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Comparative antimicrobial study using nanocopper, nanoselenium and anti-metabolite produced from curved *Halomonas variabilis*-PRS2 strain isolated from Port Said salt Marsh, Egypt

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Abstract
Extremotolerant isolatePRS2 and antimetabolite producer, identified as Gram negative curved haloalkalophiles mesophilic strain, isolated from Port Said salt marsh, Egypt. Strain PRS2 grows at temperatures ranges from 15–37°C, pHs 6.0–10.5 and salinities range 3.0–28.5 % NaCl (w/v). Non-carbohydrate fermented except little growth with glucose and fructose. Euclidean distance was 90% between isolate PRS2 verity and *Halomonas variabilis*- Phylum Proteobacteria Delta proteo bacteria and genus Halomonas. Effect of different pH values and NaCl concentration on antimetabolite production using *E.coli* organism were carried out. Highly antimetabolite production under 12%NaCl and between pHs ranges from 8 to 11. Inhibitions clearing zone (ICZ) was measured using concentrated antimetabolite alone and conjugated with copper and selenium nanoparticles. The ICZ values were done against various seven gram positive bacterial strains, six gram negative strains and seven fungal strains. Antimicrobial activity showed excellent results against all the clinical pathogens. Generally though the effect of selenium nanoparticles pluse antimetabolite were found to be excellent more pronounced against various pathogenic microbe ranged from25.6±12mm to 33± 1.5mm. Followed by the inhibitory effect of selenium single ranged from23.0 ± 1.2mm to26.4 ± 0.63mm.The effect of copper nanoparticles pluse antimetabolite against microbial pathogen ranged from 17.6±0.58mm to 25.6±0.63mm. While nanocopper particles single and antimetabolite single exhibits moderate results against microbial pathogen. Ranged from 13.4+1.5mm to21.4±0.63mmand17.3± 0.78mm to24.6± 0.82mm respectively.

Keywords: antimicrobial. Nano copper, Nano selenium and haloalkalophilic *Halomonas varibili*s

Introduction

Soda lakes contain high concentrations of sodium carbonates resulting in a stable elevated pH, which provide a unique habitat to a rich diversity of haloalkaliphilic bacteria and archaea. Both cultivation-dependent and -independent methods have aided the identification of key processes and genes in the micro bialy mediated carbon, nitrogen, and sulfur biogeochemical cycles in soda lakes. In order to survive in this extreme environment, haloalkaliphiles have developed various bio energetic and structural adaptations to maintain pH homeostasis and intracellular osmotic pressure. The cultivation of a handful of strains has led to the isolation of a number of extrem enzymes and antibiotics, which allow the cell to perform enzymatic or inhibition reactions at these extreme conditions. These products potentially contribute to biotechnological applications Dimitry *et al.* (2014) [9]. Resistance to antimicrobial drugs has become more widespread over the last decades resulting in a significant threat to public health. Infections caused by antibiotic-resistant bacteria need higher doses of drugs, additional toxic treatments and extended hospital stays, and ultimately result in increased mortality (Gadakh and Van Aerschot, 2015) [14]. To prevent or overcome antimicrobial resistance, non-antibiotic therapies will be necessary to treat bacterial infections and alternative strategies, using Nano particles that show promise for the management of resistant infections are already under investigation (Beyth *et al*, 2015; Gill *et al*, 2015) [4, 29, 17].

The emergence of Nano science and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution. Surfaces of copper nanoparticles affect interact directly with the bacterial outer membrane, causing the membrane to rupture and killing bacteria Mercy *et al,* 2015 [28]. In the current situation, one of the most promising and novel therapeutic agents are the nanoparticles. The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials. They focus on the role of various metallic nanoparticles as potential antimicrobials and the possible mechanism of their inhibitory actions. Ravishankar and Jamuna (2011) [34]. Nurit beyth *et al* (2015) [29] found that, the increasing application of nanoparticles as antimicrobials in industries, medicine, cosmetics, textiles and food packaging which requires the assessment of the toxicity and risks associated with these particles will also be reviewed. Nanoparticles used as antibacterial interaction with phosphorus moieties in DNA, resulting in inactivation of DNA replication. Reacts with sulfur-containing proteins, leading to the inhibition of enzyme functions disruption of cell membrane. Also they used as

Antifungal disruption of cell membrane. Advances in nanotechnology, and particularly in the preparation of metal nanoparticles, can be considered to constitute one of the keys to developing new antibiotics. Nanotechnology plays a key role in the fabrication of different nanoparticles that can exhibit novel antimicrobial properties. Selenium nanoparticles (SeNPs) have become the focus of intensive research owing to their wide range of applications in areas such as antioxidants, antibacterial activity and anticancer applications. However, so far not much has been reported on the evaluation of antifungal activity of SeNPs with an exception of a recent study Shahverdi *et al* (2010) [36]. Copper as an antimicrobial agent which is able to reduce specific harmful bacteria linked to potentially deadly microbial infections (European Copper Institute, 2008). In addition, no research has discovered any bacteria are able to develop immunity to copper as they often do with antibiotics. Mercy *et al.* (2015) [28].

The goal of this study is to review taxonomical research and applications in extreme- haloalkalophilies gram negative curved bacteria, studying physiological, biochemical characters, and 16S rRNAribosome of haloalkaliphilic strain would be also investigated. The present study was carried out to evaluate the antimicrobial activity of seleniumand copper nanoparticles conjugated with antimetabolite and it,s effect on different pathogenic clinical fungi and bacterial strains.

Materials & Methods

Soil sample was collected from Port Said salt marsh, Egyptian salty soda soil. Dilution plate method was used for the isolation of haloalkalophilic bacteria. One ml of the dilutions was plated on appropriate sterilized solid modified Horikoshi agar medium which contains (g/l): Glucose, 5.0; polypeptone, 5.0; K2HPO4, 1.0; MgSO4.7H20, 0.2; Na2CO3, 10.0; agar 25.0, all ingredients were dissolved in 900 ml tap water. Na2CO3 and glucose were sterilized separately each in 50 ml water and added to the medium before pouring (Horikoshi, 1999) [21] and incubated for 7 days at 37°C.

Determination of Phenotypic Characteristics

The morphological characteristics of bacterial isolate PRS2 has been studied on Horikoshi medium (Horikoshi, 1999) [21]. The bacterial cells cultivation and phenotypic characterization were tested. Isolate PRS2 was growth at different pH ranges, temperatures range, different salt concentrations, susceptibility to lysis, nitrogen and carbon sources utilization, and different enzymes detections. Phenotypic methods also include biotyping, and antibiogram according to (Cowan and Steel's, 1977; Cowan, 1992; Horikoshi and Grant 1998 and Horikoshi, 1999) [6, 7, 22, 21]. Furthermore, microscopic observations after different chemical treatments give detailed information on the cell morphology under different extreme conditions, was examined by transmission and scan electron microscope (RCMB) Regional Center for Mycology and Biotechnology. Depending on phenotypic and chemotaxonomic properties, eubacterial isolate PRS2 shared almost the same broad range of taxonomical characteristics according to classification of Bergey's manual of systematic bacteriology of Sneath, (1986) [38]; Holt *et al.*(1994) [20] and Vos *et al.* (2009) [45].

