Characterization of Gibberellic Acid Produced by Bacteria Isolated from Ghulkin Glacier, Hunza Valley

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

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Dedication

This thesis is dedicated to my beloved Parents. Thank you for all your endless love, sacrifices, prayers, and support.

Abbas Ud Din

Author`s Declaration

I Abbas Ud Din hereby state that my MPhil thesis titled **"Characterization of Gibberellic Acid Produced by Bacteria Isolated from Ghulkin Glacier, Hunza Valley"** is my own work and has not been submitted previously by me for taking any degree from Quaid-I-Azam University, Islamabad, Pakistan. At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my MPhil degree.

Abbas Ud Din

Certificate

This thesis submitted by ***Abbas ud din*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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List of Contents

List of Figures

List of Abbreviations

ABA	Abscisic Acid
AEG	Applied Environmental and Geomicrobiology lab
AFPs	Anti-freeze proteins
FTIR	Fourier transform infra-red spectroscopy
GA	Gibberellic acid
HSPs	Heat Shock Proteins
IR	Infra-Red
L	Liter
Mol	Mole
ml	Milliliter
Mg/ml	Milligrams per milliliter
nm	Nanometer
NMR	Nuclear Magnetic Resonance
CSPs	Cold shock Protein
pH	Potential of Hydrogen or negative log of H+ concentration
PPlase	Peptidyl-prolyl Isomerases
PGRs	Plant Growth Regulaters
rpm	Revolutions per minute
UV	Ultra-Violet
°C	Degree centigrade
µg	microgram

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Abstract

The northern area of Pakistan has an ecosystem of cryogenic nature where plants are hard to grow and provide unfriendly environment for their sustainability. Commercial biofertilizers has evolved to be a tremendous remedy for such environment but they are not viable at such low temperature .The psychrophilic microbes of northern glaciers has the ability to grow in such environment and produce metabolites that are adopted to cold environment. gibberellic acid produced by organisms are good example of biofertilizers produced by natural source. The current study aims to screen, optimized., produce ,purify and characterized the cold adapted bacteria Ghul kin Glacier, Hunza Valley, Gilgit Baltistan, Pakistan. A total of 8 strains were screened for the production of gibberellic acid on nutrient broth production media. Fermentation conditions for the maximum production of gibberellic acid of all selected isolates were optimized for which productions were carried out in broth media using the optimized conditions. Purifications was performed by solvent-solvent extraction method. Characterization of the selected strains were carried out through, Fourier Transform Infrared Spectroscopy (FTIR) and Proton based Nuclear Magnetic Resonance (H-NMR). Two strains GA19 and GB16 shoes the production of gibberellic acid Maximum gibberellic Acid production by GB16 were recorded at15°C, pH 7, and peptone as carbon source after 96hours of incubation. The optimum conditions for the maximum production of gibberellic Acid by GA9 were recorded at 15°C, pH 7, AND PEPTONE as carbon source after 120 hours of incubation. The similarity of spectral peaks between standard gibberellic Acid and extracted gibberellic Acid from the selected isolates confirmed the production of hormone. Further confirmation was done by NMR by finding the functional groups present in gibberellic acid. These cold adapted strains isolated from the Ghulkin Glacier samples were good producers of gibberellic Acid which would be used as biofertilizers for crop growth promotion in the cold climatic regions of Pakistan.

Introduction

The efficacy of extremophiles in most industries and other applications is due to the range of distinctive characteristics they exhibit. A significant motivation for accumulating research on biotechnology and extremophiles' likelihood related to microorganisms and their metabolites. And the creation of these metabolites and other products is directly related to the involvement of enzymes and proteins in the manufacturing pathways. The fact that these creatures can survive in harsh environments implies that their enzymes are likewise adapted to perform properly in such conditions. And this notion is confirmed by data retrieved and obtained over the last few years by investigating the nature of enzymes and bacteria. Resistance to high-temperature environments, chemically denaturing detergents, hydrogen bond breaking agents (chaotropic), extreme pH, and organic solvents (Gaur, 2010) coined the term "extremozymes" they are holding prodigious ability to work as catalyst for industry working in extreme environments in which the enzymes form the normal microorganisms lost their natural ability and structure (Hough & Danson, 1999).

Extremophiles are classified into several types based on the extreme environments they prefer to inhabit or tolerate, such as (acidophiles) who prefer an acidic environment with a pH range of 1 to 5, (alkaliphiles) who prefer a pH range of 9 or higher, (halophiles) who prefer a salty environment with a high salt concentration, (thermophiles) who prefer a temperature range of 60°C to 80°C, (hyperthermophilic) There are many more of them, such as barophilic, oligotrophic, endoliths, xerophilic, and several that can live in more than one stress state, known as polyextremophiles. Following on from the preceding debate, we will proceed with the examination of psychrophiles.

Psychrophiles are microorganisms that can thrive ideally or lower than 0°C, 15°C, and 20°C, appropriately, while psychrotolerant microorganisms can survive temperatures of up to or above 25°C (Helmke & Weyland, 2004). According to research, because they live in frigid locations, they ensure that these ecosystems have an adequate supply of critical nutrients and a proper nutrient regeneration process (Deming, 2002). One of the habitats for microorganisms is arctic sea ice, where bacteria exist at -20°C, implying that microorganisms have the potential to live in a habitat that combines -20°C and a

liquid surface (Junge, 2004). The rapid growth of psychrophiles is connected with their adaptations to low temperature conditions. However, this is especially true in surroundings with a substantial supply of energy sources for the cell. And, contrary to psychrotrophs, some obligatory psychrophiles outcompete psychrotrophs in this occurrence, implying that they have greater mineralization ability under cold circumstances (Harder & Veldkamp, 1971). A number of psychrophiles have been identified in Pakistan. In this regards, (Rafiq, 2017) has isolated several of the bacterial strains recovered from Pakistan's Siachen glaciers which were identified as the genus Pseudomonas, Alcaligenes, Jonthenobacterium, Rhodococcus, Carnobacterium, Arthrobacter, Bacillus, Lysinibacillus, Staphylococcus, and Planomicrobium. While (Rafiq, 2017) identified microorganisms such as Actinobateria, Bacteriodetes, Firmicutes, and Proteobacteria, (Shen, 2012) investigated Rongbuk glacier and reported four main groups of bacteria including actinobacteria, firmicutes, alpha-proteobacteria, and gamma-proteobacteria (Shivaji, 2011) isolated many phyla of bacteria from another glacier named Pindari glacier in Himalayas by using 16S rRNA sequencing gene libraries.

Depending on the physiology and physiological action of the hormone on plants, plants can create a wide range of hormones. These can be similar in that they have comparable physiological effects, but their chemical structure cannot be the same and will differ from one hormone to the next. Initially, the hormones studied were only of five primary types: abscisic acid, auxins, brassinosteroids, cytokinins, and ethylene (Weier, 1970). Though, throughout time, the list of these hormones has grown and many additional known hormones have been added to it, including brassinosteroids, jasmonates, salicyclic acid, and strigolactones. In addition, there are many other comparable chemicals that have yet to be identified as plant hormones since they exhibit the same qualities but have yet to be categorised as bonafide hormones.

Gibberellins or Gibberellic acid is a diterpenoid plant growth hormone from the tetracyclic diterpenoid plant hormone family that has the effect of speeding up the elongation of dwarfism in plants, promoting blooming, assisting in stem and root elongation, and allowing the plant to grow fruits. Gibberellins were discovered from a fungi called Gibberella fujikuroi, and the researcher who discovered them was a

Japanese scientist named Eiichi Kurosawa, who caused some abnormalities in rice plants (Grennan, 2006), but after further investigation, the scientists discovered that they were also produced by the plants and played an important role in the plant's life cycle. In growing seed of a peach gibbberalic acid are produced in large quantity. are Different other plant sources are Onion bulbs, spinach, and ferns . In total, 136 distinct gibberellic acid compounds have been discovered in plants, fungi, and bacteria. Many plants have numerous versions of the gibberellic acid hormone, which regulates various aspects of plant growth (Hoad, 1995).

Gibberellin-producing bacteria include Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia, and Bacillus (Helliwell, Sullivan, et al., 2001; Nelson et al., 2004). Gibberalic acid -like activity have been examined in soil yeasts and bacteria specifically like Arthrobacter globifomze, as well as in bacterium cultures recovered from pine seedling roots in notable quantities (Yanni et al., 2001). Many bacterial cultures, particularly pseudomonads, produced a GA-like substance in quantities ranging from a few micrograms of GAs-equivalents per litre of media (Singh et al., 2013). In recent times Microbiomes have been widely investigated in cold environment, with a focus on culture-dependent and culture-independent techniques. Novel psychrotrophic microbes have shown multifunctional plant growth promoting (PGP) properties at low temperatures such as Arthrobacter nicotianae, Brevundimonas terrae, Paenibacillus tylopili, and Pseudomonas cedrina, have been identified in the cold deserts of the NW Himalayas. and. Psychrotrophic PGP microbes have been shown to promote plant growth either directly through biological N2 fixation, solubilization of minerals such as phosphorus, potassium, and zinc, production of siderophores and plant growth hormones (Indole acetic acid and gibberellic acid), or indirectly through inducing resistance against plant pathogens (Yadav et al., 2016).

Plant growth regulators such as gibberellic acid, jasmonic acid, and indole acitic acid have been found to increase abiotic stress in plants (Zhang et al., 2020). Among the numerous PGRs, gibberellic acid (GA3) works as a hormone stimulator, promoting several physiological and biochemical processes in plants (Vishal & Kumar, 2018). The administration of gibberellic acid (GA3) to carrot plants at various phases of growth enhances leaf growth while inhibiting root growth. Exogenous GA3 injection produces

plant bolting divergence by instituting a short thickened condensed stem, however flowers are rarely initiated (Jung et al., 2020). Gibberellic acid has been shown to boost germination percentage and seedling growth while overcoming the preventative effects of salt stress on germination. Plant hormones are active elements of the signal cascade that initiates plant stress responses. (GA3) increased abiotic stress tolerance by inducing and raising endogenous salicylic acid levels (Kohli et al., 2013). Gibberellic acid (GA3) has been used to increase plant length or height, increase blossom quantity, and induce early flowering. SPINDLY, a Gibberellic Acid Signalling Negative Regulator, Is Involved in Plant Abiotic Stress Response (Qin et al., 2011). In the current study, the procedure was modified somewhat for screening and quantification, with 1ml of supernatant obtained from a 24 hour old broth infected with each of the selected strains and 200ul. The reagent zinc acetate was introduced. After 2 minutes, 200ul of potassium ferrocyanide was added and centrifugation at 2000rpm for 15 minutes was conducted. Following that, 1ml of supernatant was taken, 1ml of 30%HCL was added, and the mixture was incubated at 20c0 for 75 minutes. The amount of GA was determined by measuring the absorbance at 254nm using a (Shimadzu-UV1601) UV-VIS Spectrophotometer (Sharma et al., 2018).

It is crucial to understand the physiological activities that occur during plant development. Gibberellic acid (GAS) is now the least expensive and most physiologically effective of the gibberellins for commercial use. The importance of using this hormone are based on reality that it is easily produced in large quantities and has advanced level of biological activity in different types of plants that humans value (Turner, 1972).

Aim

To study screening, optimization, production and characterization of gibberellic acid produced by bacteria isolated from Ghulkin glacier Hunza valley.

Objectives:

• Screening of Gibberellic Acid producing psychrophilic bacteria .

• Optimization of fermentation conditions for maximum production of gibberellic acid by the selected bacterial strains.

• Production and Purification of gibberellic acid by solvent-solvent extraction method.

• Characterization of gibberellic acid produced by selected bacterial isolates.

