Evaluation of Antimicrobial Potential of Cold-adapted Bacterial Isolates from Passu Glacier

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A thesis submitted in partial fulfillment of the requirements for the Degree of

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In

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By

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Faculty of Biological Sciences
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Islamabad
2023

Dedicated to My

Parents

and

My Sons Affan and Sarim

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LIST OF ABBREVIATIONS

µg	Microgram	MBC	Minimum Bactericidal Concentration
µL	Microliter	mg	Milligram
AFP	Anti-Freeze Proteins	MHA	Muller Hinton Agar
AMR	Antimicrobial Resistance	MHB	Muller Hinton Broth
ARD	Antibiotic Resistance Diseases	MIC	Minimum Inhibitory Concentration
ATCC	American Type Culture Collection	min	Minutes
AWD	Agar Well Diffusion	mL	Milliliter
CFS	Cell Free Supernatant	MS	Mass Spectroscopy
CFU	Colony Forming Unit	MW	Molecular Weight
Cips	Cold Induced Proteins	NB	Nutrient Broth
cm	Centimeter	nm	Nano Meter
DMSO	Dimethyl Sulfoxide	NRPS	Non-ribosomal Peptide Synthetases
DNA	Deoxy Ribonucleic Acid	OD	Optical Density
DPPH	Diphenyl Picrylhydrazyl	PCR	Polymerase Chain reaction
EPS	Extracellular Polymeric Substances	PDB	Protein Data Bank
FA	Fatty Acid	PUFA	Poly Unsaturated Fatty Acids
FTIR	Fourier Transform Infra-Red	P-value	Probability Value
GC	Gas chromatography	QToF	Quadrupole Time of Flight
H	Hydrogen	RNA	Ribonucleic Acid
hrs	Hours	rpm	Revolution Per Minute
HTP	High Temperature Passu	rRNA	Ribosomal Ribonucleic Acid
INP	Ice nucleating Protein	SD	Standard Deviation
kcal	Kilocalorie	sec	Second
kg	Kilogram	TA	Technical agar
km	Kilometer	TSB	Tryptic Soya Broth
LB	Luria Bertani	UV	Ultraviolet
LC	Liquid Chromatography	Vis	Visible
LTP	Low Temperature Passu	WHO	World Health Organization
m	Meter	ZOI	Zone of Inhibition

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Abstract

Microbial resistance to antibiotics has been a global concern nowadays and scientists are looking for new antimicrobial compounds from extreme environments like glaciers, snow, marine, deserts, hot springs etc. For this purpose, the study is designed to unearth the antimicrobial metabolites producing strains from unexplored Passu glacier and characterize its metabolites. Among the eleven isolates, four good antimicrobials producers HTP12, HTP13, HTP36, and LTP10 were selected based on results of agar diffusion assay against test strains. These isolates were identified by 16S rRNA sequencing and phylogenetic analysis. Effects of temperature, pH, incubation period, culture medium, carbon sources, nitrogen sources and salts on antimicrobial activity of four isolates were determined. The antimicrobial metabolites from LTP10 were extracted by liquid-liquid extraction technique and were dried. The stability of extract activity was evaluated at 25-65ºC and pH 4-10. The crude extract was then evaluated by antibacterial, antifungal, antioxidant, cytotoxic, MIC and MBC assays and characterized by FTIR, GC-MS and LC-MS techniques. Molecular docking and in-silico ADMET analysis of major compounds of extract was performed using online webservers CB-Dock, pkCSM, and swissADME. The isolates HTP12, HTP13, HTP36 and LTP10 were identified as *Alcaligenes faecalis, Pseudochrobactrum saccharolyticum, Alcaligenes pakistanensis* and *Alcaligenes pakistanensis* respectively. The optimum antibiotic production by isolates was found at 25ºC and pH 7.0 after 96 hours of incubation in Luria-Bertani broth. Carbon sources, salts, and nitrogen sources have decreased antimicrobial activity of isolates except yeast extract which has positive effect on antimicrobial activity. Based on optimization study, best antibiotic producer LTP10 was selected for further evaluation. Ethyl acetate extract of LTP10 has shown best antimicrobial activity against clinical isolates. There was no significant decrease in activity of extract up to 45ºC and pH 5.5-8.5. Antibiotic discs (penicillin, imipenem, cefepime) has significantly increased antimicrobial activity of LTP10 extract by synergistic effect. MIC and MBC values of extract against tested gram-positive and gram-negative strains was found in the range of 0.4-3.2 mg/ml. Brine shrimp lethality assay indicated non-cytotoxic nature while DPPH assay revealed antioxidant nature of LTP10 metabolites. FTIR analysis proved the presence of O-H, C-H, C=C, C-O, N-O, S=O, C-N and C-O functional groups denoting presence of important organic metabolites. GC-MS chromatogram of ethyl acetate and chloroform

extracts of LTP10 validated the presence of about 14 important antimicrobial compounds. Thirteen metabolites with reported antimicrobial activity have been identified based on LC-MS analysis of ethyl acetate extract. Molecular docking and in-silico bioactivity analysis of four major metabolites 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid, dianhydroaurasperone C, L-alpha-Amino-1H-pyrrole-1-hexanoic acid and L,L-Cyclo(leucylprolyl) has predicted that these compounds could be further considered for development into antibiotic drugs. These findings have revealed the significance of antimicrobial isolates from Passu glaciers. Due to immense increase in the antimicrobial resistance, investigation of such type of unexplored microbial habitat, is urgently required.

INTRODUCTION

The environments that consist of conditions that are hard to live for most of the organisms are defined as extreme environments. These include environments like glaciers, deserts and rocks, oceans, high mountains, polar regions, salt lakes, volcanoes, and upper atmosphere. A large variety of organisms (e.g., bacteria, fungi, insects, plants, and animals) have capability to endure in these severe environments as more than 80% of surface of earth is obscured by extreme environments (Jorquera *et al*., 2019). Extremophiles are those organisms which live optimally under extreme conditions (Sayed *et al*., 2020) and have progressed and developed several mechanisms and strategies to live under extreme environments. These harsh conditions include extremes of temperature, salt content, pressure, acidity, sugars, nutrient content, radiation, oxygen, water content, and carbon dioxide (Jorquera et al., 2019).

Extreme environments offer very low levels of nutrients and are also known as oligotrophic environment. Microorganisms existing in such environments have extraordinary characteristics to produce different active metabolites. Oligotrophic environment affects growth and lifestyle of microorganisms. Microorganisms present in such environments have to compete and fight for their survival. So, for this purpose these microbes utilize their potential to produce antimicrobial compounds/metabolites against other microbes (Rampelotto, 2013).

Extremophiles are currently considered as new opportunity for exploring new biologically active metabolites and their applications in various fields (Jorquera et al., 2019). A continuous search for finding new compounds from bacteria at several locations with harsh conditions has been part of the scientific research (Mahajan and Balachandran, 2017). Thus, uncovering new antimicrobial compounds reservoirs would show an essential part in the exposure of new drug candidates, rushing in development processes of drugs and mainly solving the problem of drug resistant up to certain limit as well. Recently scientists are exploring variety of extreme ecosystems; such as deserts, glaciers, hot springs, deep sea, mountains and forest (Selvameenal *et al*., 2009), since from family cleansers to molecular diagnostics,

extremophiles or their metabolites have changed a lot of our local and working lives (Irwin and Baird, 2004).

Psychrophilic microorganisms are the cold loving extremophiles (Feller *et al*., 2017) and are further classified into two major types; one is considered true psychrophiles and the other one is called psychrotrophs. True psychrophiles are the microorganisms having the ability to grow well at temperature less than 0°C. Several scientists have attempted to classify psychrophiles as the organisms which grow under 5°C and introduced the term cryophile, which is used for those microorganisms that can multiply below 0°C (Coker, 2019). The higher and optimum temperature for their growth is 20°C and 15°C respectively (Moyer and Morita, 2007). Psychrotrophic or psychrotolerant bacteria are defined as the microorganisms which have the ability to multiply below 7 °C, although their maximal and optimal growth temperatures are above 20°C (Romanenko *et al*., 2008; Yuan *et al*., 2017).

Psychrophiles and psychrotrophs or psychrotolerants can live and multiply at reduced temperatures which clearly indicates that they have overwhelmed major obstacles intrinsic to very low temperature ecosystems. These tasks include decreased membrane fluidity, decreased enzymatic action, lower rates of transcription, translation and cell multiplication, cold denaturation of proteins, modified passage of important products, intracellular ice formation and inappropriate protein folding (D'Amico *et al*., 2006).

Genotypic and/or phenotypic characteristics of cold-adapted microorganisms have been evolved to transcend the negative impacts of low temperatures and enabled them to grow in these harsh ecosystems (De Maayer *et al*., 2014). Decreased membrane fluidity is counteracted by increase in unsaturated and polyunsaturated fatty acids in membranes that has been considered to have an important role in enhancing membrane fluidity by decreasing number of interactions in membrane (Siliakus *et al*., 2017). Psychrophilic microorganisms have evolved cold-adapted enzymes which increase their activity by reduction of ion pairs and H bonds, increased interaction with solvent and higher accessibility to active site. In order to enable efficient transcription & translation, cold-shock proteins maintain single strand of RNA by destabilizing secondary structures in target RNA. Trehalose biosynthesis in cold adapted microorganisms prevent denaturation and aggregation of protein. Anti-freeze

proteins (APFs) bind to ice crystals that block the growth of ice crystals and exopolysaccharide lowers freezing point of water (Tendulkar *et al*., 2021; Collins *et al*., 2019; Mackelprang *et al*., 2017; D'Amico *et al*., 2006).

The first antibiotic, salvarsan also known as Arsphenamine, was used in 1910. The use of antibiotics in just over 100 years has immensely transformed modern medicine and causes average human life to be extended by 23 years. In 1928, after the discovery of penicillin, golden era of antibiotic discovery from natural sources has started that peaked in the mid-1950s. Since then, there is a steady drop in discovery and development of new antibiotics and at the same time many human pathogens have evolved drug resistance which ultimately headed to the current antimicrobial resistance crisis (Hutchings *et al*., 2019). Most of the novel antibiotics have been acquired from isolates of natural habitats (Bérdy, 1980). Remarkably, microorganisms, including extremophiles, have been found to be producers of important bioactive compounds including antifungals, antibacterial, and antitumor molecules (Coker *et al*., 2016).

Generally, antibiotics are produced as secondary metabolites by multi-step biosynthetic pathways using intermediates of primary metabolism. Enzymes are involved in catalysing these biosynthetic pathways for each antibiotic (Demain *et al*., 1983). It is evident from different investigations that the organism synthesizes antibiotics after it has conceded the rapid growth phase. However, it is not universal that antibiotic formation must go after logarithmic growth (Katz and Demain, 1977). It is evident from a number of research studies that secondary metabolites produced by bacteria can be used as weapons in microbial combat. Thus the antimicrobials producing strains gets an advantage over other microbial competitors present in the same ecological niche. Secondary metabolites having antimicrobial activity can play a substantial part in predation or protection against predators (Tyc *et al*., 2017).

Among the groups of natural products, the group of microbial bioactive secondary metabolites and antibiotics has the widest range of specific and complex chemical structures, containing unique and diverse functional groups (Berdy, 2005). Antibiotics comprise compounds of molecular weight from 150 to 5000 which includes nearly all the organic chemical functional groups (carbonyl, carboxyl, hydroxyl) as well as all the organic structures (alicyclic chains, aromatic rings, aliphatic chains,

carbohydrates, polypeptides, and heterocycles). The only common property of all antibiotics is that they are solid organic compounds. The presence of several polar groups in antibiotics enables them to interact with bacterial macromolecules and thus results in bacterial growth inhibition (Lancini *et al*., 1995).

The synthesis of antibiotics is a distinctive characteristic of microorganisms which is dependent on their culture conditions. In order to enhance the growth and production of antibiotics by microorganisms, the physical and nutritional parameters of the culturing conditions are manipulated. For antibiotic production, microorganisms are dependent on certain parameters like temperature, pH, agitation and medium composition i.e., nitrogen, carbon and inorganic salts (Bundale *et al*., 2015; Satapathy and Mohapatra, 2017; Adeyemo *et al*., 2020). Therefore, formulating a suitable culturing medium is of vital significance in the production of secondary metabolites (Gao *et al*., 2009).

Modern biotechnology has the urgent tasks to explore new naturally available antimicrobial compounds and to characterize them (Esikova *et al*., 2002). A very important condition revealing an emergency state in discovery of novel antibiotics is antimicrobial resistance. Antimicrobial resistance is a dangerous threat to global public health because it results in the spread of microorganisms particularly bacteria that are very challenging to be eradicated with already available antibiotics. There has been extensive warnings from international health authorities that we are on the edge of a post-antibiotic era as approximately 25,000 deaths are reported each year alone in Europe due to antibiotic-resistant bacteria (Klug *et al*., 2021). In January 2018, the WHO issued a report on global antibiotic resistance crisis (Hug *et al*., 2018). Similarly, there is decrease in number of pharmaceutical companies which are engaged in the discovery and development of antibiotics which has also urged search for novel antimicrobial compounds (Harbarth, 2015). Recently the gap in the detection of new antibiotics has broadened due to the fact that new antibiotic drug classes have scarcely been introduced amongst others (Draenert *et al*., 2015).

The temperature range of nearly 85% of Earth is permanently or seasonally below 5°C (Hassan *et al*., 2016; Hoshino and Matsumoto, 2012; Margesin and Miteva, 2011). The cold environment of Earth has been effectively inhabited by a huge array of organisms which are collectively termed as cold-adapted organisms or

psychrophiles (Margesin and Collins, 2019). These low temperature environments range from high mountains to deep sea and from Antarctica to Arctic zone including polar ice-cap areas, polar surface, and permafrost (Shevchenko *et al*., 2021; Hassan *et al*., 2016). Studies suggested that in spite of huge number of microorganisms inhabiting polar regions, not many have been assessed for the production of noteworthy secondary metabolites. Cold-habitat microorganisms are still mostly unexploited source of new or substantially different antimicrobial compounds. Therefore, screening of cold loving microorganisms for screening of novel antimicrobial compounds is need of the day (Borchert *et al*., 2017; Tian *et al*., 2017).

Psychrophiles, due to their resistance to low temperature and successive applications in medical field, have gained the attention of scientists to extensively study and try to uncover newer antimicrobial metabolites from psychrophiles (Tendulkar *et al*., 2021). From last two decades, considerable number of novel natural biomolecules with several activities, like antibacterial, antitumor, antiviral and so on, were reported from polar microorganisms, sponges, lichen, molluscs, and moss. Natural metabolites from the Antarctic and the Arctic microorganisms have been the focus of a number of review articles (Tian *et al*., 2017). In the late 1990s and early 2000s scientists have discovered antibiotic secreting marine species, most of which were psychrophilic bacteria and algae (Stonik *et al*., 2020). Next-generation sequencing (NGS) was used for analysis of permafrost microbes from lakes in Canada which revealed the existence of novel psychrophiles, with commercially relevant enzymes and polymers. A few of these possess potential medicinal activities like antibacterial, anticancer, antiviral, and drug-delivery. (Finore *et al*., 2020). Table 1.1 shows some of the important antimicrobial compounds which have been reported from psychrophiles in last two decades.

Table 1.1: Antimicrobial compounds reported from psychrophiles and psychrotrophs

Compound	**Microorganism**	**Potential use**	**Reference**
Cyclo-(L-Trp-L-Phe), cyclic dipeptide	*Aspergillus sydowii*	Antibacterial agent	Li *et al*., (2018)
3-methylbutyl hexanoate	*Candida sake*	Antifungal agent	Arrarte *et al*., (2017)
Rhamnolipid	*Pseudomonas* sp.	Antibacterial agent	Tedesco *et al*., (2016)
Nitrosporeusines A and B	*Streptomyces nitrosporeus*	Antiviral agent	Philkhana *et al*., (2015)
Arcticoside	*Streptomyces* sp.	Antifungal agent	Moon *et al*., (2014)
Chetracins	*Oidiodendron truncatum*	Antibacterial and antiviral agent	Li *et al*., (2012)
Tropodithietic acid	*Pseudovibrio* sp.	Antibacterial agent	Penesyan *et al*., (2011)
Volacein	*Janthinobacterium* sp.	Antibacterial agent	Mojib *et al*., (2010)
Flexirubin	*Flavobacterium* sp.	Antibacterial agent	Mojib *et al*., (2010)
Geomycin	*Geomyces* sp.	Antifungal and antibacterial agent	Li *et al*., (2008)
Lysozyme	*Manduca sexta*	Antibacterial agent	Sotelo-Mundo *et al*., (2007)
Frigocyclinone	*Streptomyces griseus*	Antibacterial agent	Bruntner *et al*., (2005)

In Pakistan, there are plenty of areas that have cold ecosystems such as Karakoram, Himalaya and Hindu Kush ranges which are found in northern areas of Pakistan (Hassan *et al*., 2018; Rahman *et al*., 2008). These ranges have variety of glaciers varying in size including Siachen Glaciers, Passu glacier, Baltoro Glacier, Batura

Glacier, Biafo Glacier, Nubra Glacier, Rakaposhi Glacier, Saltoro Glacier, Gasherbrum Glacier, Hispar Glacier and Concordia Glacier.

In this study, we analysed the antimicrobial activity of selected isolates from Passu glacier situated in the Karakorum mountain range in Passu village (Upper-Hunza, Gigit-Baltistan). The distance of Passu glacier from Gilgit is about 150 km and has height of more than 7000m. At the back side of the glacier Passu Peak is sited. This glacier relates to Batura Glacier and many other glaciers of that area. Passu village is well known for its golden colour peaks that form pyramidal structure called “Passu cones” or “Passu Cathedral” that are 6106 meters in height.

The present study was carried out to find and search for new antimicrobial compounds from the untapped sites in the cold environment in Pakistan. A very little information is present in literature reporting the antimicrobial compounds from glaciers inhabiting microorganisms especially rare information is available from Karakorum Mountain range. Due to immense increase in the antimicrobial resistance, investigation of such type of unexplored microbial habitat, is urgently required.

Aim and Objectives

Aim

The aim of the study is to unearth antimicrobial metabolites producing microorganisms from Passu glacier and to purify and characterize the compounds produced.

Objectives

The objectives of the study are:

- Screening of antimicrobial metabolites production from already available bacterial isolates from Passu glacier
- To identify the antibiotic producing isolates through 16S rRNA gene sequencing and their phylogenetic analysis
- To study the effect of various culture conditions on the growth and antimicrobial production of the bacterial isolates
- To extract and characterize the antimicrobial compounds by solvent extraction, FTIR, GC/MS, LC/MS
- In-silico analysis of selected antimicrobial metabolites by molecular docking and ADMET studies

LITERATURE REVIEW

2.1 Extremophiles and extreme environments

Extreme environment is the favorite habitat for microorganisms termed as extremophiles (MacElroy, 1974), while extremotolerants are those organisms which can withstand harsh environments (Sayed *et al*., 2020). If an organism easily grows in more than one extreme conditions, it is called as a polyextremophile (Rothschild and Mancinelli, 2001). In comparison to extremotolerant microorganisms, it is important to remember that extremophiles have vastly adapted to the severe conditions of extreme environment, and these have the ability to perform their metabolic and biochemical functions easily (Merino *et al*., 2019). So, we can say that microorganisms inhabiting extreme conditions can be categorized as extremotolerants which can survive in strict environmental conditions, yet their optimum growth occur under ordinary circumstances and extremophiles which undoubtedly require the mandatory extreme environments for their existence (Singh *et al*., 2019). Furthermore, numerous environments are present on our Earth surface and especially undersurface, that indicate one or more extreme chemical or physical conditions. Hence it is understandable that the most plentiful organisms on the ground might be extremophiles and, in specific, polyextremophiles (Capece *et al*., 2013).

Extremophiles are categorized according to the obligatory ecological requirements for their growth. Psychrophiles need reduced temperature for their optimum growth, thermophiles and hyperthermophiles are the one which require high temperature, acidophiles are the microorganisms which require acidic pH, alkaliphiles require basic condition, barophiles require high pressure for growth, halophiles grow in high concentrations of sodium chloride (Singh *et al*., 2019), metalophiles can grow and survive in an environment that is full of high concentrations of heavy metals, and anoxiphiles occupy the ecosystem that is deprived of oxygen (Margesin *et al*., 2002)

All the three domains of life (i.e., archaea, bacteria, and eukarya) are present at numerous severe environments throughout the world. A number of studies, over the years, has discovered that most of the organisms which inhabit extreme environments are the microorganisms with great percentage of them are archaea, although they also consist of many algae, fungi, and protozoa (i.e., eukarya) and some multicellular

organisms (Rampelotto, 2013; Singh *et al*., 2019). In the last decade, many researchers have isolated the extremophiles in pure culture, profiled their metabolites and explored the extreme conditions in which they survive. Extremophiles secrete extremolytes and extremozymes which have industrial, pharmaceutical, and environmental applications (Raddadi *et al*., 2015). Extremophiles also produce metabolites to survive in an oligotrophic environment and compete with other microorganisms. The general cellular components that are analyzed and used in all the restorative and therapeutic uses of extremophiles relate to how the extremophiles utilize defensive components to survive in challenging circumstances and how their metabolic structures are engaged in with these survival shapes. Extremophiles have had the ability to live in unprecedented and ruthless conditions for the most part due to their flexibility. The adaptation mechanisms of such extremophiles would help researchers to fathom their survival frameworks, which along these lines would understand the technique by which their sub-nuclear segments, for instance, proteins and genes could be altered and used for restorative ramifications (Babu *et al*., 2015).

Over the last years, scientists have remained fascinated by the remarkable organisms that occupy extreme ecosystems (Rampelotto, 2013). Moreover, the border conditions in which life can flourish have been driven in every feasible way, including wider swaths of pressure, temperature, pH, salinity, radiation, nutrient limitation, and energy (Merino *et al*., 2019). Extremophilic microorganisms not only flourish in such a vast range of constraints on our planet, but also have the ability to live in the extreme environments of space, an ecosystem with vacuum pressure, intense radiation, microgravity, and exceptionally changing temperature (Horneck *et al*., 2010; Yamagishi *et al*., 2018). The meaning of “extreme conditions” has robust anthropocentric standards, rather than microbial standards, and could be the reason of misunderstanding (Rothschild and Mancinelli, 2001). Several extreme environments are present on our planet including polar regions, glaciers, hot springs, deep sea, hypersaline environments, deserts, permafrost, snow, etc. (Rothschild and Mancinelli, 2001). Here we will focus on the cold/psychrophilic environments.

2.2 Cold environments

Cold environment is one of the types of extreme environment. Microorganisms has utilized frozen water as habitat from high-altitude glaciers, colored pink with 'watermelon' or 'blood' snow to the permafrost in the polar regions. However, it should be noted that some ice comprises of liquid brine inclusions that serve as the real habitat for the microorganism. Secondly, certain ice ecosystems like permafrost have "a community of survivors". It is not likely that the microbial population of such ecosystem truly choose this environment, instead they have found themselves entrapped in the ice and survived because of their increased resistance as compared to others that have experienced as similar fate (Rothschild and Mancinelli, 2001).

Cryosphere is the collection of psychrophilic environments which represents world's greatest segment of frozen environments including ice sheets, snow, glaciers, ice caps, ice lake, ice-covered parts of the deep-sea, permafrost, lakes, and icy rivers (Musilova *et al*., 2015; Kudryashova *et al*., 2013), in arctic and Antarctic regions (NOAA 2018), lakes and glaciers of nonpolar mountains (Walsh *et al*., 2016; Salazar and Sunagawa 2017), and human-made freezers (Ahmad *et al*., 2010) and fridges (Flores *et al*., 2013). Cold environment is tough because of reduced temperature alongside with at minimum one of these conditions, i.e., minimal water and nutrients accessibility, UV rays, osmotic pressures, and freeze-thaw cycles, and still, these are of environmental and ecological significance (Rafiq *et al*., 2019).

Glaciers consist of substantial amount of shifting ice that stretches gradually around the ground. Usually, glaciers are constant forms of frost that comprise mainly of re-crystallized ice and exhibits proof of depressed slope or outward passage because of gravity. Microorganisms perform major role in carbon cycling, subglacial weathering, and other nutrients in a glacier ecosystem e.g., algae perform the function of primary maker that nourish heterotrophic inhabitants on glaciers, such as bacteria, fungi, insects, and copepods (Rafiq *et al*., 2017). Ice in the glaciers is deeply entrapped with organic carbon which is metabolized by microorganisms and produce methane (Boyd *et al*., 2010), which is a greenhouse gas. The production of methane gas from carbon could have important role in climate change (Wadham *et al*., 2012). Subglacial microbes perform mineral weathering (Montross *et al*., 2013) and provide minerals

and other nutrients for surrounding life forms (La Farge *et al*., 2013). Researchers have found that glaciers might be a good environment to sustain active and diverse populations of microorganisms and macro-organisms (Hodson *et al*., 2008; Anesio and Laybourn 2012). Culture dependent and culture independent methods were used by a large number of scientists for the occurrence of microbes in polar and non-polar glaciers (Mikucki and Priscu, 2007; Kim *et al*., 2008). Bacteria survive in snow of glacier in both vegetative and dormant forms and are adapted to this distinctive biome by several distinct mechanisms (Margesin *et al*., 2008).

The temperature of many caves, which are glaciated and underground, is permanently about 10°C to below 0°C. There have been reports of large number of bacteria in the troposphere at around 10,000 m, where the temperature might be less than 4°C. the temperature is roughly 5°C or below in the area beneath the thermocline. Hence, the deep sea is always icy-cold. Barophiles, which are also psychrophilic or psychrotrophic, might be isolated from the deepest parts of the seas (deeps and trenches). The point where ice is being melt or formed in the ocean, represents the icy environment of the sea with related microbial population (Morita and Moyer, 2000).

2.2.1 Karakoram Mountain range

The Karakoram mountains are one of the renowned mountain systems of our planet, along with four peaks having a height of more than 8000 m. More importantly, Karakoram is one of the largest regions, covered with glaciers, beyond the Antarctic and the Arctic regions. These mountains are amongst the very limited regions of the earth where a small increase in mass of glaciers has been detected during the previous decade (Ambrosini *et al*., 2017).

Pakistan has few of the longest and largest mid-latitude glaciers of world present in the northern areas of the country. These are situated in the Karakoram, Himalaya, and Hindu Kush mountains spread over an area of approximately 15,000 km^2. The glaciers present in these mountainous areas are important source of water supply in the region (Williams *et al*., 2010).

2.2.2 Exploring cold environments for antimicrobial compounds

Our planet Earth is covered mostly by cold biosphere. About 71% surface of the planet Earth is marine, while 14% is in the polar region (arctic and Antarctic regions). Approximately greater than ninety percent of the ocean environment is cold having temperature of 5℃ or lower (Moyer *et al*., 2007). Antarctica is a pristine, unique, and severe environment, deemed as the iciest, driest, and breeziest region on the earth (Asencio *et al*., 2014). Consequently, bacteria inhabiting Antarctic region have been suggested as a favorable source for new antimicrobic secondary metabolites (Núñez-Montero *et al*., 2019). In addition, Arctic region is the other important location that is considered to be the future of the drug industry, which has provoked the increase of bioprospecting in the Arctic region. The harsh environment of the Arctic, reaching at a temperature of -50℃ in the midwinter, and adaptations of microorganisms in the region, keep the key to novel compounds and medicines of the future (Page and Willahan, 2018). Many scientific expeditions have observed organisms in the Arctic region that have developed exceptional and extraordinarily effective chemical defences that might be extremely beneficial for the development of new antibiotics and drugs (Krause, 2018).

Cryosphere (part of the Earth's surface where water is frozen) consist of large portions of glaciers and ice sheets on the continents and comprise most of the Earth's freshwater. Glaciers cover almost 10% of the Earth permanently cold environments (Anesio and Laybourn 2012; Laybourn *et al*., 2012) major part of which exist in polar region. Apart from the polar region, Hindu Kush, Karakoram, and Himalaya (HKKH) region comprise of the major glacial reservoir and consist of almost 54,252 small and large glaciers covering an area of 60,000 km^2. Thus, a number of scientists refer it as "third pole" (Rafiq *et al*., 2019). The psychrophilic environments, present in polar regions including Antarctica and Greenland, are widely examined for microbes variety, environmental and biogeochemical processes, and its effect on climate change (Christner *et al*., 2002; Skidmore *et al*., 2005; Lanoil *et al*., 2009; Cowan *et al*., 2014; Ganzert *et al*., 2014; Glaring *et al*., 2015; Rafiq *et al*., 2019). In contrast, less interest has been taken in the non-polar psychrophilic environments including Karakoram Mountain region which is the major glaciated area beyond polar region. Recently the momentum to discover the 3rd pole for biogeochemical processes, microbial diversity,

and for search of novel bioactive compounds has been increased (Chaturvedi *et al*., 2005; Xiang *et al*., 2005, 2009; Liu *et al*., 2009; Reddy *et al*., 2010; Zhang *et al*., 2010; Shen *et al*., 2012; Rafiq *et al*., 2016; Rafiq *et al*., 2017; Rafiq *et al*., 2019).

2.3 Psychrophiles and psychrotrophs

The terms psychrophilic and psychrotrophic have been proposed to describe distinct abilities of microbes to multiply at low temperatures, (Morita, 1975; Reichardt and Morita, 1982; Jay *et al*., 2005). Psychrophiles have been defined as the microbes having the ability to multiply at temperatures under 0°C, with optimum growth temperature of 15°C or below, while maximum growth occurs at temperature of about 20°C. Some experts also called psychrophiles as obligate psychrophiles or cryophiles (Ferroni and Kaminski, 1980; Sandle and Skinner, 2013). Facultative psychrophiles or psychrotrophs or psychrotolerants are defined as those microorganisms having optimum growth temperature of 20°C and maximum growth temperature of 25°C (Morita, 1975; Scherer and Neuhaus, 2006; Moyer *et al*., 2016; Hilgarth *et al*., 2018).

Psychrotrophs or psychrotolerants are present in the same icy ecosystems as psychrophiles but in larger quantities (Moyer *et al*., 2007). They might also be present in low temperature environments which vary over the psychrophilic range, primarily because of the seasonal change in the radiant energy from the sun. Therefore, temperatures reach as high as approx. 28°C on the icy surfaces in either southern or northern polar areas. That is why psychrophiles are not found in these temperature varying conditions (Morita and Moyer, 2000). Facultative psychrophiles or psychrotrophs can live at temperature range of 0°C to approx. 40°C. Generally, these microorganisms are unable to multiply at low temperatures (below 15°C), though they are able to carry out basic functions. These microorganisms get developed to survive cold environments, where adaptation has needed a huge range of structural, sequence and physiological modifications. On the other hand, they are less specialized physiologically as compared to true psychrophiles and normally does not exist in the very low temperature environments (Morita and Moyer, 2004).

Different factors affect the arctic soils' microbial diversity e.g., extreme temperatures, low contents of available nutrients, low annual precipitation, low soil moisture, and freeze-thaw cycles (Mannisto and Haggblom, 2006). Instead of these restrictions,

psychrophilic microorganisms of Gram-positive bacteria (for example, *Micrococcus Arthrobacter*, and *Bacillus* species), Gram-negative bacteria (for example, *Pseudoalteromonas, Polaribacter, Psychrobacter, Moraxella, Polaromonas, Moritella, Psychroflexus*, *Pseudomonas* and *Vibrio* species), Archaea (such as *Methanococcoides*, *Methanogenium*, and *Halorubrum* species), yeast (*Cryptococcus* and *Candida* species), fungi (such as *Cladosporium* and *Penicillium*) and microalgae (*Chloromonas*) are present in these environments and exhibit prominent cold-adaptive features (Feller and Gerday, 2003). *Pseudomonas fluorescens*, *Listeria monocytogenes*, *Pseudomonas maltophilia*, and *Vibrio marinus* are some of the examples of facultative psychrophiles. These 'cold tolerant' microorganisms are frequently known as psychrotolerant microorganisms (Helmke and Weyland, 2004). A large number cultivable of isolates (about 60%) from arctic tundra soils belong to *Pseudomonas* species. The continental environments of Antarctic vary from Arctic ecosystem, with temperature below zero, typically as low as -60°C (more icier than arctic), reduced nutrient accessibility, and more dry (Margesin and Miteva, 2011). Antarctic soils are mostly dominated by bacteria as compared to archaea. Culture-independent study to determine microbial diversity on Antarctic Dry Valley soils, which are mostly called as cold deserts, showed the dominance of actinobacteria, while culture-dependent study indicated that approximately 80% of the microbial strains belonged from *Streptomycetes* (Babalola *et al*., 2009).

2.3.1 Challenges for psychrophiles

The survival of microorganisms in a cold environment is greatly dependent upon cold environments and other restrictive factors (Margesin and Miteva 2011; De Maayer *et al*., 2014). The metabolic flux of psychrophilic microorganisms is slow because these are uncovered to reduced thermal energy and elevated viscosity which are the two major physical tasks to cold-loving microorganisms (D'Amico *et al*., 2006). The growth of ice crystals in these environments due to freezing temperature damages the cells, hinders the action of enzymes and ultimately reduces flexibility of cytoplasm and plasma membranes. Hence, the regular functions of psychrophiles are foiled in cold atmosphere with no appropriate adjustment tools (Raymond *et al*., 2007).

The flexibility of the plasma membrane is crucial for its structural integrity and normal functioning of cell. It has long been recognized that one of the most

substantial impacts of low temperature is on membrane fluidity (De Maayer *et al*., 2014). The lipid composition of the plasma membrane is vital for its physical properties and thus it is not unexpected that this changes with the thermal habitat of the microorganism (D'Amico, 2006). Low temperature cause freezing of the cell wall and cell membrane which decreases the movements of molecules across the plasma membrane of psychrophiles. Likewise, the enzymes would be unable to perform biochemical processes of a cell in a frozen cytoplasm (Chandler, 2018).

Low temperatures affect the formation of enzymes which are unable to attain their triggering energy essential to process a reaction (Chandler, 2018). The major challenge cold-tolerant microbes have to deal with is the adverse impact of decreased temperatures on biochemical reactions. Indeed, with many unchanged enzymes, a reduction in temperature from 37°C to 0°C causes 16-80-fold decrease in activity (Collins and Margesin, 2019). A few of the major obstacles to protein formation at reduced temperatures consist of decreased action of transcriptional and translational enzymes, inappropriate folding of protein, and stability of DNA and secondary structures of RNA (D'Amico *et al*., 2006; De Maayer *et al*., 2014).

Temperature below zero-degree cause formation of ice which might result in osmotic stress, cryoinjury, loss of water, and even rupturing of cell and death (Klähn and Hagemann, 2011; Collins and Margesin, 2019). Naturally the formation of ice is slow inside the cell as compared to outside of the cell because of less volume and tightly packed nature of cell interior. So, in natural environment the formation of ice is usually very slow intracellularly and typically restricted to external side of the cell (Fonseca *et al*., 2016).

INPs (ice-nucleating proteins) are larger membrane attached proteins that accelerate the growth of ice. These proteins are responsible to begin synthesis of diverse ice crystals at very low temperatures and are considered to provide a model for organizing and stabilizing of water moieties in an icy shape (Lorv *et al*., 2014; Pandey *et al*., 2016; Pummer *et al*., 2015). A diversity of organisms produce INP's in the form of large complexes allowing greater activity (Bar Dolev *et al*., 2016a).

Microorganisms inhabit in the ice veins and ice sheets in the glaciers that serve as microenvironment (Thomas and Dieckmann, 2002). Various physicochemical

pressures are faced by these microorganisms like less water activity and pH, reduced diffusion rates of solutes, and membrane injury due to formation of ice crystals. Under the stressful conditions of decreased liquid water, very low temperature, elevated solar energy, and nutrient shortage, different structural and functional strategies are revealed by psychrophiles for their existence (Garcia-Lopez and Cid, 2017).

2.3.2 Survival strategies of psychrophiles

In the previous few decades, substantial attention has been paid to the mechanisms of cold adaptation by psychrophiles, especially because of biotechnological potential of these microorganisms and their metabolites. (De Maayer *et al*., 2014). Psychrophiles are dependent on various adaptive strategies for maintenance of important functions at very low temperatures and to endure and multiply in cold environments (Rodrigues and Tiedje, 2008). In addition to low temperature, to which psychrophiles have adapted, other aspects of low temperature conditions are dehydration, radiation, extreme UV, acidic or basic pH, increased osmotic stress, and decreased nutrient supply (De Maayer *et al*., 2014).

In order to maintain the properties of cell membranes at reduced temperatures, psychrophiles exhibit homeoviscous adaptation through alteration of lipid bilayer composition of fatty acids (FAs) (Siddiqui *et al*., 2013; Collins *et al*., 2019). These FAs decrease the packing compactness of lipid bilayer, resulting in decrease of temperature for liquid to gel phase shift and thus maintain lipid bilayers functional even at reduced temperature. Similarly, upregulation of genes for different proteins of biosynthesis of cell membrane and its fatty acids desaturation, branched-chain FAs formation and cis-isomerization were stated for many cold-adapted microorganisms (Medigue *et al*., 2005; Methé *et al*., 2005; De Maayer *et al*., 2014; He *et al*., 2015; Goordial *et al*., 2016). Likewise, it has been observed that there is great number of genes for proteins having a role in decomposition of plasma membrane rigidifying molecules and may have importance in decreasing membrane stiffness in cold environments (Collins *et al*., 2019). In addition, some psychrophiles have been observed to have upregulated lipid bilayer embedded transport proteins and counteract the decreased rates of diffusion across the plasma membrane at reduced temperatures (De Maayer *et al*., 2014).

Pigments, particularly carotenoids, are suggested to have a role in plasma membrane flexibility modification. Cold-adapted microorganisms reported from glaciers, marines, and high elevation soils are commonly involved in pigment production (Dieser *et al*., 2010; Shen *et al*., 2018). Similarly, there are some studies which have reported an enhanced production of these pigments in cold conditions (Pandey *et al*., 2018).

Recent reports from various studies have shown the importance of cell wall adaptation mechanisms in addition to cell membrane adaptations at low temperatures. Few psychrophiles have been reported to have upregulated genes for biogenesis of peptidoglycan and condensed peptidoglycan layer at cold temperatures (Rodrigues *et al*., 2008; Mykytczuk *et al*., 2013). *Planococcus halocryophilus* Or1 has been reported to have condensed cell wall, that is accomplished by somewhat distinctive mechanism which involves cell wall related hydrophobic accumulations made up of peptidoglycan, choline, and calcium carbonate (Mykytczuk *et al*., 2013). Such tactics for condensing and stabilizing cell wall will ultimately result in strong physical barrier which can prevent cold adapted microorganisms against cell deformation by ice growth, freeze-thaw cycle, and elevated osmotic stress at decreased temperatures (Collins *et al*., 2019).

Psychrophiles react to various cold associated harmful tests by making of a range of new tools involving, similar solutes, ice-binding proteins (INPs and antifreeze), biosurfactants and extracellular polymeric bodies (Collins *et al*., 2019). Compatible solutes are small molecular weight, non-lethal organic osmolytes, with sucrose, trehalose, sorbitol, glycine, mannitol, and glycerol being frequently described (Mykytczuk *et al*., 2013; Ghobakhlou *et al*., 2015; Goordial *et al*., 2016). Buildup of these biological osmolytes donates to rebuilding osmotic equilibrium and thus reduces water loss and cell contraction in freezing up. More importantly, chilling point of liquid is decreased by these osmolytes (Fonseca *et al*., 2016).

AFPs (anti-freeze proteins) are known to have non-colligative properties and have binding ability to ice resulting in inhibition of ice growth (Lorv *et al*., 2014; Bar Dolev *et al*., 2016b; Collins *et al*., 2019). These proteins usually bind irreversibly to definite ice-crystals, ultimately inhibiting secondary nucleation processes and forming a distinctive ice shape. Because of kelvin effect, ice surface expansion between the

adsorbed AFPs results in formation of curves at ice face which is energetically unfavorable for further ice growth. This results in thermal hysteresis which decreases chilling point under normal melting point and thus formation of ice crystals is stopped (Voets, 2017).

INPs are considered to neutralize damage due to reduced temperature by guiding ice nucleation to outside the cell and thus inhibit the synthesis of harmful ice inside the cell through removal of water from inside the cell (Lorv *et al*., 2014). Moreover, INPs facilitates less damaging small ice crystals outside the cell as compared to large crystals and loss of latent heat of crystallization can also be helpful in avoiding further damage (Pummer *et al*., 2015).

