In Vitro Evaluation of Bioactive Metabolites from UV-resistant Bacterial Strain Isolated from Cholistan Desert, Pakistan.

By

Afaq Ahmad

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad
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Dedication

In gratitude for her unwavering support and encouragement, I dedicate this thesis to my best friend Asma Mukhtiar whose beliefs in my dreams have continuously propelled me forward towards achieving my goals.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Afaq Ahmad

Certificate

This thesis submitted by Afaq Ahmad titled, "*In Vitro Evaluation of Bioactive Metabolites from UV-resistant Bacterial Strain Isolated from Cholistan Desert, Pakistan*" is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

Supervisor: _____
(Prof. Dr. Aamer Ali Shah)

External Examiner: _____
(Prof. Dr. Ijaz Ali)

Chairman: _____
(Prof. Dr. Naeem Ali)

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List of Abbreviations

UVR	Ultraviolet radiation
ROS	Reaction oxygen species
MAAs	Mycosporine like amino acids
BER	Base excision repair
DSBs	Double strand breaks
NER	Nucleotide excision repair
FA	Fanconianemia pathway
TS	Translesion synthesis
NHEJ	Non- homologous ends joining
ESDSA	Extended synthesis dependent strand annealing
TGY	Trypton glucose yeast extract agar
TSI	Triple sugar iron
SIM	Sulfur indole motility
CMC	Carboxymethyl cellulose
TLC	Thin layer chromatography
DPPH	2,2-diphenyl-1-picrylhydrazyl
BST	Brine shrimp lethality test
FC	Folin ciocalteau
MHA	Mueller hinton agar
DMSO	Dimethylsulfoxide
PDA	Potato dextrose agar
FTIR	Fourier transform infra-red
O. D	Optical density
Rf	Retention factor

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Abstract

Living organisms that are inhabiting extreme environments are termed as extremophiles which are classified into different groups. Among others, radiophiles include those organisms that thrive in environments of high radiations. Exposure to excessive amount of radiation can have dire consequences for an organism and can damage vital cellular structures including DNA, proteins, and lipids. Microorganisms that are living under intense amount of UV radiation have adopted several measures to resist negative impacts of UV radiation and produce several primary and secondary extremolytes which uplift the chances of survival for a microorganism. These extremolytes are involved in the provision of either a protective shield against the incoming UV radiation or participate in maintaining the integrity of cell regulatory machinery. In the current study, a bacterium designated as strain CH-8 was isolated from Cholistan desert of Pakistan. Strain CH-8 was found resistant to UV radiation and H_2O_2 with 60% survivability at 2712 J/m^2 and 78% at 10 mM respectively. The bacterium was identified by morphological, biochemical, and molecular methods. 16S rRNA gene sequencing results indicated that strain CH-8 is 99.85% similar to *Streptomyces sp.* strain TRM46222, therefore the bacterium was named as *Streptomyces sp.* strain CH-8. Strain CH-8 showed black color pigmentation and its intracellular metabolites were extracted using methanol as a solvent. The total phenolic and total flavonoid assays of intracellular metabolites revealed the total phenolic content as 149 mg GAE/g DW and total flavonoid content as 2.082 mg QE/g DW. The metabolites from *Streptomyces sp.* strain CH-8 were then partially purified through silica gel column chromatography and evaluated for bioactive potential through various assays. The fractions I-8 and I-12 exhibited significant antioxidant (77% and 73.62%) as well as DNA damage prevention activities. Moreover, the fractions inhibited hemolysis of RBCs in anti-hemolysis assay and had a considerable cytotoxic activity. Furthermore, purified fractions also had a remarkable antibacterial activities and prevented the growth of *Staphylococcus aureus* and *Escherichia coli*. These findings suggested that extract of *Streptomyces sp.* strain CH-8 has antioxidant, radioprotective and antitumor potential which requires further evaluation.

1. Introduction

Recent advancements in science have proved that the range of life is not limited, and it is possible that life may have ranges far beyond our imagination. Organisms that are living in extreme environment have adapted themselves to those environments. Organisms have been found in deep-sea hydrothermal vents, hot springs, nuclear waste contaminated areas, soda lakes and hot and cold deserts and are called “extremophiles”. These extremophiles have been divided into various groups including thermophiles, psychrophiles, acidophiles, alkaliphiles, halophiles, barophiles, metallophiles and radiophiles (Cowan et al., 2015). Radiophiles are very often found in environments having high oxidative stress and radiations.

One of the extreme parameters of harsh conditions is electromagnetic energy in the form of UV- radiation. The wavelength of UV light falls in the range of 100 nm to 400 nm and on the basis of their wavelength spectrum, UV light can be divided into three different types. Each type of UV light affects living organism in different manners, interfering with various processes that can be dangerous and lethal to life. Wavelength of UVA covers the spectrum of wavelength between 315 nm to 400 nm, while that of UVB covers between 280 nm to 315 nm. However, UVC has range of 100 nm to 280 nm (WORLD HEALTH ORGANIZATION, n.d.).

Among these three types, UVC has the highest energy and is far more lethal than the rest of the two, but luckily UVC is screened by the atmosphere and do not reach to the earth’s surface (Molina-Menor et al., 2023). A great amount of UVB is filtered, but still UVB in a small concentration manages to reach the surface of earth and affects organisms through various mechanisms. UVB has been reported to cause mutations in the DNA upon its absorption leading up to the formation of pyrimidine dimers and has been associated with different types of skin cancers. UVB is also involved in killing of bacterial strains that are present in the air (Madronich et al., 2018). Contrary to UVC and

UVB, UVA has a high penetration power and contributes 95% to the UV light that manages to reach the surface of earth after filtration through the atmosphere. UVA has a strong penetration power and can travel deep into the cells, damaging lipids, proteins and DNA (Wurtmann & Wolin, 2009).

It is believed that UV exposure had been a challenge to cell repair processes and overall survival on the Early Archaean Earth (Cockell, 1998). However, several bacterial strains including *Bacillus subtilis* and *Deinococcus radiodurans* have been isolated, thriving under such extreme conditions (Battista, 1997; Nicholson et al., 2000). This has kindled interest of scientists to inquire resistance of life to UV-radiation throughout the evolution of life (Cockell, 2000).

Moreover, it has been forecasted that at the end of 21st century, UVB radiation will rise, but the intensity of the increase will vary from place to place. It is also predicted that this increase in UVB radiation will range between 5% to 10% in temperate latitudes and 20% in high altitudes (Pérez et al., 2017). This increase will bring some dire repercussions for the earth and its inhabitants.

UVR has been reported as a detrimental abiotic factor that has profound effects on the microorganisms at different levels and is involved in damaging vital cellular biomolecules including DNA, proteins and lipids (Jung et al., 2017). UVR can either directly or indirectly affect the cellular structures. In direct damage to DNA, it involves double strand breaks and single strand breaks. The damage can also be due to pyrimidine dimerization that leads to the inhibition of DNA replication and transcription (Argueso et al., 2008). UVR also has the potential to indirectly damage cellular structure through the formation of reaction oxygen species (ROS) which later drastically affect cellular biomolecules (Wurtmann & Wolin, 2009). An investigative study has reported that ROS can also interact with different cellular constituents including DNA, proteins and lipids. As these molecules are the building blocks of cellular membranes, any negative impact

on their integrity can jeopardize permeability of membrane and can eventually lead to bacterial death (Barrera, 2012).

Microorganisms have been reported to exist in harsh environmental conditions and those organisms that manage to live in harsh environmental conditions are termed as extremophiles. Extremophiles have adopted different mechanisms to survive in harsh, inhospitable and extreme environmental conditions (Coker, 2019).

One class among these extremophiles is of radiophiles. Radiophiles are those organisms that are posing resistance to UVR. They have been found across the globe and in all three domains of life. These resistant organisms have developed measures to prevent the deleterious effects of UVR mediated oxidative stress which might affect the normal cellular processes. It has been reported that bacterial cells have the potential to survive in extremely harsh environmental conditions (Cowan et al., 2015). It is worth mentioning that variation in response to UVR has been observed in different bacterial strains. Bacterial strains have adopted various kind of physiological and biochemical mechanisms in order to counter balance the deleterious effects of UV- radiation including efficient DNA repair mechanism and defense against UV induced reactive oxygen species (Gao & Garcia-Pichel, 2011; Pérez et al., 2017). For example, members of the *Deinococcaceae* bacterial family have been observed to tolerate a UVR dose of over 12,000 Gy (J/Kg) or 1000J/m^2 (Daly, 2009).

Moreover, UVR resistant organisms also have been reported to pose resistance by adopting several other mechanisms. There are organisms which produce pigments as a first line of defense against UVR, but production of pigments is not sufficient and cannot prevent the damage to DNA. Along with this, bacterial strains have also adopted repairing strategies for DNA damage and are involved in the process of protecting vital cellular structures against the impact of reactive oxygen species (ROS) by inactivating them through various mechanisms. Among those mechanisms, certain cellular systems are involved. The prominent among them is enzymatic system. In enzymatic system,

several enzymes for example catalase, superoxide dismutase and peroxidases ensure a sustainable metabolic process in harsh conditions. Along with these enzymes, scavengers like Vitamin C, B and E, glutathione and cysteine also play their respective roles in counterbalancing the drastic effects of UV radiation (Gabani & Singh, 2013; Rastogi et al., 2010; Zhang et al., 2009).

Deinococcus radiodurans has a condensed genome in which DNA is tightly linked which results in the protection of DNA from any unwanted negative effects of ROS. Through this unique condensation of nucleic acid, *Deinococcus radiodurans* not only protects its DNA, but also promotes its DNA repairing mechanisms (Jin et al., 2019).

It is true that UV resistant bacteria are distributed across the globe but are most abundant in some specific areas including UV- sources, solar panels, and deserts (Molina-Menor et al., 2023). Desert areas are considered one of the extreme environments and have been considered to pose significant challenges to the survival of organisms including microorganisms. There are many limiting factors in desert environment that pose challenge to the survival of microorganisms, but the most prominent of them are extreme temperature and continuous exposure to the sunlight (Rampelotto, 2010). Various bacterial strains that are resistant to UV-radiation have been isolated from different deserts. Resistant bacterial strains belonging to genera *Deinococcus*, *Geodermatophilus* and *Hymenobacter* have been isolated from Sonoran Desert (Rainey et al., 2005). Similarly, several other radiation resistant bacterial species have been isolated from Sahara Desert (Baqué et al., 2013). Moreover, two *Deinococcus thermus* strains have also been isolated from Lut desert of Iran (Mohseni et al., 2014).

Extremolytes are small organic molecules that are concentrated in microorganisms surviving in extreme environmental conditions. Extremolytes protect cellular structures of extremophiles by stabilizing and forming protective water layers (Becker & Wittmann, 2020a). Extremolytes constitute almost 25 % of dry cell weight (Raddadi et al., 2015).

In search of compounds that are resistant to enhanced levels of radiation, various extremolytes have been isolated from numerous radiation resistant organisms which include scytonemin, mycosporine like amino acids (MAAs), melanin, bacterioruberin, carotenoid, lycopene and astaxanthin. Synthesis of these extremolytes indicates the biochemical adaptations of extremophiles (Becker & Wittmann, 2020; Kochhar et al., 2022).

These extremolytes also play their respective roles and contribute to the overall resistance of extremophiles. For example, some of the extremolytes form a kind of protective water sheet around the cellular structures which prevents deleterious effects of harsh environmental conditions of the habitat where bacterial strain is existing (Sahle et al., 2018). There are other extremolytes as well which are directly involved in the scavenging activity and protect the vital cellular structures of bacterial strain from the damage of UV-radiation and oxidative stress(O. Singh & Gabani, 2010). It is worth mentioning that many extremolytes that are involved in the process of resistance against UV- radiation work against different stresses at one time and behave like multi-functional agents (Czech et al., 2018). One of the most important extremolytes that are efficiently involved in antioxidant activities are flavonoid metabolites. These metabolites either interact with reactive radicals and result in the formation of stable and inactive radicals or directly scavenge free radicals (Sajjad et al., 2023).

Another class of extremolytes that has a versatile nature in counter balancing UV-radiation is of carotenoids. Although carotenoids are not involved in playing any role in normal cellular growth of bacterial strain, but lacking them can negatively impact the ability of bacteria to survive because these are the compounds that are responsible for tracking the reactive oxygen species including singlet oxygen and peroxyl radicals (Stahl & Sies, 2003; Tatsuzawa et al., 2000). Moreover, carotenoids have also been involved in protecting strands of bacterial DNA from any kind of oxidative damage. In the same manner, they also protect different membranes of bacteria from the process of lipid

peroxidation and prevent carbonylation of several proteins (Zhang & Omaye, 2000). *Deinococcus radiodurans*, the widely recognized UV- resistant bacteria, produces keto carotenoid, deinoxanthin which enhances its overall resistance mechanism against oxidative stress in the presence of UV-radiation because of its ability to quench reactive oxygen species and render them incapable of causing any damage to cellular structures of bacterial strain (Sajjad et al., 2017). Another study has suggested that carotenoids from bacteria, isolated from Antarctic Sea surface could be responsible for the resistance of isolated bacteria against solar radiation (Agogué et al., 2005).

Extremolytes have great potential and have been termed as "unexploited gold mine". These extremolytes have promising opportunities for different fields including cosmetic, medical and food industry. The response of MAAs upon exposure to UV radiation render them useful not only in UV-protective sunscreen in the cosmetic industry but their applicability as preventative agents of UV radiation induced cancers have also been suggested (De la Coba et al., 2009).

MAAs have the ability to work as biological antioxidant. Their absorption gradient ranges from 268 nm to 362 nm which makes them the strongest UV-radiation absorbing compounds and can provide protection against UVA and UVB as well and because of this potential they can also prevent melanoma(Geraldes & Pinto, 2021). It has been suggested that MAAs compounds have the potential to be used for therapeutic purposes. For example, scytonemin has been proposed as an important compound that can be used for the synthesis of a novel pharmacophore to produce protein kinase inhibitors such as antiproliferative and ant-inflammatory drugs. Moreover, Bacterioruberin, a compound produced by radioresistant microbes, has been suggested to have applications in prevention of human skin cancer because of its involvement in the process of repairing damaged DNA strands caused by ionizing UV- radiation (O. Singh & Gabani, 2010). Another compound, deinoxanthin, which has been reported to be synthesized by *D. radiodurans* has the capability to induce apoptosis of cancer cells and could be used as

chemo preventive agent (Choi et al., 2014). One of the extremolytes, ecotine, is presently used in skin care products(Pastor et al., 2010).

It is worth mentioning that extremolytes also have the potential to be used for the stabilization of proteins and nucleic acids. These abilities of extremolytes have made them suitable candidates for therapeutic usages. Extremolytes, due to their abilities to stabilize proteins, present an attractive solution for the stabilization and storage of sensitive proteins in the absence of other protein stabilizers (Avanti et al., 2014). Extremolytes have also been reported to be involved in inhibition of protein misfolding which make them a vital candidate for drugs development against various diseases (Kanapathipillai et al., 2005; Ryu et al., 2008).

Extremolytes have a wide scope in food industries as well. According to a study, carotenoids can be used as additives and antioxidants in food industries (Saini & Keum, 2019). There are several benefits associated with usage of carotenoids in food industries. For example, it can enhance immunity and has the ability to protect consumers from different physiological disorders, including cancers (Mel´endez-Martínez, 2019).

Main focus of this study was the isolation of UVR bacterial strain from Cholistan Desert, Pakistan and then invitro evaluation of bioactive metabolites from isolated UVR resistant bacterial strain. Cholistan Desert in Pakistan is an unexplored ecological place and has the potential to host various resistant microorganisms, thanks to the climate of that area which is categorized as harsh, sub-tropical region with relatively low level of precipitation, low humidity and high rate of evaporation (Fatima et al., 2019). It is believed that in the past years that date to about 5000 years ago, Cholistan received heavy monsoon downpours, but with the passage of time, change in climate resulted a shift in monsoon winds away from the areas which eventually resulted a great decrease in downpouring and ultimately converted the area into a desert. One of the most distinguishing features of Cholistan desert is the consecutive occurrence of dry years in cluster even for 4 to 6 years consecutively which has resulted in the great variation in

daily temperature. In summers, there is a variation in mean temperature, varying from 35 to 50ºC during May to June. However, during winters mean temperature varies from 15 to 20 ºC during December to February (Arshad et al., 2007).

Aims and Objectives

Aim

In Vitro evaluation of bioactive metabolites from UV resistant bacterial strain isolated from Cholistan Desert of Pakistan.

Objectives:

- Isolation and screening of UV resistant bacteria from Cholistan desert.
- Determination of strains survivability against various stresses.
- Optimization of growth conditions.
- Extraction and purification of extremolytes from isolated bacterial strains.
- Invitro evaluation of extracted extremolytes.

2. Literature Review

Life has been evolving since its emergence on this planet earth. However, prokaryotes remained dominant in most of the history. It is worth mentioning here, that in previous century boundaries of life that were limiting it have been pushed further resulting in possibility of life at intense temperatures, pH, pressure, salinity, energy, and radiation. Over the past several decades, isolation and identification of extremophiles have been focused by several scientists and it has provided prominent insights in assessing the boundaries of life. Research studies in this particular field not only has brought advancement in molecular biology and medicines (Merino et al., 2019) but has also transformed the understanding of evolutionary pattern of life on earth and on other planetary bodies (Schulze-Makuch, 2013).

2.1. Extreme Environments

Organisms are grouped into different categories, based on the habitat where they are residing. Mesophilic organisms are those organisms that have a limited range of life limiting parameters including water, pH, temperature, and pressure. Mesophilic organisms, in the longer run, make these parameters as standards and existence of conditions beyond these boundaries at some places of earth are termed as extreme environmental conditions. Environments with such conditions are termed as extreme environments (Nair, 2017). Organisms that thrive in such extreme environments are termed as extremophiles which will be discussed briefly in the paragraphs that are following.

2.2. Microorganisms of Extreme Environmental Conditions

Microorganisms that manage to live in extreme environments are termed as extremophiles which are further divided into different categories, based on their environments where they are living. Microorganisms that are living in extreme environmental conditions have developed certain modifications in their cellular systems

which make them able to withstand against harsh environmental conditions and ensure their survival. For example, halophiles have developed water cages and acidophiles have reduced pore size (Coker, 2019). Moreover, barophiles have the ability to thrive in environments of extreme pressures and in the similar manner, radiophiles have the adaptations to cope up with intense amount of radiations (Satyanarayana et al., 2005). Extremophiles that manage to live in more than one extreme environment are termed as polyextremophiles (Gupta et al., 2014).

2.3. Spectrum of Sunlight and Various Types of Ultraviolet Radiations

Electromagnetic radiations are brought towards the earth by sunlight and are further divided into two types: Ionizing and non-ionizing radiations. Ionizing radiation includes gamma rays and X-rays while non-ionizing radiation includes ultraviolet light. UVR travels from sun towards the earth in the form of electromagnetic radiation having wavelength ranging from 100-400 nm and energies ranging from 3 to 124 eV (Rastogi et al., 2010). According to a study, one of the extreme parameters of harsh conditions is electromagnetic energy in the form of UV- radiation (Rothschild & Mancinelli, 2001). The wavelength of UV light falls in the range of 100 nm to 400 nm and based on their wavelength spectrum, UV light can be divided into three different types.

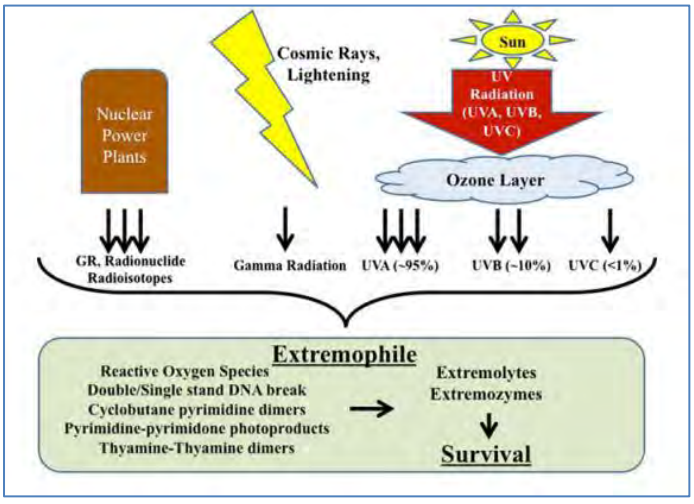

Fig 1: Impact of radiation on extremophiles (Gabani & Singh, 2013).

UVA constitutes 95% of the radiations that reach towards the earth surface and has a wavelength ranging between 320-400 nm. Although UVA has a higher wavelength and lower energy, but the penetration power of UVA is much greater than the rest of the two UVR types (Maverakis et al., 2010). UVB constitutes 5% of the total radiations that make up to the earth and has a wavelength ranging between 280-315 nm. UVB has relatively higher energy than UVA but has a less penetration power comparatively. UVC is carrying the highest energy among all types and has a wavelength ranging between 100-280 nm. However, UVC is restricted and filtered by ozone and doesn't enter into earth's atmospheric boundaries (Gabani & Singh, 2013).