Gram classification examination

Methods used to clarify the Gram classification of these bacterium included modified Gram's stain (Paik, 1980) [31]; KOH test (Wallace and Gates (1986) [46]; aminopeptidase activity test and spore examination (Cowan and Steel's, 1977and Cowan 1992) [6, 7].

Phenotypic and taxonomical analyses

The bacterial cells cultivation and phenotypic characterization were tested. Isolate PRS2 was growth at different pH ranges, temperatures range, different salt concentrations, susceptibility to lysis, nitrogen and carbon sources utilization, and different enzymes detections. Phenotypic methods also include biotyping, and antibiogram according to (Cowan and Steel's, 1977; Cowan, 1992; Horikoshi and Grant 1998 and Horikoshi, 1999) [6, 7, 22, 21]. Depending on phenotypic and chemotaxonomic properties, eubacterial isolate PRS2. shared almost the same broad range of taxonomical characteristics according to classification of Bergey's manual of systematic bacteriology of Sneath, (1986) [38]; Holt *et al.*(1994) [20] and Vos *et al.* (2009) [45].

Cluster Analyses

Studying phylogenetic relationships of the investigated isolate PRS2 with other similar reference strains were evaluated by using statistical cluster analysis with joining (tree clustering) by clustering method which the phenotypic characters were amalgamated by unweighted pair-group average method analysis (UPGAMA). However, complete linkage was the method for studying character profiles using statistics for windows, release 4.5f, state Soft, Inc.1993 software. Euclidean distances (similarity matrix) were used as the distance metric in c lab.).

Production of secondary metabolites antimicrobial agent

For the production of secondary metabolites antimicrobial agent, Horikoshi liquid medium with pH10 was used. The medium supplemented with 12% NaCl, was distributed into 250-ml Erlenmeyer flasks each containing 100 ml the medium was then autoclaved at 121°C for 15 minutes. The medium (100 ml) was inoculated with one ml bacteria suspension, then after four days adjustment metabolic product at pH9 then concentrated metabolite at 40ºC suspension using microcentricon. Concentrated antimetabolite were determined according to the methods described in the A.O.A.C. (1990) [1].

- Primary antibacterial test for antimetabolite was carried out using *Bacillus subtilis, Escherichia coli Micrococcus luteus and Enterobacter aerogenes.*
- In order to determine the effects of pH and NaCl concentration on production of antimicrobial compound the strain was grown on Horikoshi gar medium supplemented with different values of pH

(from 5 to 11), NaCl concentrations (0-28%) separately using *E.coli* organsium.

- The antimicrobial activity of samples was determined using agar well diffusion method (Scott 1989) [35]. The antimetabolite product was tested in vitro by ICZ values for their antibacterial and antifungle activity which were done against seven gram positive bacteria, six gram negative bacteria and seven fungi using Sabour and Dextrose Agar medium. Ampicillin, gentamycine and Amphotricine B were used as standard drugs for Gram positive, Gram negative and antifungal activity respectively. DMSO was used as solvent control.

Test organisms used in antimicrobial activity

The antimetabolite product, Copper and selenium nanoparticles were tested in vitro by ICZ values for their antibacterial and antifungle activity were done against Gram Positive Bacteria, *Streptococcus pneumoniae* (RCMB O1001O),*Staphylococcus aureus* (RCMB 010023), *Staphylococcus epidermidis* (RCMB 010024), *Streptococcus pyogenes* (RCMB 010015) *Corynebacterium diphtheriae* (RCMB O100846),and *Bacilli's anthracis* (RCMB 0100693) and Gram negative bacteria *Pseudomonas aeruginosa(RCMB 010043)*, *Serratia marcescens* (RCMB 010075) *Proteous vulgaris (*RCMB 01O085),*Klebsiella pneumoniae* (RCMB 0010093) using nutrient agar medium.Also ICZ was carried out against *Aspergillus fumigatus (RCMB 02568),Aspergillus clavatus(*RCMB 02572), *Aspergillu sniger* (RCMB 02581) *Aspergillus flavus* (RCMB 02557),and hazard fungi were tested also, *Trichophyton mentagrophytes (*RCMB 09258),*Trichophyton rubrum* (RCMB 09274) *Microsporum canis* (RCMB 08835).

Copper and Selenium nanoparticles preparation

Copper and selenium nanoparticles chemically prepared previously (Nano tech lab)**,** UV-Vis analysis and TEM image of selenium and copper nanoparticles synthesized were determined.

Copper Materials and synthesis

Polyvinyl pyrrolidone (PVP, K-30), sodium hypophosphite monohydrate (NaH2PO2・H2O), copper sulfate pentahydrate (CuSO4・5H2O), ethylene glycol, acetone, and 2-(2-butoxyethoxy) ethanol were all analytical grade and used without further purification. Copper nanoparticles were synthesized by the following procedure: 1.11 kg PVP and 400 g sodium hypophosphite were mixed into 4l ethylene glycol inside a round-bottom flask while vigorously stirring at room temperature under ambient atmosphere. The mixture was heated to 90◦C at a rate of 5◦C/ min−1. Then, one l of a 1 M solution of copper sulfate in ethylene glycol at 90 ◦C was rapidly added into the PVP/sodium hypophosphite solution while stirring vigorously. As reduction occurred, the color of the suspension turned from green to henna within 2–3 min, indicating the formation of copper nanoparticles. The reaction was quenched and the suspension was rapidly cooled by adding chilled deionized (DI) water. The copper nanoparticles were separated and washed with DI water by centrifugation, while using acetone as a non-solvent, in order to remove excess PVP and sideproducts. The resulting precipitates were dried under vacuum at 40 ◦C for 2–3 h. (Youngil *et al.* 2008) [51].

Selenium synthesis

Synthesis of selenium nanoparticles was done by reducing selenious acid solution with ascorbic acid in the presence of polysaccharides, such as chitosan (CTS), konjac glucomannan (KGM), acacia gum (ACG), and carboxymethyl cellulose (CMC) etc. The monodispersed spherical selenium colloid particles obtained were very stable in solution according to Sheng *et al*. (2004) [37].

- Concentrated antimetabolite product Immobilized with selenium and copper nanoparticles according to Sheng *et al*. (2004) [37] and Youngil *et al.* 2008 [51] methods. Then antimicrobial assay experiment was done using separately nanoparticles or conjugated with antimetabolite against pathogenic gram positive, gram negative bacteria and fungi. Treatments carried out as the following (1) antimetabolite product. (2) Nano copper particles (3) Nano copper particles pluse antimetabolite product. (4) Nano selenium particles. (5) Nano selenium particles pluse antimetabolite product.