EPS (Extracellular polymeric substances) are high molecular mass biopolymers that are produced by different microorganisms into their extracellular environment (Collins *et al*., 2019). Arctic and Antarctic ice ponds have been studied by metagenomic analysis for a number of genes responsible for biosynthesis of EPS (Varin *et al*., 2012) and low-temperature modified microorganisms were reported to secrete huge quantities of EPS at below zero temperatures (Marx *et al*., 2009; Mykytczuk *et al*., 2013; Feng *et al*., 2014; Caruso *et al*., 2018). A defensive shield around the cells is formed by the hydrous EPS gel matrix and is considered to provide protection against reduced temperatures by acting as barrier to diffusion of solutes and formation of ice (Krembs *et al*., 2011; Ewert and Deming, 2013; Deming and Young, 2017; Caruso *et al*., 2018).

Many of the low-temperature adapted enzymes reported until now are described for greater bioactivity at lower optimum temperatures when compared with enzymes of mesophiles and thermophiles. These are called as cold enzymes and they effectively decrease the activation energy and dependency of biochemical reactions on temperature by decreased activation enthalpy (D'Amico *et al*., 2006).

Cold induced proteins (Cips) are produced by cold modified microorganisms in reaction to a sharp decrease in temperature and their production is directly related to severity of cold shock. Another class of Cips is cold shock proteins (Csps) which are small proteins, binding to nucleic acids and having a length of 65-75 AAs (amino acids). Csps protect against some deleterious effects of reduced temperature, helping

in cell adaptation. Csps synthesis decreases after the sudden response of cold shock and biogenesis of other proteins increases. This results in the ability of cells to multiply at reduced temperatures, though at a slower pace (Keto-Timonen *et al*., 2016).

2.3.3 Psychrophiles – source of novel antibiotics

Scientists are extensively examining and pointing to unearth novel antimicrobial compounds from psychrophiles, because of their resistance to low temperatures and important applications in medical field. Multi-drug resistance to a huge number of antibiotic compounds, obtained from different sources, emphasizes to study this feature. Cold-adapted microorganisms exhibited a countless potential for the production of antimicrobial compounds (Tendulkar *et al*., 2021).

Polar regions such as Arctic and Antarctic represent special ecosystems, which inhabit microorganisms as an abundant supply of different biochemical compounds and new natural bioactive substances. In order to survive in these continuous low temperature environments (Santiago *et al*., 2015), microorganisms need a range of biological modifications that are vital for existence.

The likelihood of identifying novel functional metabolites with medicinal importance rises as a result of the frequent changes to gene regulation and metabolic pathways that go along with these adaptations. From polar species, a significant number of novel biological natural compounds with varied actions, such as anti-bacteria, anti-tumor, and anti-virus, were identified between 2001 and 2016. Several reviews of natural goods made from Arctic or Antarctic species have been written (Tian *et al*., 2017). Three new cyclic acylpeptides known as mixirins A, B, and C were discovered in the culture broth of the marine bacterium *Bacillus* sp., which was discovered in the sea mud near the Arctic pole (Zhang *et al*., 2004).

Seven known diketopiperazines, two new linear peptides, and a new diketopiperazine called cyclo-(D-pipecolinyl-L-isoleucine) were all found in the CFS of the Antarctic psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC125 (Mitova *et al*., 2005). Free radical scavenging abilities were demonstrated by Peptide 17 and a

known phenyl-containing diketopiperazine, with the phenyl group being necessary for action (Tian *et al*., 2017).

The *Salegentibacter* strain T436 was isolated from the depth of an Arctic Ocean sea ice floe, along with fifteen other known aromatic nitro compounds. Four additional aromatic nitro compounds were also reported from this strain. The newly discovered natural products had minimal cytotoxic and antimicrobial effects (Zereini *et al*., 2007). Additional research on the same bacterial isolate produced an additional seven new aromatic nitro compounds (Schuhmann *et al*., 2009).

The medium-sized spirotetronates known as lobophorins have a core ring structure with 13 carbon atoms (Vieweg *et al*., 2014). In 2013, Pan and colleagues isolated a *Streptomyces* species from a South China deep-sea sediment sample and discovered two novel families of chemicals, called lobophorins H and I (Pan *et al*., 2013). Particularly, lobophorin H demonstrated substantial efficacy against *Bacillus subtilis*, which was equivalent to ampicillin's activity (Pan *et al*., 2013).

Two novel secondary metabolites, arcticoside (30) and C-1027 chromophore-V (31), as well as three previously known substances, C-1027 chromophore-III, fijiolides A and B, were found during the development of an Arctic maritime actinomycete strain of *Streptomyces*. Compounds 30 and 31 inhibited *Candida albicans'* isocitrate lyase, an enzyme essential to *C. albicans'* pathogenicity. Additionally, with IC50 values of 0.9 and 2.7 M, respectively, compound 31 showed substantial cytotoxicity against colorectal carcinoma HCT-116 cells and breast cancer MDA-MB231 cells (Moon *et al*., 2014). *Pseudomonas* BNT1 Antarctic strain extracts were purified using bioassays, which resulted in the production of three rhamnolipids, including two novel ones (21, 22), which were found to act against pathogenic strains (Tedesco *et al*., 2016).

Antifungal compounds produced by Antarctic strains of *Candida sake* inhibited the growth of 5 apple pathogens, including 3-methylbutyl hexanoate, 3-methylbutylpentanoate, 2-methylpropyl hexanoate, and pentylhexanoate. This study is the first to examine the inhibitory effects of volatile organic compounds (VOCs) made by yeasts against *Alternaria* species that cause postharvest apple rot, and it is also the first to document the production of VOCs by C. sake (Arrarte *et al*., 2017).

A new alkaloid known as acremolin C (1) was discovered while studying the antibacterial properties of an Antarctic bacterium in a static culture of the fungus *Aspergillus sydowii* SP-1. The structure was subsequently described using spectroscopic methods. Also removed and identified were four well-known substances. In biological studies, compounds 2, 4, and 5 showed significant inhibitions of both MRSA and MRSE (Li *et al*., 2018).

2.3.4 Psychrophiles from glaciers

From samples of glacier silt, seashore mud, glacier melted ice, and *Deschampsia antarctica* rhizosphere collected from Collins glacier, Antarctica, culturable psychrophilic microorganisms were recovered. Amplified Ribosomal DNA Restriction Analysis was used in a culture-dependent molecular method for taxonomy categorization. According to the analysis, Beta-proteobacteria (35.2%) predominated the bacterial profile, which also included Gamma-proteobacteria (18.5%), Alpha-proteobacteria (16.6%), Gram-positive with high GC content (13%), *Cytophaga-Flavobacterium-Bacteroides* (13%), and Gram-positive with low GC content (3.7%) (García *et al*., 2011).

For a variety of reasons, the microorganisms found in glacial ice are heavily researched worldwide. However, compared to their arctic and Antarctic counterparts, tropical glaciers have gotten far less attention due to the challenges of reaching and working at elevations of more than 5000 m above sea level. In the current study, we isolated and characterized a total of 45 pure isolates derived from direct plating of melted ice that was collected at the base of a small glacier that was rapidly retreating at an altitude of about 4900 m in Mount Humboldt (Sierra Nevada National Park, Mérida State, Venezuela). Many of the isolates generated and excreted cold-active extracellular enzymes (proteases and amylases), and the majority were either psychrophilic or psychrotolerant (Ball *et al*., 2014).

The variety of cultivable bacteria in Pakistan's Siachen Glacier, which has not before been researched, was investigated. About 33 (66%) of the 50 isolates were Gram negative, whereas 17 (34%) were Gram positive. About half of the isolates could grow between 4-37°C and produced pigment. Gram negative bacteria were found to be dominated by Proteobacteria, particularly γ- and β-proteobacteria, and

Flavobacteria, according to 16S rRNA gene sequences. The majority of the proteobacteria (51.51%, 17) belonged to the genus *Pseudomonas*. Four (12.12%) *Alcaligenes* and four (12.12%) *Janthinobacterium* strains made up the - proteobacteria. The two most prevalent genera in the phylum Actinobacteria, which includes Gram positive bacteria, are *Rhodococcus* (23.52%, 4) and *Arthrobacter* (23.52%, 4). (Rafiq *et al*., 2017).

On northern Ellesmere Island in the Canadian Arctic, sediments and soil near the front of a melting glacier were used to extract two strains of psychrophilic basidiomycetous yeast. These isolates seemed to be a new species, according to analysis of the rDNA's internal transcribed spacer (ITS) and big subunit D1/D2 regions. The unique species' closest relatives were *Vishniacozyma globispora* and *V. dimennae*, which shared 82.2 and 81.6 percent of their sequence similarities, respectively (Tsuji *et al*., 2019).

The Kanchenjunga Biosphere Reserve, a UNESCO World Heritage Site and one of the major biological hotspot areas in the Eastern Himalayas, includes the northern Sikkim district that is home to the Changme Khangpu Glacier. Based on the 16S amplicon sequences, the top four dominating phyla were Proteobacteria (56%), Firmicutes (16%), Actinobacteria (12%), and Bacteroidetes (8%), in that order. Gram positive bacteria predominated (72%) followed by Gram negative bacteria (32%), according to PLFA analysis. The predominant fatty acids detected in the soil sample from a moraine were PUFA (61%). This is the initial research on moraine soil from Sikkim's glaciers, and it is the first of its type (Sherpa *et al*., 2021).

2.4 Secondary metabolites

Secondary metabolites or natural products are a diverse range of naturally occurring metabolic byproducts that are not necessary for the vegetative growth of the producing organisms but are nonetheless recognized as differentiating substances with adaptive roles (Thirumurugan *et al*., 2018). The normal growth and development of an organism does not need the presence of organic substances referred to as secondary metabolites. While primary metabolites are essential to the life of the species, the absence of secondary metabolites leads to a long-term decline in the organism's capacity to survive, typically contributing to organism defense rather than immediate death. These compounds are part of an extremely diverse class of organic molecules

generated by a variety of species, including plants, fungi, bacteria, algae, and mammals (Costa *et al*., 2012).

Secondary metabolites, such as antibiotics, are substances that naturally exist and are essential for the survival of the organisms that produce them. Secondary metabolites serve as metal transporters, symbiotic agents between microorganisms and higher animals like plants and nematodes, sexual hormones, competitive weapons employed against other bacteria, fungi, amoebae, plants, etc (Demain and Fang 2000; Thirumurugan *et al*., 2018). Humans extract a wide variety of secondary metabolite secretions for their own health, to fill in gaps in the food pyramid, to boost agricultural productivity, and to have a lasting impact on the economy and they are a source of antibiotics (Thirumurugan *et al*., 2018).

There are over 2,140,000 secondary metabolites that have been identified, and they are frequently categorized based on the wide variety of their structure, function, and biosynthesis. Terpenoids and steroids, fatty acid-derived compounds and polyketides, alkaloids, non-ribosomal polypeptides, and enzyme cofactors are some examples of the five main types of secondary metabolites (McMurry, 2015, Thirumurugan *et al*., 2018).

The primary suppliers of secondary metabolites include several marine creatures, bacteria, fungus, and plants (Berdy, 2005). Secondary metabolites produced by microorganisms have peculiar structures and low molecular masses. According to Demain (1999), the structurally varied metabolites exhibit a wide range of biological actions, including antibacterial agents, enzyme inhibitors, antitumor, immunosuppressants, antiparasitic agents, stimulators of plant growth, herbicides, insecticides, and anti-helmintics. They are created when the bacteria are in their late development phase. The synthesis of secondary metabolites in microorganisms is regulated by specific regulatory mechanisms because it is often suppressed in the logarithmic phase and decreased in stationary growth stages (Feher and Schmidt, 2003).

2.5 Antibiotics

When present in modest quantities, low-molecular-weight microbial compounds called antibiotics stop the growth of new germs. Low molecular weight is defined as a molecule having a certain chemical structure and a relative mass of no more than a few thousand. When we claim that an antibiotic "inhibits the growth of other germs," what we really mean is that it either prevents the microbe from reproducing either temporarily or permanently. When the inhibition is prolonged, the antibiotic activity is described as being bactericidal, or more generally, cidal (e.g., fungicidal). If inhibition disappears once the antibiotic is removed from its medium, it is said to have a bacteriostatic (fungistatic) or static action. Since even naturally occurring, vital components of cells may cause harm when present in excessive concentrations, the qualification "at low concentration" is added to the definition. Every protein contains glycine, which, when present in high concentrations in the culture medium, has a strong bactericidal impact on certain bacteria. Similarly, ethanol or butanol, the fermentation byproducts of some microorganisms, cannot be referred to as antibiotics since they only have antibacterial effects at high concentrations (Lancini *et al*., 1995).

Antibiotics have been categorized using a variety of factors, including their method of action, generating organisms, and biosynthetic pathways (Berdy, 1974; Queener *et al*., 1978). These requirements are problematic due to the fact that certain microorganisms can generate a large number of antibiotics or that many modes of action can be active at once. Following that, antibiotics were divided into 13 classes based on their chemical makeup. This categorization is the most widely used and quickly adapts to newly discovered antibiotics. The groups are aminoglycosides, ansamacrolides, beta-lactams, chloramphenicol and analogues, lincosaminides, macrolides, nucleosides, oligosaccharides, peptides, phenazymes, polyenes, and tetracyclines (Chandra and Kumar, 2017).

2.5.1 Biosynthesis of antibiotics

The secondary metabolites is regulated by complex biochemical pathways. However, marked differences in expression can even be found within a single species experiencing different environmental stress (Gouvea *et al*., 2012; Sacristán-Soriano *et al*., 2012). As a result, some microorganisms can produce very different metabolites

instead of a single one, e.g., a strain of *Streptomyces* can produce a variety of 35 anthracyclines. The biosynthetic pathways for most secondary metabolites are not clearly established (Mehta *et al*., 2018).

The biosynthetic pathways for most antimicrobial secondary metabolites are derived from precursors of primary metabolisms (Gonzalez *et al*., 2003). In a complex reaction scheme, most antimicrobial secondary metabolites are synthesized from one or a combination of different biosynthetic pathways which are overviewed below:

- Sugar derived metabolites: examples of secondary metabolites in this group are streptomycin, neomycin and kanamycin.
- Shikimic acid derived metabolites: e.g., ansamycin and rifamycin
- Aliphatic amino acid derived metabolites: e.g., penicillin, cephalosporins and cephamycins.
- Aromatic amino acid derived metabolites: e.g., actinomycin, indolmycin, lincomycin, novobiocin, and polymyxin.
- Chorismic acid derived Metabolites: e.g., candicidin, nystatin and chloramphenicol.
- Acetyl-CoA and malonyl-CoA derived metabolites: e.g., erythromycin, vancomycin and tetracyclines (Mehta *et al*., 2018).

Antibacterial glycopeptides that are antimicrobial non-ribosomal peptide synthetases produce the heptapeptides known as GPAs (NRPS). NRPS complexes are multimodular enzyme factories that put amino acids together in an orderly fashion. The primary sequence of the peptide product is determined by the arrangement of the modules in the NRPS; each module represents one amino acid (Yim *et al*., 2014; Marahiel and Essen, 2009).

A number of *Bacillus* species generate peptide antibiotics that are either produced by ribosomal or non-ribosomal mechanisms. By use of a multienzyme thiotemplate method, the antibiotics gramicidin, tyrocidine, and bacitracin are not ribosomally produced. However, the process by which surfactin and mycobacillin are made non-ribosomally appears to be different from that of the multienzyme thiotemplate. Other antibiotics, like subtilin, are ribosomally produced and are gene-encoded (Nakano and Zuber, 1990).

Antibiotics having a broad range and distinctive structural characteristics are known as nucleosides. Nucleoside antibiotic biosynthesis is controlled on several different levels. Due to their distinctive structural characteristics, nucleoside antibiotics may have undergone peculiar enzyme reactions throughout the biosynthesis process (Niu and Tan, 2015).

Huge modular non-ribosomal peptide synthetases are involved in the biosynthesis of the lipopeptide calcium-dependent antibiotic (CDA), a family of compounds that, like polyketides, has drawn a lot of interest from researchers studying natural products. Although the control of CDA biosynthesis has not received much attention, it is probable that the SARP CSR encoded by the *cdaR* activates the genes responsible for producing CDA (Liu *et al*., 2013).

Streptomyces are vital microorganisms, producing greater than 70% of commercially significant antibiotics. Autoregulators hormones are involved in production of these antagonistic compounds. However, there is little knowledge about the signaling molecules involved in regulating antibiotic synthesis in many other members of this genus. A signaling molecule i.e. avenolide was purified from *Streptomyces avermitilis* and spectroscopic analysis and chemical synthesis confirmed it as a member of class of *Streptomyces* autoregulator. Avenolide was found to be essential in avermectin production and may correspond to another class of *Streptomyces* autoregulator, having a role in regulating antibiotic synthesis (Kitani *et al*., 2011).

Streptomyces ahygroscopicus produces tetramycin, and it is biosynthesized along the same mechanism as other polyene antibiotics using small molecular carboxylic acids as TA precursors (acetyl-CoA, malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA). Tetramycinolide is produced through the polyketide synthase pathway (PKS), followed by the carboxylation and glycosylation of TA and the C4 hydroxylation of TB from TA (Cui *et al*., 2015; Chen *et al*., 2021; Cui *et al*., 2016).

The process of antibiotic production by specific microorganisms is complex in nature but the organism synthesizing these substances must be able to protect itself from their poisonous effects. Tung found that SimR, the protein *Streptomyces antibioticus* make use of to regulate antibiotic export, can attach only to the antibiotic itself or DNA but not to both. This shows that when there is antibiotic present around, then

SimR liberates the DNA which expresses a gene, encoding a pump to remove simocyclinone from the cell (Le *et al*., 2011).

2.5.2 Nature of antibiotics

Antibiotics and other bioactive microbial secondary metabolites are the only category of natural compounds that include such a broad range of particular and complicated chemical structures, as well as a fascinating array of different, unique functional groups (Berdy, 2005). The substances in this category encompass all varieties of organic compounds, ranging in complexity from the straightforward acrylamidine (MW: 72) to the macrocyclic colubricidin (MW: 2154), which has a wide glycosylated macrolactone ring as well as pyridine and pyrrole moieties. The macrocyclic versipelostatin also has intriguing novel structures, and the polycyclic tetropetalone-A (Komoda *et al*., 2003) comprises one of the most complex heterocyclic ring systems with quinone and glycosidic functions (Park *et al*., 2002).

In general, bioactive microbial metabolites have a number of structural properties with antibiotics. The most characteristic structural elements are without a doubt the cyclopeptide/cyclopeptolide, depsipeptide, and other macrocyclic lactone/lactam ring systems, notably in the actinomycetales products. The most common class of antibiotics chemically comprises of various peptide-type compounds made from amino acids, ranging from low-molecular-weight proteides, which make up around 5000 molecules, to straightforward amino acid derivatives, such as b-lactams. The second largest group is composed of varied macrolactones, polylactones, and ansalactones, which also contain simple lactones with 8–12 members, macrolides, further macrocyclic lactones with 18–60 membered rings, and other condensed macrolactones (Berdy, 2005).

In comparison to traditional antibiotics, antimicrobial peptides have a number of distinct benefits, such as a slower establishment of resistance, broad-spectrum antibiofilm efficacy, and the capacity to modify the host immune response in a way that is beneficial. A variety of bioactive tiny proteins known as antimicrobial peptides (AMPs) constitute the body's first line of defense against pathogens (Magana, 2020).

2.5.3 Mechanisms of action and resistance of antibiotics

Based on the function that is impacted, antimicrobial agents can be classified according to their mode of action. These generally include the following: inhibition of the synthesis of cell walls or nucleic acids, inhibition of ribosome function, inhibition of cell membrane function, and inhibition of folate metabolism. Antimicrobials are one of the most effective medical treatments, but a rising tide of microorganisms that are resistant to antibiotics is reducing their effectiveness. There are two methods to define resistance: when germs inherently lack the antimicrobials' target sites and are thus resistant to the drugs, this is known as intrinsic resistance and other is called as acquired resistance when sensitive bacteria develops resistance against antibiotics (Dowling *et al*., 2017). In Table 2.1, the most widely used antibiotic classes' modes of action and resistances are described.

Table 2.1 Mechanism of action and resistance of commonly used antimicrobial agents (Dowling *et al*., 2017).

Antibiotics class	Mode of action	Mechanism of Resistance
Beta-lactam antibiotics	Obstructs cell wall biosynthesis. Binds penicillin binding proteins enzymes that assist in peptidoglycans synthesis.	By beta-lactamase production mainly through *bla* genes. Changes cell wall protein enzymes to prevent binding to PBPs.
Cephalosporins	Obstructs cell wall biosynthesis. Binds penicillin binding proteins enzymes that assist in peptidoglycans synthesis.	By enzymes cephalosporinases that attack beta lactam ring.
Beta-lactamase inhibitors	Attaches to beta-lactamase enzymes.	Through ESBLs (extended spectrum beta lactamases).
Fluoroquinolone	Interferes with DNA synthesis by binding to DNA gyrase or topoisomerase II and IV.	By alteration of DNA gyrase enzyme (gyrA and gyrB). Mutations of outer membrane porins proteins, thus decreasing permeability. By efflux pump mechanism.
Aminoglycosides	rRNA- binds to 30S subunit, causing genetic code to misread. Hinders protein biosynthesis. Change in permeability of cell membrane.	Aminoglycosides are inhibited by the process of adenylation, phosphorylation, and acetylation.
Folate pathway inhibitors	Purine synthesis for DNA. Inhibits synthesis of folic acid.	Resistance occurs because of chromosomal mutations - more commonly plasmid mediated resistance. Folic acid synthesis pathway stopped by resistant dihydrofolate reductase (*dfr* gene).
Tetracycline	Ribosomal RNA binds to 30S subunit and restricts amino acid transfer. Hinders protein biosynthesis.	Inducible efflux E. coli etc. (*tetA, tetB, tetC*). Binding site changes (*tetO, tetM* genes)

2.5.4 Factors affecting antibiotic production

Different environmental conditions, such as temperature, pH, oxygen availability, nutrients (nitrogen, phosphorous, and carbon source), growth rate, etc., affect the

production of secondary metabolites during fermentation. Maximizing the metabolite yield requires optimizing the manufacturing medium (Gao *et al*., 2009; Singh *et al*., 2017). This may be done using a variety of methods, including the traditional "one factor at a time" approach and more recent statistical and mathematical methods like the artificial neural network (ANN), genetic algorithm (GA), etc. Every approach has benefits and downsides of its own, yet despite these, some strategies are used to get the greatest outcomes. The desired outcomes are also achieved through the combination of several optimization strategies (Singh *et al*., 2017).

In order to maximize the yield and activity of the intended product, medium optimization studies are frequently conducted in the chemical, food, and pharmaceutical sectors. There is currently relatively little information known on the involvement of factors and their levels in regulating the synthesis of metabolites (such as acids and antibiotics) by various strains. Researchers looked into the dietary needs for the formation of secondary metabolites in order to increase productivity of the metabolites (such as antibiotics, etc.), and they discovered that these requirements varied from strain to strain (Shih *et al*., 2002; Singh *et al*., 2012; Singh *et al*., 2017).

In one study, the antifungal activity of *Aspergillus fumigatus* against soil samples was evaluated. To maximize the formation of biologically active antifungal compounds, inhibiting bacteria were grown under a variety of conditions, including pH, medium, temperature, and incubation duration. According to the results of the optimization for the antifungal compound, based on the type of culture medium, pH, temperature, and incubation duration, the SCB medium with pH 7 at 30°C for 5 days produced the most antifungal compounds (Azish *et al*., 2020).

In the Karbala Province of Iraq, *Streptomyces* sp. LH9 was previously isolated from arid soil. This strain generated antibiotics that were effective against *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli,* and four other harmful bacteria. The vital nutritional requirements of carbon, nitrogen, and phosphate in fermentation medium were optimized using various amounts of these sources. All of the dietary factors that were examined had an effect on the quantity of antibacterial metabolites produced by *Streptomyces* sp. LH9 (Al-Ghazali and Omran, 2017).

In one investigation, *Stenotrophomonas* sp. was identified by partial 16S rRNA sequencing as the ABN18 bacterial strain, which was isolated from sewage effluent and capable of producing antibiotic substances. The production of antibacterial substance was subjected to different environmental conditions. To maximize the generation of antibacterial substances, the incubation conditions were optimized for temperature, pH, and time at 33.17°C, 7.45, and 88.8 hours, respectively (Sarkar *et al*., 2021).

Another study focused on the *Aspergillus flavus* MTCC 13062 strain that was obtained from the Hisar solid waste disposal site's antibacterial characteristics (India). Response Surface Methodology (RSM) was utilized to increase the synthesis of bioactive chemicals by *Aspergillus flavus* MTCC 13062 at pH 7.5, 28°C, and a seven day incubation period. By 22%, 75%, and 50%, respectively, respectively, this markedly improved the antibacterial activity against *Escherichia coli, Staphylococcus aureus,* and *Candida albicans* (Dudeja *et al*., 2021).

2.5.5 Why to explore novel antibiotics

Bioprospecting is the process of organizing research to identify organisms from which pharmaceutical medications and other goods with a marketable potential might be produced, frequently by examining microscopic flora and fauna and the traits they display (Page and Willahan, 2018). The major reason for exploring novel antibiotics is the antibiotic resistance.

Currently, 23,000 fatalities come from diseases linked to antibiotic resistance that affect more than 2 million North Americans each year (CDC 2017). According to Cassini *et al*., (2019), over 33,000 fatalities directly result from over 700,000 instances of antibiotic-resistant illnesses each year in Europe, with a total estimated cost of over €1.5 billion (Garau, 2010). Despite a 36% rise in antibiotic use in humans from 2000 to 2010 (Laxminarayan *et al*., 2016), almost 20% of fatalities globally are now thought to be caused by infectious illnesses (Martens and Demain, 2017). Nosocomial infections rose to prominence as a major cause of morbidity and death (Akova 2016), leading to longer hospital stays and higher healthcare expenses (McFee 2009). Furthermore, according to Theuretzbacher (2012), multidrug-resistant bacteria currently account for over 15% of nosocomial infections; for some of these diseases,

there are no effective antibiotics available (Frieri *et al*., 2017). Future prospects are not any better; for instance, a government-commissioned research in the United Kingdom anticipated that by 2050, antibiotic-resistant diseases will be responsible for 10 million annual deaths worldwide (Ribeiro *et al*., 2019).

It is a serious and complicated problem that the world is seeing an increase in resistance to common antibiotics. Since everyone may develop infections, which may be resistant to the most commonly used (and frequently misused) antibiotics, regardless of age, country, class, or even medical history, many scientists are immediately exploring for alternatives (Page and Willahan, 2018). The danger is larger now because of the misuse of common medications, which frequently results from unneeded prescriptions, easy access made possible by the pharmaceutical industry, the presence of these substances in the food we eat and the extensive use of antibiotics as growth promoters in livestock animals. The WHO describes this as leading to a "post-antibiotic age, in which ordinary infections and mild injuries can once again kill." This will make both basic and complex disorders and illnesses more difficult to cure (Dugassa and Shukuri, 2017; WHO, 2018). By 2050, infections will kill more people annually than cancer, according to one research, if nothing is done to stop the global abuse of antibiotics and the subsequent increase of antibiotic-resistant bacteria (de Kraker *et al*., 2016). Therefore, more study and the creation of new alternatives must start as soon as possible; else, mankind would have to deal with an uncontrollable epidemic that might annihilate human populations (Page and Willahan, 2018).

Antimicrobial resistance (AMR) is still having a significant negative impact on the world's health and economy, although COVID-19 is predicted to mask it for a time. The improper use of antibiotics during the COVID-19 pandemic will have catastrophic implications on the control of AMR (Ghosh *et al*., 2021). The COVID-19 pandemic is serving as a sharp reminder of the necessity of being ready before a public health emergency strikes and the critical function of antibiotics. Effective antibiotics are essential for contemporary medicine as well as for helping to protect immunocompromised patients during an epidemic. Unfortunately, the severity of the threat presented by antibiotic-resistant bacteria outweighs our degree of preparedness (Kim *et al*., 2020).

It is estimated that $200 million is needed for a molecule to reach commercialization (Renwick *et al*., 2016). A new antibiotic might be so effective that it is only used as a last resort therapeutic, leading to limited commercialization, or it might be so ineffective because of AMR, adding to the complexity. In any case, the bottom line suggests comparable risks with significantly lower investment returns compared to other medications (Smith, 2019), making the business of discovering antibiotics unappealing (Ribeiro *et al*., 2019).

Only two of the approximately 38 businesses that have antibiotics in clinical development are among the top 50 pharmaceutical companies in terms of sales. The huge pharmaceutical corporations that formerly controlled this industry are no longer studying more than 95% of the items now under development; instead, tiny businesses are doing so. Additionally, more than 70% of the businesses are regarded as pre-revenue, which denotes that they do not yet have any previously produced, commercialized, and sold items on the market (Trusts, 2021).

Pew and other groups concerned about antibiotic resistance push for legislation to solve the technical, legal, and financial barriers to the creation of new medicines (Trusts, 2021). Pew supports a set of incentives to overcome the financial difficulties that businesses have while marketing antibiotics (Trusts, 2021; Kwon and Powderly, 2021). These include novel financing mechanisms, innovative antibiotics based on public health value, and reimbursement amendment that would compensate hospitals by guarantors at a reasonable rate for usage of novel drugs. A regulatory advancement is the limited-population antibacterial drug route (LPAD), which was permitted by the 21st Century Cures Act, which passed and was signed into law in 2016. The most urgently needed antibiotics may now be developed more easily while still meeting FDA safety and efficacy guidelines. Additionally, concerted initiatives to advance fundamental scientific research and promote collaboration between academia and industry are necessary for a healthy pipeline (Trusts, 2021).

2.5.6 Golden- and post-antibiotic era

The antibiotic revolution began in 1928 with Sir Alexander Fleming's (1881–1955) discovery of penicillin. The first penicillin, penicillin G, was refined by Ernst Chain and Howard Florey in 1942, although it wasn't generally accessible outside of the

Allied military until 1945 (Adedeji, 2016). The age of antibiotics began with this. Since half of the currently used medications were discovered during this time period (Davies 2006), the 1950s to 1970s have been referred to as the "golden age" of novel antibiotic discovery (Aminov, 2010). Since that time, no new classes of antibiotics have been developed. After that, the strategy for finding new antibiotics was to modify current antibiotics (Aminov, 2010).

According to a report issued by the World Health Organization, the "post-antibiotic" future is quickly approaching (Kwon and Powderly, 2021). The WHO study, which compiles information from 129 member states to demonstrate widespread antimicrobial agent resistance around the world, offers no cause for optimism. In the prologue to the study, WHO associate director general for health security Keiji Fukuda states, "A post-antibiotic future - in which ordinary diseases and slight traumas might kill - is instead a very real potential for the twenty-first century."

The emergence of resistance to carbapenems, the "antibiotics of last resort," is arguably the most concerning trend, according to Timothy Walsh, a medical microbiologist at Cardiff University in the UK and a report adviser. Ultimately, the absence of worldwide statistics on antibiotic resistance may be the report's most unexpected conclusion. Although the study recommends the creation of a worldwide monitoring network, it is doubtful that more funding would be provided. At the Bill and Melinda Gates Foundation in Seattle, Washington, epidemiologist Keith Klugman says, "I don't know that there are the resources to deal with it. It's a major problem" (Reardon, 2014).

2.5.7 Screening techniques for antimicrobial activity

Disk-diffusion, well diffusion, broth or agar dilution are well-known and frequently used screening techniques for antimicrobial activity detection, but others, like flow cytofluorometric and bioluminescent methods, are less popular because they require specialised equipment and additional testing for reproducibility and standardization, even though they can provide quick results of the antimicrobial agent's effects (Balouiri *et al*., 2016).

Agar disk-diffusion testing is the preferred method for routine antibiotic susceptibility testing in many clinical microbiology laboratories (Balouiri *et al*., 2016; Chouhan *et al*., 2017). Several recognised and authorised standards for testing bacteria and yeasts are currently published by the Clinical and Laboratory Standards Institute (CLSI) (CLSI and Wayne, 2004; CLSI, 2012). In this well-known procedure, agar plates are inoculated with a standardised inoculum of the test microorganism. On 6 mm-diameter filter paper discs, the test material is then added in the necessary concentration before being deposited on the agar surface. The appropriate settings are used to incubate the Petri plates. An antimicrobial drug that diffuses into the agar normally inhibits the test microorganism's germination and development, and the diameters of the inhibitory growth zones are then calculated. But compared to other methods, the disk-diffusion assay has several advantages, such as simplicity, low cost, the ability to test a wide range of bacteria and antimicrobial medicines, and the simplicity with which the results may be understood (Mbewana-Ntshanka *et al*., 2021; Saedi *et al*., 2020).

The antimicrobial gradient technique, which combines the concepts of diffusion and dilution processes, is used to compute the MIC value. The possibility of creating a gradient in the concentration of the antimicrobial agent under investigation in the agar medium serves as the foundation. This method has a commercial application in The Etests (BioMérieux). In the technique, an agar surface that has already been infected with the test microorganism is covered with a strip that has been impregnated with an antimicrobial agent in a gradient of increasing concentration from one end to the other (Balouiri *et al*., 2016).

The agar well diffusion method is commonly used to evaluate the antibacterial effect of plant or microbial extracts (Magaldi *et al*., 2004; Valgas *et al*., 2007). Similar to the disk-diffusion method, the agar plate is inoculated by spreading technique. The antimicrobial extract is then added to the well after a volume (20–100 L) of the required concentration has been aseptically inserted through a hole with a diameter of 6–8 mm. The incubation procedure is then carried out with the test microorganism on an appropriate agar plate. The studied microbial strain cannot develop because of the antibiotic component, which disperses throughout the agar substrate (Balouiri *et al*., 2016; Tardugno *et al*., 2018).

The disk-diffusion method's process is comparable to the agar plug diffusion method, which is frequently used to emphasise the competition between microorganisms (Elleuch *et al*., 2010). Making an agar culture of the target strain using tight streaks on the plate surface requires using the strain's proper culture media. Microbial cells release chemicals that spread across the agar media as they proliferate. Once the test microorganism has been incubated, an agar-plot or cylinder is sliced aseptically using a sterile cork borer and placed on the agar surface of another plate (Balouiri *et al*., 2016).

The cross-streak technique is used with microorganisms to swiftly detect antagonism (Lertcanawanichakul and Sawangnop, 2008). The target microbial strain is inoculated through a onetime streak in the middle of the agar plate. After an incubation period, the microorganisms are added onto the plate in onetime streak perpendicular to the centre streak. Antimicrobial interactions are evaluated by evaluating the size of the inhibitory zone after further incubation (Nayaka *et al*., 2020).

The least often employed approach is agar diffusion, which is also familiar as the agar contact method. It involves transferring the antimicrobial substance by diffusion from the TLC chromatogram to an agar plate that has formerly been contaminated with the target pathogen. After some time, the chromatogram is taken off to allow for diffusion, and the agar plate is then incubated. Where the antimicrobial compounds come into touch with the agar layer is where the growth inhibition zones form (Marston, 2011).

The micro- or macro-dilution of broth is an easiest method for testing an antimicrobial's susceptibility. The method involves producing two-fold dilutions of the antimicrobial metabolite (for example, 1, 2, 4, 8, 16, and 32 mg/mL) in a liquid growth medium provided through tubes with a minimum capacity of 2 mL (macrodilution), or with lower concentrations using 96-well microtitration plate (microdilution). A microbial inoculum prepared in the same medium is then injected into each tube or well after a standardised microbial suspension has been diluted to 0.5 McFarland scale. After well-mixing (typically without agitation), the inoculation tubes or 96-well microtitration plate are incubated, depending on the test microorganism (Romulo *et al*., 2018; Tardugno *et al*., 2018; Patel *et al*., 2021).

2.5.8 Extraction of antimicrobial compounds

The initial stage in separating the desired natural products from the crude materials is extraction. For solvent extraction, the choice of the solvent is essential. The selection of solvents should take into account selectivity, solubility, cost, and safety. According to the principle of similarity and inter-miscibility (like dissolves like), solvents whose polarity values are close to those of the solute's are likely to perform better, and vice versa. With longer extraction times within a specific time frame, the extraction efficiency rises (Zhang *et al*., 2018).

Secondary metabolites are usually extracted from microbial cultures by solvent extraction procedure. This method has several characteristics that closely resemble chromatographic separations. A liquid-liquid extraction is often performed by taking two immiscible phases, one of which is typically aqueous (culture broth), and the other of which is typically an organic solvent (e.g., ethyl acetate, chloroform, hexane, dichloromethane etc). Compounds in the system will spread between the two phases as the two phases are placed into a device known as a separating funnel. There are two terminology that are used to describe this distribution: the partition coefficient and the distribution coefficient. The ratio of the solute concentration in the organic phase to the solute concentration in the aqueous phase is known as the distribution coefficient. A more accurate way to determine if you have succeeded is to use the partition coefficient, which is the ratio of the moles of solute in the two phases (Wenzel, 2020). However, solid-phase extraction (SPE) is now the most widely used technique for the treatment of aqueous samples and extracts in laboratories (Ramos, 2020).

Due to its benefits in lowering working time and boosting yield, more modern technologies such supercritical fluid extraction (SFE) (Cocks *et al*., 1995; Bader *et al*., 2020) and Ultrasound aided extraction (UAE) have drawn increased attention (Zheng *et al*., 2021). According to Sosa-Hernández *et al*., (2018), supercritical fluid extraction (SFE) is an analytical technique used to separate high-value bioactive chemicals from complicated matrixes. Supercritical fluids, which are used in SFE, display both gas-like and liquid-like properties above their critical points, such as increased transport capabilities and low surface tension (Silva *et al*., 2020).

The ultrasound aided extraction (UAE) method creates cavitation bubbles by passing kilohertz-range (20 kHz to 100 kHz) sonic pulses into the solution. An increase in the mass transfer of valuable bioactive chemicals through cell membranes into the solution is caused by the cavitation bubbles burst at the surface of the intricate sample matrix (Tiwari, 2015).

2.5.9 Characterization of antimicrobial compounds

Recently, the metabolomics technique has been utilized to discover metabolites in order to find secondary metabolites (Rochfort, 2005). Liquid chromatography-mass spectrometry (LC-MS) is used to detect unidentified molecules from complicated samples for the identification of complex metabolites. According to Villas-Bôase *et al*., (2005) and Lee *et al*., (2011), this method has a high throughput and is extremely sensitive for the detection and identification of unidentified chemicals in biological samples. In order to detect recognized and unidentified compounds from culture extract, Bibi *et al*., (2018) used LC-MS analysis. All six bacteria strains' culture extracts included a number of active compounds, according to an LC-MS study. By comparing the findings of an LC-MS study with those from the NIST database, the identification of the metabolites was made.

The complete examination of the biochemical composition of cells, tissues, or biofluids through the analysis of extracts is known as metabolomics (Oliver *et al*., 1998). Typically, NMR- and/or MS-based analytical methods have been used in metabolomics research to investigate the metabolite composition of experimental materials. Recent years have seen a lot of interest in LC-QToF-MS (Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry), particularly for microbial metabolic fingerprinting research (Oliver *et al*., 1998; Hodson *et al*., 2007; Dunn *et al*., 2011; Ng *et al*., 2014; Bose *et al*., 2015).

The detection, separation, and identification of metabolites are crucial processes in metabolomics; there are several analytical techniques that can evaluate metabolites in biological materials. Despite some success in profiling metabolites in microbial samples using nuclear magnetic resonance-based methods (Bundy *et al*., 2005; Lin *et al*., 2008), mass spectrometry (MS)-based tools are currently the most popular in

metabolomics due to their higher sensitivity and specificity in identifying analytes (Smart *et al*., 2010).

Gas chromatography mass spectrometry (GC-MS) has solidified its position as a major technical platform for secondary metabolite profiling in both plant and non-plant species during the past few years (Kanthal *et al*., 2014). Because of its great separation power and ability to accurately identify hundreds of organic compounds in a single study, gas chromatography combined with MS (GC-MS) has been widely utilized in metabolomics (Roessner *et al*., 2000; Villas-Bôas *et al*., 2005; Dunn *et al*., 2005; Koek *et al*., 2006). GC-MS systems are stable, strong, affordable, highly sensitive, and—most importantly—simple to operate. Additionally, the utilization of chemical libraries for the identification of metabolites is made possible by the very repeatable mass spectra produced by the electron impact ionization employed by typical GCMS equipment (Schauer *et al*., 2005). GC-MS instruments, however, only examine volatile chemicals (Smart *et al*., 2010).

MATERIALS AND METHODS

3.1 Materials used in the study

3.1.1 Culture media

Luria-Bertani broth (LB), Nutrient broth (NB), Mueller-Hinton agar (MHA), Tryptic soya broth (TCA), Technical agar (TA), and Mueller-Hinton broth (MHB) from Sigma-Aldrich are the culture media used in the study.

