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OPTIMIZATION OF INDOLE-3-ACETIC ACID BIOSYNTHESIS IN *BACILLUS SUBTILIS* STRAIN ESU181

MASTER OF SCIENCE

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ABSTRACT

OPTIMIZATION OF INDOLE-3-ACETIC ACID BIOSYNTHESIS IN *BACILLUS SUBTILIS* STRAIN ESU181
MSC THESIS
NİL BAŞAK TOP KILIÇ
BOLU ABANT IZZET BAYSAL UNIVERSITY
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xiii + 51

Biofertilizers are produced as an alternative to chemical fertilizers and pesticides to ensure sustainability in agriculture, which has gained importance especially in recent years. It is necessary to investigate the native bacterial species in the production of biofertilizers, to determine the mechanisms that affect plant growth in the detected species, and to investigate the conditions under which these mechanisms should be activated to obtain the highest possible crop. Indole-3-acetic acid (IAA) is a hormone that has a significant effect on plant growth. There are few optimization studies in *Bacillus subtilis* species, which can synthesize this hormone. The main purpose of this thesis is to optimize the ambient conditions for IAA synthesis in *B. subtilis* strain ESU181 isolated from local einkorn roots. IAA production was observed for 96 hours at three different pH (6.5, 7.5, 8.5) and two different temperatures (30°C and 37°C). IAA measurements were made in 24-hour periods. Since IAA production can occur in two known ways, tryptophan dependent and independent, in the experiment, the medium was arranged in two sets with and without the addition of tryptophan, while keeping the other nutrient types and amounts constant. According to the data, it was observed that the highest IAA production was at the end of 96 hours incubation period at pH 6.5, 37 °C and tryptophan added medium. In addition, it was observed that a significant amount of IAA was produced in the medium without tryptophan addition and the addition of tryptophan did not have as high an effect as expected on this strain. As the reason for the decrease in the amount of IAA observed in the data, storage of IAA in cells was put forward as an intermediate hypothesis and it was observed that no significant amount of IAA was found in cells. The data presented in this study offer the possibility of activating bacterial IAA production in agricultural applications and adaptability for large-scale production of IAA.

KEYWORDS: Indole-3-acetic acid (IAA), *Bacillus subtilis*, PGPB, Sustainable agriculture, Microbial biotechnology

ÖZET

BACILLUS SUBTILIS ESU181 SUŞUNDA İNDOL-3-ASETİK ASİT BİYOSENTEZİNİN OPTİMİZASYONU
YÜKSEK LİSANS TEZİ
NİL BAŞAK TOP KILIÇ
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
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Biyogübreler özellikle son yıllarda oldukça önem kazanan tarımda sürdürülebilirliğin sağlanabilmesi için kimyasal gübre ve pestisitlere alternatif olarak üretilmektedir. Biyogübrelerin elde edilmesinde yerli bakteri türlerinin araştırılması, tespit edilen türlerde bitki büyümesine etki eden mekanizmaların belirlenmesi ve mümkün olan en yüksek mahsulün elde edilmesi için bu mekanizmaların hangi koşullarda aktive edilmesi gerektiğinin araştırılması gerekmektedir. İndol-3-asetik asit (IAA) bitki gelişimine önemli derecede etki eden bir hormondur. Bu hormonu sentezleme yeteneğine sahip olan *Bacillus subtilis* türünde az sayıda optimizasyon çalışması mevcuttur. Bu tez çalışmasının ana amacı yerel siyez buğdayı köklerinden izole edilmiş olan *B. subtilis* ESU181 suşunda IAA sentezi için ortam koşullarının optimize edilmesidir. IAA üretimi üç farklı pH (6.5, 7.5, 8.5) ve iki farklı sıcaklıkta (30°C ve 37°C) 96 saat inkübasyon süresiyle gözlemlenmiştir. 24 saatlik periyotlarla IAA ölçümleri yapılmıştır. IAA üretimi triptofan bağımlı ve bağımsız olmak üzere bilinen iki yoldan gerçekleşebildiği için deneyde besi ortamı triptofan ilaveli ve ilavesiz olarak, diğer besin türü ve miktarları sabit tutularak iki set halinde düzenlenmiştir. Verilere göre en yüksek IAA üretiminin, pH 6.5, 37 °C ve triptofan ilaveli ortamda 96 saat inkübasyon süresi sonunda olduğu görülmüştür. Ayrıca, triptofan ilavesiz ortamda da belirgin miktarda IAA üretildiği ve triptofan ilavesinin bu suş üzerinde beklendiği kadar yüksek etkisinin olmadığı görülmüştür. Verilerde gözlenen IAA miktarlarındaki düşüşlerin sebebi olarak, hücrede IAA depolanması ara hipotez olarak ortaya konulmuş ve yapılan deneyler sonucunda hücre içinde anlamlı miktarda IAA olmadığı gözlenmiştir. Bu çalışmada sunulan veriler, tarımsal uygulamalarda bakteriyel IAA üretiminin aktive edilmesi ve IAA'nın büyük ölçekli üretimi için uyarlanabilme imkanı sunmaktadır.

ANAHTAR KELİMELER: İndol-3-asetik asit (IAA), *Bacillus subtilis*, Bitki gelişimini teşvik eden bakteriler , Sürdürülebilir tarım, Mikrobiyal biyoteknoloji

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LIST OF ABBREVIATIONS AND SYMBOLS

ACC **:** 1-aminocyclopropane-1-carboxylate deaminase

GB **:** Glucobrassicin

IAA **:** Indole-3-acetic acid

IAAld **:** Indole-3-acetaldehyde

IAM **:** Indole-3-acetamide

IAN **:** Indole-3-acetonitrile

IAOx **:** Indole-3-acetaldoxime

IGP **:** Indole-3-glycerol phosphate

ILA **:** Indole-3-lactic acid

IND **:** Indole

INS **:** Indole synthase

IPyA **:** Indole-3-pyruvic acid

ISR **:** Induced systemic resistance

LB **:** Lysogeny (Luria-Bertani) Broth

OD **:** Optical Density

PGPB **:** Plant Growth Promoting Bacteria

PGP **:** Plant growth promotion

PGPR **:** Plant Growth Promoting Rhizobacteria

spp. **:** Species

TAM **:** Tryptamine

TOL **:** Indole-3-ethanol

TRP **:** Tryptophan

TSO **:** Trptophan side-chain oxidase

To the memory of my uncle Mehmet Aksoy

&

To my aunt Sevin Aksoy

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1. INTRODUCTION

The increasing human population and decrease in arable lands requires to get more yield per unit area within a short time in agriculture. Fertilizer is the common name for materials either natural, or synthetic, that applied to soil or plant tissues to increase productivity in agriculture.

Using chemicals to replace the lacking nutrients necessary for plant growth dates to 19th century. Different forms of phosphate, nitrogen, and potash have been the first and most common industrial fertilizers; the importance of other nutrients in crop yield such as sulfur, calcium, magnesium macronutrients along with iron and other micronutrients have also been recognized since 19th, 20th centuries (Hignett, 1985).