2.4. Changing Climate and Impact of Ultraviolet Radiation on Cellular Structures

Discovery of ozone hole opened up an avenue for research to assess the detrimental effects of increasing levels of UVR that may have the potential to affect various cellular structures (Farman et al., 1985). It has been predicted by scientists across the globe that UV-B radiation will further increase up to 5-10% by the end of 21st century (Zepp et al., 2011). Incoming UVR will affect everything including humans, animals, plants, and microorganisms.

Moreover, the usage of radioactive elements has also increased in various other sectors including research laboratories, pharmaceutical industries and energy producing industries (Pryakhin et al., 2012). Along with this, accidents in nuclear facilities which include incident of Fukushima Daiichi in 2011 and accident of Chernobyl in 1986 have further elevated the levels of radionuclides in the environment. Increased amount of radiation in the surrounding environments had aftermaths for every living organism including humans, plants, animals, and microorganisms (Gabani & Singh, 2013).

UVR affect microorganisms at community and cellular levels, damaging vital structural and functional biomolecules including DNA, lipids, DNA and proteins. It has also been reported that at high altitudes, UVR not only affects community composition but also have drastic impacts on bacterial growth and overall productivity (Pérez et al., 2017). It

was also demonstrated in another study that ionizing radiation has many lethal effects on living organisms and affects vital cellular structures including nucleic acids, proteins and lipids (Jung et al., 2017). The unwanted impact on cellular processes is caused by the damage to DNA which can be double or single strand breaks in DNA or damage because of thymine dimerization (Argueso et al., 2008).

2.5. Radiation Resistant Microorganisms

Variety of microorganisms have been isolated that are showing resistance to UVR and have adopted survival strategies to thrive in the habitats of having high intensity of radiations. UVR microorganism are greatly distributed across the globe and have been isolated from diverse habitats including desert, radioactive lakes, and nuclear facilities. Among all the isolated species *Deinococcus radiodurans*, *Rubrobacter radiotolerans,* and *Thermococcus gammatolerans* remained species of great focus by researchers of this domain(Tapias et al., 2009; Terato et al., 2011).

2.5.1. Isolated UVR Resistant Bacterial Strains

Various varieties of microorganisms that are resistant to ionizing radiation have been isolated from different environments (Rainey et al., 2005). The most prominent among them are species of genera *Deinococcus*, *Kineococcus* and *Rubrobacter* which have shown remarkable resistance to radiation (Jung et al., 2017).

a. *Deinococcus Radiodurans*

The most prominent example of UVR resistant microorganisms is *Deinococcus radiodurans*. *Deinococcus radiodurans* has been reported to pose significant amount of resistance to numerous stress conditions including UVC and oxidative stress (Slade & Radman, 2011). For the first time *Deinococcus radiodurans* was isolated from the meat which was processed for sterilization through ionizing radiation (AW, 1956). *Deinococcus radiodurans* have adopted various strategies to get rid of unwanted structural and functional changes due to UVR. Its condensed genome is responsible for

protecting it against the DNA damage caused by reactive oxygen species (ROS) which are formed because of UVR. This distinctive feature of having condensed genome not only helps in protecting its nucleic acid but also aid in DNA repairing mechanisms (Jin et al., 2019). Moreover, *Deinococcus radiodurans* Possess large number of DNA glycosylases enzymes which play a vital role in in base excision repair (BER) (Norais et al., 2009). *D. radiodurans* also have a sophisticated enzymatic machinery which play an active role in survival during harsh conditions. This efficient enzymatic machinery hosts numerous enzymes including superoxide dismutases and catalases which are involved in converting reactive oxygen species into harmless molecules, rendering *D. radiodurans* protected from unwanted oxidative damage to vital cellular structures (Jung et al., 2017). Furthermore, a novel strain of *D. reticulitermitis sp. nov.* was isolated from gut of termite which was showing an immense resistance to UVR (100 J/m2) (Chen et al., 2012).

b. *Cyanobacteria*

Cyanobacteria are regarded as contributing to the clique of ancient microorganisms and have adopted several survival strategies to deter the harmful effects of UVR and have been reported to adapt themselves even in the presence of high intensity radiations. To counterbalance the drastic impacts of UVR, *cyanobacteria* have adopted several measures and has put forwarded various defense lines (Sorrels et al., 2009).

c. *Rubrobacter radiotolerans*

Rubrobacter radiotolerans was isolated from water sample that was taken from a hot spring in Tottori Prefecture, Japan. It has been reported to pose a significant amount of resistance to radiation which is mainly associated with bioactive compounds that are synthesized by it which include bacterioruberin and monohydrobacterioruberin (Asgarani et al., 2000; Ferreira et al., 1999). Moreover, many other mechanisms are also reported which participate in resilience against radiations. For example, it contains deinoxanthin and bacterioruberin which have a strong activity against hydrogen peroxide (Asgarani et al., 2000). Moreover, the ability of this bacterial strain to resist UVR has also been

attributed to the presence of an enzyme called superoxide dismutase (Terato et al., 2011). In another study it was also reported that in the presence of UVR there is a significant reduction in the synthesis of protein in *Rubrobacter radiotolerance*, a strategy to cope with harsh environmental conditions and enhance its survivability chances (Santos et al., 2012).

d. *Hymenobacter xinjiangensis*

It was isolated from desert of Xinjiang, China. It was observed that isolated strain has posed significant amount of resistance to UVR. In addition, it was also reported that the strain had pink colored pigmentation which can be possibly involved in protection against UVR (Zhang et al. 2007b).

2.6. Impact of UVR on Vital Cellular Structures

Since the beginning of life on this planet earth UVR has been one of the serious threats to life. UVR has the potential to affect vital cellular structures and biomolecules including DNA, RNA and proteins that ultimately jeopardize survivability of living organisms on earth. Great part of our knowledge about the molecular changes and their associated effects on living organisms come from the research and studies being conducted on microorganisms. It has been reported that UVR that remained unfiltered affect microorganisms upon its reaching towards the surface of earth. It has also been suggested that UVR and most specifically UVC and UVB either inactivate or kill bacteria by photochemical alteration of cellular DNA. Upon alteration of genetic material, microorganisms lose their ability to replicate and hence their replication is impaired (Laroussi & Leipold, 2004). Among all the detrimental effects of UVR on microorganisms, formation of pyrimidine dimers is most prominent. Along with this, protein synthesis machinery of the cell is also affected by UVR. It has been reported that UVR inhibits transcription capability of the cell which results in multiple mutations and ultimately cell death. Moreover, integrity of the cellular proteins may also be disturbed upon exposure of cells to UVR. Among all the proteins, tryptophan amino acids rich

proteins are more prone to the lethality of UVR because of its aromatic structure. In addition, cysteine contains disulfide bonds which also cannot withstand UVR and are broken down upon exposure, negatively affecting the integrity of cysteine amino acid (Gayán et al., 2014).

2.6.1. Induction of DNA damage due to Ultraviolet Radiation

There are number of factors including free radicals formation and UVR that affect the integrity of DNA through various mechanisms and thus greatly influence the life sustaining activities of all organisms, including microorganisms (Taylor, 1990; Wiseman & Halliwell, 1996). Damage to DNA can be in the form of depyrimidination, depurination or can be because of the generated reactive oxygens species due to oxidative stress (Lindahl, 1993). In case of depurination or depyrimidination there is a complete detachment of purine or pyrimidine bases from the DNA stretch that can compromise the overall integrity of DNA strands.

UVR also has the potential to induce double strand breaks and these breaks have been observed by scientists for a long time (Wang & Smith, 1986). These breaks are mostly reported in cells which have been exposed to UVR for a great length of time (Baumstark-Khan et al., 2000; Slieman & Nicholson, 2000). The lesions that arise due to primary and secondary DNA breaks are responsible for the hindrance of DNA replication during cell division and transcription during protein synthesis and thus in the longer run result in the formation of double strand breaks (DSBs) in DNA at the site of replication forks (Batista et al., 2009; Limoli et al., 2002). It has been proposed that UVR does not directly produce DSBs in DNA. However, UVR produces pyrimidine dimers, and these pyrimidine dimers lead up to the blockage of replication and then form DSBs (Rastogi et al., 2010).

2.6.2. Impact of UVR on Bacterial Proteins

UVR affect cellular structures through different mechanisms and their mode of action varies from one target to another. Most of the time the impact of UVR on cellular targets is indirect as UVR generates ROS which later negatively affects cellular structures and

biochemical processes (Chamberlain & Moss, 1987; Pattison & Davies, 2006). ROS that are generated upon exposure to UVR interacts with cellular structures and biomolecules including lipids and proteins that results in the inhibition of membranes permeability and can even lead up to the death of cell (Bose & Chatterjee, 1995; Futsaether et al., 1995). One of the most lethal impact of UVR on bacterial proteins is the capability of UVR to induce carbonylation of bacterial proteins, carbonyl derivatives are generated at the amino acid side chains of threonine, lysine, arginine and proline. In a study it was reported that carbonylated proteins can be easily broken down through the process of proteolysis and can also affect several other vital processes including transcription, metabolism and protein folding (Matallana-Surget & Wattiez, 2013). Other oxidative stress-mediated protein modifications include formation of S-S bridges, protein-protein crosslinks, increased susceptibility to proteolysis, fragmentation, and loss of catalytic activity (Vanhaelewyn et al., 2020). Along with proteolysis and fragmentation, regulation of protein and their associated activities are also affected when a bacterial cell is exposed to UVR. Several studies have been conducted which have reported the concentration of certain proteins and amino acids when bacterial cell encountered UVR. According to a study there was a significant increase in the amount of CH-2 rich amino acids in the bacterial cells that were exposed to UVR, and those amino acids included proline, lysine, glutamine, and glutamic acid. Moreover, in a similar study it was reported that there is an upregulation of amino acids including isoleucine and glutamate in *E. coli* when it encounters with UVR (Berney et al., 2006). However, in algae an accumulation of proline has been observed as its counter measure to nullify the impact of UVR (Kováčik et al., 2010). Recently it was suggested that in bacterial cells, UVR affects posttranslational modifications (Matallana-Surget et al., 2012).

2.6.3. Impact of UVR on Lipids of Bacteria

Lipids have the capability to experience photo-oxidation. It has been reported that UVR transforms triplet oxygen into singlet oxygen which have a high degree of reactivity and thus significantly enhance the oxidation of polyunsaturated fatty acids. Moreover, a study

has reported that UVRs are also directly involved in causing oxidative damage to lipids (Merle et al., 2010). Similarly, in another study it was suggested that lipid peroxidation is involved in reducing the fluidity of membrane and can disrupt transmembrane gradient (Sajjad et al., 2018). It has already been discussed that the drastic effects of UVR on cellular structures are mostly associated with reactivity of generated ROS which interact and react with these vital cellular structures including cell membrane. This reactivity of ROS with cell membrane results in decreasing integrity of lipid membrane interfering with its arrangement and can even lead up to the formation of a pore across the membrane (Smith et al., 2009). In a study it was reported that UVR is involved in the induction of peroxidation of lipids that are contributing to the structural assembly of cell membrane and thus mobility of molecules across the membrane is increased (Yeo & Liong, 2013). Moreover, according to a study changes in the lipid bilayer take place when it is exposed to UVR. It was reported that UVR is involved in the induction of oxidative stress. It was also reported that unsaturated fatty acids are more prone to ROS attack as compared saturated fatty acids (Smith et al., 2009). Keeping in mind the reactivity of lipid moieties, a study has suggested that to minimize oxidative damage of bacterial cell, amount of lipoprotein should be reduced in the cell membrane of the bacterial cell because these molecules are more susceptible to UVR (Salmon et al., 1990).

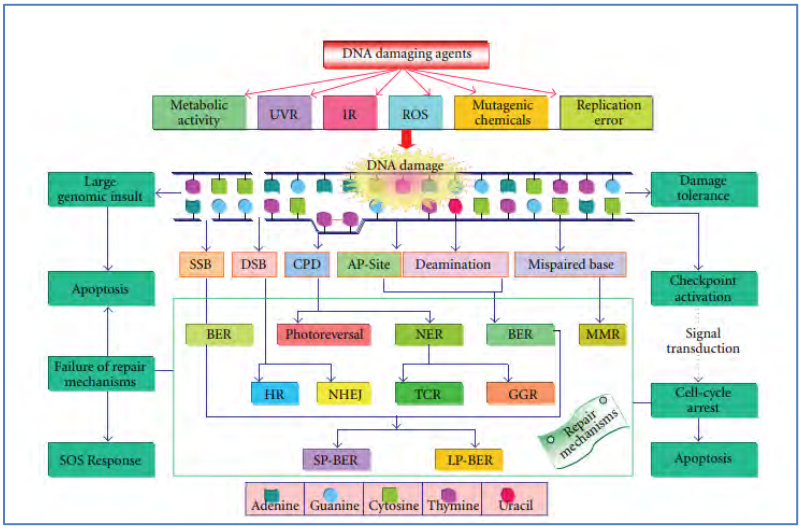

Fig 2: DNA damage and maintenance (Rastogi et al., 2010).

2.7. Radiation Resistance Mechanism in Bacteria

Microorganisms, especially bacteria that is living in extreme environments including areas of intense amounts of UVR have acquired different strategies to keep a pace with extreme nature of the environment and thus have adopted certain measures that include highly regulated protein synthesis machinery (Pérez et al., 2017). Moreover, it was also reported that to hold the line against unwanted harsh environmental conditions, UVR microorganism have acquired different sophisticated survival mechanisms, encompassing genetic changes which further affect changes in protein (Basak et al., 2020). It has been reported that UVR jeopardize the molecular structure of DNA by forming dimers in the DNA strands (Roy, 2017). UVR microorganisms have the capability to effectively repair the damaged DNA through various mechanisms that it has adopted with the passage of time. Some of the mechanisms include Nucleotide Excision Repair (XPF), Fanconianemia Pathway (FA) and Translesion Synthesis (TLS) which were observed in *Dictyostelium discoideum* helping it to withstand high radiations (Kumar et al., 2018).

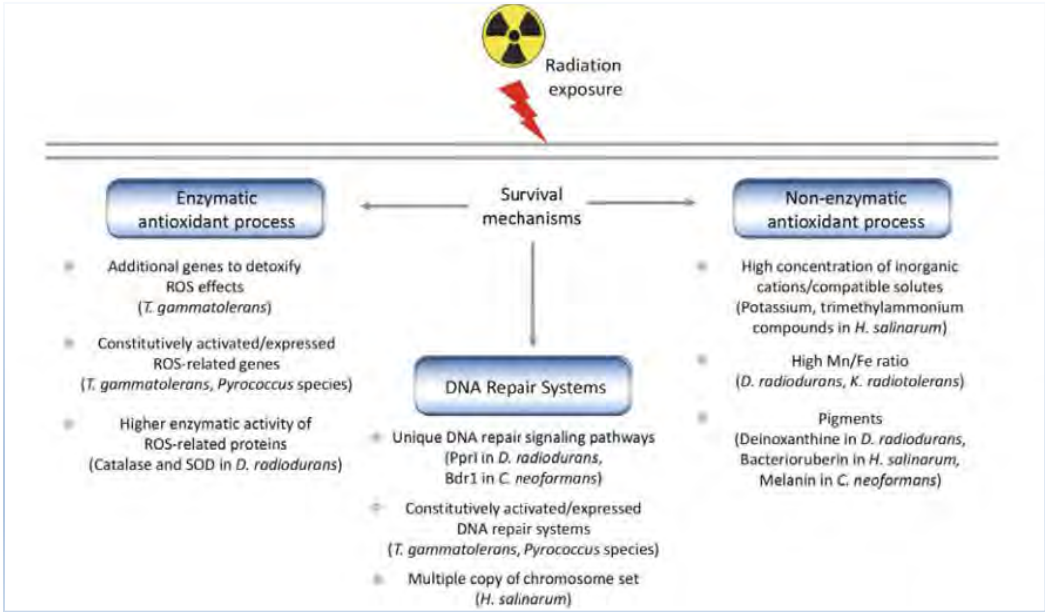

Fig 3: Radiation resistance mechanism of microorganisms (Jung et al., 2017).

2.7.1. Mechanisms of Repairing Damaged DNA

Bacteria have evolved with the passage of time and have acquired several repairing mechanisms to get rid of lesions that are formed upon exposure to UVR. The launching of a specific DNA damage repair pathway depends on the intensity, type and placement of lesions in the genome DNA damage repair pathways have been studied extensively in model organisms including *E. coli* which reported that some specified proteins keep monitoring the whole genome and upon encountering a lesion a series of event is triggered that has a sole purpose of repairing the damage DNA (Essers et al., 2006). Such mechanisms include photoreactivation, removal of lesions by glycosylase enzymes through base excision repair and removal of couple of nucleotides that are containing the lesion through nucleotide excision repair mechanisms.

a. Photoreactivation

As it name suggests, in a photoreactivation process photo reactivating enzymes "Photolyases" are involved in monomerization of cyclobutene ring of pyrimidine dimers using visible energy and hence prevent deleterious effects of UVR on genome. Photolyases have been found in various organisms including bacteria, virus, fungi, plants, and archaea (Essers et al., 2006; Kim et al., 1992). In the photoreactivation process of repairing lesions in the DNA, enzyme binds to CPDs and breakdown the cyclobutane ring between two pyrimidines with the usage of blue light and thus precent any unwanted deleterious impact of UVR on genome (Essen & Klar, 2006; Kim et al., 1992). The underlying mechanism involve capturing of blue light by photolyase which then transfer it to FADH. FADH becomes excited and then donate electrons to pyr-pyr resulting in the breakage of CPD into two units releasing the electron back to flavin molecule (Rastogi et al., 2010).

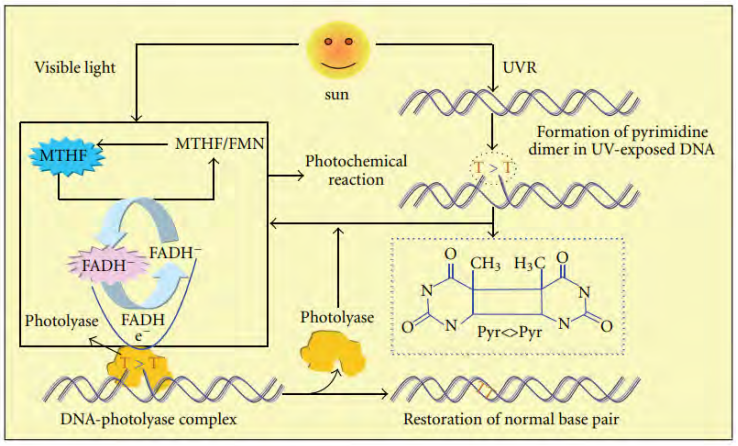

Fig 4: Photoreactivation process of repairing lesions in genome as result of exposure to UVR (Rastogi et al., 2010).

b. Excision Repair Mechanisms of Repairing Damaged DNA

Base excision repair (BER) is one of the predominant genome repairing pathways that is activated upon encountering a lesion that is formed because of hydrolytic deamination. UVR either directly affects the DNA causing formation of dimers or can induce generation of reactive oxygen species (ROS) which damage the genome and triggers BER (Almeida & Sobol, 2007; Seeberg et al., 1995). Several enzymes that are involved in BER have been reported to be existed in *Deinococcus radiodurans* (Zharkov, 2008). Moreover, other studies have also reported the existence of several glycosylase enzymes which play a critical role in ensuring stability of the genome and are involved in BER (Sarre et al., 2019).

Nucleotide excision repair mechanism is one of the most prominent mechanisms of repairing damaged DNA that has resulted upon exposure to UVR. According to various studies, in *Deinococcus radiodurans* the mechanism of NER is mediated by two Ultraviolet endonucleases α and β (Tanaka et al., 2005). Among others UvrABC and UvrDE are the leading NER mechanisms of repairing the damaged DNA (Minton, 1994).

c. Homologous Recombinational Pathway of Repairing Damaged DNA

In the life span of an organism, DNA damage can be caused because of different intrinsic and extrinsic factors (Friedberg et al., 2005). Homologous recombinational pathway is found in all living organisms including bacteria and is involved in repairing damaged DNA. However, it is one of the most widely used DNA damage repairing pathways by UVR resistant microorganisms (Shuryak, 2019). DNA damages that are repaired through this pathway can be in the form of DNA gaps or double strand breaks (Li & Heyer, 2008). In homologous recombination, an intact copy of DNA is required which serve the purpose of a template. In homologous recombination nicking of DNA takes place which leaves a 3-primer single stranded tail. Single stranded tail then invades its homologous chromatids and binds with complementary DNA sequence. This complex activity of seeking and binding with the complementary sequence is performed by Rec A proteins in bacteria (Confalonieri & Sommer, 2011). This is followed by the synthesis of displaced single stranded loop by DNA polymerase enzyme which acts at the attacking 3-primer OH and transfer the information from the homologous chromatids to the broken end (Lusetti & Cox, 2002; Pavlopoulou et al., 2016).

d. Non- Homologous Ends Joining Pathway of Repairing Damaged DNA

Contrary to the HR, non- homologous ends joining process does not require a template and can perform the process of repairing damaged DNA even if only single chromosome copy is present. NHEJ was extensively studied in eukaryotic cell and scientists were of the view that NHEJ is limited to eukaryotic cells unless the revelation of a study that was conducted on *Mycobacterium species*. Various studies that were conducted suggested the involvement of NHEJ repairing pathways in sealing DSBs in liner plasmid DNA in *Mycobacterium tuberculosis* (Gong et al., 2004) and *Mycobacterium Smegmatis* (Gong et al., 2005). In NHEJ Ku proteins are involve which binds to the ends of DSBs in the form of complexes. Then ATP- dependent DNA ligase enzymes are recruited to which perfom the activity of joining the two ends, rendering a repaired DNA (Bowater & Doherty, 2006).