Literature review

Extremophiles

Extremophiles, (from Latin *extremus* meaning "extreme" and Greek *philia* means "love") are those microorganisms that inhabit or likely thrive geochemically under extreme conditions that are not beneficial to the huge variety of life lives on earth. Contrary to these, the other organisms that live in much adequate environments may be referred to as neutrophils or mesophiles. During the decades of 1980s and 1990s many of the biologists have found that the microorganisms are so much flexible that they can tolerate the extreme environments, such as niches that are incredibly hot, acidic or even colder which can be fully uninhabitable for the mesophiles. Many scientists even believe that hydrothermal vents were the primary habitats for the life under the surfaces of ocean's (Gupta, 2014).

The word extremophile was initially coined in 1974 by (MacElroy, 1974), and they were then classified into three groups named archaea, bacteria and eukarya (Rothschild & Mancinelli, 2001) but three decades ago these extremophiles were just alienated organisms and were introduced by just few of the researchers across the world. Currently they have been emerged as the most valuable organisms for those studying enzymology and helping in various industries (Van Den Burg 2003). During the last two or three decades these studies have been advanced so much that the first conference on extremophiles has been conducted in Portugal in 1996 and a journal named "extremophiles" were introduced for the purpose in 1997. A society was also formed with the name of "international society of extremophiles" during the year 2002 for the purpose of sharing knowledge and experience in the fast expanding field of extremophiles.

The effectiveness of extremophiles in most industries and various other applications are because of the variety of idiosyncratic characters they are showing. A huge incentive compiling research on biotechnological and extremophiles' probability related to the microorganism and there metabolites. And the production of these metabolites and other products are directly associated to the involvement of the enzymes and the proteins in the pathways they are using for the production. With the saying that these

organisms can survive in extreme environments it is also inferred that their enzymes are also adapted for such conditions to work properly. And this theory is supported by the data extracted and obtained by studying the nature of enzymes and microorganisms from the past few years. Resistance to the highly thermal habitats, chemically denaturing detergents, hydrogen bonding disrupting agents (chaotropic), extreme pH and organic solvents (Gaur, 2010) Termed as the "extremozymes" they are holding prodigious ability to work as catalyst for industry working in extreme environments in which the enzymes form the normal microorganisms lost their natural ability and structure (Hough & Danson, 1999).

Extremophiles are of many types on the basis of extreme environments they are happy to inhabit or tolerate, such as (acidophiles) that inhabit acidic environment ranging from pH 1 to 5, (alkaliphiles) inhabiting pH ranging above 9, (halophiles) habitants of salty environment having high salt concentration, (thermophiles) love to live in optimum temperature from 60°C to 80°C, (hyper thermophilic) whose temperature ranges above 80°C, (psychrophiles) with optimum temperature ranges below 15°C or even tolerate temperature 20°C. There are many more of them like barophilic, oligotrophic, endoliths, xerophilic and many can even inhabit their life in more than one stress condition which can be term as the polyextremophiles. Here from the above discussion, we are going progress with the study of psychrophiles.

Psychrophiles

Schmidt-Nielsen (Schmidt-Nielsen, 1902) introduced these microorganisms as they are those which cannot only thrive life on 0°C but can also grow in number, while on the other hand Horowitz-Wlassowa and Grinberg (R. Y. Morita, 1975) used psychrobe for the actual psychrophiles and used the term psychrophiles for those which can grow at 0°C but can also grow at higher temperature. After sufficient amount of the data collected about these type of microorganisms a dictionary in the name of "Dictionary for Microbiology" were formed in 1957 that has defined them as those with ideal growth temperature of 15°C are called as the psychrophiles (Lasztity, 2009). Microorganisms that can grow optimally at or below 0°C, 15°C and 20°C respectively are called psychrophiles and those that can withstand a temperature of up to or above 25°C are called psychrotolerant (Helmke & Weyland, 2004). The sustainability of the

metabolic activities and the normal growth rate in such type of environments is very difficult as they need such a tremendous amount of the adaptation to compete such temperatures and to work out for living (Feller, 2013) and these are the actual interesting fact that are making them very much favorable for researchers to work on and get understood their behavior genetically and acclimation processes in them along with their ability of adaptations (Zakhia, 2008).

To look at the surface of earth it is easy to know that 14% is the polar region of the earth and in the remaining 71% is the oceanic region on earth in which the temperature of the 90% of the oceans is below 5°C and the oceans have the ability to maintain its constant temperature i.e., 4-5°C despite of the latitude differences and this is the reason that almost all of the barophiles are either psychotropic or psychrophilic in nature (Yayanos, 1986). Psychrotrophs can also thrive in the same environment as the psychrophiles are in, but they maintain their cell number much more than the psychrophiles and the reason is that they can compete the fluctuating temperature from the sun on the ice surfaces either they are in the north or in the southern polar region that can gain the higher temperature up to 28°C. While psychrophiles cannot survive in such a higher temperature and such a fluctuating temperature, and if the concept that life is evolved from the mesophiles or thermophile then this can also be exactly possible that the psychrophiles are also evolved from the psychrotrophs (R. Morita & Moyer, 2000). And the study reveals that as they are the residents of the cold environments than they are making it possible that these environments have sufficient amount of essential nutrients and perfect regeneration procedure of the nutrients (Deming, 2002). It is also revealed that some microbes have thrived for millions of years in the permafrost in the form of cryobiosis (Vorobyova, 1997). One of the habitat for microorganisms is the arctic sea ice in which microbes are living at -20°C and is giving the concept that microorganisms have the ability to live in a habitat that is the combination of -20°C and a liquid surface (Junge, 2004). While in contrast the growth on a temperature of -12°C is not yet reported for these microorganisms (Breezee, 2004), however the microorganisms are reported to survive in situ at -30°C and in some prediction their metabolism can also work in -40°C (Price & Sowers, 2004).

Physiological adaptations of psychrophiles

Rapid growth of the psychrophiles is associated with the adaptations made by them to the low temperature environments. Yet this is especially for those environments that consist of sufficient amount of the energy sources there for the cell. And this phenomenon is even outcompeted by some of the obligate psychrophiles contrary to the psychrotrophs suggesting that they are having more mineralization ability in the cold conditions (Harder & Veldkamp, 1971). Some of these adaptations are discussed below.

Production of antifreeze Proteins

Antifreeze proteins production is one of the best adaptation psychrophiles have made to them as they enable them to compete the cold environments and to help them live in. these are such type of proteins that cannot disturb the melting property of the solution but help the microorganisms to alter their temperature and to thrive in the conditions in colligative pattern (Davies, 2014). The term thermal hysteresis is used for this process in which the psychrophiles are altering the ice shape that is associated with the binding of the protein to the ice (Kim, 2017), alongside these the antifreeze proteins also works in the inhibition of the nucleation of the ice crystals and their growth and this phenomenon is termed as ice recrystallization inhibition (Kawahara, 2013). For the very first time these proteins were discovered in the arctic fish and after that they were reported to be present in plants, other fishes, diatoms, and microbes too. *G. Antarctica* is examined to be the first microbe that is secreting these types of proteins when grown in cold environment (Hashim, 2013). When the genome study of the yeast was done it was revealed that they have nine different genes that are responsible for encoding these proteins (GaAFP) all are giving different shapes to the crystals formed by ice (Turchetti, 2011). Each of the genome in this fungi showed low TH activity on p.05-0.08°C and much higher values for the IRI activity (Firdaus-Raih, 2018). The group of AFPs is very distinct in terms of structure of the proteins but they work the same and that is the prevention of the cell from icing or freezing. Horizontal gene transfer is said to be the process that is involved in the evolution of this gene in the organisms to full through

the odd environments (Davies, 2014). It is reported that they are folded into β-helices but these folding are in three different ways (Hashim, 2014).

Membrane fluidity

The role of first barrier to the transfer of nutrients, signaling and energy transduction from environment to the cell or vice versa is always played by the cell during any kind of stress conditions (Siliakus, 2017). When there is a low freezing temperature the cell membrane converts into rigid form and thus causing the inactivation of much type of proteins working in the membrane for transfer of proteins comprising of carrier and transporting proteins (Los & Murata, 2004). Delta-9 and delta-12 are some of the cold adapted fatty acid desaturases that are known to be upregulated at about -12°C temperature. They are reported to be responsible for the addition of the first and second double bond to the structure of the fatty acid if both are present in a cell (Bharudin, 2018). The fatty acid profile shows that masses of the fatty acids are in the form unsaturated fatty acids and the have many double bonds except the oleic acid that are of single double bond chains. Surprisingly, these polyunsaturated fatty acids number is increased by about 1-2% that fuels the fluidness of the cell membrane when the temperature is freezing like -12°C (Bharudin, 2018; Firdaus-Raih, 2018). The same behavior were also reported in many other bacteria like *Shewanella* sp.(GA-22) (Gentile, 2003) and archaea *Methanococcoides burtonii* (Nichols, 2004).

Proteins Associated with Stress

Stressors in the environments can cause stress and it can be of extreme level like they may be uttermost downshift of the temperature, decrease in nutrients availability, radiation, intemperate UV, or can be high osmotic pressure (Maayer, 2014). For competing these stressing environments internally and externally, psychrophiles have to prepare some combating proteins system that can prevent them from these kinds of stresses, and for the purpose many types of proteins are formed by them already comprising of heats shock proteins (HSPs), cold shock proteins (CSPs), cold active enzymes and the molecular chaperons that reinstate the natural structure of the abnormal or denatured proteins combating these stresses (Feller, 2013; Santiago, 2016). In *G.antarctica* the stress proteins that are reported are chaperons, Heat shock proteins

and the peptidyl- prolyl isomerases or (PPIase). While comparing the genome of psychrophilic yeasts with that of the non-psychrophilic yeast we can easily conclude that they do not have any such kind of genome sequence for these stress combating proteins. Four gene are involve in coding of Cold shock proteins, PPIase and six heat shock proteins, and it is reported that these genes are only present in the psychrophilic *P. destructants.* Hence, this can be prove that these proteins are adapted by the psychrophile for the sack of survival inside such kind of environments (Firdaus-Raih, 2018). Eighty nine possible molecular chaperon are also investigated in the microorganisms consisting of TRiC chaperon, heat shock proteins, heat shock protein70, heat shock protein40, heat shock protein20, heat shock protein90, cold shock proteins, AAA proteins, CS- domain proteins, tetra tricopeptide repeat domain proteins and ubiquitins (Yusof, 2015). Moreover, an interesting finding has been brought to the study that *G. Antarctica* produce a unique protein, the very first of its kind named as the expansion protein that helps in the softening and loosening of the cell wall when the cell needs to expand. This expansion occurs in the non-covalent bonds of the glucans matrix and the microfibrils of the cellulose causing the expansion of the cell wall (Nor, 2020).

Psychrophiles or Cold-Loving Microorganisms in Pakistan

Glaciers are the natural habitats for psychrophiles as the temperature ranges for them in the glaciers are always optimum with a little bit of the variation but not much that they cannot survive. In Tibetan plateau situated in the Hindukush-Karakoram-Himalayas mountain ranges known as the third pole covers about 104,850km of the area comprising of the 49,873km in China and about 40,000km both in Pakistan and India, and this is because of these have the highest number of glaciers and the utmost concentration of the ice and snow on them (D'Amico, 2002). These ranges are scarcely scrutinized for the prevalence of communities of microorganisms. Though (Rafiq, 2017) has isolated may of the bacterial strains sampled from the Siachen glaciers in Pakistan. After identification they were found to be the genus of *Pseudomonas, Alcaligenes, Jonthenobacterium, Rhodococcus, Carnobacterium, Arthrobacter, Bacillus, Lysinibacillus, Staphylococcus and Planomicrobium*. While (Rafiq, 2017) has isolated members of the microorganism given by, Actinobateria, Bacteriodetes,

Firmicutes And Proteobacteria. Rongbuk glacier was also investigated by (Shen, 2012) and have reported four main groups of bacteria including actinobacteria, firmicutes, alpha-proteobacteria and gamma-proteobacteria (Shivaji, 2011) has isolated many phyla of the bacteria from another glacier named as Pindari glacier in Himalayas by using the 16S rRNA sequencing gene libraries. The scarcity of and low number of bacterial isolates from such types of environments is due to the dumping of the non-biodegradable waste in utmost quantity (Rafiq, 2017). Inside the abysses of the glaciers the troops have dumped a much higher amount of ammunition waste. In estimation plastics, cadmium, cobalt and chromium comprise about 40% of the total waste that is affecting the Shyok River that connects with the Indus River near Skardu. Drinking and irrigation sources are connected from the Indus (Kemkar, 2006).

