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Screening for the Compatible Solute Producing Halobacterium Isolated From Thoothukudi Saltpans and Its Efficacy as a Formulation in Skin Moisturizer

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Abstract:- **Microorganisms living in habitats of high ionic strength produce and accumulate compatible solutes aiming at protecting themselves from hyper osmotic environmental stresses. Compatible solutes of halophiles have current applications as bio-stabilizers of proteins and genetic material. Interestingly, out of 9 strains isolated from the saltpans of Thoothukudi district, India, only one strain had the capacity to synthesize compatible solute. This solute was found to protect the cells from UV radiation. With these properties, a skin formulation has been invented using compatible solute as an element. The organism was later identified by 16S rRNA gene sequencing method. Hence, the invention is directed to a cosmetic or dermatological composition which also found to possess anti-oxidant properties. The compounds responsible for the activity were identified by GC-MS analysis.**

INTRODUCTION

Compatible solutes are a group of low-molecular mass organic compounds that act as osmolytes to help organisms survive under various stress conditions. Metabolic accumulation of compatible solute is often regarded as the second line of defense. This strategy is one of the foremost tolerance mechanism adopted by halophiles (Ventosa et al,1998 ; Waditee et al,2005). Over twenty compatible solutes have been already characterized. These fall into a few structural classes, such as sugars (trehalose, sucrose), polyols (glycerol, sorbitol, manitol, α-glucosylglycerol, mannosyl-glycerol, and mannosyl-glyceramide), N-acetylated diamino acids (e.g., N-acetylglutaminylglutamine amide), betaines (like glycine betaine and derivatives), amino acids (proline, glutamate, glutamine, and alanine) and derivatives. "The predominant compatible solutes in halophilic bacteria are the amino acid derivatives glycine-betaine and ectoine (Roberts, 2006; Oren, 2008). Most chemo heterotrophic bacteria can readily use glycine-betaine as a compatible solute if it is available in the environment. They are of particular interest as stabilizers for proteins and protectors of membranes from desiccation (Lentzen et al.,2006). Thus, these natural compounds were applied in cosmetics such as skin care products (Graf et al.,2008; Beyer et al.,2000).Hence in the present study, the invention relates to formulation containing osmolyte originating from halophilic bacterium.

MATERIALS AND METHODS

Sampling area:
In this study, saline soil sample was collected from solar salt pans of Thoothukudi district, Tamilnadu, India. The collected soil samples were placed in sterile polythene bag and containers and immediately transported to laboratory.
Isolation of halophilic bacteria from the saline soil sample:
The saline soil sample was serially diluted in the range of 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, and 10-7 in a series of test tube. The dilutions were plated in the Mineral Salt (MM63) medium with increased concentration of NaCl. The pH of the medium was adjusted to 7 – 7.4. The samples were inoculated under aseptic condition into the medium using spread plate technique and are incubated at 37°C for 48 hours.

Pure Culture Preparation:
The Mineral Salt (MM63) medium slant was prepared in test tubes and the isolated colony obtained in the petriplates were taken carefully and streaked in the medium in a zigzag manner using the inoculation loop. Then test tubes were screw capped and incubated at 37°C for 48 hours.

Morphological and biochemical tests:
Gram staining was performed by using standard procedures (Dussalt, 1955). Biochemical testing of different isolate was determined by Bergeys Manual (1994).

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Determination of the effect of UV – radiation on halophilic isolates:

The effect of UV – Radiation on nine halophilic isolates was determined by exposing the 24 hour cultures under UV light for every 5,10,15,20 and 25 minutes. The exposed culture of 0.1 ml was spread plated on Luria Bertani agar containing NaCl of 6g/100 ml and incubated at 37°C for 24 hours and observed for growth.

