Production of Indole Acetic Acid by Bacteria Isolated from Ghulkin Glacier

By

Naseer Ahmad

Department of Microbiology

Faculty of Biological Sciences

Quaid-I-Azam University

Islamabad, Pakistan

2023

Production of Indole Acetic Acid by Bacteria Isolated from Ghulkin Glacier

A thesis
Submitted in partial fulfillment of the requirements for the Degree
of
MASTER OF PHILOSOPHY

IN
MICROBIOLOGY

DRSM LIBRARY QAU
THESIS & DISSERTATION

By

Naseer Ahmad

Department of Microbiology

Faculty of Biological Sciences

Quaid-I-Azam University

Islamabad, Pakistan

2023

بسم الله الرحمن الرحيم

In the name of ALLAH, Most Gracious, Most Merciful

DEDICATION

Dedicated to
My Beloved Family
and
Friends

Author's Declaration

I ***Mr. Naseer Ahmad*** hereby state that my MPhil thesis titled "***Production of Indole Acetic Acid by Bacteria Isolated from Ghulkin Glacier***" 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.

Mr. Naseer Ahmad

Date: 31-05-2023

Plagiarism Undertaking

"***Production of Indole Acetic Acid by Bacteria Isolated from Ghulkin Glacier***" is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero-tolerance policy of the HEC and Quaid-I-Azam University towards plagiarism. Therefore, I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MPhil degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

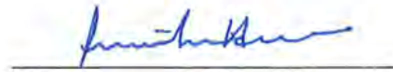
Signature:

Name: Mr. Naseer Ahmad

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled **"Production of Indole Acetic Acid by Bacteria Isolated from Ghulkin Glacier"** was conducted by **Mr. Naseer Ahmad** under the supervision of **Prof. Dr. Fariha Hasan**. No part of this thesis has been **submitted** anywhere else for any other degree. This thesis is submitted to the Department of **Microbiology**, Quaid-I-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Master of Philosophy in field of Microbiology.

Supervisor Name:



Prof. Dr. Fariha Hasan

External Examiner:

Prof Dr. Ghazala Kaukab Raja

Chairperson:

Prof. Dr. Naeem Ali

Dated:26-05-2023

List of Content

S No.	Title	Page No.
1.	List of Content	i
2.	Table of Content	ii-iv
3.	List of figures	v
4.	List of Abbreviations	vi
5.	Acknowledgments	vii-viii
6.	Abstract	ix-x
7.	Chapter 1	1-6
8.	Chapter 2	7-27
9.	Chapter 3	28-33
10.	Chapter 4	34-49
11.	Chapter 5	50-54
12.	Chapter 6	55
13.	Future Prospects	53
14.	References	54-70

Table of Contents

S No.	Title	Page No.
Chapter 1	**Introduction**	
1.1	Introduction	1
1.2	Aim	6
1.3	Objectives	6
Chapter 2	**Literature Review**	
2.1	Extremophiles	7
2.2	Psychrophiles	8
2.3	Physiological adaptations of psychrophiles	10
2.3.1	Antifreeze Protein	10
2.3.2	Membrane fluidity	11
2.3.3	Stress Related Proteins	11
2.4	Psychrophiles of Pakistan	12
2.5	Plant Hormones	13
2.6	Types of Plant Hormones	14
2.6.1	Abscisic Acid	14
2.6.2	Auxins	15
2.6.3	Brassinosteroids	15
2.6.4	Cytokinin's	15
2.6.5	Ethylene	16
2.6.6	Gibberellins	16
2.6.7	Jasmonates	16
2.6.8	Salicylic Acid	17
2.6.9	Strigolactones	17
2.6.10	Indole Acetic Acid	17
Chapter 3	**Materials and Methods**	
3.1	Strain Isolation	28
3.2	Culturing and Inoculum preparation	28
3.3	L-Tryptophan Production Media	28
3.4	Screening and quantitative examination for IAA production	28
3.4.1	Gordon and Weber (1951) method (Colorimetric assay)	28
3.5	Indole Acetic Acid Standard curve	29
3.6	Optimization of Fermentation Conditions	29
3.6.1	Effect of incubation Time	30
3.6.2	Effect of Temperature	30

3.6.3	Effect of pH	30
3.6.4	Effect of L-tryptophan concentration	30
3.6.5	Effect of Carbon Sources	31
3.7	Production and Extraction Of IAA	31
3.7.1	Centrifugation	31
3.7.2	Separation of Bacterial cell debris	31
3.7.3	Extraction of the crude IAA through separating funnel	31
3.8	Characterization of Indole acetic acid	32
3.8.1	Thin Layer Chromatography	32
3.8.2	Fourier Transform Infra-Red Spectroscopy	33
3.8.3	Nuclear Magnetic Resonance	33
Chapter 4	**Results**	
4.1	Optimization of Fermentation Condition for Indole Acetic Acid Production	34
4.1.1	Effect of incubation Time	34
4.1.2	Effect of Temperature	36
4.1.3	Effect of pH	37
4.1.4	Effect of L-tryptophan concentration	39
4.1.5	Effect of Carbon Sources	41
4.2	Thin Layer Chromatography	44
4.3	Fourier Transform Infra-Red Spectroscopy	45
4.4	Nuclear Magnetic Resonance	47
Chapter 5	**Discussion**	
5.1	Discussion	50
5.2	Screening for IAA Production	50
5.3	Optimization of Parameters for Maximum IAA Production	51
5.4	Extraction of the crude IAA	52
5.5	Thin Layer Chromatography	52

5.6	Fourier Transform Infra-Red Spectroscopy	53
5.7	Nuclear Magnetic Resonance	53
Chapter 6	**Conclusion**	55
	Future Prospects	56
	References	57

List Of Figures

S No.	Title	Page No.
Figure 3.1	Standard Curve of IAA	**29**
Figure 4.1	Optimization of incubation time for GB29	**34**
Figure 4.2	Optimization of incubation time for GB3	**35**
Figure 4.3	Optimization of incubation time for GA9	**35**
Figure 4.4	Optimization of Temperature GB29	**36**
Figure 4.5	Optimization of Temperature for GB3	**37**
Figure 4.6	Optimization of Temperature for GA9	**37**
Figure 4.7	Optimization of pH for GB29	**38**
Figure 4.8	Optimization of pH for GB3	**39**
Figure 4.9	Optimization of pH for GA9	**39**
Figure 4.10	Optimization of L-Tryp Concentration for GB29	**40**
Figure 4.11	Optimization of L-Tryp Concentration for GB3	**41**
Figure 4.12	Optimization of L-Tryp Concentration for GA9	**41**
Figure 4.13	Optimization of Carbon sources at (0.1%) for GB29	**42**
Figure 4.14	Optimization of Carbon sources at (0.1%) for GB3	**43**
Figure 4.15	Optimization of Carbon sources at (0.1%) for GA9	**43**
Figure 4.16	Thin Layer Chromatography of IAA produced by bacterial strains GB3, GA9 and GB29 compared with standard	**44**
Figure 4.17	FTIR analysis of Standard IAA	**45**
Figure 4.18	FTIR analysis of GB29	**46**
Figure 4.19	FTIR analysis of GB3	**46**
Figure 4.20	FTIR analysis of GA9	**47**
Figure 4.21	NMR analysis of GB29	**48**
Figure 4.22	NMR analysis of GB3	**48**
Figure 4.23	NMR analysis of GA9	**49**

List of Abbreviations

ABA	Abscisic Acid
ACS2	acetyl-coenzyme A synthetase
AEG	Applied Environmental and Geomicrobiology lab
AFPs	Anti-freeze proteins
ARF	Adenosine diphosphate-Ribosylation Factor
Aux1	Auxin Influx Carrier
CSPs	Chemosensory proteins
FTIR	Fourier transform infra-red spectroscopy
GaAFP	Anti-freeze proteins of G. Antarctica
HCN	Hydrogen Cyanide
HSPs	Heat Shock Proteins
IAA	Indole Acetic Acid
IR	Infra-Red
IRIs	Ice recrystallization inhibition
L	Liter
L-tryp	L-tryptophan
Mol	Mole
ml	Milliliter
Mg/ml	Milligrams per milliliter
nm	Nanometer
NMR	Nuclear Magnetic Resonance
pH	Potential of Hydrogen or negative log of H^+ concentration
R_f	Retention Factor
rRNA	Ribosomal RNA
rpm	Revolutions per minute
TLC	Thin Layer Chromatography
TRiC	Temperature-Related Intensity Change
UV	Ultra-Violet
v/v	Volume by volume
°C	Degree centigrade
µg	microgram

ACKNOWLEDGEMENTS

ALL Admirations to **Almighty Allah**, the supreme, the utmost sympathetic and His **Prophet Muhammad (P.B.U.H)**, the furthermost impeccable human being ever born in the history of earth, Who is an endless foundation of rightest direction and knowledge for all humanity as a whole.

I have great reverence and admiration for my research supervisor **Prof. Dr. Fariha Hasan**, Department of Microbiology, Quaid-I-Azam University, Islamabad for her scholastic supervision, continuous encouragement, sincere criticism, right direction and moral support throughout the study. Her motivated supervision and vibrant support during intact study program made me capable to complete this MPhil research work and Thesis.

I have no words to express my gratitude to my precious senior and Co-supervisor **Dr. Muhammad Irfan**. I am very thankful to him for his great defiance, utmost accommodated behavior, cherished suggestions, impulsive direction, right guidance and moral help.

I would like to mention **Mr. Dr Tanveer Ahmad, Mr. Hamza Abbas** and **Mr. Haseeb Khan.** Both of them were my closest friends and hostel colleagues who helped me overcome all the erroring academic and social life making me stressless to do my research. I express my deep gratitude to my extremely supportive senior lab fellows, especially **Salah Udin Snr, Asim Rehman, Sabir Nawaz, Salahuddin Daoodzai, Ubaid Ur Rehman, Abdur Rasheed Afridi** and **Alam Zeb**. I would like to present my sincerest pleasure thanks to my classmates **Hamid Khan, Muhammad Danish, Muhammad Usman, Zulqarnain Zoulfiqar, Afzal Ishaq** and **Adnan Khan**.

Not to mention friends like **Bilal Ahmed, Nizam Ud Din, Khairullah, Shad Ali Khan, Mubassir Hussain** and **Hamid Ali** will be injustice with the support from them in my hard times of life.

I am unable to find any words express my feelings of obligations for my loving parents. My father **Malak Jan Muhammad Khan** proved the ocean of Love, care for me in which I saturated myself, in fact every aspect of my life is incomplete without him. I convey my Regards and love from the deepest of my heart to my Dearest Brothers **Mansoor Ali, Imran Ali and Basit Ali.** I am utmost gravely grateful to My **Mother** and **beloved Wife** for their persistent exertions offered me to join the higher ideals of life and also for their moral support,

endurance and continuous prayers that they had made for my accomplishment. May ALLAH ALMIGHTY pervade me with the energy to fulfill their principled motivations, anticipations and further enlighten my aptitude.

Abstract

The biomes of low temperature areas specifically northern areas of Pakistan have evolved to be unfriendly for growing crops, because of their chemical and physical architectures. To make these areas cultivatable many types of biofertilizers are in use now days, but the commercial biofertilizers available are less effective in cold climatic conditions. The psychrophilic microbial flora available in the Glaciers of northern have significant potential of producing cold adapted metabolites. Indole acetic acid produced by microorganisms is a good example of biofertilizer produced by natural source, that belong to auxin family of plant hormone and play significant role in growth of plants. The current study aims to screen, optimize, produce, purify and characterize cold adapted bacteria isolated from the Ghulkin Glacier, Hunza Valley, Gilgit Baltistan, Pakistan. A total of 29 strains were screened for the production of Indole Acetic Acid on L-Tryptophan (0.2%) production media. Fermentation conditions for the maximum production of Indole Acetic acid of all selected isolates were optimized for which productions were carried out in L-Tryptophan containing broth media using the optimized conditions. Purifications were done by solvent-solvent extraction method. Characterization of the selected strains were carried out through Thin layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR) and Proton based Nuclear Magnetic Resonance (H-NMR) image was taken. Three strains GB29, GB3 and GA9 showed the production of indole acetic acid. Maximum Indole Acetic Acid production by GB29 were recorded at 5°C, pH 5, L Tryptophan (0.7%) and Maltose as carbon source after 48 hours of incubation. The optimum conditions for the maximum production of Indole Acetic Acid by GB3 were recorded at 35°C, pH 7, L Tryptophan (0.9%) and Maltose as carbon source after 72 hours of incubation. GA9 carried out maximum production at 5°C, pH 7, L Tryptophan (0.7%) and Maltose as carbon source after 72 hours of incubation. The retention factor (R_f) calculated for all the three strains were 0.27 which was equal to R_f value of standard Indole Acetic Acid loaded on TLC plate. The similarity of spectral peaks between standard Indole Acetic Acid and extracted Indole Acetic Acid from the selected isolates confirmed the production of hormone. Further confirmation was done by NMR by finding the functional groups present in the indole acetic acid. These cold adapted strains isolated from the Ghulkin

Glacier samples were good producers of Indole Acetic Acid which would be used as biofertilizers for crop growth promotion in the cold climatic regions of Pakistan.

Chapter 1

Introduction

1.1 Introduction

Microorganisms that inhabit physically or geochemically extreme conditions that are harmful to a large variety of lives on earth are known as extremophiles. In contrast those microorganisms that inhibit much favorable conditions are classified as neutrophils or mesophiles. For the purpose scientists during the years from 1980s-90s have studied hard and have identified that microorganisms have the ability to change according to the environments and can survive in very much hard condition like inhabiting hot, acidic and much colder conditions that can be intolerable for mesophilic microorganisms. Many scientists even believe that hydrothermal vents were the primary habitats for the life under the surfaces of ocean's (Gupta, Srivastava *et al.* 2014). The studies on the extremophiles have advanced so much in the decades of 1990s that a conference was for the first time held in Portugal in 1996 on the topic of extremophile and after than in 1997 there a journal published in the name of "extremophile". International society of extremophile was also coined in 2002 for the purpose to share the newly obtained knowledge about this fast-growing topic. They are having importance in the industries, and this is because of the unique properties they are possessing, one of which is they are attracting the societies towards its application as they are economical for the industry, and they can be less cost effective. Resistance to the highly thermal habitats, chemically denaturing detergents, hydrogen bonding disrupting agents (chaotropic), extreme pH and organic solvents (Gaur, Grover *et al.* 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 and Danson 1999). Extremophiles are of many types on the basis of extreme environments they are happy to inhabit or tolerate, such as acidophiles, alkaliphiles, halophiles, thermophiles, hyperthermophiles and 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.

According to (Schmidt-Nielsen 1902) those microorganisms that cannot only live at a temperature of 0°C but can also replicate and increase their number are called psychrophilic microorganisms, while Horowitz-Wlassowa and Grinberg (Morita 1975) has change the concept and use a word psychrobe for actual psychrophilic microorganisms and use the term psychrophile for those thriving at 0°C and can also live at higher temperatures. In the year 1957 a dictionary in the name of "Dictionary for Microbiology" was formed when they got sufficient amount of data about psychrophilic microorganisms which is defining them as the type of microorganisms who have their ideal growth temperature at 15°C are call 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 and Weyland 2004). Studying surface of the earth it is easy to reveal that 14% of the earth is the polar region and 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). The microorganisms are reported to survive in situ at -30°C and in some prediction their metabolism can also work in -40°C (Price and Sowers 2004). Survival of the microorganisms in such type of environments is possible after adaptations to such environment and psychrophiles have made these adaptations in their cellular bodies, some of which are; Antifreeze proteins production, Membrane fluidity, Stress related proteins (Brown, Lauro *et al.* 2012).

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, Claverie *et al.* 2002). These ranges are scarcely scrutinized for the prevalence of communities of microorganisms. Though (Rafiq, Hayat *et al.* 2017) has isolated may of the bacterial strains sampled from the Siachen glaciers in Pakistan. After identification they were found as the genus of *Pseudomonas, Alcaligenes, Jonthenobacterium, Rhodococcus, Carnobacterium,*

Arthrobacter, Bacillus, Lysinibacillus, Staphylococcus and Planomicrobium (Rafiq, Hayat *et al.* 2019).

Plant hormones or phytohormones are natural products of the plants that effect the physiological status of plants and influence their growth in very small quantity or simply we can say that they are some signaling messengers that helps in growth coordination of the plants (Fleet and Williams 2011). 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 germination of the seed are some other aspects that plant hormone are working in (Graeber, Nakabayashi *et al.* 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 (Santner, Calderon-Villalobos *et al.* 2009, Finkelstein 2010). Though in after whiles the list of these hormones and many other identified hormones contains abscisic acid, auxins, cytokinins, ethylene, brassinosteroids, jasmonates, salicyclic acid and strigolactones (Fahad, Hussain *et al.* 2015).

Indole acetic acid the member of class auxin of plant growth promoting hormone that occures most commonly. It canbe refered as a signalling molecule microorganism because of its effects on the gene expression in microorganisms (Yuan, Liu *et al.* 2008). They can be synthesis by tryptophan dependent or tryptophan independent pathways (Zhao 2010). The pathway that is adapted by the plants is tryptophan dependent through indole-3-pyruic acid (Mashiguchi, Tanaka *et al.* 2011). In plant *Arabidopsis thaliana* the production of indole acetic acid is tryptophan dependent through indole-3-acetaldoxime (Sugawara, Hishiyama *et al.* 2009). Infecting rats with *Trypanosoma brucei gambiense* it was reveal that the production of tryptophan is due the metabolic action performed by both the endogenous and the colonic microbes using the dietry tryptophan as the substrate accompanying tryptophol (Stibbs and Seed 1975).

Gracilaria dura is a macroalgal rhodophyte and *Prionitis lanceolata* is a roseobacter macroalgae that belongs to the family of algae and can produce the bacterially manipulated indole acetic acid in bud induction (Fahy, McGenity *et al.* 2006). Recently it is studied that one of the diatoms named *Pseudo-nitzschia multiseries* also play an important role in the production of the indole acetic acid (Amin, Hmelo *et al.* 2015). Brown macroalgae named *Fucus distichus* and *Ectocarpus siliculosus* are also detected with the production of the indole acetic acid. In the family of the red algae *Pyropia yezoesis* and *Bangia fuscopurpurea* are found to be responsible for the production of the indole acetic acid (Mikami, Mori *et al.* 2016). Plant growth promoting Rhizobacteria were the first to be studied for the production of the IAA via direct method and indirect method (Sarwar, Arshad *et al.* 1992). Many of the non rhizospheric bacteria are studied to be producing IAA as their secondary metabolite (Sadaf, Nuzhat *et al.* 2009).