Plant Hormones

The term phytohormones or plant hormone are naturally occuring molecules in plants use for signalling and are in eminently smaller concentration. These are having role starting from the smallest embryogenesis (Méndez-Hernández, 2019), moving from the regulation of organ sizes and leading up to the defence against the pathogens (Shigenaga & Argueso, 2016), working in the stress tolerance (Chandra, 2018) and untill the development of the reproductive system in plants (Pierre-Jerome, 2018). Contrasting to the production of hormones in animlas, plants have the capability that each of the cell can produce these hormones. The term phytohormone was used for the first time by Went and Thimann when they published their book in 1937. Coordination of cell division, growth and differentiation are some of the major functions the plant hormone are responsible for (Hooley, 1994). Control of seed dormancy and germenation of the seed are some other aspects that plant hormone are working in (Graeber, 2012). Abscisic acid, gibberellins, ethylene, indole acetic acid, cytokinins and brassinosteroids are some of the dominant hormone that plants produces to control its physiological and biochemical activities and the surprising fact is that these chemicals are also produced by the microbes present in the soil (Finkelstein, 2010; Santner, 2009). These are the only type of hormone that are not nutrients in nature but are rather chemical in nature that are responsible for the influence in growth, development differentiating the cells and tissues (Öpik, 2005). The responsive hormone

concentration for plants is extremely low as much as (10-6-10-5 mol/L) and that is the reason why they were not taken into interest for such a long time and for the first time the in late 1970s the scientists have started to divide them into categories and to study their separate activities and effect on the plant physiology (Srivastava, 2002).

Different types of Plant Hormones

Plants can produce many type of hormones depending upon the physiology and the physiological effect of the hormone on plants. These can be similar by sharing similar physiological impacts but their chemical structure cannot be the same and vary from hormone to hormone. Intially the investigated hormones were of just five major types named as abscisic acid, auxins, brassinosteroids, cytokinins and ethylene (Weier, 1970). Though in afterwhiles the list of these hormones have been expanded and many other identified hormone were also added to them in some are brassinosteroids, jasmonates, salicyclic acid and strigolactones. In addition to them there are many other similar compounds that are yet to be identified as the plant hormones as they are showing same properties but are yet to be classified as the bonafide hormones.

Abscisic Acid

Abscisic acid sometimes abbreviated as ABA or ABA hormone that helps in regulating growth,development and in stress response in plants via inhibition or promotion of plant cell division. One of the major role of this hormone is the defense against bacterial attacks. They can also help in the formation of the lateral shoot in cotyledonary nodes and increase growth of the main shoot in 17days old cultivated plants. They are the most important plant growth inhibitors and their role is the washing in and out of the degraded plants tissue during cold temperature, accomulates in the fruites during its maturation, prevent the germination of the seeds within the fruites during winter seasons and release the dormant seeds from dormancy (Feurtado, 2004).

Auxins

Cell enlargment, bud formation and initiation of the roots are some of the dominant functions that auxins are playing in the plants body. Influencing other hormones like in combination with the cytokinins the are responsible for the control of growth of the root, stem, fruites and converting the stem into flowers (Osborne & McManus, 2005).

During the process of apical dominance auxins can inhibits the growth of the buds and lower down the growth of the stem, and entertain the development of lateral advintitious roots too. In seeds they regulates a specified protein synthesis (Walz, 2002) that is responsible for the development of the flower to the fruits. The most common of the auxins found in the plants is Indole-3-acetic acid.

Brassinosteroids

These hormones were first isolated form the rapeseed in 1979 and are classified in the clas of polyhydroxysteroids, which are the on;y example of the steroid type hormones. The major functions they are performing are gravitropism, cell elongation, resistance to stress and xylum differentiation. Root inhibition and leaf abscission are also the functions of this type of hormone (Grove, 1979).

Cytokinins

Cell division and shoot formation in the plants are the two major steps that cytokinins are looking over in the plants. Delaying senescence, transport of the auxins and internodal lengts are also effected by these types of hormones. After the isolation from the yeast in the start they were named as the kinins. In combination with the auxins they works for major part of the plants life but when they combines with the ethylene they start to promote the abscission of the leaves, flower parts and fruits (Sipes & Einset, 1983).

Ehylene

Varrying from plants to plants ethylene play very important role and is considered as a multifunctional hormone of plants regulating growth and senescence in plants. These processes depends on the concentration, timing of application and specie of plants either to inhibit or promote them. They are alos called the ripening hormones as this gaseous hormone helps in the ripening of the fruits. According to the Greek philosoper Theophrastus, the sycamore fig fruits can even tolerate this hormone and resist ripening until the fruit is wounded by scraing or by the some metal tool (Theologis, 1992). This hormone is formed by the pathway called yang cycle from methionine through the intermediate 1-aminocyclopropane-1carboxylic acid and the pathway was discovered by Shang Fa Yang (D. Adams & Yang, 1979).

Gibberellins

It is a diterpenoid plants hormone from the family of tetracyclic diterpenoid plant hormone with the effect in speeding of the elongation of the dwarfism in plants to elongate faster, promote flowering, help in stem and root elongation and enable plant to grow fruits. Gibberellins were first discovered from a fungi named *Gibberella fujikuroi,* and the researcher who discovered them was a Japanese scientist named Eiichi Kurosawa, that produced some abnormality in the rice plants (Grennan, 2006), but after studying it further the scientists got to know that they were actually produced by the plants too and were playing very much role in the life cycle of plants. In seedling and adult plants the promotion of cell elongation is its main role. Transition from vegetative to reproductive growth and function of the pollens during fertilization are controlled by the gibberellins (Tsai, 1997).

Jasmonates

Due to isolation from the jasmin oil they were named as the jasmonate. They are lipid-based hormones and are responsible for attack of herbivores necrotrophic pathogens (Browse, 2005). The most active of the jasmonates is jasmonic acid that can further be metabolize to methyl jasmonate which is a volatile organic compound in nature. Crosstalk is said to be a signalling pathway in which it interact with other metabolites and show both positive and negative impacts on plants physiology. This hormone is produced by plants for the purpose to help in developmental stage including, pollen development, coiling of tendrils, ripening of fruits, senscence and response to the biotic and abiotic envirnemental factors (Lorenzo & Solano, 2005).

Salicyclic acid

It is a beta hydroxy acid that can naturally be produce by plants. They possess the ability to act as an anti-inflamatory agent and help in the process of exfoliation as an antibacterial agent. They are orderless and can be visuallize as white tan solid when expose to light. Phenolic in nature and of great medicinal interest for man, these

hormone were first extracted from *Salix alba* (white willow bark). Salicyclic acid can be use as precursor of painkiller aspirin. They can also help in defence against the attack of the pathogens like necrtrophic and herbivores. In addition to this the alos reponds to the abiotic stresses like those in droughts, high temperature, heavy metals and osmotic pressure (Rivas-San Vicente & Plasencia, 2011).

Strigolactones

Strigolactone are signalling molecules produced by plants working in two major events during plants development, one is controlling the devlopment of plants and second is the symbiotic association formation between the roots and the microorganisms present near the root nodules. Germinating the parasitic weed named *Striga lutea* was led to the discovery of the strigolactones. During the process it was noted that the roots of the host plant are producing a newly unknown type of chemical that is stimulating its germination (Xie, 2010). Shoot branching inhibition is another role is defined for the strigolactones (Gomez-Roldan, 2008). Other important roles that are played by the strigolactone are the senescence of leaf, phosphate starvation response, salt tolerance and signalling of light (Schausberger, 2018).

Gibberellic acid (GAs)

Gibberellic acid, being a plant growth hormone which stimulates cell division and growth. Several studies have been conducted in order to optimise and reduce its production costs, which might make its utilisation economically viable for various cultivars. It is derived from fungi, bacteria, and plants.

GAs were identified in a fungus called Gibberella fujikuroi, which produces gibberellic acid, also known as GA3, and other GAs commercially.(Jones & Phillips, 1966). Fungi, especially Gibberella fujikuroi, are preferred for GA3 production via submerged fermentation or solid-state fermentation (Potts et al., 1982). Gibberella moniliformis, which affects maize plants, is an additional naturally occurring source of gibberellins which is cultivated commercially for extraction (Hoad, 1995). Other fungi that produce gibberellic acid are Phaeosphaeria sp. (GA,, GA4, GAs and others), Sphaceloma manihoticola and various other species of this particular genus (GA4, GAs and others). Neurospora crassa can produce GA3 in the micrograms per kilogramme of mycelium range. (Aach et al., 1997).

Gibberellic acid occurs in abundance in the growing seed of a peach. Onion bulbs, spinach, and ferns are some other plant sources. In total, 136 distinct gibberellic acid compounds have been discovered in plants, fungi, and bacteria. Many plants have numerous versions of the gibberellic acid hormone, which regulates various aspects of plant growth (Hoad, 1995).

Gibberellins are expected to be found in all vascular plants: GAs are important in reproductive development in lower plants such as lycophytes and ferns (Helliwell, Chandler, et al., 2001), while in higher plants, GA activity has expanded to promote organ growth by increased cell elongation as well as cell division and, in many different species, activation of developmental processes such as seed germination, maturation, and blooming induction (Nelson et al., 2004).

Gibberellin-producing bacteria include Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia, and Bacillus (Helliwell, Sullivan, et al., 2001; Nelson et al., 2004). Fermentations of the microorganisms Rhizobium phaseoli (GA,, GA4, GAs,

GAza) and a species of lipoferum and A. brasilense (GA,, GAs) include nanogram quantities per litre of culture.

There have also been instances of microalgae producing GA3. Seaweeds (macroalgae) are major sources of GA3, and extracts of brown and red algae are available commercially as an alternative for chemical fertilisers (Ghosh et al., 2015).

Significant quantities of GA-like activity have been determined in soil yeasts and bacteria such as Arthrobacter globifomze, as well as in bacterium cultures recovered from pine seedling roots. (Yanni et al., 2001). Many cultures of bacteria, particularly pseudomonads, produced a GA-like substance in quantities ranging from a few micrograms of GAs-equivalents per litre of media. (Singh et al., 2013). Furthermore, the presence of GA-like compounds in many basidiomycetes has been documented. (Farooq et al., 2013; Zaheer et al., 2020).

Gibberellic acid (GAs) from microorganisms:

Different studies on the physiological or ecological role of GA formation, particularly by G. fujikuroi, has been the subjected. GA3 promotes conidial germination and growth of mycelia in G. fujikuroi and Penicillium notatum to a certain extent as evidence has also been presented from the same group (Liu et al., 2007). GAS plays a hormone-like role in fungi, not comparable to higher plants' role. G. fujikuroi secretes growth active GAS into rice, promoting growth, and GA-producing strains of E moniliforme outperform non-producing strains on oat. (Santner et al., 2009).still now , GA concentrations have been detected in cultures of bacteria very low. Whereas in the wild-type strains mostly GA-producing fungi milligrams in milligram per letre. In GA-producing bacteria there are in the amount of nanograms per litre . GC-MS identification of GAi and GA4 has been possible using extracts of axenic cultures of R. phaseoli (Zhang et al., 2020) which, like other Rhizobia or Bradyrhizobia, is known to be essential for nodule formation by its legume host plant. Bioassay reports GAS presence in Rhizobia and Bradyrhizobia species culture liquids. (Jung et al., 2020; Vishal & Kumar, 2018).