Comparatively, HR is less prone to error than NHEJ because NHEJ is a ligase dependent process. If ligases are recruited directly then the damaged DNA can be repaired without bringing any unwanted consequences for the host cell and if recruitment of ligases is hindered for a time being, then there are great chances of mutagenesis (Shuman & Glickman, 2007).

e. Extended Synthesis Dependent Strand Annealing (ESDSA)

The word "Extended" suggests the capacity of process to repair several DSBs in the cell at one time (Krisko & Radman, 2013). Extended synthesis- dependent strand annealing (ESDA) is a well-organized mechanism that is used by *Deinococcus radiodurans* to repair the damaged DNA. In this mechanism of DNA repairing process chromosomes with homologous fragments that are extending over one another are used as primers as well as a template (Zahradka et al., 2006). Several fragments of DNA are produced upon induction of DSBs in *Deinococcus radiodurans* when it is exposed to UVR. These fragments then invade one another and use homologous regions and are then extended till the end of the template, producing newly synthesized long fragment have 3-prime end. Invasion of fragment is here mediated by several copies of RecA and RadA proteins (Bentchikou et al., 2010). Long fragments that have resulted in ESDA in the initial process will repeat the process, invading other newly synthesized long fragments till the unavailability of any more 3-prime overhangs and in the end all these single stranded fragments are converted into double strands by annealing of fragments with high accuracy which are then matured into circular chromosomes via RecA dependent crossovers (Zhou et al., 2021).

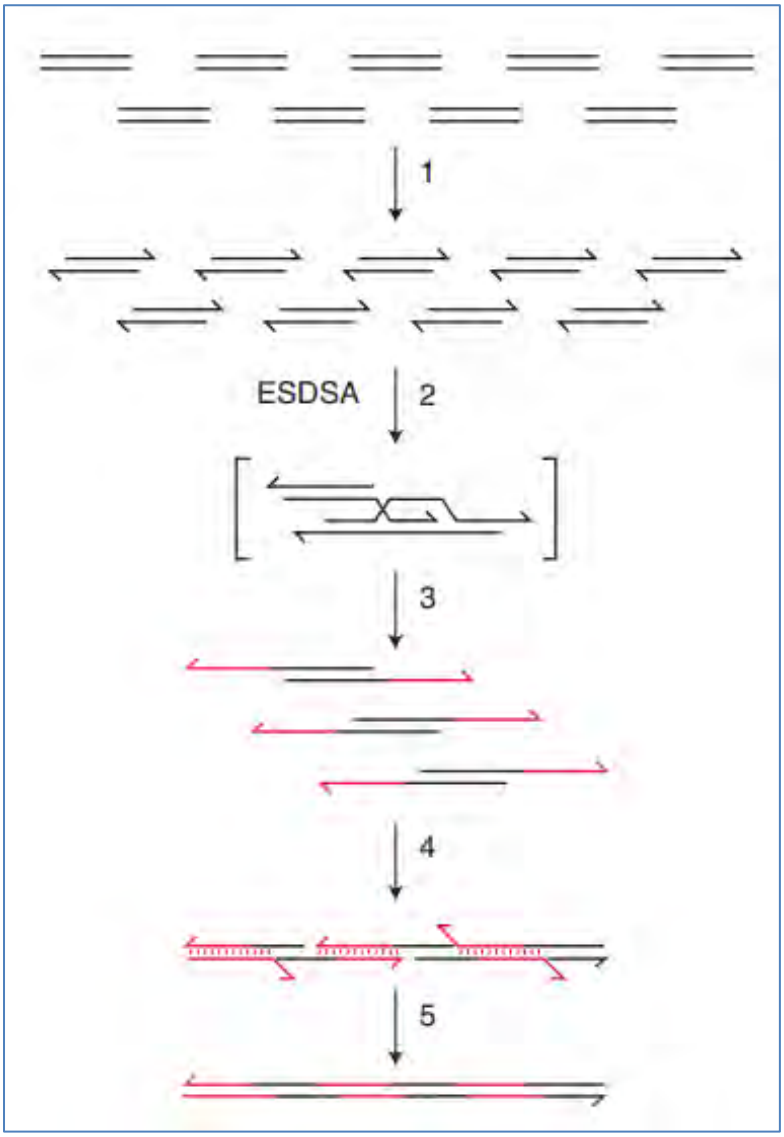

Fig 5: Extended synthesis-dependent strand annealing (ESDSA) mechanism for repairing of damaged DNA in *Deinococcus radiodurans* (Krisko & Radman, 2013).

2.7.2. Multiple Antioxidant Systems in *Deinococcus radiodurans*

Several studies that are conducted on *Deinococcus radiodurans* have reported the existence of multiple genes that are involved in the synthesis of various enzymes including phosphatases and nucleases which result in making a pool of small antioxidant molecules (Daly et al., 2010). Moreover, many antioxidants driving mechanisms have

been reported to exist in *Deinococcus radiodurans* but all of them belong to three independent groups. First group involves enhanced levels of detoxifying activity which is mediated by catalases and superoxide dismutases (Daly et al., 2010; Makarova et al., 2000). Second group involves different metabolic actions that cause a great decrease in reactive oxygen species(Ghosal et al., 2005; Liu et al., 2003). Third group involves decrease in the concentration of proteins that are having Fe-S clusters (Ghosal et al., 2005). An interesting thing that is reported by researchers is that mechanism of antioxidant defense system and the underlying machinery that is running it varies from one bacteria to another. For example, according to a study there is no impact of the inactivation of deinococcal pigment deinoxanthin on the survivability of *Deinococcus radiodurans* in extreme environments (Ji, 2010; Lemee et al., 1997). However, in *Escherichia coli* the synthesis of deinoxanthin has reportedly enhanced its resilience to oxidative damage(Misra et al., 2012).

In addition, a study has suggested the involvement of TCA cycle products in protection against radiations (Daly et al. 2010). Moreover, several other mechanisms also contribute to the ability of *Deinococcus radiodurans* to resist negative impact of radiations which include acquiring of amino acids and peptides from outside and efficient proteolysis (Slade & Radman, 2011). Similar behavior was reported in another study as well where enhanced activity of proteolysis was observed upon exposure to ionizing radiation (Daly et al., 2010).

2.7.3. Role of Extremolytes in Resilience Against UVR

Although extremolytes are not involved directly in the basic essential cellular processes, but they participate in providing a staunch resistance against UVR and their presence do affect the bacterial survival chances (O. Singh & Gabani, 2010). Radioresistant microorganisms produce extremolytes which are potent chemical scavengers and protect the cell against UV radiations and UV- induced ROS (Becker & Wittmann, 2020b). Some of the most prominent extremolytes that are produced by extremophiles across the globe are discussed in the paragraphs that are following.

a. Mycosporine-like amino acids (MAAs)

Mycosporine like amino acids (MAAs) are extracted from red algae, *cyanobacteria* and corals and have shown promising results of protection against UVR induced damage of living cells (Russo et al., 2008; Shick & Dunlap, 2002). A study has reported the isolation of a novel mycosporine which was preventing the negative implications of UVR exposure including pyrimidine dimer formation in cultured human keratinocytes (Russo et al., 2008). Moreover, MAAs are also in involved in scavenging the reactive oxygen species and thus protect the DNA from oxidative damage (Gabani et al., 2012). There are many more other MAAs including palythine, asterina, palythinol which are reported to have a significant role in protection against reactive oxygen species and UVR (Llewellyn & Airs, 2010).

b. Scytonemin

Scytonemin has been isolated from *cyanobacteria* and thanks to its complex ring structure and conjugated double bonds which render it stable against UVR. It was reported that scytonemin levels are raised when cyanobacteria are exposed to a different combination of stresses including UVA radiation and desiccation Various studies have shown that not individual factors, but a combination of several factors induce the synthesis of scytonemin in *cyanobacteria* (Dillon et al., 2002; Fleming & Castenholz, 2008).

c. Ectoine

Ecotine for the first time was isolated from *Ectothiorhodospira halochloris* (Galinski et al., 1985) and after few years derivatives of it was found in *Streptomyces parvulus* (Inbar & Lapidot, 1988). Various studies have reported that ecotine compounds are involved in the protection of halophiles against different kind of environmental stresses including desiccations, high osmotic pressure, and increased levels of radiations by keeping the cellular structures and vital molecules stabilized and protected (Ma et al., 2022). In addition, it has been also reported that ecotine enhance the fluidity of the cell membrane

and thus elevate the signaling mechanism of cell membrane which aid in the overall resilience of a cell against harsh environmental conditions (Harishchandra et al., 2010). Another study has reported that ecotine has the capability to form clusters with water molecules which greatly enhance the protection proteins and prevent their denaturation (Di Gioacchino et al., 2019; Goraj et al., 2019).

Moreover, ecotine also play a decisive role in protecting DNA against radiations. A study has reported that upon exposure to radiations, secondary particles are generated which have the potential to damage DNA. Furthermore, the damaging activity of these particles is enhanced manifold if DNA is hydrated. Ecotine molecules therefore come forward and play their role of protecting DNA. They not only replace water molecules that are surrounding DNA, but also quench reactive oxygen species in the surroundings, rendering the DNA protected (Hahn et al., 2017; Schröter et al., 2017).

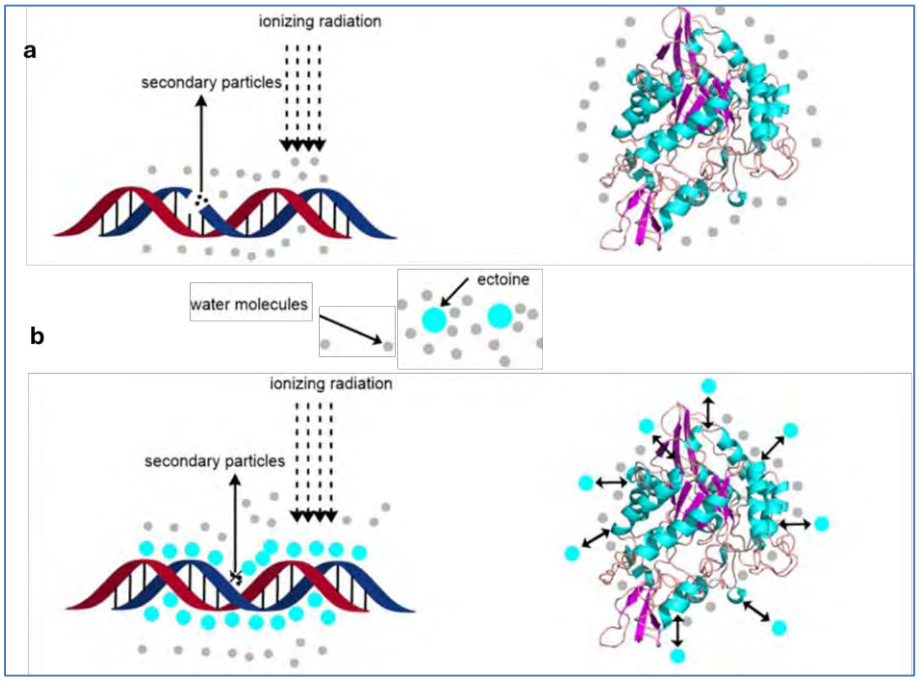

Fig 6: Mechanism of DNA protection by ecotine in the presence of radiation and DNA hydration (Ma et al., 2022).

d. Melanin

Melanin is one of the extremolytes that has been found in animals, plants, fungi, and bacteria (Solano, 2014). The structural complexity of melanin can be associated with its origin. Upon oxidation of phenolic compounds their polymerization takes place. These polymerizations result in irregular aggregations and give rise to the complex structure of melanin (Watt et al., 2009). An interesting thing is that the structural complexity of melanin aid in the resilience of the organisms that is producing it, against the harsh environments where that organism is thriving. Because of its irregular and complex structure, it has distinctive physical and chemical properties which include ROS scavenging capability and interaction with radiation. Melanin aid in protection against radiation either by absorbing radiation at first place and then releasing it in the form of heat or by coping with ROS which are produced due to radiation (Dadachova et al., 2008; Khajo et al., 2011).

2.8. Applications of Extremolytes

Extremophiles are inhabiting those habitats which are not habitable by other organisms. Such extreme habitats include, hot springs, hot and cold deserts etc. Among all the extremophiles, radiophiles are those microorganisms that have the capability to live in environments of high radiation. These microorganisms have developed an intricate survival pattern which not only make them able to survive, but to thrive in such high radiations. One of the survival patterns includes an extensive DNA repair mechanism (Raddadi et al., 2015).

2.8.1. Role of Extremophiles in Agriculture Sector

Extremophiles produce various kinds of extremolytes that have great potential in various industries because of their stability in extreme conditions. Amid this era of intense agricultural activities across the globe, a study has proposed the usage of radiophiles in agricultural sector, enhancing proper management of water by plants during times where availability of water is minimum (Gabani & Singh, 2013). Moreover, Extremophiles have

been suggested to be the best alternative for chemical-based products that are used in agricultural sector and have the capacity to contribute in making agriculture more sustainable and more productive (Chakraborty & Akhtar, 2021; Yadav, 2021).

2.8.2. Potential of Extremophiles and Extremolytes in Food industry

Extremolytes have great potential in food industries. For example, carotenoids have been used to enhance food flavors and to promote antioxidant activities of the food (Saini & Keum, 2019). According to a study, astaxanthin can be used as dietary feed supplement in Antarctica (Torregrosa-Crespo et al., 2018). Moreover, fouling had always been a major problem in food industries, particularly in dairy industries, but according to a study a biosurfactant that is synthesized by *Streptococcus thermophilus* has the potential to prevent spoilage by preventing the growth of other bacteria that may have the potential to cause fouling (Nitschke & Costa, 2007; Satpute et al., 2018).

2.8.3. Role of Extremophiles in Bioremediation of Environmental Pollutants

Greater stability during harsh condition and other such distinguishing qualities make extremophiles able to pose resistant to denaturing agents including detergents and other organic solvents (Gupta et al., 2014). Extremolytes that are produced by thriving extremophiles with their distinguishing features have usages in various industries. These extremolytes can be used against persistent toxic pollutants in the process of bioremediation and can also be used in biomedical industries (Raddadi et al., 2015). For example, according to a study *D. radiodurans* that is carrying a gene from *Pseudomonas putida* have the capability to remove toxic compounds including toluene and chlorobenzene from the surrounding environment (Kumar et al., 2010).

Another study suggested that radiophiles are best suited for a bioremediation role in managing trash that is polluted due to nuclear materials (Appukuttan et al., 2006; Brim et al., 2003). Moreover, similar study reported the treatment of radioactive waste through biosorption by extremophiles (Shukla et al., 2017). Furthermore, *D. radiodurans* has the capability of treating radioactive waste. Recently, the capability of *D. radiodurans* to

treat environmental pollutants is further enhanced by combining it with nanotechnology which has not only made it stable but efficient as well (Irwin, 2020; Jeong & Choi, 2020). Not only this, extremozymes are also widely used in various industrial sectors due their high stability in extreme physiochemical conditions. Extremozymes have been shown to pose significant resistance to variety of stresses. For instance, lipases that are acquired from *Deinococcus radiodurans* showed greater stability in high temperatures and performed their activities in a very efficient manner even in the presence of surfactants and organic solvents (Shao et al., 2014).

2.8.4. Therapeutic Applications of Extremolytes

UVR has the potential to affect skin and has the capability to cause sunburn if the skin is exposed for a short period of time and even has the ability to cause skin cancer if the skin is exposed to it for a longer duration (A. Singh et al., 2021). Extremolytes that are extracted from various extremophiles have great potential in pharmaceutical and biomedical sectors. For example, MAAs have applications in pharmaceutical industries and can be used as a preventative agent against cancers that are induced due to UVR (De la Coba et al., 2009). Moreover, a study has reported that MAAs have the potential to be used in sunscreens because of its vital role in the absorption of UVR (De la Coba et al., 2009). Furthermore, according to a study MAAs, are produced by lichens, fungi, and cyanobacteria when exposed to UV radiation, and are extremely stable at high pH and temperatures, and are primarily used as a natural bioactive ingredient in cosmetic products (Corinaldesi et al., 2017).

Another important extremolyte scytonemin has been termed as a vital candidate for drug development because of its vital role in the synthesis of novel pharmacophore to synthesize protein kinase inhibitors.

In addition, ecotine also has great implications in the therapeutics. According to a study ecotine prevents UVA induced damage of keratinocytes (Buenger and Driller (2004). An invitro study has reported that ecotine prevented human tissues from allergens, UV, and

dryness. Moreover, at pharmaceutical sector ecotine can be used to treat different diseases including Alzheimer's, respiratory related problems and infection of lungs (Ma et al., 2022). Another study has reported the usage of ecotine in preparation which was used to preserve liver during transplantation of liver (Fet et al., 2014).

Moreover, another study has suggested the role of Bacterioruberin extracted from *Rubrobacter radiotolerans* in repairing DNA damage that is induced by ionizing radiations (Asgarani et al. 2000). Furthermore, role of bacterioruberin as preventative agent of human skin has also been suggested because of its involvement In DNA repair mechanisms that are induced by UVR (O. Singh & Gabani, 2010).

2.8.5. Biotechnological Implications of Radiation-Resistant Extremolytes

Recent research in biotechnological sector has explored various applications of UVR resistant microorganisms including their role in production of bioethanol. In a study it was reported that two novel strains *C. cellulans UVP1* and *B. pumilus UVP4* that were isolated, posed significant resistance to UVR and has shown remarkable degradation of cellulose (Gabani et al., 2012).

2.8.6. Applications of Extremolytes in Textile Industries

Synthetic dyes and chemicals that are used in textile industries not only affect health of an individual but also drastically affect finishing of a product and the surrounding environment (Madhu & Chakraborty, 2017). Extremolytes therefore present an efficient, risk free and eco-friendly solution to these problems. Moreover, extremolytes also prevent the degradation of fabric during all the processes that take place in the textile industries. A noticeable feature of extremolytes has been suggested that they can even work in mild conditions and enhance the speed of a reaction by improving enzyme substrate specificity (Hari, 2020; Vashist & Sharma, 2018). Furthermore, bleaching is one of the routine processes that take place in many textile industries and may have negative impact on the environment. According to a study catalase and laccase enzymes that are produced by extremophiles can cleanup the bleach and have the potential to

breakdown H_2O_2 into H_2 and O_2 and thus minimizing the negative impact of bleaching on environment (Arputharaj et al., 2016).

3. Materials and Methods

The current study was conducted in Applied, Environmental and Geo-Microbiology (AEG) Laboratory, Department of Microbiology, Faculty of Life Sciences, Quaid-i-Azam University, Islamabad, Pakistan from February 2023 to February 2024.

3.1. Selection of Site for Sampling

For this study, Cholistan Desert of Pakistan was selected as a site of sampling. It lies between 27º 42’ and 29º 45’ North and 69º 52’ and 75º 24’ East (Arshad et al., 2007). Cholistan desert is a distinctive, unexplored ecological system that is covering an area of 26,300 km^2 in the south of district Bahawalpur, Punjab. It is located at an altitude of 89 m above sea level (Islam et al., 2016) and extends to the Thar desert in Sindh between longitudes 69°52′ to 73°24′ E and latitudes 28°42′ to 29°25′N. Average rainfall ranges between 100 mm to 200 mm.

3.2. Collection of Samples

3.3. Description of Samples

Details of the samples are given in table.

Table 1: Details of sampling.

Sample: Sand Sample	
Location:	Cholistan Desert, Punjab
Temperature:	Mean temperature is 28.33° C.
Isolated strains:	15
Soil Texture	Characterized by high salinity and alkalinity levels
UV-Index	12

3.4. Isolation of Radioresistant Bacteria Through Serial Dilution and Spread Plate Technique

The soil samples were serially diluted in normal saline, 1 gram of each soil sample was taken in 9 ml of normal saline. 5 dilutions (10^{-1} to 10^{-5}) of 1:9 were made from it. 30µl from each dilution was inoculated on basic trypton glucose yeast extract agar (TGY) medium by the spread plate method. Test TGY plates were exposed to UV-B radiation (wavelength 280 nm) for 5 min prior to incubation. Control plates were not UV irradiated. Each sample was UV irradiated in UV chamber (119 × 69 × 52 cm) that was equipped with a 20 W, 280-nm UV light at the top. Incubation was done at 37ºC for 2 to 3 days. 15 strains were isolated from test TGY plates.

3.5. UVR Chamber

UVR chamber was used in the process of isolation of UV resistant bacterial strains. All the TGY plates, having bacterial culture were irradiated in the UV Chamber (119×55×52 cm) supplied with a 20 W, 280 nm UV light at the top of the chamber. The bottom of the chamber is 52 cm far away from the UV light source. All the plates that were exposed to UV radiations were kept in dark to prevent photo-reactivation.

3.6. Primary Screening Strategy

In primary screening strategy of UV resistant bacterial strain isolation, each bacterial strain was streaked on 5 TGY plates which were then exposed to different UV doses, ranging from 1 minute to 5 minutes. After UV exposure, all the plates were covered in newspaper to prevent photoreactivation. All the plates were then incubated at 37°C for 24hrs. After incubation time, those plates were selected for further screening which were having dense growth. The selected strains were further exposed to extended UV doses, ranging from 6 minutes to 10 minutes.