Solvent extraction method:

The bacterial sample was cultured in modified nutrient broth. This broth was kept in orbital shaker for incubation for seven days in order to reach maximum production. After incubation, culture broth was centrifuged at 8000 rpm for 15 minutes and the supernatant was collected. The supernatant was mixed in three different solvent DMSO (Dimethyl Sulfoxide), Ethyl Alcohol and Phenol separately. The extracted crude compound was dried at 40°C and transferred to microfuge tubes. The crude extract thus obtained was screened for antioxidant activity.

Free Radical Scavenging Activity By DPPH Assay

Chemicals and reagents:

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid was purchased from Sigma-Aldrich. phosphate buffer (pH-7.4) and methanol. All the chemicals and solvents used were of analytical grade.

DPPH free radical scavenging activity:

DPPH solution (0.004 %), sample extracts and standard (vitamin C) was prepared in methanol. Sample extracts and standard (vitamin C) solution were prepared in different concentrations 20, 40, 60, 80 and 100 µg/ml. 0.5ml of different concentrations of standard solution or sample extracts was taken in different test tubes and then 0.5 ml of DPPH (0.004 %) solution was added and kept in dark for 30 min. and absorbance was recorded at 517 nm. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It was visually noticeable as a color change from purple to yellow. The percentage inhibition activity was calculated using the formulae below:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control - Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

16s rRNA gene sequencing

The haloalkaliphilic isolate was identified based on 16S rRNA gene sequencing. Briefly, the gDNA was extracted that served as a template for the 16S rRNA gene amplification using universal forward primer (27F) (5'-AGAGTTTGATCMTGGCTCAGTAC-3') and reverse primer (1492R) (5'- GGYTACCTTGTTACGACTT-3') (Biozone (India) Pvt. Ltd.) via standard polymerase chain reaction (PCR) protocol. The amplified gene product (1 Kb) was checked on a 1.5% agarose gel against a 1 Kb DNA ladder. The BLASTn search program was employed to find nucleotide sequence homology.

GC-MS ANALYSIS

Interpretation of mass spectrum of GC-MS was done in Analyzer Solution Research Association (Coimbatore) using the database of National Institute Standard and Technology (NIST4) and WILEY9.The spectrum of the known component was compared with the spectrum of the known components stored in the inbuilt library.

Extraction of intracellular solutes

Cytoplasmic solutes were extracted essentially as described by Canovas et al. (1997), but with the following modifications. Pseudomonas stutzeri was grown in 200 ml minimal medium M63 until late-exponential phase (OD600 1–1.2) .Cells were collected by centrifugation and washed with the same medium with no carbon source. To extract the cytoplasmic compatible solutes, pellets were resuspended in 10 ml double-distilled water and, after 10 min incubation at room temperature,cell debris was removed by centrifugation. Supernatants were extracted twice with chloroform and freeze-dried. Cell extracts were resuspended in 0.5 ml D2O (Canovas et al., 1997).

RESULTS AND DISCUSSION

Processing of soil sample collected from Thoothukudi district, Tamil Nadu has resulted in the isolation of nine halophilic bacterial strains. They were isolated in pure cultures for which Gram's staining and biochemical tests were performed (Table 1). Certain biochemical properties of microorganisms also relate to their potential in biotechnology. The nine halophilic strains were subjected to the exposure of UV radiation. The irradiated culture plated in LB medium was observed for colony growth. Out of nine strains, only one strain (DM27) was able to grow on medium even on exposure of UV light for 25 minutes. This UV resistant halophilic bacteria was chosen for further testing. Intercellular compound production of the isolate was enhanced by solvent extraction method.The solvent layer was collected and condensed to obtain the crude extract, which is brown colored, thick viscous extract.

The DPPH assay has carried out to evaluate the anti oxidant activity for the crude compound. Scavenging of DPPH, a

stable free radical, is one of the major methods commonly used to evaluate the antioxidative activity. The antioxidants react with the stable free radical i.e. 1,1-diphenyl-2-picrylhydrazyl (deep violet color) and convert it to 1,1-diphenyl-2-picrylhydrazine with discoloration. The results of DPPH assay suggest that the phenol extract of DM27 possessed maximum antioxidant activity. The DPPH scavenging potential of the crude compound of DM27 varied from 9 – 66 % at a concentration range of 20 –100 µg /ml(Table 2). The degree of discoloration indicates the free radical scavenging potentials of the sample through the hydrogen donating ability.