Indole acetic acid produced by bacteria work as a signaling molecule responsible in changing the auxin pool in plant to either optimal or to super-optimal level and ultimately causing a rapid growth in plants (Iqbal, Wagi *et al.* 2018). IAA plays an extensive role in the growth of the roots and shoots of the plants during the development processes. And all this is because of the movement of the hormone in the plant body by the interference of specific importers some of which are named as *(AUX1)* and efflux pumps *(PIN1-7)* (Prusty, Grisafi *et al.* 2004). The IAA that is produced by bacteria by demanouvering the defense system of the plant making it friendly for bacteria to live in (Spaepen, Vanderleyden *et al.* 2007). Mechanism of action of IAA start with the De novo IAA biosynthesis, the characteristic feature of the homeostasis, followed by the degradation IAA and then heading to the conjugation/deconjugation of IAA with amino acids involve in the activity (Cohen, Slovin *et al.* 2003). Ubiquitination which is a three step post transcriptional modification proceeding with the attachment of the ubiquitin complex with the substrate proteins. Step one is the action of ubiquitin-activating enzymes, second step is the action of ubiquitin-conjugating enzymes and the third step is the recognition of protein by ubiquitin ligases (Tiwari, Hagen *et al.* 2004).

Screening methods used for the detection of IAA from strains of *Rhizobium* isolated form the root nodules of *Acacia cyanophylla* is according to the method mentioned by (Bric, Bostock

et al. 1991). According to this technique the Salkowski's reagent having a composition 1ml of 0.5M $FeCl_3$ and 50ml of 35% $HClO_4$ can be used for the qualitative analysis of the sample during the protein assays in the ratio of 1:2 with the supernatant taken for the production media having L-tryptophan as the substrate for IAA made in the nutrient broth or yeast mannitol broth. One of the methods that is used to determine the production of IAA in the *Pseudomonas putida* where along with reagents used for the qualitative analysis of the IAA production by the bacteria are Salkowski's reagent and orthophosphoric acid. In this technique 2ml of the supernatant form the L-tryptophan production media to be mix the 4ml of the Salkowski's reagent and 25µl of orthophosphoric acid to be added to the mixture and to be incubated for 1hour in the dark and the absorbance to be measure 535nm by using spectrophotometer (Glickmann and Dessaux 1995). . Qualitative study shows that the color detected in the above assays was pinkish or red color confirming the visual analysis and presence of the IAA.

Indole-3-acetic acid a plant growth promoting hormone that can be produce by the microorganisms inclusive of bacteria and fungi. It plays very important role in the interaction between plants and micrbes and hence indirectly effect the growth of the plants. This hormone have a major imapct in the interaction of plants and microbes. They can also play a role as the effector molecule between the IAA producing bacteria, plants and other bacteria (Spaepen and Vanderleyden 2011). Studying the growth of *L.gibba* it was noted to be increased in the range of temperture from 5°C-30°C. While noting the growth rate it was detected that the growth was increase in fresh weight per day over 18days of culture at 15°C and at a temperature of 5°C and 10°C it took 38days to showed an increase in its growth rate in its fresh culture. The small parts of the plants like shoots have a large amount of the resultant free IAA and that leads to the decarboxylation in the seed endosperm. As the comparitive rate of the destructive and formitive IAA become equal and remains in study state depending neither on the tyrptophan nor on the tryptamine as the source of the IAA as the substrate material. In other literature it is also showen that the turnover of IAA at 25°C for $t_{1/2}$ siganls a much lower rate in comparison to all other plants at all other temperatures (Ljung, Ostin *et al.* 2001).

that IAA in large amount leads to the inhibition of seed germination and plant growth (Sindhu, Khandelwal *et al.* 2018). Alongwith these factors another most important factor is that higher

amountof the IAA show a phytotoxic effect and hence leads to the deluge amount of the abscisic acid. This is why IAA production in the industrial scale is not preferable and is limited to the lab scale production by microorganisms for plants (Grossmann 2010). *Rhodosporidiobolus fluvialis* is investigated as a potential IAA producing yeast from class basidiomycetous (Bunsangiam, Sakpuntoon *et al.* 2019). One of the reason behind not to produce IAA at large is the impact of the ratio of product concentration to the cost effect on product in comparison to the medium composition. This media must be adjust at the optimal condition including carbon and nitrogen sources, L-tryptophan as IAA precursor, temperature and pH (Chandra, Askari *et al.* 2018). It is also been investigated for its effect on the humen and horse serum which provid a data that as serum constitues one of its main component that is detoxifying agent and can help in the hydrolysis of the ester (Brown, Kalow *et al.* 1981). The kinetic and behavioral assay of interaction of IAA with both of the samples was done using butyrylthiocholine as substrate but the enzymes in the humen and horse serum varies as there is an extra negative charge on the omega loop in the horse protein and it is as much as effective as triple humen mutant. The results when interpreted it showed that IAA have a time dependent effect and inhibition effect on both the *huBChE* and *eqBChE* serums (Wierdl, Morton *et al.* 2000).

1.2 Aim

To study screening, optimization, production and characterization of indole acetic acid produced by bacteria isolated from ghulkin glacier.

1.3 Objectives:

- Screening of Indole Acetic Acid producing psychrophilic bacteria
- Optimization of fermentation conditions for maximum production of Indole acetic acid by the selected bacterial isolates.
- Production and Purification of IAA by solvent-solvent extraction method.
- Characterization of IAA produced by selected bacterial isolates.

Chapter 2

Literature Review

Literature review

2.1 Extremophiles

Extremophiles, (from Latin *extremus* meaning "extreme" and Greek *philia* means "love") are those microorganisms that inhabit or likely thrive physically or geochemically extreme conditions that are harmful 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, Srivastava *et al.* 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 and 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, Grover *et al.* 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 and 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.

2.2 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 (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 and 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 and Gerday 2003) 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, Jungblut *et al.* 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 (Morita and 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, Soina *et al.* 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, Eicken *et al.* 2004). While in contrast the growth on a temperature of -12°C is not yet reported for these microorganisms (Breezee, Cady *et al.* 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 and Sowers 2004).

2.3 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 and Veldkamp 1971). Some of these adaptations are discussed below.

2.3.1 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, Baardsnes *et al.* 2002). 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, Lee *et al.* 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, Bharudin *et al.* 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, Thomas Hall *et al.* 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, Hashim *et al.* 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, Sulaiman *et al.* 2014).

2.3.2 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, van der Oost *et al.* 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 and 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, Bakar *et al.* 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, Bakar *et al.* 2018, Firdaus-Raih, Hashim *et al.* 2018). The same behavior were also reported in many other bacteria like *Shewanella* sp.(GA-22) (Gentile, Bonasera *et al.* 2003) and archaea *Methanococcoides burtonii* (Nichols, Miller *et al.* 2004).

2.3.3 Stress Related Proteins

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 (De Maayer, Anderson *et al.* 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, Keto-Timonen, Hietala *et al.* 2016, Santiago, Ramírez-Sarmiento *et al.* 2016, Turchetti, Marconi *et al.* 2020, Baeza, Zúñiga *et al.* 2021). 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, Hashim *et al.* 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, Abu Bakar *et al.* 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, Hashim *et al.* 2020).

2.4 Psychrophiles of 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, Claverie *et al.* 2002). These ranges are scarcely scrutinized for the prevalence of communities of microorganisms. Though (Rafiq, Hayat *et al.* 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, Hayat *et al.* 2019) has isolated members of the microorganism given by, Actinobateria, Bacteriodetes, Firmicutes And Proteobacteria. Rongbuk glacier was also investigated by (Shen, Yao *et al.* 2012) and have reported four main groups of bacteria including actinobacteria, firmicutes, alpha-proteobacteria and gamma-proteobacteria. (Shivaji, Pratibha *et al.* 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, Hayat *et al.* 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, Rafiq, Hayat *et al.* 2017).

2.5 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, Ledezma-Rodríguez *et al.* 2019), moving from the regulation of organ sizes and leading up to the defence against the pathogens (Shigenaga and Argueso 2016, Bürger and Chory 2019), working in the stress tolerance (Chandra, Askari *et al.* 2018) and untill the development of the reproductive system in plants(Pierre-Jerome, Drapek *et al.* 2018). Contrasting to the production of hormones in animlas, plants have the capability that each of the cell can produce these hormones (Sochová). 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, Nakabayashi *et al.* 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 (Santner, Calderon-Villalobos *et al.* 2009, Finkelstein 2010). 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, Rolfe *et al.* 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, Eckert *et al.* 2002).

2.6 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, Stocking *et al.* 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.

2.6.1 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, Ambrose *et al*. 2004).

2.6.2 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 and 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, Park *et al*. 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.

2.6.3 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, Spencer *et al*. 1979).

2.6.4 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 and Einset 1983).

2.6.5 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 (Adams and Yang 1979, De Paepe and Van Der Straeten 2005).

2.6.6 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, Lin *et al.* 1997).

2.6.7 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 and Solano 2005).

2.6.8 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 and Plasencia 2011).

2.6.9 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, Yoneyama *et al.* 2010). Shoot branching inhibition is another role is defined for the strigolactones (Gomez-Roldan, Fermas *et al.* 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).

2.6.10 Indole Acetic Acid

Indole acetic acid the member of class auxin of plant growth promoting hormone that occures most commonly. Scientists who are investigating the plant physiology have found it the most interesting and have worked on it extensively generating a vast amount of the knowledge on this hormone (Simon and Petrášek 2011). It is a derivative of the indole having a carboxymethyl substituent and is colorless solid having high solubility in organic solvents as of its solvent nature. Indole acetic acid is mainly produced in the apical parts and mostly in the newly grown leaves of plants. They can be synthesis by tryptophan dependent or tryptophan independent pathways (Zhao 2010). The pathway that is adapted by the plants is tryptophan dependent through indole-3-pyruic acid (Mashiguchi, Tanaka *et al.* 2011). In plant *Arabidopsis thaliana* the production of indole acetic acid is tryptophan dependent through indole-3-acetaldoxime (Sugawara, Hishiyama *et al.* 2009). During the synthesis of IAA by tryptophan dependent pathway L-tryptophan is metabolised to indole-3-acetamide by the help of enzyme tryptophan-2-monooxigenaseandthen by the help of indole-3-acetamide hydrolases this product is metabolised to indole acetic acid (Matsukawa, Nakagawa *et al.* 2007). Infecting rats with *Trypanosoma brucei gambiense* it was reveal that the production of tryptophan is due the metabolic action performed by both the endogenous and the colonic microbes using the dietry tryptophan as the substrate accompanying tryptophol (Stibbs and Seed 1975).

2.7 Sources of indole acetic acid

Gracilaria dura is a macroalgal rhodophyte that belongs to the family of algea and can produce the bacterially manipualted indole acetic acid in bud induction (Fahy, McGenity *et al.* 2006). *Prionitis lanceolata* is a roseobacter another macroalgae that resides the intercellular space between the galls of Fish are said to be involved in the production or the alteration of the process of production in the host cell. The are clades of roseobacter that belongs to the class α-proteobacteria. The amount of theindole acetic acid in these are of extensive amount but it is still a mystery that if it is produced by the bacteria or by the macroalgal host because till now non of them is isolated as an uncontaminated culture (Ashen and Goff 2000). Recently it is studied that one of the diatoms named *Pseudo-nitzschia multiseries* also play an important role in the production of the indole acetic acid

and they are studies as they are having role in the up-regulation of the L-tryptophan synthesis in the responsible algae that can lead to the production of the indole acetic acid the plants and bacteria and in this way it work as sigalling molecule for increasing th rate of the growth in *Pseudo-nitzschia* (Amin, Hmelo *et al.* 2015). Brown macroalgeae named *Fucus distichus* and *Ectocarpus siliculosus* are also detected with the production of the indole acetic acid. In the family of the red algae *Pyropia yezoesis* and *Bangia fuscopurpurea* are found to be responsible for the production of the indole acetic acid (Mikami, Mori *et al.* 2016). *Nitella* from the calss Charophyta having close association with the land plants are investiagted with the production of the indole acetic acid and this is leading to the conclusion that the acestors of the land plants were having this ability of the production of the indole acetic acid. Afterwhile it was been studied that there were some specified type of genes in the genome of the Charophytes that were coding the biosynthetic pathways of the indole acetic acid production (De Smet, Voß *et al.* 2011). Plant growth promoting Rhizobacteria were the first to be studied for the production of the IAA via direct method and indirect method. Alongwith the production of the IAA they are also responsible for the production of the siderophores, HCN and some antimicrobials (Sarwar, Arshad *et al.* 1992). Many of the non rhizospheric bacteria are said to be producing IAA acetic acid secondary metabolite to inhibit the the growth of rhizosphere of variety of plants and this is because of the availability of many different types of substrates for the IAA that are present outside the rhizosphere (Sadaf, Nuzhat *et al.* 2009).

2.8 Indole acetic acid role in plants

Indole acetic is a plant hormone that can be biosynthesis by plants and many microorganisms as discussed with some example in the above mentioned topic. They play an extensive role in the growth of the roots and shoots of the plants during the development processes. And all this is because of the movement of the hormone in the plant body by the interfarence of specific importers some of ehich are named as *(AUX1)* and efflux pumps *(PIN1-7)* (Prusty, Grisafi *et al.* 2004). Gene ACS2 that is responsibel for the production of the IAA production is diagnosed to be inhibited by the IAA produced by the fungi causing wounds in the pea plants, however there is not much amount of knowledge that can suuports

this phenomenon of the IAA of causing wounds in plants but is obvious that it acts as attractant for the fungi that is pathogenic to the pea plants (Peck and Kende 1998). It is investigated that the bacterial IAA acetic acid can help in the demanouvering of the defence system of the host plant that dispiriting the signalling pathway for the IAA and in this way they can show diect resisitive ability to resist the defence of the host plant and live in friedly environment with the plant. This show the negative role of the IAA that is to be produced by the bacteria by demanouvering the defence system of the plant making it friendly for bacteria to live in (Spaepen, Vanderleyden *et al.* 2007). Although bacteria is producing IAA but still there is no such role of the hormone in the bacterial growth, instead it helps the plants to manouver and increase the fitness of the plant in plant-bacterium interaction (Patten and Glick 2002). While in the elongation period of plant growth the overproduction of the root hairs and lateral roots is stimulated by the production of IAA that leads to the removal of the saccharides from the plants cell that is a source of nutrients and is found to be involve in the colonization of the plant growth promoting rhizobacteria (Brandl and Lindow 1998).

2.9 Mechanism of Action of IAA in plants

There are three interdependent pathways that are studied for detecting the activity of the IAA in plants. These pathways are homeostasis, polar transport and auxin response. De novo IAA biosynthesis is the characteristic feature of the homeostasis, followed by the degradation IAA and then heading to the conjugation/deconjugation of IAA with amino acids involve in the activity (Cohen, Slovin *et al.* 2003). The process of IAA action start with the entry of the IAA to the nucleus of the plant cell where it start ubiquitination which is a post transcriptional modification proceeding with the attachment of the ubiquitin complex with the substrate proteins. This is a three steps process in which the substrate protein attaches with proteins of the ubiquitin complex one after the other in a well directed way. First of all they attaches with the E1 known as the ubiquitin-activating enzyme which targets protein through proteosome bonding covalently to the target protein in protein regulation process. The step is followed by the bonding of the substrate protein with the E2 that is ubiquitin-conjugating enzyme which is a short protein having 76 amino acid in it to

bind covalently with the lysine amino acid of the substrate protein. The third step is the recognition of the substrate that is to be bind to the E2 that is has already to be loaded with the ubiquitin molecules which is done by the ubiquitin ligase or E3 protein and in turn the acting mechanis of the IAA boosts up (Petroski and Deshaies 2005). The IAA proteins then binds with the auxin response factor or ARF protein which is a binding factor of TGTCTC in promotors of early auxin response gene resulting in a heterodiamer that in turn cause suppression of the ARF activity (Tiwari, Hagen *et al.* 2004). The role of the IAA signalling was also suggested in stress tolerance while investigating its ihibition ability in the photorespiratory- dependent cell death due to the photorepiratory catalase mutants (Kerchev, MÜHlenbock *et al.* 2015).

2.10 Microbial Indole acetic acid

Plant growth is detected to be enhanced by the production of IAA by the rhizobacteria that worked an important tool in this prospect. Studying the *Acacia cynophylla* for this purpose were investigated with the presence of about eighty different rhizobacterial isolates residing the root nodules of the *Acacia* plant. In this research it was also studied that they produces this hormone as their secondary metbolite by using L-tryptophan as the substrate molecule. *Rhodopseudomonas palutris* and *Dalbergia lanceolaria* were studied to produce IAA at an optimal temperature of 35°C in 2 days incubation time. Further studies showed that the bacterial isolates when were studied for the identification, they were identified as the members of *Paenibacillus, Agrobacterium, Azotobacter chroococcum, Azospirillum brasilense* and *Streptomyces mutabilisa* (El-Shanshoury 1995, Lebrazi, Niehaus *et al.* 2020). When the molecular mechanism of the tumorigenesis was studied, interestingly it was noted that it was coinciding with the pattern of IAA as the plant growth regulator during its recent discoveries at that time which were induced by the *Agrobacterium*. The source of IAA in *Agrobacterium* were shown to be the tumor that was induced by the bacterium and was suuposed to be helpig in growth of the plants till the discovery of the IAA as the plant growth regulator (Link and Eggers 1941). As a very common ability of the PGPB it can be determine that in an utmost number of the bacterial strains there is the ability to produce IAA by adapting either L-tryp dependent pathways or L-tryp independent pathway of IAA

prduction mechanism. *Azospirillum, Pseudomonas* and *Rhizobium* are some common classes of them that are studied for IAA production (Malhotra and Srivastava 2008). For commerical level production many different types of microorganisms were studied in which yeasts were supposed to be the most potent microorganims that can help to produce IAA commercially. For the purpose *Rhodosporidiobolus fluvialis* is said to be the strongest IAA producer for large scale production (Bunsangiam, Sakpuntoon *et al.* 2019). *Chlorella pyrenoidosa* belogs to algae are studeid with the production of IAA, indole propionic and idole butyric acid. The production of these were also detected with the provision of the tryptoamine instead of the tryptophan as a precusore for the production of these auxins (Czerpak, Krotke *et al.* 1999). Among the fungus there are so many species investigated toproduce IAA in which the following are the most common producers of the IAA; *Phanerochaete chrysosporium, Colletotrichum gloeosporioides* and *Aescgynomene. Penicillium sp* is also suggested based the studies that is responsible for the production of IAA (Karthikeyan, Nithya *et al.* 2010). Amongest the exogenous fungi it is been studied that *Funalia trogii* and *Lentinus sajor-caju* are the strongest producers of the IAA (ÜNYAYAR 2000).

2.11 Microbial IAA help in promoting plant growth?

Studying the role IAA produced by the microbial cells in promoting the growth of the plants its is suggested that it have no clear role in enhacing bacterial and microbial growth but can help in enhancing the fitness and health of the plants. So in this regard when it was studied deeply it was detected that when the bacterial cell invade the plants epidermal cell they start solonization inside the root hairs and that leads to the strong growth of the roots in plants in comparison to the absence of the rhizobaterium inside the root hair zones. And when the phenomenon were studied deeply it was detected that there was a mere production of the IAA in the plant roots (James, Gyaneshwar *et al.* 2002). As per the reports the bacterial IAA can loosen up the cell wall of the plant roots cells and making it easier fr bacterial cell to colonize inside the roots that is why the bacterial IAA attract more and more endophytic rhizospheric bacteria to the rootsvof the plants and hence causing more exudation of the roots leading to strengthening of the host plants (Garcia Salamone, Hynes *et al.* 2005). By

increasing the surface area of the plants roots and helping in lengthening of the roots bacterial IAA lead plants to higher acees to the nutrients and more uptake of water (Vessey 2003). One of the benefits to plants is increase in number of nodulation that is cause by the bacterial IAA as they provide more and more active sites to the other PGPRs to bind to the roots and and cause colonization of the bacterial communities (Parmar and Dadarwal 2000). The role of the bacterial IAA is obvious in the strengthening of the roots of plants but it also help in the exapnsion of the cell size in the cotyledon when the seed are in the phase of the germination, however this depends on the amount of IAA production by the endophytic bacteria (Jasim, Jimtha John *et al.* 2014). In the process of transcription the role of the endophytic bacterial IAA is also studied which can enhance the process in the nodules of plant roots where the process of nitrogen fixation occures (Defez, Esposito *et al.* 2016).