However, the soils lose their fertility in time along with sowing. Excess and uncontrolled use of chemical fertilizers cause salt accumulation in soil which results in blocking of the plants reach to nutrients, change in the structure of soil, decrease in soil pH, or, if those chemicals dissolve in water with rainfalls or irrigation, they cause eutrophication, which is the nutrient enrichment in aquatic water bodies especially in N and P, that gives harm to aquatic ecosystem. Besides the environmental causes, they impose an economic burden on farmers.

Organic fertilizers, the main component of “organic agriculture”, are safer alternatives to chemical fertilizers if they are not overused. Organic fertilizers are materials that come from living things or their remains, such as their waste products or their decomposed bodies, either fully or partially. Nutrient release is generally slow when organic fertilizers are used. This may be advantageous or disadvantageous according to demands. Manure obtained from livestock like cow/cattle, poultry, goat excreta, green manure which are cover crops derived from mostly legumes, or other crops according to need, and composts made up of agricultural organic wastes such as straw, cotton stalks, corn stalks, or decomposed wastes such as sewage sludge, slaughterhouse sludge are major types of organic fertilizers (El-Haggar, 2007; Sharma, 2017).

1.1 Biofertilizers

Biofertilizers are natural fertilizers like organic fertilizers, that consist of single or mixture of two or more microorganisms (Nambirajan, Ashok, Baskaran, & Viswanathan, 2020). Biofertilizers differ from organic fertilizers. Because organic fertilizers supply nutrients for plants directly like chemical fertilizers or by their decay (Vessey, 2003). However, biofertilizers consist of dormant or live cells of microorganisms that promote plant growth and development by various mechanisms (Kaur & Vishnu, 2022). They are commercialized within liquid, organic, inorganic, polymeric carriers or in encapsulated forms (Bashan, de-Bashan, Prabhu, & Hernandez, 2014). Being more economic and healthier, they are also more useful in agriculture. They are environment - friendly alternatives to chemical fertilizers and pesticides. Biocontrol is simpler to manage and have long-term effects (Kumar, Pandhi, Mahato, Kamle, & Mishra, 2021; Patibanda & Math, 2018).

For sustainable agriculture biofertilizers are really promising in terms of keeping and enhancing the soil quality and fertility. Besides providing nutrients necessary for plants growth and development, they increase the resistance of plants against biological and abiotic stresses. So that, increasing crop yields and quality becomes more possible and easier.

Bacteria, blue-green algae (cyanobacteria), and mycorrhizae are the well-known genera that show plant growth promoting effects and are used in biofertilizer formulations.

1.2 Plant Growth Promoting Rhizobacteria

Rhizosphere is the layer of soil that surround the plant roots (Hiltner, 1904). The rhizosphere contains a vast number of different microorganisms, with bacteria being the most prevalent (Bernard R. Glick, 1995; Hashem, Tabassum, & Fathi Abd_Allah, 2019). Most of the interactions between rhizosphere microorganisms such as bacteria, fungi, micro-algae, actinomycetes, protozoa, other invertebrates (nematodes, arthropods…) and plants occur within that zone of the soil.

PGPR are the bacterial species that colonize the rhizosphere having positive effects on the health and development of plants (Joseph Kloepper & Schroth, 1978). *Acetobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Azorhizobium*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Beijerinckia*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Methylobacterium*, *Pseudomonas*, *Rhizobium*,

Paenibacillus are among the reported species having plant growth promotion effects (Çakmakçı et al., 2017; D, Rane, Chaudhari, & B, 2009; JW Kloepper, Schroth, & Miller, 1980; Timmusk, Nicander, Granhall, & Tillberg, 1999; Ünüvar & Ünlü, 2022; Vessey, 2003). It was reported that the predominant genera of PGPR are *Pseudomonas* and *Bacillus* (Hashem et al., 2019).

Rhizobacteria can be classified according to the site of colonization as extracellular (ePGPR) and intracellular (iPGPR). Extracellular PGPR colonize the rhizosphere, the surface of the roots (rhizoplane), or the spaces between the root cortex cells while intracellular PGPR colonize inside the root cells, generally in the root nodules (Figueiredo, Seldin, Araujo, & Mariano, 2010). PGPR can also be classified according to their functions: biofertilizers, phytostimulators, rhizoremediators and biopesticides (Joshi, Andharia, Patel, & Kotadiya, 2019).

PGPR function as biofertilizers in improving soil fertility, crop yield, and quality by increasing nutrient uptake via replacing soil nutrients, making vital nutrients available for plants, and by allowing plants to reach more nutrients.

PGPR having phytostimulation effect on plants, produce plant growth-promoting hormones like auxins, cytokinins, gibberellins, and other phytohormones. These hormones stimulate cell division, growth, and differentiation. *Azospirillum, Pseudomonas,* and *Bacillus* species are examples of that kind PGPR.

Biopesticides are bacteria that control the accumulation of organisms that can damage plants. Despite biopesticides are not used primarily for growth promotion, they may have an indirect effect in enhancing growth in plants. In addition, some PGPR strains may have both features of being biopesticide and biofertilizer (Vessey, 2003).

Rhizoremediation is a type of bioremediation by which the pollutants in soil such as industrial wastes (metals, metalloids, polycyclic aromatic hydrocarbons…), antibiotics, or pesticides are degraded with the action of rhizobacteria that secrete various regulatory compounds such as metal-binding proteins, phytohormones, and siderophores. *Pseudomonas, Bacillus, Alcaligens, Arthrobacter, Achromabacter, Azospirillum, Pantoea* are among the most studied genera of PGPR that function in bioremediation (Saeed et al., 2021).

In Table 1.1, examples of bacterial genera are given with their functions in plant growth promotion.

Table 1.1. Examples of bacterial genera having different functions in plant growth promotion.

Hormone synthesis		
	Azospirillum *Rhizobium* *Acetobacter* *Herbaspirillum* *Bacillus* *Acinetobacter* *Bradyrhizobium* *Klebsiella*	(Bottini, Cassán, & Piccoli, 2004; Hussain & Hasnain, 2009; Kang et al., 2009; Sturtevant & Taller, 1989)
ACC deaminase activity		
	Alcaligenes *Bacillus* *Enterobacter* *Pseudomonas* *Variovorax*	(Vessey, 2003)
Increasing nutrient availability		
Nitrogen fixation	*Azospirillum* *Enterobacter* *Klebsiella* *Pseudomonas* *Rhizobium* *Bradyrhizobium* *Azorhizobium* *Allorhizobium* *Sinorhizobium* *Mesorhizobium* *Cyanobacteria* *Azotobacter* *Acetobacter* *Nostoc*	(Hayat, Ali, Amara, Khalid, & Ahmed, 2010; Saeed et al., 2021)

Phosphate solubilization	*Bacillus* *Acinetobacter* *Azotobacter* *Pseudomonas* *Rhizobium*	(Diaz, Baron, & Rigobelo, 2019; Saeed et al., 2021)
Biocontrol		
	Pseudomonas *Acinetobacter* *Enterobacter* *Bacillus* *Azotobacter* *Mezorhizobium* *Azospirillum*	(Hayat et al., 2010; Ramesh, Joshi, & Ghanekar, 2009; Xue et al., 2009)
Bioremediation		
	Pseudomonas *Bacillus* *Alcaligens* *Arthrobacter* *Achromabacter* *Azospirillum* *Pantoea*	(Saeed et al., 2021)

1.3 *Bacillus spp*. Use in Agriculture

Bacillus species are gram positive, rod shaped, endospore forming bacterial species, which are belong to Bacillaceae family. They are resistant to environmental conditions, also to various chemicals. *Bacilli* are very important members of rhizosphere; they affect the rhizosphere microbial community and plant growth by secreting a variety of metabolites that stimulate plant growth and inhibit pathogen infections (Hashem et al., 2019; Sui et al., 2022; Zhang, Mao, Zhuang, & Yang, 2022). *Bacillus* species can also be genetically modified for improving biopesticides and biofertilizers (Yadav, Rastegari, Yadav, & Kour, 2020a). In Table 1.2, examples of *Bacillus* species and action mechanisms in plant growth promotion are given.