3.7. Secondary Screening Strategy

Strains that shown resistance to extended UV doses were selected for further screening. 24 hours old culture of Strain CH-8, grown in TGY broth, was serially diluted in normal saline up to OD_{600} of 0.08-0.1. 20µl was taken from dilution and was spread on TGY agar plates using spread plate technique. The streaked plates were then exposed to various doses of UV up to 10 minutes and were then incubated at 37°C. After incubation time, colonies of all the plates were counted and survival curve was plotted, based on the survival rate. Survival rate of Strain CH-8 was determined by dividing the number of colonies appearing on UV-irradiated plates by the number of unirradiated culture plates.

Survival = no of colonies on UV irradiated test plates/no of colonies on control ×100

3.8. Radiant Exposure

The UV fluency rate (energy/area/time) to the test strains was calculated by using the following equation in J/m^2.

He = Ee x t in units of J/m^2

Where He is the radiant exposure that is the energy reaches a surface area due to irradiance (Ee) maintained for a time duration (t).

3.8.1. Radiant Exposure Calculation

Test TGY plates were irradiated for 10 minutes before incubation. Each test plate was irradiated in UV chamber (119×55×52 cm), equipped with a 20 W, 280 nm UVB light placed at the top of the chamber.

$$Height\ of\ the\ UV\ chamber\ H = 52cm = 0.52\ \text{m}$$

$$Power\ of\ the\ lamp\ used\ in\ chamber$$

$$= P = 20W$$

$$Length\ of\ the\ UV\ chamber, L = 55cm = 0.55\ \text{m}$$

$$Frequency, F = 50Hz$$

$$Wavelength\ used = \text{ɻ} = 280nm$$

$$Where\ intensity, I = P/A$$

$$Where\ A\ is\ the\ area = 2\Pi dl$$

$$\text{where intensity}, I = \frac{P}{2\Pi dl} = Ee$$

This intensity I is equal to irradiance or exposure per unit area of the plate to UV so

$$I=Ee$$

$$Ee = \frac{P}{2\Pi dl}$$

$$Ee = \frac{20\ w}{2(\frac{22}{7})(0.52)(0.55)}$$

$$Ee = 11.30\ W/m^2$$

Now radiant exposure He=Ee × t

That is the radiant exposure (He), which can be defined as irradiance (Ee) of the plate in time (t)

$$He = 11.30\ (t)$$

$$in\ W/m^2s$$

Where watt $W = Js/m^2$

If the exposure of the respective sample on the plate is 30 sec then,

$$He = 11.30\ Js/m^2\ (30\ s)$$

So, the Radiant exposure is

$$He = 330\ J/m^2$$

3.9. UV resistant Bacterial Strains after Screening

Out of 15 resistant isolates, 8 strains were selected based on their pigmentation and survivability against UV. The selected strains were showing resistance to UV at 280 nm for up to 5 minutes exposure in primary screening at 3390 J/m² radiant energy. Out of these 8 strains, Strain CH-8 shown resilience against UV for up to 10 minutes at 280 nm at UV fluency rate of 6780 J/m² radiant exposure. The strain was also morphologically and microscopically examined.

3.10. Cryopreservation of Bacterial Strains

Strain CH-8 was preserved in glycerol to maintain purity of the strain in viable conditions without any genetic change for the long time. For this purpose, overnight grown culture of the pure bacterial strains was used. 700µl broth of pure strains was transferred in each autoclaved cryovial and mixed with 300µl of 30% glycerol. 30% glycerol (v/v) was prepared by dissolving 30ml of the glycerol in 70ml of distilled water which was then heated at 100ºC in oven for half an hour. Preservation of Strain CH-8 was done in triplicate. The cryovials were shifted to ultra-low temperature freezer at -80ºC and stored for extended time. Viability assessment of thawed preserved cultures was done by

scrapping the bacterial culture with sterile loop and streaking at TGY plates. Streaked TGY plates were incubated at respective temperature and growth was seen after 48 hours incubation.

3.11. Strains Selection

Following a secondary screening process, Strain CH-8 was selected among all the strains for further in-depth investigation due to its remarkable resistance to UV exposure, lasting 10 minutes, and its exceptional black color pigment production capabilities.

3.12. Characterization of Bacteria Exhibiting UV Resistance

3.12.1. Characteristics of a Colony

The UV resistant isolates were cultured on TGY agar plates and subjected to morphological examination, which included both naked eye observations and microscopic analysis using Gram staining. This comprehensive assessment encompassed various aspects of cellular and colony morphology, such as shape, size, elevation, margins, surface, color, pigmentation, opacity, and overall appearance.

3.12.2. Gram Staining

Gram staining is one of the most important procedures in microbiology which helps in the identification of an unknown bacterial strain and was introduced by Danish bacteriologist Hans Christian Gram in 1882. Gram staining of Strain CH-8 involved two major steps: preparation of slide smear and staining.

In the first step which involved the formation of smear, thin smear of Strain CH-8 was prepared by transferring a bacterial colony from the petri plate onto the glass slide which was then spread with the help of a loop which eventually resulted in the formation of a thin film of around 15 mm in diameter. The glass slide was then dried by passing it carefully through flame in a circular pattern to avoid overheating of glass slide. This process resulted in the formation of smear which was ready to be processed further.

Smear formation was followed by staining which involved different steps. In the first step, crystal violet stain was poured over the smear and after 60 seconds glass slide was rinsed gently with water. It was followed by addition of iodine solution which was supposed to fix the crystal violet. After 60 seconds, Iodine was rinsed off with water and few drops of decolorizer (ethanol) was added on the slide. 5 seconds later, decolorizer was poured off and glass slide was rinsed with water. In the final step, counterstaining was done with safranin which was added to the glass slide and then rinsed off with water after 60 seconds. The smear was then air dried and was observed under microscope using oil immersion lens at 100X.

3.12.3. Biochemical Tests

3.12.3.1. Sugar Utilization Tests

a. Citrate Utilization Test

The purpose of citrate utilization test is to determine the ability of an organism to utilize citrate as a carbon source. Formation of alkaline by products will take place if the organism could utilize citrate which will turn color of media from green to blue. For determination of our isolated bacterial strains ability to utilize citrate as a carbon source, Simon citrate agar slants were prepared and were streaked with fresh bacterial culture with the help of loop. 48 hours of incubation time was given and after that slants were observed.

b. Indole Test

Indole test is used to determine the ability of an organism to breakdown amino acid tryptophan and lead up to the formation of indole in the medium. For indole test tryptophan broths were prepared in test tubes. Tubes were then inoculated with a small amount of bacterial culture and was incubated at 37°C for 24 to 48 hours. After incubation time, 3 drops of Kovács reagent were added to the test tube. *Escherichia coli*

was taken as positive control. Appearance of red color ring was the indication of tryptophan hydrolysis and presence of indole in the media.

c. Triple Sugar Iron (TSI)

It is a differential medium that is used to determine ability of organisms to ferment carbohydrate and detect H_2S production. Differentiation is based on the fermentation pattern of lactose, glucose, and sucrose. TSI agar contains three carbohydrates: glucose (0.1%), sucrose (1%), and lactose (1%). Phenol red is the pH indicator. To detect the differential fermentation ability of our isolated bacterial strains, TSI agar slants were prepared and inoculated with fresh bacterial culture with the help of inoculating needle. Bottom of slants were stabbed with needle and upper surface area was streaked in zigzag pattern. Screw caps were loosely placed on the test tubes to allow passage of air. In the end, results were recorded after incubating the test tubes at 37°C for 48 hours.

d. Sulfur Indole Motility (SIM)

Sulfur Indole Motility test is used to determine ability of bacterial strains to produce indole and hydrogen sulphide (H_2S). Motility of bacterial strains is also determined through SIM test. For this biochemical test, SIM agar test tubes were prepared and with the help of sterile inoculating needle single colonies of bacterial strains were picked and straight stabbed in the SIM agar medium. Incubation time of 24 hours was given, and test tubes were kept at 37° C. After incubation time, tubes were observed for H_2S production and motility. To determine the ability of bacterial strain to produce indole, 3 drops of Kovac´s reagent were added to the test tubes after incubation time.

3.12.3.2. Enzymes Tests

a. Amylase Test

Amylase test is used to determine the ability of isolated bacterial strains to breakdown starch into maltose with the help of extracellular alpha amylase enzyme. For this enzyme test, starch agar plates were prepared by adding 2% starch in nutrient agar medium. Plates

were then streaked with isolated colonies of fresh bacterial culture and were kept at 37°C for 48 hours. After incubation time, plates were flooded with iodine solution. Formation of clear zones was observed which was the indication of amylase production by isolated bacterial strains.

b. Protease

Protease test is used to determine the ability of bacterial strain to produce protease enzyme. Protease is a proteolytic enzyme which breakdown protein. For this enzyme test, Casein agar plates are prepared by adding 1% casein in nutrient agar medium. Spot inoculation of isolated bacterial strains was done with the help of sterile inoculating loop and incubation time of 48 hours was given at 37°C. After 48 hours plates were flooded with glacial acetic acid and zone formation was observed. Clear transparent zone formation around the bacterial colonies was the indication of protease production by isolated bacterial strains.

c. Cellulase

Carboxymethyl cellulose (CMC) agar medium is used to screen isolated bacterial strains to produce cellulase enzyme. For this purpose, CMC agar medium was prepared by dissolving 2% CMC in nutrient medium. Plates were spot inoculated with fresh culture of isolated bacterial strains using a sterile inoculating loop and were given incubation time of 48 hours at 37 ºC. After 48 hours, plates were flooded with Congo red solution (1%) and were further given incubation time of 30 minutes. After 30 minutes of incubation, excess Congo red solution was poured off and the plates were washed with 1M NaCl solution. This was followed by the observation of zones formation around the bacterial spots which was the indication of cellulose hydrolysis activity and production of cellulose enzyme by isolated bacterial strains.

d. Catalase

Catalase test is used to screen the isolated bacterial strains to produce catalase enzyme. For this test, isolated bacterial colony were picked from petri plates and were transferred to the glass slide. A drop of 3% H_2O_2 was added to the glass slide, and bubbles formation was observed, which was the indication of the presence of catalase enzyme, produced by isolated bacterial strains.

e. Gelatin Hydrolysis

Gelatin hydrolysis enzyme test is used to screen isolated bacterial strains to produce gelatinase enzyme. For this purpose, nutrient gelatin medium was prepared in test tubes by using 12% gelatin in nutrient agar medium. Test tubes were stabbed with single isolated colony of bacterial strain using needle. One test tube was left uninoculated and was kept as negative control. All the test tubes were incubated at 37°C and were checked daily for liquefaction of gelatin, comparing with liquefaction of negative control.

f. Urease Enzyme Test

Urease enzyme test is used to determine the capability of isolated bacterial strains of hydrolyzing urea to produce ammonia and carbon dioxide. The production of ammonia causes the medium to become more alkaline, leading to a shift in pH, which is indicated by the transformation of phenol red's color from a light orange hue at pH 6.8 to a magenta (pink) shade at pH 8.1. For this enzyme test urease enzyme media was dissolved in distilled water and slants were prepared by first heating the media ingredients to bring it to boil and then upon cooling to 50°C sterile 40% urea solution was added. All the slants were then inoculated with fresh bacterial cultures using a sterile needle. Slants were incubated at 37°C for 48 hours. After 48 hours, all the slants were observed, and results were noted.

3.13. Molecular Identification of Strain CH-8

For molecular identification, DNA of strain CH-8 was extracted using Thermo Scientific GeneJET Genomic DNA Purification Kit. DNA extraction was followed by 16S rRNA sequencing and phylogenetic analysis for which amplification of 16S rRNA gene sequence was performed using universal primers F27: AGAGTTTGATCMTGGCTCAG and R1492: TACGGYTACCTTGTTACGACTT. The amplicon was then sequenced at Macrogen Service Center (Geunchun-gu, Seoul, South Korea). Resultant sequences were computed, using Basic Local Alignment Search Tool (BLAST) at NCBI database for nearest relatives and homologs were analyzed for their phylogeny using Molecular Evolutionary Genetic Analysis (MEGA) 11 (Tamura et al., 2021). Neighbor joining tree was constructed on the basis of the distance matrix.

3.14. Survival Rate of Selected UV resistant Bacterial Strains

3.14.1. Survival Curve Under UV Radiations

To evaluate the potential of Strain CH-8 to withstand extended dose of UVR, Strain CH-8 was grown in TGY broth and was incubated at 37°C in shaking incubator at 130 rpm for 48 hours. Broth was then serially diluted in autoclaved normal saline up to 0.08-0.1 at $O.D_{600}$. 20µl inoculum was taken from the test tube and was spread on TGY agar plates using spread plate method. Total 5 plates were made which were then exposed to UV doses ranging from 2 minutes up to 10 minutes. All the plates were then incubated at 37° C for 48 hours. Control plate of Strain CH-8 was not exposed to UV. In this experiment, *E. coli* was used as control and was processed in the same manner as Strain CH-8. After 48 hours, survival rate of Strain CH-8 and *E. coli* was found by counting colonies on the plate and survival curve was drawn by comparing colony counting of *E. coli* with Strain CH-8.

3.14.2. Survival rate against Hydrogen Peroxide

To evaluate the potential of Strain CH-8 to withstand against hydrogen peroxide, Strain CH-8 was grown in TGY broth and was incubated at 37°C in shaking incubator at 130 rpm for 48 hours. Broth was then serially diluted in autoclaved normal saline up to 0.08-0.1 at $O.D_{600.}$ 1 ml of the cell suspension was the taken in sterile test tubes and treated with 9 ml of different molar concentrations (20mM-100 mM) of hydrogen peroxide. Control of Strain CH-8 was not exposed to hydrogen peroxide. After incubation at 37°C for 1 hour, 20 µl of the treated cell suspension was plated on TGY agar plates by spread plate method. In this experiment, *E. coli* was used as control and was processed in the same manner as Strain CH-8. After 48 hours, survival rate of Strain CH-8 and *E. coli* was found by counting colonies on the plate and survival curve was drawn by comparing colony counting of *E. coli* with Strain CH-8.

3.15. Optimization of culture conditions for Strain CH-8

3.15.1. Temperature Optimization

Strain CH-8 was grown at different temperatures including 30ºC, 37ºC, 40ºC and 45ºC in TGY broths. Broths were then kept in shaking incubator at 130rpm. Optical Density (O.D) was measured at 600 nm wavelength after every 24 hours, using UV-vis spectrophotometer and graphs were plotted.

3.15.2. Media Optimization

Strain CH-8 was grown in different growth media (Nutrient broth, Luria-Bertani broth and TGY broth). Broths were prepared of the growth medium, and Strain CH-8 was inoculated in each of the broth. All the broths were kept in shaking incubator at 130rpm. O.D was measured at 600 nm wavelength after every 24 hours using UV-vis spectrophotometer and graphs were plotted.

3.15.3. pH Optimization

Strain CH-8 was grown at different ranges of pH (4,6,8 and10). TGY broths were prepared for the purpose and desired pH of each broth was set using pH meter. Strain CH-8 was then inoculated in each broth and all the broths were then kept in shaking incubator at 130rpm. O.D was measured after every 24 hours at 600 nm using UV-vis spectrophotometer and graphs were plotted accordingly.

3.15.4. Carbon Source Optimization

Strain CH-8 was grown under provision of different carbon sources in TGY broth. Broths of TGY were prepared using Glucose, Xylose, Mannitol, Lactose and Dextrose as a carbon sources. All the broths were kept in shaking incubator at 130rpm. O.D was measured after every 24 hours at 600 nm using UV-vis spectrophotometer and graphs were plotted accordingly.

3.15.4. Optimum Salt Concentration

Strain CH-8 was grown at different salts concentrations (1% - 8%) in TGY broth medium. Broths were kept in a shaking incubator at 130rpm and O.D was measured after every 24 hours, at 600 nm using UV-vis spectrophotometer and graphs were plotted accordingly.

3.16. Extraction and Purification of Bioactive compounds

For the purification of Intracellular bioactive compounds of Strain CH-8s, Strain CH-8 was grown in 1000ml TGY broth at optimized conditions. Broth was kept in shaking incubator at 130rpm for 5 days. After incubation of 5 days color of broth turned into green from yellow and then further 3 days of incubation time was given which resulted in turning the color of broth from green to black. Broth was then taken out from shaking incubator and bacterial cells were harvested by centrifugation at 4°C, 8000 rpm for 10

minutes. The cell pellets were separated and further processed for the extraction of intracellular extremolytes.

3.16.1. Extraction of Intracellular Extremolytes

In order to extract intracellular extremolytes of Strain CH-8, Strain CH-8 was grown in 1000 ml TGY broth at optimal conditions. After proper incubation time, cells were harvested from the broth and were centrifuged at 8000 rpm for 20 minutes. Cell pellets were collected from the falcon tubes. After that, chilled methanol was added to the pellets and were placed at 4ºC for cold shock treatment for 1 hour. Heat shock was then given to the cells by placing them in water bath at 60°C for 30 minutes. To lyse bacterial cells, glass beads were added to the cell pellets and were vortexed for 15 minutes. To further enhance breakage of bacterial cells, pellets were treated with ultrasonication for 20 minutes. Mixture was then centrifuged at 8000 rpm for 15 minutes and colorful supernatant formation was observed. Supernatant was shifted to glass vials and were left in open and clean area to be air dried. The process was repeated for cell pellets multiple times till the color of supernatant became transparent. After drying, extract weight was found and was noted down.

3.16.2. Solvent Based Partial Purification of Bioactive Compounds

Purification of the intracellular crude extract of Strain CH-8 was carried out through column chromatography. For column chromatography, column was first washed and then packed with silica. Crude extract of Strain CH-8 (976 mg) was then added on the top of silica and then different solvents were passed through the column, starting from non-polar solvents, and then moving towards polar solvents. Fractions of each solvent were collected carefully. Composition of solvents used in the process are given in the table:

Table 2: Composition of mobile phase used in column chromatography.

Strain	Fraction No:	Solvents Composition
Strain CH-8	I-1	Chloroform (100%)
	I-2	Chloroform (50%), n-hexane (50%)
	I-3	Ethyl acetate (100%)
	I-4, I-5, and I-6	Methanol (100%)
	I-7, I-8, I-9, and I-10	Methanol (70%), Distill Water (30%)
	I-11, I-12, and I-13	Methanol (50%), Ethanol (50%)

3.17. Thin Layer Chromatography (TLC)

TLC is an affinity-based method that is used to separate compounds in a mixture using capillary forces. TLC was performed for Strain CH-8 partially purified intracellular extract fractions using combination of mobile phases. Aluminum plates coated with silica were used as a stationary phase. 1 cm line was drawn at the bottom of TLC plates coated with silica with the help of a pencil. Partially purified fractions were dissolved in methanol and were spotted carefully on the bottom of the TLC plate. Spotted TLC plate was then placed vertically in a beaker containing Whatman's filter paper soaked in different mobile phase concentrations. Beaker was then covered with aluminum foil to prevent evaporation. Movement of solvent was started and was keenly observed. When the solvent reached to the top, the plate was removed from the beaker and was allowed to dry. After drying, plates were visualized under UV-lamp and separated compound which appeared as spots were observed and their retention factor (R_f) values were calculated.

3.18. Bioassays of Metabolic Extracts of Strain CH-8

3.18.1. Radical Scavenging Activity by DPPH Assay

DPPH has been widely used and is one of the most popular colorimetric assays to determine radical scavenging activity of extracts. Antioxidant potential of Strain CH-8 metabolic extracts was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay using a method suggested by Hassan with slight modifications (Hassan, 2011). At first, 0.2 mM DPPH solution was prepared in methanol. Then, 1µg/ml of bacterial metabolic extract and 1µg/ml ascorbic acid solution was prepared in Eppendorf tubes. After that, 20µg/ml-100 µg/ml of the bacterial metabolic extract was added to 96 well microtiter plate and 100 µl of DPPH solution (0.2mM) was added to each well and final volume of each well was raised to 200 µl. Ascorbic acid (1µg/ml) was used as positive control and solvents in which extracts were dissolved were used as negative controls. The plate was wrapped in aluminum foil to protect reaction mixture from light as DPPH is light sensitive and was incubated in dark at 37°C for 30 minutes. After incubation absorbance was measured at 517 nm using ELISA microplate reader.

DPPH radical scavenging ability of the extracts was calculated by the following formula:

$$\% \text{ DPPH radical scavenging potential} = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} \times 100$$

3.18.2. Cytotoxic Assay

Cytotoxicity of Strain CH-8 metabolic extract was determined using brine shrimp lethality test (BST) by following protocol described by Meyer, with slight modifications (Meyer et al., 1982). 17g of sea salt and 3mg yeast extract was dissolved in 500ml distilled water in order to make artificial sea water. Water was then shifted to a container and brine shrimp eggs (Artemia salina) were added to it. The eggs were then incubated

for 48 hours under continuous illumination (100-Watt lamp) to hatch. After 48 hours, the nauplii were seen to be released from the eggshells and were rapidly moving in the container. Nauplii were collected using a micropipette and were transferred to glass tube (10 nauplii per glass tube) containing artificial seawater and different concentrations of prepared bacterial metabolic extract ranging from 20µl – 100µl. Cytotoxic effect of extracts was observed after 24 hours by counting number of alive and dead nauplii. Nauplii were termed as alive if they exhibited any movement during 15 seconds of observation. Mitomycin-C was used as positive control and DMSO was taken as negative control. The experiment was repeated three times and mean values were taken.