Azospirillum soil bacteria live in intimate connection with the roots of grasses and other higher plants and are particularly abundant in tropical environments. Azospirillum

performs dinitrogen fixation and can thus contribute to plant nitrogen requirement to some level. (Kohli et al., 2013). At the moment, determining the physiological role that GAS produced by Azospirillum bacteria in the rhizosphere of a higher plant may have, both in the bacteria and in the higher plant is difficult.

Gibberellic acid from psychrophiles

One of the most crucial direct mechanisms contributing to rapid and persistent soil colonisation, which in turn improves plant growth, is microbial synthesis of phytohormones and phytoregulators (Zhou et al., 2018). More consideration should be given to microorganisms that promote plant growth at low temperatures as well as a wide range of temperatures when selecting microbiological parts of organic fertilisers to be used in climate conditions (Ozimek et al., 2018). Detailed study has been done on Mortierella genus, growing in a wide temperature range of temperatures (Ozimek et al., 2018). In recent years, the microbiomes in cold habitats have been extensively studied, with a focus on culture-dependent and culture-independent approaches (Yadav, 2017) Novel psychrotrophic bacteria, including as Arthrobacter nicotianae, Brevundimonas terrae, Paenibacillus tylopili, and Pseudomonas cedrina, have been discovered in the harsh deserts of the NW Himalayas and have shown multifaceted plant growth promoting (PGP) properties at low temperatures (Yadav et al., 2015). Psychrotrophic PGP microorganisms have shown to promote plant growth either directly through biological nitrogen fixation, solubilization of minerals including phosphorus, potassium, and zinc, production of siderophores and plant growth hormones (Indole acetic acid and gibberellic acid), or indirectly through triggering resistance against plant pathogens (Yadav et al., 2016).

Role of gibberellic acid in plants

Excessive usage and accumulation of poisonous and hazardous industrial products wreak havoc on our soil, air, and water. Among these risks and hazardous compounds, several types of heavy metals that may be found everywhere in nature have serious negative effects on living things (Singh et al., 2013).

Heavy metals reach the plant through many routes (through foliar adsorption, specific element deposition in leaves, and root uptake), and thus contamination with these metals can affect plant structure (Zaheer et al., 2020).

Heavy metal toxicity lowers plant efficiency and slows plant development and production by weakening the antioxidant system. (Farooq et al., 2013).

Traditional measures can be used to reduce contamination from heavy metals and reduce the risk of fruit quality and safety by avoiding heavy metal uptake and movement into different edible portions of plants. To counteract the toxicity of heavy metals in soil, traditional approaches employ a mixture of thermal, physical, and chemical treatments (Liu et al., 2007) However, these methods are too expensive; so, the latest trend in agricultural sciences is to encourage and regulate plant development through the use of plant growth regulators (Santner et al., 2009).

Plant growth regulators such as gibberellic acid, jasmonic acid, and indole acitic acid have been found to increase abiotic stress in plants (Zhang et al., 2020).

Among the numerous PGRs, gibberellic acid (GA3) works as a hormone stimulator, promoting several physiological and biochemical processes in plants (Vishal & Kumar, 2018).

The application of GA3 to carrot plants at various phases of growth enhances leaf growth while inhibiting root growth. Exogenous GA3 treatment produces plant bolting divergence by implementing a short thickened condensed stem, however flowering is rarely initiated (Jung et al., 2020). Gibberellic acid has been shown to boost germination percentage as well as seedling growth while overcoming the preventative effects of salt stress on germination. Hormones made by plants are active elements of the signal cascade that initiates plant stress responses. (GA3) increased abiotic stress tolerance by inducing and raising endogenous salicylic acid levels (Kohli et al., 2013). However, a few studies have shown that foliar pretreatment with GA3 can counteract the negative effects of NaCl (Chakrabarti & Mukherji, 2003). Surprisingly, few evidence exist to demonstrate the critical functions of GAs in elevated temperatures stress response and adaptability, particularly during the reproductive stage. Paclobutrazol (PBZ), a triazole derivative, inhibits GA production in plants by inhibiting kaurene oxidase and preventing kaurene from being converted to kaurenoic acid. PBZ can improve plant resilience to cold, heat, drought, and salt. Triazole-mediated stress protection results

from hormonal changes such as an increase in cytokinins, a temporary increase in ABA, and a decrease in ethylenestress (Soumya et al., 2017). Gibberellic acid (GA3) is being used to increase plant length or height, enhances blossom quantity, and promotes early flowering (Qin et al., 2011). The SPINDLY (SPY) gene was identified by map-based cloning after being detected by EMS mutagenesis screening for mutants resistant to paclobutrazol (PAC), an inhibitor of GA synthesis in plants.(Jacobsen & Olszewski, 1993). The SPY gene expresses throughout the plant and can be found not just in all organs where spy mutant phenotypes have been seen, yet additionally in the roots, indicating that the gene plays a function in root development (Swain et al., 2002). The SPY protein is mostly found in the nucleus, where it alters components of the GA signalling pathway (Swain et al., 2002). It has been proposed that SPY plays a crucial function in GA signalling (Hartweck et al., 2002).

Mechanism of action of gibberellic acid by different pathways in plants

The treatment of germinating seeds with gibberellic acid (GA) increases the activity of many enzymes. (Bawden et al., 1959) study shows Effect of Gibberellic Acid on the Plasticity and Elasticity of Avena Stem Segments (P. A. Adams et al., 1975, p. 1). The degree of the growth stimulation triggered by gibberellic acid in Avena stem segments is likely the most ever observed for excised plant tissue. (P. A. Adams et al., 1973). Treatment of this tissue with GA reduces cell division activity below control level, and thus the hormone-stimulated growth occurs exclusively by cell elongation.

According to a study The regulatory module SnRK2-APC/CTE controls the antagonistic effect of the gibberellic acid and abscisic acid pathways. Recent research has depicted an ABA signaling pathway in which ABA binds to its receptor PYL/PYR/RCARs, then the PYL/PYR/RCAR-ABA complex binds to PP2C phosphatases that repress the SnRK2s, allowing the activated SnRK2s to phosphorylate downstream targets to activate ABA responses. (Cutler et al., 2010).

The receptor GID1 and the E3 ligase SCFSLY1/GID2 work together in the GA signaling pathway to promote the degradation of the DELLA repressor proteins in a GA-dependent manner, therefore relieving their inhibition of GA action. (Ueguchi-Tanaka et al., 2005). Although recent studies have shown that ABA can antagonize GA-promoted degradation of DELLA proteins (Achard et al., 2006) although other

study shows that Effect of gibberellic acid and calliterpenone on plant growth attributes, trichomes, essential oil biosynthesis and pathway gene expression in differential manner in *Mentha arvensis* L (Bose et al., 2013). In seed plants, the repression or activation of gibberellic acid (GA)signalling-dependent processes such as germination, elongation growth and the onset of flowering is to a large extent dependent on the presence or absence of the so-called DELLA repressors (Achard et al., 2008). DELLA repressors are characterized by their conserved N-terminal DELLA domain that is essential for GA-dependent interactions with the GIBBERELLIC ACID INSENSITIVE DWARF1 (GID1) GA receptors (and contains the conserved amino acid stretch D-E-L-L-A) (Hedden, 2003). REPRESSOR-OF-*ga1*-3 (RGA) and GIBBERELLIC ACID INSENSITIVE (GAI) are two (out of five) prominent DELLA repressors in Arabidopsis, SLENDER RICE1 (SLR1) is the only DELLA protein in rice (Djakovic-Petrovic et al., 2007).

Industrial uses of giberellic acid:

Gibberellic acid (GA) is a plant hormone that affects a variety of plant growth and development processes. Although its primary role is in plants, gibberellic acid is also used in a variety of industrial applications. Seeds contain embryos that are unable to grow into plants due to insufficient environmental conditions (Bewley, 1997) The transition from seed dormancy to germination is influenced by physical elements (light, temperature, and moisture) as well as endogenous growth regulating hormones (GA and ABA). GA promotes seed germination, whereas ABA aids in the formation and maintenance of dormancy(Debeaujon & Koornneef, 2000) GA acts in two ways: first, by enhancing embryo development potential, and second, by triggering hydrolytic enzymes. (Ogawa et al., 2003).

A germination-promoting role for GAs has also been deduced from their ability to overcome germination constraints that exist in seeds requiring after-ripenin (Metzger, 1983), light(Hilhorst & Karssen, 1988) and cold. This raised the possibility that such environmental influences could stimulate GA production during the early stages of germination.

It has also been proven that 1-3p.p.m. gibberellic acid accelerates malt modification and gives high laboratory extracts; however, treatments at these levels can result in over-modification and excessive color generation; also, the process is uneconomic. The benefits of gibberellic acid therapy remain significant at the more cost-effective treatment rate of around 0-25p.p.m., but several challenges remain.(Macey & Stowell, 1961).

It is crucial to understand the physiological activities that occur during plant development. Gibberellic acid (GAS) is now the least expensive and most physiologically effective of the gibberellins for commercial use. The economics of using this hormone are based on the fact that it is easily made in large quantities and has a high level of biological activity in a variety of plants that humans value.(Turner, 1972)

Although the use of commercially synthesised gibberellic acid in malting is perhaps the most common commercial application of this natural plant hormone, other notable uses of the hormone in industry include the following.

Grapes For more than a decade, seedless grape growers have acknowledged gibberellic acid as a safe and viable technique of lowering labour costs by managing harvesting time, boosting yields, and enhancing crop quality (Turner, 1972).

Citrus The most common application of gibberellic acid in citrus plantations is to delay the ripening of fruits on the tree(Palmer, 1974). This property of hormones stems from their ability to significantly slow the cellular breakdown associated with skin senescence.

Pears Gibberellic acid is also commonly used in Europe to boost pear crop productivity.(Turner, 1972) As with Clementines, care and judgement are essential when selecting when and how much hormone to spray trees to encourage fruit set in both normal and frost injured trees.

GA4 and GA7 have been shown to be more effective than GA3 in post poning "June drop" in apple cultivars that are particularly vulnerable to this problem. The physiological significance of an exogenous spray of A4 and A7 shortly after bloom set

can be attributed to the fact that both of these gibberellins are naturally produced by the developing apple seed (Mostafa & Saleh, 2023).

Floriculture, sometimes known as "tomorrow's green cultivation," is emerging as a future thrust industry in India. The importance of cut flowers in the international flower industry motivates farmers to start growing them in India. The role of growth regulators in floral crop production a significant part in higher production and quality flowers to market(Verma et al., 2000). Growth regulators for plants Commercial growers of ornamental plants employ them as part of their cultural practise. Plant growth regulators have a faster influence on both vegetative and floral production of flowering plants crops. GA3 at 200 ppm was observed to boost plant height, shoot length, and the maximum number of leaves per plant in rose cv First. A group of scientists investigated the effects of growth regulators on Anthurium andreanum Linden growth and blooming(N. Sangma et al., 2017).

Marine microalgae, which are primitive eukaryotic plant cells, have a strong evolutionary link with plants and are frequently employed to produce high-value products (Salama et al., 2014). The most important plant growth regulators are Indole-3- acetic acid (IAA), Gibberellic acid (GA3), Kinetin (KIN) and Abscisic acid (ABA). There is little or no evidence beyond the effects of Gibberellins in higher plants. Gibberellin was discovered to increase the amount of proteins, monosaccharides, chlorophyll a and b, and total carotenoids in Chlorella vulgaris. (Tate et al., 2013). It also promotes the growth of Chlamydomonas reinhardtii, which is used to produce biofuel. Gibberellins have a positive effect on growth and development. (Yu et al., 2016).

Material and Methods

3.1 Strains Isolation

In the current study the strains used were already isolated from the sample collected from Ghulkin Glacier, Gilgit Baltistan. These strains were then preserved at -20°C and were used for further studies. The whole research was conducted in the Applied Environmental and Geomicrobiology lab (AEG), Department of Microbiology, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad.