3.1.2 Chemicals

Chemicals and reagents used in the study were Agarose, L-leucine, Glycerol, Arginine, Barium chloride, Yeast extract, Sulfuric acid, Sodium chloride, Normal saline, Potassium chloride, Phenol, n-Hexane, Chloroform, Ethyl acetate, Isoamyl alcohol, Petroleum ether, Glycogen, Chloroform, Ammonium acetate, Sodium carbonate, Ethanol, Potassium sodium tartrate, TEN buffer, Copper sulphate, Tris-borate-EDTA, Folin phenol, Loading dye, Methanol, Hydrochloric acid, Dimethysulfoxide, Sodium hydroxide, Artificial sea water, Tryptophan, DPPH, Threonine, and Ascorbic acid.

3.2 Area of the study

The samples were previously isolated by the same research group from Passu glacier, situated in the Karakorum Mountain range in Passu village (Upper-Hunza, Gigit-Baltistan, Pakistan). The distance of Passu glacier from Gilgit is about 150 km and has height of more than 7000m. A total of 56 samples had been isolated from Passu glacier water, ice and permafrost. These isolates have been preserved in 70% glycerol and kept at -20°C. All the isolates have been previously subjected to antimicrobial activity analysis against test strains which indicated that 28 (50%) isolates had antimicrobial potential.

Figure 3.1 Location from where strains were isolated

3.3 Microorganisms used in the study

3.3.1 Bacterial isolates

Preserved cultures of twenty-eight bacteria in glycerol, isolated from Passu glacier were present at Applied Environmental and Geomicrobiology Lab, Quaid-i-Azam University, Islamabad. The isolates were coded as LTP8, LTP10, HTP9, HTP12, HTP13, HTP14, HTP19, HTP27, HTP36, HTP37, and HTP38. HTP coded isolates were isolated from sediment while LTP coded isolates from ice. These isolates were carefully chosen for the study on the basis of the previously available data of their preliminary screening for antimicrobial potential. In order to use these isolates for further study, they are revived from their preserved culture. An aliquot of 200 µL of each glycerol preserved bacterial culture was spread on LB agar plate and incubated for 3 to 5 days. Isolated strains with code HTP were incubated at 15°C while isolates LTP8 and LTP10 at 4°C. After incubation period, pure isolated colony of each isolate was picked and processed for downstream application. These eleven isolates were subjected to antimicrobial activity analysis. Four isolates (HTP12, HTP13, HTP36, and LTP10) were designated for optimization studies while isolate LTP10 was

processed for further studies which included extraction and characterization of its antimicrobial compounds. Throughout the study, initially fresh cultures of bacterial isolates were used for optimization studies and for production of antimicrobial metabolites. For comparison purpose, the cultures of the isolates were taken at log phase and stationary phase respectively (Koku *et al*., 2003) and then used to determine maximum antimicrobial compound production.

3.3.2 Test organisms

To determine the antimicrobial potential of the selected isolates mentioned above against different microorganisms, several test organisms were used in the study. These test strains included ATCC strains such as *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), and *Staphylococcus epidermidis* (ATCC 12228). In addition, clinical isolates of *Escherichia coli, Staphylococcus aureus, Salmonella enterica, Pseudomonas aeruginosa, Candida krusei* and *Candida albicans* (obtained from Pakistan Institute of Medical Sciences Hospital) were also used. The antibiotic sensitivity profile of clinical bacterial isolates was determined by disk diffusion method (Moussaoui and Alaoui, 2016).

3.4 Scheme of study

The current study is aimed at the production and characterization of antibacterial metabolites from bacterial strains. The research was performed in Applied Environmental and Geomicrobiology (AEG), laboratory of Microbiology Department, Faculty of Biological Sciences, Quaid-e-Azam University Islamabad. Figure 3.2 shows the flow chart of the study.

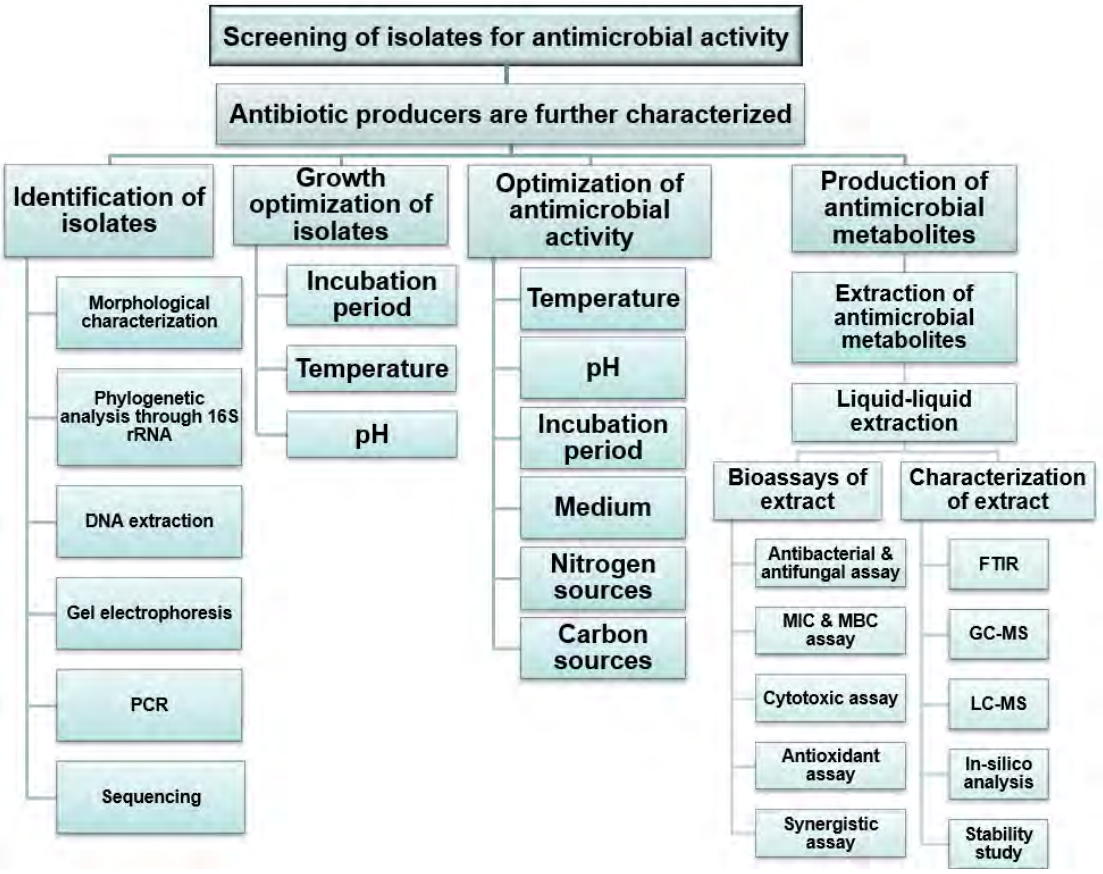

Figure 3.2 Scheme of the study

3.5 Screening of isolates for antimicrobial activity

The already mentioned eleven isolates were subjected to spot-on-lawn method for the verification and analysis of their antibacterial action. Among these 11 isolates, four isolates (HTP12, HTP13, HTP36, and LTP10) have shown best antimicrobial activity by measuring their zones of inhibition (ZOI) against ATCC strains and were subjected to secondary screening by agar-well diffusion assay. Throughout the study, zone of inhibition refers to diameter of clear zone in mm around the samples on petri plates.

3.5.1 Preparation of 0.5 McFarland standard solution

The bacterial suspensions used in antimicrobial assay are prepared by comparing to turbidity standards known as McFarland standard solutions. McFarland standards are used to facilitate the number of microorganisms used for making bacterial lawn in the specific range to standardize antimicrobial analysis of compounds. In order to obtain mixtures of specific absorbance, these solutions are prepared by combining different

concentrations of sulfuric acid and barium chloride. The absorbance of 0.5 McFarland solution is usually comparable to optical density (OD) of $1.5x10^8$ colony forming units (CFU/mL) of microbial suspension. Standard solution of 0.5 McFarland was prepared by adding 0.05 mL of $BaCl_2$ (1% w/v) in 9.95 mL of H_2SO_4 (1% v/v) with continuous stirring on magnetic stirring plate. The resulting turbid mixture was transferred to screw-cap tube. The optical density (OD) of 0.5 McFarland solution was checked by UV/Visible spectrophotometer at 600 nm and was found to be 0.085. Fresh culture of bacterial colonies was transferred through heat sterilized wire loop in the normal saline in screw-cap tubes and then their turbidity was compared with that of 0.5 McFarland standard solution by observing the tubes in front of light against white paper having black lines. The turbidity of bacterial suspension was adjusted either by adding normal saline in case the suspension density was high or by adding more bacterial colonies to suspension in case density of bacterial suspension was low. The 0.5 McFarland standard was covered with aluminium foil when not in use and stored at 25°C (Tankeshwar, 2021).

3.5.2 Primary screening by spot-on-lawn method

This technique was utilised to test bacterial isolates (HTP9, HTP12, HTP13, HTP14, HTP19, HTP27, HTP36, HTP37, HTP38, LTP8 and LTP10) that produce antibiotic compounds against ATCC bacterial strains. The test strain inoculum was made using 0.5 McFarland standards and sterile normal saline. To guarantee equal circulation of the inoculum, sterile cotton swabs were dipped in bacterial cell suspensions in normal saline and streaked across Mueller-Hinton agar (MHA) plate surfaces. On Mueller-Hinton agar plates inoculated with test strains, the bacterial cultures were spotted using a sterile wire loop. The spotted isolates were allowed to grow and produce antimicrobial chemicals on the MHA plates by being maintained at 15°C for three days. After that, the plates were transferred to incubator set at 37°C and incubated for 24 hrs. Clear ZOI on the plates around the spotted isolates were then observed (Kobras *et al*., 2022).

3.5.3 Secondary screening by agar-well diffusion assay

Using spot-on-lawn approach, the isolates HTP12, HTP13, HTP36, and LTP10 were shown to have the strongest antibacterial activity. These isolates were inoculated in a

shaker incubator at 150 rpm with 50 mL of Luria-Bertani broth in a 100 mL volumetric flask. The temperature of shaking incubator was set at 15°C, as the strains were isolated from low temperature area. After 72 hrs, 2 mL of the culture broth of each isolate was taken in 2 mL eppendorf tube in aseptic conditions in biosafety cabinet and subjected to centrifugation using Eppendorf 5417R Refrigerated Centrifuge at 5000 rpm for 10 minutes at 15°C (Pumkaew *et al*., 2019). The cell-free supernatant was examined using the agar well diffusion (AWD) test (Hwanhlem *et al*., 2017).

The test strain inoculum was made using sterile normal saline and the 0.5 McFarland standard. Muller-Hinton agar (MHA) plates were prepared, and each agar plate was inoculated with 50 µL of test microorganism and spread over entire agar plate surface with sterile cotton swab to make a uniform lawn of the test strain (Nalawade et al., 2016). Then, 5 wells with diameter of 6 mm are made on the agar plate with a sterile cork borer. A volume of 100 µL of cell free supernatant (CFS) of each isolate was added into the separate well. Similarly, 100 µL of the control (LB broth) was also poured to a well. The MHA plates were kept in LFH for two hours so that CFS diffused in the agar medium and then for 24 hrs incubated at 37°C. After one day, the MHA plates were analysed for antimicrobial activity of the isolates by measuring their zone of inhibition (ZOI) in mm using measuring scale (Balouiri *et al*., 2016). In three parallel experiments, the antibacterial activity of all four isolates was assessed. LB broth served as the negative control, while an amoxicillin (10 g) disc served as the positive control.

3.6 Identification of isolates

3.6.1 Morphological characterization

The morphology of the isolated strains HTP12, HTP13, HTP36, and LTP10 was investigated. Based on their microscopic and morphological traits, the isolates were described and identified.

3.6.2 Molecular identification of isolates

All the bacterial isolates were characterized based on 16S rRNA sequence analysis. The DNA were extracted, PCR optimization was performed and sent to Macrogen, Korea, for sequencing.

3.6.2.1 DNA extraction

The phenol-chloroform approach was used to chemically extract the DNA of isolated bacteria (Wright *et al*., 2017). The isolates (HTP12, HTP13, HTP36, and LTP10) were cultured on LB agar plates. Then these cultures were incubated for 48 hours and used for DNA extraction. After that, 200 µL of culture broth of isolate was mixed with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) in 1 mL eppendorf tube and then vortexed the mixture for 20 sec. The mixed sample was then subjected to centrifugation at room temperature for 5 minutes at 16000 rpm. After centrifugation, top aqueous layer was carefully separated and then shifted to fresh eppendorf tube. Then 100 µL of 7.5 M ammonium acetate ($C_2H_7NO_2$), 1 µL of glycogen, and 750 µL of 100% ethanol was mixed with aqueous layer. The tube was then placed in dry ice for 1 hr. At 4°C, the mixed sample was centrifuged at 16000 rpm for 30 min and the supernatant was carefully removed without disturbing cDNA pellet. 150 µL of 70% ethanol was mixed with sample and centrifuged under same conditions for 2 min and top layer was separated as much as possible. The sample was then air dried for 2 minutes. The cDNA pellet thus obtained was resuspended in TEN buffer by pipetting up and down 30 times.

3.6.2.2 Gel electrophoresis

Gel electrophoresis is a technique by which DNA can be separated by mass to charge ratio. In this basically the charge impulses move the negatively charge DNA towards positive electrodes through an agarose gel matrix. For this gel apparatus was taken that comprises gel casting tray, sample comb and electrodes. Electrophoresis buffer containing TBE (Tris-borate-EDTA) buffer was poured into gel tank about the level where the gel is depth completely. 1% agarose gel was prepared for gel electrophoresis process to obtain the DNA bands. Comb was placed in the gel and place it for few minutes at normal room temperature to solidify the gel, comb was

removed off, and wells were formed. The gel was then placed in a gel tank. Loading dye along with DNA sample was then poured in the wells and set it on time for 40 minutes. The gel is then exposed to 40V electric field. Clear bands of DNA were shown upon Transilluminator (Lee *et al*., 2012).

3.6.2.3 PCR amplification of 16S rRNA

Polymerase chain reaction (PCR) was executed by using Biometra T1 Thermocycler. Using universal primers (27F and 1492R), the extracted DNA samples were then amplified by PCR as shown in Table 3.1.

Table 3.1 Sequences of 27F primer and 1492R primers

S. No.	Primer Name	Primer Sequence
1.	27 F	5′- AGAGTTTGATYMTGGCTCAG-3′
2.	1492 R	5′-TACCTTGTTAYGACTT-3′

The PCR settings were optimized and were found to be 96°C for 5 min of initial denaturation, 35 rounds of denaturation, annealing, and extension. All the three steps took place at temperatures of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec, respectively. At 72°C for ten min, the last expansion was completed. After then, the sample was held at 4°C until the PCR results were acquired.

3.6.2.4 Phylogenetic analysis

In MEGA 4.0, the ClustalW tool was utilised to analyse the Phylogenetic analysis of Passu bacterial isolates (Mahtab *et al*., 2019). DNA sequences were discovered by phylogenetic analysis, and the equivalent sequences were retrieved from the National Center for Biotechnology Information (NCBI). After alignment of all nucleotides, a phylogenetic-tree was created using MEGA 11's Neighbour Joining technique. By using Bootstrap analysis, the importance of the constructed tree was investigated (1000 replicate).

3.7 Optimum growth conditions of isolates

Growth of the isolated strains (HTP12, HTP13, HTP36, and LTP10) was studied at different incubation period, pH, and temperature in order to find optimum conditions required for their growth. Stock cultures of isolated strains were prepared by taking single colony of each isolate from LB agar plate with sterile wire loop and inoculated in 100 mL of LB broth. Culture of each isolate was then kept in incubator at 25°C for 24 hours.

3.7.1 Effect of incubation period on growth

All the four isolates were inoculated with 1 mL of the stock culture suspension separately in 50 mL Luria-Bertani broth in 100 mL volumetric flask and then incubated at 15°C in shaking incubator at 150 rpm to determine their optimum growth incubation period. After every 24 hrs, 2 mL of the culture broth of each isolate was taken in 2 mL eppendorf tube in aseptic conditions. The culture broths of all the isolates were then studied for their growth by measuring their turbidity. The growth rate of isolates was determined after every 24 hrs by taking their optical density (OD) or absorbance at 600 nm utilizing UV Vis spectroscopy. The process of taking optical density (OD) was continued until a maximum value of OD was obtained which indicated the stationary phase of growth.

3.7.2 Effect of temperature on growth

All the four were inoculated with 1 mL of the stock culture suspension separately in 50 mL Luria-Bertani broth in 100 mL volumetric flask and then incubated in shaking incubator at 150 rpm at temperatures 5, 15, 25 and 35°C for 120 hrs to verify the influence of temperature on their growth. 2 mL of the culture broth of each isolate was transferred to 2 mL eppendorf tube in laminar flow hood in aseptic environment after every 24 hrs. The culture broths of all the isolates were then studied for their growth by measuring their turbidity. The growth rate of isolates was determined after every 24 hrs by taking their optical density (OD) or absorbance at 600 nm utilizing UV Vis spectroscopy.

3.7.3 Effect of pH on growth

In order to determine the effect of acidic, neutral, and basic conditions on the growth of isolates, initial pH of the culture broth was adjusted either with 0.1N HCl or 0.1N NaOH at pH 5.0, 7.0, and 9.0. The isolated strains (HTP12, HTP13, HTP36, and LTP10) were cultured with 1 mL of the stock culture suspension separately in 50 mL Luria-Bertani broth in 100 mL volumetric flask. The culture broths of all the isolates at various pH were then transferred to shaking incubator for 5 days. After every 24 hrs, 2 mL of the culture broth of each isolate was taken in 2 mL eppendorf tube in laminar flow hood in aseptic environment. The culture broths of all the isolates were then observed for their growth by measuring their turbidity. The growth rate of isolates was determined after every 24 hrs by taking their optical density (OD) or absorbance at 600 nm utilizing UV Vis spectroscopy.

3.8 Optimization of antimicrobial production

For optimization of antimicrobial production of all four isolates, their culture broths were subjected to different incubation time, temperatures, pH, culture media, nitrogen supplements, carbon supplements, and salts and the impact of these conditions on antimicrobial production was determined. Stock cultures of isolated strains were prepared by taking single colony of each isolate from LB agar plate with sterile wire loop and inoculated in 100 mL of LB broth. Incubation of culture of each isolate was then completed at 25°C for 24 hours and used further. In three parallel trials, the antibacterial activity of all four isolates was identified.

3.8.1 Incubation period

For the production of stock cultures of isolated strains, Luria-Bertani broth was utilised. 1 mL of the stock culture suspension of each isolate was inoculated separately in 100 mL Luria-Bertani broth in 200 mL volumetric flask and incubated in shaking incubator at 150 rpm at 15°C. After 24 hrs, 2 mL of the culture broth of each isolate was taken in 2 mL eppendorf tube in aseptic conditions in biosafety cabinet. The eppendorf tubes were then centrifuged using Eppendorf 5417R Refrigerated Centrifuge at 5000 rpm for 10 minutes at 15°C (Pumkaew *et al*., 2019). After centrifugation, cell free supernatant (CFS) of each isolate was taken and by using an

AWD experiment, the presence of antimicrobial chemicals in CFS was identified (Balouiri *et al*., 2016) using LB broth as negative control. The samples were taken from the broth after every 24 hrs, until decrease in antimicrobial activity was observed after maximum activity.

3.8.2 Incubation temperature

To determine the effect of incubation temperature on antimicrobial activity of isolates, 1 mL of each isolate's stock culture was inoculated in 100 mL LB broth separately in 200 mL volumetric flask. Each isolate, in different flasks, was then incubated in shaking incubator at 150 rpm at 5, 15, 25, and 35°C. After 96 hrs, 2 mL of the culture broth of each isolate was taken in 2 mL eppendorf tube in aseptic environment in laminar flow hood. The eppendorf tubes were then centrifuged (Eppendorf 5417R Refrigerated Centrifuge) at 5000 rpm for 10 minutes at 15°C (Pumkaew *et al*., 2019) and CFS of each isolate was subjected to AWD assay using LB broth as negative control to define the production of antimicrobial metabolites (Balouiri *et al*., 2016).

3.8.3 Incubation pH

To study the impact of pH on antimicrobial production of isolates, initial pH of LB broth was set to preferred pH by applying 0.1N NaOH or 0.1N HCl. pH of culture broth in separate flasks was adjusted to 5.0, 7.0, and 9.0. After that 1 mL of each isolate's stock culture was inoculated separately in 100 mL LB broth in 200 mL volumetric flask. Each isolate, in different flasks, was then kept in shaking incubator for 96 hrs at 25°C. Then 2 mL of the culture broth of each isolate was taken in 2 mL eppendorf tube in aseptic environment in laminar flow hood. Centrifugation (Eppendorf 5417R Refrigerated Centrifuge) of the eppendorf tubes at 5000 rpm for 10 minutes at 15°C (Pumkaew *et al*., 2019) obtained CFS of each isolate. CFS was then analysed by AWD assay using LB broth as negative control to define the production of antimicrobial metabolites (Balouiri *et al*., 2016).

3.8.4 Medium selection

Luria Bertani (LB) broth, nutrient broth (NB), and tryptic soy broth (TSB) were utilised as culture medium to ascertain the best synthesis of antibacterial chemicals by

chosen isolates. For this purpose, 1 mL of the stock culture suspension of each isolate was inoculated separately in 100 mL NB, TSB, and LB broth in 200 mL volumetric flask and incubated in shaking incubator at 150 rpm at 25°C for 96 hrs. An aliquot of 2 mL of the culture broth of each isolate was taken in 2 mL eppendorf tube aseptically. The eppendorf tubes were then centrifuged by using Eppendorf 5417R Refrigerated Centrifuge at 5000 rpm for 10 minutes at 15°C (Pumkaew *et al*., 2019) to obtain CFS and by using an AWD experiment, the presence of antimicrobial chemicals in CFS was identified using LB broth as negative control (Balouiri *et al*., 2016).

3.8.5 Effect of carbon sources

The chosen isolates were cultured in 100 mL of Luria-Bertani medium adding extra glucose and starch at 1% and 2% concentrations at 25°C for 96 hours to assess the influence of carbon source on the synthesis of antibiotic metabolites. After that 2 mL of the culture broth of each isolate was centrifuged (Eppendorf 5417R Refrigerated Centrifuge) at 5000 rpm for 10 minutes at 15°C (Pumkaew *et al*., 2019) and CFS of each isolate was subjected to AWD assay using LB broth as negative control for measuring their antimicrobial activity (Balouiri *et al*., 2016).

3.8.6 Effect of nitrogen sources

Antimicrobial compounds production was examined using LB broth supplemented with 1% concentration of arginine, tryptophan, L-leucine, threonine, and yeast extract. The isolated strains' LB broth containing nitrogen sources was cultured at 25°C for 96 hours and then centrifuged for 10 min at 5000 rpm and 15°C (Pumkaew *et al*., 2019) CFS, so obtained, was then analysed by AWD assay using LB broth as negative control (Balouiri *et al*., 2016) to find the secretion of antimicrobial metabolites.

3.8.7 Effect of salts

All four isolates were cultured in LB broth supplemented with 2 and 3% sodium chloride and 1 and 2% potassium chloride to see how salts affected the generation of antibacterial chemicals. For this purpose, 1 mL of the stock culture suspension of each isolate was inoculated separately in 100 mL LB broth and transferred to shaking

incubator at 150 rpm at 25°C for 96 hrs. An aliquot of 2 mL of the culture broth of each isolate in eppendorf tubes was then centrifuged for 10 min at 5000 rpm and 15°C (Pumkaew *et al*., 2019) to obtain CFS and by using an AWD experiment, the presence of antimicrobial chemicals in CFS was identified using LB broth as negative control (Balouiri *et al*., 2016).

3.9 Antibiotic sensitivity test of isolates

Naturally the antibiotic producing organisms should exhibit self-resistance to the antimicrobial compounds produced by themselves. It is also thought that co-existence of non-producer and producer microorganisms results in the development of resistance mechanisms in the former (Peterson and Kaur, 2018). For this purpose, all the four isolates were tested against antibiotics discs Ciprofloxacin, Ofloxacin, Nalidixic acid, Amoxicillin, Penicillin G, Carbenicillin, Oxacillin, Ceftriaxone, Cefotetan, Cephalexin, Cephalothin, Cephazolin, Cefpodoxime, Linezolid, Erythromycin, Vancomycin, Tigecycline, Metronidazole, Levofloxacin, Ticarcillin, Cefepime, Ceftaroline, Meropenem, Imipenem, Ertapenem, Norfloxacin, Piperacillin, Ceftazidime, and Aztreonam using the agar diffusion (Kirby-Bauer) method in accordance with the 2013 recommendations of Clinical and Laboratory Standards Institute (CLSI). The plates were placed in an incubator and incubated there for 72 hrs at 25°C and clear ZOI was noticed and recorded.

3.10 Antimicrobial activity of *A. pakistanensis* LTP10 against clinical isolates

The clinical isolates of *Staphylococcus aureus, Salmonella enterica, Escherichia coli, Pseudomonas aeruginosa, Candida krusei,* and *Candida albicans* were employed. Gram positive, gram negative, and fungal isolates were also used. Strains of bacteria and fungus were cultivated overnight at 37 and 35°C, respectively, prior to the experiment. *Alcaligenes pakistanensis* (LTP10) was cultured in LB broth and then incubated at pH 7 and 25 °C for four days since it showed the most antibacterial activity at these conditions. After 96 hours, the AWD assay (Balouiri et al., 2016) was performed to assess the antimicrobial activities of CFS of *Alcaligenes pakistanensis* (LTP10) against clinical strains.

3.11 Antimicrobial compounds production from *Alcaligenes pakistanensis* LTP10

The isolated strain LTP10 was selected for further studies based on the data obtained from optimization findings. Stock cultures of the isolated strain was prepared by taking single colony of LTP10 from LB agar plate with sterile wire loop and inoculated in 100 mL of LB broth. Culture of LTP10 isolate was then incubated at 25°C for 24 hrs. LB broth was used for extracellular production of antimicrobial compounds from LTP10. For this purpose, about 1000 mL of LB broth medium was prepared in 2L volumetric flask and autoclaved at 121°C and 15 psi for 20 min. The sterilized broth was then kept at 37°C for one day to check the sterility. LB broth was inoculated with 50 mL of 24 hours old stock culture of LTP10 in aseptic conditions in laminar flow hood. LB broth was then incubated in orbital shaker incubator at 150 rpm and 25°C. After 96 hrs, an aliquot of 2 mL of the broth was added to 2mL eppendorf tube and subjected to centrifugation using Eppendorf 5417R Refrigerated Centrifuge for 10 minutes at 5000 rpm to get CFS. The CFS was examined for antimicrobial activity by AWD assay against test organisms using LB broth as negative control. The clear zone of inhibition indicated the production of antimicrobial compounds.

3.12 Extraction of antimicrobial compounds from *Alcaligenes pakistanensis* LTP10

3.12.1 Optimization of solvent extraction

For the extraction of antimicrobial products from LTP10, solvent phase extraction method was used. Organic solvents including ethyl acetate, chloroform, and hexane were studied for antimicrobial metabolites extraction from supernatant. 1000 mL culture broth of LTP10 was subjected to centrifugation and each 200 mL of CFS was then mixed separately with equal volumes of n-hexane, ethyl acetate, and chloroform and agitated thoroughly. Separating funnel was used for separation of two immiscible layer and the upper layer containing bioactive compounds was collected. This process was repeatedly done for 3 times (Kislik, 2012). Then, using an AWD experiment, the antibacterial activity of the top layer of each solvent was evaluated. The extraction of

crude extract was continued using the ethyl acetate extract, which demonstrated the best activity in terms of zone of inhibition. The positive control was a disc of amoxicillin (10 mg), while the negative controls were n-hexane, ethyl acetate, and chloroform.

3.12.2 Extraction of metabolites

For extraction of metabolites from culture medium, equal quantities of LTP10 broth and ethyl acetate were mixed in a separating funnel. After extraction of metabolites by vigorous shaking, ethyl acetate layer (upper) was removed and the process was repeated multiple times to extract maximum metabolites from LTP10 broth (Tanvir *et al*., 2016). The crude extract of LTP10 was dried completely in rotatory evaporator Buchi R-200 Rotavapor System and dried crude powder was obtained.

3.13 Characteristics of dried extract of *A. pakistanensis* LTP10

3.13.1 Solubility

The dried crude extract solubility was tested with different solvents such as DMSO, methanol, chloroform, n-hexane, ethyl acetate, and petroleum ether.

3.13.2 Temperature stability

The stability of antimicrobial activity of crude extract of LTP10 was examined at different temperatures. The crude extract was exposed to 25, 35, 45, 55, and 65°C for 6 hrs. Ethyl acetate extract (5 mg/mL) was solubilized in methanol and then subjected to AWD assay to determine its antimicrobial activity using methanol as negative control.

3.13.3 pH stability

The pH stability of antimicrobial potential of crude extract of LTP10 was studied. For this purpose, the crude extract at concentration of 5 mg/mL was mixed with buffers of pH 4.0, 5.5, 7.0, 8.5, and 10 and kept at 25°C for 6 hrs. The antimicrobial activity of crude extract in different buffers was determined by AWD assay using respective buffer as negative control.

3.13.4 Extracellular Protein determination

Lowry's (1951) method was followed to estimate protein content in a crude sample. Protein was estimated by adding 1 mL sample and 1 mL solution D in a test tube by incubating at ambient temperature for ten minutes. After incubation, 100 µL of solution E was added and again incubated in dark at ambient temperature for thirty minutes in darkness. Then OD was estimated at 650nm. Reagents used for protein estimation are 2% Na2CO3 in 0.1 N NaOH (Solution A), 1% potassium sodium tartratein in H_2O (Solution B), 0.5% $CuSO_4.5H_2O$ in H_2O (Solution C), A:B:C = 48:1:1 (Solution D), and H_2O + Folin phenol = 1:1 (Solution E). The protein content of the crude extract of LTP10 was determined by standard curve development method. Bovine serum albumin (BSA) stock solution (1 mg/mL) was diluted as 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1 mg/mL and checked for protein quantification. The resulted absorbance of these dilutions were plotted in Microsoft Excel and a graph was constructed. A pattern line was added to the graph and an equation {Y = (0.9898 x X) + 0.03} was gotten from the slant of graph for measuring unknown estimation of released proteins. The whole protein content of sample was measured with reference to BSA curve.

3.14 Bioassays of ethyl acetate extract of *A. pakistanensis* LTP10

3.14.1 Antibacterial and antifungal activity against clinical isolates

Clinical isolates of *Staphylococcus aureus, Salmonella enterica, Pseudomonas aeruginosa, Escherichia coli, Candida krusei*, and *Candida albicans* were employed. Gram positive, gram negative, and fungal isolates were also used. Strains of bacteria and fungus were cultivated overnight at 37 and 35°C, respectively, prior to the experiment. By using the AWD technique (Balouiri *et al*., 2016), the crude extract of *A, pakistanensis* (LTP10) was assessed for its antimicrobial activity against clinical strains. DMSO served as the negative control. The antibiotic sensitivity profile of bacterial isolates was determined by agar-disk diffusion method.

3.14.2 MIC assay

To determine MIC of crude extract of LTP10, ATCC test strains *E. coli*, *Salmonella enterica*, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *and S. epidermidis* were utilized. To create single colonies, the test-relevant bacterial isolates (together with a control organism) were spread onto MHA plates without inhibitor. The plates were incubated overnight at 37°C. A single colony from the agar plates for each isolate was chosen, transferred to a conical flask with 50 mL MH-Broth, and cultured for 24 hrs at 37°C at 150 rpm. Then OD_{450} of these cultures were measured and dilutions were made in 5 mL MHB to OD_{450} = 0.01. MIC was assessed by microdilution technique using 96 well microtiter plate (Wiegand *et al*., 2008; Hussain *et al*., 2019). 50 µL of Muller-Hinton broth was poured into wells of first 6 rows of columns 2-11. Then MHB (100 µL) was poured into column 12 and 100 µL (12.8 mg/mL) of the crude extract was added into column 1. An aliquot of 50 µL was withdrawn from each well in column 1 and mixed this to the subsequent wells in 2nd column. The process was repeated for column 2 and 3, column 3 and 4 up to column 10. The withdrawn solution from column 10 was discarded. The bacterial suspensions in MHB were vortexed and 50 µL of each bacterial culture was poured to each well in single row in columns 1-11. The plates were covered with film to create a tight seal to prevent any evaporation. Each assay was performed in triplicate and the value repeated at least two times was considered as the MIC. The plates were kept at 37ºC in incubator for 16-24 hrs. The turbidity of bacterial cultures were measured by determining OD_{450}. The concentration of extract in mg/mL was considered was minimum inhibitory concentration which resulted in minimum growth.

3.14.3 MBC assay

After 96-well plate incubation, dilutions that showed no sign of growth were streaked with a sterile wire loop on sanitised MHA plates. After that, the plates underwent another 24 hours of incubation. After the incubation time, plates were inspected for growth. The lowest concentration that failed to form even a single colony was identified as the MBC value.

3.14.4 Synergistic effect of LTP10 extract with antibiotics

The antimicrobial activity of ethyl acetate extract of *Alcaligenes pakistanensis* (LTP10) was estimated in combination with known antibiotics to study their synergistic effect. For this purpose, disk diffusion method was used, in which sterile MHA was added to autoclaved petri dishes and was then inoculated with *S. aureus* and *E. coli*. Inoculated MHA plates were exposed to the antibiotic discs (6 mm) of imipenem (10 µg), nalidixic acid (30 µg), penicillin (10 µg), cefepime (30 µg), and metronidazole (5 µg). To evaluate synergistic effects of the combination of the crude extract and antibiotics, a second set of the same antibiotic discs were impregnated with 5 µL of crude extract (5 mg/mL), and they were likewise put on the inoculated agar surface. Before being incubated for 24 hrs at 37°C, petri plates were kept at ambient temperature for 30 minutes. Formation of a clear circular ZOI surrounding the discs, which corresponded to the lack of bacterial growth, was indicative of the action of extract (Moussaoui and Alaoui, 2016). The inhibitory zone's diameter was measured in millimetres. The strain is more vulnerable the bigger the area's diameter (Choi *et al*., 2006).

3.14.5 Cytotoxicity Assay (Brine shrimp assay)

The cytotoxicity assay of *Alcaligenes pakistanensis* (LTP10) crude extract was tested against the brine shrimp lethality by following the protocol of Alowonle (2014) with slight modifications. The preparation of artificial sea water was carried out by dissolving 34 grams of artificial sea water in 1000 mL of DW to make a concentration of 34 g/L by constant aeration and continuous stirring. After making of salt solution, it was then filtered through filter paper and poured into a hatching container consists of two compartments interlinked with each other with small holes. Then this container containing salt solution was kept under the lamp or an electric bulb. Almost one teaspoon of brine shrimp eggs (Artemia salina) were gently poured into one chamber and with aluminium foil it was then covered in order to avoid the penetration of light. The illumination is for attracting the emerging shrimps. The hatched shrimps (mature nauplii) were collected from the illuminated portion after 48 hours by using Pasteur pipette. Ten hatched shrimps were collected and transferred it into a glass vials containing 5 mL of salt solution. Then crude extract of *Alcaligenes pakistanensis* (LTP10) was mixed in DMSO and the extract was assessed at the concentrations of

100, 500, and 1000 µg in 100 µL of DMSO poured into these vials containing the active nauplii. The DMSO (100 µL) was treated as negative control. Also, three vials were left blank containing 10 active nauplii. The vials containing these mixtures were gently mixed and then incubated it for 24 hours at ambient temperature. Results were observed after 24 hours, 48 hours and 72 hours. The larvae mortality was established by calculating the amounts of dead and active nauplii. The following formula was used for estimating the mortality rate (Tanvir *et al*., 2016).

$$M = (A - B - N) / (G - N) \times 100$$

In the above equation, M indicates the number of shrimps larva (nauplii), A denotes number of dead nauplii, B represents average number of dead shrimps larva in control, N indicates number of dead shrimps before beginning and G denotes total brine shrimp larva.

3.14.6 Antioxidant assay (DPPH Method)

Natural colour of DPPH (2,2'-diphenyl-1-picrylhydrazyl) is red that contains free radicals which are stable. When the sample is treated with DPPH (2,2'-diphenyl-1-picrylhydrazyl) and it consists of antioxidants, then the free radical of DPPH will be scavenged by these antioxidants and the natural colour of DPPH from red is changed into yellow.

The DPPH solution was made by dissolving the DPPH (0.24 mg/mL) in methanol and then incubated it in dark for 30 minutes. The determination of scavenging activity was done by slight modification in method of (Barros *et al*., 2008). The DPPH assay was carried on 96 well microtiter plate. The O.D of the DPPH was ensured that it is equal to 1 at 517 nm in order to use it for standard curve of test compound. For positive control ascorbic acid was mixed in DMSO at 0.04 mg/mL concentration. The stock solution of crude extract of LTP10 that was to be tested for antioxidant activity was made in DMSO at a concentration of 100, 200 and 300 µg/mL. Then 5 µL of test sample and positive control as well as 5 µL of DMSO as negative control was taken and loaded into the well of a microtiter plate. After loading these samples 95 µL of DPPH was added into each well containing samples, positive control, and negative control. After that, the plate was shielded from light using aluminium foil and

incubated at 37°C for one hour. Then the Ultra Microplate Reader (Elx 808, BIO TEK Instruments Inc) was utilized to detect and measure the reduction in absorbance at 517 nm. The antioxidant activity was determined as a reduction in absorbance of DPPH by applying the formula,

$$\text{DPPH Scavenging activity (\%)} = (AD - AS/\ AD) \times 100$$

In the above equation, AD and AS represents absorbance value of control and sample respectively at 517 nm.

3.15 Characterization of extract of *Alcaligenes pakistanensis* LTP10

3.15.1 Analysis of ethyl acetate extract by FTIR

To distinguish different types of functional groups and chemical bonds nature present in compounds, Fourier transform infrared spectrophotometer (FTIR) is considered as the best technique (Pakkirisamy *et al*., 2017). IR analysis was performed using PerkinElmer Spectrum 65 FT-IR Spectrometer. A dried powder of the LTP10 ethylacetate extract was used for the FTIR examination. A small amount of LTP10 extract dried powder was transferred to diamond crystal present in middle of circular metallic plate. Movable pressure arm was then rotated to place it over the sample. Infra-red rays were passed from below through sample and the spectrum was obtained. An FTIR spectrum at a frequency of 4 cm^{-1} with and scanning range of 515 to 4000 cm^{-1} was used to analyse the powdered sample.

3.15.2 Analysis of extract by GC-MS

The gas chromatograph (GC) used for the GC analysis has a capillary column and a detector (FID). The GC-MS evaluation of the crude LTP10 extract was accomplished using a Shimadzu GC-17A fitted with a Shimadzu GCMSQP5050A mass selective detector and an HP-5 MS capillary column having dimensions of 30 m x 0.25 mm and film thickness of 0.25 mm. For GC-MS finding, EIA (electron ionisation apparatus) having an ionisation energy of 70 eV was utilized. The carrier gas (helium) was set flowing at 0.8 mL/min rate. The mass spectrometer transfer line and injector were both set to 270 and 250 °C, respectively. Oven's temperature was maintained at 50 °C for three minutes before being raised by 3°C/min to 240°C. By comparing each

component's average peak area to the total areas, relative percentage amount of each component was calculated. Utilizing NIST library spectral data, compounds were identified, and the fragmentation pattern of mass spectra was compared to information that had already been published in the literature (Adam, 2007; Ezhilan and Neelamegam 2012).

3.15.3 Analysis of extract by LC-MS/MS

Liquid chromatography mass spectroscopy was used to perform the metabolic profiling of the LTP10 microbial extract. The instrument used for this task was an Agilent 1290 Infinity LC system connected to an Agilent 6530 QToF-MS and armed with a quaternary pump, DAD detector, temperature-controlled column (25ºC), and cooled auto-sampler compartment (4ºC). At a speed of two spectra/sec, full-scan MS spectra covering m/z range of 100-1700 Da were obtained using both positive and negative ion modes. The extract's metabolites were separated using a reverse-phase C18 Poroshell column with a C18 guard column (2.1 × 100 mm, 2.7 µm particle size) (Agilent). The flow rate was maintained at constant rate of 0.3 mL/min. Before analysis, the column was subjected to a 40-minute equilibration process. Solvent A [Water (Milli-Q, TKA-GenPure, Germany) + 0.1% formic acid (LC-MS grade, LiChropur®, 98-100%, Sigma-Aldrich, MO, USA)] and solvent B [95% HPLC-quality acetonitrile (RCI Labscan, Bangkok, Thailand) + 0.1% formic acid] were used in a gradient to achieve separation. The gradient of the solvent was as at: 5% of solvent B for 0.5 min, 100% B for 16 minutes, 23 minutes of holding at 100% solvent B, and finally 23.1 minutes of reverting to 5% solvent B. The DAD measured absorbance at wavelengths between 210 and 635 nm. For every sample, a 10 µL injection volume was used. The drying gas, nitrogen, was employed at 250°C and at flow rate of 9 L/min (Latif *et al*., 2020).