3.18.3. Anti-hemolytic Activity Assay

For determining the anti-hemolytic activity of Strain CH-8 bacterial extract, anti-hemolytic activity assay was carried out as described by Karim, M.A (Karim et al., 2020). 10 milliliters of blood were taken from a healthy individual in EDTA tube and was centrifuged at 3000 rpm for 10 minutes. The supernatant was thrown away and the pellet was washed two times with 0.2M PBS solution (pH 7.4) and centrifuged at 3000 rpm for 10 minutes. The pellet was the resuspended in normal saline solution (0.9%). Different concentrations of the CH-8 bacterial extract, ranging from 20 to 100 µg /ml were added to 0.4 ml of erythrocyte suspension and was given incubation time of 10 minutes at 37 °C. After incubation time of 10 minutes, 0.2 ml of H_2O_2 was added to the mixture in order to induce oxidative stress. The reaction mixture was then given an incubation time of 3 hours at 37°C and after incubation time, mixture was centrifuged at 3000 rpm for 10 minutes. Absorbance of the mixture was taken at 540nm using UV-Vis spectrophotometer. In this assay ascorbic acid was used as a positive control and DMSO was considered as negative control. Combination of PBS and H_2O_2, without bacterial extract served the purpose of blank.

3.18.4. DNA Damage Prevention Assay

DNA damage prevention potential of Strain CH-8 metabolic extract was assessed using DNA nicking assay as described by (Lee et al., 2002) with some modifications. In this assay, pUC 19 plasmid was used which was extracted from competent bacterial cells of *Escherichia coli*. Reaction mixture was prepared which contained 3µl of plasmid pUC19, 10µl of purified extracts, 4µl of $FeSO_4$ (2mM), 4µl of H_2O_2 (30%) and 4µl of sodium nitroprusside (1M). The reaction mixture was then incubated at 37°C for 1 hour. After incubation time 0.8% agarose gel was prepared and then reaction mixture was added into the wells of solidified gel. Gel electrophoresis was then carried out at 90 V for 1 hour. After 1 hour gel was examined under UV-light illuminator and calls were made for bands presence. In this assay plasmid DNA alone was used as a positive control. Plasmid DNA treated with sodium nitroprusside and hydrogen peroxide was used as a negative control.

3.19. Total Phenolic Content

Total phenolic content of Strain CH-8 crude extract (Intracellular) was found using Folin ciocalteau (Fc) reagent by following procedure as described by (Kaur & Kapoor, 2002) with little changes. FC reagent's working solution was prepared in the ratio of 1:1 with distilled water. Then, 1 ml of the crude extract was added into the autoclaved test tubes. In this experiment of determining the total phenolic content of crude bacterial extract, Gallic acid was used as a standard and different concentration of Gallic acid (50 µl- 500 µl) were taken in test tubes. This was followed by the addition of 800 µl distilled water. The test tubes were thoroughly shaken to mix the reaction mixture. 1.5ml FC reagent was added to all the test tubes and were vortexed. The reaction mixtures in test tubes were then given incubation time of 15 minutes and then 1 ml of 20% Na_2CO_3 solution was added to the test tubes, containing the reaction mixture. Test tubes were then left in dark conditions for 1 hour and after 1 hour absorbance was measured at 710nm against the blank which contained all the chemical ingredients of the reaction except standard (gallic acid) or sample (crude extract). Total phenolic content was calculated from the calibration

curve and the outcomes were presented as milligrams of gallic acid equivalent per gram of dry weight.

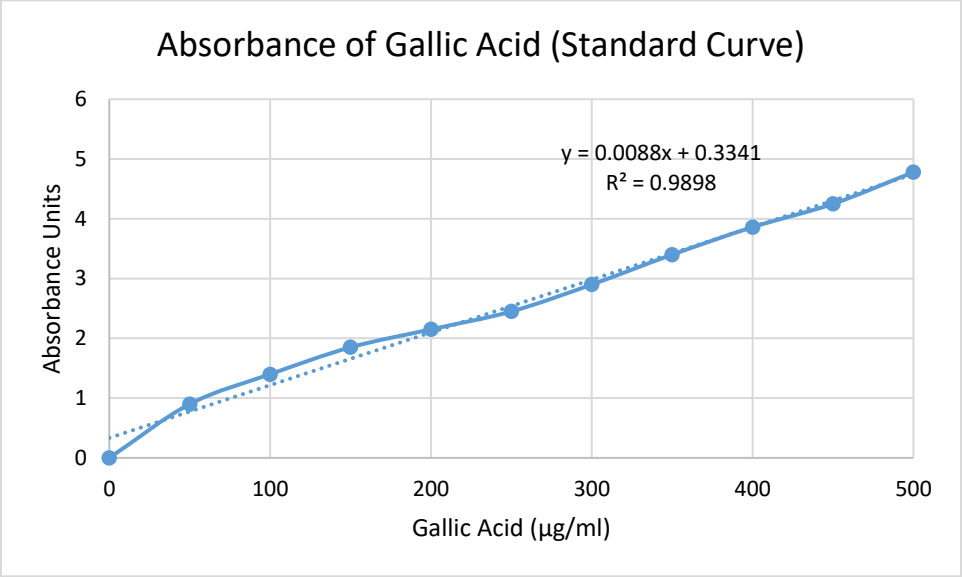

Fig 7: Gallic acid standard curve.

3.20. Total Flavonoid Content

The total flavonoid content of Strain CH-8 crude metabolic extract was determined by using aluminum colorimetric chloride assay (Fattahi et al., 2014) with some modifications. In this process, CH-8 bacterial crude extracts (Intracellular) were taken in test tubes and were diluted with 800 µl of methanol. Quercetin was used as a standard and different concentrations of Quercetin ranging 10 µl - 100 µl were taken in test tubes. Volume of all test tubes were made uniform (1000 µl) by addition of methanol. All the tubes were then vortexed and then 1ml of 5% $NaNO_2$ was added to all the test tubes. After 5 minutes, 1 ml $AlCl_3$ (10%) was added and then after 6 minutes, 2 ml of NaOH (1 M) was added. All the reaction mixtures, in the test tubes were shaken and were mixed thoroughly and were then given an incubation time of 45 minutes in the dark at room temperature. After incubation time, absorbance of all the reaction mixtures was measured at 510 nm. Methanol was used as blank in the process. Total flavonoid content of the

extracts was expressed as percentage of quercetin equivalent per 100 g dry weight of sample.

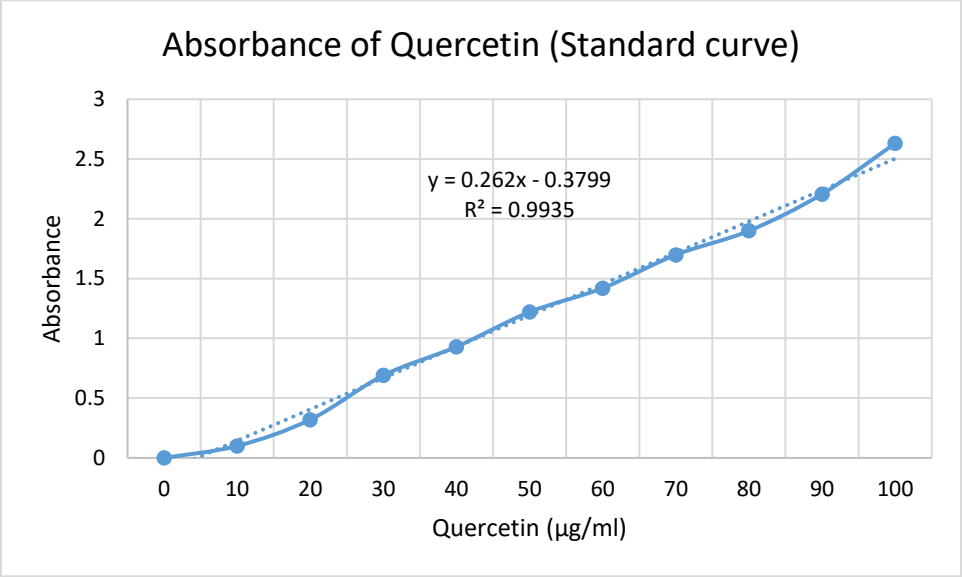

Fig 8: Standard curve of quercetin.

3.21. Antibacterial Activity Assay of Strain CH-8 Metabolic Extract

Antibacterial activity of Strain CH-8 metabolic extract was determined of crude and purified extracts. Activity of the crude extract was checked against two gram negative and two-gram positive bacterial strains. Gram negative bacterial strains were *Pseudomonas aueruginosa* and *Escherchia coli* while gram positive bacterial strains were *Staphylococcus aureus* and *Bacillus subtilis*. However, antibacterial activity of purified extract was checked against *E. coli* and *S. aureus.*

For determination of antibacterial activity of bacterial extract agar well diffusion method was used. For this purpose, O.D of the bacterial strain was set according to McFarland standard (O.D 0.08-1.00). MHA plates were already prepared, and lawns of bacterial strains were made on each plate and then wells were created using sterile glass borer. Bottom of each well was sealed with a small amount of soft agar and then 20 µl of bacterial extract was added in each well. In this assay, DMSO was used as negative

control and antibiotic (Gentamycin) was used as positive control. All the plates were then kept at 37°C and were given an incubation time of 24 hours. After 24 hours, antibacterial activity of the bacterial extract was determined by measuring the diameter of zone of inhibition of bacterial extract, using scale.

3.22. Characterization of Purified Extract of Strain CH-8

3.22.1. Fourier Transform Infra-Red (FTIR) Analysis of Purified Fraction

FTIR is used to monitoring the variations in the functional groups and characterization of the molecular structure of the compound by spectral location of their infra-red absorption. Sample of Strain CH-8 intracellular purified fractions (I-8 and I-12) were subjected to Fourier transform Infra-Red spectrometry analysis. Sample was placed over ZnSe crystal and measurement was taken over the wavelength range (400 to 4000 cm^{-1}).

4. Results

4.1. Isolation of UV resistant Bacteria

UV resistant strains were isolated after exposing all the isolated strains to different UV-doses. Time of exposure ranged from 3 minutes to 10 minutes and UV dose ranged from 2.034×10^3 to 6.780×10^3 J/m^2. Total eight isolates managed to survive UV-dose of 2.034×10^3 J/m^2 and upon subsequent exposure of these strains to further higher doses of UV, two strains managed to survive at UV energy dose of 6.780×103 J/m^2. Details of time duration of UV exposure of each isolated strain is given in table 5.

All the isolates were also examined morphologically and microscopically and among all isolated strains, Strain CH-8 was then selected for this study because of its great resistance to higher doses of UV and its distinctive black pigmentation.

Table 3: UV resistant bacterial strains selection after primary and secondary screening.

Time of Exposure	**Radiant Exposure** **He = Ee × t (J/m²)**	**UV Resistant Isolates**
3 minutes	2.034×10^3 J/m²	CH-1, CH-2, CH-3, CH-4, CH-5, CH-6, CH-7, CH-8
5 minutes	3.390×10^3 J/m²	CH-1, CH-2, CH-3, CH-4, CH-5, CH-6, CH-7, CH-8
10 minutes	6.780×10^3 J/m²	CH-8, CH-1

4.2. Morphological and Microscopic Examination of UV resistant Bacterial Isolates

All the isolated were observed through naked eyes and distinguishing morphological features of their colonies were noted down. For microscopic examination, Gram reaction method was used. Complete details of colony morphology are given in table 4.

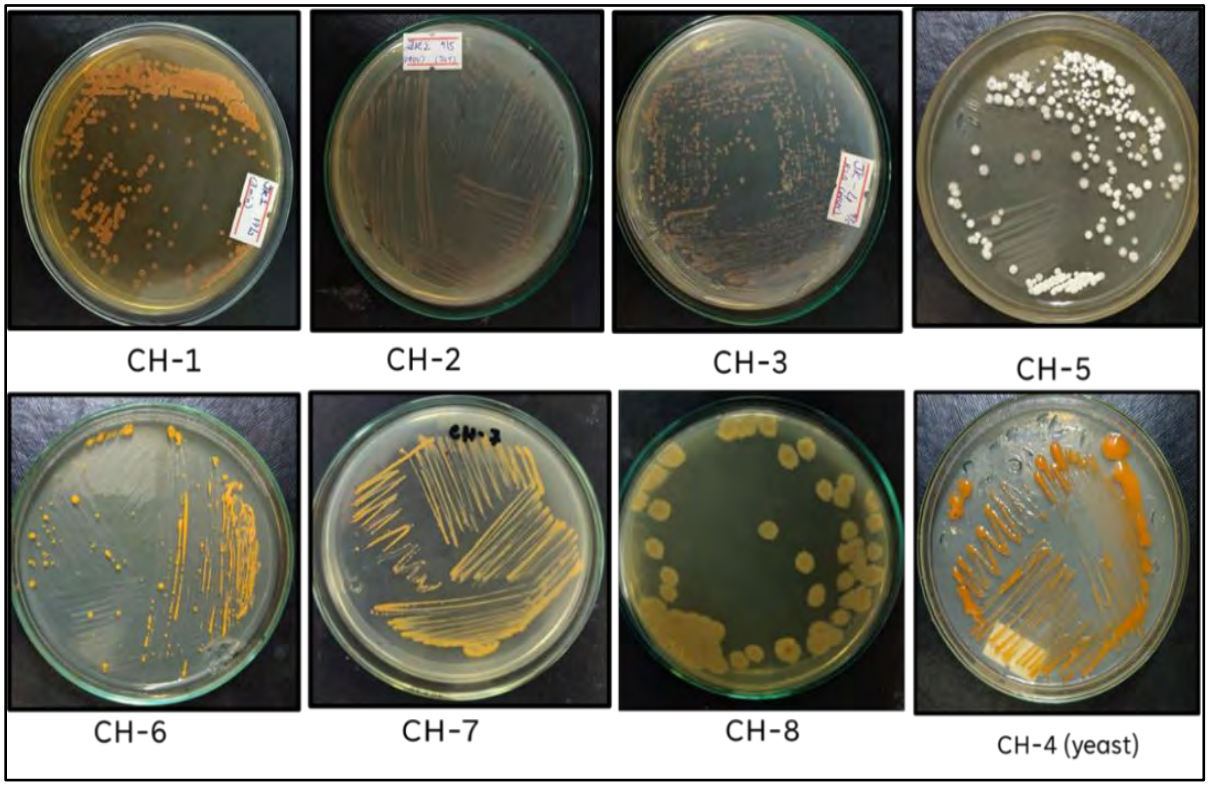

Fig 9: Colony Morphology of UV- resistant Isolates.

Table 4: Morphological characterization of UV resistant isolated bacterial and fungal strain.

Strains	Morphology
CH-1	Medium sized, white colored, opaque, dry, and slightly raised colonies having circular margins.
CH-2	Punctiform, yellow colored, opaque, flat, smooth, and shiny colonies having circular margin.
CH-3	Small sized, flat orange colored, translucent rough colonies having circular margins.
CH-4	Large sized, orange-colored smooth colonies having shiny mucoid surface and circular margins.
CH-5	Medium sized white colored slightly raised colonies having rough and dry surface and circular margins.
CH-6	Small sized, orange-colored flat colonies having rough surface and circular margins.
CH-7	Medium sized, orange-colored raised colonies having mucoid surface and circular margins.
CH-8	Large sized, brown colored flat colonies having rough surface and irregular margins.

4.2.1. Strain Selection and Identification

Among all the isolated bacterial strain, Strain CH-8 was selected because of its ability to survive against the extended dose of UV and its black color pigmentation.

Fig 10: Colony morphology of strain CH-8.

4.2.2. Microscopic Examination of Strain CH-8

After performing Gram staining for Strain CH-8. It appeared as gram positive rod as can be seen in figure 11.

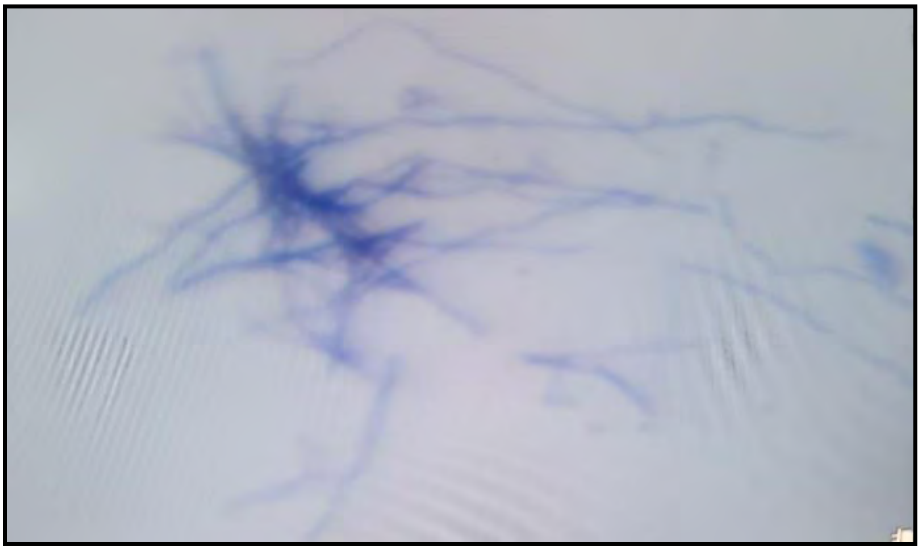

Fig 11: Gram Staining results of strain CH-8.

4.2.3. Biochemical Characterizations

Various sugar utilization and enzymes production tests of Strain CH-8 were performed for its biochemical characterizations.

a. Sugar Utilization Test

Through sugar utilization test, ability of strain CH-8 to ferment sugar was assessed through different biochemical tests including Triple sugar iron test, Simon citrate test, Sulfur indole motility test and Indole test.

b. Enzymes Test

Ability of strain CH-8 to produce enzymes was assessed through catalase, cellulase, amylase, protease, gelatinase, and urease tests. Enzymes production tests results of strain CH-8 are given below in the table 6.

Table 5: Sugar utilization tests of strain CH-8.

Bacterial Strain	Citrate	Indole	Triple Sugar Iron	SIM
CH-8	Positive	Negative	Alkaline (red) Slant /Acidic (yellow) butt, No H_2S or H_2 production	No Sulfur Production, No Indole Production, No motility

Table 6: Enzymes tests of strain CH-8.

Bacterial Strain	Catalase	Cellulase	Amylase	Protease	Gelatinase	Urease
CH-8	(+)	(-)	(+)	(+)	(-)	(-)

4.2.4. Molecular Identification and Evolutionary Relationship of Strain CH-8

Molecular identification and phylogenetic analysis of strain CH-8 was done by sequencing the amplicon and then constructing phylogenetic tree following Neighbor-Joining approach (Saitou & Nei, 1987) which showed 98.85% homology of Strain CH-8 with *Streptomyces sp.* TRM46222. The optimal tree is shown in figure 12. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum composite Likelihood method (Tamura & Nei, 1993) and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotides sequences. All ambiguous positions were removed for each sequence pair (pairwise detection option). There were a total of 1329 positions in the final dataset. Final evolutionary analysis was conducted in MEGA11 (Tamura et al., 2021).

4.3. Survival Rate of Selected UV resistant Bacterial Strains

4.3.1. Survival Curve Under UV-radiation

To evaluate the potential of Strain CH-8 to withstand extended dose of UVR, Strain CH-8 exposed to UV doses ranging from 2 minutes up to 10 minutes. In results it was found

that Strain CH-8 showed survivability of 71.25% at 1356 J/m^2, 60% at 2712 J/m^2, 46.87% at 4068 J/m^2, 32.12% at 5424 J/m^2, and 26% at 6780 J/m^2.

4.3.2. Survival Rate Under Exposure to Hydrogen Peroxide

To evaluate the potential of Strain CH-8 to withstand against hydrogen peroxide, Strain CH-8 was treated with different molar concentrations of hydrogen peroxide (10-50 mM). In results it was found that Strain CH-8 had 78% survivability at 10 mM concentration of H_2O_2, 48% survivability at 30 mM concentration of H_2O_2 and 9% survivability at 50 mM concentration of H_2O_2.