2.12 IAA from Psychrophiles

Amongest the biofertilizers IAA is the most commonly produced fertilizer or PGP by the microorganisms either it is bacteria or fungi. The role of the IAA in plants is very special and important as it is phytohormone and helpin the cell division, cell elongation, fruits ripening and in the process of senescence. The initiation of roots, leaves and flower in the plants are alsoby the role of IAA (Phillips, Skirpan *et al.* 2011). Induction of the process of lateral and advintitious roots formation in dicots and monocots respectively are also due to the interference of the IAA (McSteen 2010). Other activites that IAA performs in the plants are the vascular development, cambial growth, fostering of the thikness of the secaondary wall and manipulating the size of the xylem cells (Uggla, Moritz *et al.* 1996). Now as from the literature it is extracted that the production of the indole acetic is not only the characteristic function of the thermophiles and mesophiles but can also be produced by the psychrophiles living in mutual relation with the nodules of plants in cold environments.

2.13 Screening and Detection of IAA

As from the research it is clear that IAA can be produce by bacteria in lab and we can screen those bacterial strain for the production of IAA for which different methods are use in the literature depending upon the concentrations of the supernatants taken for the culture. These

methods also varies for the variation in the type of the strains like for example, screening methods used for the detection of IAA from strains of *Rhizobium* isolated form the root nodules of *Acacia cyanophylla* is according to the method mentioned by (Bric, Bostock *et al.* 1991). According to this technique the Salkowski's reagent having a composition 1ml of 0.5M $FeCl_3$ and 50ml of 35% $HClO_4$ can be use for the qualitative analysis of the sample during the protein assasy in the ratio of 1:2 with the supernatant taken for the production media having L-tryptophan as the substrate for IAA made in the nutrient broth or yeast mannitol broth. After mixing 2ml of the Salkowski's reagent with 1ml of the supernatant it should be incubate in the dark for at least 1hour and then the absorbance to be measure with the spectrophotometr on 535nm wavelength. One of the method that is use to determine the production of IAA by *Pseudomonas putida* where along with reagents used for the qualitative analysis of the IAA production by the bacteria are Salkowski's reagent and orthophosphoric acid. In this technique 2ml of the supernatant form the L-tryptophan production media to be mix the 4ml of the Salkowski's reagent and 25µl of orthophosphoric acid to be added to the mixture and to be incubated for 30minutes in the dark and the absorbance to be measure 535nm by using spectrophotometer (Glickmann and Dessaux 1995). According to the technique used by the (Goswami, Vaghela *et al.* 2013) the supernatant taken from the broth having 1% or 1mg/100ml L-tryptophan as the suplement for the IAA is mixed with the Salkowski's reagent in the ratio of 1:1 and to be incubated for 1hour in the dark and then to measure the absorbance or optical density at 530nm using spectrophotometer. Qualitative study showed that the color detected in the above assays was pinkish or red color confirming the visual analysis and presence of the IAA. Bacterial culture was made in the broth media having 1-2% of L-tryptophan as the suplement for all of the above processes where innoculum of different concentrations were cultured in the the above mentioned medias and and incubated according to the optimal growth time of the respective bacterial strains. After completion of the incubation time sample of different amount were centrifuge at 10,000rpm for 10minutes to take out the supernatant for purpose of further analysis.

2.14 Applications of Cold active IAA

Indole-3-acetic acid a plant growth promoting hormone that can be produce by the microorganisms inclusive of bacteria and fungi. It plays very important role in the interaction between plants and micrbes and hence indirectly effect the growth of the plants. This hormone have a major imapct in the interaction of plants and microbes. They can also play a role as the effector molecule between the IAA producing bacteria, plants and other bacteria (Spaepen and Vanderleyden 2011). Studying the growth of *L.gibba* it was noted to be increased in the range of temperture from 5°C-30°C. While noting the growth rate it was detected that the growth was increase in fresh weight per day over 18days of culture at 15°C and at a temperature of 5°C and 10°C it took 38days to showed an increase in its growth rate in its fresh culture. Similarly studying the number of fronds per day flask to flask showed the same trend in its amount increasing with time in relation to the temperature. But in in normal case if anyone look into the growth of plants he/she can easily conclude that the growth of plants decreases due to the decrease in the temperature like at 5°C and 10°C. But shifting the plants or the temperature for the plants from 5°C and 10°C to 25°C or room temperature the growth increases and it continue to increase till the temprature reaches to 35°C at a time of 4days period but further increase in temperature up to 40°C causes death of the plants in a span of the 2days time (Rapparini, Tam *et al.* 2002). After comparing the growth at a lower temperature of 5°C to 15°C the record showed a correlation with the temperature. But investigating the correlation at 15°C and 20°C there was nothing of any interest noted in the variability correlation at the given two temperatures. Still after the huge variation in the ranges of the temperature there noted two to three different groups formed by the IAA at the given two temperatures in respect of the mgnitude and the indication in the research. But above the temperature level of 20°C it can be noted that there is no such change in the correlation of the IAA with the temperature (Rapparini, Tam *et al.* 2002). The small parts of the plants like shoots have a large amount of the resultant free IAA and that leads to the decarboxylation in the seed endosperm. As the comparitive rate of the destructive and formitive IAA become equal and remains in study state depending neither on the tyrptophan nor on the tryptamine as the source of the IAA as the substrate material. In other literature it is also showen that the turnover of IAA at 25°C for $t_{1/2}$ siganls a much

lower rate in comparison to all other plants at all other temperatures (Ljung, Ostin *et al.* 2001).

2.15 Industrial Applications of the IAA

Among the family of the auxins IAA is the most common that is phytohormone and play an imprtant role in the developmental stages of plant cells including cell division, elongation of the cell, expansion and differentiation of the cell and also in the development of the fruits. Homeostasis of the IAA is very important for maintaining normal and optimal growth but if the process of IAA production face any disturbance it will cause the inhibition of many physiological processes in the plants. From the studies it have been noticed that IAA in large amount leads to the inhibition of seed germination and plant growth (Sindhu, Khandelwal *et al.* 2018). Alongwith these factors another most important factor is that higher amountof the IAA show a phytotoxic effect and hence leads to the deluge amount of the abscisic acid. This is why IAA production in the industrial scale is not preferable and is limited to the lab scale production by microorganisms for plants (Grossmann 2010). According to the previous data present almost every type of microorganism can produce IAA amongest the bacteria, fungi and actinomycetes. In this regards, researchers have use them for large scale production for the purpose of the agricultural use to demoralize the chemical herbicides and use IAA as a safe source as herbicide. *Rhodosporidiobolus fluvialis* is investigated as a potential IAA producing yeast from class basidiomycetous (Bunsangiam, Sakpuntoon *et al.* 2019). One of the reason behind not to produce IAA at large is the impact of the ratio of product concentration to the cost effect on product in comparison to the medium composition. This media must be adjusted at the optimal condition including carbon and nitrogen sources, L-tryptophan as IAA precursor, temperature and pH (Chandra, Askari *et al.* 2018). IAA is been investigated for its effect on the humen and horse serum which provid a data that as serum constitues one of its main component that is detoxifying agent and can help in the hydrolysis of the ester (Brown, Kalow *et al.* 1981). The kinetic and behavioral assay of interaction of IAA with both of the samples was done using butyrylthiocholine as substrate but the enzymes in the humen and horse serum varies as there is an extra negative charge on the omega loop in the horse protein and it is as much as

effective as triple humen mutant. The results when interpreted it showed that IAA have a time dependent effect and inhibition effect on both the *huBChE* and *eqBChE* serums (Wierdl, Morton *et al.* 2000).

Chapter 3

Materials and Methods

Materials 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.

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 (Lebrazi, Fadil *et al.* 2020).

3.3 L-Tryptophan Production media

After the completion of the incubation time of the inoculum in broth, the strains were inoculated in the L-tryptophan production media as L-tryptophan is a precursor of IAA via bacterial production. The media prepared was composed of 0.2% L-tryptophan in nutrient broth and sterilized at 121°C for 20minutes in autoclave. 5% inoculum was transferred from the inoculum containing broth to the L-tryp production media and were incubated again for 48hours (Phetcharat and Duangpaeng 2012).

3.4 Screening and quantitative examination for IAA production

Estimation of IAA production was conducted by using a standard method, as given below:

3.4.1 Gordon and Weber (1951) method (Colorimetric assay)

For screening and quantification this method was adapted with slight modification in which 1ml of supernatant was taken from a 48hrs old broth inoculated with each of the selected strains and 2ml of Salkowski's reagent was added to it. In addition, we added 25µl of

orthophosphoric acid and incubated the solution for 30minutes in dark at room temperature. The color detected after the incubation was pink that indicates the presence of IAA. Determination of the quantity of IAA was done by measuring the absorbance by using (Shimadzu-UV1601) UV-VIS Spectrophotometer at 530nm and the results were studied in comparison to the standard curve.

3.5 Indole Acetic Acid Standard curve

To make a standard curve for the IAA, 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 530nm and then standard curve chart was made in the Microsoft Excel sheet.

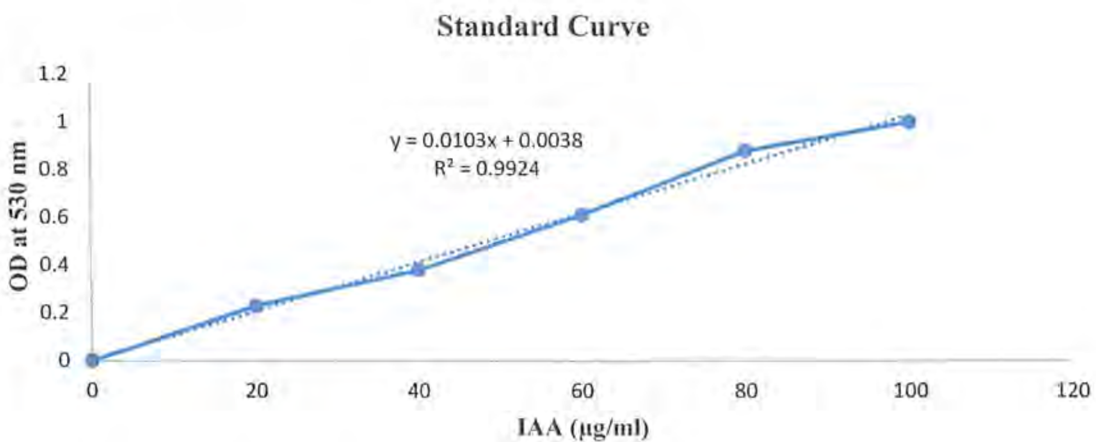


Figure 3.1. Standard Curve of IAA

3.6 Optimization of Fermentation Condition

To produce maximum amount of IAA, bacteria must be grown in their optimal conditions. For this purpose, the fermentation conditions optimized were incubation time, temperature, pH, L-tryptophan concentrations, and carbon sources at 0.1%. GB3, GB29 and GA9 were cultured separately in Erlenmeyer flaks at each of these conditions and were incubated in shacking incubators. Samples were collected from each of the flask and were processed for further estimation (Patten and Glick 2002).

3.6.1 Effect of Incubation time

L-tryptophan production media was studied for IAA production for 0-120hours at 15°C and agitation speed of 140rpm in shaking incubator. Six samples 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 indole acetic acid production(Patten and Glick 2002).

3.6.2 Effect of Temperature

L-tryptophan production media was studied for IAA 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 48hours for GB29 and 72hours incubation for GB3 and GA9. Estimation of protein was then performed to determine the indole acetic acid production(Patten and Glick 2002).

3.6.3 Effect of pH

IAA production at different pH levels ranging 5, 7 and 9 at a temperature of 5°C for GB29 and GA9 and 35°C for GB3 with agitation speed of 140rpm in shaking incubator was studied in L-tryp production media. Samples at 48hours for GB29 and 72hours for GB3 and GA9. Estimation of protein was then performed to determine the indole acetic acid production (Chandra, Askari *et al.* 2018).

3.6.4 Effect of L-tryptophan concentration

L-tryptophan production media was studied for IAA production at different concentrations of L-tryp ranging 0.1%, 0.2%, 0.5%, 0.7%, 0.9% with optimum pH level 5 for GB29 and pH level 7 for GB3 and GA9 at a temperature of 5°C for GB29 and GA9 and 35°C for GB3 with agitation speed of 140rpm in shaking incubator. Samples at 48hours for GB29 and 72hours for GB3 and GA9. Estimation of protein was then performed to determine the indole acetic acid production (Chaiharn and Lumyong 2011).

3.6.5 Effect of Carbon concentration

IAA production in L-tryp production media containing various sources of carbon which were glucose, sucrose, fructose and maltose at a concentration of 0.1% with an optimum concentration of L-tryp, 0.7% for GA9 and GB29, 0.9% for GB3 with optimum pH level 5 for GB29 and pH level 7 for GB3 and GA9 at a temperature of 5°C for GB29 and GA9 and 35°C for GB3 with agitation speed of 140rpm in shaking incubator. Samples at 48hours for GB29 and 72hours for GB3 and GA9. Estimation of protein was then performed to determine the indole acetic acid production (Chandra, Askari *et al.* 2018).

3.7 Production and Extraction of IAA

To produce IAA in higher amount all of the optimized fermentation conditions were applied and the same procedure was adapted, as adapted for screening and optimization process but the media concentration was kept much higher than that for the screening amounting 500ml for each strain in Erlenmeyer's flasks and were kept in the shaking incubator for the incubation at their respective optimized conditions with the agitation speed of 140rpm (Chaiharn and Lumyong 2011). After the completion of incubation, extraction was done in the following steps:

3.7.1 Centrifugation

After the completion if the incubation the L-tryp production media was centrifuged at a speed of 10,000rpm for 12 minutes so that the cell debris and the high molecular weight substance in the media settle down to the supernatant at the top for extraction (Widawati 2020).

3.7.2 Separation of bacterial cell debris

The process of centrifugation has enabled us to separate the pellet and supernatant of the media after passing the media through the centrifuge cycle. So after centrifugation, supernatants were collected in separate labeled flask for each strain (Panigrahi, Mohanty *et al.* 2020).

3.7.3 Extraction of the crude IAA through separating funnel

To extract the crude IAA from the supernatant two times washing was done for the supernatant with ethyl acetate. In which 200ml of the supernatant was mixed with equal amount of the

ethyl acetate from the supernatant of each strain and was kept in the separating funnel for three hours to allow the organic solvent to bind all the organic debris with it and let the inorganic part settle down in the funnel. After 3 hours' time the inorganic part was collected, and the process was repeated and the organic solvent along with the crude extract was kept preserved. When the repeating process was completed, the extract was kept in open air allowed to dry. Dried extract was preserved for further analyses at 20°C in 3ml methanol in labelled vial for extract of each strain. The extract was measured by measuring the vials before and after the transfer of the extract into it. The net weight was calculated for the weight of the extract (Panigrahi, Mohanty *et al.* 2020).

3.8 Characterization of Indole acetic acid

For the confirmation of the production of IAA by GB3, GA9 and Gb29 the extract was characterized by the following processes.

3.8.1 Thin Layer Chromatography

Thin layer chromatography or (TLC) is a purification process of the crude proteins in which silica gel or aluminum oxide coated layer was used to run the extract in the solvent on it and separately observe the bands formed by different components in the extract. The same principle was adapted to separate the components of the extract. The stationary phase chemically silica gel coated on aluminum plate used was readymade provided by the university supplier. It was cut according to the sample size which was 2µl from each extract and a standard IAA of the same size also loaded to calculate the retention factor (R_f) value for the exact location of the IAA. Standard IAA usually is solid in nature so it should be converted to liquid state by adding methanol making the exact dilution as that is of our extract. For this purpose, 1.56mg IAA was added to 1ml of methanol and the same dilution was made for the extract samples with 1.56mg/ml. The plate taken was 12cm high with 1.5cm sample loading and solvent cutoff lines each. Samples were loaded at a spacing of 1cm each so adding standard IAA we were having four samples and the plate width taken was 5cm. After loading the samples, the plate was kept for 5 minutes to make it air dried. Mobile phase for this process was a mixture of three different organic solvents consisting of chloroform, ethyl acetate and formic acid all of these were taken in the ratio of 77:22:1 v/v respectively (Bishnu, Dhurva *et*

al. 2020). When all the preparations were done the mobile phase solvent mixture were transferred to a beaker and the plate were kept vertically and gently so that the mobile phase started movement uniformly and carried the sample with uniform speed from all points. The beaker was kept covered with aluminum foil so that the solvent would not evaporate out of the beaker. Left the chamber for about 50 minutes to let the mobile phase move above the stationary phase until the cutoff line reached. The plate was then allowed to dry before visualization. Visualization was performed with the UV visualizer with 254nm wavelength (Rahman, Sitepu *et al.* 2010). Retention factor was calculated for each component visualized using the formula.

Retention factor (R_f) = distance travelled by chemical of interest / distance covered by solvent

The values were compared with that of standard IAA and the results were confirmed (Parvin, Rahman *et al.* 2020).

3.8.2 Fourier Transform Infra-Red Spectroscopy

Fourier transform infra-red spectroscopy (FTIR) analysis for the samples processed through TLC was done with (Perkin Elmer Spectrum 64) in the general lab of Department of Microbiology, Quaid-I-Azam University Islamabad to confirm the presence of IAA in the extract by the absorbance of the spectrum. The extract preserved in the methanol solvent were loaded and the analysis was done by adjusting the transmission mode of the instrument from 400-4000cm^{-1} (Panigrahi, Mohanty *et al.* 2020).

Nuclear Magnetic Resonance

To confirm the presence of IAA, proton-based NMR or H-NMR was done in department of chemistry, Quaid-I-Azam university Islamabad with (Bruker's NMR-300) facility. In this process the solvent used were D-chloroform because in H-NMR the machine collects the spectral peaks from the vibration of the protons present in the compounds present in the samples. The peaks that can confirm the presence of IAA in H-NMR were of aromatic rings, methines and methylene groups (Chitra, Selvi *et al.* 2019).

Chapter 4

Results

Results

4.1 Optimization of Fermentation Condition for Indole Acetic Acid Production

The fermentation conditions optimized were incubation time, temperature, pH, L-tryptophan, and carbon Source. GB3, GB29 and GA9 were cultured separately in Erlenmeyer flaks at each of these conditions and were incubated in the shacking incubators. Samples were collected from each of the flasks and were processed for further estimation. The maximum productions detected in each phase are discussed below.