Results and Discussion

During the last few years, interest in saline desert microbes has increased due to investigations on novel bioactive metabolites, especially antibiotics and enzymes. Many of these metabolites possess antimicrobial activities and have the potential to be developed as therapeutic agents. Desert bacteria and actinomycetes are a prolific but under-explored source for the discovery of novel secondary metabolites. This report high lights the screening and production of an antimicrobial agent from a new halotolerant alkalophilic isolate. The emerging infectious diseases and the development of drug resistance in the pathogenic bacteria and fungi at an alarming rate is a matter of serious concern. Therefore, there is a pressing demand to discover novel strategies and identify new antimicrobial agents from natural and inorganic substances to develop the next generation of drugs or agents to control microbial infections. Prior to the extensive use of chemo therapeutics in modern health care system, the nano sized inorganic antimicrobials such as silver and copper were used Gajjar *et al* (2009) [15]. In the past decade, study of the toxicological properties of nanomaterials and/or nanoparticles has opened up a new research field known as Nano toxicology. Research on nanotoxicity is of extremely high scientific, social and economic value Nanomaterial- induced reactive oxygen species play a key role in cellular and tissue toxicity (Pumera, 2011 and Yan *et al.*, 2013) [32, 50].

Strain PRS2 isolated from soil sample was collected from Porsaid salt marsh soil, Egypt. It was sand-loam sality soil textures, which had high NaCl percentage. The cilimatic conditions and Egyptian soil texture could yield many species of bacteria, have adapted to grow and thrive under the extreme environmental conditions, in addition to other species, which tolerate under these conditions.

Physiological characters and growth requirements

Isolate PRS2 was Gram negative, Smooth, flat, opaque dark brown and circular with entire margins: Colony colour change under sever condition to pale creamy colony colour. Deep creamy colour colony (0.2-0.7ml) grow in sand-loam sality soil textures. Non-spore forming mono polar flagelle, motile and did not possess endospores, also capsules are not formed aerobic. Isolate PRS2 can tolerate NaCl range 3-28.5% and pH range 6-10.5 and temperature range15-37 ºC with optimum range 25-32ºC.Produced oxidase, catalase, also reduced urea. Tributyrin, tweens 40 and 80 were hydrolysed, while nitrate reduction was negative. Gelatin, starch, casein and pectin were not hydrolysed, also hydrogen sulphid and indole were not produced. Isolate PRS2 was non-oxidized carbohydrate except glucose and fructose. The good growth of isolate PRS2 occurred under aerobic condition, with shaking incubation at 200 rpm for 72 to 120 h, or static for 72 to 144 hr. in Sato broth media adjusted at pH10 and containing 15% NaCl at 37ºC. Could not grow in mineral medium without organic constituents (peptone, amino acid, glucose or yeast extract). Good growth of the isolate with darker creamy colonies pigmentation was enhanced on the media containing 0.2 -0.5% yeast extract, 1ml of mineral salt solution and when peptone was replaced by 0.1 to 1% casoamino acid or by different amino acids as nitrogen sources,. Also the isolate could be survived when ammonium sulphate, sodium nitrate and urea used as sole nitrogen source on the media. The phylogenetic relationship among the investigated isolate PRS2 and reference strains *Halomonas variabilis* based on their morphological, physiological and biochemical profiles was illustrated approximately 90% similarty through the phylogenetic relationship. Many reports were published for haloalkalophiles bacteria isolated from Egyptian soda soil (Tindall *et al.* 1984 [43]; Weisser and Truper, 1984 & 1985 and Lowe *et al.* 1993) [43, 48-49, 25]. Wadi El-Natrun has crystalline deposite reflecting total dissolved solids of up to 40% (w/v). *Methanohalo philuszhilinae* isolated from Bosa Lake of the Wadi El-Natrun by Mathrani *et al.* (1988) [27]. Also, Ghanem *et al.* (1990) [16] reported two haloalkalophilic Bacillus species isolated from the side brines of Wadi Natrun in Egypt soda lakes are intriguing ecosystems harboring extremely productive microbial communities in spite of their extreme environmental conditions. This makes them valuable model systems for studying the connection between community structure and abiotic parameters such as pH and salinity. For the first time Sorokin, *et al* (2008) [39], apply high-throughput sequencing to accurately estimate phylogenetic richness and composition in five soda lakes, located in the Ethiopian Rift Valley. The lakes were selected for their contrasting pH, salinities and stratification and several depths or spatial positions in each lake. Interestingly, diversity appeared uncorrelated or positively correlated to pH and salinity, with the most "extreme" lakes showing the highest richness. Together, pH, dissolved oxygen, sodium- and potassium concentration explained approximately 30% of the compositional variation between samples.

Table 1: Phenotypic and characteristics of isolate No. PRS2 species and *Halomonas variabilis.* As reference

No	General characteristics	Reference Halomonas variabilis	Unknown isolates (PRS2)
1-	Morphology		
a)	Morphology of colony	Light brown colour	Smooth, flat, dark creamy opaque and circular with entire margins: Colony colour change under sever condition to pale colony colour
b)	Colony pigment	Light brown colour	Deep creamycolour (0.2-0.7ml)
	i) At early stage		pale creamy colour
	ii) At stationary phase		Deep dark creamy colour
c)	Habitat		Sand-loam sality soil textures
d)	Growth		Chemotrophic complex media
2-	Cell morphology (shape)		
a)	At early stage	Rods or vibrio	Littele curved rod or curved
b)	At stationary phase and end growth	with coccoid head bodyin old culture	Most are vibrio some coccoid head body in old culture
3-	Cell dimension (um)	0.5-0.8 µm x 1-3 µm	0.5-0.7 µm x 1-3.5 µm
4-	Gram classification:		
a)	Gram stain	Gram negative	Gram negative
b)	KOH test	G-ve	G-ve
c)	Amino peptidase activity test	G-ve	G-ve
5-	Spore forming	ND	Non spore forming
6-	Flagellation (position)	Mono polar flagella	Mono polar flagella
7-	Motility	motile	Motile
8-	Pleomorphism	ND	Negative condition
9-	O_2requirment	Aerobic	Aerobic
10-	▪ NaCl range at 35ºC ▪ At 55ºC ▪ Optimum	3-28.5% -ve ND	3-27.5% -ve 10-20%
11-	pH range		
	▪ At 35ºC ▪ At 50ºC	6.5-8.5 -ve	6-10.5 -ve