Table 1.2. Examples of *Bacillus* species and action mechanisms in plant growth promotion.

B. subtilis	➢ IAA synthesis	(Singh, Raina, Singh, Ghosh, & Heflish, 2017)
	➢ Cytokinin synthesis	(Hussain & Hasnain, 2009)
	➢ Gibberellin synthesis	(Malfanova et al., 2011)
	➢ Abscisic acid synthesis	(Ji et al., 2020)
	➢ Jasmonic acid synthesis	(Ji et al., 2020)
	➢ Salicylic acid synthesis	(Ji et al., 2020)
	➢ VOC synthesis (Induction of systemic resistance)	(Ryu et al., 2004; Tahir et al., 2017)
	➢ Production of fungal cell degrading enzymes	(Lim & Kim, 2009)
	➢ Production of antibiotics	(Malfanova et al., 2011)
	➢ Nitrogen fixing	(Kumari, Prabha, Singh, Kumari, & Kiran, 2018)
	➢ Phosphate solubilization	(Lim & Kim, 2009)
	➢ Siderophore synthesis	(Ghazy & El-Nahrawy, 2021; Singh et al., 2017)
B. cereus	➢ IAA synthesis	(Pandya & Desai, 2013)
	➢ Cytokinin synthesis	(Akhtar et al., 2021)
	➢ Gibberellin synthesis	(X. Wang et al., 2020)
	➢ Abscisic acid synthesis	(Akhtar et al., 2021)
	➢ (ACC) deaminase synthesis	(Akhtar et al., 2021)
	➢ Phosphate solubilization	(Akhtar et al., 2021)
	➢ Production of siderophores	(Akhtar et al., 2021)
	➢ Organic acid synthesis	(Akhtar et al., 2021)
B. flexus	➢ Nitrogen fixing	(Yousuf et al., 2017)

B. thuringiensis	➢ IAA synthesis	(Gerayeli, Baghaee-Ravari, & Tarighi, 2018)
	➢ Production of fungal cell degrading enzymes	(Shrestha, Sultana, Chae, Kim, & Lee, 2015)
B. amyloliquefaciens	➢ IAA synthesis	(Asari et al., 2017)
	➢ Cytokinin synthesis	(Asari et al., 2017)
	➢ Gibberellin synthesis	(Kim et al., 2017)
	➢ Abscisic acid synthesis	(Kim et al., 2017)
	➢ ACC deaminase activity	(Zafar-ul-Hye, Danish, Abbas, Ahmad, & Munir, 2019)
	➢ Production of VOCs (Induction of systemic resistance)	(Abdul et al., 2017)
	➢ Phosphate solubilization	(Diaz et al., 2019)
	➢ Production of antimicrobial compounds	(Malfanova et al., 2011)
B. artrophaeus	➢ Production of VOCs (Induction of systemic resistance)	(Abdul et al., 2017)
B. licheniformis	➢ Gibberellin synthesis	(Gutiérrez-Mañero et al., 2001)
	➢ Cytokinin synthesis	(Hussain & Hasnain, 2009)
	➢ Siderophore synthesis	(Woo, Woo, & Kim, 2007)
B. megaterium	➢ IAA synthesis	(Chakraborty, Chakraborty, & Basnet, 2006)
	➢ Cytokinin synthesis	(Ortíz-Castro, Valencia-Cantero, & López-Bucio, 2008)
	➢ Nitrogen fixing	(Yousuf et al., 2017)
	➢ Phosphate solubilization	(Chakraborty et al., 2006)
	➢ Siderophore synthesis	(Chakraborty et al., 2006)

	➢ Defence related gene expression (Induction of systemic resistance)	(Chakraborty et al., 2006)
B. altitudinis	➢ IAA synthesis	(Sun et al., 2017)
B. mucilaginosus	➢ K and SiO_2 solubilization	(Liu et al., 2006)
B. edaphicus	➢ Potassium solubilization	(Sheng & He, 2006)
B. circulans	➢ Potassium solubilization	(Zeng et al., 2012)
	➢ Nitrogen fixing	(Yousuf et al., 2017)
B. pumilus	➢ Gibberellin synthesis	(Gutiérrez-Mañero et al., 2001)

1.3.1 *Bacillus subtilis*

Bacillus subtilis is characterized by its high genetic diversity and ability to adapt to multiple environmental conditions (Earl, Losick, & Kolter, 2008). It has rapid growth, short fermentation cycle, and protein secretion ability, thus, has great importance in different fields in science and industry, having various potential biotechnological applications. Many kinds of industrial enzymes, medicinal proteins can be produced by using *B. subtilis* as a host. It is a model organism for genetic engineering purposes, as well as biofilm studies (Su, Liu, Fang, & Zhang, 2020).

B. subtilis is of great relevance in agriculture due to its numerous uses and its capacity of promoting plant growth as a rhizobacterial species. *B. subtilis* increase plants resistance against abiotic and biotic stresses. It enhances plant growth by indirect mechanisms as a biocontrol agent, through eliminating pathogens with its antagonistic effects (i.e., by secretion of antibiotics, hydrolytic enzymes like chitinase, siderophores, lipopeptides, nanoparticles, VOCs), and by triggering systemic resistance in plants (Alfiky, L'Haridon, Abou-Mansour, & Weisskopf, 2022; Gerayeli et al., 2018; Karunya, Reetha, Saranraj, & Milton, 2011), and by direct mechanisms such as fixing nitrogen, solubilizing phosphate and iron, producing phytohormones such as auxins, cytokinins, gibberellins, abscisic acid, jasmonic acid and salicylic acid, HCN synthesis, and ACC deaminase activity (Hashem et al., 2019). *B. subtilis* is also one of the species that serve in

reducing environmental pollution by bioremediation (Mahapatra, Yadav, & Ramakrishna, 2022).

B. subtilis produce surfactin, a biosurfactant. Biosurfactants have a wide range of applications in various industries, such as enhanced oil recovery, paints and cosmetics, food processing, and the production of industrial products, also having potential use as a bioremediator (Parthiban & Manimekalan, 2023).

B. subtilis form biofilms to colonize the roots by benefiting from the walls of the plant cells. The major component of biofilm, the EPS (extracellular polymeric substances) matrix, is an interface between host plant and bacteria through which signaling, and transfer of solutes occur which play role in plant growth promotion, nutrition, and induction of systemic resistance (Pieterse et al., 2014).

B. subtilis is one of the notable species in PGPR, having the ability of IAA biosynthesis. In Ünüvar, Zencirci, and Ünlü (2022), it was shown that *B. subtilis* inoculation to wheat increases root length significantly. So, it can be used as a biofertilizer supporting plant growth and development. Besides, IAA can be produced in large scale by using *B. subtilis* species.