4.1.1 Effect of Incubation time

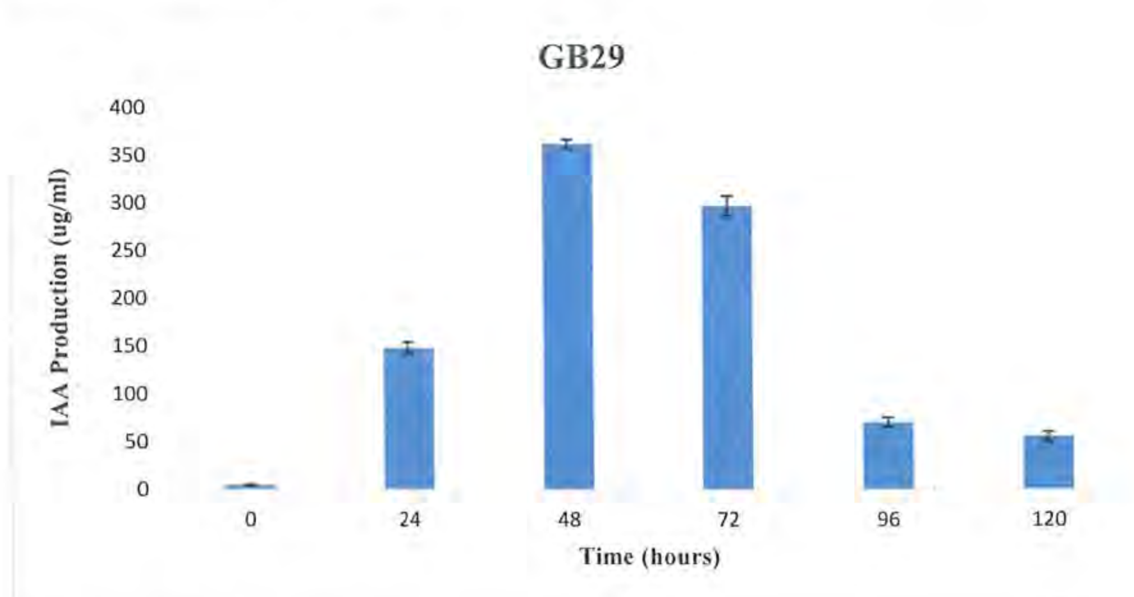


Figure 4.1. optimization of incubation time for GB29

The incubation period is the most important factor for the estimation of maximum quantity of metabolites production. The maximum production of IAA was detected at 48hrs of incubation for GB29 with an amount of about 361.83μg/ml and showed the decrease of about 94%, 81%, 32%, 69% and 75% respectively in production at 0hrs, 24hrs, 72hrs, 96hrs and 120hrs. The maximum production with amount of about 116.23μg/ml of the IAA was detected at 72hrs of incubation for GB3 and the decrease of about 87%, 53% and 87% respectively at 0hrs, 24hrs, 48hrs and both 96hrs and 120hrs was detected. The same 72hrs of incubation time was

detected as the optimum time for GA9 to produce maximum amount of about 134.74μg/ml IAA with a decrease of about 94%, 81%, 32%, 69%, and 75% in production at incubation times 0hrs, 24hrs, 48hrs, 96hrs and 120hrs, respectively.

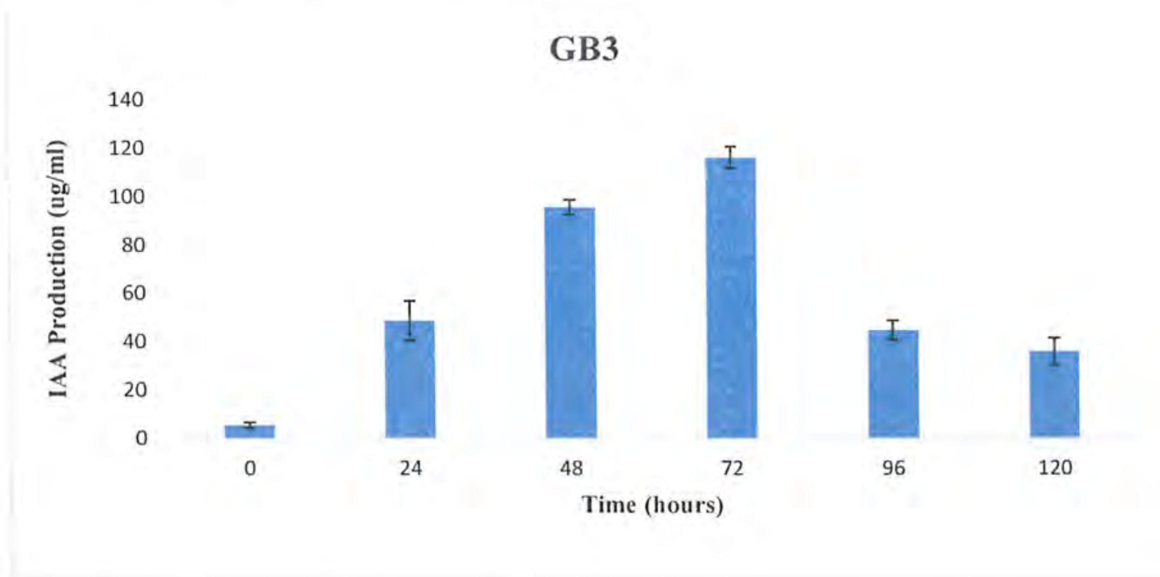


Figure 4.2. optimization of incubation time for GB3

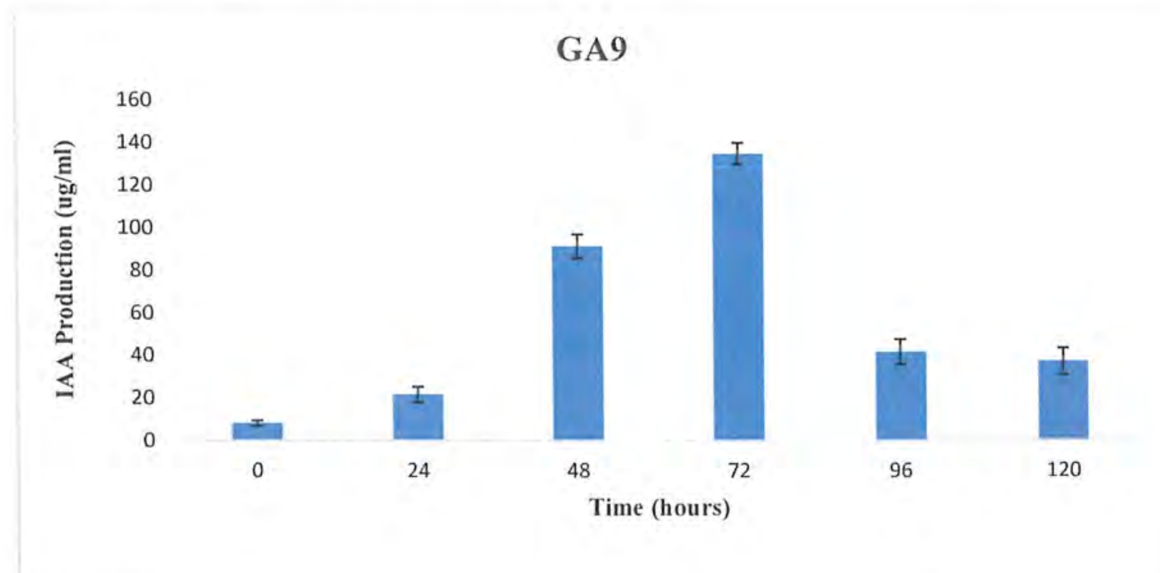


Figure 4.3. optimization of incubation time for GA9

4.1.2 Effect of Temperature

Temperature can affect the production of plant hormone and that is why growth can also retard. For this purpose, the optimum temperature detected to be 5°C for strain GB29. The highest amount of 350.44µg/ml IAA production was estimated with a decrease of about 93%, 97% and 87% in production at 15°C, 25°C and 35°C respectively when incubated for 48hours. GB3 showed the highest amount of production at 35°C with an amount of 130.14µg/ml having a percent decrease of about 91%, 88% and 84% at 5°C, 15°C and 25°C respectively. 5°C was also the optimum temperature for strain GA9 to produce about 223.48µg/ml showing decrease in production at 15°C, 25°C and 35°C with 92%, 96% and 91%. Both GB3 and GA9 were incubated 72 hours.

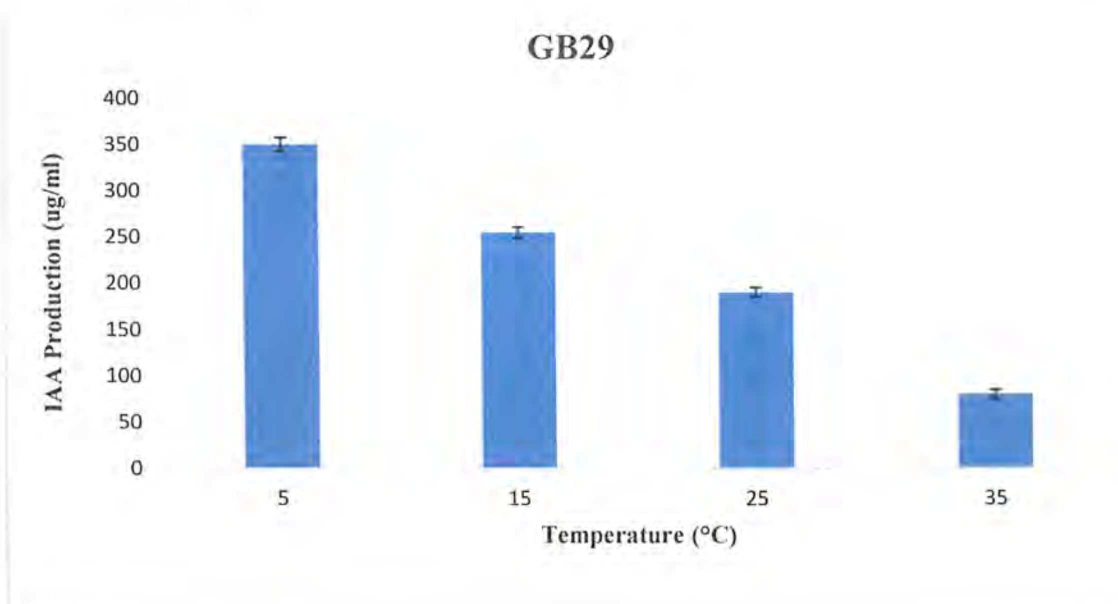


Figure 4.4. optimization of temperature for GB29

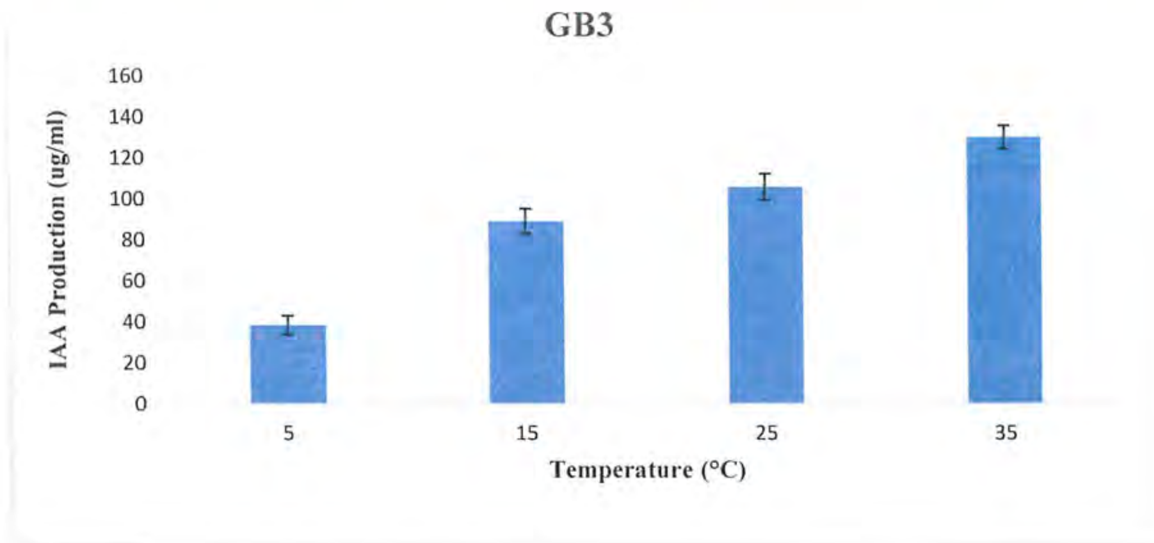


Figure 4.5. optimization of temperature for GB3

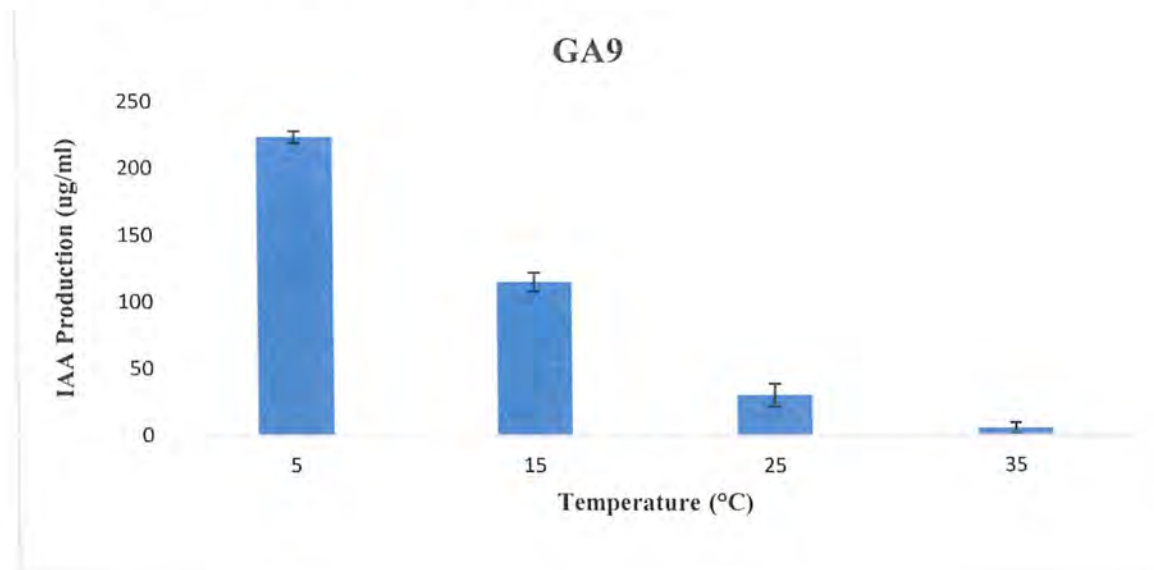


Figure 4.6. optimization of temperature for GA9

4.1.3 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 GB29 has shown the highest production

with an amount of 342.73µg/ml at pH range of 5 with 43% and 84% decrease in production at pH 7 and 9, respectively. The highest amount of production for GB3 was 124.28µg/ml at 7 pH and the percent decrease of production at 5 and 9 pH was estimated to be 60% and 19% respectively. Similarly at an optimum pH of 7 GA9 showed the highest amount of IAA production amounting 139.3µg/ml and the decrease estimated at 5 and 9 pH was 51% and 37%.