3.16 In-silico analysis of Compounds

3.16.1 Molecular docking study of important compounds

Molecular docking was used to study the nature of the interactions between the target proteins and antimicrobial compounds. This technique uses classical force field theory to forecast the best position and orientation of a ligand (drug) with protein (target) to

form a stable complex (Sá *et al*., 2020). The molecular docking assays were performed for test organisms' proteins or enzymes that were used in the study. Important antimicrobial compounds from LCMS analysis were subjected to molecular docking study. The 3D chemical structures of 15 compounds (ligands) were downloaded from Pubchem database (Rahman *et al*., 2021) as sdf files. The 12 target proteins (3D crystal structures) of test microorganisms were obtained from PDB (Protein Data Bank) (Rahman *et al*., 2021) as pdb files. The virtual interaction between ligands and proteins was performed using a docking webserver named as CB-dock. CB-Dock evaluated the binding affinity of ligand towards the target protein cavities in terms of vina score (kcal/mol) (Cao and Li, 2014; Liu *et al*., 2020). On the basis of preliminary results of CB-dock, four ligands 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid (compound19), Dianhydroaurasperone C (compound5), L-alpha-Amino-1H-pyrrole-1-hexanoic acid (compound15), and L,L-Cyclo(leucylprolyl) (compound24) were selected for further evaluation. The chemical structure of meropenem and trimethoprim was obtained from Pubchem database to be used as control. The selected target proteins dihydrofolate reductase (PDB ID: 3DRC), peptidoglycan transpeptidase (PDB ID: 7JWL), transglycosylase (PDB ID: 2OLU), Nitric oxide synthase (PDB ID: 2ANO), and D-alanine-D-alanine Ligase (PDB ID: 2DLN) were prepared by removing their water and ligand molecules if present and adding polar hydrogens by using discovery studio software. In the next step, the 4 ligands were docked against the purified target proteins using CB-Dock to study the interactions of ligands with target proteins and to determine the best pose of interaction. The result of docking was downloaded as pdb file and visualised in BIOVIA Discovery Studio (Rahman *et al*., 2021). The amino acid residues interacting with ligand molecule, number of hydrogen bonds and other interacting bonds was determined using Discovery studio.

3.16.2 In-silico analysis of compounds ADMET properties and drug-likeness

Early ADMET property prediction of compounds has shown to be highly helpful in the drug discovery and development process (Rehman *et al*., 2018). An online web server pkCSM (Pires *et al*., 2015) was used to predict human intestinal absorption (%), skin permeability (logKp), BBB permeability (logBB), CYP450 inhibitor, total renal clearance (logmL/min/kg), AMES toxicity, minnowtoxicity (logmM),

hepatotoxicity, and skin sensitisation of four metabolites namely 7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid, L-alpha-Amino-1H-pyrrole-1-hexanoic acid, dianhydroaurasperone C, and L,L-Cyclo(leucylprolyl). For this purpose, the canonical SMILES of compounds were obtained from Pubchem webserver and analysed on pkCSM webpage. For comparison, ADMET properties were also determined for well-known antibiotics meropenem and ciprofloxacin. The Lipinki's RO5 (rule of five) and a few additional qualities were anticipated using the drug-likeness property test by the swissADME server (Chenafa, *et al*., 2021).

3.17 Statistical analysis

By calculating the standard deviation (stdev) and p-value, difference in activity under various cultural situations was examined by statistical analysis. For the purpose of calculating the statistical importance of variations in ZOI under various culture circumstances, the p-value was computed employing T.Test in Microsoft Excel 365 (Deepak *et al*., 2020). Three times each experiment was run. Mean ± SD (n + 3) was used to depict the values. The significance threshold is $p < 0.05$, and the confidence level is 95%.

RESULTS

In the present study, 11 isolates from Passu glacier coded as HTP and LTP, were designated based on preliminary data available. These isolates were then subjected to antimicrobial activity against test strains. The isolate LTP10 was discovered to exhibit stronger antibacterial action than the other compounds.

4.1 Revival of isolates

The bacterial isolates LTP8, LTP10, HTP9, HTP12, HTP13, HTP14, HTP19, HTP27, HTP36, HTP37, and HTP38 preserved in glycerol, were spread on LB agar plates. After incubation for specific duration, as shown in table 4.1, growth of these isolates was observed. The pure isolated colonies of these isolates were picked and processed for downstream application.

Table 4.1 Revival of isolates

Isolate	Incubation temperature	Incubation period	Colony morphology
HTP9	15ºC	72 hrs	Whitish, medium, irregular edges
HTP12	15ºC	72 hrs	Whitish, small, flat
HTP13	15ºC	72 hrs	Beige, medium, circular
HTP14	15ºC	72 hrs	Beige, circular, irregular margins
HTP19	15ºC	72 hrs	Offwhite, medium, flat
HTP27	15ºC	72 hrs	Beige, irregular edges
HTP36	15ºC	72 hrs	Offwhite, circular, irregular margins
HTP37	15ºC	72 hrs	Whitish, medium
HTP38	15ºC	72 hrs	Offwhite, flat
LTP8	4ºC	120 hrs	Whitish, circular
LTP10	4ºC	120 hrs	Offwhite, circular, low convex

4.2 Screening of bacterial isolates for antimicrobial activity

4.2.1 Primary screening by spot-on-lawn method

Spot-on-lawn assay was used to test the selected 11 bacteria for antagonistic activity against the ATCC test microorganisms *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Staphylococcus epidermidis, Salmonella enterica,* and *Pseudomonas*

aerugenosa. The obvious zone of inhibition surrounding the isolates' spots, measured in millimetres after 72 hours of incubation, served as evidence of the antibacterial activity as shown in table 4.2. A zone of inhibition of more than 15 mm was considered as strong antimicrobial activity, 10-15 mm was considered as moderate antimicrobial activity, and 5-9 mm was considered as low antimicrobial activity. The results revealed that isolate LTP10 has shown strong activity against *Staphylococcus aureus* and *Bacillus subtilis* while moderate activity against *Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella enterica.* Similarly isolates HTP12, HTP13, and HTP36 have also been found to have good antimicrobial activity.

Table 4.2 Spot-on-lawn assay results for antimicrobial activity of Passu isolates against ATCC strains

Isolates	*Staphylococcus aureus*	*Bacillus subtilis*	*Staphylococcus epidermidis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Salmonella enterica*
HTP9	+	-	-	+	-	-
HTP12	+++	++	+	++	++	+
HTP13	++	++	+	+	+	-
HTP14	++	++	+	+++	++	+
HTP19	++	-	+	+++	++	-
HTP27	-	++	-	-	-	+
HTP36	++	++	+	++	+++	-
HTP37	+	-	+	+	-	-
HTP38	+	-	-	++	-	+
LTP8	++	+++	++	++	+++	-
LTP10	+++	+++	++	++	++	++

Inhibition zone: +++ = strong (> 15 mm), ++ = moderate (10-15 mm), + = low (5-9 mm), - = no activity

4.2.2 Secondary screening by agar well diffusion assay

Against test strains, four isolates LTP10, HTP12, HTP13, and HTP36 revealed the highest ZOI. The zones of inhibition for these 4 bacteria were then measured in millimetres and reported in figure 4.1 as part of the secondary screening procedure using the agar well diffusion test. All the isolates have shown good antimicrobial activity against test organisms with HTP36 showing 21 ± 2.0 mm ZOI against *Bacillus subtilis* while LTP10 showing 19.3 ± 1.5 mm ZOI against *Staphylococcus aureus*. Positive control amoxicillin (10 µg) has shown 21-28 mm ZOI against tested

strains while negative control LB broth did not produce any zone against tested strains.

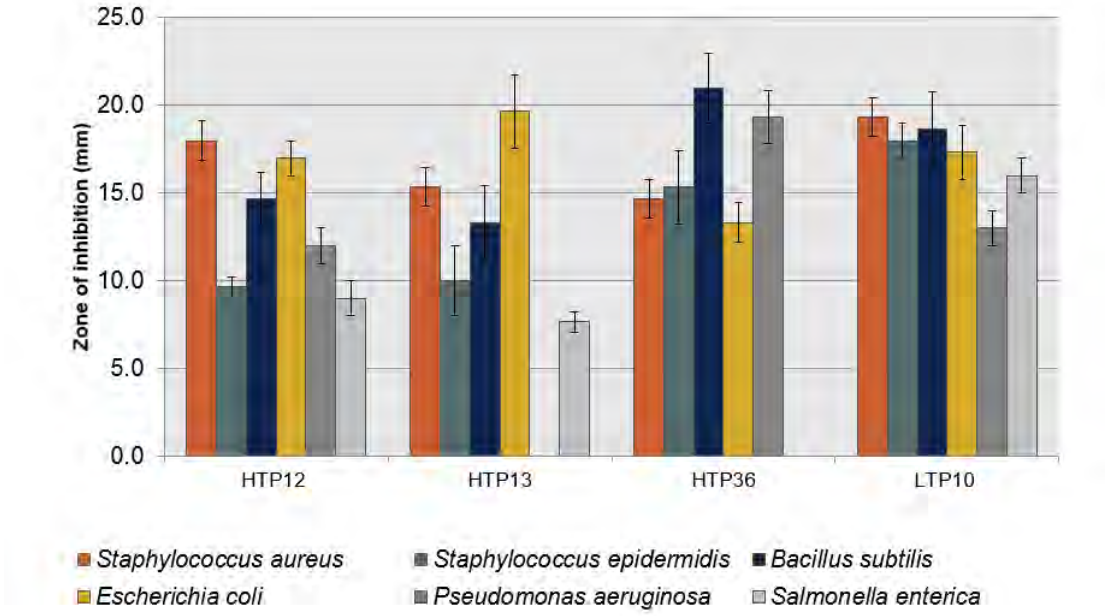

Figure 4.1 Antibacterial activity of study isolates measured in mm (ZOI) by AWD method against ATCC test organisms. The error bars denoted the SD of triplicate ZOI measurements.

4.3 Identification of isolates

4.3.1 Morphological characterization

The isolated strains, LTP10, HTP12, HTP13, and HTP36 were found to be gram negative, aerobic and rod shaped. The colonies of isolate HTP12 was found to be small, whitish, and flat surface. Colony morphology of the isolate HTP13 revealed beige colour circular colonies while HTP36 and LTP10 showed offwhite circular colonies as given in Table 4.3.

Table 4.3 Morphological and microscopic characteristics of the isolates

Isolate	Colony morphology	Microscopic characteristics
HTP12	Offwhite, small, irregular edges, flat	Gram -ive, rod shaped
HTP13	Beige, medium, circular	Gram -ive, rod shaped
HTP36	Offwhite, circular, irregular margins	Gram -ive, rod shaped
LTP10	Offwhite, circular, low convex	Gram -ive, rod shaped

4.3.2 Phylogenetic analysis

The BLAST analysis of the four total strains revealed three distinct strains with 99% or greater similarity to their respective specie. According to Table 4.4 and Figure 4.2, the isolated strains LTP10, HTP12, HTP13, and HTP36 were recognised as *Alcaligenes pakistanensis*, *Alcaligenes faecalis, Pseudochrobactrum saccharolyticum,* and *Alcaligenes pakistanensis*, respectively.

Table 4.4 Phylogenetic analysis results for identification of study isolates

Isolates	Isolate accession number	Homologous species with accession number	Groups	Identity (%)
HTP 12	OP352247	*Alcaligenes faecalis* (KF641844)	Proteobacteria Betaproteobacteria Burkholderiales Alcaligenaceae	99.8
HTP13	OP352248	*Pseudochrobactrum saccharolyticum* (KX977558)	Proteobacteria Alpha Proteobacteria Rhizobiales Brucellaceae Pseudochrobactrum	99.0
HTP36	OP352249	*Alcaligenes pakistanensis* (AB968096)	Proteobacteria Betaproteobacteria Burkholderiales Alcaligenaceae	100.0
LTP 10	OP352250	*Alcaligenes pakistanensis* (AB968096)	Proteobacteria Betaproteobacteria Burkholderiales Alcaligenaceae	99.6

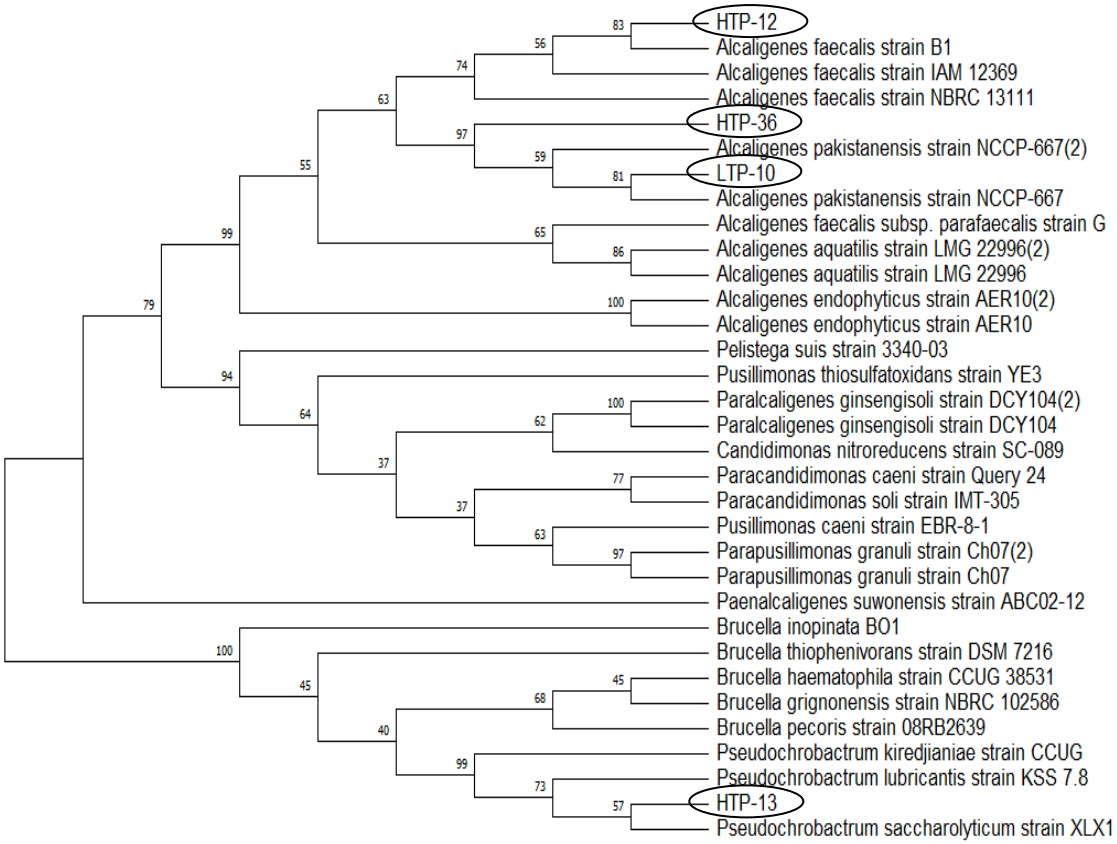

Figure 4.2 Phylogenetic analysis of cold-adapted bacterial isolates used in the study with other related strains obtained from NCBI webserver

4.4 Optimum growth conditions of bacterial isolates

By incubating the isolates of *A. faecalis* HTP12, *Pseudochrobactrum saccharolyticum* HTP13, *A. pakistanensis* HTP36, and *A. pakistanensis* LTP10 at several incubation periods, temperatures, and pH levels, the best conditions for growth were identified. It was found that different temperatures, incubation periods and pH had greatly effected growth of these isolates.

4.4.1 Effect of incubation period on growth

As shown in figure 4.3, there was increase in growth of isolates upto 72 hrs and then a decline in growth was observed. However, the difference in growth of the isolates at 24, 48 and 72 hrs was significant with p-value found to be less than 0.05 ($P < 0.05$), while after 72 hrs the differnce in growth was not significant statistically ($P > 0.05$). As isolates used in the study were cold-adapted and have slow growth rate, therefore 72 hrs as optimum incubation period was considered good for selected isolates.

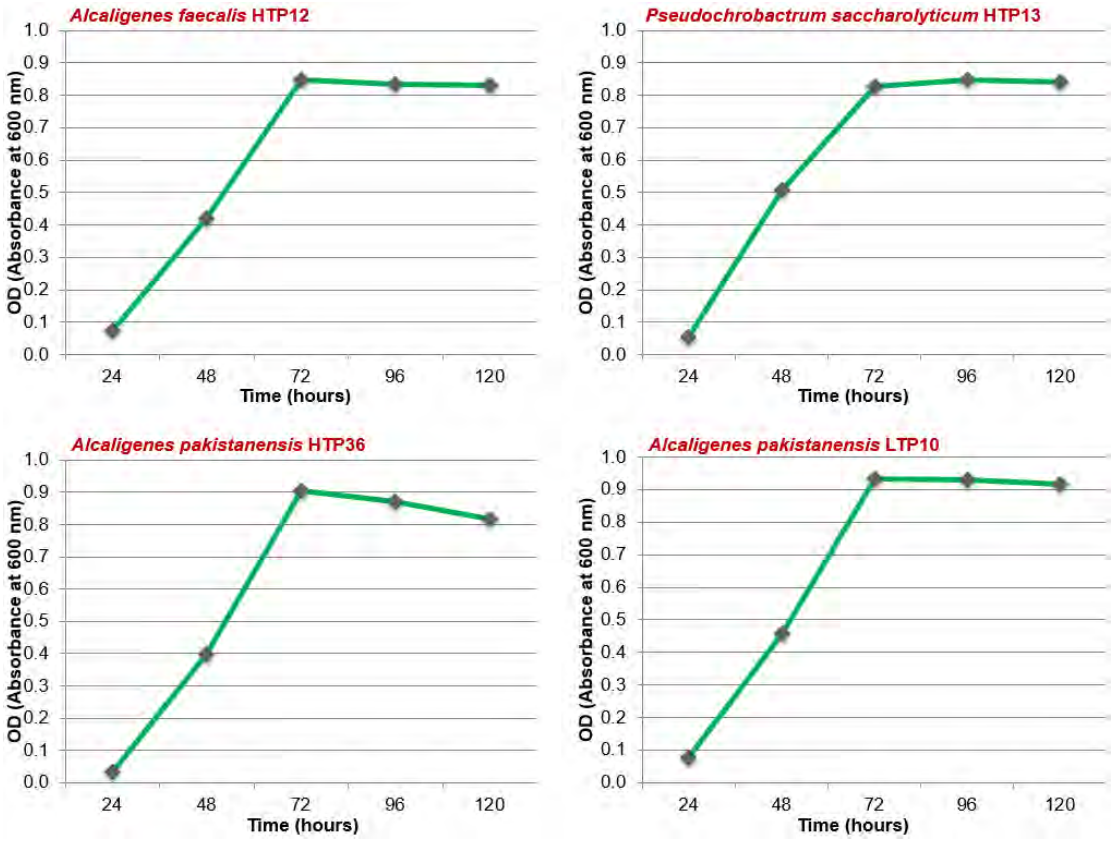

Figure 4.3 Optimization results of different incubation periods on the growth of study isolates. Data points represent the optical density (absorbance) of isolates culture broth at 600 nm.

4.4.2 Effect of temperature on growth

Optimum temperature for growth of isolates *A. faecalis* HTP12, *Pseudochrobactrum saccharolyticum* HTP13, and *A. pakistanensis* HTP36 was found to be 25ºC and for *A. pakistanensis* LTP10 it was determined to be 15ºC after 72 hrs of incubation as shown in figure 4.4. Moreover, the difference in growth of the all four isolates between 25ºC and 5ºC, 25ºC and 15ºC, and 25ºC and 35ºC was significant with p-value found to be less than 0.05 ($P < 0.05$). Similarly, the difference in growth of the isolate *Alcaligenes pakistanensis* LTP10 between 15ºC and 5ºC, 15ºC and 25ºC, and 15ºC and 35ºC was significant with p-value found to be less than 0.05 ($P < 0.05$).

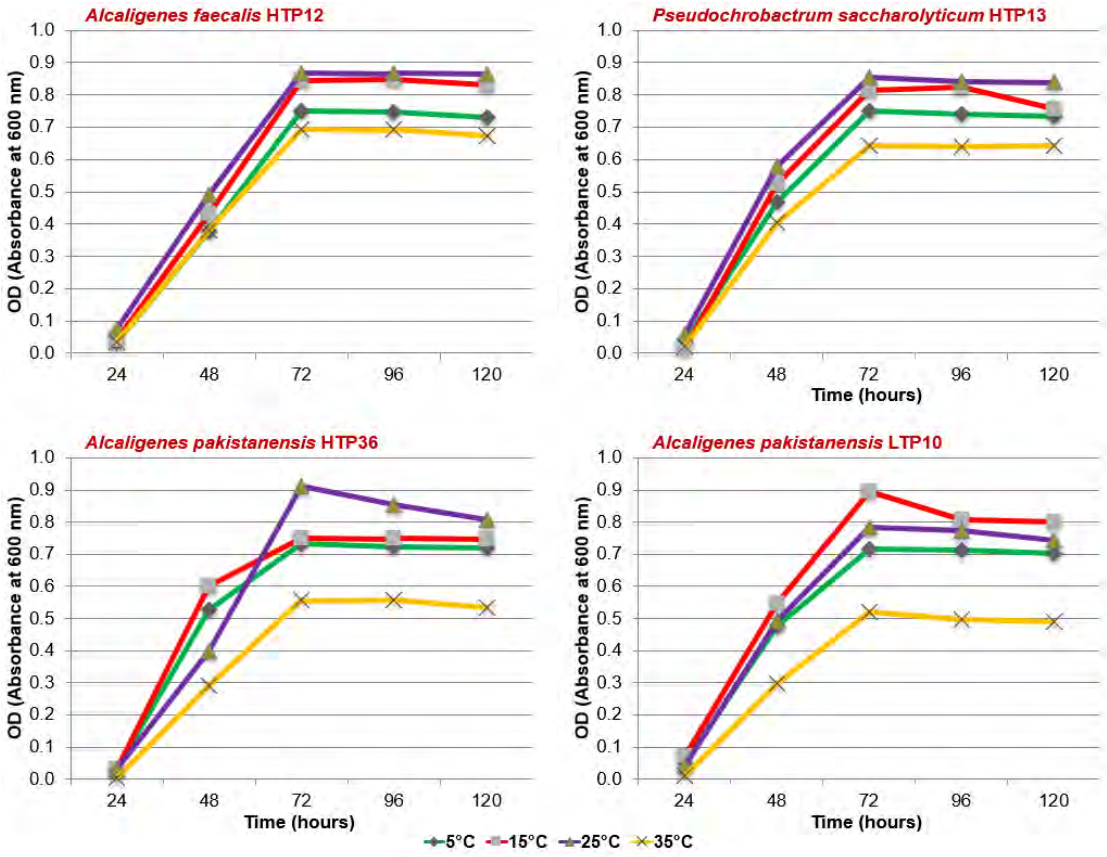

Figure 4.4 Optimization results of different temperatures on the growth of study isolates. Data points represent the optical density (absorbance) of isolates culture broth at 600 nm.

4.4.3 Effect of pH on growth

The optimum pH for growth of all isolates was observed to be 7.0 after 72 hrs of incubation as shown in figure 4.5. Moreover, the difference in growth of the isolates between pH 7.0 and 5.0, and 7.0 and 9.0 was significant with p-value found to be less than 0.05 ($P < 0.05$).

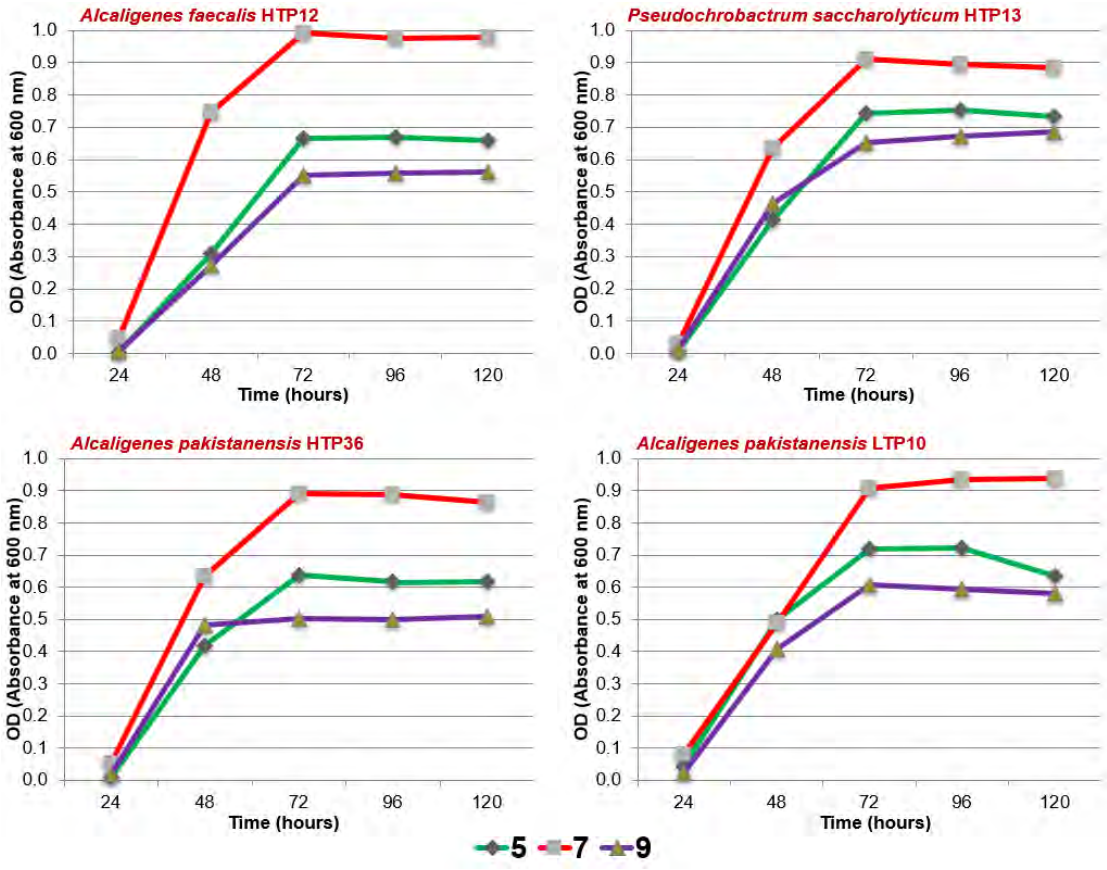

Figure 4.5 Optimization results of different pH on the growth of study isolates. Data points represent the optical density (absorbance) of isolates culture broth at 600 nm.

4.5 Optimization of antimicrobial compound production

AWD method was employed to confirm that isolated strains produce antimicrobial chemicals. *Alcaligenes pakistanensis* LTP10 had the greatest effectiveness against the various test organisms utilised in the study as evidenced by its zones of inhibitions in mm. Different cultural factors, such as the choice of growth medium, the length of incubation, the temperature, the pH, and the supplements of carbon, nitrogen, and salt sources were taken into account.

4.5.1 Effect of incubation period

After 96 hours of incubation, all four isolates had shown their maximal antibacterial activity. The statistical difference between the antibacterial effect at various incubation periods was statistically significant ($P < 0.05$). As indicated in Figure 4.6, the maximal antibacterial activity of *A. pakistanensis* LTP10 against *S. aureus* (ATCC

25923) was 20.0 ± 1.0 mm, while that of *A. pakistanensis* HTP36 against *Bacillus subtilis* (ATCC 6633) was 20.3 ± 2.1 mm.

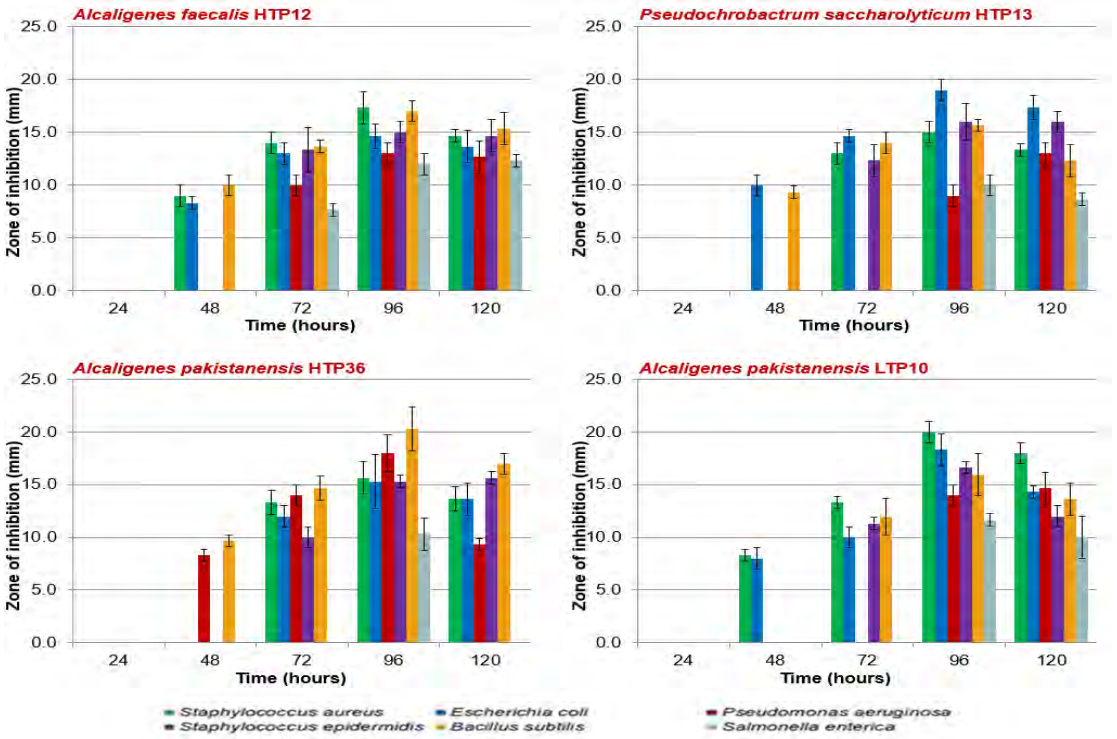

Figure 4.6 Antibacterial activity of study isolates measured in mm (ZOI) against ATCC test organisms at different incubation periods. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.5.2 Effect of culture medium

In order to assess the maximal antibacterial activity of certain isolates, NB, TSB, and LB broths were utilized. All four isolates shown distinct antibiotic activity in various culture conditions, which was statistically significant ($P < 0.05$). All four Luria-bertani broth isolates have been shown to exhibit maximal activity against test organisms in their cell-free supernatants. As shown in Figure 4.7, *Alcaligenes pakistanensis* LTP10 had the greatest activity against *S. aureus* and *E. coli*, with ZOI of 20.7 ± 1.5 mm and 21.0 ± 1.0 mm, respectively.

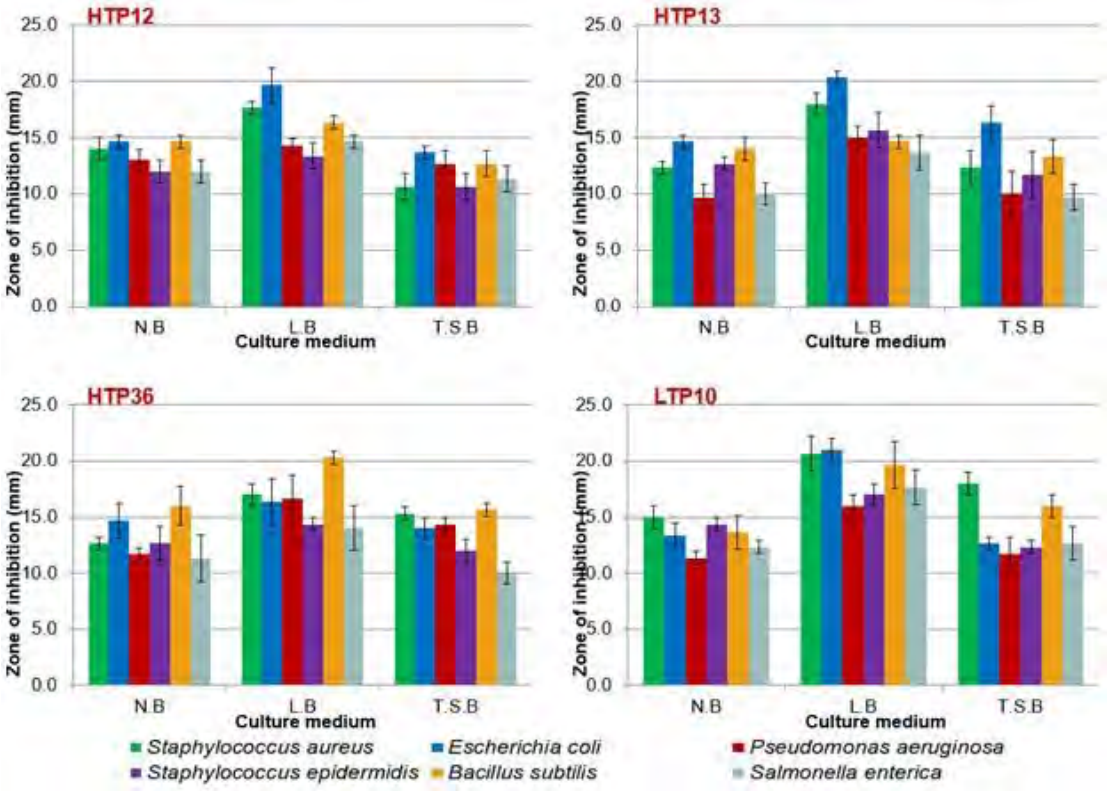

Figure 4.7 Antibacterial activity of study isolates measured in mm (ZOI) against ATCC test organisms in different culture media. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.5.3 Effect of Temperature

All four isolates shown distinct antibacterial activity at various temperatures, which was statistically significant ($P < 0.05$). After 4 days of incubation in LB broth, isolated strains displayed their peak antibacterial activity at 25°C. Alcaligenes pakistanensis LTP10 had a maximal ZOI of 20.3 ± 2.1 mm against *S. aureus* as depicted in figure 4.8.

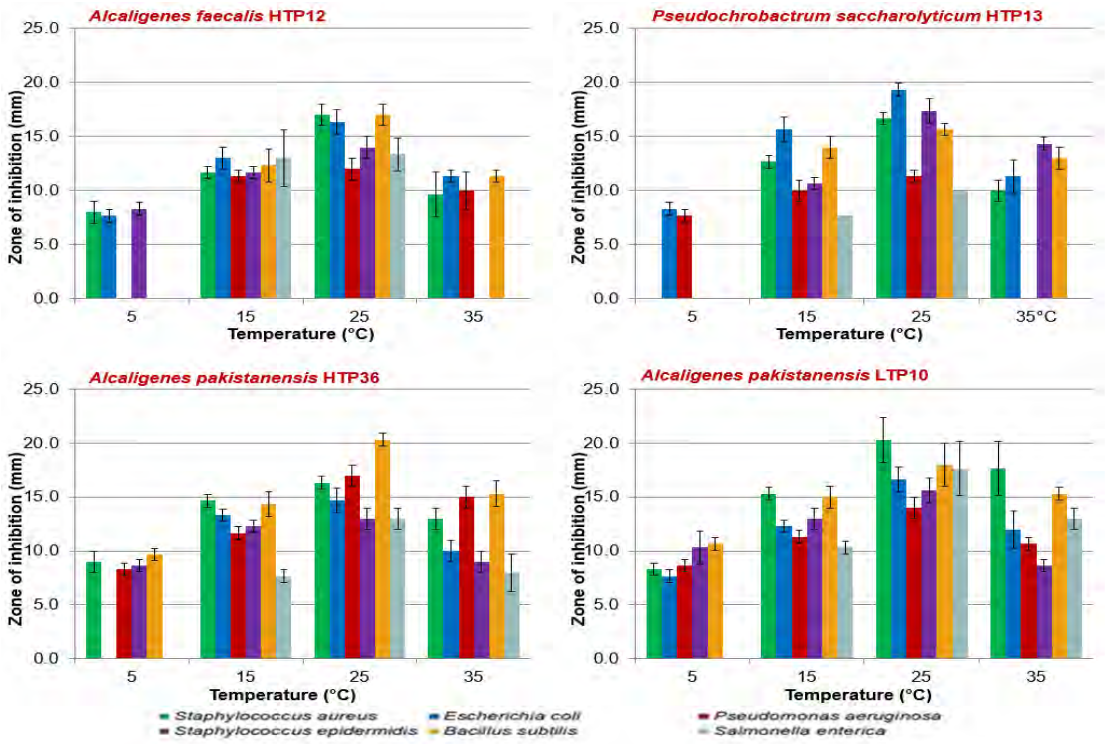

Figure 4.8. Antibacterial activity of study isolates measured in mm (ZOI) against ATCC test organisms at various temperatures. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.5.4 Effect of pH

Statistical difference between the antibacterial potential at various pH levels was significant ($P < 0.05$). Maximum antibacterial effect was found for all four isolates at pH 7. *Alcaligenes pakistanensis* (LTP10) had the highest antibacterial action against *S. aureus* with a 23.3 ± 0.6 mm ZOI (Figure 4.9).

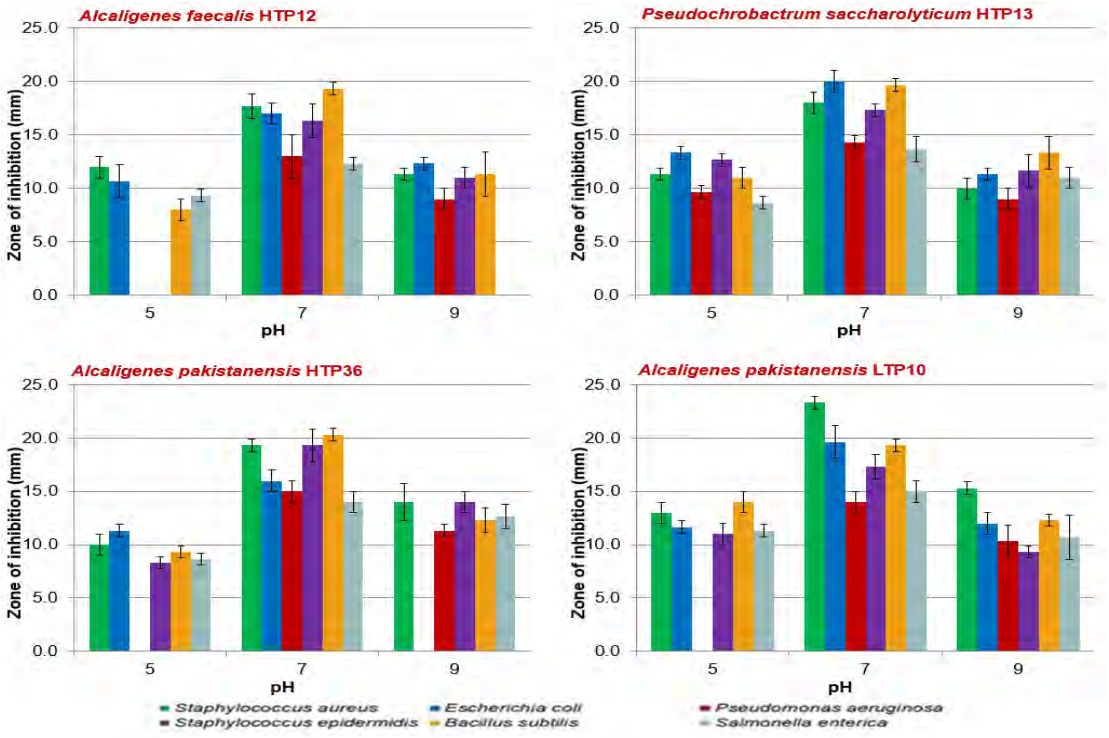

Figure 4.9 Antibacterial activity of study isolates measured in mm (ZOI) against ATCC test organisms at different pH. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.5.5 Effect of carbon sources

Antibacterial activity of all four strains was shown to be negatively impacted by the supplementation of LB broth with carbon sources. By adding glucose and starch at concentrations of 1% and 2%, as shown in table 4.5, the antibacterial action against S. *aureus* and *E. coli* was reduced by 2 to 8 mm in ZOI.

Table 4.5 Effect of glucose and starch on the antibacterial activity of study isolates measured in mm (ZOI) against ATCC test organisms. The values after ± denoted the SD of triplicate ZOI measurements.