Selection of isolates

8 strains GA27,GB29,GB16,GB23,GB3,GA9,GB11,GB19 were selected for screening of gibberellic acid.

3.2 Culturing and Inoculum preparation

All the available strains were refreshed on nutrient agar plates having composition 20g/L and amount of media prepared were 25ml/plate incubated for 48hours. After completion of the incubation time a loop full of colonies picked from the nutrient plate, shifted to nutrient broth and then incubated for 48hours giving strain enough time to grow at a temperature of 15°C and agitation speed of 140rpm (de Oliveira et al., 2017)

3.3 Nutrient broth as Production media

After the completion of the incubation time of the inoculum in broth, the strains were inoculated in the nutrient broth production media having (yeast extract ,peptone and sodium chloride as their basic composition). The media prepared was then sterilized at 121°C for 20minutes in autoclave. 5% inoculum was transferred from the inoculum containing broth production media and were incubated again for 24hours. (de Oliveira et al., 2017)

3.4 Screening and quantitative examination for gibberellic acid

For screening and quantification a method was adapted with slight modification in which 1ml of supernatant was taken from a 24hrs old broth inoculated with each of the selected strains and 200ul. Zinc acetate reagent was added. After 2 minutes 200ul of potassium ferrocyanide was added and centrifugation was performed at 2000rpm for 15 minutes. After that 1ml of supernatant has been taken and 1ml of 30% HCL was added and mixture was incubated at $20C^0$ for 75 minutes. Determination of the quantity

of IAA was done by measuring the absorbance by using (Shimadzu-UV1601) UV-VIS Spectrophotometer at 254nm.(Sharma et al., 2018).

GIBBERELLIC Acid Standard curve

To make a standard curve for the Gibberellic acid, 10ml of stock solution was prepared and then six dilutions of different concentrations were made. After preparing dilutions these were studied for the absorption at a wavelength of 254nm and the results were studied in comparison to the standard curve nm and then standard curve chart was made in the Microsoft Excel sheet as shown in figure 1.

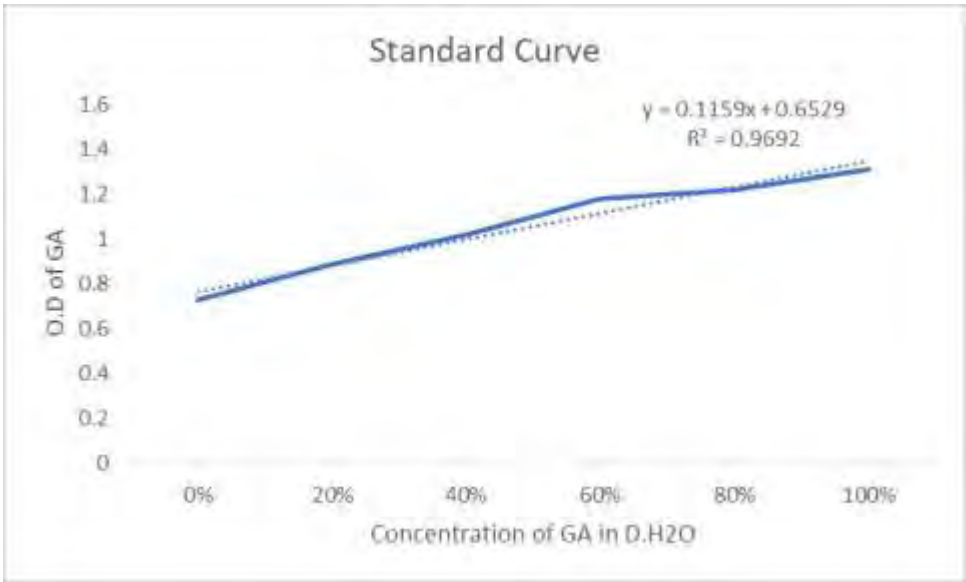

Figure 1 Standard Curve for Gibberellic Acid

3.6 Optimization of Fermentation Condition

To produce maximum amount of gibberellic acid, bacteria must be grown in their optimal conditions. For this purpose, the fermentation conditions optimized were incubation time, temperature and pH concentrations. Strains were cultured separately in shaking incubator samples collected from each flask was further proceeded for estimation.(karakoç & aksöz, 2006)

3.6.1 Effect of Incubation time

Nutrient broth production media was studied for gibberellic acid production for 0-120hours at 15°C and agitation speed of 140rpm in shaking incubator. Eight samples GA27, GB29, GB16, GB23, GB3, GA9, GB11, GB19 at different times were taken from inoculation time, Day1, Day2, Day3, and Day4 to Day5 of incubation. Estimation of protein was then performed to determine the gibberellic acid production (karakoç & aksöz, 2006).

3.6.2 Effect of Temperature

Nutrient broth production media was studied for gibberellic acid production at different temperatures like 5°C, 15°C, 25°C and 35°C with agitation speed of 140rpm in shaking incubator. Samples at different optimum times were taken as on 96 and 120hrs for 8 strains GA27, GB29, GB16, GB23, GB3, GA9, GB11, GB19 Estimation of protein was then performed to determine the gibberellic acid production (Isa & Mat Don, 2014)

3.6.3 Effect of pH

Gibberellic acid production at different pH levels ranging 5, 7 and 9 at a temperature such that 25°C for GB19 and $15c^0$ for GA9, GB16, GB11, GB19 and GB3 with agitation speed of 140rpm in shaking incubator was studied in nutrient broth production media. Samples at 96hours and 120hrs for respective strains were taken. Estimation of protein was then performed to determine the gibberellic acid production(Isa & Mat Don, 2014)

Nutrient broth as carbon source

Nutrient broth contains essential components that act as carbon source for the production of gibberellic acid beef extract and peptone act as carbon source for gibberellic acid production.(Silpa et al., 2018)

3.7 IAA Production and Extraction

To produce more gibberellic acid, all of the optimized fermentation conditions were used, and the same procedure as for screening and optimization was used, but the media's concentration was kept much higher than for screening, amounting to 200ml for each strain in Erlenmeyer's flasks and placed in the shaking incubator for incubation at their respective optimized conditions with the agitation speed of 140rpm.(Sharma et al., 2018).

The process of centrifugation

After incubation, the nutrient broth production media was centrifuged at 8000 rpm for 12 minutes to allow the cell debris and high molecular weight substance in the media to settle to the supernatant at the top for extraction (Silpa et al., 2018)

Bacterial cell debris separation

After operating the product through the centrifuge cycle, we were able to distinguish between the pellet and supernatant using the centrifugation procedure. Following the centrifugation supernatants were collected in separate labelled flasks for each strains .(Kumar & Lonsane, 1990)

Extraction of crude gibberellic acid using a separating funnel

to extract crude gibberellic acid from the supernatant, the supernatant was washed twice with ethyl acetate. 200ml of supernatant was combined with an equal amount of the ethyl acetate was extracted from the supernatant of each strain and maintained in the funnel used for separation for a period of three hours in order for the organic solvent to bind all of the organic debris and allow the inorganic component to settle in the funnel. After 3 hours, the inorganic component was recovered, and the method was repeated, with the organic solvent and crude extract saved. When the process was finished, the extract was left to dry in the open air. For each strain, dried extract was stored at 20°C in 3ml ethanol in a labelled vial for subsequent analysis. The amount of extract was determined by measuring each vial prior to and subsequent to the extract was transferred into them. The gross weight was calculated.(Costa et al., 2018)

Gibberellic acid Characterization

The extract was characterized by the following methods to determine the synthesis of GA by GA9 and Gb16.

Fourier Transform Infra-Red Spectroscopy

The absorbance of the spectrum was used to establish the presence of gibberellic acid in the extract using Fourier transform infrared spectroscopy (FTIR) analysis in the general lab of Department of Microbiology, Quaid-I-Azam University Islamabad. The methanol extract was loaded, and the analysis was performed by altering the transmission mode of the instrument from(400-4000cm)(McGovern et al., 2002).

Nuclear Magnetic Resonance

I establish the presence of gibberellic acid, proton-based NMR or H-NMR was performed at Quaid-I-Azam University Islamabad's Department of Chemistry using a (Bruker's NMR-300) facility. The solvent employed in this technique was D-chloroform since the H-NMR machine gathers spectrum peaks from the vibration generated by the proton present in the chemicals contained in the samples.(Marchettini et al., 2002).

Results

4.1 Optimization Of Fermentation Condition For Gibrallic acid Production

Effect of incubation time

The fermentation process that were optimized were incubation duration, temperature,and ph for *Acinetobacter radioresistens* (GB16)) and *Arthrobacter flavus* (GA9) they were cultivated individually in Erlenmeyer flasks under each of those conditions and incubated in shacking incubators. Samples were taken from each flask and processed for further estimate. The maximum outputs obtained from each phase are explored further below.

The duration of incubation is the most critical component in determining the greatest amount of metabolites produced. The maximum production was shown by *Acinetobacter radioresistens* (GB16) on 96hrs with the amount of 3.891ug/ml while as the maximum production was seen on 120hrs for *Arthrobacter flavus* (GA9) that was 3.947ug/ml, that shows the decrease of 31% at 24hrs ,24% percent at 48hrs ,17 % percent at 72hrs and 31 % at 120hrs of incubation. Similarly, *Arthrobacter flavus* (GA9) shows the decrease of 29% percent at 24hrs, 29 percent at 48hrs ,22 % percent at 72 hours and 18% at 96 hrs of incubation as shown in figure 2 and figure 3.

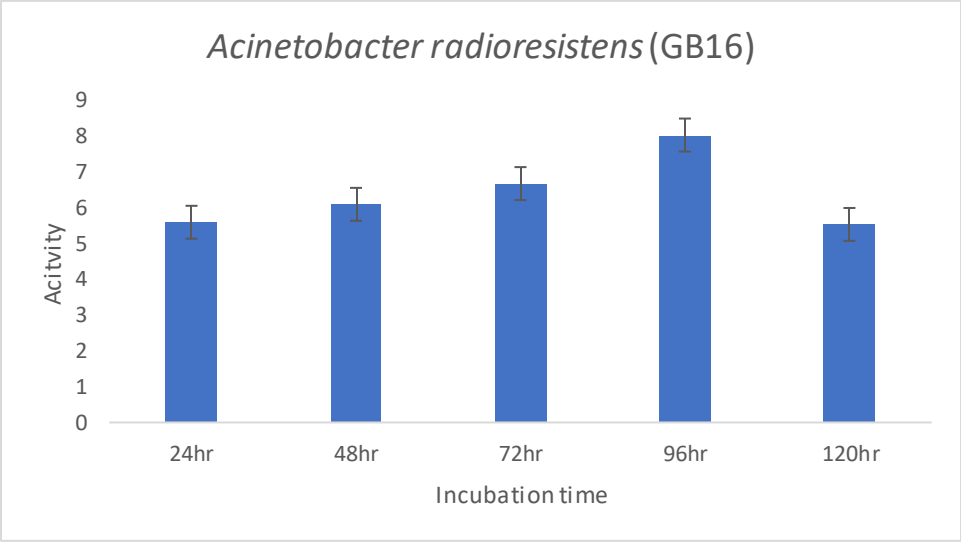

Figure 2 Shows incubation time optimization for *Acinetobacter radioresistens* (Gb16)

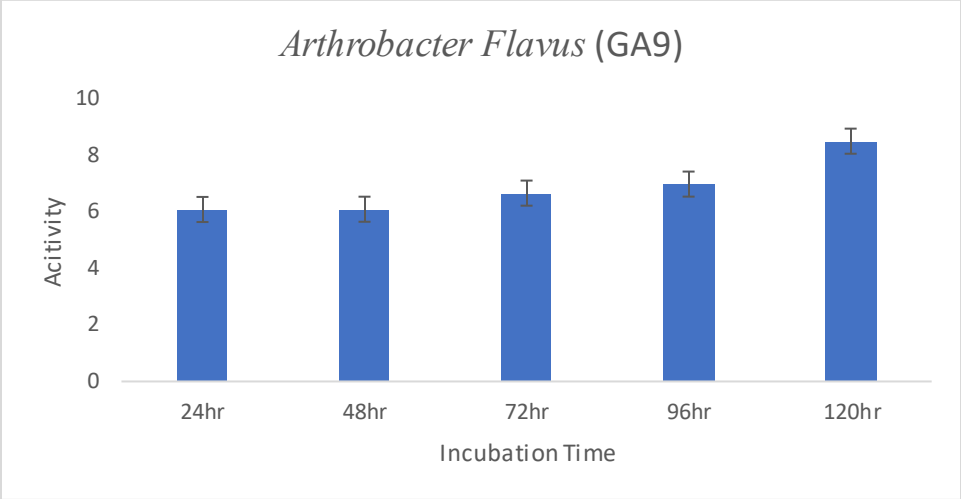

Figure 3 Shows incubation time optimization for *Arthrobacter flavus* (GA9)

Effect of Temperature

Because temperature can influence plant hormone production, growth can be slowed.That is why temperature must be considered as a major element while considering the production of hormones .for this purpose the optimum temperature recorded was 15c for *Acinetobacter radioresistens* (GB16) with the maximum production of 3.532ug/ml by incubating it for 96hrs .same temperature of 15c was seen to be the optimum temperature for Arthrobacter Flavus (GA9).with the maximum production of 3.502ug/ml at the incubation time of 120hrs. the decrease in activity was seen 9% at 5c, 18 % at 25c,while 22%at 35c for *Acinetobacter radioresistens* (GB16),for Arthrobacter flavus (GA9) the decrease in activity was seen 18% at 5c,11% at 25c, and 13% at 35c. This is schematically represented in the following figure 3 and

figure 4.

