hours, 1ml of the broth containing fungus was poured into 99 ml of distilled water. The fungus sample was serially diluted in the sterile petridishes at concentrations ranging from 10^{-2} to 10^{-7} and incubated for 48 hours for the formation of the unknown fungus. After incubation, 5 pure single colonies from different concentrations of serially diluted plates were selected and inoculated into 5 respective test tubes containing PDA broth and observed for 48 hours after which the pure cultures were grown and the fungus was identified using morphological tests. The fungus grown in each petriplate was identified using Lactophenol Cotton Blue stain which was thickly smeared onto the clean glass slide and was covered with a coverslip and viewed under microscope.

Confirmation of the isolated 5 fungal samples was analysed for Urease using urea agar, incubated for 24 hours and the results were observed.

Isolation of Bacteria from Cooked Meat Waste

About 10 grams of cooked meat waste was crushed with sterilized water using mortar and pestle. Serial dilution of the preparation was carried out after which *Bacillus cereus* and *Escherichia coli* were isolated based on the Gram-staining and biochemical characterization. The isolated food-borne pathogens were checked for its antibacterial activity against crude liquid sample of *Pseudomonas monteilii*.

Antibacterial Activity of *Pseudomonas monteilii*

The antibacterial activity of crude extract of *Pseudomonas monteilii* was evaluated by using agar well diffusion assay. Muller-Hinton agar medium was prepared and poured into sterile petriplates and were allowed to solidify. After solidification, Gram-positive (*Bacillus cereus*) as well as Gram-negative bacteria (*Escherichia coli*) were swabbed onto the respective marked petridishes using sterile cotton swabs and allowed to incubate for few minutes. Using well cutter, wells were cut appropriately as for the required concentrations of crude sample to be loaded. The crude sample was added into the appropriate cut wells and incubated for 24 hours for visualization of clear inhibitory zones around the wells.

RESULTS AND DISCUSSION

Antioxidants Activity

Table 3: Hydroxyl Radical Scavenging Activity Values of *Pseudomonas monteilii.*

Concentration (µL)	Absorbance	Percentage of Inhibition (%)
Control	0.1881	0
12.5	0.1590	15.47
25	0.1149	38.92
50	0.1011	46.25
100	0.0871	53.69
200	0.0800	57.47

IC 50 Value – 122.474µL (Calculated using ED50 PLUS V1.0 Software)

Table 4: DPPH Assay Values of *Pseudomonas monteilii.*

Concentrations (µL)	Absorbance	Percentage of Inhibition (%)
Control	0.3554	0
1.25	0.3011	15.28
2.5	0.2896	18.51
5	0.2009	43.47
10	0.1987	44.09
20	0.1522	57.18

IC 50 Value- 14.6219µL (Calculated using ED 50 PLUS V1.0 Software)

Many synthetic antioxidants including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) are widely used in the food industry for preservation and to retard lipid oxidation (23) and (24). However, the dosages of the synthetic antioxidants are under strict regulation due to their potential health hazards and toxic effects (25) and (26). Therefore, there has been a large amount of interest in researching safe antioxidants from natural sources as an alternative to synthetic antioxidants (27).

Formation of Fungus in Homemade Pickle

After 4 days of incubation in laboratory conditions, formation of a white slimmish fungus was observed at the top layer of the homemade pickle.

Fig. 1: Appearance of white slimmish layer at the top of the pickle.

Isolation of Fungus from Homemade Pickle

The fungal isolates from homemade pickle produced *Aspergillus niger* and Yeast. *Aspergillus niger* was produced in the form of greenish-blackish moss like colonies surrounded by a white layer, while clustered single white colonies showed the presence of yeast.

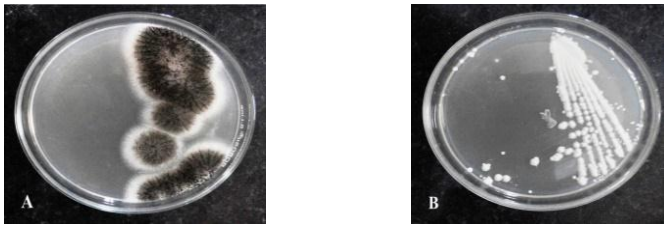

Fig. 2(A): Appearance of moss green-black colonies.
Fig. 2(B): Appearance of clustered white single colonies.