Isolates	**Zone of inhibition (Mean value in mm ± SD) of isolates in against *S. aureus* & *E. Coli***									
	Staphylococcus aureus					***Escherichia coli***				
	***Control**	**Glucose (1%)**	**Glucose (2%)**	**Starch (1%)**	**Starch (2%)**	***Control**	**Glucose (1%)**	**Glucose (2%)**	**Starch (1%)**	**Starch (2%)**
A. feacalis HTP12	15 ± 1.5	13 ± 1.3	10 ± 1.2	14 ± 0.6	13 ± 0.7	18 ± 1.5	13 ± 1.2	15 ± 0.5	15 ± 0.5	14 ± 1.0
P. saccharolyticum HTP13	17 ± 2.0	14 ± 0.7	11 ± 1.5	15 ± 1.7	14 ± 1.8	20 ± 1.3	15 ± 2.0	16 ± 1.4	17 ± 0.3	16 ± 1.2
A. pakistanensis HTP36	16 ± 1.0	14 ± 0.6	12 ± 0.5	13 ± 1.3	13 ± 2.0	14 ± 0.5	12 ± 0.5	10 ± 1.5	14 ± 0.6	12 ± 0.8
A. pakistanensis LTP10	21 ± 1.3	16 ± 1.0	15 ± 0.3	15 ± 1.2	16 ± 1.0	21 ± 0.3	17 ± 0.8	16 ± 0.3	18 ± 0.4	13 ± 0.6

*Control; culture of isolate in LB broth

4.5.6 Effect of nitrogen sources

The synthesis of antimicrobials by isolated strains is affected differently by the adding various nitrogen supplements (yeast extract and amino acids). The antibacterial activity of isolates was lowered by all amino acids used in analysis but elevated by yeast extract. With the addition of 1% yeast extract, *A. pakistanensis* LTP10 exhibited a rise of 3 mm against *E. coli*. Similar to this, adding 1% yeast extract improved the antibacterial activity of *A. pakistanensis* HTP36 against *S. aureus* by 2 mm as given in table 4.6.

Table 4.6 Effect of nitrogen supplements on the antibacterial activity of study isolates measured in mm (ZOI) against ATCC test organisms. The values after ± denoted the SD of triplicate ZOI measurements

Isolates	**Zone of inhibition (Mean value in mm ± SD) of isolates in against *S. aureus* & *E. coli***					
	Staphylococcus aureus					
	****Control**	***Trp**	***Thr**	***Leu**	***Arg**	***Y.E.**
HTP12	16 ± 0.5	12 ± 1.5	--	11 ± 1.2	13 ± 0.7	16 ± 2.0
HTP13	17 ± 1.2	14 ± 0.5	13 ± 1.5	15 ± 0.6	--	16 ± 1.2
HTP36	18 ± 0.6	13 ± 0.6	14 ± 0.5	12 ± 0.8	10 ± 0.3	20 ± 0.3
LTP10	20 ± 0.3	14 ± 1.2	--	13 ± 1.1	16 ± 1.2	21 ± 0.6
Escherichia coli						
HTP12	17 ± 1.2	--	15 ± 1.5	14 ± 0.5	10 ± 0.6	15 ± 1.3
HTP13	19 ± 0.5	16 ± 1.0	14 ± 1.5	--	--	21 ± 1.5
HTP36	15 ± 1.7	10 ± 0.5	--	13 ± 1.0	12 ± 0.3	16 ± 1.0
LTP10	19 ± 0.5	--	12 ± 0.5	--	16 ± 0.5	22 ± 1.7

*Trp = Tryptophan, Thr = threonine, Leu = L-leucine, Arg = Arginine, Y.E. = Yeast extract
**Control; culture of isolate in LB broth

4.5.7 Effect of salts

Separate amounts of potassium chloride (1% and 2%) and sodium chloride (2% and 3%) were added to culture medium. The antibacterial activity of isolates was decreased or decreased as a result of high salt concentrations in culture medium, as shown in Table 4.7.

Table 4.7 Effect of NaCl and KCl on the antibacterial activity of study isolates measured in mm (ZOI) against ATCC test organisms. The values after ± denoted the SD of triplicate ZOI measurements.

Isolates	Zone of inhibition of isolates in (Mean value in mm ± SD) against *S. aureus* & *E. coli*					
	Staphylococcus aureus			*Escherichia coli*		
	*Control	NaCl (2%)	NaCl (3%)	Control	KCl (1%)	KCl (2%)
HTP12	17 ± 0.4	13 ± 0.5	10 ± 0.6	15 ± 0.6	10 ± 0.7	--
HTP13	16 ± 0.3	12 ± 0.8	--	16 ± 1.3	12 ± 1.3	9 ± 0.3
HTP36	19 ± 1.3	12 ± 1.0	--	15 ± 0.5	11 ± 0.5	8 ± 0.6
LTP10	21 ± 1.6	15 ± 1.2	10 ± 1.3	20 ± 0.5	14 ± 1.7	--

*Control; culture of isolate in LB broth containing 1 % NaCl (w/v)

4.6 Antibiotic sensitivity test of isolates

To assess the antibiotic sensitivity of isolated bacteria, antibiotic discs were utilised. *Alcaligenes pakistanensis* LTP10 was found to be resistant to Ciprofloxacin, Ofloxacin, Norfloxacin, Amoxicillin, Penicillin G, Oxacillin, Ceftazidime, Cefpodoxime, Ceftaroline, Meropenem, Ertapenem, Aztreonam, Linezolid, Erythromycin, Vancomycin, Tigecycline, and Metronidazole and sensitive to Levofloxacin, Nalidixic acid, Carbenicillin, Ticarcillin, Piperacillin, Ceftriaxone, Cefotetan, Cephalexin, Cephalothin, Cefepime, Cephazolin, and Imipenem, while isolate *Alcaligenes pakistanensis* (HTP36) was resistant to Cefepime, Cefpodoxime, Ceftaroline, Meropenem, Ertapenem, and Metronidazole and sensitive to Levofloxacin, Ciprofloxacin, Ofloxacin, Norfloxacin, Nalidixic acid, Amoxicillin, Penicillin G, Carbenicillin, Ticarcillin, Oxacillin, Piperacillin, Ceftriaxone, Cefotetan, Cephalexin, Cephalothin, Ceftazidime, Cephazolin, Imipenem, Aztreonam, Linezolid, Erythromycin, Vancomycin, and Tigecycline as depicted in table 4.8.

Table 4.8 Antibiotic resistance profile of study isolates against various antibiotic

Isolate	Resistant to antibiotics	Sensitive to antibiotics
Alcaligenes faecalis HTP12	Ciprofloxacin, Ofloxacin, Nalidixic acid, Amoxicillin, Penicillin G, Carbenicillin, Oxacillin, Ceftriaxone, Cefotetan, Cephalexin, Cephalothin, Cephazolin, Cefpodoxime, Linezolid, Erythromycin, Vancomycin, Tigecycline, Metronidazole	Levofloxacin, Ticarcillin, Piperacillin, Cefepime, Ceftazidime, Ceftaroline, Meropenem, Imipenem, Ertapenem, Aztreonam
Pseudochrobactrum saccharolyticum HTP13	Amoxicillin, Penicillin G, Ceftriaxone, Cefotetan, Cefepime, Ceftazidime, Cefpodoxime, Ceftaroline, Meropenem, Ertapenem Aztreonam, Metronidazole	Levofloxacin, Ciprofloxacin, Ofloxacin, Norfloxacin, Nalidixic acid, Carbenicillin, Ticarcillin, Oxacillin, Piperacillin, Cephalexin, Cephalothin, Cephazolin, Imipenem, Linezolid, Erythromycin, Vancomycin, Tigecycline
Alcaligenes pakistanensis HTP36	Cefepime, Cefpodoxime, Ceftaroline, Meropenem, Ertapenem, Metronidazole	Levofloxacin, Ciprofloxacin, Ofloxacin, Norfloxacin, Nalidixic acid, Amoxicillin, Penicillin G, Carbenicillin, Ticarcillin, Oxacillin, Piperacillin, Ceftriaxone, Cefotetan, Cephalexin, Cephalothin, Ceftazidime, Cephazolin, Imipenem, Aztreonam, Linezolid, Erythromycin, Vancomycin, Tigecycline
Alcaligenes pakistanensis LTP10	Ciprofloxacin, Ofloxacin, Norfloxacin, Amoxicillin, Penicillin G, Oxacillin, Ceftazidime, Cefpodoxime, Ceftaroline, Meropenem, Ertapenem, Aztreonam, Linezolid, Erythromycin, Vancomycin, Tigecycline, Metronidazole	Levofloxacin, Nalidixic acid, Carbenicillin, Ticarcillin, Piperacillin, Ceftriaxone, Cefotetan, Cephalexin, Cephalothin, Cefepime, Cephazolin, Imipenem

4.7 Antimicrobial activity of CFS of *A. pakistanensis* LTP10 against clinical isolates

Clinical bacterial and fungal isolates were used to study the antibacterial efficacy of CFS of best antibiotic producing strain *A. pakistanensis*. After incubation under optimal parameters, it was discovered that the CFS of *A. pakistanensis* LTP10 culture medium had decent activity against all tested bacterial and fungal clinical isolates but no activity was observed against *Candida krusei*, as given in figure 4.10. The maximum ZOI of 17.0 ± 1.0 mm was found against *Staphylococcus aureus* followed by 15.7± 0.6 mm against *Escherichia coli.* Negative control LB broth did not produce any zone against tested strains.

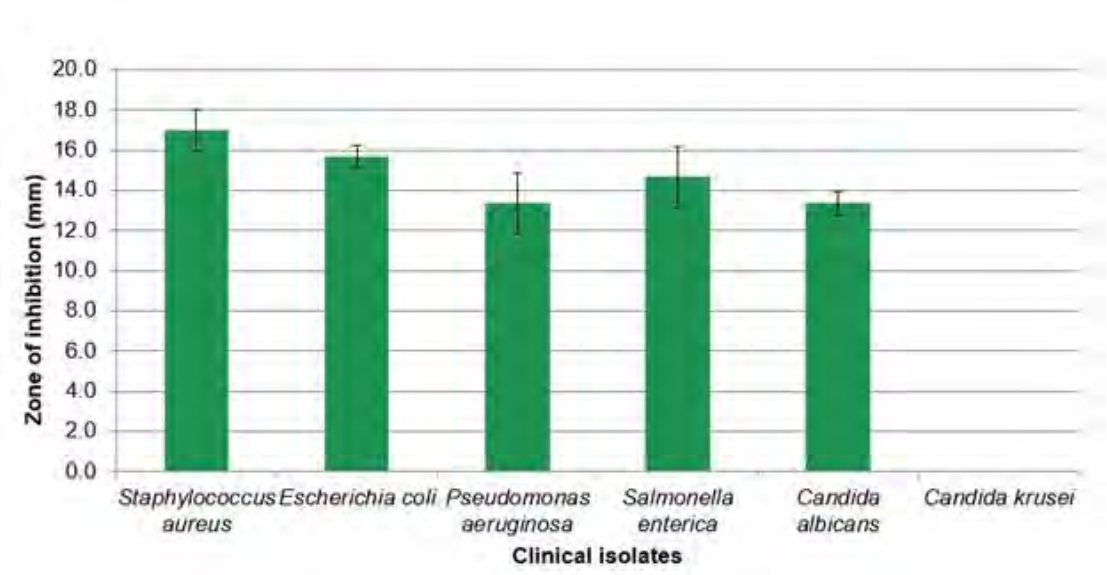

Figure 4.10 Antibacterial activity of CFS of *Alcaligenes pakistanensis* LTP10 measured in mm (ZOI) against clinical isolates. The error bars denoted the SD of triplicate ZOI measurements.

4.8 Production and extraction of antimicrobial compounds from *Alcaligenes pakistanensis* LTP10

The extracellular production of antimicrobial metabolites by *A. pakistanensis* LTP10 was accomplished in LB broth. After 96 hrs of incubation at 25ºC and initial pH 7.0 of culture medium, the CFS was evaluated for antagonistic activity against ATCC test strains. After confirmation of antimicrobial activity, CFS was further subjected to extraction of the required metabolites.

4.8.1 Extracts preparation using different solvents

The solvents used for the extraction of antimicrobial metabolites from CFS of LTP10 were n-hexane, ethyl acetate, and chloroform. After the extraction process, the extract of different solvents were assessed for antagonistic activity by AWD assay as shown in figure 4.11. The results showed that ethyl acetate extract has shown maximum activity in mm (ZOI) against ATCC test strains *S. aureus, E. coli, S. epidermidis,* and *Salmonella enterica* followed by chloroform. The difference between activity of ethyl acetate extract and chloroform extract was significant statistically as evident from p-value ($P < 0.05$). However, n-hexane had shown no or little activity against test strains. Based on these results, ethyl acetate was further used for the extraction of antimicrobial metabolites from CFS of *Alcaligenes pakistanensis* (LTP10). Positive control amoxicillin (10 µg) has shown 23-30 mm ZOI against tested strains while negative controls hexane, ethyl acetate, and chloroform did not produce any zones against tested strains.

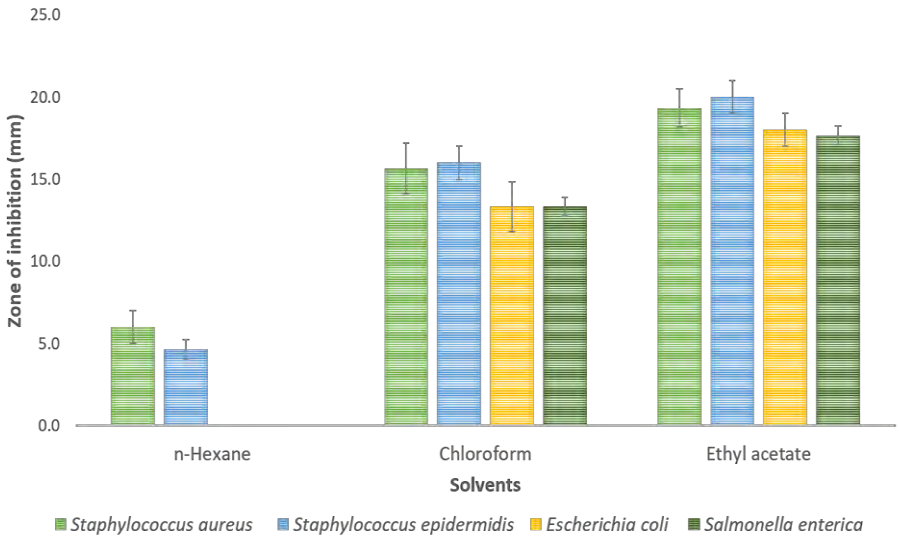

Figure 4.11 Antibacterial activity of extracts of *A. pakistanensis* LTP10 in various solvents measured in mm (ZOI) against ATCC test organisms. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.9 Characteristics of extract of *Alcaligenes pakistanensis* LTP10

4.9.1 Solubility

The dried crude powder was mixed with several organic solvents. It was found that crude powder was very soluble in methanol and DMSO, soluble in ethyl acetate, less soluble in chloroform and very slightly soluble in petroleum ether and n-hexane which indicated the polar nature of crude extract of LTP10.

4.9.2 Temperature stability

The dried ethyl acetate extract of LTP10 was kept at 25, 35, 45, 55, and 65°C for 6 hrs. After that crude powder was evaluated for antimicrobial activity to confirm the stability of antimicrobial compounds. It was found that there was no significant change in antimicrobial activity of the extract when treated from 25 to 45°C ($P > 0.05$), however at 55 and 65°C there was decrease in antimicrobial activity ($P < 0.05$) as shown in figure 4.12.

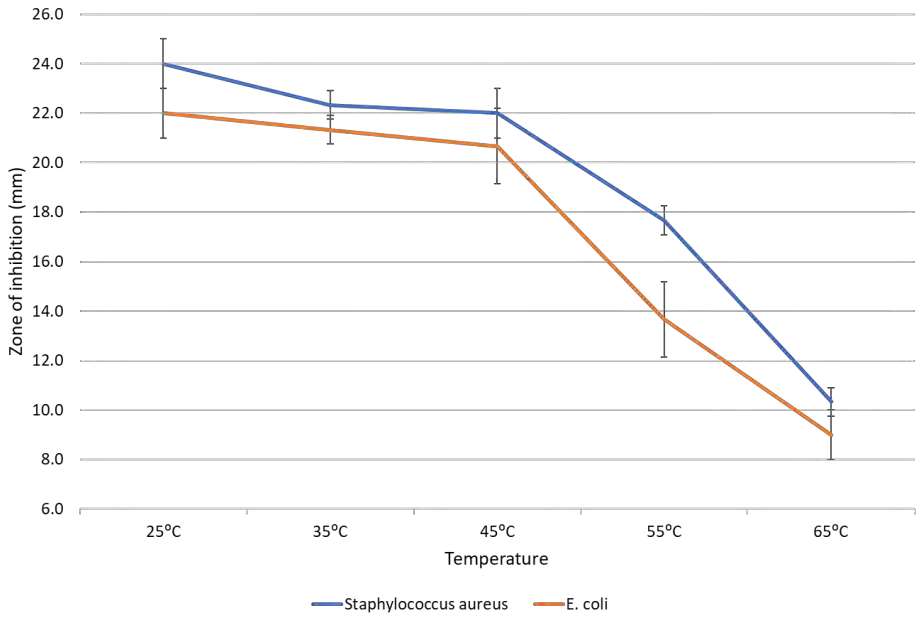

Figure 4.12 Antibacterial activity of ethyl acetate extract of *A. pakistanensis* LTP10 measured in mm (ZOI) against ATCC test organisms at temperatures 25-65°C. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.9.3 pH stability

The dried crude extract of LTP10 was assessed for antimicrobial action to confirm the stability of antimicrobial compounds at pH range 4 to 10. It was found that there was no significant change in antimicrobial activity of the extract when evaluated at pH 5.5, 7.0, and 8.5 ($P > 0.05$), however at 4.0 and 10.0 there was decrease in antimicrobial activity ($P < 0.05$) as shown in figure 4.13.

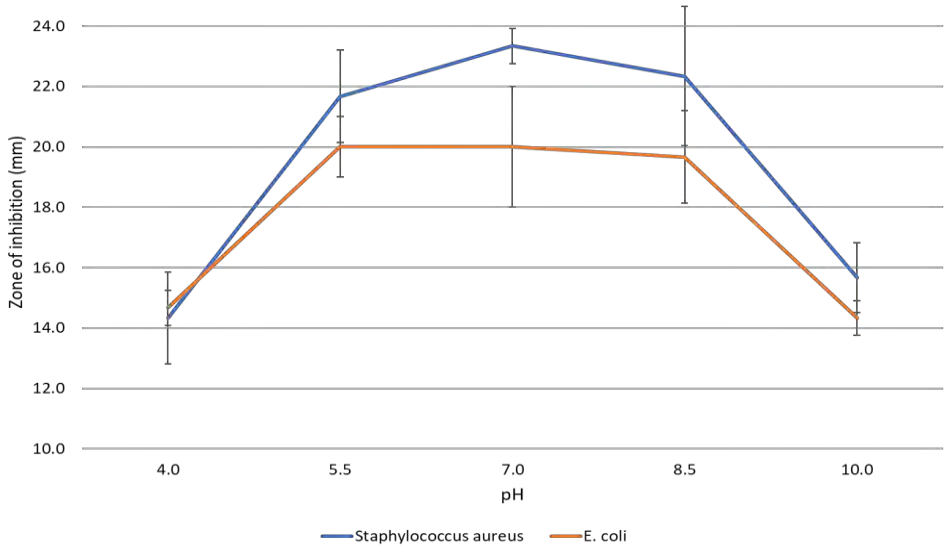

Figure 4.13 Antibacterial activity of ethyl acetate extract of *A. pakistanensis* LTP10 measured in mm (ZOI) against ATCC test organisms at pH 4-10. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.9.4 Extracellular protein estimation

The equation $Y = (0.9898 \times X) + 0.03$, developed from standard BSA curve, was used to determine protein estimation of crude extract of LTP10, where Y indicates concentration of unknown protein in sample and X indicates optical density of unknown protein in sample. The optical density of crude extract of LTP10 at 650 nm was 1.0719. So according to equation, the conc. of protein was calculated as;

$$Y = (0.9898 \times 1.0719) + 0.03 = 1.0910 \text{ mg/mL}$$

4.10 Bioassays of ethyl acetate extract of *A. pakistanensis* LTP10

4.10.1 Antibacterial and antifungal assay against clinical isolates

The antimicrobial activity of dried crude extract of LTP10 was tested against clinical isolates, as shown in figure 4.14, by AWD assay. The antibiotic resistance profile of bacterial isolates was determined by agar-disk diffusion method and all four clinical bacterial isolates were resistant to penicillin-G, tertracycline, ampicillin, and erythromycin, intermediate resistant to ciprofloxacin and susceptible to ceftriaxone and mreopenem. It was evident from the results that crude extract of LTP10 showed greatest activity against *Staphylococcus aureus* with zone of inhibition 24.3±0.6 mm and against *Escherichia coli* with zone of inhibition 22.0±1.0 mm as shown in figure 4.14 and 4.15. The activity was also present against *Salmonella enterica, Candida albicans,* and *Pseudomonas aeruginosa* with 21.3±1.2, 17.6±1.5 and 16.0±2.0 mm zone of inhibition respectively. However, there was no activity of extract against *Candida krusei*. Negative control DMSO did not produce any zone against tested strains.

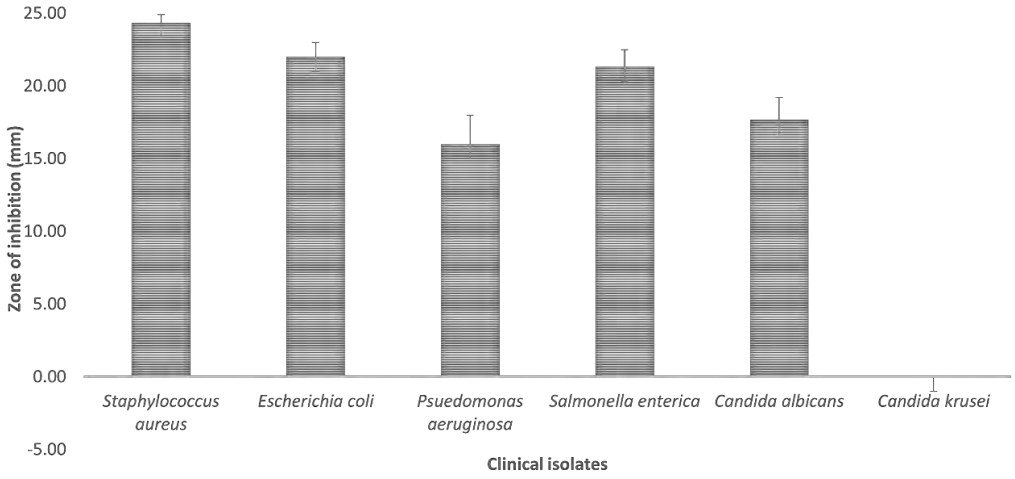

Figure 4.14 Antibacterial activity of ethyl acetate extract of *A. pakistanensis* LTP10 measured in mm (ZOI) against clinical isolates. The error bars denoted the SD of triplicate ZOI measurements.

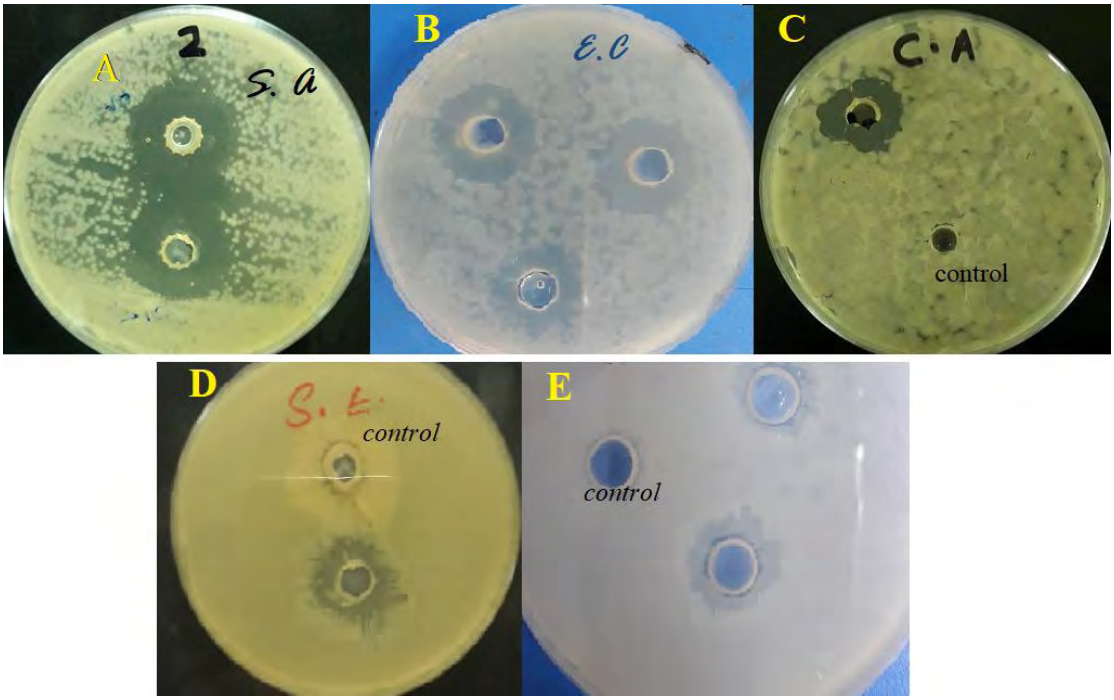

Figure 4.15 Antibacterial activity of ethyl acetate extract of *A. pakistanensis* LTP10 against clinical isolates. A = *Staphylococcus aureus*, B = *Escherichia coli*, C = *Candida albicans*, D = *Salmonella enterica*, E = *Pseudomonas aeruginosa*

4.10.2 MIC assay

The antimicrobial effectiveness of the crude extract of LTP10 against tested strains was examined by evaluating the MIC. In this study, MIC of the extract was determined only against those test strains which were susceptible to extract as evident from their clear zone of inhibition by agar diffusion assay. The MIC of LTP10 extract against *Staphylococcus aureus* was found to be 0.4 mg/mL and against *Escherichia coli* was 1.6 mg/mL. Similarly, MIC against other bacteria (*B. subtilis, S. epidermidis, P. aeruginosa* and *S. enterica*) were found to be 0.8, 0.8, 3.2 and 1.6 mg/mL respectively as shown in Table 4.9.

Table 4.9 MIC values of ethyl aceate extract of LTP10 against test strains

Test organisms	MIC (mg/mL)
Staphylococcus aureus	0.4
Escherichia coli	1.6
Bacillus subtilis	0.8
Staphylococcus epidermidis	0.8
Pseudomonas aeruginosa	3.2
Salmonella enterica	1.6

4.10.3 MBC assay

To observe the bactericidal effect of ethyl acetate extract of LTP10, the dilutions in MIC test which did not show any growth were selected. These dilutions were inoculated on MHB to check for any growth. After 24 hrs of incubation, it was observed that *Staphylococcus aureus* showed no growth from any of the dilutions and so its MBC was found to be same as MIC i.e., 0.4 mg/mL. Similarly, MBC values of LTP10 extract against *Bacillus subtilis* and *Staphylococcus epidermidis* was found to 1.6 mg/mL as shown in table 4.10. However, *E. coli, P. aeruginosa* and *Salmonella enterica* had shown growth in all dilutions indicating non-bactericidal behaviour of LTP10 extract against these microorganisms.

Table 4.10 MBC values of crude extract of LTP10 against test strains

Test organisms	MBC (mg/mL)
Staphylococcus aureus	0.4
Escherichia coli	--
Bacillus subtilis	3.2
Staphylococcus epidermidis	3.2
Pseudomonas aeruginosa	---
Salmonella enterica	---

4.10.4 Synergistic effect of LTP10 extract with antibiotics

The crude extract of LTP10 was evaluated for its synergistic effect with known antibiotics (penicillin, imipenem, cefepime, nalidixic acid and metronidazole) against

test strains *Staphylococcus aureus* and *Escherichia coli*. As shown in figure 4.16, there was increase in the activity of LTP10 extract (5 mg/mL) when combined with penicillin 10 µg, imipenem 10 µg, and cefepime 30 µg. However, there was no or little increase in activity of LTP10 when combined with nalidixic acid 30 µg and metronidazole 5 µg. These results had shown the synergistic effect of LTP10 with different antibiotics. The difference in antimicrobial activity of LTP10 extract alone and combined with antibiotic discs (penicillin, imipenem, cefepime) was found to be significant statistically as evident from p-value ($P < 0.05$).

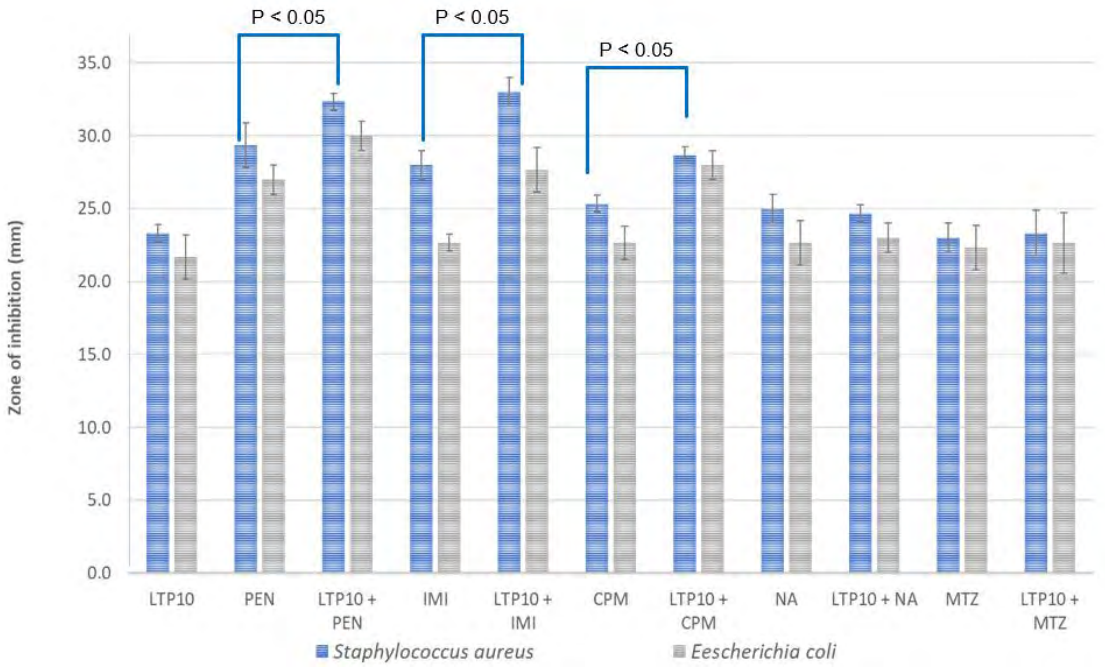

Figure 4.16 Antibacterial activity of ethyl acetate extract of *A. pakistanensis* LTP10 with and without antibiotics measured in mm (ZOI) against *S. aureus* and *E. coli*. The error bars denoted the SD of triplicate ZOI measurements. P-value was found to be less than 0.05.

4.10.5 Brine shrimp cytotoxicity assay

The crude extract of the LTP10 was tested against 10 active nauplii of brine shrimp by following protocols as mentioned earlier. The analysis were performed in triplicates. The cytotoxic study determined that the extent of lethality was proportional to the concentration of the extract. After 72 hrs of observation all the nauplii were survived in the control and negative control as shown in table 4.11. There was no mortality observed for crude extract after 48 hours. The maximum

mortality of 40% were observed at a concentration of 1000 µg of crude extract after 72 hours, however no mortality was observed at conc. of 100 µg and 500 µg. The results indicated non-cytotoxic nature of the metabolites of the crude extract. Percent survival rate of brine shrimps against cytotoxic activity of crude extract of LTP10 was found to be 100% after 72 hours at 100 and 500 µg concentration, while it was observed as 100% at 1000 µg concentration of extract up to 48 hrs. However, after 72 hrs, percent survival rate was decreased to 60%. These results indicated the non-cytotoxic nature of antimicrobial metabolites.

Table 4.11 Cytotoxic activity of crude extract of LTP10 with different concentrations

Sample	Conc.	Avg. number of surviving nauplii			Nauplii killed	Mortality after 72 h
		24 hr	48 hr	72 hr		
LTP10 Extract	100 µg	30	30	30	0	0 %
	500 µg	30	30	30	0	0 %
	1000 µg	30	30	18	12	40 %
Control*	100 µL	30	30	30	0	0 %
DMSO**	100 µL	30	30	30	0	0 %
Blank***	100 µL	30	30	30	0	0 %

* = Ethyl-acetate was used as control
** = DMSO was used as negative control
*** = Physical control, contains 10 active nauplii without extract

4.10.6 Antioxidant assay

The antioxidant assay was performed by treating the crude extract of LTP10 with DPPH and measuring absorbance of resulting solution at 517 nm. Antioxidant activities of ascorbic acid and crude extract of LTP10 was calculated in percent inhibition as shown in table 4.12, by using following formula.

$$\text{Inhibition (\%)} = (AD - AS/\ AD) \times 100$$

Where AD indicates absorbance of DPPH and AS represents absorbance of sample. The absorbance of DPPH was found to be 0.4583. The antioxidant activity of crude extract of LTP10 was determined at concentrations of 100, 200 and 300 µg. The results has revealed that antioxidant activity of LTP10 was proportional to its

concentration with 68.16% inhibition at 300 µg concentration. Ascorbic acid was used as positive control while DMSO was used as negative control. DMSO has shown 15.19% inhibition of free radical DPPH and was subtracted from the percent inhibition shown by various concentration of crude extract of LTP10.

Table 4.12 Antioxidant activity of crude extract of LTP10 against DPPH

Sample	Concentration	Absorbance (517 nm)	Antioxidant activity (% inhibition)
Ascorbic acid*	0.04 mg/mL	0.9166	89.42 %
DMSO**	---	0.0970	15.19 %
LTP10 extract	100 µg/mL	0.7774	47.85 %
	200 µg/mL	0.3388	60.18 %
	300 µg/mL	0.2258	68.16 %

* = Positive control, ** = Negative control

4.11 Characterization of extract of *Alcaligenes pakistanensis* LTP10

The compounds in the *Alcaligenes pakistanensis* LTP10 extract were identified by GCMS and LCMS techniques which are commonly used for metabolic profiling. The combined information of compounds are given section 4.11.2 and 4.11.3.

4.11.1 Analysis of ethyl acetate extract by FTIR

To recognize the important functional groups of the bioactive metabolites in the ethyl acetate extract of LTP10, the FTIR spectrum was obtained. For this purpose, extract was exposed to infra-red radiadtions in FTIR instrument resulting in seperation of metabolites' functional groups due to absorption of different infra-red radiations. The analysis of FTIR spectrum has shown the existence of important peaks at 3275, 2924, 1631, 1540, 1444, 1380, 1235 and 1071cm-^{1} corresponding to O-H, C-H, C=C, C-O, N-O, S=O, C-N and C-O functional groups respectively (Figure 4.17 and Table 4.13).

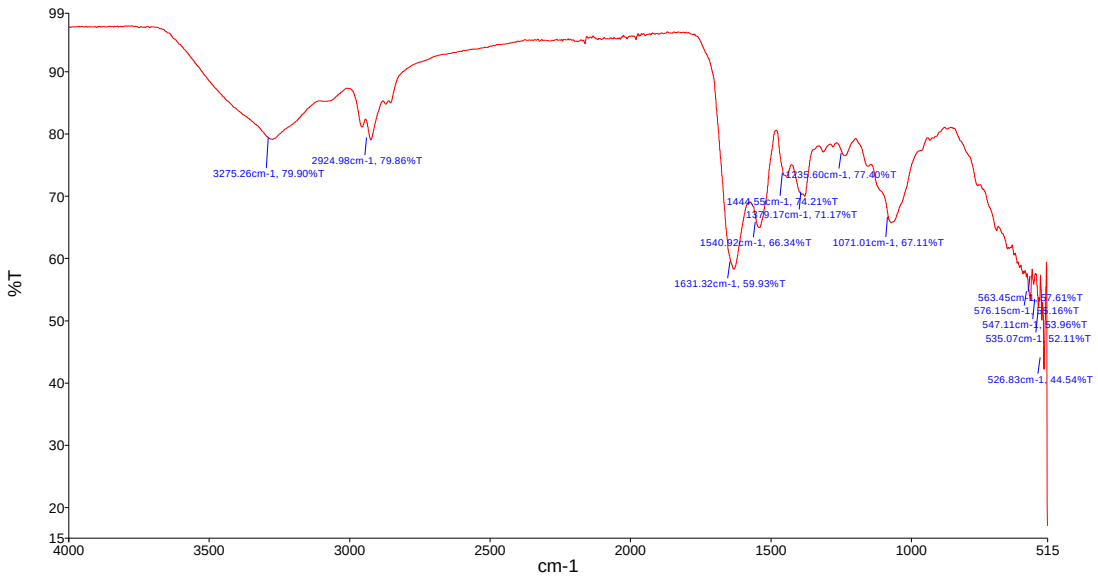

Figure 4.17 FTIR spectrum of ethyl acetate extract of *Alcaligenes pakistanensis* LTP10

Table 4.13 FTIR spectroscopic data of ethyl acetate extract of *Alcaligenes pakistanensis* LTP10 and their functional *groups*

Frequency (cm-1)	Bond strength	Functional Group	Compound Class
3275	strong, broad	O-H stretching	alcohol
2924	medium	C-H stretching	alkane
1631	medium	C=C stretching	alkene
1540	strong	N-O stretching	nitro compound
1444	medium	C-H bending	alkane
1380	strong	S=O stretching	sulfate
1235	medium	C-N stretching	amine
1071	strong	C-O stretching	primary alcohol

4.11.2 Analysis of extract by GC-MS

Gas chromatography / mass spectroscopy was done for the evaluation of ethyl acetate and chloroform extracts of LTP10. The GC-MS analysis of extract of *Alcaligenes pakistanensis* LTP10 showed the presence of more than 30 peaks which indicated the presence of microbial compounds as shown in figure 4.18. The mass spectra obtained as a result of GC-MS analysis was automatically compared with the NIST database and eventually 22 metabolites were characterized and recognized as shown in table 4.14). Few of the important metabolites were Phenol,2,4-bis(1,1-dimethylethyl),

Pentadecanoic acid,14-methyl-,methyl ester, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), Oleic Acid and Heptadecanoic acid, hepta-decylester having reported bioactivities like antibacterial, antifungal, anticancer etc. The mass spectra and chemical structures of these compounds are shown in figure 4.19.