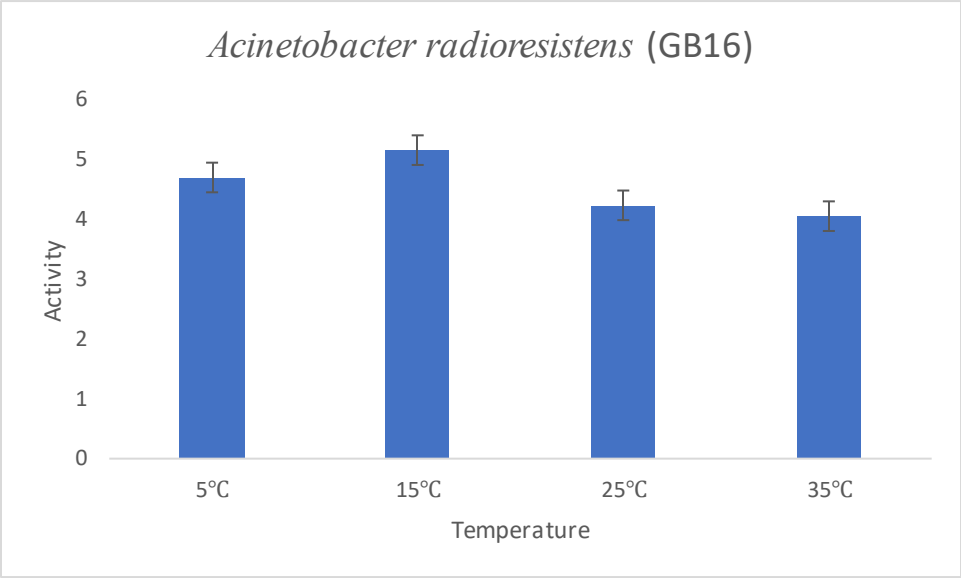

Figure 4 Shows temperature optimization for *Acinetobacter radioresistens* (Gb16)

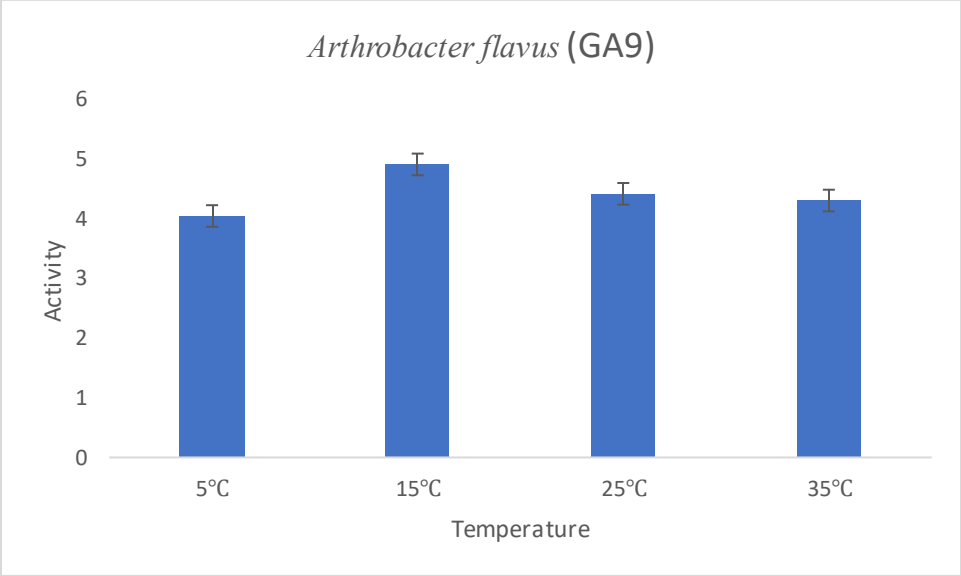

Figure 5 Shows Temperature optimization for *Arthrobacter flavus* (GA9)

EFFECT OF PH

Both acidic and basic environments drastically affects the growth of plants by disturbing their metabolic pathways. That is why optimization was done to find out the

best production pH of media for isolates. At optimum time and temperature *Acinetobacter radioresistens* (GB16) has shown the highest production

with an amount of 3.348µg/ml at pH range of 7. The highest amount of production for ARTHROBACTER FLAVUS (GA9) was 3.352ug/ml at 7 Ph, at ph 7,the decrease in activity was seen for *Acinetobacter radioresistens* (GB16) that was 42%% at ph5 ,while 57% at ph 9 , sameway the decrease was seen upto 54% at ph 5 and 35% at ph 9 for *Arthrobacter flavus* (GA9) as shown in figure 5 and figure 6.

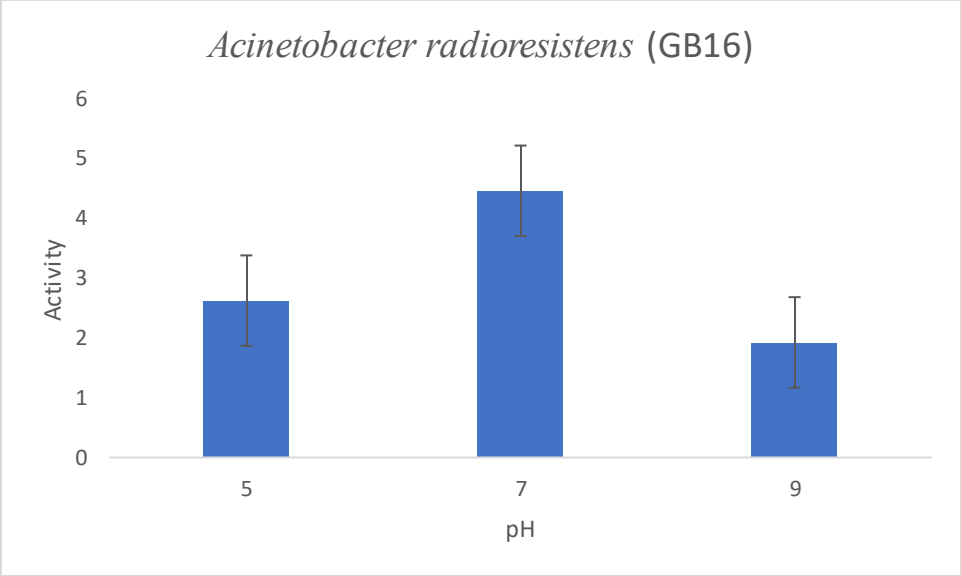

Figure 6 Shows pH optimization for *Acinetobacter radioresistens* (Gb16)

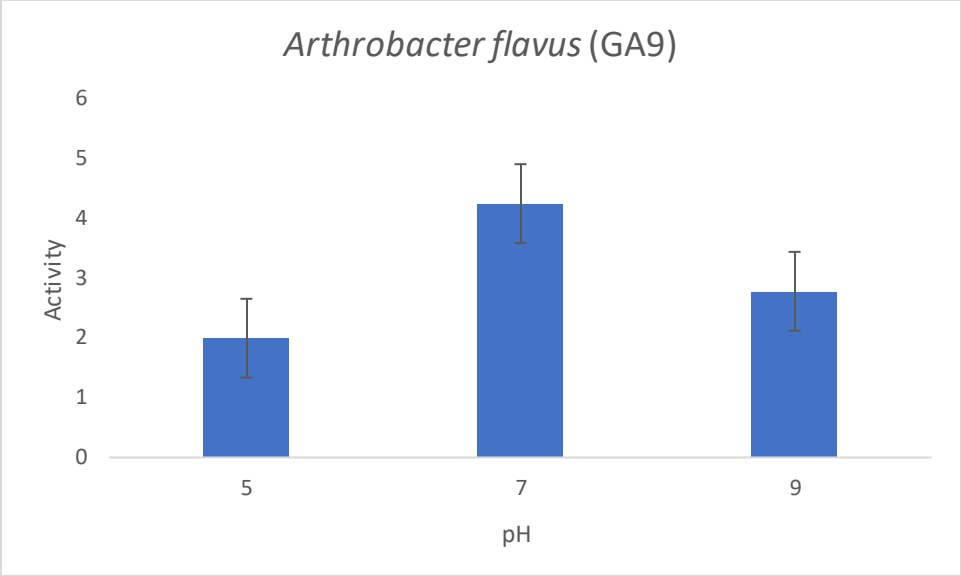

Figure 7 Shows Temperature optimization for *Arthrobacter flavus* (GA9)

Fourier Transform Infra-Red Spectroscopy

The presence of GA was further confirmed by conducting FTIR to show the similarity of bonds on the basis of absorbance and transmission of the IR spectrophotometric waves. It was noted that there were absorbance and transmission peaks at 2973.84cm^{-1} confirming the presence of C-H bonds in standard GA and in both samples. The spectrum with peak 1087 cm^{-1} confirms the C-O stretching present in all the samples loaded with attached methyl group. Stretching of C=O of carboxylic acid can be confirmed by the spectral peak ranging 1046 cm^{-1}. The secondary alcoholic stretching C-O is confirmed with the presence of the spectrum 1117 cm^{-1}. The above-mentioned absorption and transmission spectral peaks confirmed the presence of the GA which was our desired hormone to be produced by the selected strains.