	▪ pH range in broth ▪ Optimum	6.5-8.5 7.5	7-10.5 7 -9.5
12-	Temperature range	15-37ºC	15-37 ºC
	▪ Optimum		25-32 ºC
13-	Temperature tolerance at 65ºC	ND	Not survive
14 -	Growth period (at pH 10 and 20% NaCl		
	▪ At 4 ºC	-ve	-ve
	▪ At 35 ºC	ND	72.- 120h
15-	Effect of replaced of NaCl with some minerale: K^+ Mg^{+2} Ca^{2+} Biochemical tests	ND	+ve and colony colour pale with 3-5 g Mg^{2+} and 100g K^+ while negative with Ca^{2+}
16-	Oxidase	+	++
17-	Nitrate reductions	-	-
18-	Catalase	+	++
19-	Indole	-	-
20-	H_2S production	-	-
21-	Urease	+	+
22-	Gelatin liquefaction	-	-
23-	Starch hydrolysis	-	-
24-	Casein hydrolysis	ND	-
25-	Hydrolysis of tributarne	+	~~+~~
26-	Hydrolysis of Tween 40	+	+
27-	Hydrolysis of Tween 80	+	+
28-	Methyl red	-	-
29-	Voges-Proskauer	ND	-
30-	(Minimal medium)		
	Growth with NH_4Cl		+w
	Growth without glucose	+	+
	Growth without peptone	-	-
	Growth without Mg ions	-	-
	Growth without yeast extract	ND	-
	Enzymes production		
31-	▪ Neutral amylase ▪ Alkaline amylase	ND	- -
32-	▪ Neutral lipase ▪ Alkaline lipase	ND	- -
34-	▪ Neutral phosphatase ▪ Alkaline phosphatase	ND	- -
35-	▪ Neutral protease ▪ Alkaline protease	ND	+ +
36-	▪ Neutral cellulase ▪ Alkaline cellulase	ND	- -
37-	▪ Growth at pH6- pH 7.5 and 3% NaCl	ND	+(with change pH to 9.5)
	▪ Growth at pH6- pH 7.5 (in broth or agar) on 15% NaCl	ND	+ (with change pH to 9.5)
	▪ Growth in Na_2CO_3 1%	ND	+
	▪ Growth in Na_2CO_3 5%	+	+
39-	▪ Growth with Casoamino acid 1%	ND	++
40-	Growth with tryptone	ND	-
41-	Mineral salt solu. requirement	ND	++
42	Growth with vitamins solution and yeast Extract Folic acid pyridoxine riboflavin Biotin Cyano-cobalamin	+ +w + ~~+~~w ~~+~~ w ND ND ND	+/-or week +++ +w +w w+ +ve +ve +ve
43-	Cell lysis in H_2O ▪ SDS	- ND	- -

44-	Carbohydrate utilization	non oxidized carbohydrate	Approximately non-oxidized carbohydrate except glucose and fructose
	Xylose	-	-
	▪ Arabinose	-	-
	▪ Rhamnose	-	-
	▪ Ribose	-	-
	▪ Glucose	-	+w
	▪ Fructose	-	+w
	▪ Mannose	-	-
	▪ Lactose	-	-
	▪ Sucrose	-	-
	▪ Maltose	-	-
	▪ Raffinose	-	-
	▪ Trehalose	-	-
	▪ Starch	-	-
	▪ Cellulose	-	-
	▪ Salicin	-	-
	Glycerol	-	-
	Mannitol	-	-
	▪ Cholesterol	-	-
45-	Antibiotic sensitivity		
	Rifampicin	ND	+++
	Vibramycin	ND	-
	puromycin	ND	++
	Tobramycin	ND	-
	Sulfamethin	ND	+
	Triple sulfa	ND	+
	Sulphonamides	ND	-
	Nitrofurantion	ND	-
	Bactracin	ND	-
	Chloramphincol	ND	++
	Erythromycin	ND	++
	Novobiocin	ND	+
	Pencillin	ND	+W
	cephalosporin	ND	+W
46-	Antifungl effect		
	Furamazone	ND	-
	Nizarol	ND	-
	Lamyzol	ND	-
47-	Nitrogens sources requirement	ND	UTILIZE amino acid
	Alanine	ND	+w
	Asparagine	ND	++
	Cysteine	ND	+
	Cystine	ND	+++
	Glutamic acid	ND	+
	Glutamine	ND	+w
	Histidine	ND	+
	Isoleucine	ND	++
	Lysine	ND	+
	Methionine	ND	+
	Phenyl – alanine	ND	++
	serine	ND	+
	Threonine	ND	+
	Tryptophan	ND	+w
	Tyrosine	ND	++
	Glycine	ND	+
	Peptone	ND	++
	Urea	ND	+
	$NaNO_3$	ND	+
	$(NH4)_2SO4$	ND	+

ND= not detect
+W = positive weak

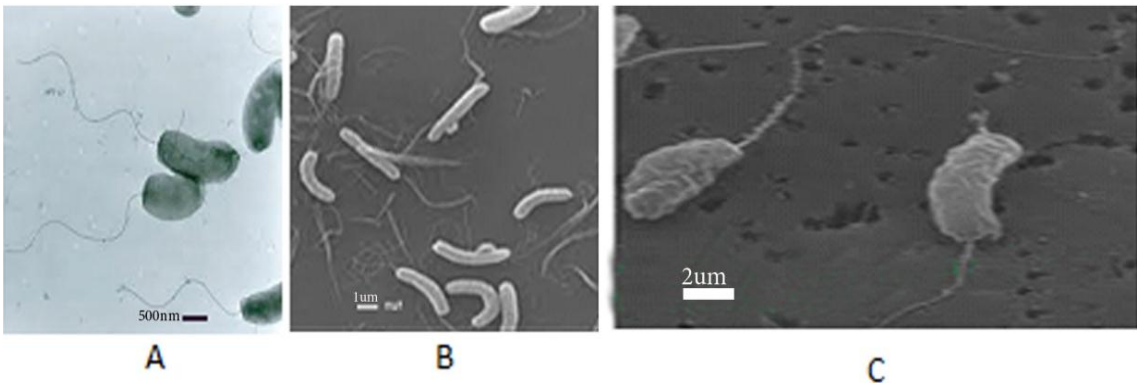

Plate 1: Isolate PRS2 G-ve curved bacterial under normal and extreme saline conditions. (a & b) Transimation electron microscop at15%NaCl and pH0 (1um and500nm) (C) Scaning electron microscopy Coarse curvy mono flagelate cells under very high salinity 28% and NaCl after storage three months (2um)

Electron microscopy examination

On electron microscopy revealed the cell shape of isolate PRS2 under normal and highly extreme saline conditions, it was clear that, rod mono flagelate cells forms under normal condition and coarse curvy under highly extreme salinity conditions plates (1) While plate (2) showed that, cells of isolate PRS2 adapted themselves, when they survive under osmoregulant factors on distilled water or SDS as hypotonic conditions. The cells adaptation was occurred in distilled water or SDS detergent without susceptibility to lysis after 6, 24hrs. It was clear that, turbidity of bacterial