1.4 Plant Growth Promotion Mechanisms in PGPR

PGPR have different mechanisms that increase plant growth. These are classified into two groups: i) direct mechanisms, ii) indirect mechanisms.

Direct mechanisms include direct interactions between PGPR and plants, such as the supply of growth-promoting molecules like auxins, gibberellins, cytokinins, abscisic acid, and brassinosteroids, the induction of the synthesis of these molecules in plants, increasing nutrient availability for plants by solubilizing inaccessible forms of nutrients, and producing siderophores that aid in the transportation of certain nutrients (such as ferric iron). Other than making growth promoting molecules available for plant uptake, PGPR may also increase nutrient uptake significantly by altering root growth and morphology (Vessey, 2003).

By indirect mechanisms, PGPR can support plant growth, via the inhibiton of plant pathogens by acting as biocontrol agents, and/or increasing or accelarating plants response to biotic and abiotic stress conditions by acting as priming agents (Mashabela, Piater, Dubery, Tugizimana, & Mhlongo, 2022).

1.4.1 Direct Mechanisms

1.4.1.1 Biological Nitrogen Fixation

Nitrogen is one of the most necessary elements in plant growth. It participates in the structure of nucleic acids and amino acids. Meeting the nitrogen needs of plants is quite important for productivity in agriculture. However, atmospheric dinitrogen cannot be used directly in biochemical processes of plants since N_2 gas is too stable. So, it must be converted to the suitable forms for plants' uptake. Nitrogen is typically assimilated by plants in the form of nitrate (NO_3^-) or ammonium (NH_4^+). These are the forms of nitrogen produced through the nitrogen fixation process which is a part of the biogeochemical nitrogen cycle. Soil bacteria, either free living or symbiotic, take place in nitrogen fixing and release ammonium (Bhattacharjee, Singh, & Mukhopadhyay, 2008; Tavares Nunes Monteiro, Nogueira, Neto, Nascimento, & Freitas, 2021).

1.4.1.2 Phosphate Solubilization

Phosphorus comes after nitrogen among the most essential minerals for the growth of plants. It takes place in the structure of nucleic acids, phospholipids, and ATP, and plays role in several biochemical reactions of which one of them is the photosynthesis (Schachtman, Reid, & Ayling, 1998). However, only a small part of the phosphorus in soil is in soluble form, available for plant use. Plants can uptake P in hydrogen phosphate (HPO_4^{-2}) and dihydrogen phosphate ($H_2PO_4^-$) forms. Various species of PGPR have been shown for converting the insoluble phosphates into plant-available forms by different mechanisms. These are called phosphate solubilizing bacteria (PSB) (Rodríguez & Fraga, 1999). However, a PGPR having the ability of solubilizing phosphate may not make an increase in phosphorus content of the host (Vessey, 2003). So, it is important to match the effective PGPR and host plant strains.

1.4.1.3 Siderophore Production

Siderophores are small molecules which have three iron binding groups and make chelates with Fe^{3+}. Iron is another growth limiting nutrient for plants, having many metabolic functions. In alkaline soil or in soil highly exposed to chemicals, iron deficiency is a potential problem for plants. Because iron is generally in insoluble forms and cannot be assimilated by plants. Siderophore synthesizing PGPR help plants to uptake iron by making possible the formation of siderophore-Fe (III) complex in soil. Fe^{3+} is transferred inside the root cells by iron transporters.

Siderophores also play role in plants defense against pathogens and reducing heavy metals in soil (Ali & Vidhale, 2013).

1.4.1.4 Phytohormone Production

Phytohormones are a kind of phytostimulators which are the regulatory molecules of plant growth and development. Phytostimulators consist of amino acids, vitamins, minerals, and hormones. In case of growth limiting environmental stress, plants secrete regulatory hormones to overcome. Some soil microorganisms can also produce phytohormones as secondary metabolites that plants can uptake. Major types of phytohormones are auxins, cytokinins, gibberellins, ethylene, and abscisic acid. Phytohormones make significant effects even they are applied in quite small amounts.

1.4.1.5 ACC Deaminase Production

ACC (1-aminocyclopropane-1-carboxylate) is the precursor of ethylene, which is a phytohormone secreted during pathogenic infection or other environmental stresses. High intensity ethylene secretion leads to growth inhibition in plants. Many PGPB can synthesize ACC deaminase enzyme which degrades the ACC excluded from the plant tissue, thus, inhibit the synthesis of stress ethylene (B. R. Glick, 2012).

1.4.2 Indirect Mechanisms

The impact of PGPR on soil quality may be due to interaction of bacteria with their surroundings. Their activity can change environmental conditions by various responses that may affect other members of the environment which in turn affect the plant growth eventually. PGPR produce biocontrol agents such as antibiotics, fungal cell wall degrading enzymes (protease, β-1,3-glucanase, chitinase), HCN, bacteriocins, siderophores, lipopeptides and volatile organic compounds. Also, there are other mechanisms such as competition, induced systemic resistance (ISR), and quorum quenching that prevent the growth inhibition caused by phytopathogens (Olanrewaju, Glick, & Babalola, 2017; Orozco-Mosqueda, Rocha-Granados, Glick, & Santoyo, 2018).

1.5 Auxin

Auxin is the first identified growth hormone which is produced naturally by plants. In 1880, Charles Darwin and his son firstly mentioned a substance that plays role in phototropism, which then will be isolated by Fritz W. Went in 1926 and called as auxin (Eckardt, 2001; Leyser, 2010).

The first identified auxin is indole-3-acetic acid. Its presence in plants was shown in the study of Haagen-Smit, Leech, and Bergen (1941). Auxins provide cell growth, cell division and cell differentiation in plants. Plays an important role in majority of the mechanisms related to the growth and development of plants such as embryogenesis, gametogenesis, seedling growth, leaf formation, flower development, vascular patterning (Kasahara, 2016; Zhao, 2012).

While natural auxins include the active auxins indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA), phenylacetic acid (PAA) and the inactive auxin precursors indole-3-butyric acid (IBA), and indole-3-propionic acid (IPrA), indole-3-acetic acid (IAA) is the most widespread in literature, and auxin and IAA have been attributed by each other. Figure 1.1 illustrates the chemical structure of IAA.

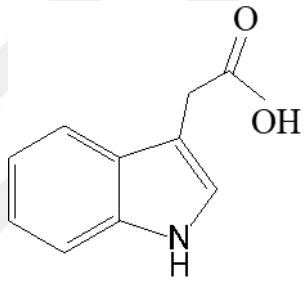

Figure 1.1. Structure of indole-3-acetic acid.

IAA, being the main auxin in plants, is synthesized not only in plants but also in other kingdoms. It is assumed that IAA is generated in more than 80% of prokaryotes including archaea and bacteria (Carrillo-Carrasco, Hernandez-Garcia, Mutte, & Weijers, 2023). In addition, according to Patten and Glick (1996) about 80% of rhizosphere microbes can synthesize and release auxin as a secondary metabolite (Olanrewaju et al., 2017).