Antifungal Activity of crude *Pseudomonas monteilii* against Isolated Fungus

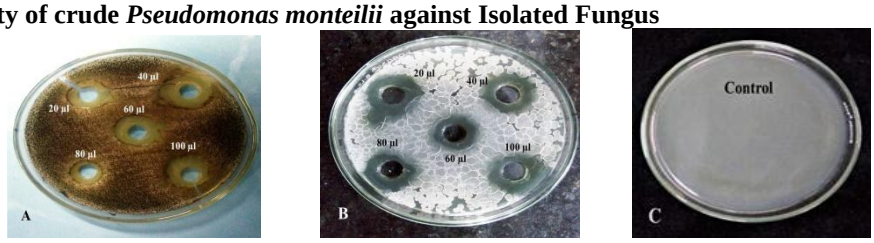

Fig. 3(A): Inhibitory zones against Aspergillus niger
Fig. 3(B): Inhibitory zone formation against Yeast
Fig. 3(C): Control

Antifungal Activity of crude *Pseudomonas monteilii* against Isolated Fungus.

Table 5: MIC values of *Pseudomonas monteillii* against Fungal Isolates.

Fungal Isolates	Minimum Inhibitory Concentration (MIC) (mm)				
	20µl	40µl	60µl	80µl	100µl
Aspergillus niger	09±0.30	12±0.30	07.5±0.20	09.5±0.30	08±0.30
Yeast	07±0.30	08±0	10±0.20	12±0.30	13±0

Collection of Cooked Meat Waste

The cooked meat waste was collected from a restaurant nearby Manonmaniam Sundaranar University Campus, Tirunelveli and stored in a sterile plastic container.

Fig.4: Cooked Meat Waste.

Isolation of Bacterial Food Pathogens from Cooked Meat Waste

Food pathogens *Bacillus cereus* and *Escherichia coli* were isolated from the cooked meat waste and grown in specific media namely PYD (Peptone Yeast Dextrose) Agar and Eosin Methylene Blue Agar media respectively.

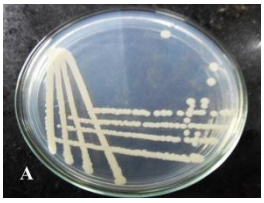

Fig. 5(A): Isolation of *Bacillus cereus.*

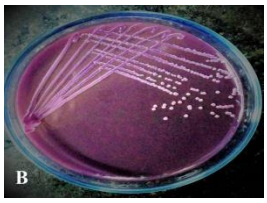

Fig. 5(B): Isolation of *Escherichia coli.*

Antibacterial Activity of crude *Pseudomonas monteilii* against Isolated Food Pathogens

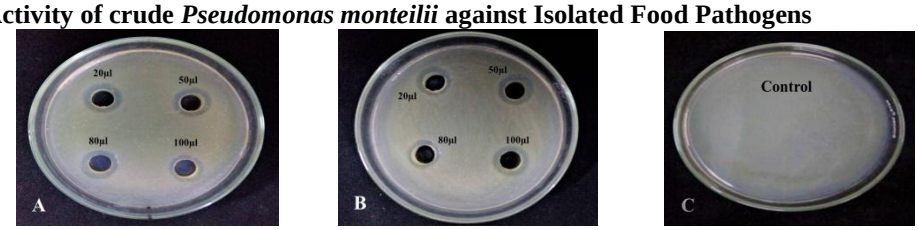

Fig. 6(A): Inhibitory zone formation against *Bacillus cereus.*
Fig. 6(B): Inhibitory zonal activity against *Escherichia coli.*
Fig. 6(C): Control.

Antibacterial activity of crude *Pseudomonas monteilii* against Isolated pathogens

Table 6: MIC zone formation by *Pseudomonas monteilii* against Food Pathogens.

Bacterial Isolates	Minimum Inhibitory Concentration (MIC) (mm)			
	20µl	50µl	80µl	100µl
Bacillus cereus	08±0.20	06±0.30	11±0	14±0.30
Escherichia coli	07±0.30	09±0.30	10±0.20	12±0.30

CONCLUSION

Bioactive secondary metabolites isolated from *Pseudomonas monteilii* had the potential to exhibit antioxidants, antibacterial as well as antifungal activity. This proves that the extracted crude protein sample was potentially effective and could be used a biopreservative such as bacteriocins and hence can be used to increase the shelf life of preserved food stuffs avoiding health hazards through incorporation of chemical antimicrobial agents.

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