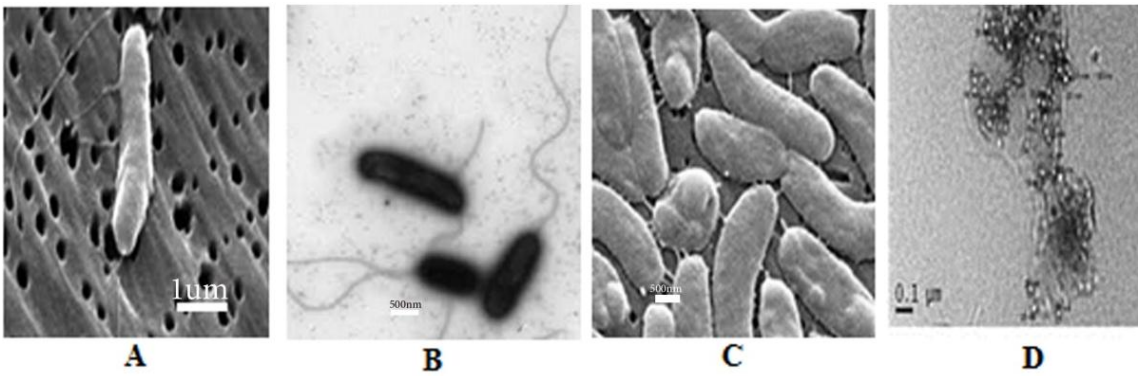

Plate 2: Isolate PRS2 G-ve curved bacterial under normal and highly osmolarity condtions under transimation and scan electron microscopy. (A) Scan electron microscopy at 15%NaCl and at pH0 (1um). (B) Osmolarity after 6h.in distilled water 500nm.(TEM). (C) Osmolalarity after sowllen cells, 24h.in distilled water, 500nm. (SEM). (D) Osmolarity after 24h. Using SDS 100nm (TME).

Solution was highly increased after six hour incubation period by visual observation in distilled water whilst swollen of cells began after 24hrs. While osmolality on PRS2 by SDS. It was clear that, turbidity of bacterial solution was highly by visual observation and under electron microscopy cells was reduced with very thin cells. So that, this is connected with the unusual osmoregulation of this organism which permit the intracellular and extracellular ionic concentration to remain constant. It can be concluded from the variety of the morphological and growth requirements discussed previously that, the behaviors' of the extreme isolate adapted morphologically and physiologically according to the type of extreme environmental factors. The environmental influence as salinity by Na^+ concentration, pH and oxygen tension, and complex compound requirement, play important roles in the selection of this identified haloalkalophilic isolate. These environmental parameters could be controlled and affected on internal pressure and salt concentration in this haloalkalophile isolate, which affected cytoplasm survival strategies, and cell wall composition. The pigmentation system of colony be affected by the variety of physiological properties may be correlated to especially survival system inside the cells for adaptation under sever conditions.

These results are parallel to the results obtained by Atlas & Barth (1987) [3]; Atlas (1988) [2]; and Hassan (1992) [19]. The data are inagreement also with Dusch & Lanyi (1990) [10], Horikoshi (1999) [21] and Oren (2002) [30], they reported that, in haloalkalophilic bacterial pigmentation systems at high pH effect on chloride transport through the cells, and this caused change in pigmentation degree. These data agree with Javor (1984) [23] who replaced Na^+ by K^+ and chloride by sulfate, on the growth of certain haloalkalophiles strains, he found that, Na^+ can replaced by K^+, in chloride form only in the media. Sulphate was inhibitorier, while good growth occurred with traces of magnesium sulfate. The results are also parallel with reports of Tindall *et al*. (1980) [44] and Edgerton & Brimblecombe (1981) [11] they recorded that, halophiles (including haloalkalophiles) bacteria classified according their level of Mg^{2+} requirement for their growth, and the requirements of Na^+ and Mg^{2+}

levels reflected the saline habitat ions constituent and concentration.

Phylogenetic data

Cluster Analyses for studying phylogenetic relationships of isolate No.PRS2 with other Halomonas strains by 16S ribosomes was carried out (Fig1). The constructed dendrogram asserted relationship of strain PRS2 and *Halomonas variabilis*. So, on the basis of phenotypic properties, eubacterial isolate PRS2 shared almost the same broad range of taxonomical characteristics lead to consider PRS2 is a Haloalkalophiles, mesophilic under gram negative groups. Euclidean distance was 90% between isolate PRS2 varity and *Halomonas variabilis*-Phylum Proteobacteria Delta-proteobacteria and genus Halomonas, different varaity of *Halomonas variabilis* according to classification of Bergey's manual of systematic bacteriology of Holt *et.al.* (1994) [20] and Brenner *et.al* (2005) and Vos *et al.* (2009) [45].

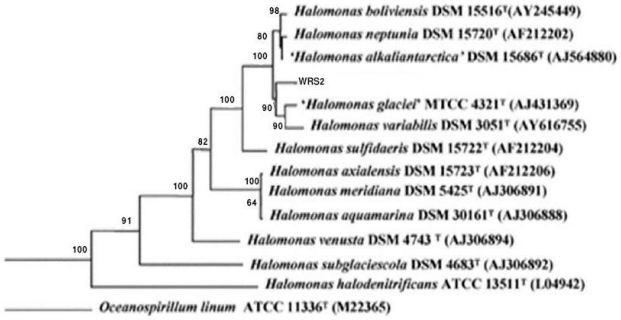

Fig 1: Dendogram of isolate No.PRS2 and reference strains based on the similarity matrix of phylogenetic data.

Antibiotic assay for antimetabolic product

The primary effectiveness of antimetabolic product Plate (3) was tested against four clinical pathogens *Bacillus subtilis*, *Micrococcus luteus*, *Enterobacter aerogenes* and *Escherichia coli*. They showed good antimicrobial activity against the four clinical pathogens ranged from 15 to 25mm under concentrated antimetabolic product and first dilution of antimetabolic product 10^{-1}.Also, effect of different pH values and different NaCl concentration on production of antimetabolic product using *E.coli* organism were carried out (Plate 4).It was clearly that antimetabolic production have broad spectrum activity under 10% NaCl and between pHs ranges from 8 to 11 ranged from1.4 to 2.7mm. Production of antimetabolic product was tested at pH9 and under different NaCl concentrationI. T was observed highly production of antimetabolic product at 12% salt concentration, it was 2.9 mm. A moderately haloalkaliphilic streptomycete strain with high antimicrobial activity was isolated from the saline-alkaline soils of Ararat Plain, Armenia and phenotypically identified as *Streptomyces roseosporus* A3. The isolate exhibited optimal growth at 5% NaCl and pH 9 at 37°C and had high antimicrobial activity against Gram-positive bacteria and yeasts. Optimum salt and pH value for antibiotic production was 2% NaCl and pH 9, respectively. The antimicrobial compound was extensively synthesized at stationary stage of growth and had high stability against proteinase Hakobyan and Panosyan (2012) [18]. Recently, Tambekar and Dhundale (2013) [42] investigated the study deals with isolation, production and partial characterization of antibacterial substance producing bacteria from the alkaline Lonar Lake. Total twenty nine bacilli were isolated by using different enrichment media, out of which BW1 selected for antibacterial study and subjected to phenotypic and biochemical characteristics and identified as *Oceano bacillus iheyensis*on the basis of 16S rDNA sequencing. The strain was found to be potential antibacterial against *E. coli* and moderate against *K. pneumoniae, S. typhi, S. aureus*and poor against *P. aeruginosa, P.vulgaris* and *E aerogenes.* The study reveals that the haloalkaliphilic. *iheyensis* produce broad spectrum of antimicrobial agents which can be exploited for biotechnological potential and improve as promising sources for new antibacterial compound.