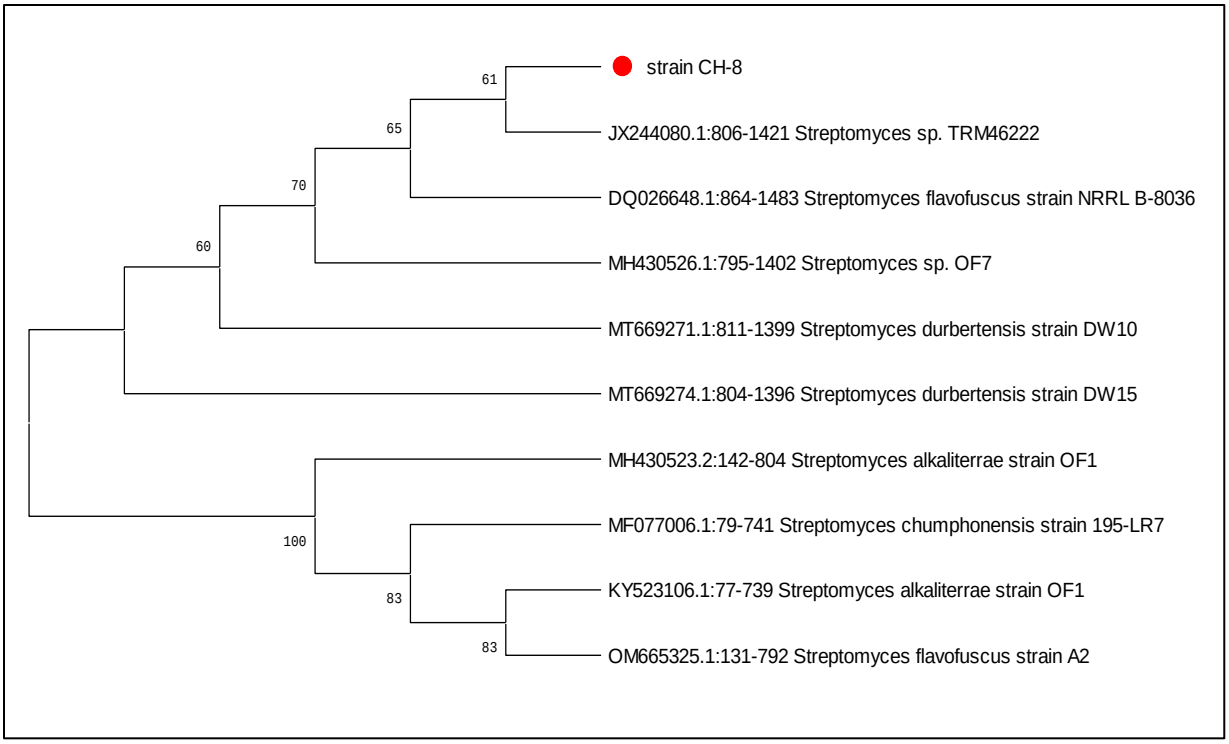

Fig 12: Phylogenetic analysis of strain CH-8.

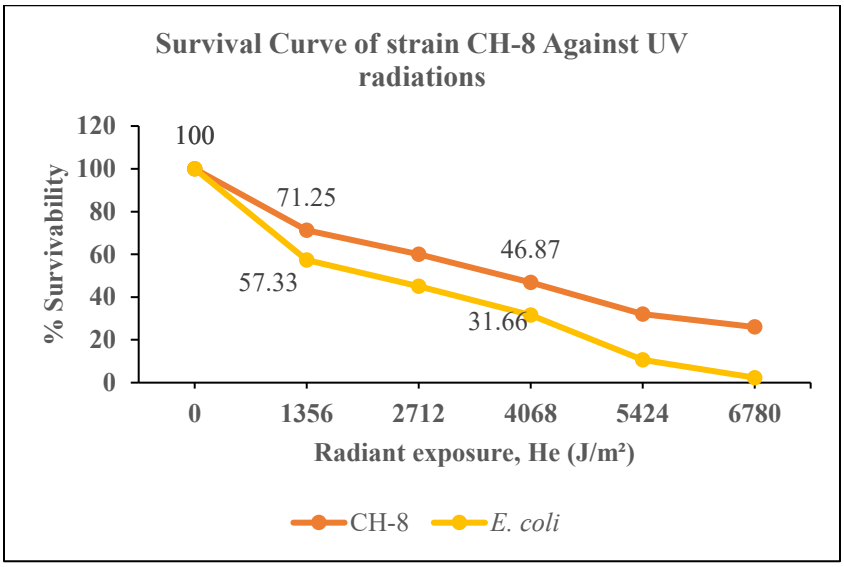

Fig 13: Percentage survivability strain CH-8 against UV-radiation in comparison to *E. coli.*

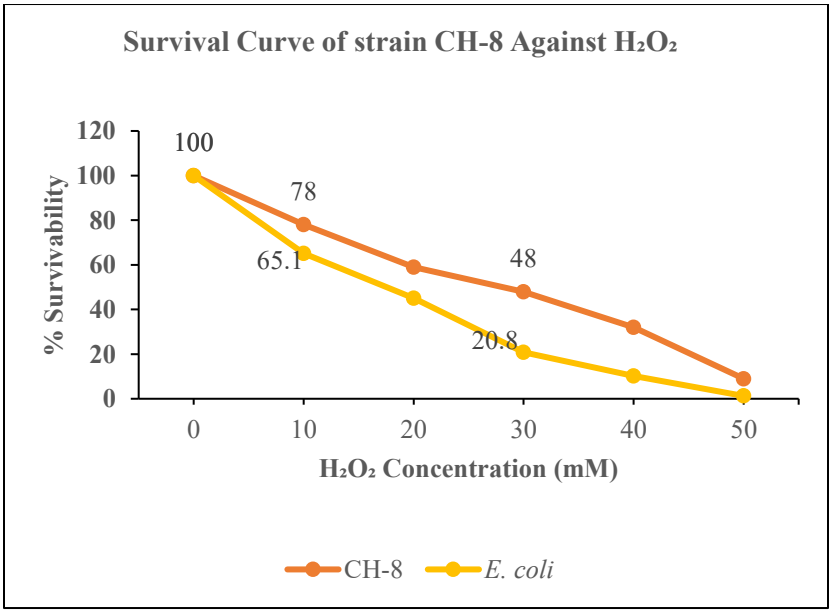

Fig 14: Percentage survivability of strain CH-8 against H_2O_2 in comparison to *E. coli.*

4.4. Optimization of culture conditions for *Streptomyces sp.* strain CH-8

4.4.1. Temperature Optimization

Temperature of *Streptomyces sp.* strain CH-8 was optimized by allowing *Streptomyces sp.* strain CH-8 to grow in TGY broth at different temperatures (30ºC, 37ºC, 40ºC and 45ºC). O.D was taken after every 24 hours in triplicate and graphs were formulated. It was observed that *Streptomyces sp.* strain CH-8 exhibited maximum growth at 37ºC as given in the figure 15.

4.4.2. Media Optimization

Streptomyces sp. strain CH-8 was grown in different growth media (Nutrient broth, Luria-Bertani broth and TGY broth). O.D was taken after every 24 hours, and it was noted that the *Streptomyces sp.* strain CH-8 grown well in TGY medium as shown in figure 16.

4.4.3. pH Optimization

Streptomyces sp. strain CH-8 was grown at different ranges of pH (4, 6, 8, and 10) in TGY medium. O.D was taken after every 24 hours and graphs for different pH were plotted. It was observed that *Streptomyces sp.* strain CH-8 give maximum growth at 8 pH as shown in figure 17.

4.4.4. Carbon Source Optimization

Streptomyces sp. strain CH-8 was grown under provision of different carbon sources in TGY broth. Different sources of carbon were provided including Glucose, Xylose, Mannitol, Lactose and Dextrose. O.D was taken after every 24 hours and graphs were designed accordingly. It was noted that *Streptomyces sp.* strain CH-8 favor Lactose as carbon source Figure 18.

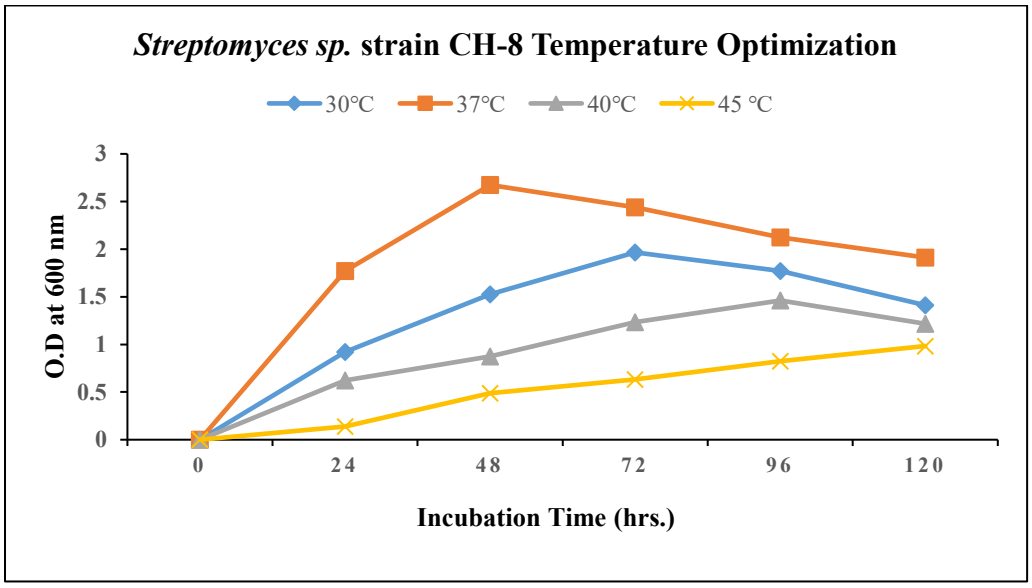

Fig 15: Effect of temperature on growth of *Streptomyces sp.* strain CH-8.

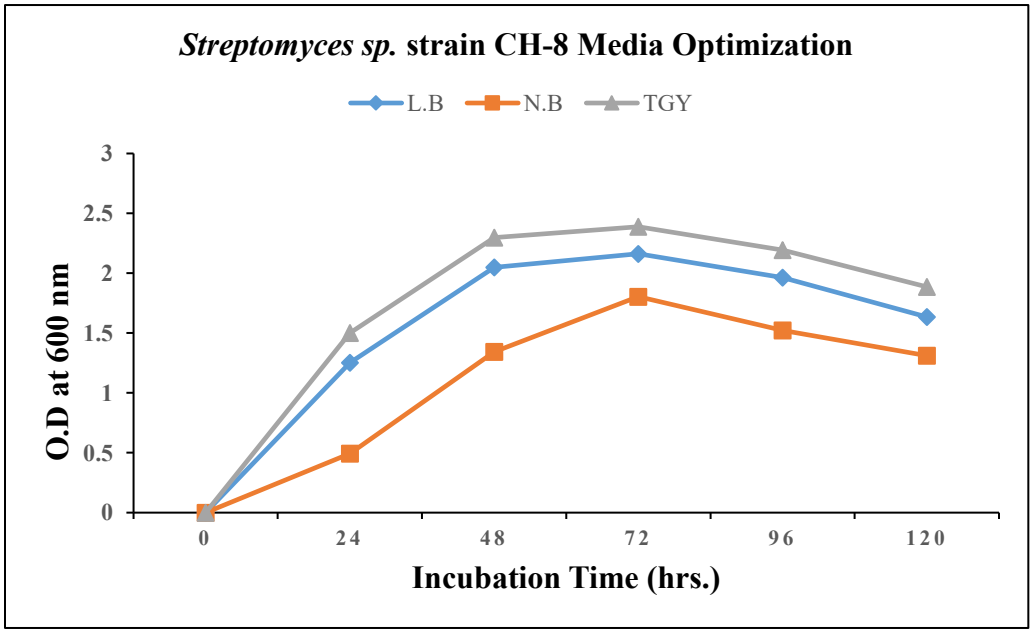

Fig 16: Effect of various media on growth of *Streptomyces sp.* strain CH-8.

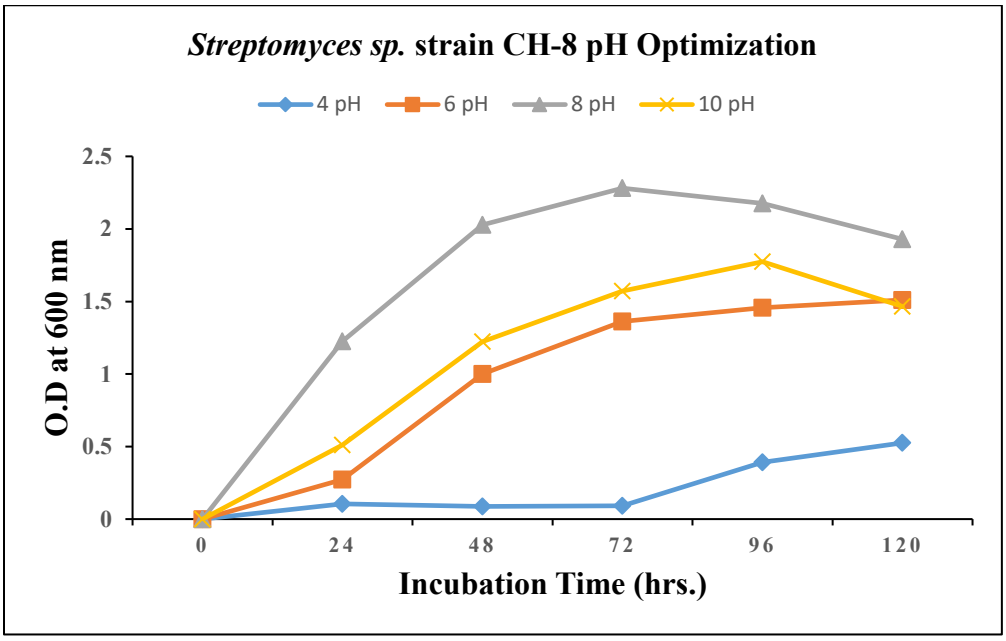

Fig 17: Effect of pH on growth of *Streptomyces sp.* strain CH-8.

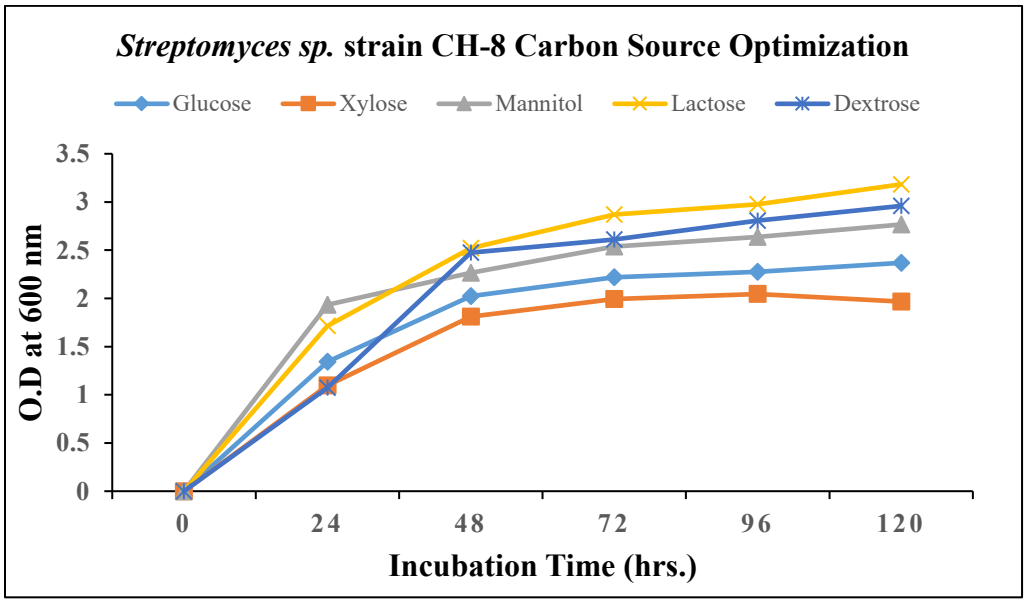

Fig 18: Effect of various carbon sources on the growth of *Streptomyces sp.* strain CH-8.

4.4.5. Optimum Salt Concentration

Streptomyces sp. strain CH-8 was grown at different salts concentrations (1% - 8%) in TGY broth medium. O.D was taken after every 24 hours, and it was observed from the graph that *Streptomyces sp.* strain CH-8 grows well at salt concentration of 4% as shown in Figure 19.

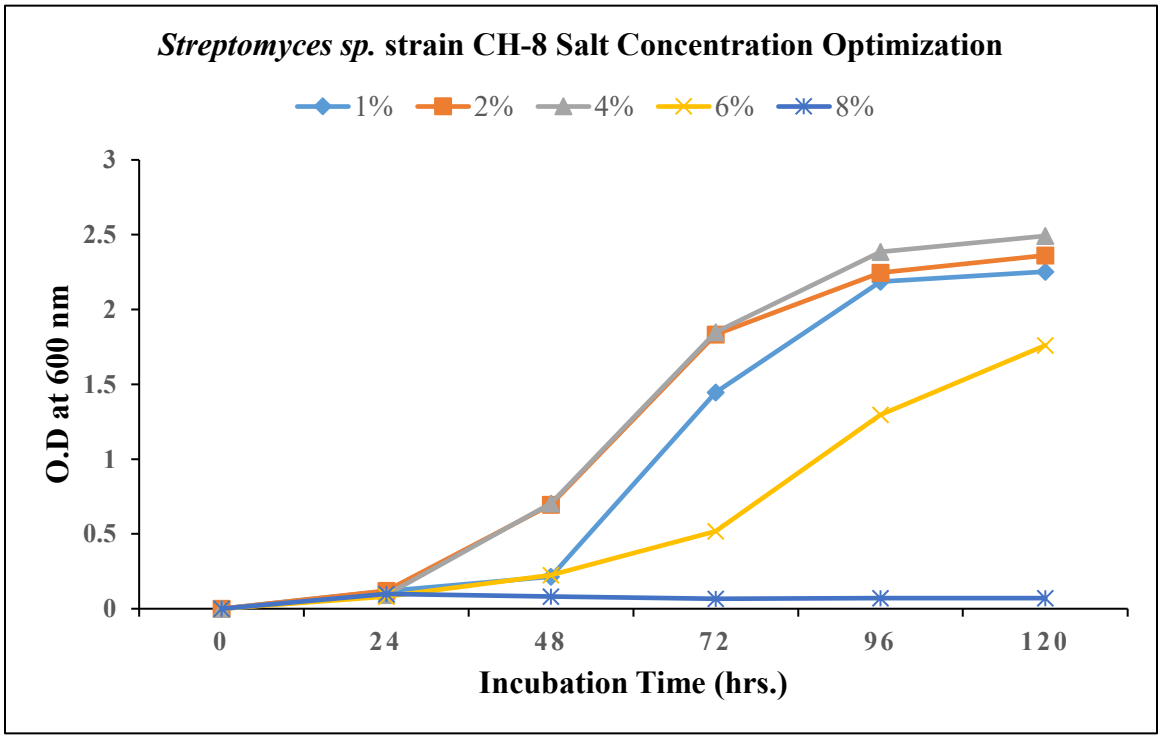

Fig 19: Effect of different salt concentrations on the growth of *Streptomyces sp.* strain CH-8.

4.5. Solvent Based Partial Purification of Bioactive Compounds

Intracellular metabolite extract of *Streptomyces sp.* strain CH-8 was partially purified through column chromatography using different solvents as mobile phase. Details of mobile phase composition and amount of purified extract is given in table 7. However, partially purified fractions that were collected are given in fig 20.

Table 7: Intracellular metabolic extracts from *Streptomyces sp.* strain CH-8 with their solvents.

Strain	Fraction No:	Solvent Fractions	Purified Extract Amount
Strain CH-8	Intracellular Metabolic Extract-5	Methanol (100%)	60mg
	Intracellular Metabolic Extract-6	Methanol (100%)	30mg
	Intracellular Metabolic Extract-7	Methanol (70%), Distill Water (30%)	30mg
	Intracellular Metabolic Extract-8	Methanol (70%), Distill Water (30%)	50mg
	Intracellular Metabolic Extract-9	Methanol (70%), Distill Water (30%)	130mg
	Intracellular Metabolic Extract-10	Methanol (70%), Distill Water (30%)	130mg
	Intracellular Metabolic Extract-11	Methanol (50%), Ethanol (50%)	150mg
	Intracellular Metabolic Extract-12	Methanol (50%), Ethanol (50%)	90mg
	Intracellular Metabolic Extract-13	Methanol (50%), Ethanol (50%)	40mg

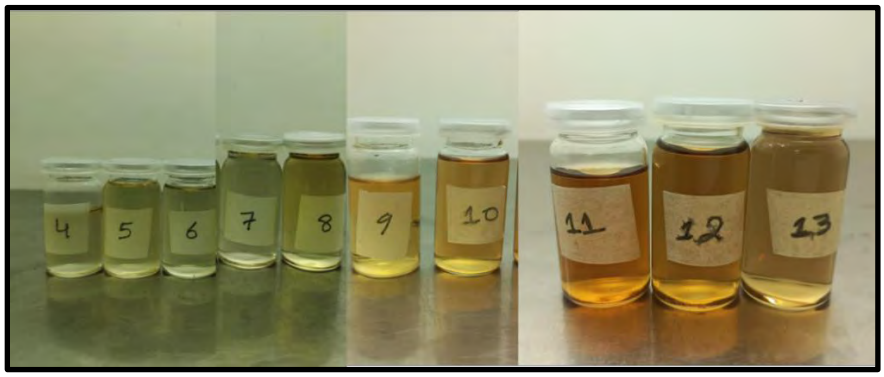

Fig 20: Solvent based partially purified intracellular metabolite extracts from *Streptomyces sp.* Strain CH-8.

4.6. Thin Layer Chromatography of Purified Fractions

TLC of partially purified intracellular extract fractions of *Streptomyces sp.* strain CH-8 was performed using silica gel as a stationary phase and combination of chloroform and methanol (9:1) as mobile phase. R_f values of bands of different compounds are given in table 8 and their movement can be seen in fig 21.