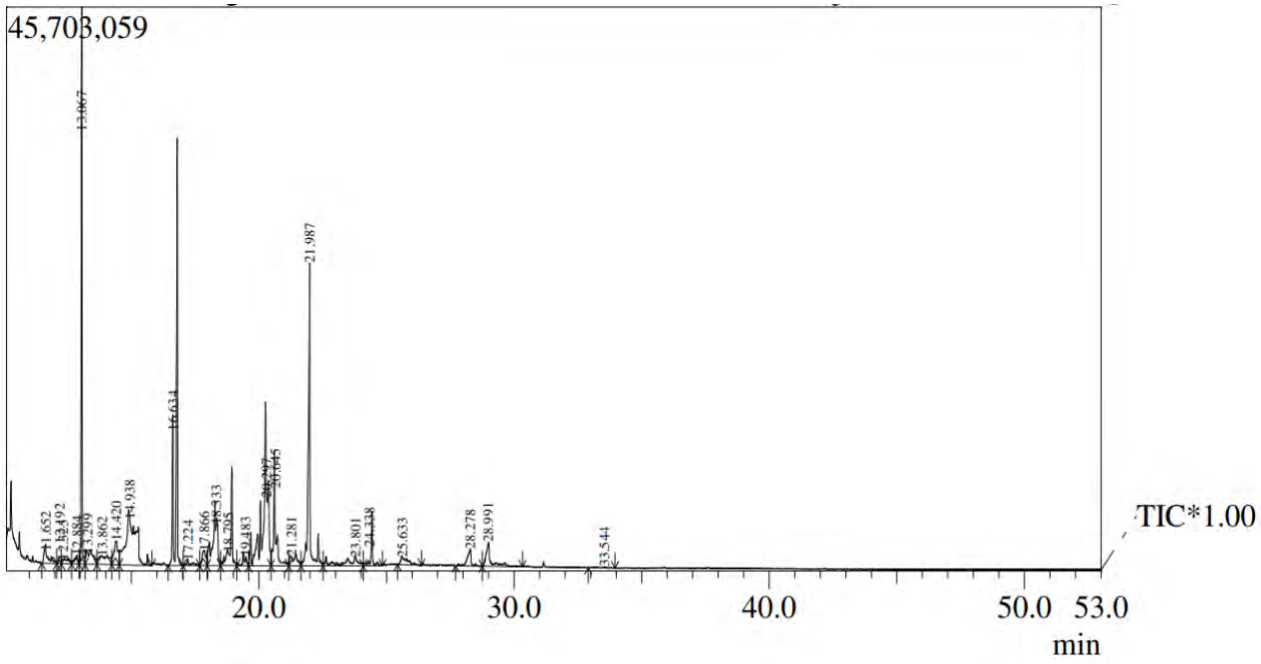

Figure 4.18 GC/MS chromatogram of ethyl acetate extract of *Alcaligenes pakistanensis* LTP10

Table 4.14 Details of compounds detected in ethyl acetate extract of *Alcaligenes pakistanensis* LTP10

S#	Formula	Area (%)	RT (min)	Tentative compound	Activity reported	Reference
1	$C_6H_9NO_3$	1.55	11.625	DL-Proline, 5-oxo-, methyl ester	antitumor	Yu *et al*., 2005
2	$C_9H_{18}O$	0.71	12.192	trans-3,4-Epoxynonane	not reported	NA
3	$C_5H_{12}N_2O_2S$	0.6	12.884	S-[2-Aminoethyl]-dl-cysteine	antibacterial	NA
4	$C_{14}H_{22}O$	11.27	13.067	Phenol, 2,4-bis(1,1-dimethylethyl)	antifungal antibacterial	Abd Sharad *et al*., (2018)
5	$C_9H_{19}NO$	2.09	13.299	Azacyclodecan-5-ol	not reported	NA
6	$C_{11}H_{22}$	2.18	14.421	2-Octene, 2,3,7-trimethyl	not reported	NA
7	$C_{12}H_{16}N_4O_5$	11.65	14.938	Pyrimidine-2,4(1H,3H)-dione	antimicrobial	Singla and Bhat 2011
8	$C_{17}H_{34}O_2$	11.9	16.634	Pentadecanoic acid, 14-methyl-, methyl ester	antibacterial antifungal	Ohiri and Bassey 2016
9	$C_{28}H_{56}O_2$	0.58	17.224	Heptacosanoic acid, methyl ester	not reported	NA
10	$C_{12}H_{24}O_3$	1.31	17.866	Dodecanoic acid, 3-hydroxy-	antifungal antibacterial	Mun *et al*., 2019
11	$C_9H_{14}O_2$	6.12	18.333	6-Oxabicyclo[3.1.0]hexan-3-one	not reported	NA
12	$C_{11}H_{18}N_2O_2$	4.13	18.795	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	antioxidant	Rangel-Sánchez et., 2014
13	$C_{17}H_{24}O_3$	0.89	19.483	7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	antimicrobial	El-Fayoumy *et al*., 2021
14	$C_{22}H_{44}O_2$	14.98	20.297	Heneicosanoic acid, methyl ester	not reported	NA
15	$C_{18}H_{34}O_2$	5.32	20.645	Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester	not reported	NA
16	$C_{15}H_{30}O_2$	1.48	21.281	Pentadecanoic acid	antioxidant	Henry *et al*., 2002
17	$C_{19}H_{40}O$	11.51	21.987	1-Nonadecanol	antibacterial	Nair *et al*., 2019
18	$C_{11}H_{16}O_4$	2.49	23.801	9,9-Dimethoxybicyclo [3.3.1]nona-2,4-dione	cytotoxic	Dassamiour *et al*., 2022
19	$C_{17}H_{32}O_2$	1.83	24.338	7-Hexadecenoic acid, methyl ester, (Z)-	antimicrobial antioxidant	Fagbemi *et al*., 2022
20	$C_{18}H_{34}O_2$	1.17	25.633	Oleic Acid	antibacterial	Dilika *et al*., 2000)
21	$C_{14}H_{16}N_2O_2$	1.48	28.278	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	antifungal	Kannabiran, 2016
22	$C_{34}H_{68}O_2$	0.1	33.544	Heptadecanoic acid, heptadecyl ester	antibacterial	Gacem *et al*., 2020

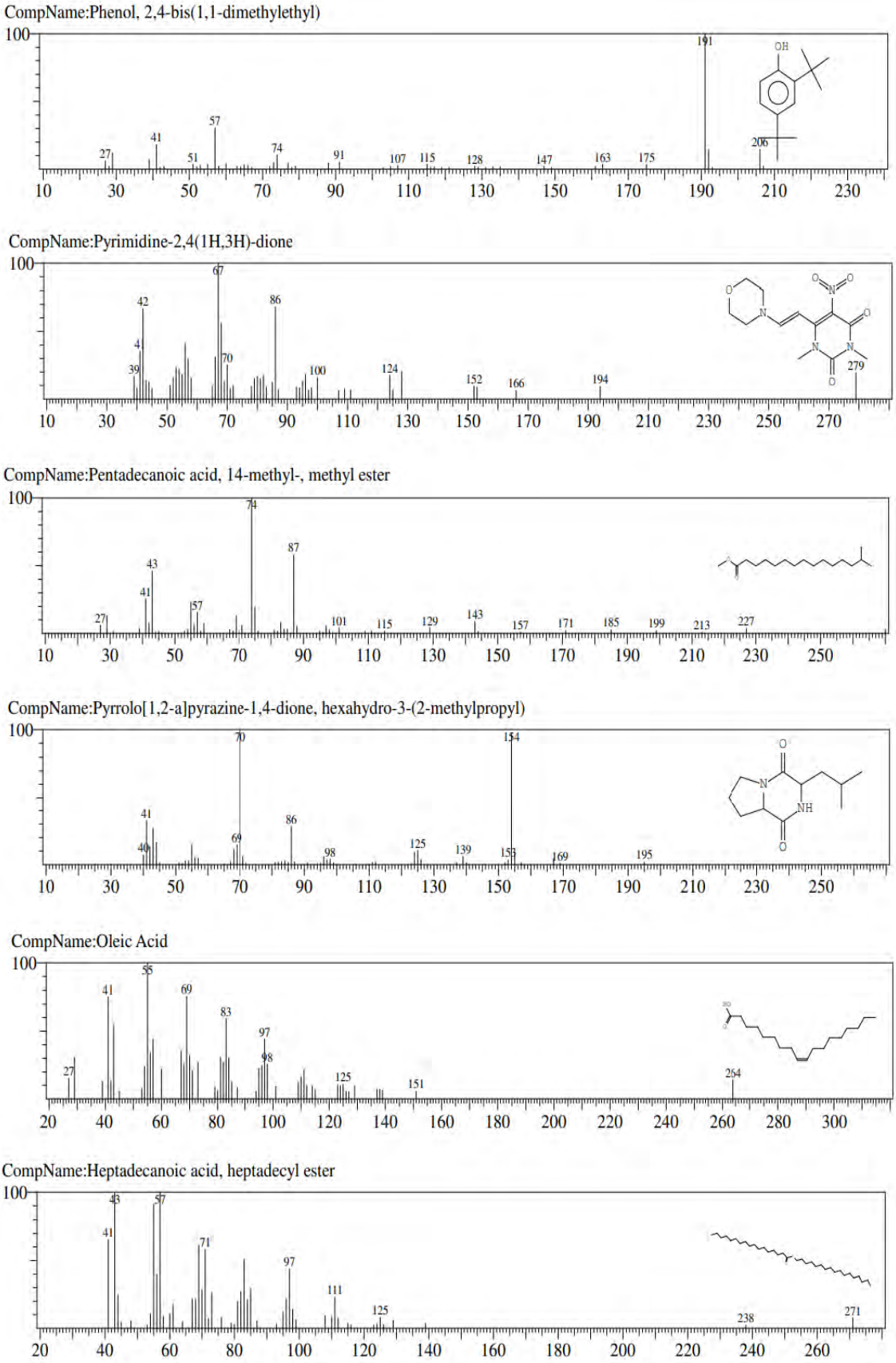

Figure 4.19 Mass spectra and chemical structures of important compounds identified in ethyl acetate extract of *Alcaligenes pakistanensis* LTP10

Similarly, GC-MS study of chloroform extract of LTP10 has shown presence of nine peaks as shown in figure 4.20. By comparing the mass spectra of the constituents of crude extract with the NIST library, 7 metabolites were identified as shown in table 18. Among these compounds, 3 compounds namely Phenol, 2,4-bis(1,1-dimethylethyl), Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl) and Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl) were found in both ethyl acetate extract and chloroform extract. The mass spectra and chemical structures of biologically important compounds of chloroform extract are shown in figure 4.21.

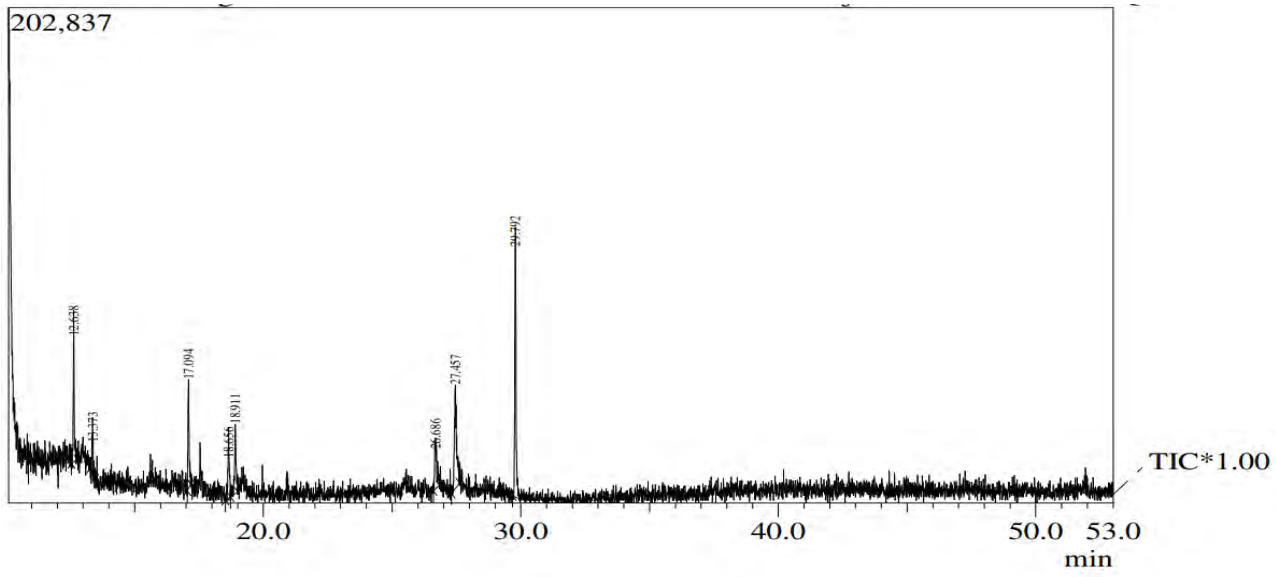

Figure 4.20 GC/MS chromatogram of chloroform extract of *Alcaligenes pakistanensis* LTP10

Table 4.15 Details of metabolites detected in chloroform extract of *Alcaligenes pakistanensis* LTP10

S#	Formula	Area (%)	RT (min)	Tentative compound	Activity reported	Reference
1	$C_{14}H_{22}O$	11.58	12.638	Phenol, 2,4-bis(1,1-dimethylethyl)	antibacterial	Abd Sharad *et al*., (2018)
2	$C_8H_{15}BrO_2$	3.87	13.373	Butanoic acid, 2-bromo-, butyl ester	not reported	NA
3	$C_{11}H_{22}$	10.34	17.094	2-Decene, 3-methyl-, (Z)	antimicrobial	Passari *et al*., 2017
4	$C_{11}H_{18}N_2O_2$	9.13	18.911	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	antioxidant	Rangel-Sánchez et., 2014
5	$C_{14}H_{16}N_2O_2$	9.15	26.686	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	antifungal	Kannabiran, 2016
6	$C_{33}H_{37}N_5O_5$	19.2	27.457	Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)	antimicrobial	Zaman *et al*., 2021
7	$C_{24}H_{38}O_4$	29.24	29.792	Di-n-octyl phthalate	antimicrobial	Passari *et al*., 2018

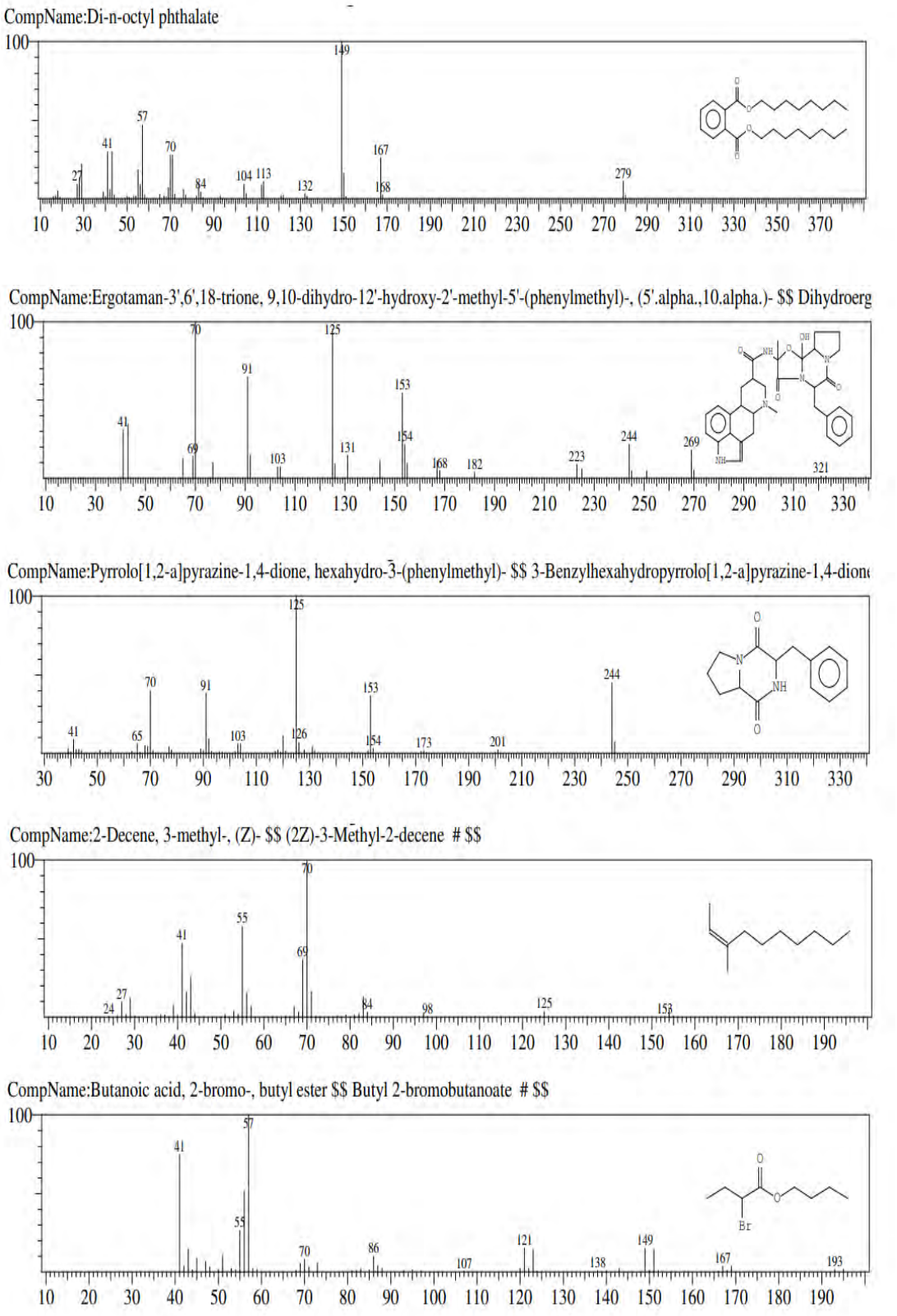

Figure 4.21 Mass spectra and chemical structures of important compounds identified in chloroform extract of *Alcaligenes pakistanensis* LTP10

4.11.3 Analysis of ethyl acetate extract by LC-MS

The ethyl acetate extract of LTP10 was further analysed by liquid chromatography – mass spectrometry. The analysis revealed several important bioactive metabolites which are shown by peaks in LC/MS chromatogram as shown in figure 4.22. Chemical formula, measured mass (m/z), reference mass, retention time and tentative compound names are given in table 4.16. LCMS analysis determined the identification of metabolites in ethyl acetate extract of LTP10 on the basis of similarity in the structure and molecular weight of metabolites with compounds in database. Similarly, the structure of compound has been predicted by comparison of distinguishing fragmentation patterns by means of a mass bank database. A total of twenty-five important compounds were identified in the ethyl acetate extract over 25 min elution time. Among these compounds, important compounds were 7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid 9 (compound19), L,L-Cyclo(leucylprolyl) (compound24), L-alpha-Amino-1H-pyrrole-1-hexanoic acid 9 (compound15), Noruron (compound1), Streptidine (compound2), Etomidate (compound18), N-(3-(Aminomethyl)benzyl)acetamidine (compound10), Nicotine-1'-N-oxide (compound14), N-Carboxyethyl-γ-aminobutyric acid (compound23), Dianhydroaurasperone C (compound5), [R-(Z)]- (compound17), and Asp-Phe methyl ester (compound21) most of which have already reported activities as given in table 4.17. The mass spectra of compounds with major peaks are given in figure 4.23.

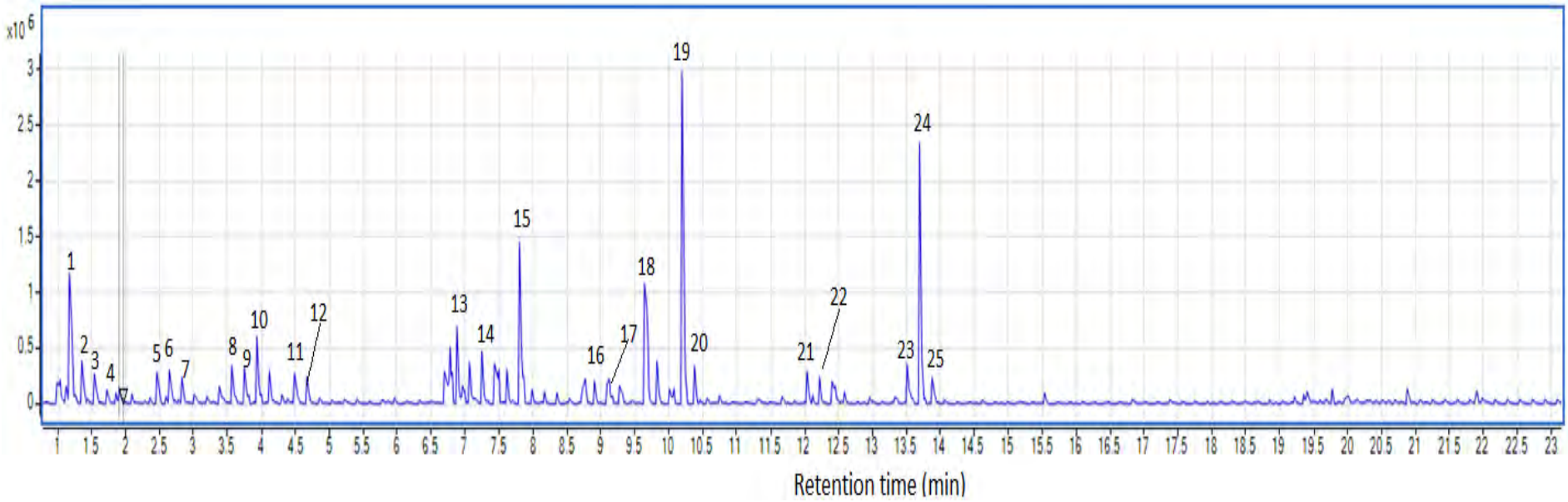

Figure 4.22 LC/MS chromatogram of ethyl acetate extract of *Alcaligenes pakistanensis* LTP10

Table 4.16 LC/MS analysis of compounds of ethyl acetate extract of *Alcaligenes pakistanensis* LTP10

Compound	Formula	Mass (m/z)*	Ref. mass*	RT (min)*	Tentative compounds
1	$C_{13}H_{22}N_2O$	222.1726	222.1732	1.17	Noruron
2	$C_8H_{18}N_6O_4$	262.1386	262.1390	1.35	Streptidine
3	$C_{13}H_{18}O_2$	206.1305	206.1307	1.54	2-Phenylethyl 3-methylbutanoate
4	$C_{27}H_{35}N_3O_6$	497.2535	497.2526	1.82	N1,N10-Diferuloylspermidine
5	$C_{31}H_{24}O_{10}$	556.1374	556.1369	2.46	Dianhydroaurasperone C
6	$C_{10}H_8N_2$	156.0681	156.0687	2.64	3-Indoleacetonitrile
7	$C_{14}H_{21}NO_4$	267.1466	267.1471	2.83	Metoprolol acid
8	$C_{10}H_{16}N_2O$	180.1257	180.1263	3.56	Rilmenidine
9	$C_{16}H_{29}N_3O_6$	359.2051	359.2056	3.75	Leucyl-isoleucyl-aspartic acid
10	$C_{10}H_{15}N_3$	177.1256	177.1266	3.93	N-(3-(Aminomethyl)benzyl)acetamidine
11	$C_8H_8O_3$	152.0474	152.0473	4.48	4-Hydroxyphenacyl alcohol
12	$C_{15}H_{27}N_3O_6$	345.1894	345.1900	4.67	Valyl-aspartyl-isoleucine
13	$C_{11}H_{12}N_2O_2$	204.0890	204.0899	6.88	L-Tryptophan
14	$C_{10}H_{14}N_2O$	178.1103	178.1106	7.25	Nicotine-1'-N-oxide
15	$C_{10}H_{16}N_2O_2$	196.1209	196.1212	7.80	L-alpha-Amino-1H-pyrrole-1-hexanoic acid
16	$C_{12}H_{24}N_2O_7$	308.1588	308.1584	8.93	1-[(5-Amino-5-carboxypentyl)amino]-1-deoxyfructose
17	$C_{12}H_{22}O_3$	214.1576	214.1569	9.18	5-Dodecenoic acid, 3-hydroxy-, [R-(Z)]-
18	$C_{14}H_{16}N_2O_2$	244.1207	244.1212	9.64	Etomidate
19	$C_{24}H_{34}O_4$	386.2439	386.2457	10.19	7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid
20	$C_5H_{11}NO_2$	117.0790	117.0790	10.38	Isoamyl nitrite
21	$C_{14}H_{18}N_2O_5$	294.1217	294.1216	12.04	Asp-Phe methyl ester
22	$C_{22}H_{26}O_6$	386.1731	386.1729	12.22	3,5-Di-O-methyl-8-prenylafzelechin-4beta-ol
23	$C_7H_{13}NO_4$	175.0838	175.0845	13.51	N-Carboxyethyl-γ-aminobutyric acid
24	$C_{11}H_{18}N_2O_2$	210.1360	210.1368	13.69	L,L-Cyclo(leucylprolyl)
25	$C_9H_{12}FN_3O_3$	229.0862	229.0863	13.88	Endalin

*RT = retention time in minutes; m/z = measured mass-to-charge ratio; Ref. mass = exact mass-to-charge ratio

Table 4.17 List of compounds from LC/MS analysis with reported bioactivities

Compound	**Activity**	**Reference**
L,L-Cyclo(leucylprolyl)	Antibacterial	Gowrishankar *et al*., 2015
	Antioxidant	Deepak *et al*., 2020
	Antifungal	Yan *et al*., 2004
Etomidate	Antibacterial	Sa *et al*., 2020
	Antifungal	Sa *et al*., 2019
L-Tryptophan	Antimicrobial	Feng *et al*., 2020
N-(3-(Aminomethyl)benzyl)acetamidine	Immunomodulator	Mertas *et al*., 2014
Streptidine	Antibacterial	Latorre *et al*., 2007
Noruron	Herbicidal	Gonzalez *et al*., 2003
N-Carboxyethyl-γ-aminobutyric acid (spermidic acid)	Antibacterial	Hwang-Bo *et al*., 2019
	Antioxidant	Hwang-Bo *et al*., 2019
Rilmenidine	Anticancer	Vucicevic *et al*., 2016
3-Indoleacetonitrile	Antiviral	Zhao *et al*., 2021
	Antibacterial	Kashyap *et al*., 2022
Asp-Phe methyl ester	Antibacterial	Yu *et al*., 2022
2-Phenylethyl 3-methylbutanoate	Antibacterial	Jirovtez *et al*., 2008
Endalin	Antiviral	Gosselin *et al*., 1994
Dianhydroaurasperone C	Antimicrobial	Magdy *et al*., 2017
N1,N10-Diferuloylspermidine	Antibacterial	Wang *et al*., 2020
5-Dodecenoic acid, 3-hydroxy-, [R-(Z)]-	Antimicrobial	Mun *et al*., 2019

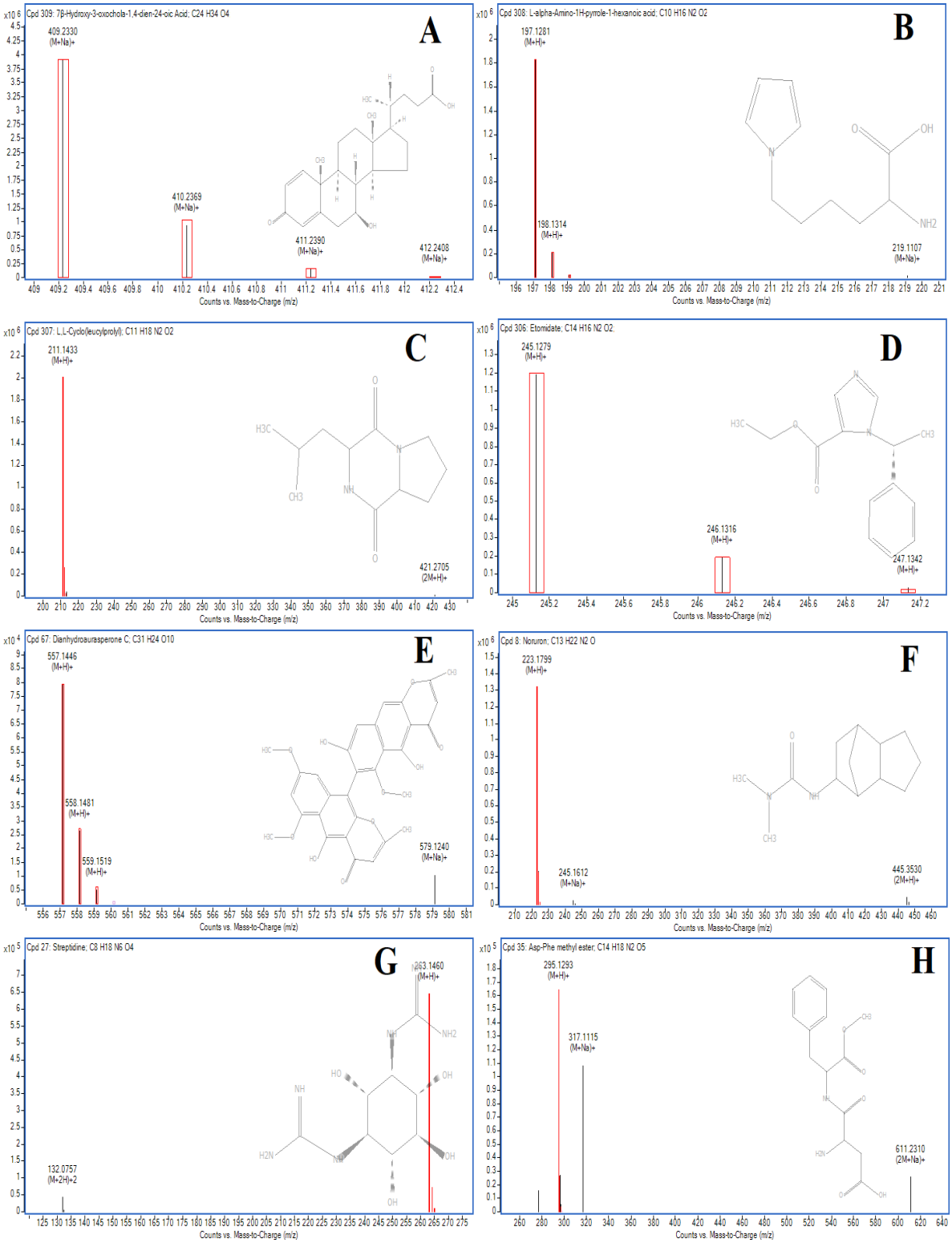

Figure 4.23 Mass spectra and chemical structures of important metabolites detected in ethyl acetate extract of *Alcaligenes pakistanensis* LTP10. A = 7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid, B = L,L-Cyclo(leucylprolyl), C = L-alpha-Amino-1H-pyrrole-1-hexanoic acid, D = Etomidate, E = Dianhydroaurasperone C, F = Noruron, G = Streptidine, H = Asp-Phe methyl ester.

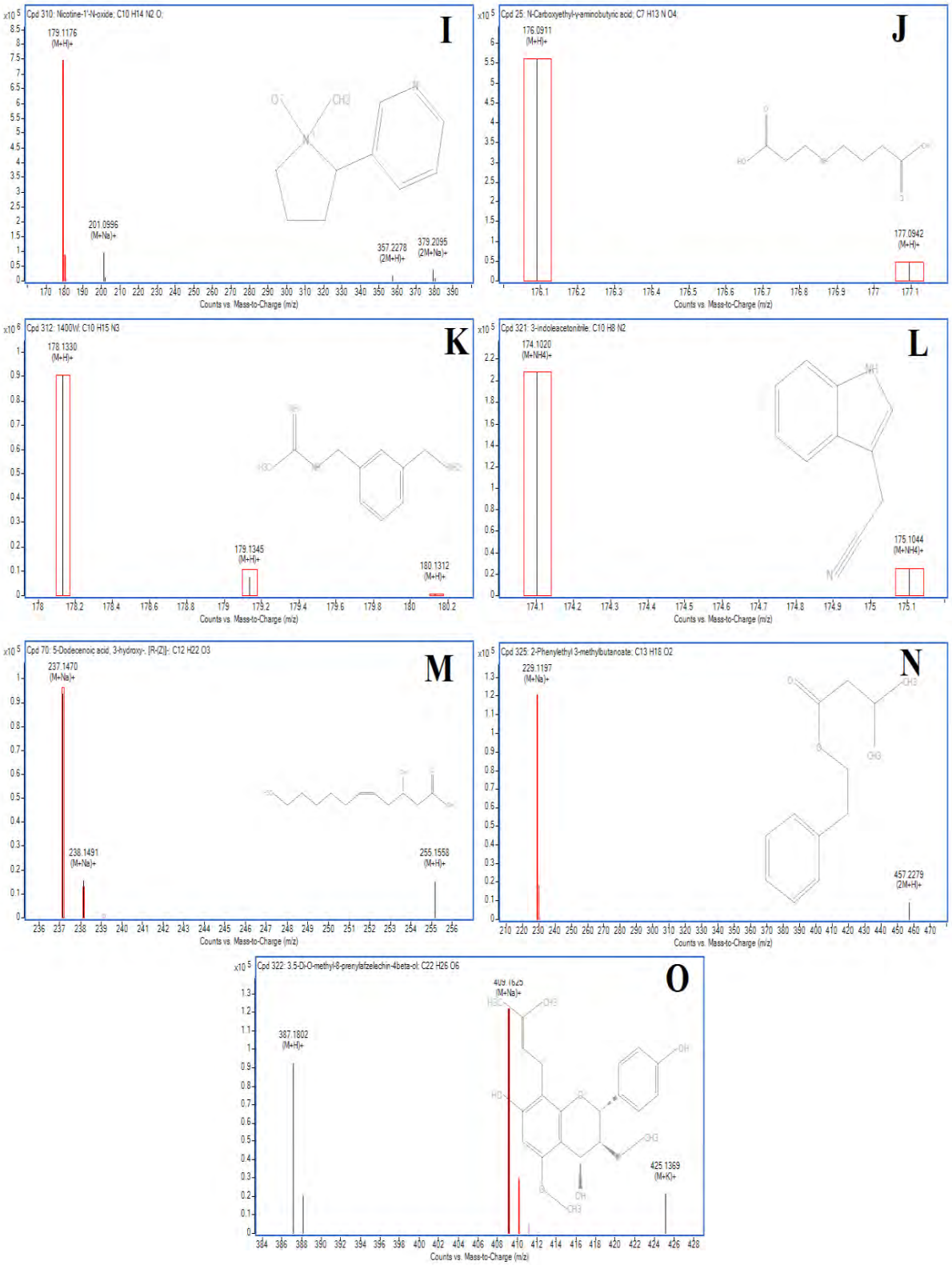

Figure 4.23 (continued) I = Nicotine-1'-N-oxide, J = N-Carboxyethyl-γ-aminobutyric acid, K = 1400W, L = 3-Indoleacetonitrile, M = 5-Dodecenoic acid, 3-hydroxy-, [R-(Z)]-, N = 2-Phenylethyl 3-methylbutanoate, O = 3,5-Di-O-methyl-8-prenylafzelechin-4beta-ol.

4.12 In-silico analysis of compounds

4.12.1 Molecular docking study of important compounds

The selected compounds were docked against target proteins by blind docking technique using CB-Dock and results were obtained in terms of vina score (kcal/mol) which indicated binding affinity of ligand with protein molecule. The more negative the value the greater will be the affinity of ligand and protein. Binding affinity of 15 ligand molecules with different target proteins are given in table 4.18. The results indicated that dianhydroaurasperone C, 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid, and L,L-Cyclo(leucylprolyl) has shown stronger affinity with their respective target proteins from microorganisms. Dianhydroaurasperone C has shown greatest affinity of -10.2 kcal/mol towards enzyme dihydrofolate reductase of *E. coli*. Similarly, the compound has shown binding affinity of -9.3, -9.7, and -9.0 kcal/mol against target proteins of DNA Gyrase, nitric oxide synthase, and peptidoglycan transpeptidase respectively. 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid has shown binding affinity of -9.3, -9.1 and -8.6 kcal/mol against Nitric oxide synthase, dihydrofolate reductase, and lipid A palmitoyltransferase respectively. The binding affinity of L,L-Cyclo(leucylprolyl) was found to be -7.5 and -6.9 kcal/mol towards D-alanine-D-alanine ligase and transglycosylase enzymes. The binding affinity of L-alpha-Amino-1H-pyrrole-1-hexanoic acid was found to be -6.2, -6.1 and -5.5 kcal/mol towards transglycosylase, D-alanine-D-alanine Ligase and DNA Gyrase enzymes respectively. All the target proteins or enzymes used are common target sites for most of the commonly used antibiotics. The results indicated great binding interactions of these ligand molecules with bacterial target enzymes. Overall, the binding affinity results of all the ligands tested ranged from -10.2 to -5.0 kcal/mol confirming their antimicrobial potential.

Table 4.18 Molecular docking study of compounds with target proteins showing their binding affinity by using CB-Dock

Compound / Ligand	Target microorganism	Target protein	PDB ID	Binding affinity (kcal/mol)
7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid	*Escherichia coli*	DNA Gyrase	1KZN	-8.4
	Staphylococcus aureus	Transglycosylase	2OLU	-8.5
	Escherichia coli	Lipid A palmitoyltransferase	1THQ	-8.6
	Escherichia coli	Dihydrofolate reductase	3DRC	-9.1
	Bacillus subtilis	Nitric oxide synthase	2AN0	-9.3
Dianhydroaurasperone C	*Escherichia coli*	Dihydrofolate reductase	3DRC	-10.2
	Staphylococcus aureus	DNA Gyrase	3U2D	-9.3
	Bacillus subtilis	Nitric oxide synthase	2AN0	-9.7
	Pseudomonas aeruginosa	Peptidoglycan transpeptidase	7JWL	-9.0
	Staphylococcus aureus	Transglycosylase	2OLU	-8.5
L,L-Cyclo(leucylprolyl)	*Escherichia coli*	D-alanine-D-alanine Ligase	2DLN	-7.0
	Staphylococcus epidermidis	Oxidoreductase	3GBH	-6.5
	Staphylococcus aureus	Transglycosylase	2OLU	-7.5
	Escherichia coli	Lipid A palmitoyltransferase	1THQ	-6.5
Etomidate	*Escherichia coli*	Lipid A palmitoyltransferase	1THQ	-7.2
	Staphylococcus aureus	Transglycosylase	2OLU	-7.5
	Bacillus subtilis	Nitric oxide synthase	2AN0	-8.1
	Escherichia coli	Dihydrofolate reductase	3DRC	-7.2
	Pseudomonas aeruginosa	Peptidoglycan transpeptidase	7JWL	-6.4
Noruron	*Escherichia coli*	Dihydrofolate reductase	3DRC	-6.8

Table 4.18 (continued)

Compound / Ligand	Target microorganism	Target protein	PDB ID	Binding affinity (kcal/mol)
Streptidine	*Escherichia coli*	Adenyl transferase	1GN8	-7.1
	Staphylococcus aureus	Transglycosylase	2OLU	-7.0
	Bacillus subtilis	Nitric oxide synthase	2AN0	-6.8
Asp-Phe methyl ester	*Escherichia coli*	Penicillin-Binding Protein 3	6HZQ	-6.8
	Bacillus subtilis	Nitric oxide synthase	2AN0	-7.9
L-alpha-Amino-1H-pyrrole-1-hexanoic acid	*Staphylococcus aureus*	Transglycosylase	2OLU	-6.2
	Escherichia coli	DNA Gyrase, isomerase	1KZN	-5.5
	Escherichia coli	D-alanine-D-alanine Ligase	2DLN	-6.1
N-(3-(Aminomethyl)benzyl)acetamidine	*Bacillus subtilis*	Nitric oxide synthase	2AN0	-7.4
N-Carboxyethyl-γ-aminobutyric acid	*Escherichia coli*	Penicillin-Binding Protein 3	6HZQ	-5.1
Nicotine-1'-N-oxide	*Escherichia coli*	Penicillin-Binding Protein 3	6HZQ	-5.2
	Staphylococcus aureus	Transglycosylase	2OLU	-7.2
2-Phenylethyl 3-methylbutanoate	*Escherichia coli*	DNA Gyrase	1KZN	-6.0
	Staphylococcus aureus	Transglycosylase	2OLU	-7.3
3,5-Di-O-methyl-8-prenylafzelechin-4beta-ol	*Escherichia coli*	Penicillin-Binding Protein 3	6HZQ	-7.2
	Staphylococcus aureus	Transglycosylase	2OLU	-8.2
	Pseudomonas aeruginosa	Peptidoglycan transpeptidase	7JWL	-7.9
3-Indoleacetonitrile	*Staphylococcus aureus*	DNA Gyrase	3U2D	-6.4
5-Dodecenoic acid, 3-hydroxy-, [R-(Z)]-	*Escherichia coli*	Penicillin-Binding Protein 3	6HZQ	-5.0
	Staphylococcus aureus	Transglycosylase	2OLU	-6.7
	Pseudomonas aeruginosa	Peptidoglycan transpeptidase	7JWL	-5.5

On the basis of above results, four compounds 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid, dianhydroaurasperone C, L-alpha-Amino-1H-pyrrole-1-hexanoic acid and L,L-Cyclo(leucylprolyl) with high affinity against target proteins were selected for further evaluation. Molecular docking technique was used to determine their range of binding affinity energy at different sites of proteins, ligand interacting amino acid residues, number of hydrogen bonds, hydrophobic bonds, and other bonds as shown in table 4.19 CB-Dock was used to determine the top five binding sites of protein with ligand based on binding affinity and best binding pose of ligand-protein interaction. The binding affinity of 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid (compound19) against transglycosylase (penicillin binding protein) of *Staphylococcus aureus* was in the range -8.5 to -6.4 kcal/mol over 5 different binding sites, indicating its strong interaction with the target protein. The same is evident from 2 hydrogen bonds, 2 pi-alkyl bonds and 1 van der Waal interaction at the top binding site with lowest energy values. The hydrogen bond contact residues were found to be LYS583 and THR584 (figure 4.24). The binding affinity of dianhydroaurasperone C (compound5) against dihydrofolate reductase of *Escherichia coli* was found to be in the range -10.2 to -6.5 kcal/mol over 5 different binding sites, indicative of its strong contact with the target protein. The same is evident from 2 hydrogen bonds, 3 pi-alkyl bonds and 3 van der Waal interactions at the best binding site of dihydrofolate reductase with lowest energy values. The hydrogen bond contact residues were noted to be TRP22 and ILE94 (figure 4.25). Compound15 (L-alpha-Amino-1H-pyrrole-1-hexanoic acid) has shown good interaction with D-alanine-D-alanine ligase of *Escherichia coli* with binding affinity range of -6.1 kcal/mol. The 5 hydrogen bonds contacted with residues SER150, SER274, GLY276, TYR216, and ASN272 (figure 4.26). Compound24 (L,L-Cyclo(leucylprolyl)) has shown good interaction with D-alanine-D-alanine ligase of *Escherichia coli* with binding affinity range of -7.0 to -4.6 kcal/mol. The hydrogen bond contact residues were noted to be LYS144 and TYR210 (figure 4.27). There were 2 hydrogen bonds, 4 pi-alkyl bonds and 1 van der Waal bond between compound24 and D-alanine-D-alanine ligase at the best binding site of interaction. Antibiotics meropenem and trimethoprim were used as control to determine their interaction against target enzymes transglycosylase (penicillin binding protein) and dihydrofolate reductase respectively. The results of their docking study were found to be similar to compounds tested against same target proteins as shown in table 26.