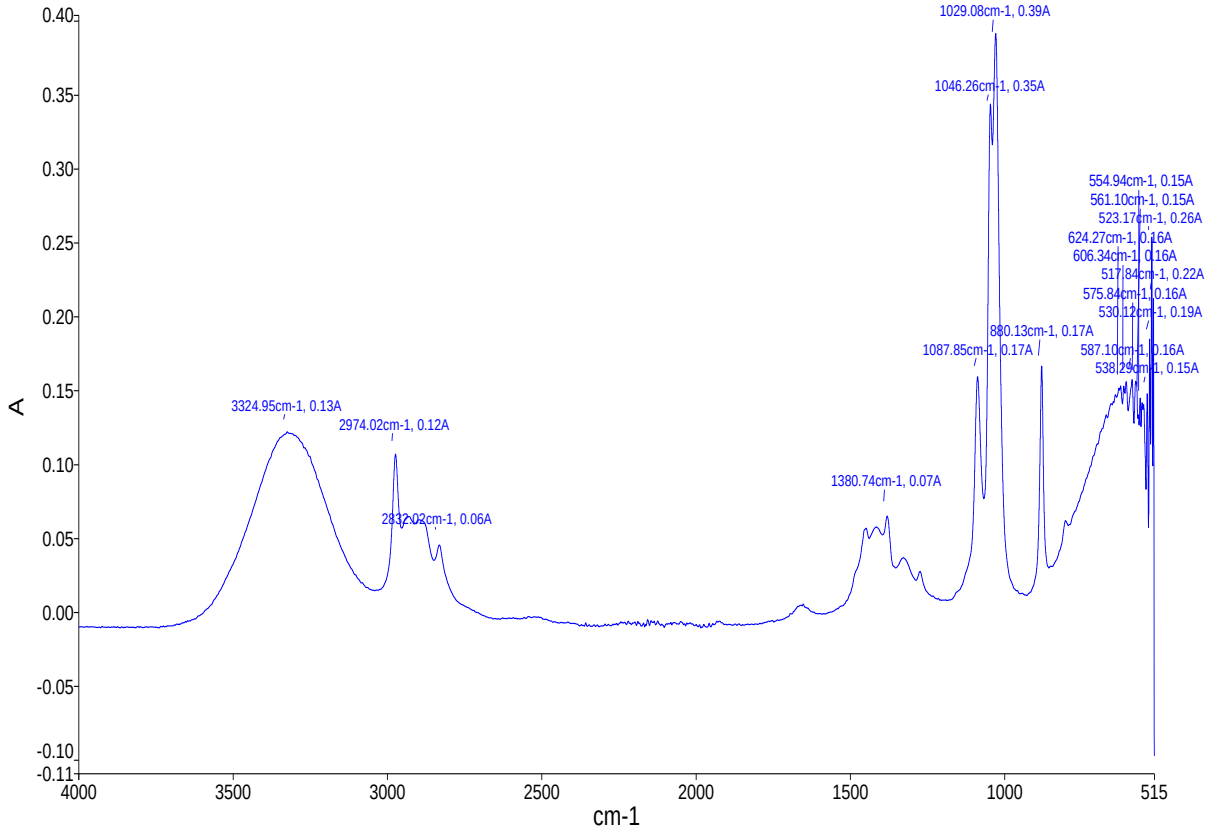

Figure 8 Shows Standard Curve for GA

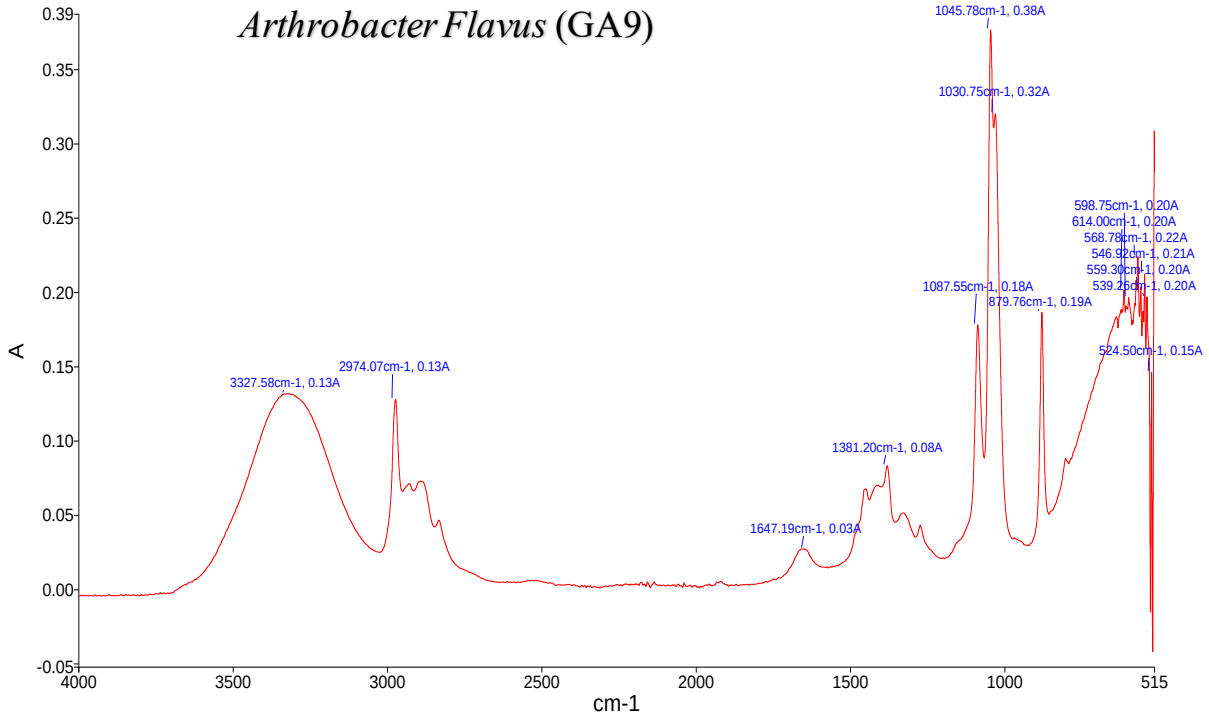

Figure 9 Shows Figure: FTIR analysis of GA produced by *Arthrobacter flavus* (GA9)

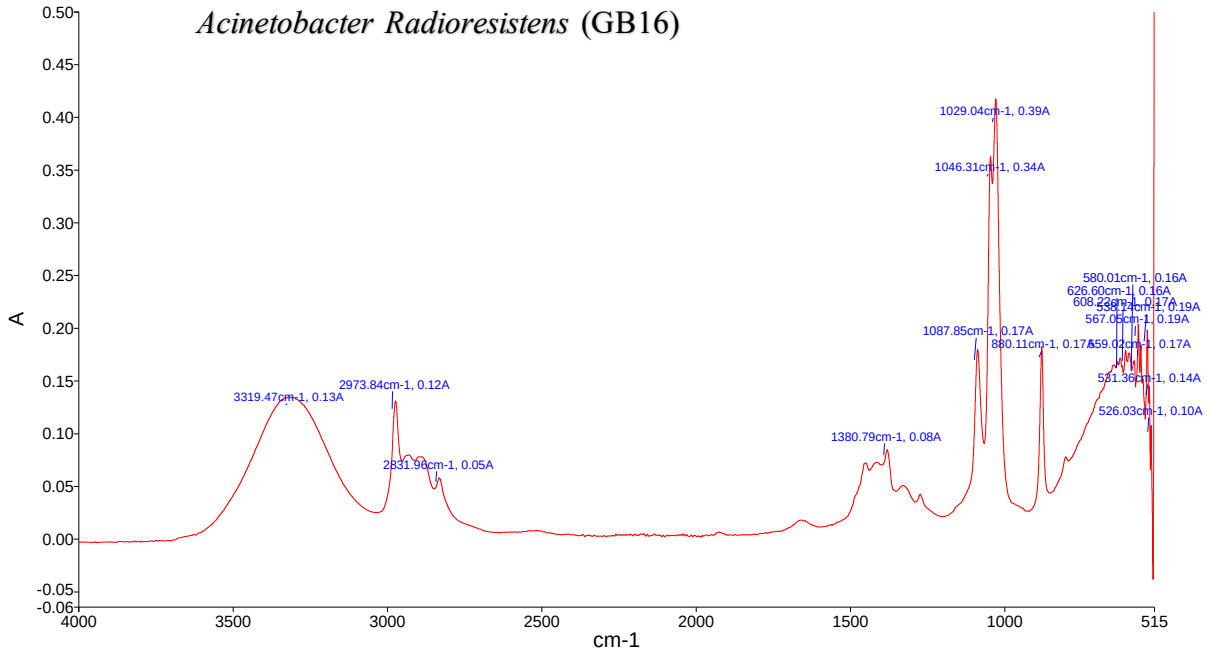

Figure 10 Shows Figure: FTIR analysis of GA produced by *Acinetobacter radioresistens* (GB16)

Nuclear Magnetic Resonance

The confirmation of the presence of GA in the extracts of ACINETOBACTER RADIORESISTENS (GB16) and ARTHROBACTER FLAVUS (GA9) was done by performing the proton-based nuclear magnetic resonance (H-NMR). In which the parts per million (ppm) obtained were ranging in different ppm regions and confirming the presence of different functional groups. As the chemical structure of GA has three different functional groups having proton in bonding with carbon or any other element that is why we have compared the ppm with the reference ppm and obtained our desired results. 5.091ppm shows the presence of methylene group in which the peak was in quartet with the same topology. 4.064-4.109ppm corresponds to the presence of the methine group bonding with the carbonyl group. Chemical shift in the range of 7.367-7.381ppm confirms the presence of aromatic ring.

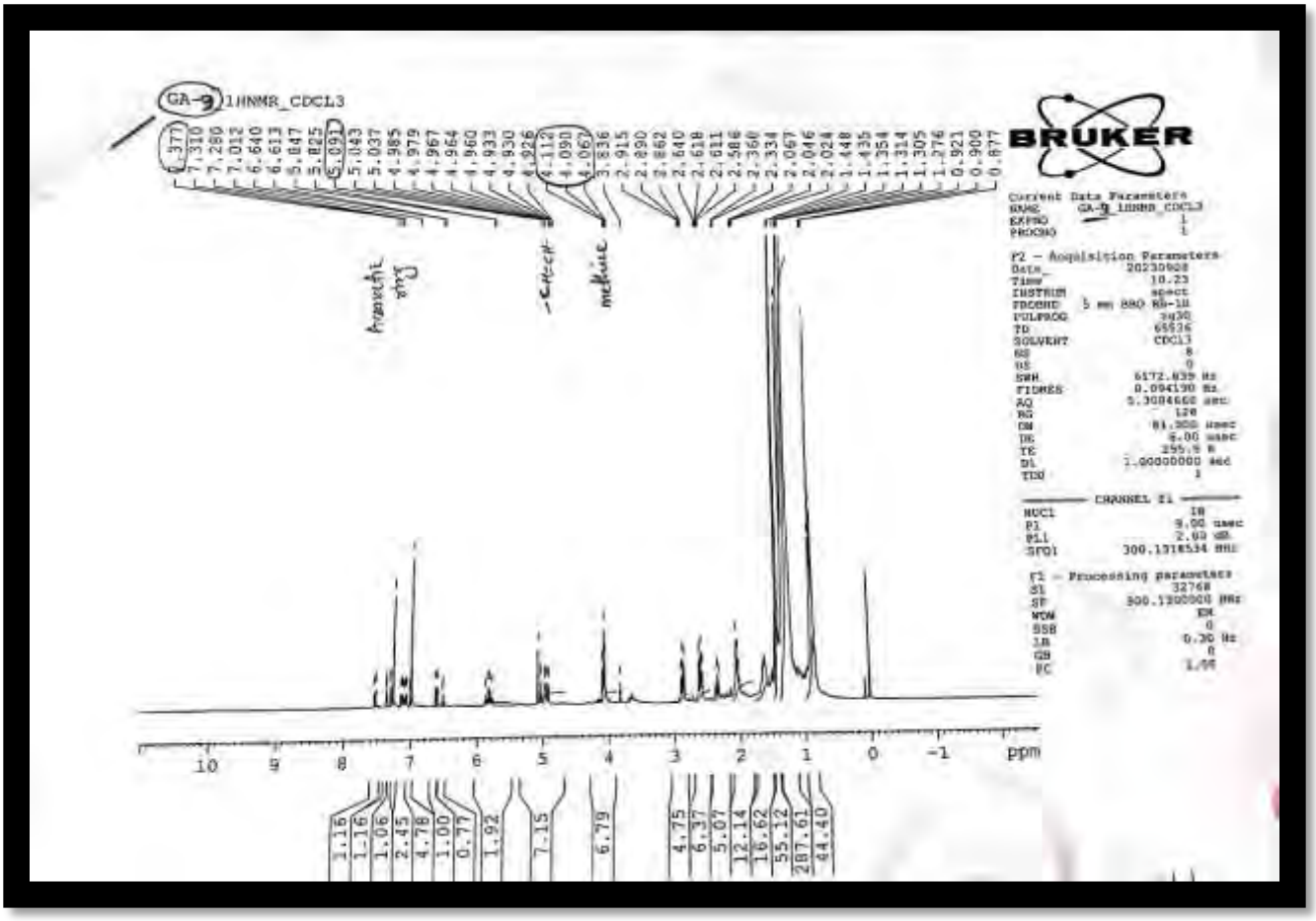

Figure 11 Shows H-NMR analysis of GA produced by *Arthrobacter flavus* (GA9)