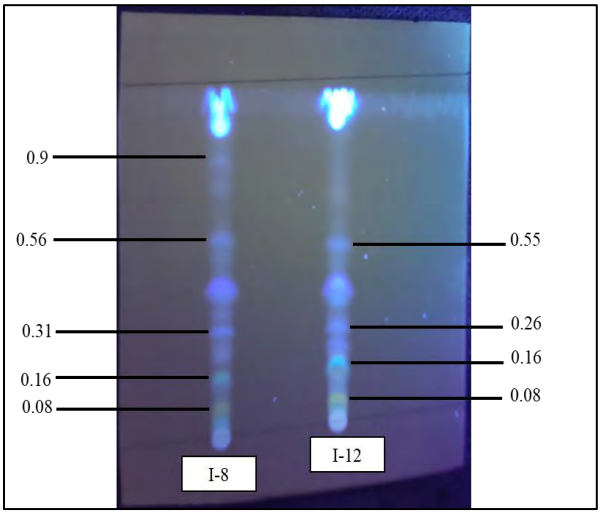

Fig 21: TLC plates showing bands of fractions I-8 and I-12 of intracellular extract of *Streptomyces sp.* strain CH-8 compounds under UV 254 and 365 nm.

Table 8: Details of mobile phases used for each fraction and Rf values of bands, appeared on TLC plate.

No. of Fraction	Mobile Phase (Solvents)	Rf Value
I-8	Chloroform: Methanol (9:1)	0.08, 0.16, 0.26, 0.55
I-12	Chloroform: Methanol (9:1)	0.08, 0.16, 0.31, 0.56, 0.9

4.7. Total Phenolic Content

Total phenolic content of *Streptomyces sp.* strain CH-8 crude extract (Intracellular) was found using Folin ciocalteau (Fc) reagent. Total phenolic content was calculated from the calibration curve and the outcomes were presented as milligrams of gallic acid equivalent per gram of dry weight. Total phenolic content of *Streptomyces sp.* strain CH-8 crude extract (intracellular) was 149 mg GAE/g DW.

4.8. Total Flavonoid Content

The total flavonoid content of *Streptomyces sp.* strain CH-8 crude extract (Intracellular) was determined by using aluminum colorimetric chloride assay. Total flavonoid content of the extracts was expressed as percentage of quercetin equivalent per 100 g dry weight of sample. Total flavonoid content of *Streptomyces sp.* strain CH-8 intracellular crude extract was 2.082 mg QE/g DW.

4.9. Bioassays of Metabolic Extracts of *Streptomyces sp.* strain CH-8

4.9.1. Radical Scavenging Activity by DPPH Assay

Antioxidant potential of intracellular crude extract and purified fractions from *Streptomyces sp.* strain CH-8 was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. In case of crude extract 62% scavenging activity of DPPH free radical was observed. Whereas I-8 (Water-Methanol) fraction showed 77% and I-12 fraction (Methanol-Ethanol) showed 73.62% scavenging activities of DPPH free radicals.

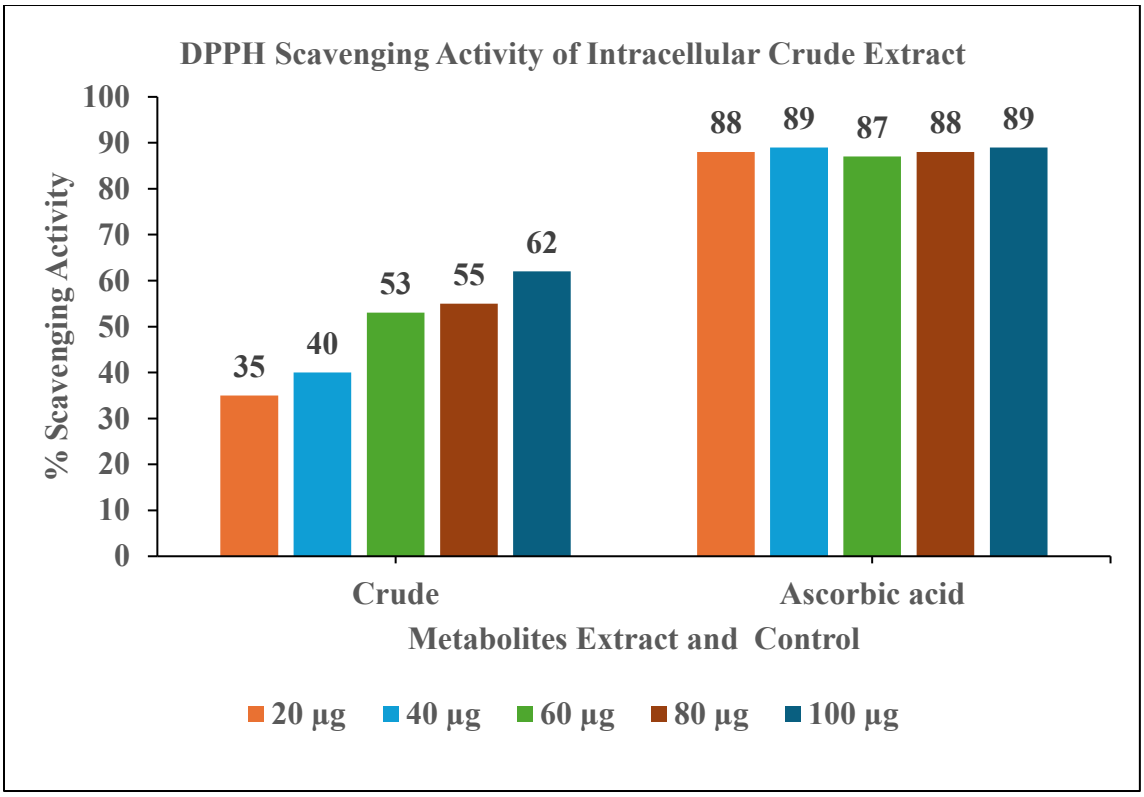

Fig 22: DPPH Scavenging activity of intracellular crude extract and fractions I-8 and I-12 of *Streptomyces sp.* strain CH-8.

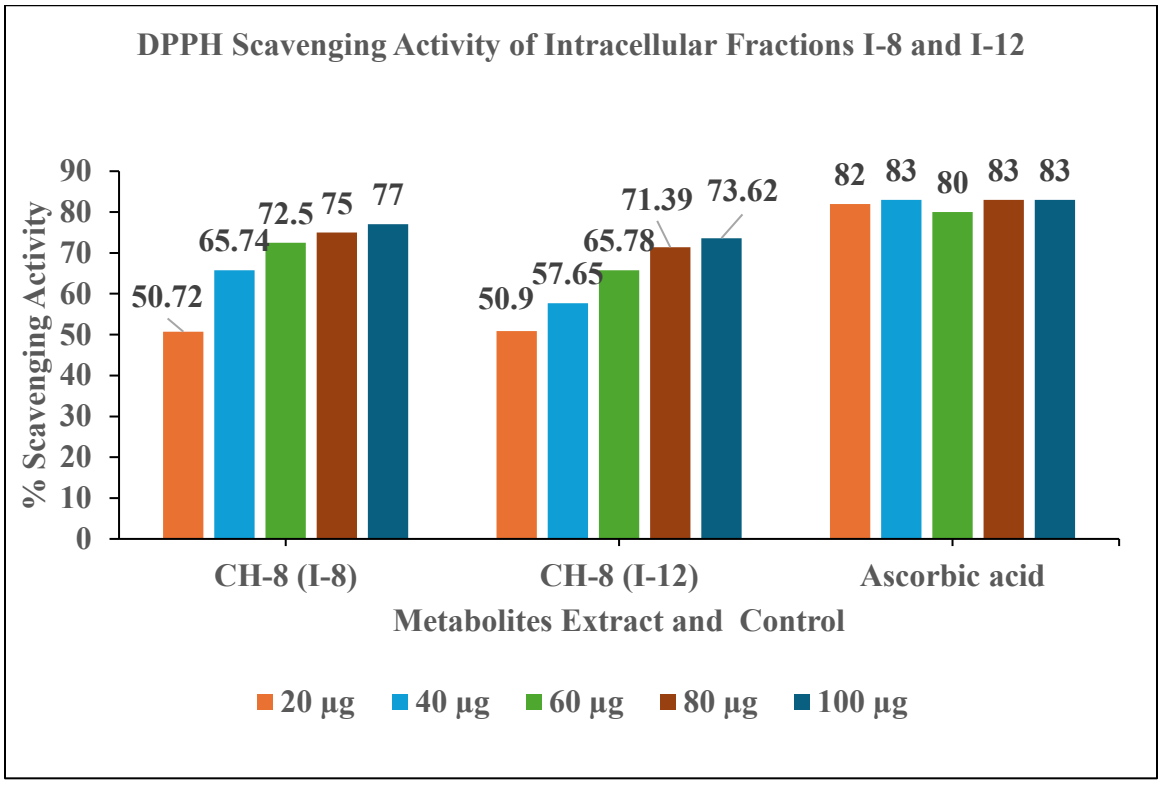

Fig 23: DPPH scavenging activity of intracellular extract fractions of *Streptomyces sp.* strain CH-8.

4.9.2. Cytotoxic Assay

Cytotoxicity of intracellular crude extract and purified fractions of *Streptomyces sp.* strain CH-8 was determined by using brine shrimps (*Artemia salina*). Maximum toxicities of the extracts were observed when the concentration was increased to 100µg. Percentage of survivability at 100µg was observed as 30% of the crude extract. However, percentage survivability at 100µg of purified fraction I-8 was observed as 10% while 30% survivability was observed of purified fraction I-12. Mitomycin-C was used as positive control and DMSO was used as negative control.

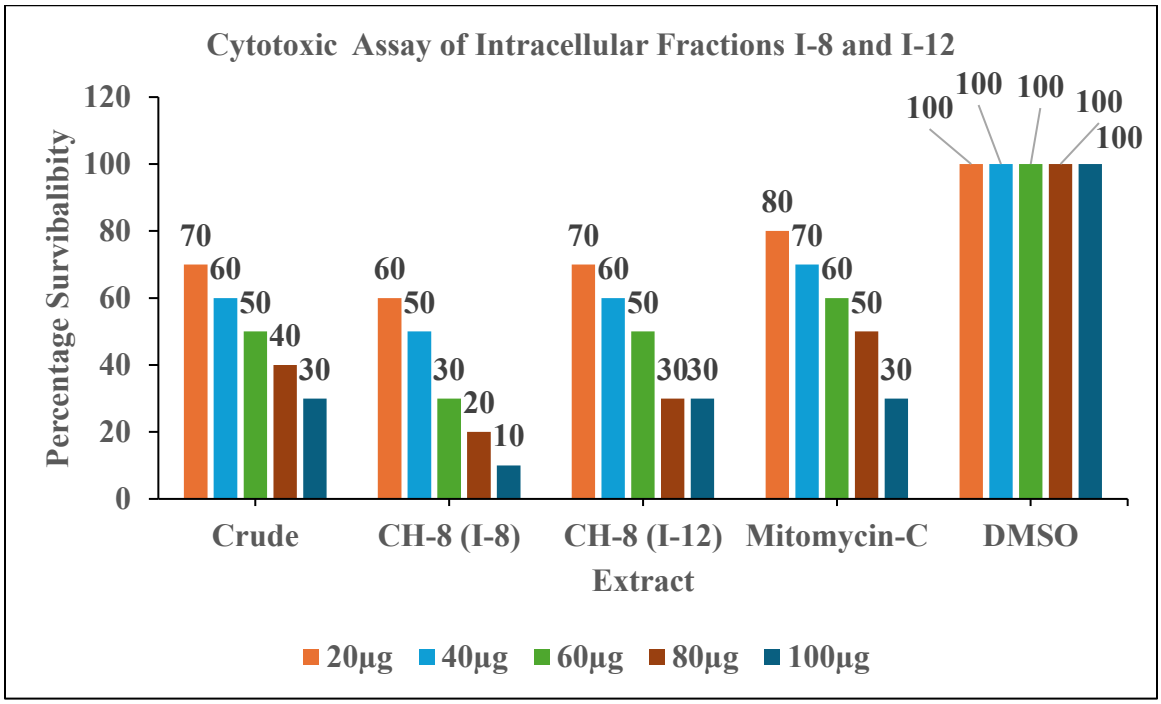

Fig 24: Percentage survivability of brine shrimp nauplii against intracellular crude extract and fractions (I-8 and I-12) of intracellular extracts from *Streptomyces sp.* strain CH-8 after 48 hours of incubation. Mitomycin C was taken as positive control and DMSO was used as negative control.

4.9.3. Anti-hemolysis Assay

Anti-hemolysis assay of crude extract and purified fractions of *Streptomyces sp.* strain CH-8 was performed using human RBCs. In results it was observed that crude extract had 43% inhibitory activity while 54% inhibitory activity of purified fraction I-8 and 35.6% inhibitory activity of purified fraction I-12 was observed at 100µg concentration. Ascorbic acid was used as positive control and was giving inhibitory activity of 72.3% at 100 µg.

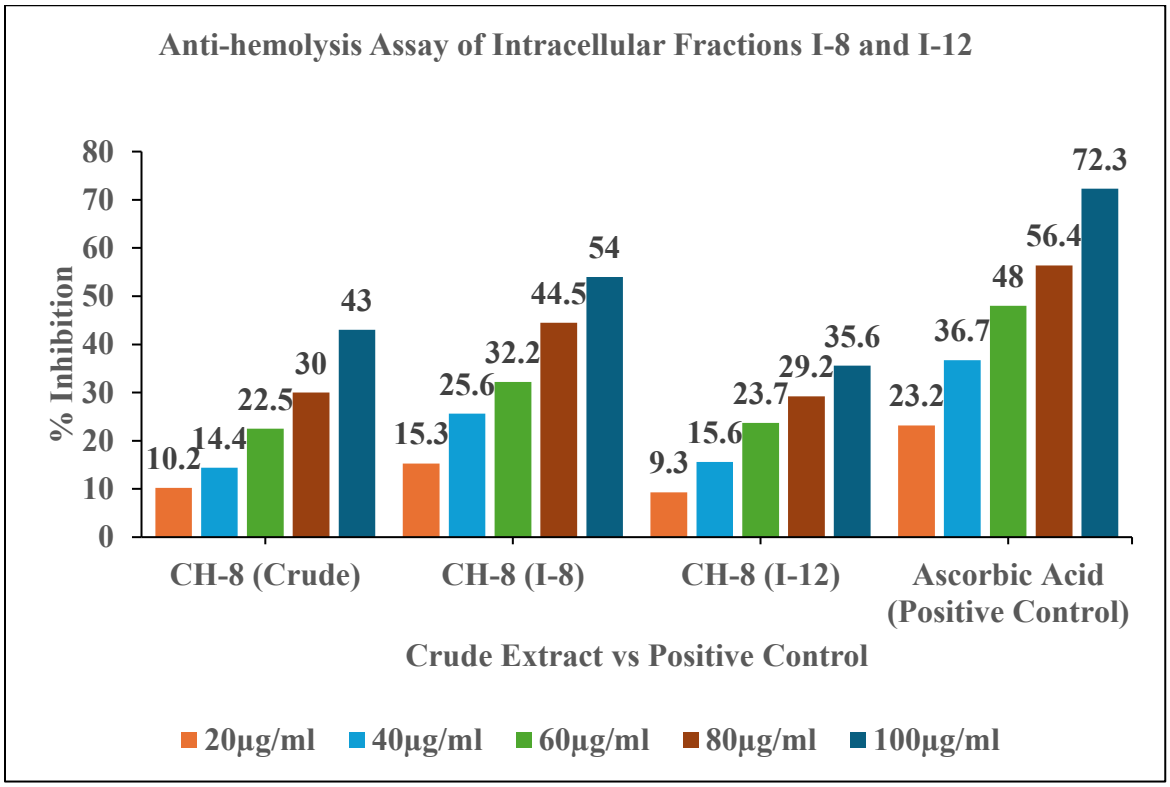

Fig 25: Anti-hemolysis assay of intracellular crude extract and purified fractions I-8 and I-12 of *Streptomyces sp.* strain CH-8 using ascorbic acid as positive control.

4.9.4. DNA Damage Prevention Assay

DNA damage prevention assay of crude extract and purified fractions of *Streptomyces sp.* strain CH-8 was performed using plasmid pUC18. In results, it was observed that plasmid DNA was broken down by • OH as can be seen in lane N.C in fig 26. However, DNA damage by H_2O_2 and sodium nitroprusside was prevented in the presence of intracellular crude extract and purified fractions I-8 and I-12 as can be seen in fig 26.

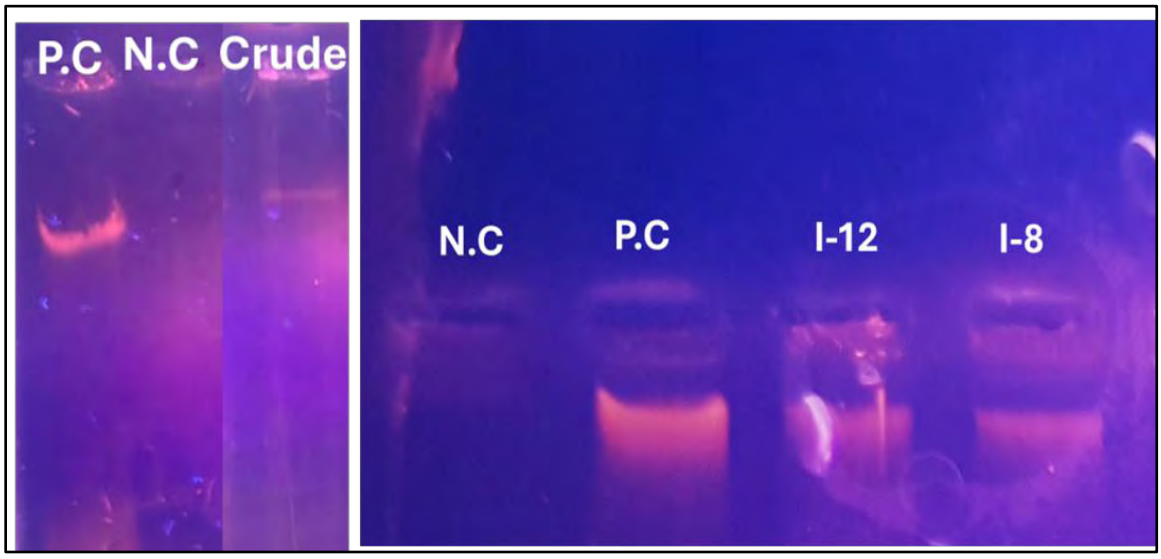

Fig 26: DNA damage prevention assay. Left: Lane P.C is positive control consisting of plasmid DNA only. Lane N.C is negative control consisting of plasmid DNA treated with H_2O_2, FeSO4 and sodium nitroprusside. Right: Lane P.C is positive control consisting of plasmid DNA only. Lane N.C is negative control consisting of plasmid DNA treated with H_2O_2, FeSO4 and sodium nitroprusside. Lane I-8 and lane I-12 are of the intracellular fractions from *Streptomyces sp.* strain CH-8.

4.10. Antibacterial Activity

Antibacterial activity of intracellular crude extract of *Streptomyces sp.* strain CH-8 was performed against gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). Among all the strains, *Streptomyces sp.* strain CH-8 crude extract was more active against *Staphylococcus aureus* and *Bacillus subtilis* and details of zones of inhibition in comparison with antibiotic gentamycin are given in table 9. However, no activity was seen against any gram-negative strains.

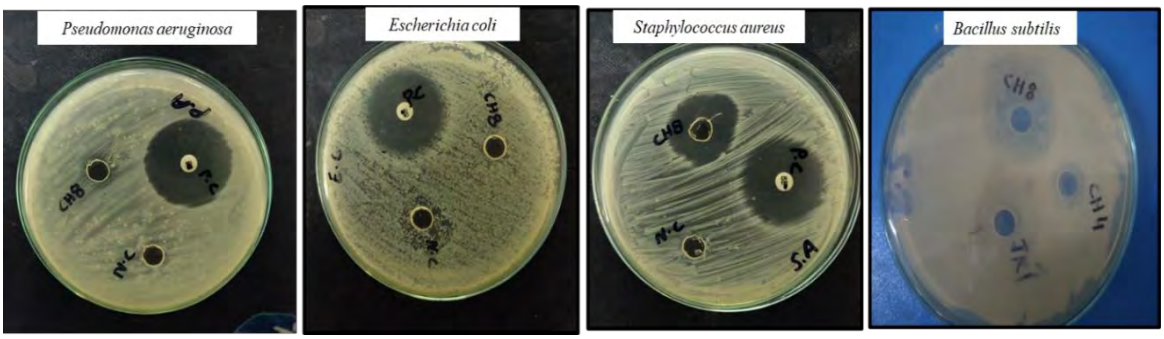

Fig 27: Antibacterial activity of crude metabolites from *Streptomyces sp.* strain CH-8. Gentamycin was used as positive control and DMSO was used as negative control.

Table 9: Zones length of antibacterial activity of crude metabolites from *Streptomyces sp.* strain CH-8. and positive control.