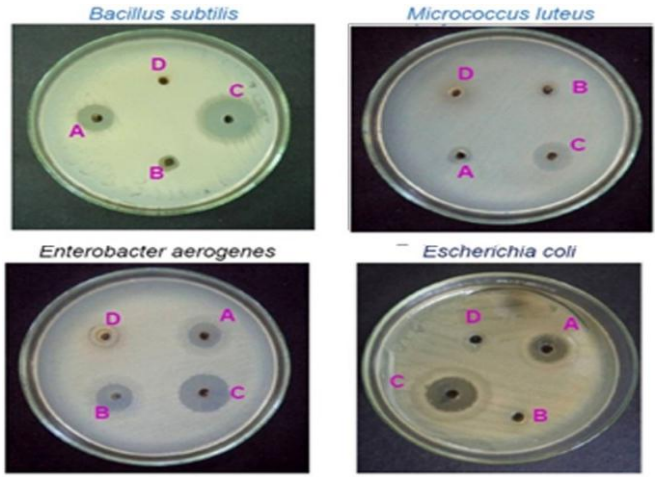

Plate 3: Effect of different concentrations of antimetabolic production media contain 10% NaCl and pH9 (A) first dilution of antimetabolic product10^{-1} (B) second dilution10^{-2} (C) concentrated antimetabolic product (D)Third dilution10^{-3} (1*) Bacillus subtilis* (2) *Micrococcus luteus.* (3)*Enterobacter aerogenes* (4) *Escherichia coli*

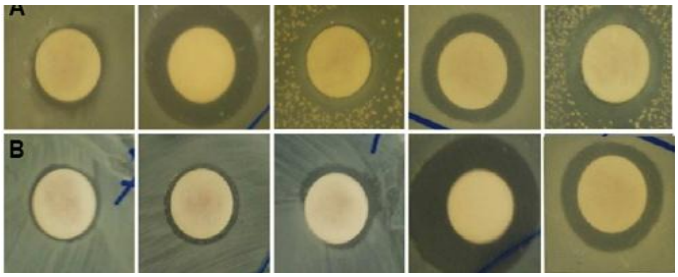

Plate 4: Effect of different pH values and different NaCl concentrations in production of antimetabolic product using *E.coli* organism (A) different pHs values with 10%NaCl: pH7,pH 8, pH9,pH10 and PH11 (B) Different NaCl concentration at pH9: 28%,22%, 18%, 12% and 3%.

Also, Bivin and Stoeckeniusi (1986) [5] studies, the antimicrobial production of marine actinomycete isolated from the Sundarbans region of the Bay of Bengal, India, was maximum with 5% NaCl and pH 7-9, while optimum growth of isolate was at 20% NaCl.

Characterization of copper and selenium nanoparticles

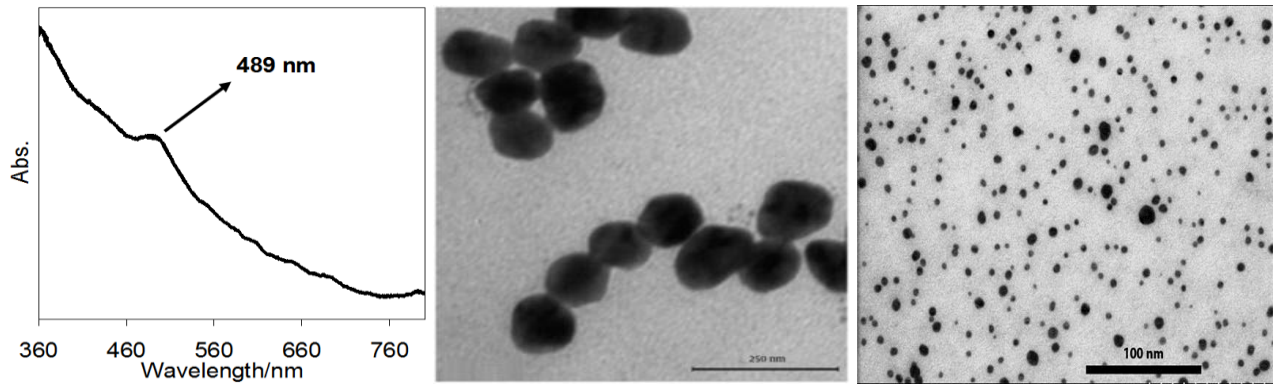

Plate 5: UV-Vis spectrum of copper Nanoparticles show w.v. at 489nm and transmission electron microscopy (TEM) images of nanocopper particles 25nm and 10 nm.

The particle size distribution was 10-25 nm and images of nanocopper particles evaluated were irregular spherical in shape from the TEM micrographs (plate5). The UV analysis of the fractions indicated that the first peak corresponded to the copper.

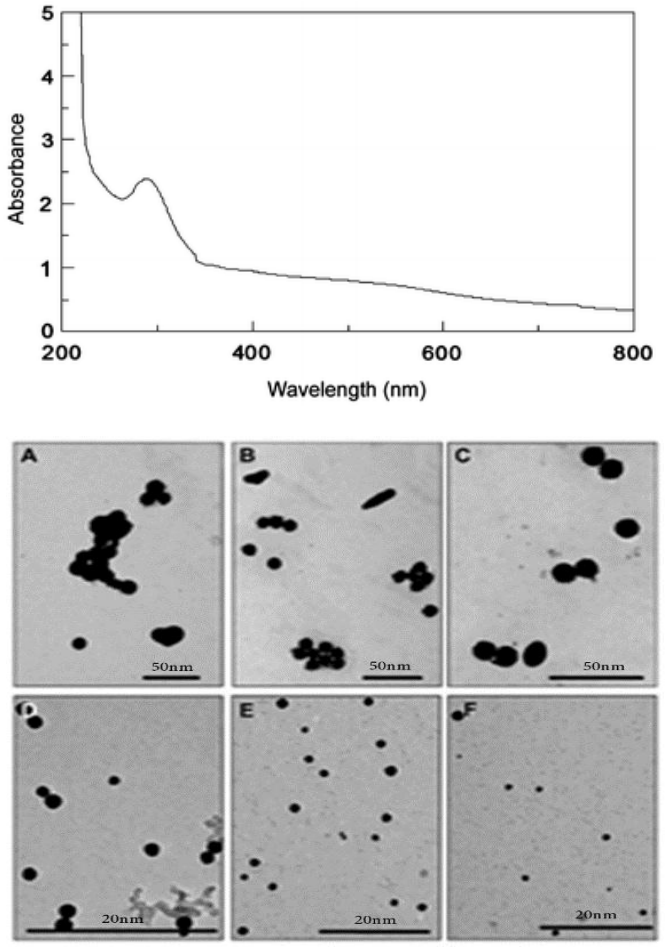

Plate 6: UV-Vis spectrum of selenium Nanoparticles show w.v. at 220nm Transmission electron microscopy (TEM) images of nanoselenium particles at different sizes from a to f),20- 50nm.