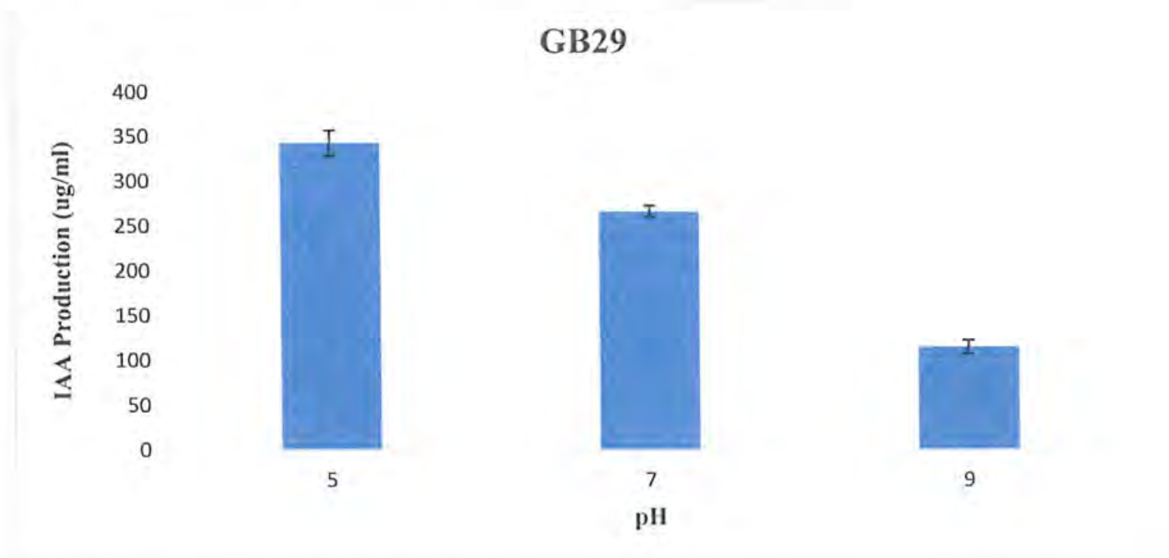


Figure 4.7. optimization of pH for GB29

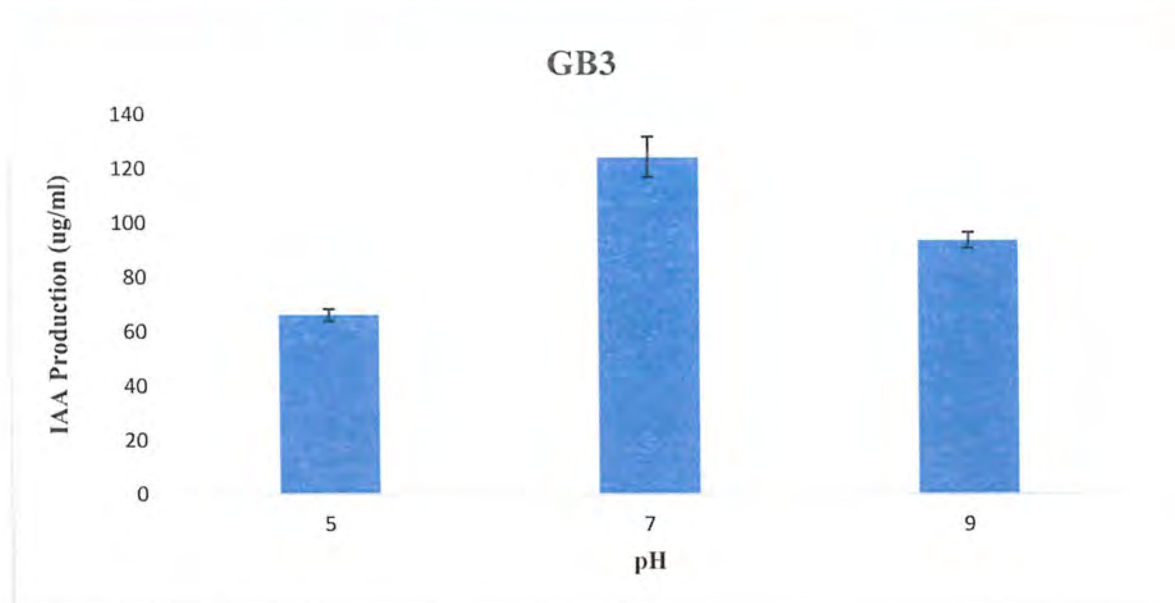


Figure 4.8. optimization of pH for GB3

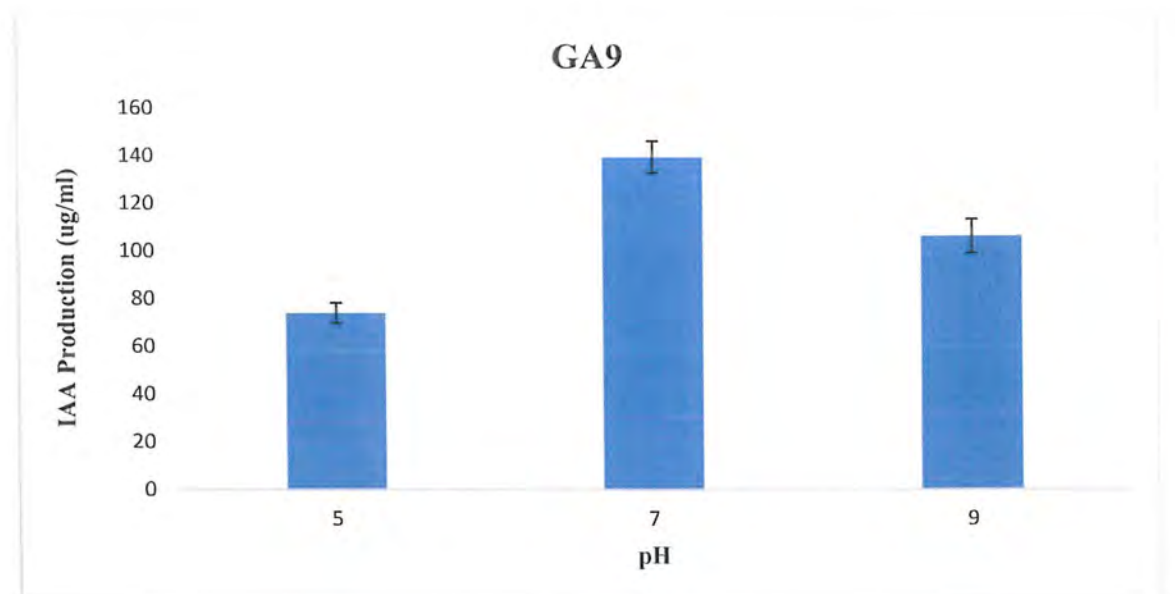


Figure 4.9. optimization of pH for GA9

4.1.4 Effect of L-tryptophan Concentration

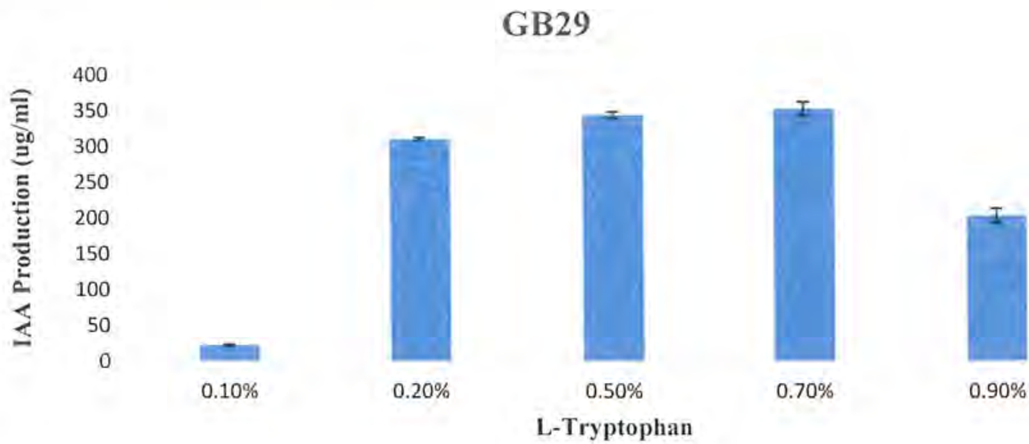


Figure 4.10. optimization of L-Tryp Concentration for GB29

Indole acetic acid producing bacteria always use L-tryptophan to carry out its metabolic pathway and produce IAA., because it works as precursor for IAA. Results showed that GB29 at L-tryp concentration 0.7% in media produced highest amount of IAA about 353.029µg/ml showing a decrease of 97%, 72%, 5% and 1% respectively at concentrations of 0.1%, 0.2%, 0.5% and 0.9%. Optimum concentration of L-tryp for GB3 detected was 0.9% where it produces maximum IAA with an amount of about 440.084µg/ml showing a decrease of 83%, 64%, 18% and 12% respectively at concentrations of 0.1%, 0.2%, 0.5% and 0.7%. GA9 at L-tryp concentration of 0.7% in media produce highest amount of IAA about 241µg/ml showing a decrease of 85%, 1%, 29% and 48% respectively at concentrations of 0.1%, 0.2%, 0.5% and 0.9%. When they were grown in optimum time, temperature, pH.

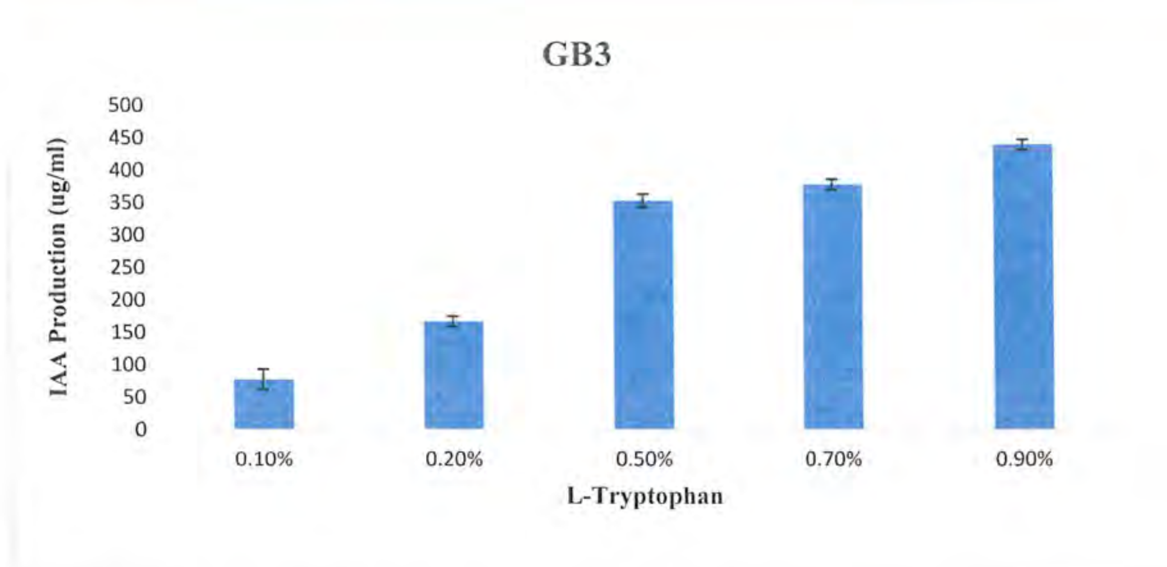


Figure 4.11. optimization of L-Tryp Concentration for GB3

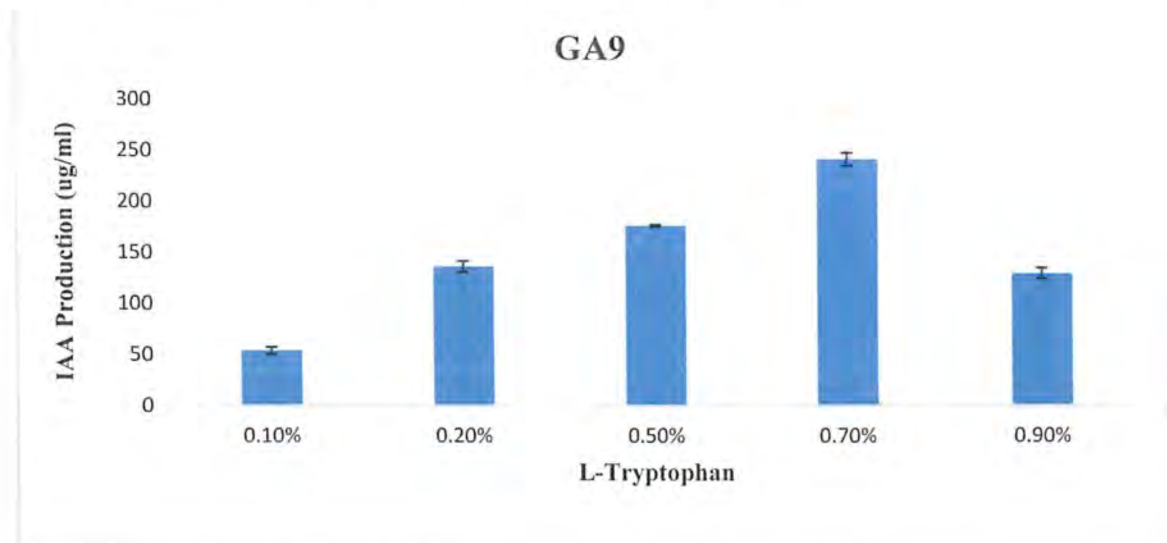


Figure 4.12. optimization of L-Tryp Concentration for GA9

4.1.5 Effect of Carbon Sources

IAA production estimated with different with glucose, sucrose, fructose, and maltose as carbon sources with optimum pH 5 for GB29 and pH 7 for GB3 and GA9 at a temperature of 5°C for GB29 and GA9 and 35°C for GB3 with agitation speed of 140rpm in shaking

incubator. Optimum L-tryp concentration for GA9 and GB29 was 0.7% and 0.9% for GB3 was kept constant. Samples at different optimum times were taken as on 48hours for GB29 and 72hours incubation for GB3 and GA9. Estimation of maximum production showed maltose as the optimum carbon source for all the three strains. GB29 showed maximum of 361.7996µg/ml of the IAA production with 36%, 62% and 86% decline in production when glucose, sucrose and fructose were used as carbon sources, respectively. 133.1261µg/ml of IAA was produced by GB3 as the maximum production with a decline of 6%, 8% and 12% respectively for glucose, sucrose, and fructose as carbon source. The highest amount of IAA produced by GA9 was 154µg/ml and 17%, 22% and 27% decline was reported when glucose, sucrose and fructose were used as carbon source in production media.

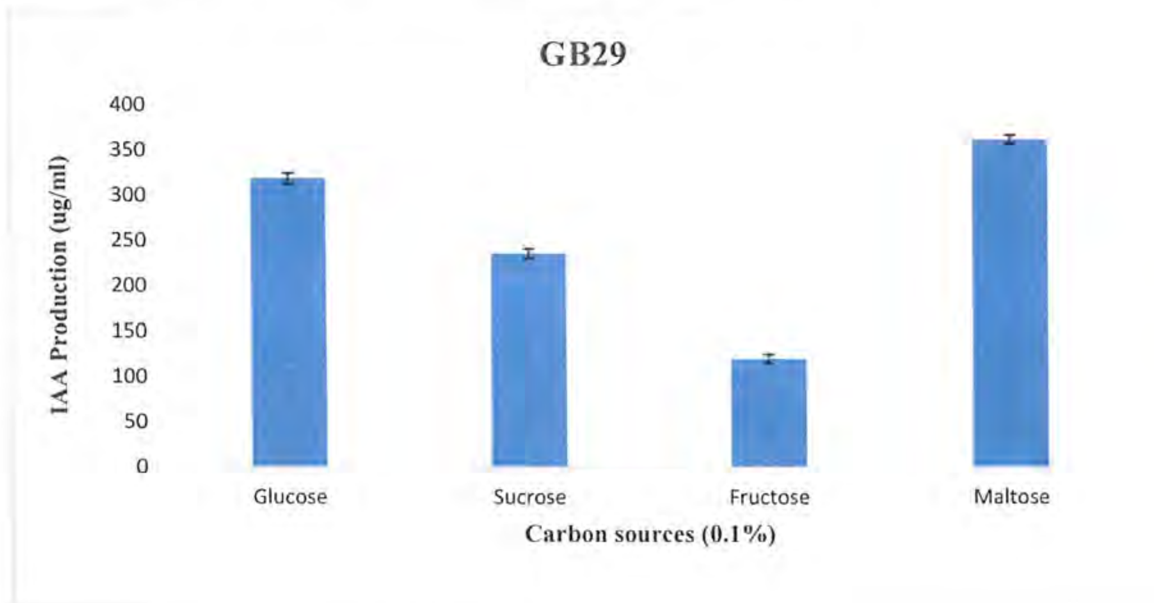


Figure 4.13. optimization of Carbon sources at (0.1%) for GB29

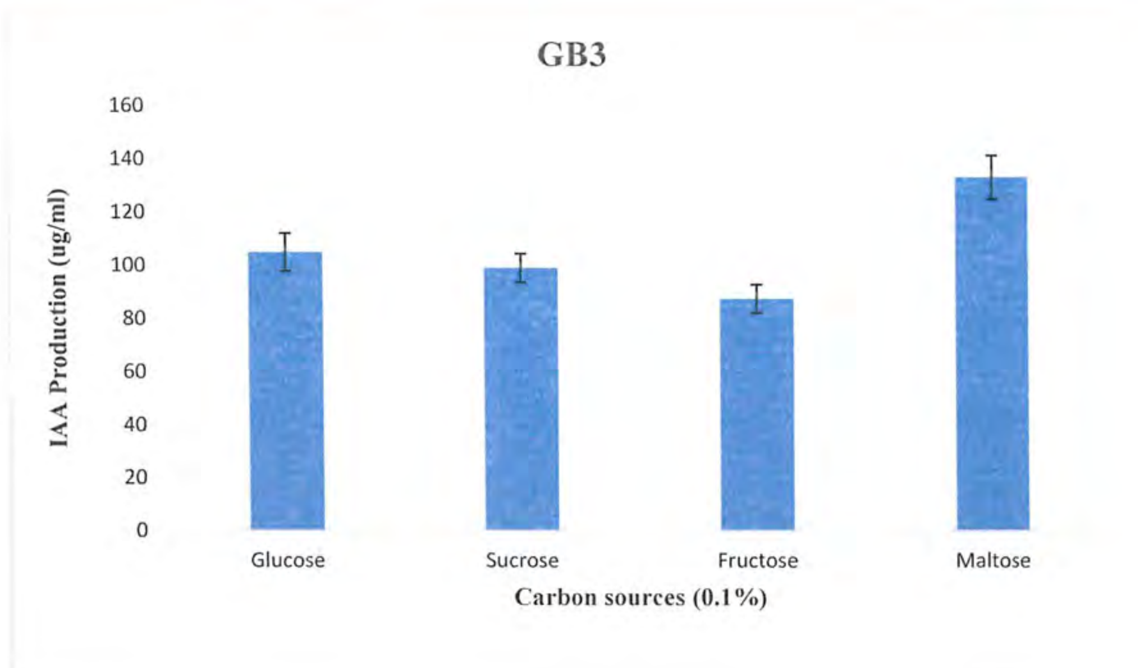


Figure 4.14. optimization of Carbon sources at (0.1%) for GB3

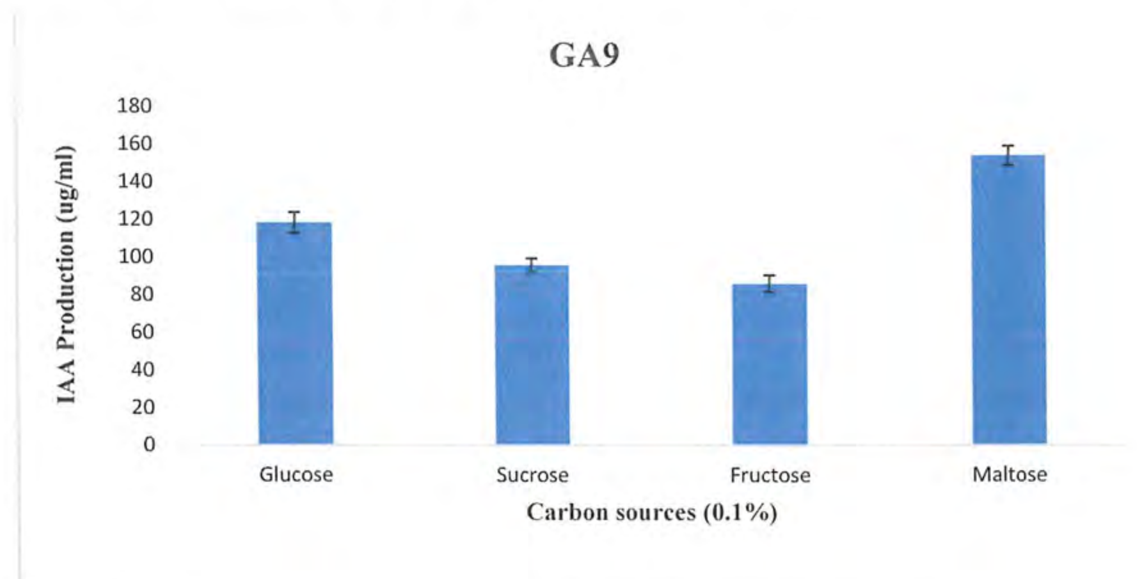


Figure 4.15. optimization of Carbon sources at (0.1%) for GA9

4.2 Thin Layer Chromatography

Indole acetic acid is bacterial metabolite that can be produced by bacteria along with many other metabolites. Thin layer chromatography was done to separate IAA from the rest of the compounds in the crude extract from the strain by loading 2μl of the methanolic solution from standard and all the three samples i.e., (GA9, GB3 and GB29). The plate were analyzed the UV visualizer to calculate the R_f for all of the above and the results calculated were 0.27 each for GA9, GB3 and GB29 which were almost the same as that for the standard confirming the presence of the IAA which were further confirmed by FTIR.