1.5.1 Indole-3-Acetic Acid Synthesized by PGPB

Bacteria have their own purpose to synthesize IAA to increase their chances of survival. There are related studies on different strains that report, higher IAA levels in bacteria obtained by treating with exogenous IAA or genetically modifying them to get IAA-overproducing strains, makes them more resistant to harsh conditions (D. Duca, Rose, & Glick, 2014).

IAA synthesized by root associated beneficial bacteria has many significant roles as a signaling molecule in plant-microbe interactions. Phytostimulation and

pathogenesis are the two important mechanisms in plants that bacterial IAA effects on. Bacterial IAA shows its phytostimulatory effect as enhancing embryo, root, and flower development, stem elongation, leaf expansion, shoot branching, vascular formation, and differentiation. It increases root hair formation, and root length, initiates lateral root formation, so that root surface area increases. As a result, nutrient and water uptake of plants is enhanced. In addition, loosens cell walls of the root tissue allowing more root exudation that provides more nutrient for bacterial colonization. However, additional IAA supplied by PGPR may alter the IAA level to a supraoptimal level if the endogenous IAA level in the roots is optimal and can make inhibitory effect on plant growth (B. R. Glick, 2012).

Pseudomonas, *Rhizobium*, *Xanthomonas*, *Azospirillum*, and *Bacillus* are among the genera that were reported to synthesize IAA (Yadav, Rastegari, Yadav, & Kour, 2020b).

In case of pathogenesis, bacterial IAA has been reported to be a signaling molecule having influence on plants defense mechanisms. Studies on manipulation of auxin signaling can be benefited for enhancing plant growth (Spaepen, Vanderleyden, & Remans, 2007; S. Wang & Fu, 2011).

1.5.2 IAA Biosynthesis Pathways

There are several methods used for proposing biochemical pathways of IAA biosynthesis in plants or other organisms including: Isotope labelling of IAA precursors, quantification of IAA and its intermediates, measuring the activities of enzymes with known or predicted functions in pathways, genetic analysis covering the identification of genes potentially involved in pathways and determining their role by gene knockout or overexpression experiments, comparative genomics to locate the conserved genes related to IAA synthesis in different species and make hypotheses on the biosynthetic pathways.

1.5.3 IAA Biosynthesis in Plants

Based on the studies by above mentioned methods, it is assumed that IPyA pathway is the first complete and universally conserved IAA biosynthesis pathway in plants (Kasahara, 2016; Zhao, 2012).

The two-step reaction for IAA biosynthesis in plants involves the deamination of TRP to IPyA by tryptophan aminotransferase (TAAs) followed by the irreversible decarboxylation of IPyA to IAA, which is a rate-limiting step, catalyzed by flavin-containing monooxygenases (YUCs). In plants, TRP is

synthesized in the chloroplast by the conversion of chorismate into anthranilate and follows as in Figure 1.2 (Dong-Wei, 2016; Kasahara, 2016).

Other proposed pathways (IAM, IAN, IAOx, TAM) as seen below needs to be further investigated as well as the TRP-independent pathway proposed by Wang et. al. in which indole (IND) is synthesized as an IAA precursor by cytosol-localized indole synthase (INS).

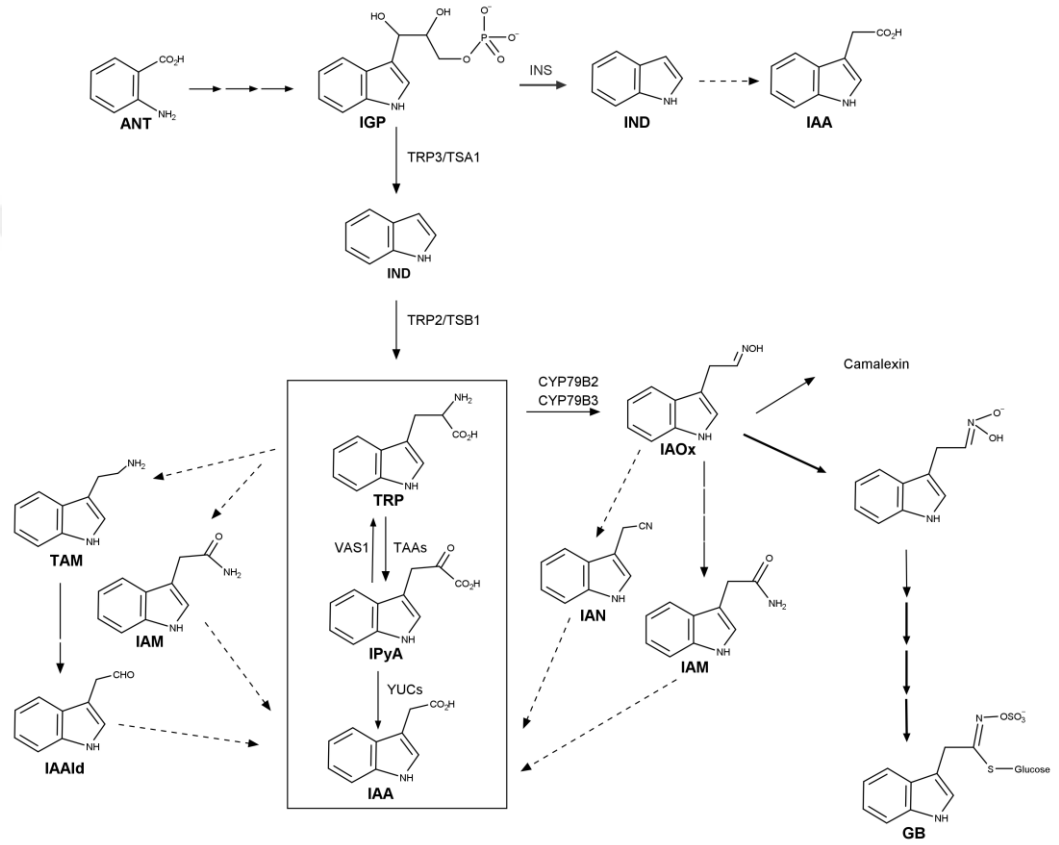

Figure 1.2. Putative IAA biosynthesis pathway in plants, adapted from Kasahara (2016).

1.5.4 IAA Biosynthesis in Bacteria

Proposed IAA synthesis pathways in microorganisms show a similar pattern to plants, mainly relying on tryptophan and involving intermediates such as indole-3-pyruvic acid (IPyA), indole-3-acetaldoxime (IAOx), indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), and indole-3-acetaldehyde (IAAld). Moreover, a TRP-independent IAA biosynthetic pathway in bacteria was suggested firstly for *Azospirillum brasilense* in Prinsen et. al. (Li et al., 2018; Prinsen, Costacurta, Michiels, Vanderleyden, & Van Onckelen, 1993).

1.5.4.1 TRP-dependent IAA biosynthesis

Putative pathways for TRP-dependent IAA biosynthesis in bacteria are illustrated in Figure 1.3.