Table 4.19 Binding affinity range, interaction bonds and hydrogen contact amino acid residues of target proteins with antimicrobial compounds

Compound	Target protein	*Binding affinity range (kcal/mol)	**Hydrogen contacts residues	**H-bonds	**Pi-Alkyl bonds	**van der Waal bonds
7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid	Transglycosylase (PBP2)	-8.6 to -6.4	LYS583, THR584	2	2	1
	Nitric oxide synthase	-9.3 to -5.7	ASN60, ARG61	2	8	1
	PBP activator	-6.6 to -5.0	LYS146	1	2	0
Dianhydroaurasperone C	Dihydrofolate reductase	-10.2 to - 6.5	TRP22, ILE94	2	3	3
	Peptidoglycan transpeptidase (PBP)	-9.0 to -6.9	ARG152, ARG273 (2)	3	6	3
L-alpha-Amino-1H-pyrrole-1-hexanoic acid	D-alanine-D-alanine Ligase	-6.1 to -4.1	SER150, SER274, GLY276, TYR216, ASN272	5	2	1
L,L-Cyclo(leucylprolyl)	D-alanine-D-alanine Ligase	-7.0 to -4.6	LYS144, TYR210	2	4	1
	Transglycosylase (PBP2)	-7.7 to -4.2	--	0	1	1
Meropenem (control)	Transglycosylase (PBP2)	-8.8 to -5.5	ASN456 (2), LYS583, GLN641, THR586, GLN645	6	4	0
Trimethoprim (control)	Dihydrofolate reductase	-6.8 to -4.6	ILE5, ALA7, TYR100 (2), MET20	4	4	2
Meropenem (control)	D-alanine-D-alanine Ligase	-6.4 to -5.3	--	0	1	1

* = Binding affinity range is the affinity of ligand molecule with target protein at 5 different binding sites, obtained from CB-Dock
** = Results are based on interaction of protein best binding site with ligand

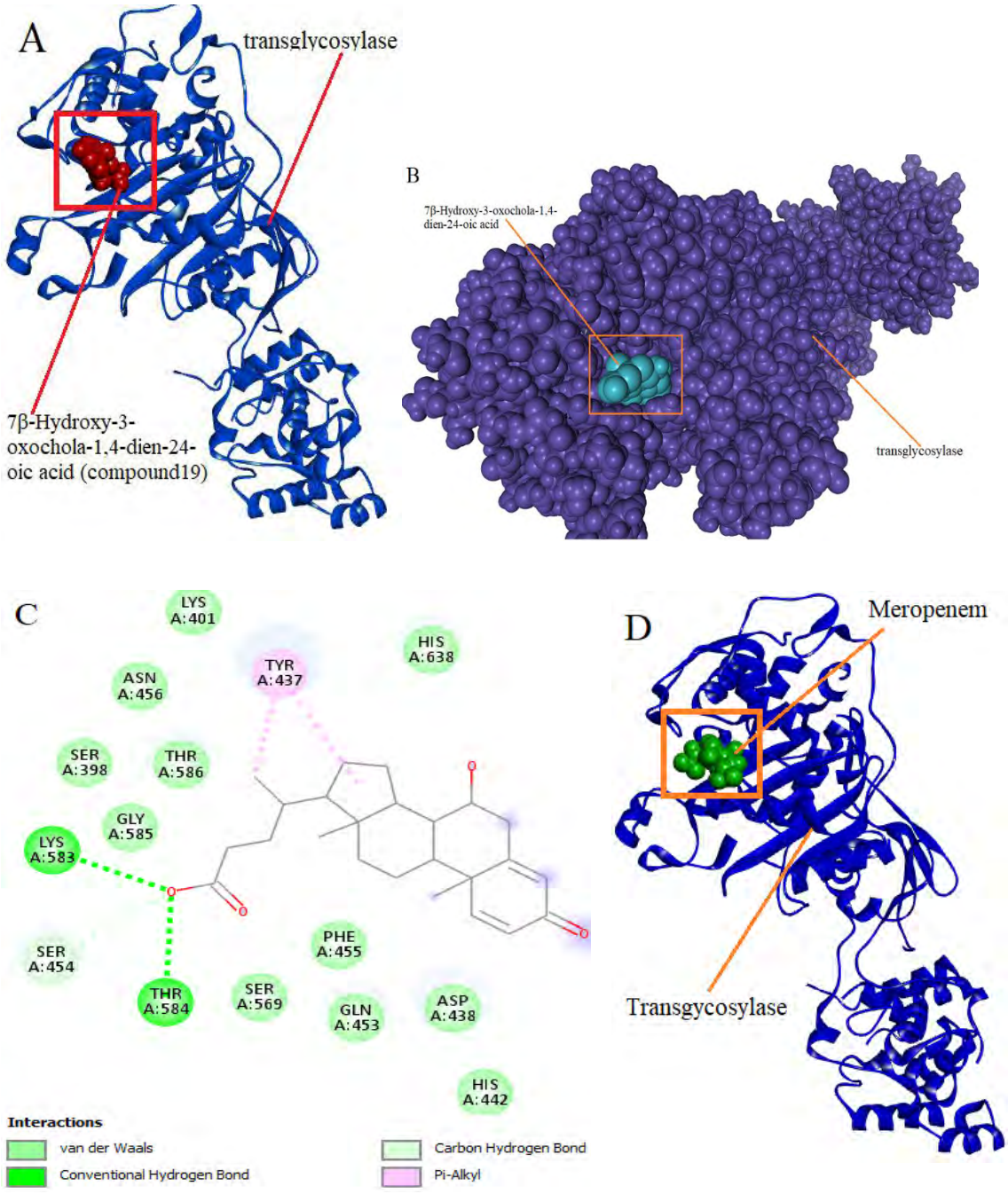

Figure 4.24 Docking results of transglycosylase (penicillin binding protein) of *Staphylococcus aureus* with 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid (compound19). Three-dimensional visualization of interaction (A & B), Amino acid contact residues and types of interactions (C). Interaction of meropenem with transglycosylase at the same binding site as 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid (D).

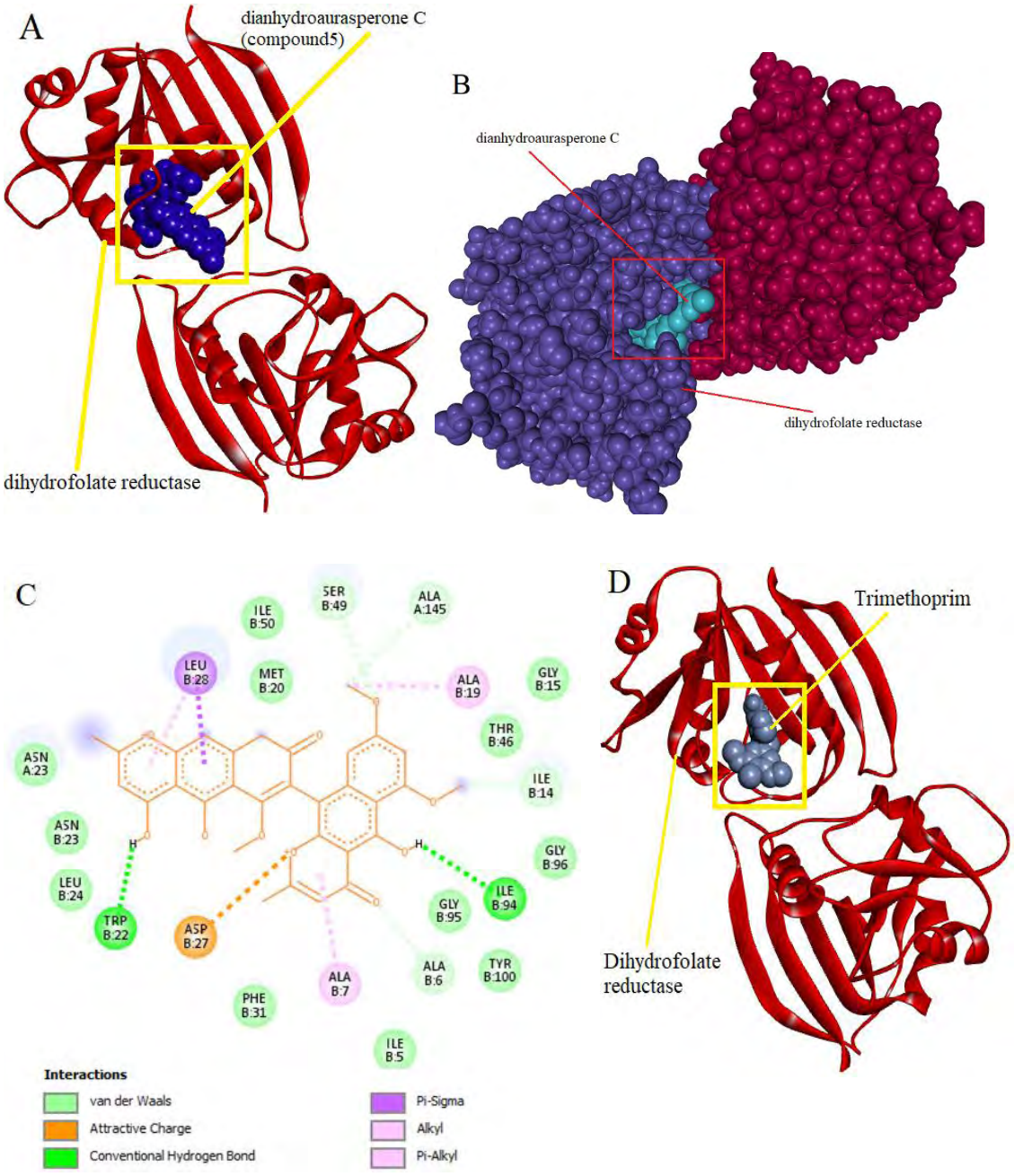

Figure 4.25 Docking results of dihydrofolate reductase of *Escherichia coli* with dianhydroaurasperone C (compound5). Three-dimensional visualization of interaction (A & B), Amino acid contact residues and types of interactions (C). Interaction of trimethoprim with dihydrofolate reductase at the same binding site as dianhydroaurasperone C (D).

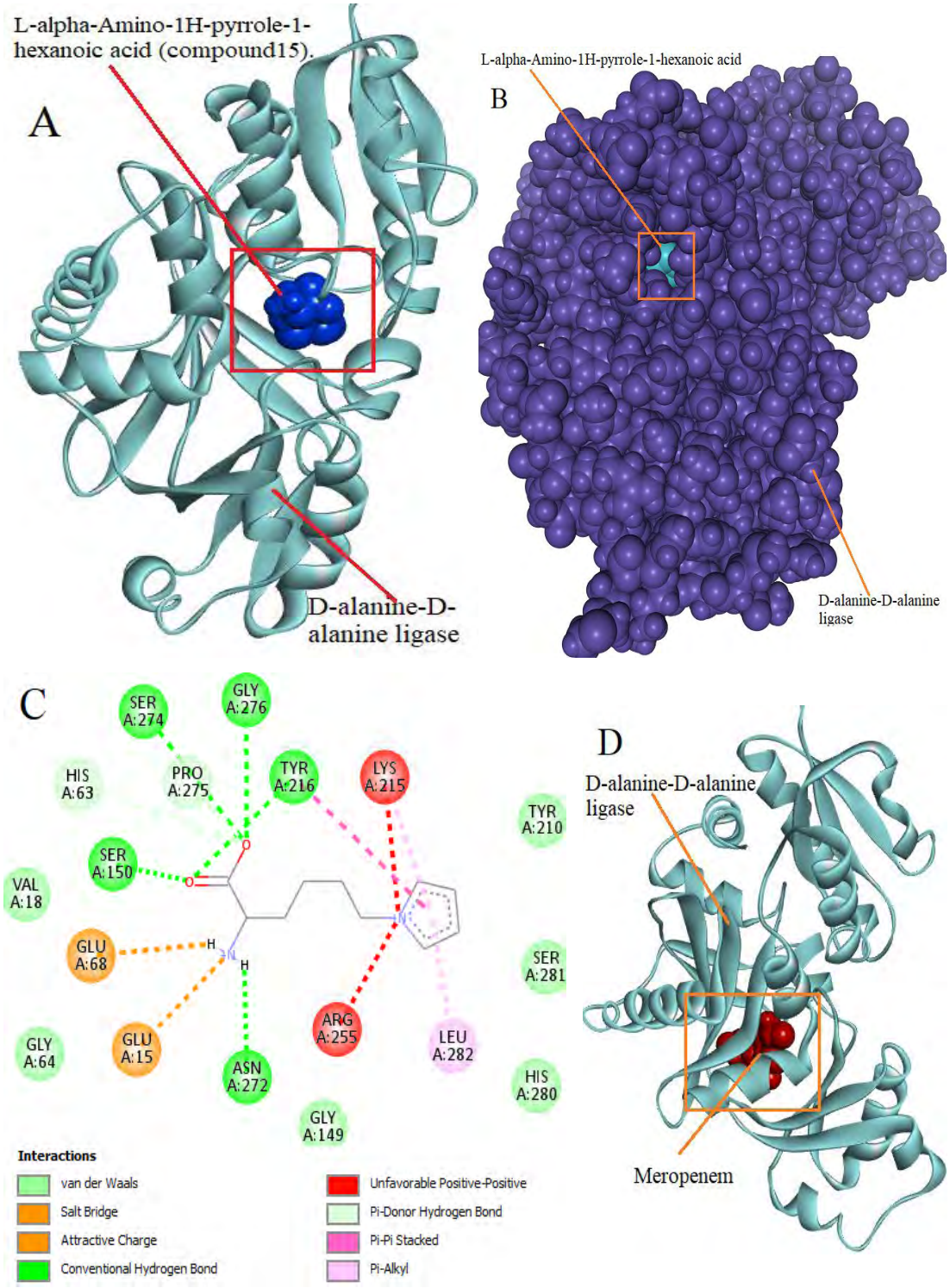

Figure 4.26 Docking results of D-alanine-D-alanine ligase of *Escherichia coli* with L-alpha-Amino-1H-pyrrole-1-hexanoic acid (compound15). Three-dimensional visualization of interaction (A & B), Amino acid contact residues and types of interactions (C). Interaction of mreopenem with D-alanine-D-alanine ligase in the same binding cavity as L-alpha-Amino-1H-pyrrole-1-hexanoic acid (D).

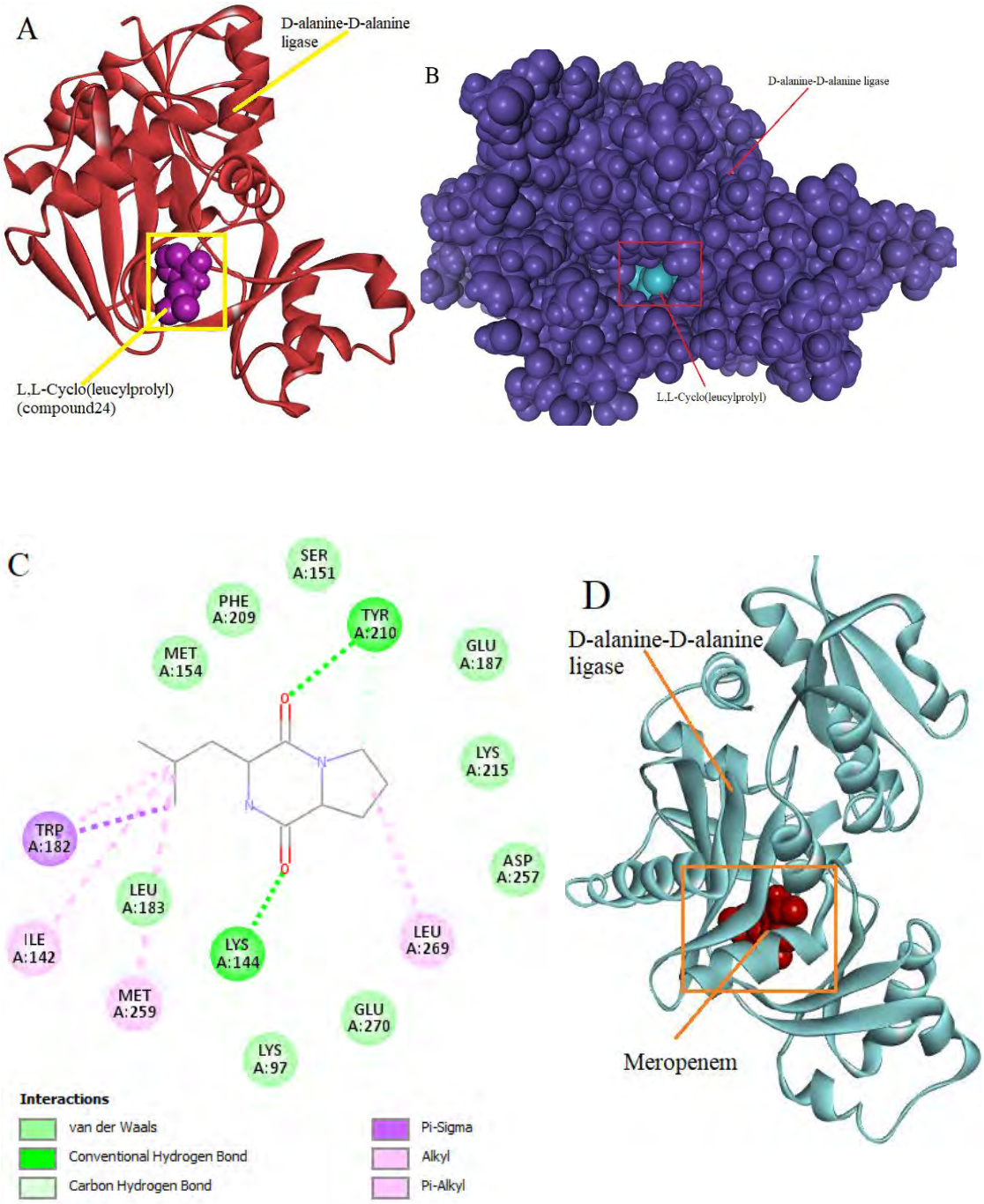

Figure 4.27 Docking results of D-alanine-D-alanine ligase of *Escherichia coli* with L,L-Cyclo(leucylprolyl) (compound24). Three-dimensional visualization of interaction (A & B), Amino acid contact residues and types of interactions (C). Interaction of mreopenem with D-alanine-D-alanine ligase in the same binding cavity as L-alpha-Amino-1H-pyrrole-1-hexanoic acid (D).

4.12.2 In-silico analysis of compounds ADMET properties and drug-likeness

A computational study of the best compounds was performed for the assessment of ADMET properties using online webserver pkCSM and is given in table 26. For comparison, ADMET properties were also determined for well-known antibiotics meropenem and ciprofloxacin. Human intestinal absorption (HIA) of all compounds analyzed were more than 30% indicating its easy absorption in human GIT. The required limit for skin permeability was also below the minimum limit of -2.5 logKp for all the 4 compounds. Blood brain barrier permeability (BBBP) for all compounds was less than minimum limit of 0.3 logBB required for a substance to be BBB permeable, indicating incapability of compounds to cross BBB. The results show that CYP450 was inhibited only by compound dianhydroaurasperone C. AMES toxicity result has shown non-mutagenic nature of the compounds. Based on minnowtoxicity test a compound will have acute toxicity if the result of test was less than -0.3 logmM. The results of minnowtoxicity prediction for all compounds were above -0.3 logmM confirming non-toxic nature of the compounds. Hepatotoxicity prediction was negative only for dianhydroaurasperone Ca and L,L-Cyclo (leucylprolyl). The predicted results of all four compounds were comparable to commonly used antibiotics meropenem and ciprofloxacin as shown in table 4.20.

Table 4.20 Pharmacokinetic and toxicity properties prediction of best compounds

Compound	HIA* (%)	SP* (log Kp)	BBBP* (logBB)	C450I*	TC* (logmL/ min/kg)	AMEST*	MT* (log mM)	HT*
7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid	97.87	-2.732	0.208	No	0.551	No	0.904	Yes
L-alpha-Amino-1H-pyrrole-1-hexanoic acid	72.40	-2.736	-0.099	No	0.803	No	1.889	Yes
Dianhydroaurasperone C	100.00	-2.735	-1.871	Yes	0.641	No	1.582	No
L,L-Cyclo (leucylprolyl)	85.36	-2.635	-0.152	No	1.21	No	2.268	No
Meropenem	34.83	-2.735	-0.754	No	0.365	No	3.695	Yes
Ciprofloxacin	96.47	-2.734	-0.587	No	0.633	No	1.194	Yes

* = HIA, Human intestinal absorption; SP, Skin permeability; BBBP, Blood Brain Barrier permeability, C450I, CYP450 inhibitor; TC, Total clearance; AMEST, AMES toxicity; MT, Minnowtoxicity; HT, Hepatotoxicity

All the four compounds were also analyzed by Lipinski's rule of five (RO5) using swissADME prediction tool. The Lipinski rule states that for a compound to qualify drug-likeness criteria, it should have molecular weight less than 500 g/mol, lipophilicity less than 5 MlogP, number of hydrogen bond donors less than 5, and hydrogen bond acceptors less than 10. All the values are in multiple of 5, and hence the name "rule of 5". The predictions of Lipinski's rule of five for all four compounds are given in table 4.21. There were no violations to any rule by all compounds, except dianhydroaurasperone C which violated the rule of molecular weight less than 500 g/mol, however qualifying the rule of 5. The analysis has shown that all the compounds follow the druglikness properties according to Lipinski rule of five.

Table 4.21 Lipinski rule of five of best compounds

Compound	MW* (g/mol)	LP* (MLog P)	NH or OH*	N or O*	Violations	Follow Lipinski's rule
7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid	386.24	3.58	2	4	0	Yes
L-alpha-Amino-1H-pyrrole-1-hexanoic acid	196.12	-2.01	2	3	0	Yes
Dianhydroaurasperone C	556.14	0.50	3	10	1	Yes
L,L-Cyclo(leucylprolyl)	210.14	0.64	1	2	0	Yes

* = MW, Molecular weight; LP, Lipophilicity; NH or OH; Hydrogen bond donors; N or O, Hydrogen bond acceptors

SwissADME tool was also used for graphical representation of the absorption and distribution parameters by the improved version of the Edan–Egg model named BrainOrIntestinaLEstimateD (BOILED) permeation predictive model (BOILED-Egg). BOILED-Egg graphical expression for the compounds 7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid (compound19), L-alpha-Amino-1H-pyrrole-1-hexanoic acid (compound15), dianhydroaurasperone C (compound5) and L,L-Cyclo(leucylprolyl) (compound24) is shown in figure 4.28. The diagram has shown that all four molecules were unable to cross blood brain barrier (BBB) and could be easily absorbed in human GIT except compound5.

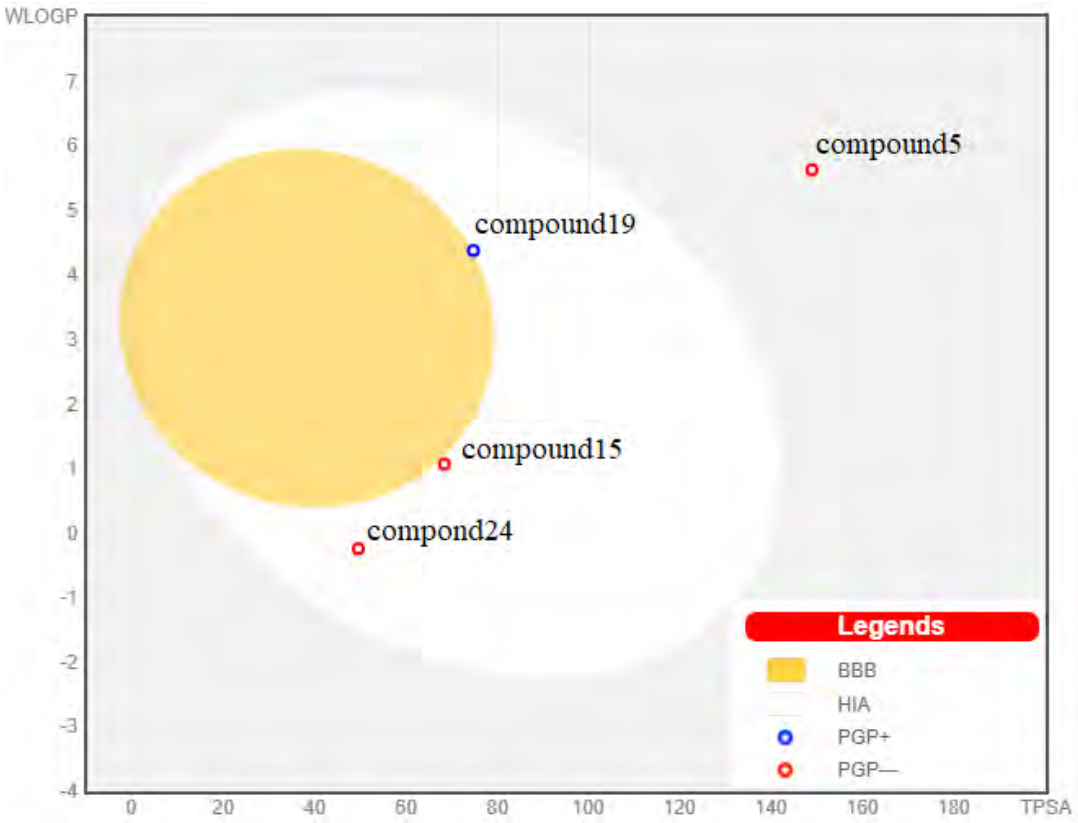

Figure 4.28 BOILED-Egg graph of compounds. BOILED- Egg's white and yellow yolks stand for HIA and BBB, respectively. Red dots represent chemicals that were anticipated not to be removed from the central nervous system (CNS) by the P-glycoprotein, whereas blue dots represent molecules for which it was projected that the P-glycoprotein would do so.

DISCUSSION

Secondary metabolites produced by microorganisms have been identified as the main source of novel chemicals for therapeutic discovery and development (Pan *et al*., 2019). The important bioactive chemicals found in these metabolites can be employed to treat microbial illnesses (Dantas *et al*., 2008). Antibiotic resistance in bacteria is a rising worldwide concern that requires rapid attention due to its multifaceted spread. Antibiotic resistance in bacteria occurs by target alteration, the synthesis of enzymes that break down antibiotics, changes in porins, and the overexpression of efflux pumps, etc (Kongkham *et al*., 2020). However, the rise of multidrug resistance bacteria poses the biggest threat to clinical settings and hospitals (Dantas *et al*., 2008). Additional motivation to investigate this topic is provided by the development of antimicrobial substances from other sources in response to antibiotic resistance and drug resistance (Tendulkar *et al*., 2021). Researchers and scientists are increasingly motivated to find novel antimicrobial compounds from uncharted territory in order to address the issue of medication resistance up to a certain point (Selvameenal *et al*., 2009). Most cryptic gene clusters, which are dormant under normal conditions, are activated to create microbial secondary metabolites (Singh *et al*., 2019). In this context microorganisms inhabiting low temperature environments are suitable for producing important bioactive metabolites. Psychrophilic bacteria are recognized as a potential source of new antibiotic metabolites, according to Sanchez *et al*., (2009). These microorganisms need a wide range of biochemical and physiological modifications in order to live under the continual effect of low temperatures, strong winds, limited nutrients, and intense UV radiation, or combinations of these characteristics (Santiago *et al*., 2015). The likelihood of discovering distinct functional metabolites with pharmacological significance is increased by the frequent occurrence of alterations to both gene regulation and metabolic pathways in conjunction with these adaptations (Tian *et al*., 2017). This study focuses on bacterial isolates from low temperature environment to analyze and optimize their antimicrobial activity and to study the nature of antimicrobial metabolites produced by these microorganisms.

Agar well diffusion test was used in the current study to confirm the production of antimicrobial metabolites by the bacterial isolates from Passu glaciers (Wefky *et al*., 2009; Abd-Elnaby *et al*., 2016). To identify the bacteria that produced antimicrobial

compounds in that uncharted region, isolates from the Passu glacier were chosen. At such a low temperature, the psychrophiles and psychrotrophs are subjected to harsh conditions like nutrient shortage, oxidative stress, increase concentration of salts, struggle, and stressed environments, which enliven the genes accountable for the synthesis of antimicrobial compounds. A new antibiotic called Frigocyclinone was discovered by Bruntner *et al*., in 2005 and generated by an Antarctica-isolated strain of *Streptomyces griseus*. Similarly, several antibacterial compounds produced by psychrophiles and psychrotrophs have been reported in different studies like Cyclo-(L-Trp-L-Phe)-cyclic dipeptide produced by *Aspergillus sydowii* (Li *et al*., 2018), Rhamnolipid by *Pseudomonas* sp. (Tedesco *et al*., 2016) and Flexirubin produced by *Flavobacterium* sp. (Mojib *et al*., 2010). The producing organisms are able to compete and live in such an environment because to their antimicrobial metabolites (Oyedele and Ogunbanwo, 2014).

The antimicrobial activity of CFS of bacterial isolates (HTP12, HTP13, HTP36, LTP10) against ATCC strains *Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Salmonella enterica,* and *E. coli* was measured by agar well diffusion method. Similarly, Clements *et al*., (2019) also determined activity of the crude extract of *Serratia marcescens* (1.00 mg/mL) against ATCC bacterial and fungal strains. Agar well diffusion assay is very well-known method for determining antimicrobial activity of microorganisms, plants, and their compounds. The result was measured in terms of zone of inhibition in mm. According to Potbhare *et al*., (2020), Using the agar well diffusion method, the fungus-mediated production of silver nanoparticles was tested for its antibacterial effectiveness against the human pathogenic bacteria *Escherichia coli, Bacillus subtilis, Staphylococcus aureus,* and *P. aeruginosa* and results were shown in mm indicating zone of inhibition. By using agar well diffusion, Gummuluri *et al*., (2019) reported the efficacy of *Morinda citrifolia's* ethanolic extract against *Enterococcus feacalis*. The results were assessed in terms of the zone of inhibition in millimetres. Fandi *et al*., (2014) demonstrated antibacterial activity of several thermophiles isolated from Jordan hot springs against pathogenic strains of *S. aureus, B. subtilis, E. coli*, and fungi by measuring zone of inhibition in mm.

The isolates HTP12 and LTP10 have been found to have good antimicrobial potential as evident from their clear zone of inhibition against *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella enterica* while HTP13 and HTP36 were active against all test strains mentioned except *Pseudomonas aeruginosa* and *Salmonella enterica* respectively. Best activity and larger ZOIs were seen in *Alcaligenes pakistanensis* LTP10. Various studies on the antibacterial activity of *Alcaligenes* species have been published. Several clinical isolates and ATCC strains of *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were reported to be susceptible to *Alcaligenes faecalis* HTP6 (*S. aureus*, *E. faecalis*, *Candida albicans* and *Aspergillus fumigatus*) (Rafiq *et al*., 2016). Khim *et al*., (2019) reported antagonistic activity of *Alcaligenes faecalis* isolated from crocker range of Sabah against gram negative plant pathogen *Erwinia psidii.* Literature confirmed that *A. faecalis* has shown activity against fungal pathogens. According to one investigation, *A. faecalis* inhibited the development of 13 different plant pathogens on either complicated L-agar medium or synthetic MM-agar media (Honda *et al*., 1999). *Serratia* sp. and *Enterobacter* sp., which are multidrug resistant to sulfamethoxazole, ampicillin, azithromycin, and tetracycline, were both susceptible to the antibacterial activity of *Alcaligenes* sp. HPC 1271. (Kapley *et al*., 2016). These studies suggest the great potential of *Alcaligenes* sp. for antimicrobail potential. Although the bacterium genus *Alcaligenes* is not often linked to antibiotics, this work emphasises the value of searching the microbial diversity for novel antimicrobial drugs.

Out of the eleven antimicrobial compounds producing isolates from the Passu glacier, four bacterial isolates were chosen for this investigation. By morphological characterization and 16SrRNA sequencing, the isolates with the codes HTP12, HTP13, HTP36, and LTP10 were determined to be *Alcaligenes faecalis*, *Pseudochrobactrum saccharolyticum*, *Alcaligenes pakistanensis*, and *Alcaligenes pakistanensis*, respectively. According to several research, 16S rRNA sequencing and its phylogenetic analysis are used to identify microbial isolates. In stressed cells, intracellular RNA degrades more quickly and is more unpredictable outside the cell than DNA. RNA-based techniques have therefore been proposed to investigate the active microbial fraction in environmental matrices (Revetta *et al*., 2010). The 16S rRNA gene, a molecular marker for recognition of microbial species, is a useful tool for identification

of bacterial isolates. Several clinically relevant bacterial isolates have been identified by use of 16S rRNA gene (Srinivasan *et al*., 2015). Revetta *et al*., (2010) reported use of 16S rRNA-based sequence analyses for identification of microorganism in water. Another study reported the identification of two isolates as *Kocuria* sp. and *Streptomyces intermidus* respectively using 16S rRNA gene sequencing (Passari et., 2018).

To ascertain the ideal incubation period, temperature, and pH for their development, the isolated strains were incubated at various pH values, incubation times, and culture conditions. The chosen strains were discovered to develop at temperatures as low as 5°C and as high as 35°C, with maximal growth occurring at 15° to 25°C in Luria-Bertani broth. In contrast to three isolates *Alcaligenes faecalis* (HTP12), *Pseudochrobactrum saccharolyticum* (HTP13), and *Alcaligenes pakistanensis* (HTP36), the optimal temperature for growth of *Alcaligenes pakistanensis* (LTP10) was determined to be 15°C. These bacteria were classified as facultative psychrophiles, which are cold-adapted organisms with an ideal growth temperature above 15 °C (Yuan *et al*., 2017). Facultative psychrophiles or psychrotrophs or psychrotolerants are defined as those microorganisms having optimum growth temperature of 20-25°C and maximum growth temperature of 25-40°C (Morita, 1975; Scherer and Neuhaus, 2006; Moyer *et al*., 2016; Hilgarth *et al*., 2018). Psychrotrophs or psychrotolerants are present in the same icy ecosystems as psychrophiles but in larger quantities (Moyer *et al*., 2016). After three days of incubation, psychrophilic bacteria showed optimal growth at a temperature of around 15°C, according to Margesin *et al*., (1994). After a 72-hour incubation period, the isolates displayed their maximal development at neutral pH 7. It has been evident from various studies that optimal pH for growth of psychrophiles/psychrotrophs was found to be 7 (neutral). Shcherbakova *et al*., (2005) has shown that novel psychrophilic anaerobic spore-forming bacterium grew at the pH range from 4.5 to 8.5 with optimal pH of 6.5–7.2. The pH range for growth was discovered to be between 6.5 and 7.9 by Franzmann *et al*., (1997) after they isolated the psychrophile Methanogenium frigidum sp. nov. from the permanently freezing Ace Lake in the Vesfold Hills of Antarctica. Psychrophiles exhibited their greatest development in environments with a neutral pH and temperatures about 15°C, according to Heather and Vanderzant, (1957).

Secondary metabolite generation by bacteria is influenced by a variety of physical parameters, including nutrition, temperature, pH, moisture, and light (Tyc *et al*., 2017). The influence of many factors, including pH, temperature, incubation time, and culture medium, was also taken into consideration in the current study effort for the creation of active metabolites. After 96 hours of incubation, the greatest antimicrobial metabolite synthesis by all four isolates was detected at 25°C and pH 7. The generation of antimicrobials decreased with rising temperatures and shifting pH levels. It was established by Awais *et al*., (2007) and Gebreel *et al*., (2008) that *Bacillus* sp. generated bacitracin at a pH 7 and temperature range of 25°C to 30°C. According to Oyedele and Ogunbanwo, (2014), *Bacillus subtilis* had its most hostile behavior after 96 hours of incubation. The influence of temperature on the production of cyanobacterial secondary metabolites was studied by incubating samples at different temperatures (15 °C, 25 °C, 35 °C) and pH 6.0 to 9.0 and it was observed that compounds were produced in higher concentrations at the temperature of 35 °C and pH 7.5 (Lalić *et al*., 2020). The results from these studies clearly indicates the importance of temperature and pH effect on the production of bioactive compounds from microorganisms.

The medium formulation is a crucial element in the design of effective laboratory studies. The ingredients needed for development and the synthesis of secondary metabolites must be present in the components of the culture medium (Abd-Elnaby *et al*., 2016). Carbon and nitrogen supply typically make up the majority of the culture media. The carbon source supplies the building blocks for biomass as well as the carbon units for secondary metabolites (Singh *et al*., 2017; Pan *et al*., 2019). The isolated bacteria were cultured for 96 hours at 25°C and pH 7 after being inoculated in LB broth with additional carbon sources like glucose (1% and 2%) and starch (1% and 2%), respectively. When *Staphylococcus aureus* and *Escherichia coli* were exposed to the cell free supernatant from *Alcaligenes pakistanensis* (LTP10) culture broth with 1-2% glucose and starch, the zone of inhibition was reduced by 2–5 mm. These results are according to the report by Shoda, (2020) who stated that *Alcaligenes faecalis* does not use carbs and instead uses acetate, propionate, butyrate, and other organic acids as its only source of carbon and energy. Similarly, glucose, which is often a great supply of carbon for development, prevents bacteria and other microbes from producing numerous antibiotics. To explain the detrimental effects of carbon catabolite on the

formation of secondary metabolites, many mechanisms have been proposed in bacteria (Sánchez *et al*., 2010).

Additionally, the impact of nitrogen sources on isolates' ability to produce antibiotics was investigated. The creation of vital proteins, nucleic acids, and N-containing components for secondary metabolites all depend on the availability of nitrogen. It is well known that the kind of carbon and nitrogen sources employed has a big impact on microbial secondary metabolism (Singh *et al*., 2017; Pan *et al*., 2019). Yeast extract, L-leucine, tryptophan, threonine, arginine, and as nitrogen supplies were added individually to Luria-Bertani broth that had been infected with isolates. The mixture was then cultured for 96 hours at 25°C and pH 7 before being added to the nitrogen sources. *Alcaligenes pakistanensis* (LTP10) boosted its activity against *Escherichia coli* by 3 mm when cultured in Luria-Bertani broth with 1% yeast extract, whereas *Alcaligenes pakistanensis* HTP36 raised its activity against *Staphylococcus aureus* by 2 mm. All four isolates—HTP12, HTP13, HTP36, and LTP10—have decreased antibacterial activity with the addition of tryptophan, L-leucine, threonine, and arginine. The effects of various nitrogen and carbon sources on *Thermococcus* sp. growth and the synthesis of antimicrobial metabolites were examined by Rinker and Kelly in 2000. Narayana and Vijayalakshmi, (2008) reported that production of antimicrobial metabolites by *Streptomyces albidoflavus* was enhanced when culture medium was supplemented separately with soyabean meal and yeast extract. Increasing concentration of inorganic salts (NaCl and KCl) up to 2-3% had negative effect of antimicrobial production of all four isolates used in the study. It was noted by Gesheva *et al*., (2005) that inorganic salts in the medium inhibited synthesis of the AK-111-81 macrolide antibiotic by *Streptomyces hygroscopicus*.

The isolates employed in this investigation, HTP12, HTP13, HTP36, and LTP10, have demonstrated resistance to a variety of antibiotic classes. These isolates were tested against 28 antibiotics discs. LTP10 was resistant to 17 antibiotics, HTP12 was resistant to 18 antibiotics and HTP13 was resistant to 12 antibiotics while HTP36 was resistant to only 6 antibiotics. The resistance to greater number of antibiotics by LTP10 and HTP12 make them candidates for good antibiotic producers. The bacteria that produce antibiotics employ a variety of intricate processes to defend themselves from their own antibiotics. Antibiotic target alteration, antibiotic variation, efflux of antibiotics,

bypassing antibiotic targets, protecting antibiotic target, and antibiotic confiscation by unique proteins are the main strategies of self-defense in bacteria that produce antibiotics (Peterson and Kaur, 2018). Passari *et al*., (2018) evaluated antibiotic producing strains *Kocuria* sp. and *Streptomyces intermidus* for their antibiotic resistance pattern using 21 standard antibiotic discs. *Kocuria* sp. showed resistance activity against 10 out of 21 antibiotics while *Streptomyces intermidus* showed resistance for 15 antibiotics and found susceptible for 5 antibiotics. These results indicated that these two isolates *Kocuria* sp and *Streptomyces intermidus* could be a good candidate for the discovery of antibiotics. It's noteworthy to note that the genes for antibiotic production are almost always clustered with genes for self-resistance, and their expression is co-regulated (Mak *et al*., 2014). *Streptomyces coelicolor,* producer of important biologically active metabolites, also have resistance genes for the antagonistic compounds they secrete; often these are related to and are coregulated with the antibiotic biosynthesis genes (Nodwell, 2007). *Alcaligenes pakistanensis'* (LTP10) tolerance to a variety of antibiotics ensures its higher potential for antibiotic synthesis.