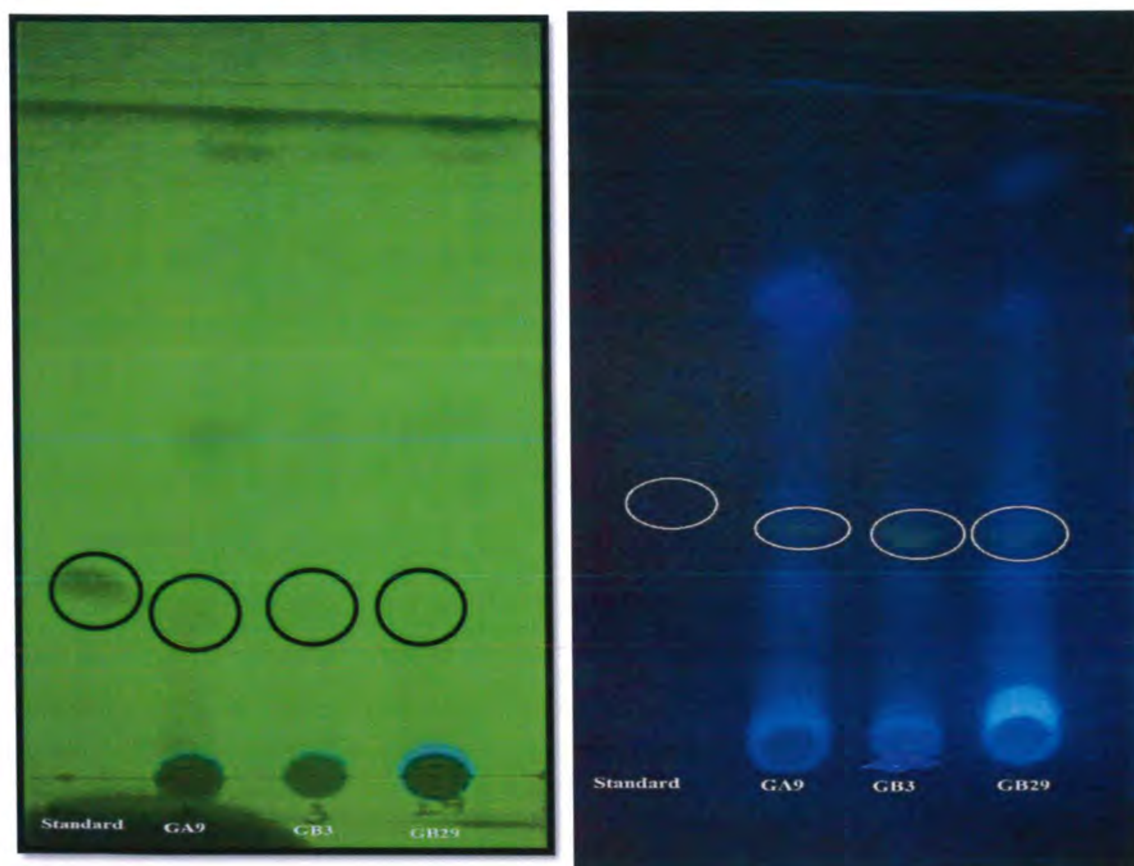


Figure 4.16. Thin Layer Chromatography of IAA produced by bacterial strains GB3, GA9 and GB29 compared with standard

4.3 Fourier Transform Infra-Red Spectroscopy

The presence of IAA was further confirmed by conducting FTIR to show the similarity of bonds on the basis of absorbance and transmission of the IR spectrophotometer waves. It was noted that there were absorbance and transmission peaks at 3310cm^{-1} confirming the presence of –OH and –NH bonds in standard IAA and in all the three samples. Peaks with value 2943cm^{-1} and 2831 cm^{-1} shows the C-H stretching the IAA. The spectrum with peak 1449 cm^{-1} confirms the C-H stretching of the methyl group present in all the samples loaded. Stretching of C=O can be confirmed by the spectral peaks 1022 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 IAA which was our desired hormone to be produced by the selected strains.

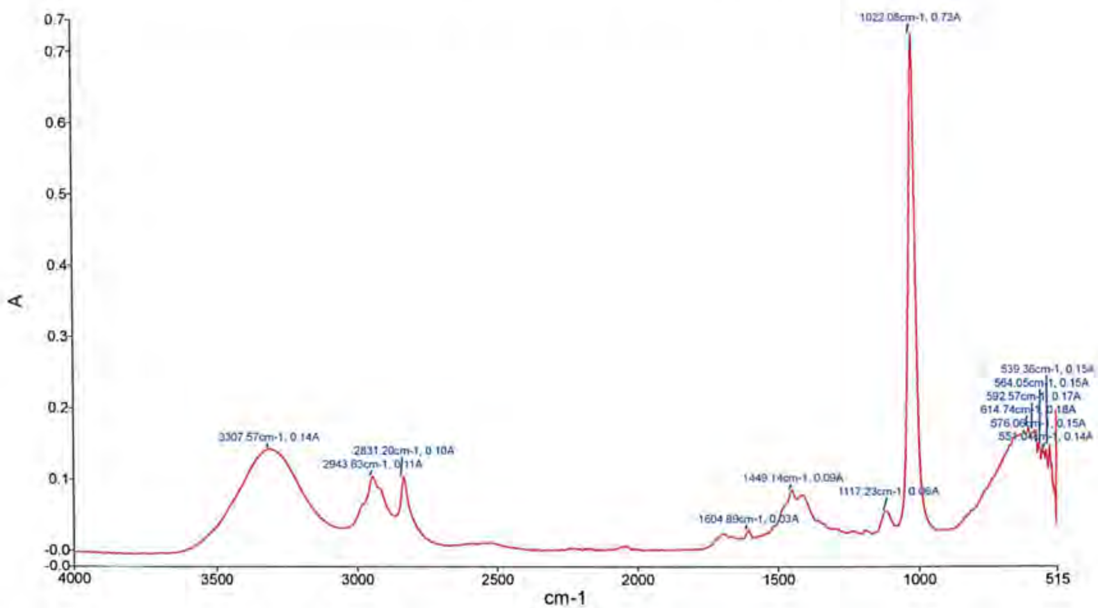


Figure 4.17. FTIR analysis of Standard IAA

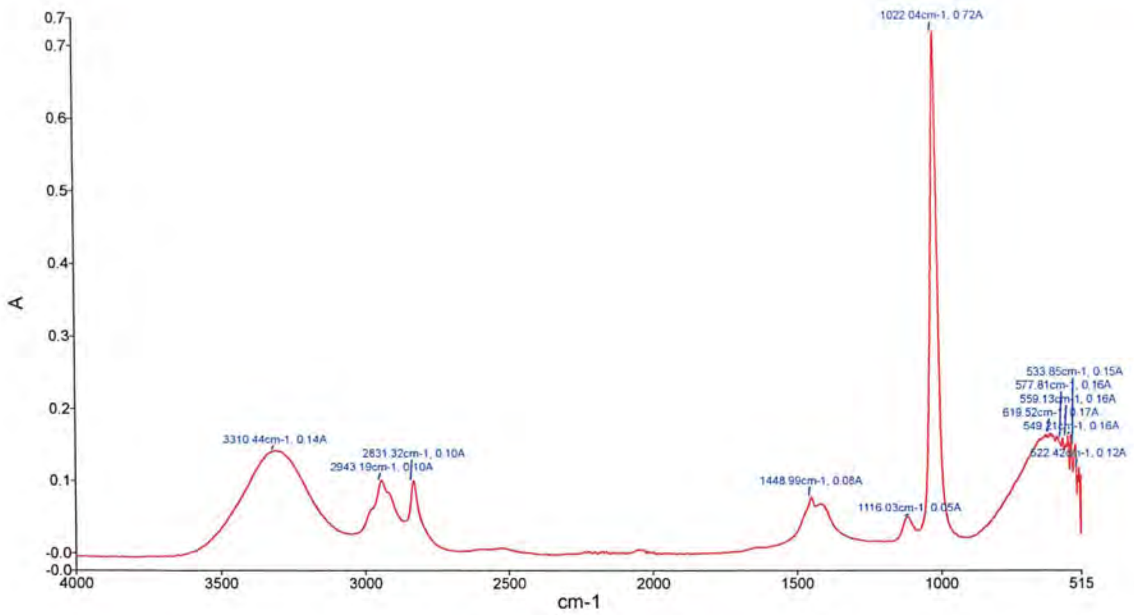


Figure 4. 18. FTIR analysis of IAA produced by GB29

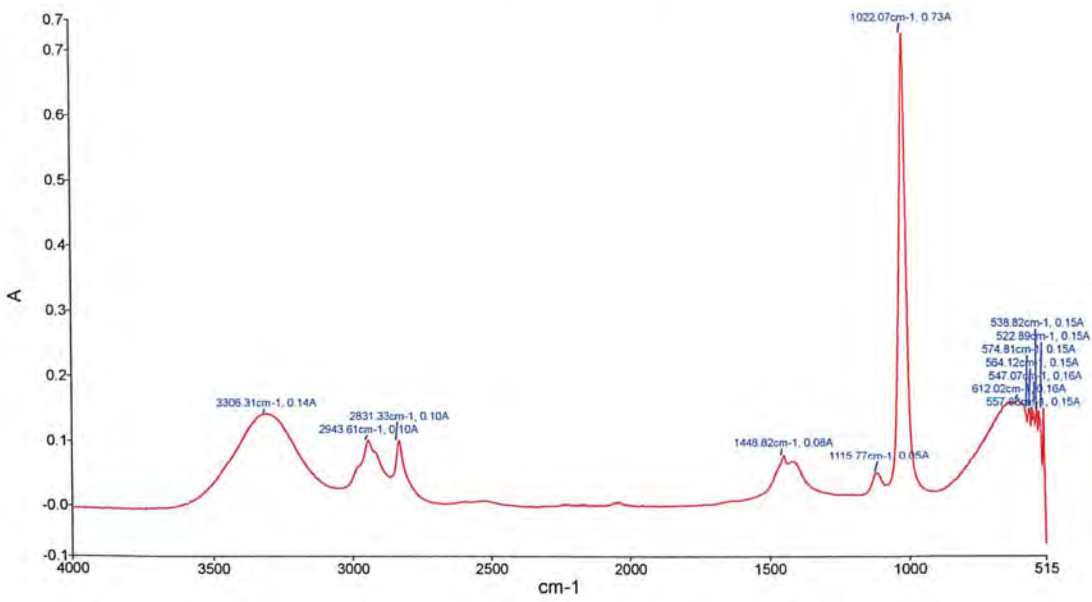


Figure 4.19. FTIR analysis of IAA produced by GB3.

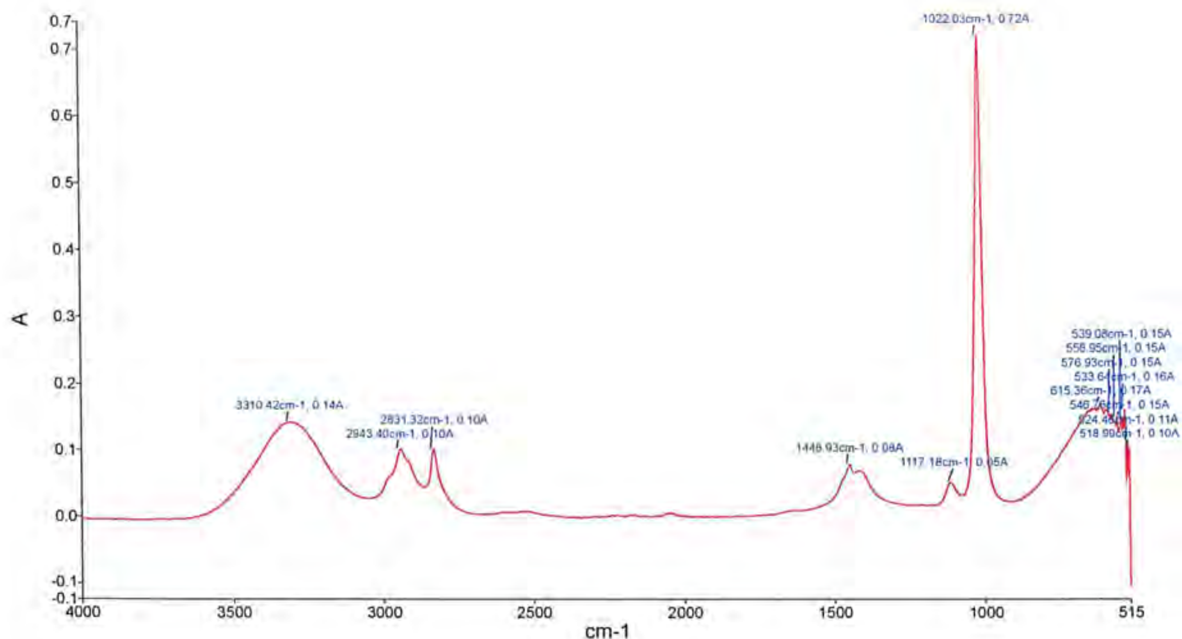


Figure 4.20. FTIR analysis of IAA produced by GA9.

4.4 Nuclear Magnetic Resonance

The confirmation of the presence of IAA in the extracts of GB29, GB3 and 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 IAA 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. 3.510ppm 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 carboxyl group. Chemical shift in the range of 7.011-7.373ppm confirms the presence of aromatic ring with a nitrogen atom. Having an adjacent proton.

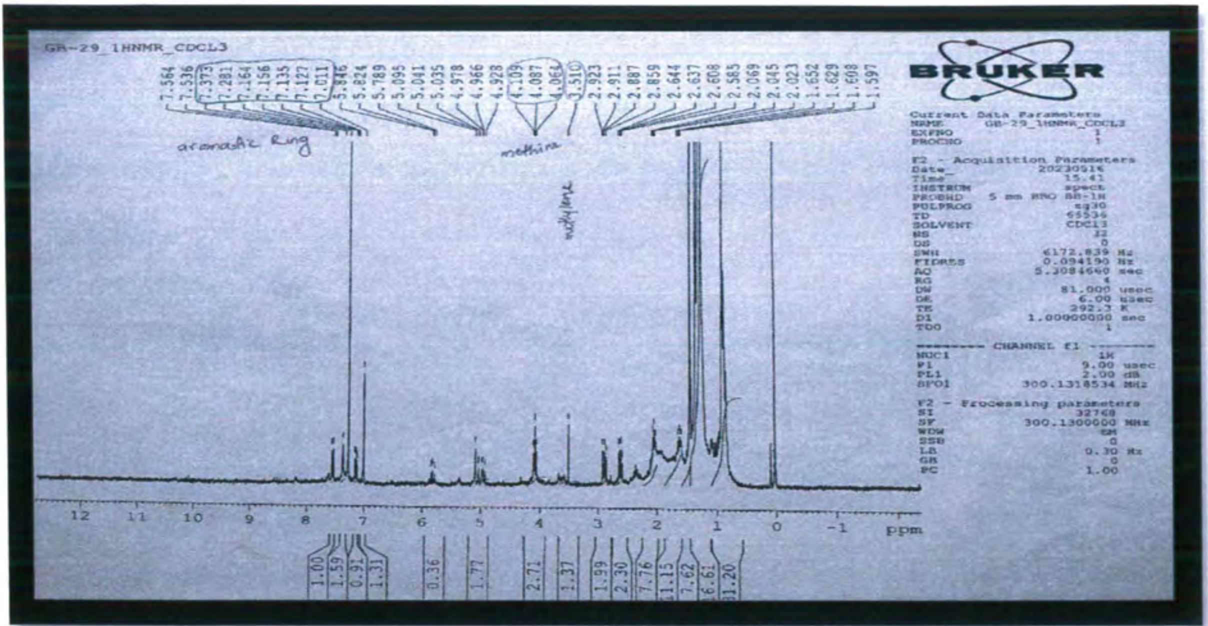


Figure 4.21: H-NMR analysis of IAA produced by GB29

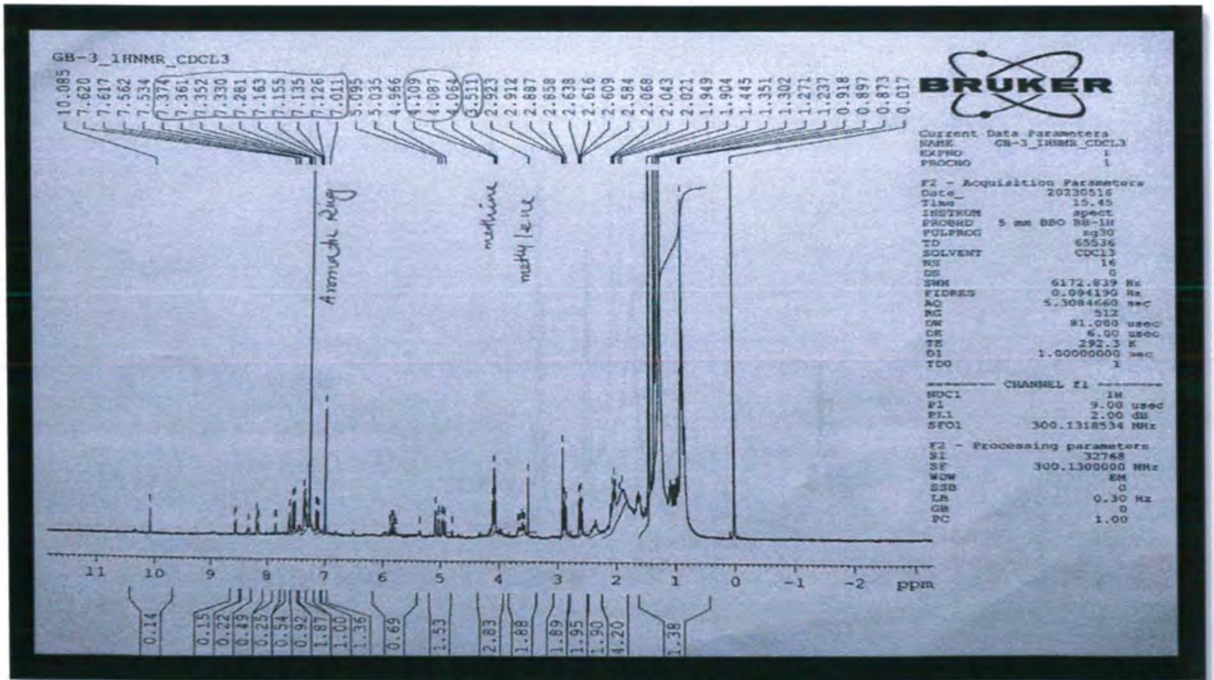


Figure 4.22: H-NMR analysis of IAA produced by GB3

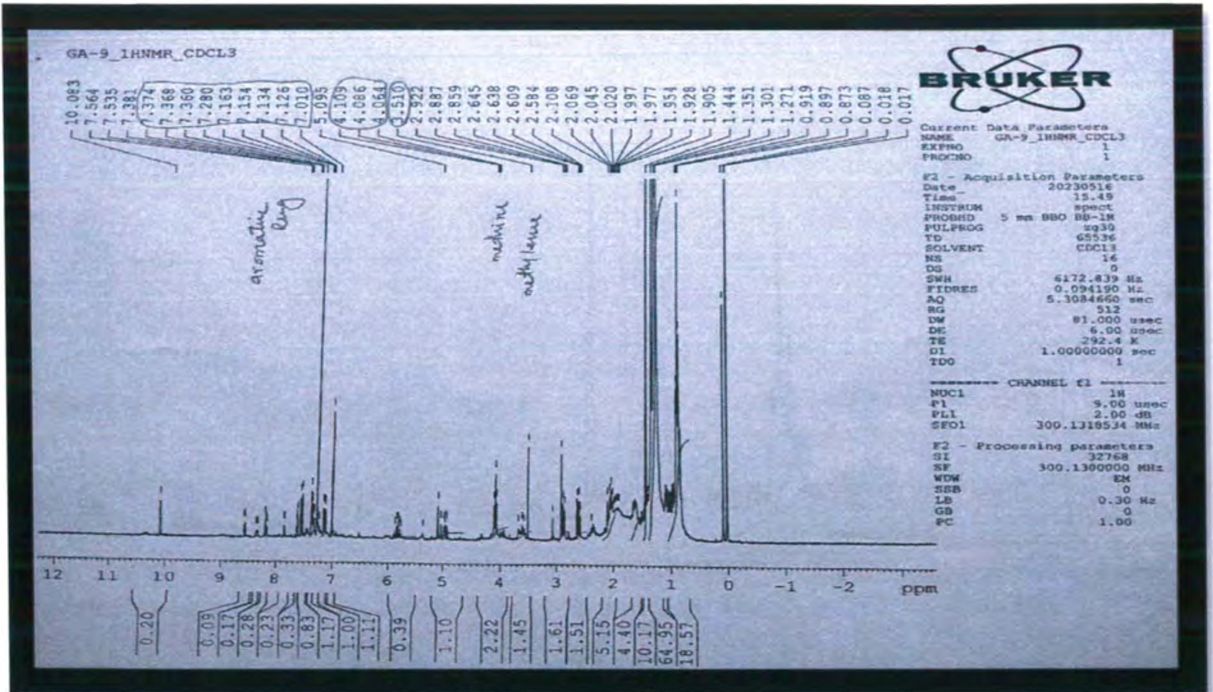


Figure 4.23: H-NMR analysis of IAA produced by GA9

Chapter 5

Discussion

5.1 Discussion

The cold adapted microorganisms are categorized as the psychrophilic microorganisms. In contrast to the hormones and enzymes produced by the organisms of higher temperature areas it has been suggested that the hormones of these cold adapted microorganisms are more sustainable and are more effective in the biotechnological, agricultural and many other economic and ecological fields (Bhatia, Ullah *et al.* 2021). IAA is the hormone that is essential for plant growth as they belong to the auxin family of hormones. Still there isn't much research done to produce this hormone by using cold adapted bacteria and fungi because of the presence of so much sources of the IAA available (Khan, Gurmani *et al.* 2016). Psychrophilic bacteria and yeasts have a good potential to produce IAA at lab scale and at industrial scale with many industrial applications (PADRILAH 2017) in the agriculture industry as bio-fertilizers, herbicides and treatment of human and horse serum. These can also help to reduce the use of artificial fertilizers to reduce contamination of the soil by the chemical in these fertilizers. Psychrophilic IAA 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. These 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 (Rafiq, Hayat *et al.* 2017) from where the sample can be collected to isolate psychrophilic and psychotropic bacteria and fungi.