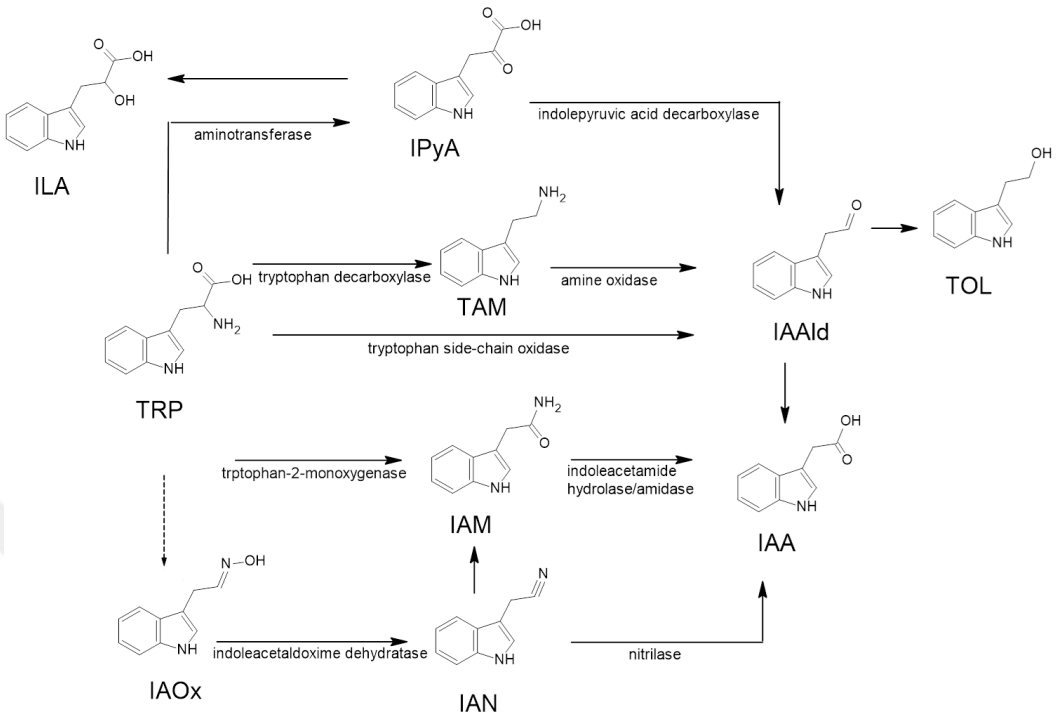

Figure 1.3. Putative pathways for bacterial IAA biosynthesis, adapted from Li et al. (2018).

In this pathway, L-tryptophan is the main precursor of IAA. Rhizosphere bacteria typically have the capacity to synthesize IAA. However, their endogenous tryptophan level is low, so they can produce high amounts of IAA through TRP-dependent pathway only if they have an exogenous source of tryptophan (D. R. Duca & Glick, 2020). Rhizobacteria can get tryptophan from their host plants since tryptophan is one of their root exudates. In D. R. Duca and Glick (2020), it was stated that a single PGPB strain could have multiple and interconnected pathways allowing the synthesis of IAA by the alternative route when the other was lost. TRP-dependent pathways proposed as results of genetic and biochemical studies are:

i. Indole-3-pyruvic acid (IPyA) pathway

In the first step, an aminotransferase deaminates L-tryptophan to form IPyA. Following this, IPyA is converted to IAAld by decarboxylase enzyme, which is further transformed into IAA by the action of indole-3-acetaldehyde dehydrogenase/oxidase/mutase (D. Duca, Lorv, Patten, Rose, & Glick, 2014).

ii. Indole-3-acetonitrile (IAN)/Indole-3-acetaldoxime (IAOx) pathway

This pathway starts with the transformation of TRP to IAOx, which is then converted to IAN by an indoleacetaldoxime dehydratase (Kato, Yoshida, & Asano, 2005). However, further research is needed to confirm the TRP-IAOx conversion step for bacteria. Subsequently, IAN is converted to IAA in a single step catalyzed by nitrilase or in two steps, including the transformation of IAN into IAM, and then IAM into IAA (D. Duca, Lorv, et al., 2014; Li et al., 2018).

iii. Indole-3-acetamide (IAM) pathway

IAM pathway has been stated to be the best characterized IAA synthesis pathway in bacteria. IAM pathway is a two-step pathway: TRP is converted to IAM by the activity of trptophan-2-monoxygenase, and then IAM is converted to IAA by the activity of IAM hydrolase. IAM pathway was stated to be in connection with IAN pathway. Such that, IAN is converted to IAM catalyzed by nitrile hydratase/nitrilase activity (D. Duca, Lorv, et al., 2014; Li et al., 2018; Spaepen et al., 2007).

iv. Tryptamine (TAM) pathway

Although this pathway has been reported to be common to plants and fungi (Patten & Glick, 1996), there are studies reporting TAM pathway for some bacterial species (Hartmann, Singh, & Klingmüller, 1983; Perley & Stowe, 1966). In this pathway TRP is converted to TAM by tryptophan decarboxylase activity, and then, TAM is converted to IAAld by amine oxidase, and IAAld is converted to IAA by a non-specific aldehyde oxidase (Carreño-Lopez, Campos-Reales, Elmerich, & Baca, 2000).

v. Trptophan side-chain oxidase (TSO) pathway

In TSO pathway, tryptophan side chain monooxygenase catalyzes the conversion of TRP to IAAld, which is then converted to IAA. Studies have been reported for some *Pseudomonas* strains related to this pathway (D. Duca, Lorv, et al., 2014; Oberhänsli, Dfago, & Haas, 1991; Suzuki, He, & Oyaizu, 2003; Whistler, Corbell, Sarniguet, Ream, & Loper, 1998). In addition, ILA as the enzymatic reduction product of IPyA, and ILA and TOL as the enzymatic reduction products of IAAld have been reported which were proposed as the storage compounds of IPyA and IAAld (D. Duca, Lorv, et al., 2014; Li et al., 2018; Spaepen et al., 2007).

2. AIM AND SCOPE OF THE STUDY

The variables in producing bacterial inoculants that will be used as biofertilizers are of great importance for both industrial-scale production and the use of products in agriculture.

In this study, we hypothesize that the IAA biosynthesis capacity of the bacterial strain *Bacillus subtilis* ESU181, which were isolated from the Iza wheat rhizosphere (Ünüvar et al., 2022), differ significantly at varying pH, temperature and additional L-tryptophan in culture media giving the highest IAA concentration in the optimum combination of these parameters. So, we plan to determine the optimum parameter combination for IAA biosynthesis.

3. MATERIALS AND METHODS

3.1 Materials

B. subtilis strain ESU181 obtained from the stock culture of the study of Ünüvar et al. (2022) was used in this thesis. In Table 3.1, chemicals used in the study are given.

Table 3.1. List of the chemicals used in the study.

No.	Chemical	Supplier	Cat No.
1	Acetone	Sigma	179124
2	Agar	Liofilchem	611001
3	IAA Standard	Duchefa Biochemie	10901.0005
4	Iron (III) chloride hexahydrate (FeCl3.6H2O)	Merck	1.03943.0250
5	L-tryptophan	Merck	1.08374.0010
6	Sodium chloride (NaCl)	Sigma-Aldrich	31434-1KG-R
7	Perchloric acid (HClO4)	Merck	1.09065.1000
8	Peptone	Bioshop	PEP403.1
9	Yeast Extract	Bioshop	YEX401.1

3.2 Methods

3.2.1 Determination of Indole-3-acetic acid (IAA) Biosynthesis Capacity of *Bacillus subtilis* Strain ESU181

The bacteria were revived from the frozen culture stock and were grown in two sets as in Table 3.2. Both sets were designed in two parts as the culture media (LB) is with and without being supplemented with 0.1% (w/v) L-tryptophan. In each set, bacteria were grown at three different pH values for 4 days.