Nanoparticles was at w. v. 489nm. The particle size distribution was 20- 50nm and images of selenium Nanoparticles evaluated were regular or irregular spherical in shape from the TEM micrographs (plate 6). The UV analysis of the fractions indicated that the first peak corresponded to the selenium nanoparticles was at w. v. 220 nm.

Antimicrobial activity of antimetabolic produced by *Halomonas variabilis* PRS2 conjugated with copper and selenium nanoparticles

Antimicrobial activity showed excellent results against various seven gram positive bacterial strains, six gram negative strains and seven fungal strains. The treatments done as the following table (2 and 3) zone of inhibition values inthe results demonstrate that, good antimicrobial activity against all the clinical pathogens, generally though the effect of selenium nanoparticles pluse antimetabolic product was found to be excellent more pronounced. against various bacterial and fungal pathogens ranged from25.6±0.12mm to33± 1.5mm.Followed by The inhibitory effect of selenium single ranged from23.0 ± 1.2mm*to*26.4 ± 0.63 mm. Approximately the same results was clear the effect of copper nanoparticles pluse antimetabolic product against microbial pathogens which ranged from 17.6±0.58 mm to 25.6±0.63 *mm.* While Nano copper particles single and antimetabolic product exhibits moderate results against microbial pathogen ranged from13.4+1.5mm to 21.4±0.63mm and 17.3± 0.78mm to 24.6± 0.82 mm respectively. While, *Streptococcus pyogenes* (RCMB 010015) not effected by antimetabolic product, copper nanoparticles and antimetabolic productplusecopper nanoparticles. These results are parallel with Sundrarajan (2012) [41] who found that, all the samples have shown antimicrobial activity against some of the used test organisms with different results and different diameter of the inhibition zones among each other. It was found also that, the most effective sample was the extracellular NPs of isolate *Halovibrio varibilis species* which gave the highest result. It has also found that, varied results were obtained among G +ve and G –ve bacteria which may referred to the size of the produced nanoparticles.

Haloalkaliphilic bacterial species have much interest because of their ability to produce extracellular metabolites. The suitability of secondary microbial products produced from realizable bacterial taxa was

significant to discover novel chemicals for the improvement of new therapeutic agents. The various nanoparticles, metal nanoparticles assume special importance because they are easier and cheaper to synthesis and are most promising in application. A series of inhibetors materials, including antimetabolic product, nano copper and nano selenium compounds, can inhibit microbial growth this may be due to mechanism of the biocidal action of nanoparticles involves the interaction between microbial cells and nanoparticles which was stronger. The small size and the high surface to volume ratio i.e., large surface area of the nanoparticles enhances their interaction with the microbes The reason could be that gets tightly adsorbed on the surface of the microbial cells so as to disrupt the membrane, thereby leading to the leakage of intracellular components, thus killing the bacterial cells. Nanoparticles could be used as antibacterial Interaction with phosphorus moieties in DNA, resulting in inactivation of DNA replication. Reacts with sulfur-containing proteins, leading to the inhibition of enzyme functions disruption of cell membrane. Also they used as Antifungal disruption of cell membrane (Yan *et al.*, 2013 and Mercy *et al* 2015) [50, 28].

Table 2: Antibacterial activity of antimetabolic produced by *Halomonas variabilis* PRS2 conjugated with copper and selenium nanoparticles

Sample / Tested microorganisms	Antimetabolic product (A P)	Nano copper	A P +Nano copper	Nano selenium	(A P + selenium Nano	St.
Gram Positive Bacteria						***Ampicillin***
Streptococcus pneumoniae (RCMB O1001O)	18. 3± 0.92	19.6 ± 1.5	24.6 ± 1.2	25.6 ± 1.5	29.6 ± 1.2	23.8± 0.63
Staphylococcus aureus (RCMB 010023)	17.9± 1.9	20.0 ± 1.2	23. 2± 1.5	23.0 ± 1.2	33± 1.5	32.4± 1.5
Staphylococcus epidermidis (RCMB 010024)	18.6± 1.9	17.6± 0.63	22.3 ± 1.2	25.6± 0.63	28.3 ± 1.2	25.4± 1.2
Streptococcus Pyogenes (RCMB 010015)	NA	NA	NA	24+ 1.5	27.4± 1.5	26.4± 1.5
Corynebacterium diphtheriae (RCMB O100846)	17. 3± 0.78	14.6 ±1.2	19.1±1.2	24.6 ±1.2	30.1±1.2	22.3± 0.63
Bacilli's anthracis (RCMB 0100693)	19. 8± 0.93	21.3 ± 1.5	22.8±	25.3 ± 1.2	29.8±0.72	25.4± 1.2
Methiciltin-Resistant Staphylococcus aureus MRSA 2658 RCMB	24.6± 0.82	18.3±0.72	21.4 ±1.2	26.3±0.72	31.4 ±1.2	22.3± 1.2 Vancomycine
Gram negative bacteria:						Gentamicin
Pseudomonas aeruginosa(RCMB 010043)	19.3±0.68	19.3 ± 1.2	23.8± 0.63	26.3 ± 1.2	28.8± 0.63	17.3± 0.58
Serratia marcescens (RCMB 010075)	18.3 ± 1.9	15.9 ± 0.72	23.7 ± 0.63	25.9 ± 0.72	29.7 ± 0.63	23.4± 1.5
Proteous vulgaris (RCMB 01O085)	NA	15.6±0.58	19.3 ± 1.2	25.6±0.58	29.3 ± 1.2	19.9± 1.2
Klebsiella pneumoniae (RCMB 0010093)	18.3 ± 0.76	16.3±0.63	24.2 ± 0.72	24.3±0.63	28.2 ± 0.72	26.3± 0.63
Neisseria gonorrhoeae (RCMB 010034)	NA	15.2±0.63	18.6±1.2	25.2±0.63	25.6±1.2	20.6± 0.72
Mycobacterium tuberculosis (RCMB 010094-8),	19.6± 0.66	13.4+1.5	19.9± 1.2	23.4+1.5	29.9± 1.2	23.2+2.1

Table 3: Antifungal activity of antimetabolic produced by *Halomonas variabilis* PRS2 conjugated with copper and selenium nanoparticles