In this study the strains studied for the production of the indole acetic acid were GB29, GA9 and GB3 named after the sample collected from the Ghulkin glacier.

5.2 Screening for IAA Production

Screening of strain to select those that can produce IAA was done by following the conventional method of culturing on nutrient agar, making inoculum and incubation in the L-tryptophan production media to produce IAA. At the end of the completion of the incubation period hormone assay was done by following the (Glickmann and Dessaux 1995) method of adding 2ml of Salkowski's reagent and 25µl ortho-phosphoric acid to 1ml of the supernatant collected by the centrifugation of the incubated L-tryp broth at 15°C in shaking incubator with a shaking speed of 140rpm. Another method proposed by the (Goswami, Vaghela *et al.* 2013)

can also be adapted to screen the microorganisms for the production of the IAA in which 1ml of Salkowski's reagent made of 1ml of 0.5M $FeCl_3$ and 50ml of 35% $HClO_4$ can be added to 1ml of the supernatant and then to be incubated at room temperature in dark for an hour because of its sensitivity to the light. The incubated supernatant turns pinkish or red in color showing the presence of the IAA because of its ability to change its color in the presence of the acids. Optical density to be measured at a wavelength of 530nm and the results to be noted. But in the process adapted by (Glickmann and Dessaux 1995) optical density to be measure after completion of incubation in dark at a wavelength of 533nm which is almost the same. The second process was adapted initially but the results were not sufficient enough, so we have changed our process of screening and adapted the early discussed process which enabled us to get fine enough results.

5.3 Optimization of Parameters for Maximum IAA Production

The optimization of culture conditions is extremely important for maximum production of IAA. Production of the IAA 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 IAA. 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 (Bessai, Bensidhoum *et al.* 2022). In this study we have grown our strain at different ranges of temperature, pH, L-tyrp concentration and carbon sources and samples were taken on 0-120hrs with an interval of 24hrs each to study the optimum conditions for our strains. We have found out that the best production by GB29 was at 5°C temperature, 5 pH, 0.7% L-tyrp and presence of maltose as carbon source incubated for 48hrs. Maximum production by GB3 was at 35°C temperature, 7 pH, 0.9% L-tyrp and presence of maltose as carbon source incubated for 72hrs. Highest amount of IAA production by GA9 was at 5°C temperature, 7 pH, 0.7% L-tyrp and presence of maltose as carbon source incubated for 72hrs. Similar study has been done for *Pseudomonas putida* by (Bharucha, Patel *et al.* 2013) showing the optimum conditions of 0.2% L-tryp concentration, 7.5 pH and sucrose as carbon source incubated for 96hrs. (Panigrahi, Mohanty *et al.* 2020) has also adapted the

same steps for *Ocimum sanctum* detecting 37°C temperature, 7 pH and sucrose as a source of carbon content incubated for 48hrs.

5.4 Extraction of crude IAA

Ethyl acetate washing is the most effective and commonly followed step for the extraction of the crude IAA 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. 200mlof the L-tryp media inoculated with microorganisms was centrifuged at 10,000rpm for 12minutes after the completion of the incubation at optimum condition and supernatant acidified to 2.5 pH with HCl 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. (Bharucha, Patel *et al.* 2013) has taken 300ml of the L-tryp media inoculate and have centrifuged it at 5000rpm for 15 minutes acidified to 2.5 pH with HCl and extract was preserved at 4°C in 3ml methanol for further study. (Panigrahi, Mohanty *et al.* 2020) has washed the supernatant three times with ethyl acetate and preserved it at -20°C in methanol. (Tallapragada, Dikshit *et al.* 2015) adapted the similar method as done by other researchers but the drying process was different and was done in hot air oven at 50°C and the preserved in 0.50ml of methanol at 20°C.

5.5 Characterization by TLC

Thin layer chromatography is a separating process of components in a mixture on the basis of retention ability while moving with the mobile phase with capillary movement. The mobile phase used in this process was a mixture of chloroform, ethyl acetate and formic acid with the ratio of 77:22:1 v/v and the stationary phase use were silica gel pasted on aluminum plate. The resultant R_f value was 0.27 for all the samples equal to that of the standard IAA used in the concentration of 1.56mg/ml in methanol. The same study has been done by the (Bishnu, Dhurva *et al.* 2020) by using two different mobile phases one was a solution of chloroform,

methanol and formic acid and the second was the solution of chloroform, ethyl acetate and formic acid in the ratio of 77:22:1 v/v respectively. The R_f calculated was 0.42 with the plate height of 6cm while the plate we used was 12cm high. (Suresh, Soundararajan *et al.* 2019) used isopropanol, ammonia, and ethyl acetate as solvent in 10:1:1 v/v as solvent. The R_f calculated was 0.45 with plate height of 6cm. Keeping the plate height 4.7cm (Raval and Saraf 2020) calculate that the R_f value for the indole acetic acid was about 1 for all of his samples and standard too. By comparing with the above-mentioned studies, it can be confirmed that the results obtained from our technique were complete enough for the confirmation of IAA presence.

5.6 Fourier Transform Infra-Red Spectroscopy

The FTIR analysis of IAA is the best way for studying the chemical structure of its surface and confirmation by the presence of its bonds. The peaks seen at around 3100-3600 cm^{-1} are due to O-H and N-H bond vibration (Panigrahi, Mohanty *et al.* 2020). It also shows that the peaks at 2800-2989cm^{-1} show the C-H stretching in the graph with symmetric CH_2 at 2874 cm^{-1} and 2958cm^{-1} shows asymmetric stretching of the CH_2. Our results shows that there is a peak at 2943cm-1 and a peak at 2831cm^{-1}. Bands with value 1150-1284cm^{-1} corresponds to the presence of the –C-O stretching according to (Panigrahi, Mohanty *et al.* 2020) and in comparison we have a band nearly the same value of 1117cm^{-1} in our results. (Husain, Ansari *et al.* 2011)reported that between 3000 and 3500 cm^{-1} observed peaks were due to the stretching of O-H and -NH groups of the hormone. Thus, the observed peak at 3310 cm^{-1} is due to the -OH group present in IAA. Furthermore, the peak observed at 1634 cm^{-1} was due to Amide II of the hormones while the peaks obtained at 1022cm^{-1} were due to the stretching of C-OH groups (Amente and Chimdessa 2021). Comparing all these bands obtained from the FTIR analysis with those in the previous study and with the bands obtained from the FTIR of standard IAA it is confirmed that IAA is produced by all the three (GB29, GB3 and GA9) strain.

5.7 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, where the chemical shift ranging

3.510ppm corresponded to the presence of the methylene functional group. In similar research methylene was detected in range between 3.5-4ppm by (Chitra, Selvi *et al.* 2019). (Jagannath, Konappa *et al.* 2019) has reported the presence of the methine in the range of 4-4.7ppm while the chemical shift in our samples was detected in the range of 4.064-4.109ppm that fell in exactly the sample region. According to (Chitra, Selvi *et al.* 2019) and (Jagannath, Konappa *et al.* 2019) aromatic rings were detected to be present in the range of shifts between 7-7.5ppm and after analyzing our own sample it was noted that there was a chemical shift ranging between 7.011-7.374ppm confirming the presence of the aromatic having a nitrogen with adjacent proton. Analyzing these facts, it was confirmed that there was IAA in the extract with many other impurities.

Chapter 6

Conclusion

Conclusion

GB29, GB3 and GA9 isolated form the Ghulkin glacier of the Gilgit Baltistan, Pakistan is investigated in the current study to be the competent producing psychrophiles of IAA and other PGPRs. Incubating GB29 for 48hrs while both GA9 and GB3 for seventy-two produce the highest amount of IAA. GB29 at 5 pH and GA9 at 7 pH and at 5°C, temperature while GB3 at 35°C with seven pH produces its highest amount of IAA at the above-mentioned incubation time. Keeping maltose as the carbon source in the L-tryp production media with concentration of 0.7% both for GA9 and GB29 and 0.9% for GB3 give rise to high production. Crude IAA extracted by the separating funnel in ethyl acetate solvent can be purified by thin layer chromatography and can be confirmed by finding its retention factor (R_f) and then processing through FTIR spectroscopy. The bonds comparison of IAA produced by selected strains and sample can be compared for confirmation and can also be compared with the studies done by past research. Bond wise confirmation of the IAA presence was done by performing H-NMR in $CDCl_3$ that has given us different chemical shifts that corresponds for different bonds in IAA and all other metabolites present in the extract.

Future Prospects

Future Prospects

- Indole Acetic Acid (IAA) produced by GB29, GB3 and GA9 strains, can further be characterized by their three-dimensional structure.
- Manipulation of the gene responsible for cold active IAA through genetic engineering and clone in other organisms to get hormones with desired properties for industrial processes.
- Different properties of IAA produced by GB29, GB3 and GA9 strains can also be explored for many other applications, like its anti-pathogenic activity.
- Development of low cost and novel optimization strategies for maximum production of IAA and minimizing the final cost of the hormone by applying new techniques.
- Development in the confirmation of use of IAA as anti-cancer agent.

References

References

Adams, D. and S. Yang (1979). "Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene." Proceedings of the National Academy of Sciences **76**(1): 170-174.

Amente, G. and E. Chimdessa (2021). "Control of browning in plant tissue culture: A review." Journal of Scientific Agriculture **5**: 67-71.

Amin, S., *et al.* (2015). "Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria." Nature **522**(7554): 98-101.

Ashen, J. B. and L. J. Goff (2000). "Molecular and ecological evidence for species specificity and coevolution in a group of marine algal-bacterial symbioses." Applied and Environmental Microbiology **66**(7): 3024-3030.

Baeza, M., *et al.* (2021). "Identification of stress-related genes and a comparative analysis of the amino acid compositions of translated coding sequences based on draft genome sequences of Antarctic yeasts." Frontiers in microbiology **12**: 623171.

Bessai, S. A., *et al.* (2022). "Optimization of IAA production by telluric bacteria isolated from northern Algeria." Biocatalysis and Agricultural Biotechnology **41**: 102319.

Bharucha, U., *et al.* (2013). "Optimization of indole acetic acid production by Pseudomonas putida UB1 and its effect as plant growth-promoting rhizobacteria on mustard (Brassica nigra)." Agricultural research **2**(3): 215-221.

Bhatia, R. K., *et al.* (2021). "Psychrophiles: a source of cold-adapted enzymes for energy efficient biotechnological industrial processes." Journal of Environmental Chemical Engineering **9**(1): 104607.

Bharudin, I., *et al.* (2018). "Unravelling the adaptation strategies employed by Glaciozyma antarctica PI12 on Antarctic sea ice." Marine environmental research **137**: 169-176

Bishnu, M. K., *et al.* (2020). "Quantification of indole-3-acetic acid from Bambusa tulda Roxb. seedlings using high performance liquid chromatography." African Journal of Biotechnology **19**(10): 781-788.

Brandl, M. and S. Lindow (1998). "Contribution of indole-3-acetic acid production to the epiphytic fitness of Erwinia herbicola." Applied and environmental microbiology **64**(9): 3256-3263

Breezee, J., *et al.* (2004). "Subfreezing growth of the sea ice bacterium "Psychromonas ingrahamii"." Microbial Ecology **47**(3): 300-304

Bric, J. M., *et al.* (1991). "Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane." Applied and environmental microbiology **57**(2): 535-538.

Brown, M. V., *et al.* (2012). "Global biogeography of SAR11 marine bacteria." Molecular systems biology **8**(1): 595.

Brown, S., *et al.* (1981). "The plasma cholinesterases: a new perspective." Advances in clinical chemistry **22**: 1-123.

Browse, J. (2005). "Jasmonate: an oxylipin signal with many roles in plants." Vitamins & Hormones **72**: 431-456.

Bunsangiam, S., *et al.* (2019). "Biosynthetic pathway of indole-3-acetic acid in basidiomycetous yeast Rhodosporidiobolus fluvialis." Mycobiology **47**(3): 292-30

Bürger, M. and J. Chory (2019). "Stressed out about hormones: how plants orchestrate immunity." Cell host & microbe **26**(2): 163-172.

Chaiharn, M. and S. Lumyong (2011). "Screening and optimization of indole-3-acetic acid production and phosphate solubilization from rhizobacteria aimed at improving plant growth." Current microbiology **62**(1): 173-181.

Chandra, S., *et al.* (2018). "Optimization of indole acetic acid production by isolated bacteria from Stevia rebaudiana rhizosphere and its effects on plant growth." Journal of Genetic Engineering and Biotechnology **16**(2): 581-586.

Chitra, G., et al. (2019). "pH-sensitive biopolymeric hydrogel-based on indole-3-acetic acid for wound healing and anti-cancer applications." SN Applied Sciences 1: 1-15.

Cohen, J. D., *et al.* (2003). "Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis." Trends in plant science **8**(5): 197-199.

Czerpak, R., *et al.* (1999). "Comparison of stimulatory effect of auxins and cytokinins on protein, saccharides and chlorophylls content in Chlorella pyrenoidosa Chick." Polskie Archiwum Hydrobiologii **46**(1).

D'Amico, S., *et al.* (2002). "Molecular basis of cold adaptation." Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences **357**(1423): 917-92.

Davies, P. L. (2014). "Ice-binding proteins: a remarkable diversity of structures for stopping and starting ice growth." Trends in biochemical sciences **39**(11): 548-555.

Davies, P. L., *et al.* (2002). "Structure and function of antifreeze proteins." Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences **357**(1423): 927-935.

De Maayer, P., *et al.* (2014). "Some like it cold: understanding the survival strategies of psychrophiles." EMBO reports **15**(5): 508-517.

De Paepe, A. and D. Van Der Straeten (2005). "Ethylene biosynthesis and signaling: an overview." Vitamins & Hormones **72**: 399-430.

De Smet, I., *et al.* (2011). "Unraveling the evolution of auxin signaling." Plant physiology **155**(1): 209-221.

Defez, R., *et al.* (2016). "Overproduction of indole-3-acetic acid in free-living rhizobia induces transcriptional changes resembling those occurring in nodule bacteroids." Molecular Plant-Microbe Interactions **29**(6): 484-495.

Deming, J. W. (2002). "Psychrophiles and polar regions." Current opinion in microbiology **5**(3): 301-309.

El-Shanshoury, A. (1995). "Interactions of Azotobacter chroococcum, Azospirillum brasilense and Streptomyces mutabilis, in relation to their effect on wheat development." Journal of Agronomy and Crop Science **175**(2): 119-127.

Fahad, S., *et al.* (2015). "Phytohormones and plant responses to salinity stress: a review." Plant growth regulation **75**(2): 391-404.

Fahy, A., *et al.* (2006). "Heterogeneous aerobic benzene-degrading communities in oxygen-depleted groundwaters." FEMS microbiology ecology **58**(2): 260-270.

Feller, G. (2013). "Psychrophilic enzymes: from folding to function and biotechnology." Scientifica **2013**.

Feller, G. and C. Gerday (2003). "Psychrophilic enzymes: hot topics in cold adaptation." Nature reviews microbiology **1**(3): 200-208.

Feurtado, J. A., *et al.* (2004). "Dormancy termination of western white pine (Pinus monticola Dougl. Ex D. Don) seeds is associated with changes in abscisic acid metabolism." Planta **218**(4): 630-639.

Finkelstein, R. R. (2010). The role of hormones during seed development and germination. Plant hormones, Springer: 549-573.

Firdaus-Raih, M., *et al.* (2018). "The Glaciozyma antarctica genome reveals an array of systems that provide sustained responses towards temperature variations in a persistently cold habitat." PLoS One **13**(1): e0189947.

Fleet, C. and M. Williams (2011). "Gibberellins. Teaching tools in plant biology: lecture notes." Plant Cell **110**.

Garcia Salamone, I. E. d., *et al.* (2005). "Role of cytokinins in plant growth promotion by rhizosphere bacteria." PGPR: biocontrol and biofertilization: 173-195.

Gaur, R., *et al.* (2010). "Purification and characterization of a solvent stable aminopeptidase from Pseudomonas aeruginosa: Cloning and analysis of aminopeptidase gene conferring solvent stability." Process Biochemistry **45**(5): 757-764.

Gentile, G., *et al.* (2003). "Shewanella sp. GA-22, a psychrophilic hydrocarbonoclastic antarctic bacterium producing polyunsaturated fatty acids." Journal of applied microbiology **95**(5): 1124-1133.

Glickmann, E. and Y. Dessaux (1995). "A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria." Applied and environmental microbiology **61**(2): 793-796.

Gomez-Roldan, V., *et al.* (2008). "Strigolactone inhibition of shoot branching." Nature **455**(7210): 189-194.

Goswami, D., *et al.* (2013). "Plant growth promoting potentials of Pseudomonas spp. strain OG isolated from marine water." Journal of plant interactions **8**(4): 281-290.

Graeber, K., *et al.* (2012). "Molecular mechanisms of seed dormancy." Plant, cell & environment **35**(10): 1769-1786.

Grennan, A. K. (2006). "Gibberellin metabolism enzymes in rice." Plant physiology **141**(2): 524-526.

Grossmann, K. (2010). "Auxin herbicides: current status of mechanism and mode of action." Pest Management Science: formerly Pesticide Science **66**(2): 113-120.