Anti Strains	**Zone Length of Positive Control**	**Zone Length of Crude metabolites**
Staphylococcus aureus	30 mm	24 mm
Escherichia coli	30 mm	-
Pseudomonas aeruginosa	30 mm	12 mm
Bacillus subtilis	30 mm	27 mm

However, antibacterial activity of fractions I-8 and I-12 was determined against *Escherichia coli* and *Staphylococcus aureus* and their details are given in table 10.

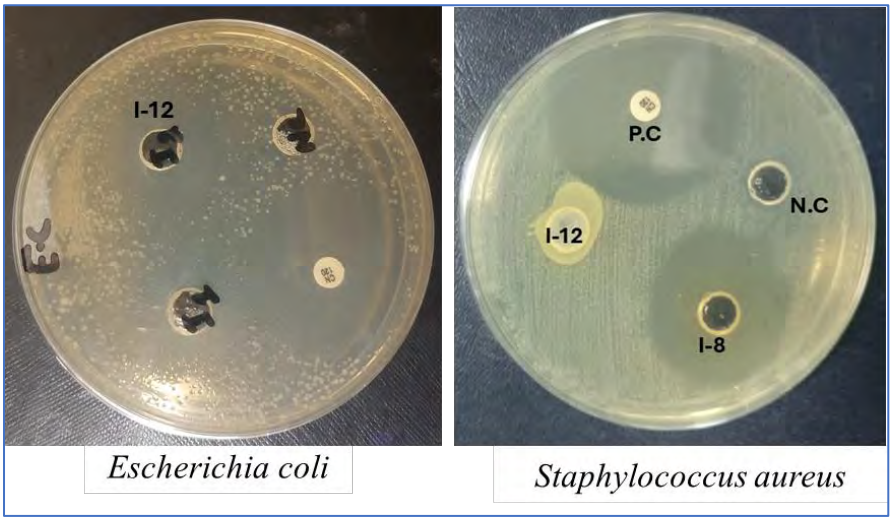

Fig 28: Antibacterial activity of intracellular fractions I-8 and I-12 of *Streptomyces sp.* strain CH-8 intracellular extract. Gentamycin was used as positive control and DMSO was used as negative control.

Table 10: Zones length of antibacterial activity of fractions I-8 and I-12 and positive control.

Anti Strains	**Positive Control**	**Zone Length of Strains Extract**	
		I-8	**I-12**
Staphylococcus aureus	30 mm	24 mm	-
Escherichia coli	30 mm	12 mm	16 mm

4.11. Fourier Transform Infra-Red (FTIR)

FTIR analysis of intracellular extract of partially purified fractions I-8 of *Streptomyces sp.* strain CH-8 revealed different peaks. Peak at 3452 cm^{-1} represented O-H stretch. Peak at 2997 cm^{-1} and 2914 indicated stretching of C-H bonds (flavonoids and alkanes). Peak at 1661cm^{-1} suggested C-H bending and presence of aromatic compounds. Peak at 1436 cm^{-1} represented O-H bending (carboxylic acid group). Peak at 1406 cm^{-1} indicated O-H bending (alcohols). Peak at 1310 cm^{-1} revealed C-N stretching (aromatic amines) and peak at 1019 cm^{-1} suggested presence of C=O linkages (ether, polyphenols, and flavonoids).

Similarly, FTIR analysis of intracellular extract of partially purified fractions I-12 of *Streptomyces sp.* strain CH-8 revealed different peaks. Peak at 3450 cm^{-1} represented O-H stretch. Peak at 2996 cm^{-1} and 2913 indicated stretching of C-H bonds (flavonoids and alkanes). Peak at 1665 cm^{-1} suggested C-H bending and presence of aromatic compounds. Peak at 1436 cm^{-1} represented O-H bending (carboxylic acid group). Peak at 1406 cm^{-1} indicated O-H bending (alcohols). Peak at 1310 cm^{-1} revealed C-N stretching (aromatic amines) and peak at 1019 cm^{-1} suggested presence of C=O linkages (ether, polyphenols, and flavonoids).

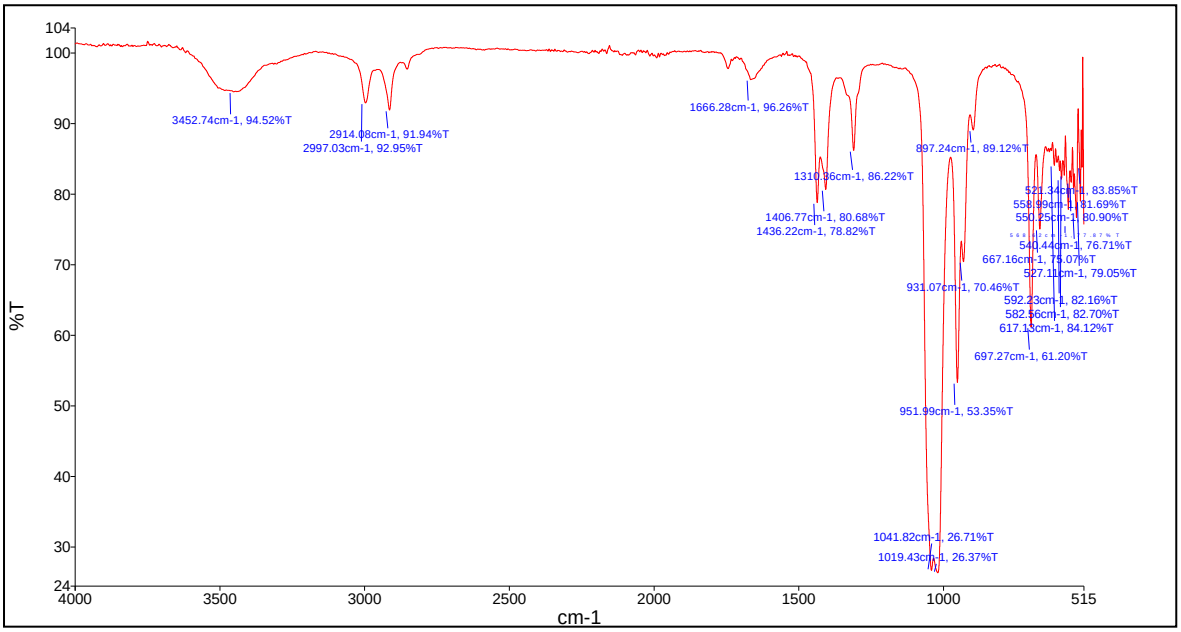

Fig 29: FT-IR Analysis of I-8 Fraction of *Streptomyces sp.* strain CH-8.

Table 11: *Streptomyces sp.* strain CH-8 purified fraction I-8 wave numbers of revealed peaks in FT-IR and their corresponding compounds groups.

Peaks at wavenumber	Group and compound class
3452 cm^{-1}	stretching of OH^- group (alcohol)
2997 cm^{-1} and 2914 cm^{-1}	stretching of C-H bonds (flavonoids and alkanes)
1661 cm^{-1}	C-H bending (aromatic compounds)
1436 cm^{-1}	O-H bending (carboxylic acid group)
1406 cm^{-1}	O-H bending (alcohols)
1310 cm^{-1}	C-N stretching (aromatic amines)
1019 cm^{-1}	C = O linkages (ether, polyphenols and flavonoids)

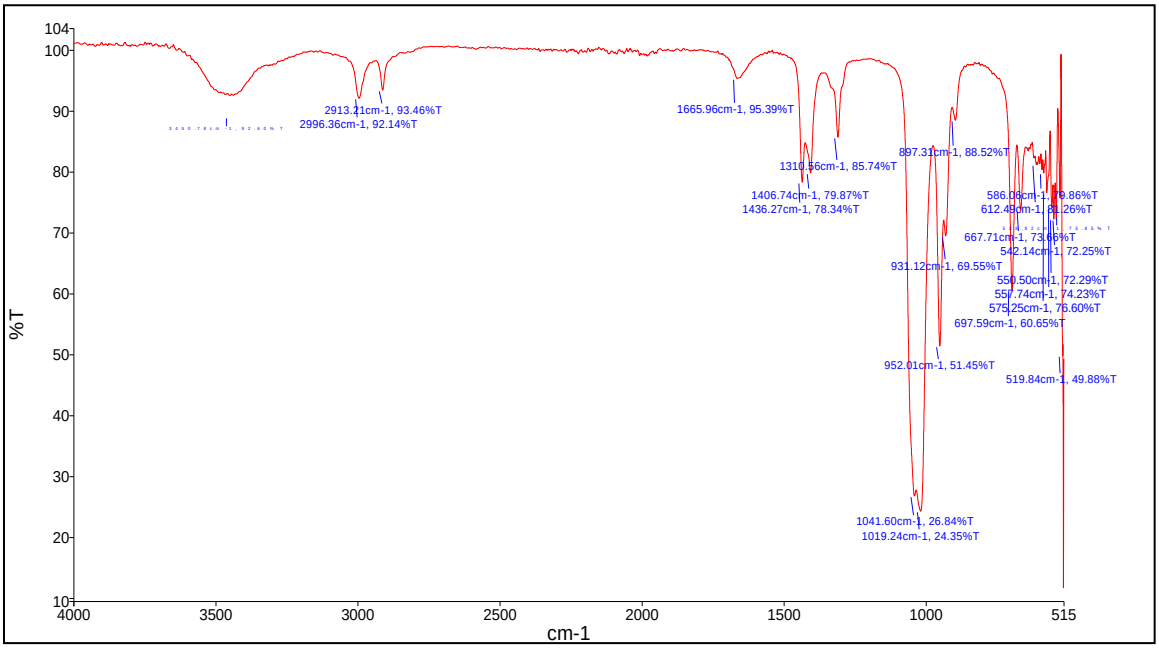

Fig 30: FT-IR Analysis of I-12 Fraction of *Streptomyces sp.* strain CH-8.

Table 12: *Streptomyces sp.* strain CH-8 extract purified fraction I-12 wave numbers of revealed peaks in FT-IR and their corresponding compounds groups.

Peaks at wavenumber	Group and compound class
3450 cm^{-1}	stretching of OH^- group (alcohol)
2996 cm^{-1} and 2913 cm^{-1}	stretching of C-H bonds (flavonoids and alkanes)
1665 cm^{-1}	C-H bending (aromatic compounds)
1436 cm^{-1}	O-H bending (carboxylic acid group)
1406 cm^{-1}	O-H bending (alcohols)
1310 cm^{-1}	C-N stretching (aromatic amines)
1019 cm^{-1}	C = O linkages (ether, polyphenols and flavonoids)

5. Discussion

The aim of this study was to examine the bioactive metabolites extracted from a strain initially named as Strain CH-8 which was isolated from Cholistan desert of Pakistan. Desert can be considered as one of the extreme environments for the living organisms of all kinds because of the existence of several life limiting parameters including decreased nutrient availability, high temperature and prolonged exposure to high UVR.

There are mainly two counter measures that are adopted by microorganisms to survive in environments of high UVR. They either protect themselves or bring forth sophisticated repairing mechanisms. In the process of protecting themselves from radiation, microorganisms try to prevent or minimize the direct and indirect impact of UVR on vital cellular structures by using their antioxidant systems. In contrast, repairing system does not prevent the access of radiation to cellular structures and biomolecules but participate in repairing the damage that is done by incoming UVR (Williamson et al., 2007). Moreover, another similar study proposed three mechanisms including prevention, tolerance and repairing mechanisms which work collectively to cope up with UVR (Agar et al., 2004).

UVR not only damages DNA but also have the potential to affect other cellular biomolecules including lipids and proteins and can even render a bacterial strain inactivated. The impact of UVR on these vital biomolecules varies from one bacteria to another. Different researchers are of the view that damaging impact of UVR on DNA is lower in gram positive bacteria as compared to its impact on the DNA of gram-negative bacteria and this variation has been attributed to the extra layers of peptidoglycan in gram positive bacteria (Jagger, 1985). Strain CH-8 was found as Gram positive and its resilience against the UV for a prolonged time can be associated with its thick cell wall.

Another study also has reported the abundance of gram-positive bacteria as compared to gram negative in Talikman desert (Liu et al., 2022). In contrast, a study that was conducted in Thar desert reported that there are more gram-negative bacteria than gram

positive bacteria (Chowdhury et al., 2007). Variation in the abundance of bacterial strains that are belonging to different group but are residing in similar ecological environment suggests the intricate patterns of their adaptation. As it has been discussed that gram positive bacterial strains are having a thick peptidoglycan which give them an edge over gram negative bacterial strains, but isolation of gram-negative bacterial strain from harsh ecological environment suggests the sophisticated regulatory cellular mechanisms that ensure their survival.

16S rRNA gene sequencing of Strain CH-8 revealed its similarity with *Streptomyces sp.* strain TRM46222 Several studies have been conducted involving isolation of *Streptomyces sp.* from deserts which make deserts an ideal place for the isolation of *Streptomyces sp.* In a study a total of 49 *Streptomyces sp.* have been reported to be isolated from desert and savanna soil in Sudan (Hamid et al., 2020).

Moreover, it has been reported in various studies that reactive oxygen species that are induced due to exposure of bacterial strain to UVR have drastic impacts on the overall regulatory machinery of a bacterial cell and affects bacterial cell division, repairing mechanisms and cell recovery (Szumiel, 2015). The lethal effects that are associated with ROS generation include formation of lesions in genome and damages to cellular membranes. In this study, intracellular crude extract of *Streptomyces sp.* strain CH-8 and its partially purified fractions were assessed for their potential to neutralize ROS. Moreover, cytotoxicity, antihemolytic and antibacterial assessment of partially purified fractions was also performed. Researchers have been trying to understand the underlying mechanisms of oxidative stress that is induced by reactive oxygen species (ROS) and to seek for counter measures that organisms adopt to counterbalance ROS (Vilchez & Manzanera, 2011).

Initially, Strain CH-8 was exposed to extended dose of UVR and H_2O_2 and was found resilient against these stresses which suggested the existence of extensive mechanisms that prevented drastic impacts of UVR and H_2O_2 on cellular activities of Strain CH-8. Moreover, Strain CH-8 was found as catalase positive and hence its survivability against

ROS can also be attributed to activity of ROS quenching enzymes catalase and peroxidase (Hara et al., 2004). Many researchers have reported that resistance to radiation can be possibly associated with bacterial ability to resist H_2O_2 (Sajjad et al., 2017).

Furthermore, colored compounds that have been extracted from various bacterial strain are reported to have the potential to scavenge superoxide anions (Albrecht et al., 2000; Zhang & Omaye, 2000). *Streptomyces sp.* strain CH-8 has been observed to produce black colored pigments and the remarkable DPPH scavenging activities of its crude extract and purified fractions can also be associated with its pigmented, black colored compounds. It was also reported in another study that microorganisms produce colored metabolites which are not considered as essential for basic cellular processes, but can uplift chances of survival of a microorganism in harsh environmental conditions by either providing them protection from external damaging factors or mediating the regulating machinery of the cell (Fiedor & Burda, 2014).

To evaluate the antioxidant potential of crude and purified extracts of *Streptomyces sp.* strain CH-8, DPPH activity was performed in a concentration dependent manner. It was found that intracellular crude extract was giving 62% scavenging activity while purified fractions were showing scavenging activity of 77% and 73.62% respectively. Comparatively, DPPH activity of the crude extract and purified fractions is higher as compared to the scavenging activity of previously reported bacterial strains (Sajjad, Ahmad, et al., 2018). Phylogenetic analysis has demonstrated that *Streptomyces sp.* strain CH-8 shares 98. 22% homology with *Streptomyces sp.* strain TRM46222. A similar investigative study was conducted in which it was observed that *Streptomyces sp.* strain MUSC 14 showed 24.71% DPPH scavenging activity at 4 mg/mL concentration (Kemung et al., 2020).

Phenolic compounds have caught the attention of various scientists because of their distinctive qualities including their ability to scavenge free radicals and their anti-inflammatory effects (Kaur & Kapoor, 2002; Manthey et al., 2001) and this potential of phenolic compounds to quench free radicals has been associated with redox properties of

phenolic compounds (Pietta, 2000; Soobrattee et al., 2005). Moreover, phenolic compounds also interact with reactive oxygen and reactive nitrogen and ultimately result in breaking of cycle of the generation of new radicals (Heim et al., 2002). Total phenolic content of *Streptomyces sp.* strain CH-8 crude extract (intracellular) was found as 149 mg\g. It has been discussed in previous paragraphs that CH-8 crude extract and purified fractions had a significant DPPH scavenging activity which provides an insight to further look deep into the involvement of phenolic compounds in staunch resistance of *Streptomyces sp.* strain CH-8 against oxidative stress and the ability of its extracted compounds to quench free radicals. A similar study has reported the existence of strong relationships between total phenolic content and antioxidant potential of *Streptomyces sp.* particularly of phenol, 2,4-bis(1,1-dimethylethyl). Along with posing a strong antioxidant potential phenol, 2,4-bis(1,1-dimethylethyl) is also involved in inhibiting bacterial growth (Kemung et al., 2020).

Flavonoids contents of crude extract was found was as 2.082 mg\g. Flavonoids contents are reported to play a prominent role in showing resistance to oxidative stress that is induced due to UVR. It was reported in a study that flavonoids bind with DNA and forms DNA duplex which protects DNA from oxidative damage (Tiwari & Mishra, 2017).

Assessment of cytotoxic effects of *Streptomyces sp.* strain CH-8 extracts were performed using brine shrimps. Usage of this technique to assess cytotoxicity of extract has many benefits which include cost effectiveness, give quick results and its easy implementation. In the results of this assay, it was observed that purified extracts had considerably toxic effects when their concentration was increased which defined the potential of purified extracts as anticancer agents. Thus, further evaluation of purified extracts is required to evaluate their anticancer activities in cell lines (Veeramuthu et al., 2017).

To evaluate the potential of *Streptomyces sp.* strain CH-8 intracellular crude extract and purified fractions to prevent free radical induced damage of erythrocytes, anti-hemolysis assay was performed. Hemolysis means the breaking down of red blood cells, releasing all the internal components. RBCs are very reactive to oxidative stress because of the

presence of high amount polyunsaturated fatty acids which are prone to oxidative damage (Liao et al., 2016; Nabavi et al., 2013). Moreover, presence of hemoglobin further elevates chances of RBCs to undergo oxidation (Welbourn et al., 2017). Hemolysis is therefore considered as a visible sign of damage that can be done to the RBCs by the free radicals (Chansiw et al., 2018). Some compounds have been reported to have the potential of scavenging free radicals and thus preventing hemolysis (Surendra et al., 2016). In results it was found that at 100µg concentration, crude extract prevented 45% hemolysis, purified fraction I-8 prevented 54% and purified fraction I-12 prevented 35% as compared to positive control ascorbic acid, which prevented 72.3% hemolysis.

In DNA damage prevention assay, it was noted that crude intracellular extract and purified fractions prevented the damaging effects of H_2O_2 and sodium nitroprusside and kept the pUC18 plasmid DNA safe and protected. Similar results were also reported in a study where antioxidant rich metabolites that were extracted from extremophiles prevented damage to DNA (O. Singh & Gabani, 2010).

Antibacterial activity of crude extract and purified fractions was performed against gram positive and gram-negative bacterial strains and in results it was found that crude extract was showing significant activities against gram positive bacteria *S. aureus* but was not active against any gram-negative bacteria. However, when partially purified fractions were tested against gram positive bacteria *S. aureus* and *E. coli,* they were surprisingly giving activity against both the strains.

Total phenolic content was found as 149.4 µg\mg and it has been reported in a study that phenolic compounds target cytoplasmic membranes leading to formation of gap in lipid bilayer (Kyrikou et al., 2005). Formation of gap will then increase the mass transfer across the cell membrane and will increase the access of phenolic compounds to their suitable targets inside the cell.

In FTIR results it was found that I-8 fraction had peaks at 2997 cm^{-1} and 2914 indicated stretching of C-H bonds (flavonoids and alkanes). Similarly, similar peaks were observed

in I-12 fraction at 2996 cm^{-1} and 2913 indicating C-H bonds which indicate the presence flavonoids content and need further characterization.

6. Conclusion

Based on the outcomes of this study it can be concluded that strain CH-8 intracellular metabolic fractions have a significant contribution in overall resistance of strain CH-8 against oxidative stress and contribute to resilience of strain CH-8 against reactive oxygen species and DNA damage inducing factors. Moreover, through molecular identification it was shown that Strain CH-8 shares 98.85% homology with Streptomyces sp. TRM46222 and various studies that involved *Streptomyces sp.* have observed the close association of extracted metabolites with strong antioxidant and antibacterial activities. Thus, further evaluation of purified fractions is necessary to exploit their potential at medicinal and industrial level.

7. Future Prospects

Findings of current research study suggest significant opportunities for further exploration and application.

- In vivo assessment of purified fraction is required to assess their true antioxidant potential and to explore their role in therapeutic sector.
- Further evaluation of cytotoxicity of purified fractions is required to explore their true potential as anti-cancer agents.
- Identification and assessment of genes that are responsible for resistance to UVR will provide an insight into underlying molecular mechanisms of resistance which will be useful in sterilization processes.
- Moreover, identification and isolation of genes that are responsible for the production of extremolytes of having antioxidant potential will enhance the mass production of these bioactive compounds and their subsequent usage in pharmaceuticals and cosmetic industries. For example, they can be used in sunscreen products.
- Role of bioactive compounds to prevent DNA damage and relieve oxidative stress also have great implications in agricultural sector. Further study is required to examine their role in resilience of plant against oxidative stress and UVR.

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