Sample / Tested microorganisms	antimetabolic product(A P)	Nano copper	A P +nano copper	Nano selenium	(A P + selenium Nano	St.
FUNGI					**Amphotericin B**	
Trichophyton mentagrophytes (RCMB 09258)	18.4 ± 0,92	20.3 ± 0.63	22.3 ± 1.2	24.3 ± 0.63	29.3 ± 1.2	24.3± 0.63
Trichophyton rubrum (RCMB 09274)	NA	14.6 ± 1.5	18.4± 1.2	24.6 ± 1.5	31.4± 1.2	20.3± 0.72
Microsporumcanis (RCMB 088354)	19. 2± 1.7	21.2 ± 0.58	24.3±0.63	26.2 ± 0.58	28.3±0.63	26.4± 1.5
Aspergillus fumigatus (RCMB 02568)	18.6 ± 0:78	19.3± 1.2	23.4 ± 1.5	25.3± 1.2	29.4 ± 1.5	23.7± 0.58
Aspergilluscla vatus (RCMB 02572)	17.3 ± 1.8	18.6 ± 0.58	18.7± 1.2	24.6 ± 0.58	28.7± 1.2	19. 7± 1.2
Aspergillus niger (RCMB 02581)	19.2 ± 0.185	21.4 ± 0.63	25.6±0.63	26.4 ± 0.63	25.6±0.63	28.7± 1.2
Aspergillus flavus (RCMB 02557)	NA	16.3 ± 1.2	17.6±0.58	26.3 ± 1.2	26.6±0.58	25.4± 0.72

Mean zone of inhibition in mm ± Standard deviation beyond well diameter (6 mm) produced on a range of clinically pathogenic microorganisms. The test was done depicted in the table: using the diffusion agar technique, Well diameter: 6.0 mm in VACSERA lab. Antimicrobial unit test organisms *NA: No activity, data are expressed in the form of mean ±SD.

Several authors reported that, because of the large surface area of the nanoparticles, it could be tightly adsorbed on the surface of the bacterial cells so as to disrupt the membrane, which would lead to the leakage of intracellular components, thus killing the bacterial cells. The detailed mechanism for the activity of metal nanoparticles is still under debate. One possible explanation of the antibacterial effect is that the ions released by the nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death. There is also potential for multiple adverse interactions such as oxidative stress and inflammatory responses. Such cellular processes may lead to cell death via cell necrosis or apoptosis (Qi L *et al* 2004 and Wei *et al* 2009) [33, 47].

Also, Mercy *et al.* (2015) [28] studied that, the biologically synthesized CuNps shows excellent

antibacterial activity. The CuNPs also exhibited potent antifungal effects on fungi tested, probably through destruction of membrane integrity, therefore it may be concluded that CuNPs has considerable antifungal activity, deserving further investigation for various biomedical application such as catheters, topical antimicrobial gel formulation, food packaging materials, food processing equipments.

Our results are parallel with Eswarapriya and Jegatheesan (2015) [13] in which selenium nanoparticles (SeNPs) has been used for a wide range of applications including antibacterial, antioxidant and anticancer applications. The effects of SeNPs on fungal strains remain for the most part unknown to date. The antifungal effectiveness of SeNPs was tested against two important clinical fungal genera, *Candida* and *Aspergillus*. The antifungal efficacy was determined by disc diffusion method and sensitivity in terms of zone of inhibition formed around the disc. Results of this study provided the first evidence of SeNPs was effective against the fungal strains tested.

In a recent study El-Batal *et al* (2016) [12] reported that, the possible synthesis of selenium nanoparticles (SeNPs) in aerobic optimized conditions using *Bacillus laterosporus* bacterial strainA microtiterplate assay was used to evaluate the ability of SeNPs to inhibit the biofilm formation of *Pseudomonas aeruginosa*. Evaluating the antimicrobial activity of some antimicrobial agents upon addition of SeNPs was performed that SeNPs inhibit the biofilm formation of *Pseudomonas aeruginosa* with a percentage reduction of 99.7%. SeNPs increase the antibacterial activity of fucidic acid by 13.6% and 28.5% against *Escherichia coli* and *Staphylococcus aureus* respectively. But with Gentamycin sulphate, no change in the antibacterial activity.

our result is inaccordance with Cremonini *et al.* (2016) [8] found that, Antimicrobial activity of SeNPs against clinical isolates of *P. aeruginosa* and Candida spp were tested. This is the first report confirming that biogenic SeNPs are potentially suitable as antimicrobial agents against clinical strains isolated from patients with chroniclung diseases. They also show that the biogenic SeNPs can inhibit biofilm synthesis by *P. aeruginosa* and the two Candida species, and can efficiently disaggregate the mature exopolysaccharide matrix produced by these microorganisms. The antimicrobial potential of these biogenic SeNPs is greater than that of synthetic SeNPs, probably due to the presence of a bacterial protein layer coating the surface of the biogenic particles.

Our results of nano copper agree with Subhankari and Nayak (2013) [40] who determined the antimicrobial efficacy of green and chemical synthesized Cu nanoparticle against various bacterial and fungal pathogens. Various microbiological tests were performed around the outside of the bacterial cell membrane and it is essential to the survival of bacteria. Surfaces of copper nanoparticles affect interact directly with the bacterial outer membrane, causing the membrane to rupture and killing bacteria. Antibacterial activity ofcopper nanoparticles synthesized by electrolysis was evaluated by using standard Zone of Inhibition (ZOI) microbiology assay. The sample copper nanoparticles prepared in electrolysis method showed 15 mm diameter of inhibition zone against *E. Coli*.

Also, Jeyaraman *et al.* (2012) [24] found that, The antimicrobial activity was carried out using Copper nanoparticles against *Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa*, fungus like *Aspergillus flavus, Aspergillus niger and Candida albicans.* The copper nanoparticles showed more inhibitory activity in bacteria than the fungus and it also showed more zone of inhibition *in E.coli* (26 mm) than *C. albicans* (23 mm).

Maqusood *et al*. (2014) [26] found that, the structural and antimicrobial properties of copper oxide nanoparticles (CuO NPs) synthesized by a very simple precipitation technique. CuO NPs showed excellent antimicrobial activity against various eight bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus faecalis, Shigella flexneri, Salmonella typhimurium, Proteus vulgaris,* and *Staphylococcus aureus*). Moreover, *E. coli* and *E. faecalis* exhibited the highest sensitivity to CuO NPs while *K. pneumonia* was the least sensitive. Consequently, CuO NPs have potential for external uses as antibacterial agents in surface coatings on various substrates to prevent microorganisms from attaching, colonizing, spreading, and forming biofilms in indwelling medical devices. This study suggests that mechanisms of antimicrobial response of CuO NPs in different species of bacteria should be further investigated.

Conclusion

Results from this study signify that antimetabolic produced from haloalkalophilic bacteria defined phenotypically *Halomonas varibilis* PRS2. It can be concluded from the variety of the morphological and growth requirements, the behaviors' of the extreme isolate adapted morphologically and physiologically according to the type of extreme environmental factors. The environmental influence as salinity by Na^+concentration, pH and oxygen tension, and complex compound requirement, play important roles in the selection of this identified haloalkalophilic. The pigmentation system of colony be affected by the variety of physiological properties may be correlated to especially survival system inside the cells for adaptation under sever conditions. Antimicrobial activity showed excellent results against various seven gram positive bacterial strains, six gram negative strains and seven fungal strains. The results confirmed that, it have antimicrobial activity against all the clinical pathogens. Generally though the effect of selenium nanoparticles pluse antimetabolic product was found to be excellent more pronounced followed by the inhibitory effect of selenium single and copper nanoparticles pluse antimetabolic produced.

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