Table 3.2. Cell Growth Parameters.

	Set 1		**Set 2**	
Temperature	30 °C		37 °C	
Culture medium	LB	LB (with tryptophan)	LB	LB (with tryptophan)
pH	6.5 ± 0.2 7.5 ± 0.2 8.5 ± 0.2			
Nutrient composition	Peptone 10 g/L Yeast extract 5 g/L NaCl 5 g/L			

1 ml of supernatants were taken every 24 h periods. The samples were centrifuged at 3000xg at 4 °C for 10 minutes. Supernatants were mixed with 2 ml of freshly prepared Salkowski reagent.

Salkowski reagent:

0,5 M $FeCl_3$ solution 6 ml

Distilled water 147 ml

% 70 perchloric acid 147 ml

The mixtures were left in the dark at room temperature for 30 minutes. Then, the IAA amounts were measured by the UV/VIS spectrophotometer at 535 nm. Bacterial cell densities were also measured at the end of each 24 hours of incubation period at 600 nm. IAA readings were optimized for 1 OD of bacterial cell density. And the IAA concentrations were calculated in µg/ml from the standard curve plotted by using the IAA standard with concentrations of 0.3125, 0.625, 1.25, 2.5, 5, 10, and 15 µg/mL (Table 3.3, Figure 3.1).

3.2.2 Analysis for Intracellular IAA Determination

Pellets, stored at -80 ℃, from the first part of our experiments were used to detect if any IAA present inside the bacterial cells.

500 µl of LB was added to each precipitate, and the cells were lysed by 10 sec. ultrasonication followed by ice bath (10 cycles). Finally, after adding 600 µl of LB, the sample was centrifuged at 20,000xg, 4 °C for 1 minute and 1 ml of the supernatant was used for the IAA test. IAA tests were done by the same method in 3.2.1(Ehmann, 1977; Sarker & Al-Rashid, 2013).

Table 3.3. Data for plotting the standard curve.

LB standard				
IAA Concenration (µg/mL)	Abs (1)	Abs (2)	Absorbance	SD
0,3125	0,0098	0,0105	0,0102	0,0005
0,625	0,0185	0,0189	0,0187	0,0003
1,25	0,0407	0,0410	0,0409	0,0002
2,5	0,0890	0,0839	0,0865	0,0036
5	0,1516	0,1640	0,1578	0,0088
10	0,3411	0,3416	0,3414	0,0004
15	0,5075	0,4966	0,5021	0,0077

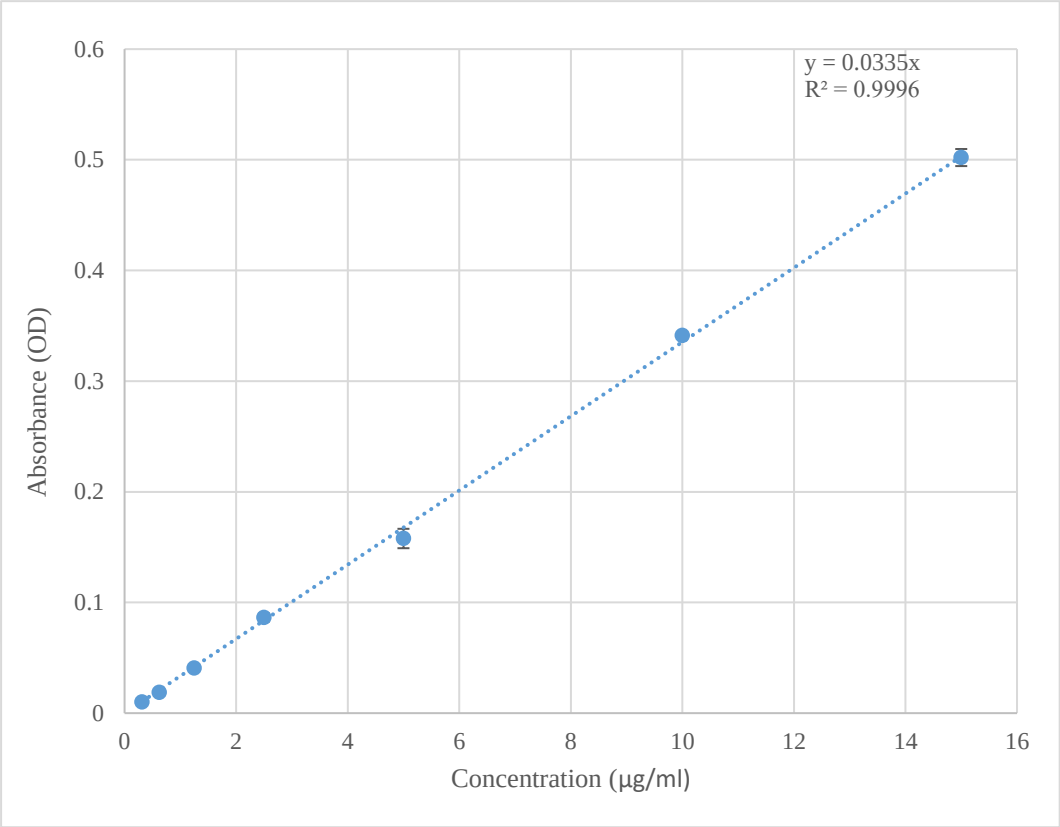

Figure 3.1. Standard curve for quantifying IAA in µg/ml.

3.2.3 Statistical Analysis

2way ANOVA along with Tukey's multiple comparison tests and Pearson correlation were performed by using Graphpad Prism 9.5.1.733 with serial number GPS-2730056-TGUD-7A7AD.

4. RESULTS

4.1 Quantitative Analysis for IAA Biosynthesis Capacity of *Bacillus subtilis* Strain ESU181

In Figures 4.1-8, results of the quantitative IAA analysis were given for two sets, regarding pH effect on IAA synthesis in different incubation times.

Set 1 (30 ℃):

The results for 24 h of incubation period indicate that the effect of pH change had a significant impact on IAA biosynthesis in TRP added media. After the 24th hour the pH effect was not observed as significant in TRP added media.

In the LB media a significant change was observed by the increase of pH from pH 6.5 to pH 7.5 only at the 24th hour, along with a significant change by the increase of pH to pH 8.5 starting from the 48th hour with a greater difference at the 96th hour.

Set 2 (37 ℃):

In TRP added media, statistically, the significant impact of pH was observed at the 24th hour. Whereas, in LB, the significant impact of pH was at the 24th and 96th hours.