Grove, M. D., *et al.* (1979). "Brassinolide, a plant growth-promoting steroid isolated from Brassica napus pollen." Nature **281**(5728): 216-217.

Gupta, G., *et al.* (2014). "Extremophiles: an overview of microorganism from extreme environment." International Journal of Agriculture, Environment and Biotechnology **7**(2): 371.

Harder, W. and H. Veldkamp (1971). "Competition of marine psychrophilic bacteria at low temperatures." Antonie van Leeuwenhoek **37**(1): 51-63.

Hashim, N. H. F., *et al.* (2013). "Characterization of Afp1, an antifreeze protein from the psychrophilic yeast Glaciozyma antarctica PI12." Extremophiles **17**(1): 63-73.

Hashim, N. H. F., *et al.* (2014). "Molecular cloning, expression and characterisation of Afp4, an antifreeze protein from Glaciozyma antarctica." Polar biology **37**(10): 1495-15.

Helmke, E. and H. Weyland (2004). "Psychrophilic versus psychrotolerant bacteria-occurrence and significance in polar and temperate marine habitats." Cellular and molecular Biology **50**(5): 553-561.

Hooley, R. (1994). "Gibberellins: perception, transduction and responses." Signals and Signal Transduction Pathways in Plants: 293-319.

Hough, D. W. and M. J. Danson (1999). "Extremozymes." Current opinion in chemical Biology **3**(1): 39-46.

Husain, Q., *et al.* (2011). "Immobilization of Aspergillus oryzae β galactosidase on zinc oxide nanoparticles via simple adsorption mechanism." International Journal of Biological Macromolecules **49**(1): 37-43.

Iqbal, M., *et al.* (2018). "Phyllospheric Bacterial Treatments Improve Growth in Helianthus annuus L." RADS Journal of Biological Research & Applied Sciences **9**(1): 30-40.

Jagannath, S., et al. (2019). "production, characterization of indole acetic acid and its bioactive potential from endophytic fungi of Cymbidium aloifolium L." Journal of Biologically Active Products from Nature 9(5): 387-409.

James, E. K., *et al.* (2002). "Infection and colonization of rice seedlings by the plant growth-promoting bacterium Herbaspirillum seropedicae Z67." Molecular Plant-Microbe Interactions **15**(9): 894-906.

Jasim, B., *et al.* (2014). "Studies on the factors modulating indole-3-acetic acid production in endophytic bacterial isolates from P iper nigrum and molecular analysis of ipdc gene." Journal of applied microbiology **117**(3): 786-799.

Junge, K., *et al.* (2004). "Bacterial activity at− 2 to− 20 C in Arctic wintertime sea ice." Applied and Environmental Microbiology **70**(1): 550-557.

Karthikeyan, S., *et al.* (2010). "Indole-3-acetic acid from contaminant fungus and potential application for cell cultures of Alternanthera sessilis." International Journal of Pharma and Bio Sciences **1**(4).

Kawahara, H. (2013). "Characterizations of functions of biological materials having controlling-ability against ice crystal growth." Advanced Topics on Crystal Growth; InTech: Rijeka, Croatia: 119-143.

Kemkar, N. A. (2006). "Environmental peacemaking: Ending conflict between India and Pakistan on the Siachen Glacier through the creation of a transboundary peace park." Stan. Envtl. LJ **25**: 67.

Kerchev, P., *et al.* (2015). "Activation of auxin signalling counteracts photorespiratory H2O2-dependent cell death." Plant, cell & environment **38**(2): 253-265.

Keto-Timonen, R., *et al.* (2016). "Cold shock proteins: a minireview with special emphasis on Csp-family of enteropathogenic Yersinia." Frontiers in microbiology 7: 1151.

Khan, S. U., *et al.* (2016). "Exogenously applied gibberellic acid, indole acetic acid and kinetin as potential regulators of source-sink relationship, physiological and yield attributes in rice (Oryza sativa) genotypes under water deficit conditions." Int J Agric Biol **18**(1): 139-145.

Kim, H. J., *et al.* (2017). "Marine antifreeze proteins: structure, function, and application to cryopreservation as a potential cryoprotectant." Marine drugs **15**(2): 27.

Lasztity, R. (2009). "Micro-organisms important in food microbiology." Food Quality And Standards-Volume III **10**: 26.

Lebrazi, S., *et al.* (2020). "Screening and optimization of indole-3-acetic acid production and phosphate solubilization by rhizobacterial strains isolated from Acacia cyanophylla root nodules and their effects on its plant growth." Journal of Genetic Engineering and Biotechnology **18**(1): 1-12.

Link, G. K. and V. Eggers (1941). "Hyperauxiny in crown gall of tomato." Botanical Gazette **103**(1): 87-106.

Ljung, K., *et al.* (2001). "Developmental regulation of indole-3-acetic acid turnover in Scots pine seedlings." Plant Physiology **125**(1): 464-475.

Lorenzo, O. and R. Solano (2005). "Molecular players regulating the jasmonate signalling network." Current opinion in plant biology **8**(5): 532-540.

Los, D. A. and N. Murata (2004). "Membrane fluidity and its roles in the perception of environmental signals." Biochimica et Biophysica Acta (BBA)-Biomembranes **1666**(1-2): 142-157.

MacElroy, R. D. (1974). "Some comments on the evolution of extremophiles." Biosystems **6**(1): 74-75.

Malhotra, M. and S. Srivastava (2008). "An ipdC gene knock-out of Azospirillum brasilense strain SM and its implications on indole-3-acetic acid biosynthesis and plant growth promotion." Antonie Van Leeuwenhoek **93**(4): 425-433.

Mashiguchi, K., *et al.* (2011). "The main auxin biosynthesis pathway in Arabidopsis." Proceedings of the National Academy of Sciences **108**(45): 18512-18517.

Matsukawa, E., *et al.* (2007). "Stimulatory effect of indole-3-acetic acid on aerial mycelium formation and antibiotic production in Streptomyces spp." Actinomycetologica **21**(1): 32-39.

McSteen, P. (2010). "Auxin and monocot development." Cold Spring Harbor perspectives in biology **2**(3): a001479.

Méndez-Hernández, H. A., *et al.* (2019). "Signaling overview of plant somatic embryogenesis." Frontiers in plant science **10**: 77.

Mikami, K., *et al.* (2016). "Comprehensive quantification and genome survey reveal the presence of novel phytohormone action modes in red seaweeds." Journal of applied phycology **28**(4): 2539-2548.

Mohite, B. (2013). "Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth." Journal of soil science and plant nutrition **13**(3): 638-649.

Morita, R. and C. Moyer (2000). "Origin of psychrophiles." Schulze, and GD Tilman (ed.), Encyclopedia of biodiversity **4**: 917-924.

Morita, R. Y. (1975). "Psychrophilic bacteria." Bacteriological reviews **39**(2): 144-167.

Nichols, D. S., *et al.* (2004). "Cold adaptation in the Antarctic archaeon Methanococcoides burtonii involves membrane lipid unsaturation." Journal of Bacteriology **186**(24): 8508-8.

Nor, N. M., *et al.* (2020). "Functional and structural analyses of an expansin-like protein from the antarctic yeast Glaciozyma antarctica PI12 reveal strategies of nutrient scavenging in the sea ice environment." International journal of biological macromolecules **144**: 231-241.

Öpik, H., *et al.* (2005). The physiology of flowering plants, Cambridge University Press Cambridge.

Osborne, D. J. and M. T. McManus (2005). Hormones, signals and target cells in plant development, Cambridge University Press.

Padrilah, s. n. b. (2017). "toxicity effects of copper on partially-purified cholinesterase and various tissues of clarias gariepinus (burchell, 1822)."

Panigrahi, S., *et al.* (2020). "Characterization of endophytic bacteria Enterobacter cloacae MG00145 isolated from Ocimum sanctum with Indole Acetic Acid (IAA) production and plant growth promoting capabilities against selected crops." South African Journal of Botany **134**: 17-26.

Parmar, N. and K. Dadarwal (2000). "Stimulation of plant growth of chickpea by inoculation of fluorescent pseudomonads." J Appl Microbiol **86**: 36-44.

Parvin, W., *et al.* (2020). "Identification, Determination and Quantification of Indole-3-Acetic Acid Produced by Pseudomonas aeruginosa UPMP3 and Its Effect on The Growth of Oil Palm (Elaeis guineensis Jacq)." World Journal of Agricultural Research **8**(3): 75-83.

Patten, C. L. and B. R. Glick (2002). "Role of Pseudomonas putida indoleacetic acid in development of the host plant root system." Applied and environmental microbiology **68**(8): 3795-3801.

Peck, S. C. and H. Kende (1998). "Differential regulation of genes encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase in etiolated pea seedlings: effects of indole-3-acetic acid, wounding, and ethylene." Plant molecular biology **38**(6): 977-982.

Phetcharat, P. and A. Duangpaeng (2012). "Screening of endophytic bacteria from organic rice tissue for indole acetic acid production." Procedia Engineering 32: 177-183

Petroski, M. D. and R. J. Deshaies (2005). "Function and regulation of cullin–RING ubiquitin ligases." Nature reviews Molecular cell biology 6(1): 9-20.

Phillips, K. A., *et al.* (2011). "vanishing tassel2 encodes a grass-specific tryptophan aminotransferase required for vegetative and reproductive development in maize." The Plant Cell **23**(2): 550-566.

Pierre-Jerome, E., *et al.* (2018). "Regulation of division and differentiation of plant stem cells." Annual review of cell and developmental biology **34**: 289.

Price, P. B. and T. Sowers (2004). "Temperature dependence of metabolic rates for microbial growth, maintenance, and survival." Proceedings of the National Academy of Sciences **101**(13): 4631-4636.

Prusty, R., *et al.* (2004). "The plant hormone indoleacetic acid induces invasive growth in Saccharomyces cerevisiae." Proceedings of the National Academy of Sciences **101**(12): 4153-4157.

Rafiq, M., *et al.* (2017). "Recovery of metallo-tolerant and antibiotic resistant psychrophilic bacteria from Siachen glacier, Pakistan." PLoS One **12**(7): e0178180.

Rafiq, M., *et al.* (2019). "Geochemistry and bacterial recovery from Hindu Kush Range glacier and their potential for metal resistance and antibiotic production." Geomicrobiology Journal **36**(4): 326-338.

Rahman, A., *et al.* (2010). "Salkowski's reagent test as a primary screening index for functionalities of rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil." Bioscience, biotechnology, and biochemistry **74**(11): 2202-2208

Rapparini, F., *et al.* (2002). "Indole-3-acetic acid metabolism in Lemna gibba undergoes dynamic changes in response to growth temperature." Plant Physiology **128**(4): 1410-1416.

Raval, V. H. and M. Saraf (2020). "Biosynthesis and purification of indole-3-acetic acid by halotolerant rhizobacteria isolated from Little Runn of Kachchh." Biocatalysis and Agricultural Biotechnology **23**: 101435.

Rivas-San Vicente, M. and J. Plasencia (2011). "Salicylic acid beyond defence: its role in plant growth and development." Journal of experimental botany **62**(10): 3321-3338.

Rothschild, L. J. and R. L. Mancinelli (2001). "Life in extreme environments." Nature **409**(6823): 1092-1101.

Sadaf, S., *et al.* (2009). "Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs." African Journal of Agricultural Research **4**(11): 1312-1316.

Santiago, M., *et al.* (2016). "Discovery, molecular mechanisms, and industrial applications of cold-active enzymes." Frontiers in microbiology **7**: 1408.

Santner, A., *et al.* (2009). "Plant hormones are versatile chemical regulators of plant growth." Nature chemical biology **5**(5): 301-307.

Sarwar, M., *et al.* (1992). "Tryptophan-dependent biosynthesis of auxins in soil." Plant and Soil **147**(2): 207-215.

Schausberger, P. (2018). "Herbivore-associated bacteria as potential mediators and modifiers of induced plant defense against spider mites and thrips." Frontiers in plant science **9**: 1107.

Schmidt-Nielsen, S. (1902). "Ueber einige psychrophile Mikroorganismen und ihr Vorkommen." Zentr. Bakteriol. Parasitenkd. Infektionsk. Hyg. Abt. **9**: 145-147.

Shen, L., *et al.* (2012). "Variation of culturable bacteria along depth in the East Rongbuk ice core, Mt. Everest." Geoscience Frontiers **3**(3): 327-334.

Shigenaga, A. M. and C. T. Argueso (2016). No hormone to rule them all: Interactions of plant hormones during the responses of plants to pathogens. Seminars in Cell & Developmental Biology, Elsevier.

Shivaji, S., *et al.* (2011). "Bacterial diversity of soil in the vicinity of Pindari glacier, Himalayan mountain ranges, India, using culturable bacteria and soil 16S rRNA gene clones." Extremophiles **15**(1): 1-22.

Siliakus, M. F., *et al.* (2017). "Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure." Extremophiles **21**(4): 651-670.

Simon, S. and J. Petrášek (2011). "Why plants need more than one type of auxin." Plant Science **180**(3): 454-460.

Sindhu, S., *et al.* (2018). Bioherbicidal potential of rhizosphere microorganisms for ecofriendly weed management. Role of rhizospheric microbes in soil, Springer: 331-376.

Sipes, D. L. and J. W. Einset (1983). "Cytokinin stimulation of abscission in lemon pistil explants." Journal of Plant Growth Regulation **2**(1): 73-80.

Sochová, L. "The role of plant hormones for efficient fertilization processes at high temperatures in Arabidopsis and oilseed rape."

Spaepen, S. and J. Vanderleyden (2011). "Auxin and plant-microbe interactions." Cold Spring Harbor perspectives in biology **3**(4): a001438.

Spaepen, S., *et al.* (2007). "Indole-3-acetic acid in microbial and microorganism-plant signaling." FEMS Microbiology Reviews **31**(4): 425-448.

Srivastava, R. M., *et al.* (2002). "Cooling rate evaluation for bulk amorphous alloys from eutectic microstructures in casting processes." Materials Transactions **43**(7): 1670-1

Stibbs, H. H. and J. R. Seed (1975). "Short-term metabolism of [14C] tryptophan in rats infected with Trypanosoma brucei gambiense." Journal of Infectious Diseases **131**(4): 459-462.

Sugawara, S., *et al.* (2009). "Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in Arabidopsis." Proceedings of the National Academy of Sciences **106**(13): 5430-5435.

Suresh, A., *et al.* (2019). "Evaluation and characterization of the plant growth promoting potentials of two heterocystous cyanobacteria for improving food grains growth." Biocatalysis and Agricultural Biotechnology **17**: 647-652

Tallapragada, P., *et al.* (2015). "Isolation and optimization of IAA producing Burkholderia seminalis and its effect on seedlings of tomato." Songklanakarin Journal of Science & Technology **37**(5).

Theologis, A. (1992). "One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening." Cell **70**(2): 181-184.

Tiwari, S. B., *et al.* (2004). "Aux/IAA proteins contain a potent transcriptional repression domain." The Plant Cell **16**(2): 533-543.

Tsai, F.-Y., *et al.* (1997). "A comparative study of the effects of abscisic acid and methyl jasmonate on seedling growth of rice." Plant Growth Regulation **21**(1): 37-42.

Turchetti, B., *et al.* (2020). "DNA methylation changes induced by cold in psychrophilic and psychrotolerant Naganishia yeast species." Microorganisms **8**(2): 296.

Turchetti, B., *et al.* (2011). "Psychrophilic yeasts from Antarctica and European glaciers: description of Glaciozyma gen. nov., Glaciozyma martinii sp. nov. and Glaciozyma watsonii sp. nov." Extremophiles **15**(5): 573-586.

Uggla, C., *et al.* (1996). "Auxin as a positional signal in pattern formation in plants." Proceedings of the national academy of sciences **93**(17): 9282-9286.

ÜNYAYAR, S. (2000). "Production of Gibberellic Acid and Cytokinin By Phanerochaete chrysosporiumME446 Immobilized on Polyurethane Foam." Turkish Journal of Biology **24**(3): 513-520.

Van Den Burg, B. (2003). "Extremophiles as a source for novel enzymes." Current opinion in microbiology **6**(3): 213-218.

Vessey, J. K. (2003). "Plant growth promoting rhizobacteria as biofertilizers." Plant and soil **255**(2): 571-586.

Vorobyova, E., *et al.* (1997). "The deep cold biosphere: facts and hypothesis." FEMS Microbiology Reviews **20**(3-4): 277-290.

Walz, A., *et al.* (2002). "A gene encoding a protein modified by the phytohormone indoleacetic acid." Proceedings of the National Academy of Sciences **99**(3): 1718-1723.

Weier, T. E., *et al.* (1970). "Botany. An introduction to plant biology." Botany. An introduction to plant biology.

Widawati, S. (2020). Isolation of Indole Acetic Acid (IAA) producing Bacillus siamensis from peat and optimization of the culture conditions for maximum IAA production. IOP Conference Series: Earth and Environmental Science, IOP Publishing

Wierdl, M., *et al.* (2000). "Isolation and characterization of a cDNA encoding a horse liver butyrylcholinesterase: evidence for CPT-11 drug activation." Biochemical pharmacology **59**(7): 773-781.

Xie, X., *et al.* (2010). "The strigolactone story." Annual review of phytopathology **48**: 93-117.

Yayanos, A. A. (1986). "Evolutional and ecological implications of the properties of deep-sea barophilic bacteria." Proceedings of the National Academy of Sciences **83**(24): 9542-9546.

Yuan, Z.-C., *et al.* (2008). "Transcriptome profiling and functional analysis of Agrobacterium tumefaciens reveals a general conserved response to acidic conditions (pH 5.5) and a complex acid-mediated signaling involved in Agrobacterium-plant interactions." Journal of Bacteriology **190**(2): 494-507.

Yusof, N., *et al.* (2015). "In silico characterisation of the Glaciozyma antarctica genome: mining the molecular chaperones." Malaysian Applied Biology **44**(1): 161-165.

Zakhia, F., *et al.* (2008). Cyanobacteria in cold ecosystems. Psychrophiles: from biodiversity to biotechnology, Springer: 121-135.

Zhao, Y. (2010). "Indole-3-acetic acid (IAA), the main auxin in higher plants, has profound effects on plant growth and development." Annu Rev Plant Biol 61: 49-64.

Production of Indole Acetic Acid by Bacteria Isolated from Ghulkin Glacier

ORIGINALITY REPORT

6%	1%	5%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

	Source	%
1	Sylia Ait Bessai, Leila Bensidhoum, El-hafid Nabti. "Optimization of IAA production by telluric bacteria isolated from northern Algeria", Biocatalysis and Agricultural Biotechnology, 2022 Publication	3%
2	Amitav Bhattacharya. "Soil Water Deficit and Physiological Issues in Plants", Springer Science and Business Media LLC, 2021 Publication	<1%
3	Nitin Baliyan, Sandhya Dhiman, Shrivardhan Dheeman, Sandeep Kumar, Dinesh Kumar Maheshwari. "Optimization of indole-3-acetic acid using response surface methodology and its effect on vegetative growth of chickpea", Rhizosphere, 2021 Publication	<1%
	Craig L Moyer, Richard Y Morita. "Psychrophiles and Psychrotrophs", Wiley, 2007 Publication	<1%