Liquid-liquid separation technique is used for separation of metabolites of interest into an immiscible solvent (Pinu and Villas-Boas, 2017). For this purpose, the CFS of LTP10 was extracted with n-Hexane, ethyl acetate and chloroform followed by their antimicrobial evaluation. The results indicated that ethyl acetate has shown good antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli,* and *Salmonella enterica* followed by chloroform extract. However n-Hexane has shown no or little antimicrobial activity. Marrez *et al*., (2019) reported the use of solvents including hexane, chloroform, and ethyl acetate for extraction of antimicrobial metabolites of *Scenedesmus obliquus* and evaluated their antimicrobial activity by disc diffusion method. Khim *et al*., (2019) evaluated the use of hexane, diethyl ether, chloroform, ethyl acetate, acetone and methanol for antimicrobial compounds extraction. Bose *et al*., (2015) described the use of ethyl acetate for extraction of antibiotics from culture of obligate marine actinobacterial species *Salinispora arenicola.* Kapley *et al*., (2016) reported that ethyl acetate extract of *Alcaligenes* sp. HPC 1271 has shown clear ZOI against both gram negative and gram positive microbes.

Most significantly, microbial metabolites should be able to endure diverse conditions in their natural settings. The most important variables that might either favorably or unfavorably affect the stability of the metabolites are light, pH, and temperature (Jayaprakashvel and Mathivanan, 2011). The dried ethyl acetate extract of LTP10 was evaluated for its temperature and pH stability by determining its antimicrobial activity at various temperatures (25°C, 35°C, 45°C, 55°C, 65°C) and pH (4.0, 5.5, 7.0, 8.5, 10.0). It was found that the antimicrobial metabolites of LTP10 extract of were quite stable over a range of temperature as shown by its antimicrobial activity at 25°C, 35°C, and 45°C with p-value more than 0.05 ($P > 0.05$) while there was decrease in activity at 55°C and 65°C which is significant statistically when compared to activities at 25°C, 35°C, and 45°C ($P < 0.05$). These results indicated the stable nature of antimicrobial metabolites of LTP10. Similarly, antimicrobial activity at pH 5.5, 7.0, and 10.0 was similar as evident from p-value more than 0.05 ($P > 0.05$) while there was decrease in activity at pH 4.0 and 10.0 which is significant statistically when compared to activities at pH 5.5, 7.0, and 10.0 ($P < 0.05$). Overall, these results indicated the stable nature of antimicrobial metabolites of LTP10 however there was decrease or loss in activity at very high temperatures and highly acidic or basic conditions. At pH levels between 1 and 8, the crude metabolites' antifungal efficacy against *Fusarium graminearum* and *Alternaria alternata* growth stayed almost stable (>75%) but drastically diminished under alkaline conditions (Zhao *et al*., 2010). *Magnaporthe grisea* is the cause of rice blast, and Kavitha *et al*., (2005) have identified and proven the thermostability of an antifungal peptide from *Bacillus subtilis*.

Using an agar well diffusion test, the antibacterial activity of the dried crude extract of LTP10 was assessed against clinical isolates of *Escherichia coli, Staphylococcus aureus, Salmonella enterica, Pseudomonas aeruginosa, Candida krusei*. and *Candida albicans*. Results indicated that crude extract has shown greatest activity against *Staphylococcus aureus* while showing good activity against *Escherichia coli, Salmonella enterica,* and *Pseudomonas aeruginosa*. No antimicrobial activity of extract was noted against *Candida krusei*, however good activity was found against *Candida albicans*. Clements *et al*., (2019) also determined activity of the crude extract of *Serratia marcescens* (1.00 mg/mL) against a panel of clinical bacterial and fungal strains including *Pseudomonas aeruginosa,* and *Candida albicans*. Inturri *et al*., (2019) reported the use of varoius clinical isolates including *Escherichia coli, Pseudomonas*

aeruginosa, Salmonella enterica, Candida albicans and *Candida krusei* for determining inhibitory activity of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 against these clinical isolates.

The synergistic effect denotes that a chemical combination's activity is better than the sum of its constituent compounds' individual effects. Antibiotics and secondary metabolites work together to combat many MDR pathogens (Shin *et al*., 2018). For this reason, the synergistic antimicrobial effect of crude extract of LTP10 with few known antibiotics was determined by agar disc diffusion method. It was found that antimicrobial activity of the extract increased when combined with penicillin, imipenem, and cefepime as compared to extract alone against *Staphylococcus aureus* and *Escherichia coli.* The difference in activity of extract of LTP10 alone and in combination was found significant statistically ($P < 0.05$). Keawchai *et al*., (2021) described the synergistic effect of a secondary metabolite obtained from ethyl acetate–hexane extract of *M. fragrans* seeds with ampicillin against *E. coli* which greatly enhances the potential of ampicillin against *E. coli*. Hussain *et al*., (2019) noted the synergistic antimicrobial effect of dinactin, a potent microbial metabolite, with drugs of tuberculosis against *Mycobacterium tuberculosis*.

MIC and MBC of ethyl acetate extract of LTP10 was evaluated against ATCC test strains. MIC of extract was found in the range of 0.4 to 3.2 mg/ml for different strains. The lowest MIC (0.4 mg/ml) was against *S. aureus* while highest MIC (3.2 mg/ml) was against *P. aeruginosa*. MBC of extract was in the range of 0.4 to 3.2 mg/ml with lowest MBC (0.4 mg/ml) against *S. aureus* and (3.2 mg/ml) against *S. epidermidis* and *B. subtilis*. However, *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella enterica* have shown growth even after treatment with highest concentraion of extact (12.8 mg/ml). MIC and MBC against *S. aureus* was found to be same (0.4 mg/ml) while MBC was three folds in case of *S. epidermidis* and *B. subtilis*. These results indicated the bactericidal nature of LTP10 metabolites against gram-positive bacteia while bacteriostatic nature against gram negative baceria. Similarly, Khim *et al*., (2019) reported MIC value of more than 2 mg/ml of ethyl acetate and chloroform extracts of *Alcaligenes faecalis* against *E. psidi*. Inturri *et al*., (2019) determined MIC of cell free supernatants of *Bifidobacterium longum* and *Lactobacillus rhamnosus* against ATCC strains including *E. coli*, *S. aureus*, and *P. aeruginosa* by broth dilution method.

According to Shalayel *et al*., (2017), the MIC of ethyl acetate extract of *Mentha piperita* against all Gram-negative pathogens was noted as 5–40 mg/ml with lowest MIC (1.25 mg/ml) value against *Streptococcus pyogenes*.

One of the most useful test species for cytotoxicity testing is brine shrimp. Brine shrimp lethality assays are cheap, continuously available, simple, reliable and extensively used in research (Arumugam *et al*., 2019). In this study cytotoxic nature of crude extract was evaluated by brine shrimp lethality assay. All three concentrations of LTP10 extract did not kill any shrimp (nauplii) after 24 hrs of incubation. Even after observing for three days, there was no killing on nauplii at 100 and 200 µg concentrations while only 40% lethality of shripms was observed at 300 µg of extract. Jaki *et al*., (2008) reported cytotoxic activity of methanolic extracts (500 ppm) of *Cyanobacteria* against brine shrimp as significant (lethality ≥ 60%). Qin *et al*., (1999) noted significant cytotoxic effect of tenuazonic acid from extract of *Alternaria* No.28 with mortality rates greater than 68% at a concentration of 10 µg/mL. Santos *et al*., (2019) reported that isolated biosurfactant metabolite from *Streptomyces* sp. DPUA1566 did not display any lethality to brine shrimp after 24 h. These results indicated the non-cytotoxic nature of crude extract of LTP10 metabolites, which has shown good antimicrobial activity.

Utilizing the stable free radical diphenilpycrylhydrazil is a typical way to measure antioxidant activity (DPPH). Because the DPPH approach is simple, quick, sensitive, and only needs a tiny sample extract, it is chosen. The antioxidant activity against DPPH of crude extract of LTP10 was found to be concentration dependent. The highest inhibition was found to 68.16% at 300 ug of extract. The results indicated good antioxidant potential of secondary metabolites of LTP10. Aullybux *et al*., (2021) reported that exopolysacharide, microbial metabolite produced by *Alcaligenes feacalis*, at the maximum EPS concentration (1 mg/ml), exhibited DPPH scavenging activity of up to 70.5%. Through testing its capacity to scavenge DPPH, the antioxidant activity of *A. faecalis* AU02 culture supernatant was discovered to be 84% (Annamalai *et al*., 2011). According to studies, the antioxidant activity of microorganisms linked with sponges increased free radical scavenging by 40–46% (Balakrishnan *et al*., 2015).

Fourier Transform Infrared (FTIR) has been created as a technique for the simultaneous and quantitative measurement of organic components, including chemical bonds, as well as organic content, such as protein, carbohydrate, and lipid (Nandiyanto *et al*.,

2019). In this study, ethyl acetate extract of *A. pakistanensis* LTP10 was subjected to FTIR analysis and the peaks at 3275, 2924, 1631, 1540, 1444, 1380, 1235. and 1071 cm^{-1} in FTIR spectrum confirmed the presence of O-H, C-H, C=C, C-O, N-O, S=O, C-N and C-O functional groups respectively. These functional groups indicates the presesnce of organic compounds belonging to alcohols, aliphatic hydrocarbons, and nitrogen containing organic compouns. *Alcaligenes faecalis'* crude ethyl acetate extract was FTIR-analyzed to reveal the presence of functional groups O-H, C-H, C-O, C=O, C=C, and N-O (Rafiq *et al*., 2016). Similarly, FTIR analysis was used for methanolic extract of *Cnestis ferruginea* and different functional groups were found in FTIR spectrum of extract (Acheampong *et al*., 2018). Pharmawati and Wrasiati, (2020) performed the FTIR analysis of crude extract of Enhalus acoroides and found functional groups such as hydroxyl groups, secondary amines, alkanes, fatty acids, and phenols.

Gas Chromatography-Mass Spectroscopy analysis were carried out to determine the antimicrobial secondary metabolites produced by *Alcaligenes pakistanensis* LTP10. Both ethyl acetate extract and chloroform extract were subjected to GC-MS analysis as both extracts have shown antimicrobial activity. Gas chromatogram of ethyl acetate extract has shown presence of 22 compounds while that of chloroform extract revealed presence of 7 compounds. Three metabolites namely Phenol,2,4- bis(1,1-dimethylethyl), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylpropyl) were found in both extracts. The major metabolites in ethyl acetate and cholorom extracts have known bioactivities. For example, Phenol,2,4-bis(1,1-dimethylethyl) (Abdullah *et al*., 2011;Rangel-Sánchez et., 2014), Pyrimidine-2,4(1H,3H)-dione (Singla and Bhat, 2011), Pentadecanoic acid,14-methyl-,methyl ester (Ohiri and Bassey, 2016), 1-Nonadecanol (Nair *et al*., 2019), Ergotaman-3',6',18-trione (Zaman *et al*., 2021), and Di-n-octyl phthalate(Passari *et al*., 2018), 2-Decene, 3-methyl-(Z) (Passari *et al*., 2017) and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) (Kannabiran, 2016) have reported good antimicrobial activities. Similarly, compounds S-[2-Aminoethyl]-dl-cysteine (NCBI, 2022), Dodecanoic acid,3-hydroxy- (Mun *et al*., 2019), 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (El-Fayoumy *et al*., 2021), 7-Hexadecenoic acid, methyl ester,(Z) (Fagbemi *et al*., 2022), Heptadecanoic acid,heptadecyl ester (Gacem *et al*., 2020), and Oleic acid (Dilika *et al*., 2000) has antimicrobial activities reported. Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-

methylpropyl) (Ser *et al*., 2015) and Pentadecanoic acid (Henry *et al*., 2002) have reported antioxidant activities. DL-Proline, 5-oxo-, methyl ester (Yu *et al*., 2005) and 9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione (Dassamiour *et al*., 2022) have reported cytotoxic activities. A study by Khim *et al*., (2019) reported a total of nine metabolites by GC-MS analysis of diethyl ether extract of *Alcaligenes faecalis*. The metabolites were (1) di[1-(3,4- methylenedioxyphenyl)-2-propyl]amine, (2) clindamycin, (3) N-formylmaleamic acid, (4) malonic acid, (5) sabinene hydrate, (6) methyl salicylate, (7) methylethylketon, (8) gamma-Crotonolactone and (9) valproic acid and some metabolites detected in this extract were known to have antibacterial activities. Abd Sharad *et al*., (2018) also reported Phenol,2,4-bis(1,1-dimethylethyl) among twenty compounds in chloroform extract of *Alcaligenes feacalis* by GC-MS analysis. The results of GC-MS chromatography in this study has revealed that most metabolites identified from different extracts of *Alcaligenes pakistanensis* have antimicrobial effects against different types of microorganisms while few have antioxidant activities reported. So these results also confirmed the results of antimicrobial and antioxidant assays performed in the study.

The analysis of ethyl acetate extract by LC-MS/MS technique has revealed the presence of important metabolites. The subsets of metabolites that are covered by GC-MS and LC-MS are very different. For instance, LC-MS mostly covers polar molecules that are prevalent in secondary metabolites, whereas GC-MS prefers volatile metabolites (El Sayed *et al*., 2020; Saravanakumar *et al*., 2021). The LC/MS run reviewed vast diverse compounds found in the cell free ethyl acetate extract of ethyl acetate. LC/MS was used to help identify the inhibitory compounds produced by *A*. *pakistanensis*. Most of these metabolites have antimicrobial activities reported in literature. Correlation of the inhibitory activity of these compounds was shown by their molecular docking studie. One of the major peak compound L,L-Cyclo(leucylprolyl) (compound24) was reported to have antibacterial, antifungal, antioxidant and anticancer activities (Yan *et al*., 2004; Gowrishankar *et al*., 2014; Gowrishankar *et al*., 2015; Deepak *et al* 2020). Similarly etomidate (compound18) was reported to have antibacterial and antifungal activities (Sá *et al*., 2019; AV Sá *et al*., 2020). Compounds nicotine-1'-N-oxide (compound14), streptidine (compound2), 3-Indoleacetonitrile (compound6), 2-Phenylethyl 3-methylbutanoate (compound3), Dianhydroaurasperone C (compound5), N1,N10-Diferuloylspermidine (compound4), Asp-Phe methyl ester (compound21), N-

Carboxyethyl-γ-aminobutyric acid (compound23), and 5-Dodecenoic acid, 3-hydroxy-, [R-(Z)]- (compound27) had shown antimicrobial activities reported in different studies (Jirovetz *et al*., 2008; Magdy *et al*., 2017; Mun *et al*., 2019; Hwang-Bo *et al*., 2019; Wang *et al*., 2020). 3-Indoleacetonitrile (compound6) and endalin (compound25) were reported to have antiviral activity (Gosselin *et al*., 1994; Zhao *et al*., 2021). The compound noruron (compound1) was known for its herbicidal activity (González *et al*., 2003). Menchén *et al*., (2001) has noted the anti-inflammatory actvity of compound N-(3-(Aminomethyl)benzyl)acetamidine (compound10). Two compounds L,L-Cyclo(leucylprolyl) (compound24) and N-Carboxyethyl-γ-aminobutyric acid (compound23) have also been reported to have antioxidant activity (Hwang-Bo *et al*., 2019; Deepak *et al* 2020). According to our research, Kapley *et al*., (2016) identified the nucleoside antibiotic tunicamycin from A. faecalis HPC 1271 using solvent extraction using ethyl acetate and metabolite profiling using HPLC and LC-MS.Eltokhy *et al*., (2021) performed the LC/MS analysis of the ethyl acetate extract of *A. faecalis* isolate MZ921504 and established the occurrence of antimicrobial metabolites ectoine, burkholderic acid, bacillibactin, and quinolobactin. The results of LC-MS analysis in this study has confirmed that most metabolites produced by *Alcaligenes pakistanensis* have antimicrobial activities against different types of microorganisms while some have antioxidant activities reported as well. Therefore, these results were in accordance to the results of antimicrobial and antioxidant assays performed in the study and revealed the importance of *Alcaligenes* species for production of important antimicrobial compounds.

Molecular docking is frequently used to forecast the affinities and mechanisms of binding of ligands with proteins. Traditionally, blind docking is accomplished on the entire protein exterior to determine the most feasible binding mode and cavity on protein (Liu *et al*., 2020). As most of the currently used antibiotics' mechanisms of action are based on targeting enzymes and proteins, the antimicrobial compounds in ethyl acetate extract of LTP10 were analyzed by molecular docking. For this purpose, an online webserver CB-Dock was used which determines the interactions of ligands with target proteins and claculates their binding affinity (Liu *et al*., 2020). CB-Dock results of 15 compounds, obtained from LC-MS analysis, has shown that most of these had good binding affinity with target proteins. Their binding affinity ranged from -10.2 to -5.0 kcal/mol. The highest binding affinity was shown by Dianhydroaurasperone C

(compound5), 7β-Hydroxy-3-oxochola-1,4-dien-24-oic acid (compound19) and L,L-Cyclo(leucylprolyl) (compound24) as -10.2, -9.3, and -7.5 kcal/mol respectively, indicating very good binding affinity score as the more negative the value the more efficient will be the binding. As proteins may have more than one binding site, CB-Dock was used to determine the binding interaction of antimicrobial compounds with minimum of five binding sites of target bacterial enzymes. The target proteins used in the study were selected on the basis of test microorganisms used in this study and their susceptibility to most of the antibiotics like penicillin-binding proteins, DNA gyrase, and dihydrofolate reductase etc. The hydrogen and other interacting bonds between ligand and protein was noted. Stabilizing energetically-favored ligands depends heavily on weak intermolecular interactions like hydrogen bonds and hydrophobic interactions (Patil *et al*., 2010). These results of binding affinity and presence of intermolecular bonds at site of interaction between antimicrobial metabolites and target bacterial enzymes has supported the antimicrobial activities of metabolites obtained by agar well diffusion assays and the results of LC-MS analysis.

Drug discovery is very fascilitated by timely prediction of pharmacokinetic properties of compounds such as absorption, distribution, metabolism, excretion, and toxicity properties (Rehman *et al*., 2018). Different online prediction tools are available for this purpose lke pkCSM (Pires *et al*., 2015) and swiss ADME (Chenafa *et al*., 2021). PkCSM was used to predict ADMET properties and druglikeness properties were determined by using swissADME. The predicted results of these analysis has shown that all the four best compounds could be good antibacterial drug-candidates. The results indicated the non-toxic nature of the compounds which was also evident from the results of Brine shrimp cytotoxic assay performed for ethyl acetate extract of LTP10. The in silico and in vitro findings showed that compound 19 and 24 were the most active against bacterial strains. A similar study of the invitro and insilico analysis of benzimidazo-1,2,3-triazole based compounds was performed by Rashdan *et al*., (2021) and predicted the animicrobial nature of these compounds.

The purpose of the study was to analyze the antimicrobial potential of bacterial isolates from Passu glacier. It was concluded from the results of antimicrobial activity by agar-well and agar-disk diffusion assys, characterizations of metabolites by GC-MS, LC-MS and insilico study of metabolites against target proteins that isolates used had good

antimicrobial action and can be used for production of important antimicrobial metabolites. However, It is advised that in subsequent research, the extract be subjected to additional analytical processing in order to completely isolate and identify the antimicrobial metabolites. This processing should involve larger-scale fermentation in order to produce adequate amounts of the metabolites, followed by additional purification and fractionation techniques.

Conclusions

This research work was designed to search for microorganisms from Passu glacier capable of producing antimicrobial metabolites. It was concluded that:

- The Passu glacier contains diversity of antibiotic producing bacterial strains.
- The Isolated strains were categorized as psychrotrophs based on results of optimization of growth temperature.
- *Alcaligenes* sp. was found to be a good producer of antibacterial metabolites active against ATCC and clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica*.
- The resistance to number of antibiotics by *Alcaligenes pakistanensis* LTP10 favors its antimicrobial activity as resistant microbes have greater potential of antimicrobial metabolites production.
- The antimicrobial metabolites produced by *Alcaligenes pakistanensis* LTP10 were found to be safe as shown by its non-cytotoxic nature.
- The secondary metabolites of *Alcaligenes pakistanensis* LTP10 have good antioxidant activity.
- *Alcaligenes pakistanensis* LTP10 produced important antimicrobial metabolites 7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid, L,L-Cyclo(leucylprolyl), and L-alpha-Amino-1H-pyrrole-1-hexanoic acid as evident from the results of GC-MS and LC-MS analysis.
- The antimicrobial compounds of *Alcaligenes pakistanensis* LTP10 showed stability at high temperature (up to 45°C) and at wide pH range (5.5-8.5).
- In-silico analysis of selected compounds has predicted the interaction of major metabolites with enzymes of target strains that could be possible reason of their antimicrobial activity.

Future Prospects

- It is recommended that extreme habitats, such as Passu glacier, should be explored, to locate microorganisms with the capacity to produce novel antimicrobial chemicals.
- The antimicrobial compounds dianhydroaurasperone C and L,L-cyclo(leucylprolyl could be further studied against fungal isolates as several metabolites have reported antifungal activity.
- The major compounds 7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid, L,L-Cyclo(leucylprolyl), and L-alpha-Amino-1H-pyrrole-1-hexanoic acid should be further purified by techniques like column chromatography, TLC and HPLC.
- The compounds 7β-Hydroxy-3-oxochola-1,4-dien-24-oic Acid and L-alpha-Amino-1H-pyrrole-1-hexanoic acid should be further characterized by different analytical methods.
- The antibacterial compounds produced could be further studied for its cytotoxic activity against normal human cell line.
- The *Alcaligenes pakistanensis* (LTP10) could be further grown at pilot scale to determine the potential yield of antimicrobial compounds produced and at large scale to produce large amount of important antimicrobial compounds for biotechnological use.

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APPENDIX

Table A.1 Zone of inhibition in triplicate of study isolates by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average)

Isolate	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
HTP12	16	19	19	18.0	1.7	10	9	10	9.7	0.6
HTP13	15	15	16	15.3	0.6	12	10	8	10.0	2.0
HTP36	14	17	13	14.7	2.1	16	17	13	15.3	2.1
LTP10	18	21	19	19.3	1.5	17	18	19	18.0	1.0
	Bacillus subtilis					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
HTP12	13	15	16	14.7	1.5	16	18	17	17.0	1.0
HTP13	14	15	11	13.3	2.1	19	22	18	19.7	2.1
HTP36	21	23	19	21.0	2.0	12	14	14	13.3	1.2
LTP10	18	17	21	18.7	2.1	17	16	19	17.3	1.5
	Psuedomonas aureginosa					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
HTP12	11	12	13	12.0	1.0	8	9	10	9.0	1.0
HTP13	0	0	0	0.0	0.0	8	8	7	7.7	0.6
HTP36	18	21	19	19.3	1.5	0	0	0	0.0	0.0
LTP10	12	14	13	13.0	1.0	15	17	16	16.0	1.0

Table A.2 Zone of inhibition in triplicate of HTP12 at different incubation periods by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average)

Hours	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	9	8	10	9.0	1.0	0	0	0	0.0	0.0
72	14	13	15	14.0	1.0	11	14	15	13.3	2.1
96	16	17	19	17.3	1.5	14	15	16	15.0	1.0
120	14	15	15	14.7	0.6	15	16	13	14.7	1.5
	Bacillus subtilis					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	9	10	11	10.0	1.0	8	8	9	8.3	0.6
72	13	14	14	13.7	0.6	13	14	12	13.0	1.0
96	18	17	16	17.0	1.0	14	14	16	14.7	1.2
120	15	14	17	15.3	1.5	14	15	12	13.7	1.5
	Psuedomonas aureginosa					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	0	0	0	0.0	0.0	0	0	0	0.0	0.0
72	9	10	11	10.0	1.0	8	8	7	7.7	0.6
96	12	13	14	13.0	1.0	11	12	13	12.0	1.0
120	14	13	11	12.7	1.5	12	13	12	12.3	0.6

Table A.3 Zone of inhibition in triplicate of HTP13 at different incubation periods by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average)

Hours	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	0	0	0	0.0	0.0	9	10	11	10.0	1.0
72	13	14	12	13.0	1.0	15	14	15	14.7	0.6
96	14	15	16	15.0	1.0	19	20	18	19.0	1.0
120	13	14	13	13.3	0.6	16	18	18	17.3	1.2
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	0	0	0	0.0	0.0	0	0	0	0.0	0.0
72	0	0	0	0.0	0.0	12	14	11	12.3	1.5
96	9	10	8	9.0	1.0	15	15	18	16.0	1.7
120	13	12	14	13.0	1.0	15	16	17	16.0	1.0
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	9	10	9	9.3	0.6	0	0	0	0.0	0.0
72	13	14	15	14.0	1.0	0	0	0	0.0	0.0
96	16	15	16	15.7	0.6	10	11	9	10.0	1.0
120	12	14	11	12.3	1.5	9	8	9	8.7	0.6

Table A.4 Zone of inhibition in triplicate of HTP36 at different incubation periods by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average)

Hours	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	0	0	0	0.0	0.0	0	0	0	0.0	0.0
72	12	14	14	13.3	1.2	12	13	14	12.0	1.0
96	14	17	16	15.7	1.5	15	18	13	15.3	2.5
120	15	13	13	13.7	1.2	14	15	12	13.7	1.5
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	8	9	8	8.3	0.6	0	0	0	0.0	0.0
72	13	15	14	14.0	1.0	9	10	11	10.0	1.0
96	17	20	17	18.0	1.7	15	15	16	15.3	0.6
120	9	10	9	9.3	0.6	16	15	16	15.7	0.6
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	10	10	9	9.7	0.6	0	0	0	0.0	0.0
72	14	14	16	14.7	1.2	0	0	0	0.0	0.0
96	21	22	18	20.3	2.1	9	10	12	10.3	1.5
120	16	17	18	17.0	1.0	0	0	0	0.0	0.0

Table A.5 Zone of inhibition in triplicate of LTP10 at different incubation periods by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average)

Hours	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	8	8	9	8.3	0.6	7	8	9	8.0	1.0
72	13	14	13	13.3	0.6	9	10	11	10.0	1.0
96	21	19	20	20.0	1.0	17	18	20	18.3	1.5
120	17	18	19	18.0	1.0	14	14	15	14.3	0.6
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	0	0	0	0.0	0.0	0	0	0	0.0	0.0
72	0	0	0	0.0	0.0	11	12	11	11.3	0.6
96	13	14	15	14.0	1.0	17	16	17	16.7	0.6
120	15	16	13	14.7	1.5	11	12	13	12.0	1.0
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
24	0	0	0	0.0	0.0	0	0	0	0.0	0.0
48	0	0	0	0.0	0.0	0	0	0	0.0	0.0
72	11	11	14	12.0	1.7	0	0	0	0.0	0.0
96	16	14	18	16.0	2.0	11	12	12	11.7	0.6
120	12	15	14	13.7	1.5	10	12	8	10.0	2.0

Table A.6 Zone of inhibition in triplicate of HTP12 in different culture media by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Medium	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	13	14	15	14.0	1.0	15	14	15	14.7	0.6
L.B	18	17	18	17.7	0.6	20	21	18	19.7	1.5
T.S.B	10	10	12	10.7	1.2	14	13	14	13.7	0.6
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	12	13	14	13.0	1.0	13	12	11	12.0	1.0
L.B	15	14	14	14.3	0.6	14	12	14	13.3	1.2
T.S.B	12	12	14	12.7	1.2	10	10	12	10.7	1.2
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	14	15	15	14.7	0.6	13	12	11	12.0	1.0
L.B	16	17	16	16.3	0.6	15	14	15	14.7	0.6
T.S.B	12	12	14	12.7	1.2	10	12	12	11.3	1.2

Table A.7 Zone of inhibition in triplicate of HTP13 in different culture media by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Medium	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	12	13	12	12.3	0.6	14	15	15	14.7	0.6
L.B	17	19	18	18.0	1.0	21	20	20	20.3	0.6
T.S.B	11	12	14	12.3	1.5	16	15	18	16.3	1.5
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	9	9	11	9.7	1.2	12	13	13	12.7	0.6
L.B	15	16	14	15.0	1.0	16	14	17	15.7	1.5
T.S.B	10	12	8	10.0	2.0	11	14	10	11.7	2.1
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	15	14	13	14.0	1.0	10	11	9	10.0	1.0
L.B	15	14	15	14.7	0.6	14	15	12	13.7	1.5
T.S.B	13	15	12	13.3	1.5	9	11	9	9.7	1.2

Table A.8 Zone of inhibition in triplicate of HTP36 in different culture media by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Medium	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	13	12	13	12.7	0.6	13	16	15	14.7	1.5
L.B	16	17	18	17.0	1.0	14	17	18	16.3	2.1
T.S.B	15	15	16	15.3	0.6	13	14	15	14.0	1.0
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	12	12	11	11.7	0.6	13	14	11	12.7	1.5
L.B	15	16	19	16.7	2.1	14	14	15	14.3	0.6
T.S.B	14	15	14	14.3	0.6	11	12	13	12.0	1.0
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	15	15	18	16.0	1.7	9	12	13	11.3	2.1
L.B	20	21	20	20.3	0.6	14	12	16	14.0	2.0
T.S.B	15	16	16	15.7	0.6	10	11	9	10.0	1.0

Table A.9 Zone of inhibition in triplicate of LTP10 in different culture media by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Medium	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	15	16	14	15.0	1.0	12	14	14	13.3	1.2
L.B	21	22	19	20.7	1.5	20	22	21	21.0	1.0
T.S.B	19	17	18	18.0	1.0	12	13	13	12.7	0.6
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	11	12	11	11.3	0.6	14	15	14	14.3	0.6
L.B	16	15	17	16.0	1.0	16	18	17	17.0	1.0
T.S.B	12	10	13	11.7	1.5	12	12	13	12.3	0.6
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
N.B	12	14	15	13.7	1.5	12	12	13	12.3	0.6
L.B	22	19	18	19.7	2.1	16	18	19	17.7	1.5
T.S.B	16	15	17	16.0	1.0	14	13	11	12.7	1.5

Table A.10 Zone of inhibition in triplicate of HTP12 at different temperatures by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Temp	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	7	8	9	8.0	1.0	7	8	8	7.7	0.6
15°C	12	11	12	11.7	0.6	12	14	13	13.0	1.0
25°C	16	17	18	17.0	1.0	17	15	17	16.3	1.2
35°C	9	8	12	9.7	2.1	11	11	12	11.3	0.6
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	0	0	0	0.0	0.0	8	8	9	8.3	0.6
15°C	11	12	11	11.3	0.6	11	12	12	11.7	0.6
25°C	11	12	13	12.0	1.0	14	13	15	14.0	1.0
35°C	9	12	9	10.0	1.7	0	0	0	0.0	0.0
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	0	0	0	0.0	0.0	0	0	0	0.0	0.0
15°C	11	12	14	12.3	1.5	14	15	10	13.0	2.6
25°C	16	17	18	17.0	1.0	15	13	12	13.3	1.5
35°C	12	11	11	11.3	0.6	0	0	0	0.0	0.0

Table A.11 Zone of inhibition in triplicate of HTP13 at different temperatures by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Temp	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	0	0	0	0.0	0.0	8	9	8	8.3	0.6
15°C	13	12	13	12.7	0.6	15	17	15	15.7	1.2
25°C	16	17	17	16.7	0.6	20	19	19	19.3	0.6
35°C	9	10	11	10.0	1.0	11	13	10	11.3	1.5
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	7	8	8	7.7	0.6	0	0	0	0.0	0.0
15°C	10	11	9	10.0	1.0	10	11	11	10.7	0.6
25°C	11	12	11	11.3	0.6	18	18	16	17.3	1.2
35°C	0	0	0	0.0	0.0	14	15	14	14.3	0.6
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	0	0	0	0.0	0.0	0	0	0	0.0	0.0
15°C	15	14	13	14.0	1.0	7	8	8	7.7	0.6
25°C	15	16	16	15.7	0.6	10	9	11	10.0	1.0
35°C	12	13	14	13.0	1.0	0	0	0	0.0	0.0

Table A.12 Zone of inhibition in triplicate of HTP36 at different temperatures by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Temp	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	0	0	0	0.0	0.0	8	9	8	8.3	0.6
15°C	13	12	13	12.7	0.6	15	17	15	15.7	1.2
25°C	16	17	17	16.7	0.6	20	19	19	19.3	0.6
35°C	9	10	11	10.0	1.0	11	13	10	11.3	1.5
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	7	8	8	7.7	0.6	0	0	0	0.0	0.0
15°C	10	11	9	10.0	1.0	10	11	11	10.7	0.6
25°C	11	12	11	11.3	0.6	18	18	16	17.3	1.2
35°C	0	0	0	0.0	0.0	14	15	14	14.3	0.6
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	0	0	0	0.0	0.0	0	0	0	0.0	0.0
15°C	15	14	13	14.0	1.0	7	8	8	7.7	0.6
25°C	15	16	16	15.7	0.6	10	9	11	10.0	1.0
35°C	12	13	14	13.0	1.0	0	0	0	0.0	0.0

Table A.13 Zone of inhibition in triplicate of LTP10 at different temperatures by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutreint broth, LB Luria Broth, TSB = Tryptic Soya Broth)

Temp	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	9	8	8	8.3	0.6	8	7	8	7.7	0.6
15°C	15	15	16	15.3	0.6	12	13	12	12.3	0.6
25°C	21	22	18	20.3	2.1	16	18	16	16.7	1.2
35°C	15	18	20	17.7	2.5	10	13	13	12.0	1.7
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	9	8	9	8.7	0.6	9	12	10	10.3	1.5
15°C	11	11	12	11.3	0.6	13	14	12	13.0	1.0
25°C	15	14	13	14.0	1.0	15	15	17	15.7	1.2
35°C	11	10	11	10.7	0.6	9	9	8	8.7	0.6
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5°C	11	10	11	10.7	0.6	0	0	0	0.0	0.0
15°C	16	14	15	15.0	1.0	10	10	11	10.3	0.6
25°C	18	20	16	18.0	2.0	18	20	15	17.7	2.5
35°C	15	16	15	15.3	0.6	12	13	14	13.0	1.0

Table A.14 Zone of inhibition in triplicate of HTP12 at different pH by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutrient broth, LB Luria Broth, TSB = Tryptic Soya Broth)

pH	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	12	11	13	12.0	1.0	11	12	9	10.7	1.5
7	17	19	17	17.7	1.2	17	16	18	17.0	1.0
9	11	12	11	11.3	0.6	12	13	12	12.3	0.6
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	0	0	0	0.0	0.0	0	0	0	0.0	0.0
7	13	15	11	13.0	2.0	16	15	18	16.3	1.5
9	9	10	8	9.0	1.0	11	12	10	11.0	1.0
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	8	7	9	8.0	1.0	9	10	9	9.3	0.6
7	19	20	19	19.3	0.6	12	12	13	12.3	0.6
9	12	13	9	11.3	2.1	0	0	0	0.0	0.0

Table A.15 Zone of inhibition in triplicate of HTP13 at different pH by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutrient broth, LB Luria Broth, TSB = Tryptic Soya Broth)

pH	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	11	12	11	11.3	0.6	13	14	13	13.3	0.6
7	17	18	19	18.0	1.0	20	21	19	20.0	1.0
9	11	10	9	10.0	1.0	11	12	11	11.3	0.6
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	9	10	10	9.7	0.6	13	12	13	12.7	0.6
7	14	15	14	14.3	0.6	17	18	17	17.3	0.6
9	8	9	10	9.0	1.0	12	13	10	11.7	1.5
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	10	12	11	11.0	1.0	9	9	8	8.7	0.6
7	20	19	20	19.7	0.6	13	15	13	13.7	1.2
9	13	12	15	13.3	1.5	11	12	10	11.0	1.0

Table A.16 Zone of inhibition in triplicate of HTP36 at different pH by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutrient broth, LB Luria Broth, TSB = Tryptic Soya Broth)

pH	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	10	9	11	10.0	1.0	11	11	12	11.3	0.6
7	19	20	19	19.3	0.6	16	17	15	16.0	1.0
9	12	15	15	14.0	1.7	0	0	0	0.0	0.0
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	0	0	0	0.0	0.0	8	9	8	8.3	0.6
7	15	14	16	15.0	1.0	21	18	19	19.3	1.5
9	11	12	11	11.3	0.6	13	15	14	14.0	1.0
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	9	10	9	9.3	0.6	8	9	9	8.7	0.6
7	20	21	20	20.3	0.6	13	15	14	14.0	1.0
9	11	13	13	12.3	1.2	12	12	14	12.7	1.2

Table A.17 Zone of inhibition in triplicate of LTP10 at different pH by Agar Well Diffusion assay against ATCC test strains (SD = Standard deviation, Avg = Average, NB = Nutrient broth, LB Luria Broth, TSB = Tryptic Soya Broth)

pH	**Zone of inhibition (mm)**									
	Staphylococcus aureus					***Escherichia coli***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	12	13	14	13.0	1.0	12	12	11	11.7	0.6
7	24	23	23	23.3	0.6	18	21	20	19.7	1.5
9	15	16	15	15.3	0.6	13	11	12	12.0	1.0
	Psuedomonas aureginosa					***Staphylococcus epidermidis***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	0	0	0	0.0	0.0	11	12	10	11.0	1.0
7	15	14	13	14.0	1.0	18	16	18	17.3	1.2
9	10	9	12	10.3	1.5	9	10	9	9.3	0.6
	Bacillus subtilis					***Salmonella enterica***				
	1st	**2nd**	**3rd**	**Avg**	**SD**	**1st**	**2nd**	**3rd**	**Avg**	**SD**
5	13	15	14	14.0	1.0	11	12	11	11.3	0.6
7	19	20	19	19.3	0.6	16	14	15	15.0	1.0
9	12	13	12	12.3	0.6	10	9	13	10.7	2.1

EFFECT OF VARIOUS CULTURE CONDITIONS ON THE ANTIMICROBIAL ACTIVITY BY BACTERIA FROM PASSU GLACIER, PAKISTAN

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Abstract. Microorganisms inhabiting cold environments have an incredible potential to produce secondary metabolites including antibacterial, antifungal and antiviral compounds. The aim of the study was to evaluate the possibility of using cold adapted bacteria isolated from Passu glacier for the production of antimicrobial metabolites. Agar well diffusion assay was used to select four best antimicrobial compound producers coded as HTP12, HTP13, HTP36 and LTP10. These strains were identified as *Alcaligenes faecalis*, *Pseudochrobactrum saccharolyticum*, *Alcaligenes pakistanensis* and *Alcaligenes pakistanensis*, respectively, by their phylogenetic analysis. The media, temperature, incubation time and pH were optimized for antimicrobial compound production. The effect of nitrogen sources, carbon sources and salts on antibiotic production was also determined. *Alcaligenes pakistanensis* (LTP10) showed the best antimicrobial activity in Luria Bertani broth, at 25°C, pH 7 and incubation time of 96 hrs against various bacterial ATCC strains and clinical isolates. Yeast extract increased antibiotic activity of *Alcaligenes pakistanensis* (LTP10) while glucose, starch, tryptophan, threonine, L-leucine, arginine, NaCl and KCl decreased the activity. This study concludes that the psychrophilic bacteria are abundant, undiscovered, and good producers of antimicrobial metabolites under optimum conditions. This will lead to the discovery of potent and novel metabolites which could be of medical and industrial importance.
Keywords: *psychrophilic bacteria, Alcaligenes pakistanensis, agar well diffusion assay, antimicrobial metabolites, antibiotic production optimization*

Introduction

The environment which is not suitable and is considered severe for the survival of human beings is referred to as extreme environment and most of the world's extreme environments are low temperature environments. These low temperature environments provide harsh conditions for the survival and growth of organisms but still harbour a large persistent community of microbes. These persistent microbes have adaptations to cope with the challenges of low temperature environments for their survival and successful colonization (Margesin et al., 2002; Furhan, 2020). There are a lot of microorganisms reported that have the ability to adapt and even show best growth and survival in harsh conditions like low or absent oxygen, high salt concentration, less nutrient availability and oxidative stress which occurs due to low temperature environments (D'Amico et al., 2006; Yarzábal, 2016). Cold-loving microbes have adapted several different mechanisms such as production of high amounts of fatty acids, proteins and non-polar carotenoids for excess fluidity of membrane, possess cold adapted enzymes and containing cold acclimation proteins (caps), antifreeze

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