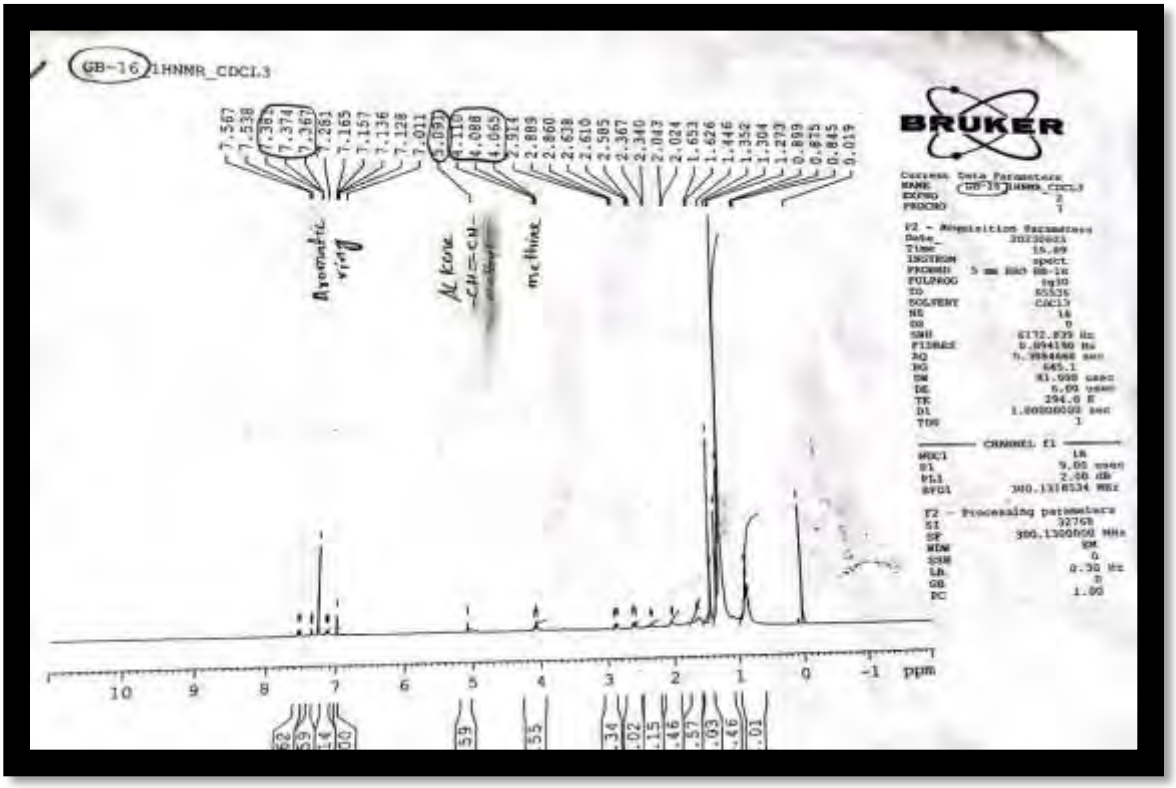

Figure 12 Shows H-NMR analysis of GA produced by *Acinetobacter radioresistens* (GB16)

Discussion

The psychrophilic bacteria are cold-habitable microorganisms. Low temperatures have a deleterious impact on the morpho-anatomical, chemical, functional, and genetic content of plants, reducing crop output. (Vega-Celedón et al., 2021) the use of cold-adapted enzymes can minimize undesirable chemical reactions that can occur at higher temperatures, the enzymes can be rapidly inactivated by heating, and they can be used to transform substrates that require enzyme reactions to be performed at low temperature because substrates are heat-sensitive (cavicchioli et al., 2011). Gibberellic acid is a diterpenoid carboxylic acid that is from the gibberellins family and acts as a natural plant growth hormone.(Camara et al., 2018).as for it is concerned with psychrophiles there is lot of research to be done on gibberellic acid.. Gibberellic acid is reported to be cost effective, easy to produce, low affinity of contamination of the soil and helps to enable plants to grow in cold environments because of the ability to tolerate and resist low temperature. microorganisms are commonly the habitants of the glaciers therefore in Pakistan, the researchers have great opportunity to collect their sample and isolate these strains. Some of them are Batura and Ghulkin glaciers from where the sample can be collected to isolate psychrophilic and psychotropic bacteria and fungi.(Rafiq et al., 2017)

In this study the strains studied for the production of the Gibberellic acid were GB16, and GA9 named after the sample collected from the Ghulkin glacier.

Screening for gibberellic acid Production

Screening of strains to select those that can produce gibberellic acid was done by following the conventional method of culturing on nutrient agar, making inoculum and incubation in nutrient broth production media to produce gibberellic acid. At the end of the completion of the incubation period hormone assay was done that was shown by (Sharma et al., 2018) in which 200ul. Zinc acetate reagent was added. After 2 minutes 200ul of potassium ferrocyanide was added and centrifugation was performed at 2000rpm for 15 minutes. After that 1ml of supernatant has been taken and 1ml of 30% HCL was added and mixture was incubated at $20C^0$ for 75 minutes. Determination of the quantity of GA was done by measuring the absorbance by using (Shimadzu-UV1601) UV-VIS Spectrophotometer at 254nm. Another method shown

by(HOLBROOK et al., 1961)by adding Dilute Hydrochloric Acid, 30%. Zinc Acetate Solution, Phosphate Buffer and zinc acetate reagent. While measuring it on 254nm of wavelength.

Optimization of Parameters for Maximum gibberellic acid Production

The optimization of culture conditions is extremely important for maximum production of gibberellic acid. Production of the GA is dependent upon the growth rate of the bacteria which can be maximum when grows in optimum conditions e.g., Incubation time, temperature, pH etc. Hence various conditions were provided to bacteria to grow and produce maximum amount of the GA. The main aim of this step was to allow bacteria to growth at their optimum conditions and to enhance their production ability. Conventional methods were used for such optimization. The capability of production of organisms depend on selection of successful substrate and growth conditions (Machado et al., 2002). In this study we have grown our strain at different ranges of temperature, pH, and incubation time and samples were taken on 24 -120hrs with an interval of 24hrs each to study the optimum conditions for our strains. The best production was seen on 96 hrs on temperature of $15c^0$ for *Acinetobacter radioresistens* (GB16)) while for *Arthrobacter flavus* (GA9) at temperature of 15 c^0 for 120hrs.as studies been done on *Endophytic Bacillus cereus* shown by (Baliyan et al., 2022).similarly the optimum production of GA was seen on PH7 for both strains while growing in nutrient broth the same study was done on Fusarium moniliform that shows the maximum production on PH 7 (Rangaswamy, 2012).

Extraction of crude gibberellic acid

Ethyl acetate washing is the most effective and commonly followed step for the extraction of the crude GA from the inoculated broth with the selected strains. This process follows the principle of like dissolves like because hormones are hydrophobic in nature and the solvent used is also a hydrophobic and volatile compound. In contrast the liquid part of the supernatant is hydrophilic in nature and that is why the solvent while evaporating drags the extract with itself forming a dense layer of it between the supernatant and the solvent. 200ml nutrient broth media inoculated with microorganisms was centrifuged at 10,000rpm for 12minutes after the completion of

the incubation at optimum condition and supernatant was washed with ethyl acetate twice in the separating funnel. The extract collected was evaporated keeping it in open beaker and was preserved at -20°C by adding 3ml of methanol to each sample. Evaporation of solvent from the extract can also be done by the rotary evaporator at 40°C. The process is also followed by almost all the researchers and used the rotavapor to evaporate the solvent study conducted by .(Qian et al., 2017) used rota evaporator and the dried extract was preserved in 10ml methanol.

Fourier Transform Infra-Red Spectroscopy

FTIR shows similarity of bonds on the basis of absorbance and transmission of the IR spectrophotometric waves. It was noted that there were absorbance and transmission peaks at 2973.84cm^{-1} confirming the presence of C-H bonds in standard GA and in both samples. The spectrum with peak 1087 cm^{-1} confirms the C-O stretching present in all the samples loaded with attached methyl group. Stretching of C=O of carboxylic acid can be confirmed by the spectral peak ranging 1046 cm^{-1}. The secondary alcoholic stretching C-O is confirmed with the presence of the spectrum 1117 cm^{-1}. The above-mentioned absorption and transmission spectral peaks confirmed the presence of the GA which was our desired hormone to be produced by the selected strains. According to the results of FTIR conducted by (Monrroy & García, 2022)shows the result of 2927 cm^{-1} confirm the presence of C-H .the spectrum with peak 1038cm^{-1} correspond to C-O stretching Moreover, the band at 1038 cm^{-1} corresponds to the cellulose (polysaccharide) C–O stretching. While investigating these results shows that the strains used for the production of GA was gibberellic acid producing strains.

Nuclear Magnetic Resonance

In chemistry NMR is a precise analysis to confirm the chemical formula of the compounds either by the vibration of protons or carbon atoms in the compound. In this study proton-based nuclear magnetic resonance (H-NMR) was performed, The confirmation of the presence of GA in the extracts of *Acinetobacter radioresistens* (GB16) and *Arthrobacter flavus* (GA9) was done by performing the proton-based nuclear magnetic resonance (H-NMR). In which the parts per million (ppm) obtained were ranging in different ppm regions and confirming the presence of different functional groups. As the chemical structure of GA has three different functional groups

having proton in bonding with carbon or any other element that is why we have compared the ppm with the reference ppm and obtained our desired results. 5.091ppm shows the presence of methylene group in which the peak was in quartet with the same topology. 4.064-4.109ppm corresponds to the presence of the methane group bonding with the carbonyl group. Chemical shift in the range of 7.367-7.381ppm confirms the presence of aromatic ring. According to study done by the(Yan et al., 2014) the range 5.12ppm shows thee presence of methylene group , 4.85 ppm was observed for methine group attached with carbonyl group confirm the presence of GA in given sample.

CONCLUSION

GB16 and GA9 isolated form the Ghulkin glacier of the Gilgit Baltistan, Pakistan is studied to find out its ability to produce gibberellic acid. Incubating GB16 for 96hHrs while GA9 for 120Hrs to produce the maximum amount of gibberellic acid.GB16 and GA9 at 7 pH and at 15℃ shows the maximum amount of GA production. At the above-mentioned incubation time. Keeping peptone as the carbon source in the nutrient broth media gives rise to maximum production. Crude GA was extracted by the separating funnel in ethyl acetate solvent. The conformation of GA was studied through FTIR spectroscopy and peaks were compared with the standard GA as control, that confer the presence of GA. Moreover confirmation of the GA presence was done by performing H-NMR in $CHCl_3$ that has shown different chemical shifts that corresponds for different types of bonds in GA and all other metabolites present in the extract.

Future prospective

- Gibberellic acid produced by GB16 , and GA9 strains, can further be characterized by their three-dimensional structure.
- Manipulation inside the gene that is responsible for cold-active GA through genetic engineering to obtain hormones with desired properties for industrial uses.
- Different properties of GA produced by GB16, and GA9 strains can also be studied for many other applications, like its anti-pathogenic activity.
- Development of less cost and novel optimization strategies for high production of GA and minimizing the final cost of the hormone through new techniques.

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4433

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