16S rRNA gene sequence analysis revealed that the isolated haloalkaliphile DM27 is a member of the genus Pseudomonas. The closest phylogenetic relative of the isolate was Pseudomonas stutzeri, with a sequence similarity of 90% (Table 3).The results of GC-MS analysis shows alkene compounds which is tabulated(Table 4)

The intracellular osmolyte extracted was now used as a component in skin moisturizer preparation. The cosmetic and dermatological preparations are produced in that one or several compounds of the group of osmolytes, the physiologically compatible salts of compounds of the osmolytes in combination with the jajoba oil.

CONCLUSION

The photoprotective formulations containing extracts were photostable after irradiation. Thus the Pseudomonas stutzeri has the potiential for cosmetic material because it has stabilizing compound that can prevent for free radical by anti oxidant content. The osmolytes and the jajoba oil contained in cosmetic and dermatological formulations are to be applied topically. They may, for example, be used in the form of solutions, suspensions, emulsions, pastes, ointments, gels, creams, lotions, oils and sprays.

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Table 1 Morphological & Biochemical tests of isolates

S.NO	Morphological & Biochemical tests	DM07	DM17	DM27	DM37	DM47	DM57	DM67	DM77	DM87
1.	Staining	-	+	-	-	-	+	-	-	+
2.	Motility	+	-	+	-	-	-	-	+	-
3.	Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
4.	Indole	+	+	-	+	-	-	+	+	+
5.	Methyl Red	+	-	+	-	+	-	+	-	-
6.	Voges Proskauer	-	-	-	-	-	+	+	+	-
7.	Citrate utilization	-	+	+	+	-	-	-	-	+
8.	Catalase test	-	+	+	+	-	+	-	+	+
9.	Oxidase test	-	+	+	-	-	+	+	+	-
10.	Urease test	+	-	+	-	-	-	-	-	-
11.	L-Arginine	+	+	-	-	+	-	+	+	+
12.	D-Fructose	-	+	+	-	+	-	-	+	+

Table 2 Anti- Oxidant activity

Treatment	Dose (µg/ml)	Absorbance @517 nm	% activity against DPPH radicals
Controls	----	DPPH control= 0.987	----
Vit C	100	0.168	82.97
Sample B	20	0.892	9.625
Sample B	40	0.740	23.72
Sample B	60	0.512	46.28
Sample B	80	0.400	58.17
Sample B	100	0.322	66.07

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Table 3 16S rRNA gene sequencing analysis

Strain ID	Strain name / Genus	Number of similar nucleotides of 16S rRNA gene	Accession number of 16SrRNA gene	Closely related taxa	Sequence similarity (%) of 16SrRNA gene
DM27	*Pseudomonas*	1150	ABI26690	*Pseudomonas stutzeri*	90%

Table 4 List of components identified from the sample using GC-MS

RT	**Name**	**Molecular Weight**	**Molecular Formula**	**Nature of the compound**	**Activity**
14.13	n-heptacosane	1701.20	$C_{76}H_{52}O_{46}$	Alkanes	Antioxidant activity, antimicrobial activity
25.68	n-octacosane	394.772	$C_{16}H_{32}O_2$	Paraffin alkane hydrocarbon	Lubricant, anticorrosive agent
27.56	n-dotriacontane	450	$C_{18}H_{32}O_2$	Paraffin alkane hydrocarbon	Lubricant, anticorrosive agent
28.71	Methyl Hydroperoxide	48.041	$C_{24}H_{38}O_4$	Atmospheric oxidant	Antioxidant activity
30.57	isopytol	296.53	$C_{15}H_{14}O_6$	Acyclic diterpene alcohol	Strong Antioxidant activity
32.77	n-hexdecane	226.448	$C_{16}H_{34}$	Methylated phenols	Emulsifying ability