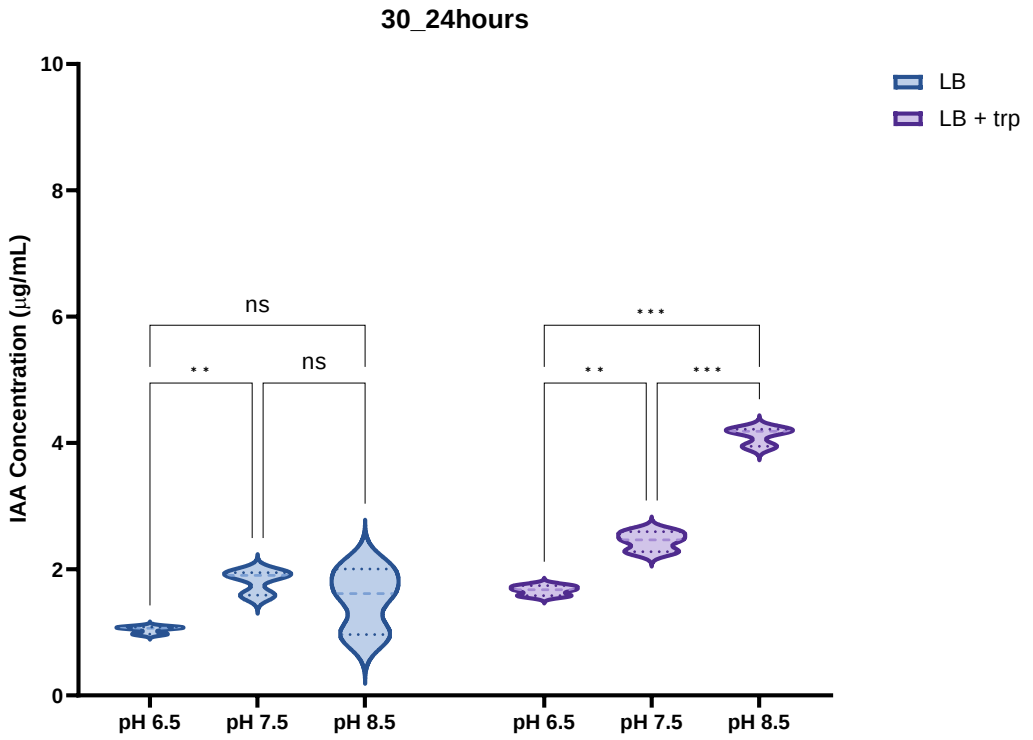

Figure 4.1. IAA biosynthesis at 30 °C with 24 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

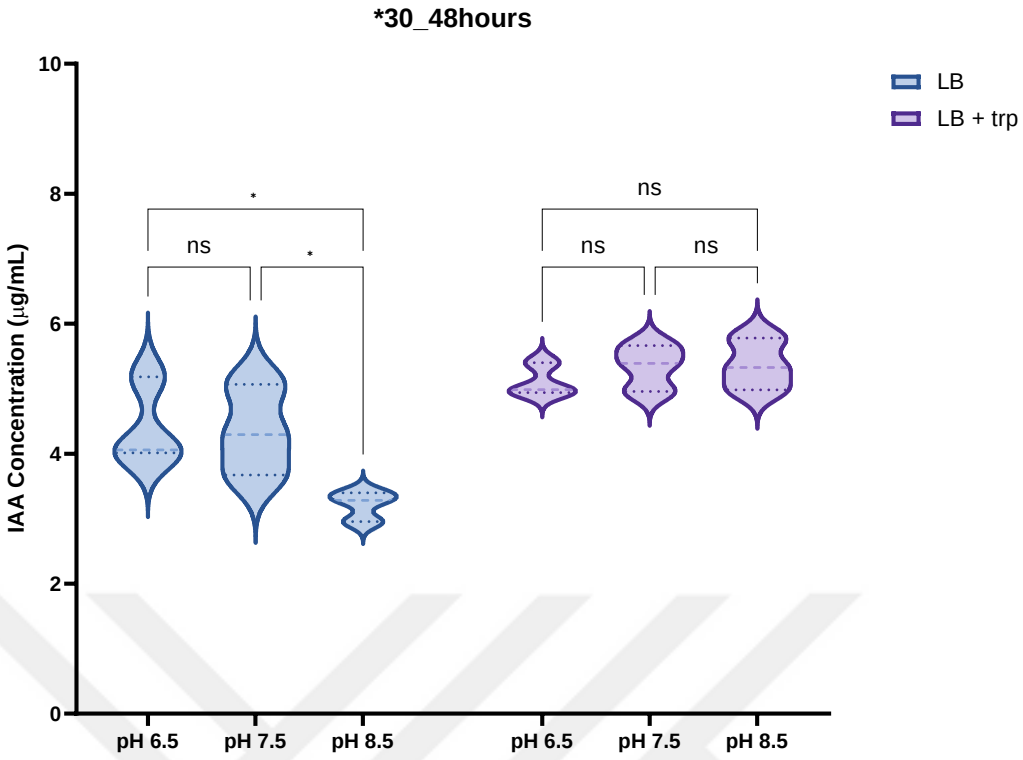

Figure 4.2. IAA biosynthesis at 30 °C with 48 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

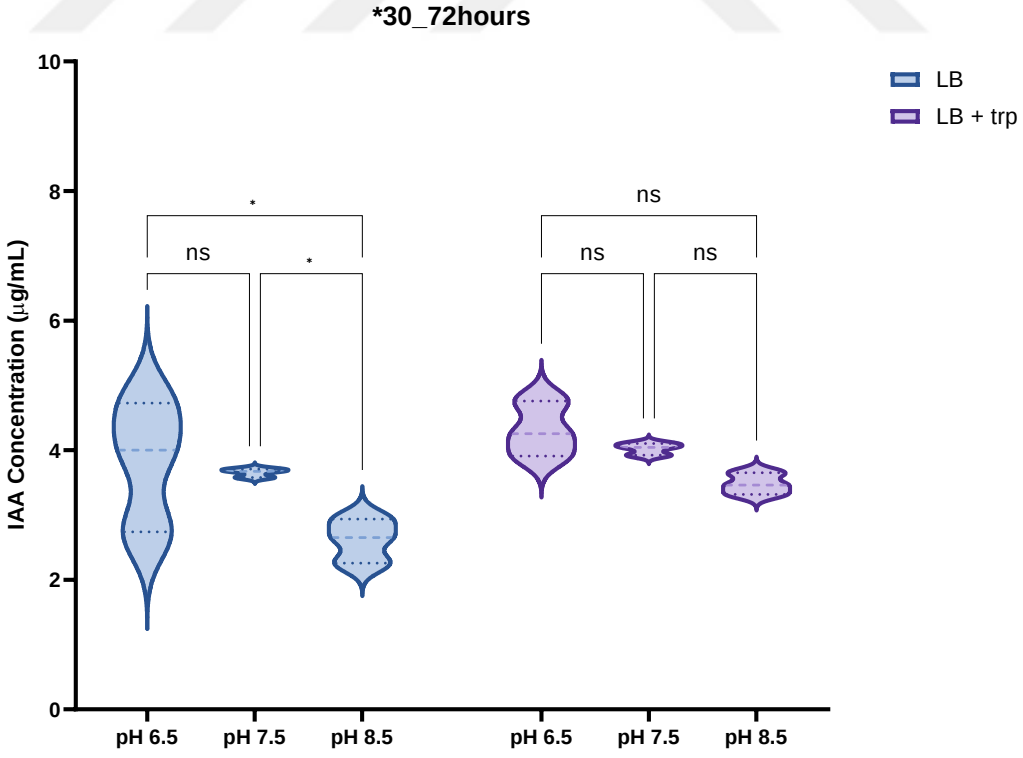

Figure 4.3. IAA biosynthesis at 30 °C with 72 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

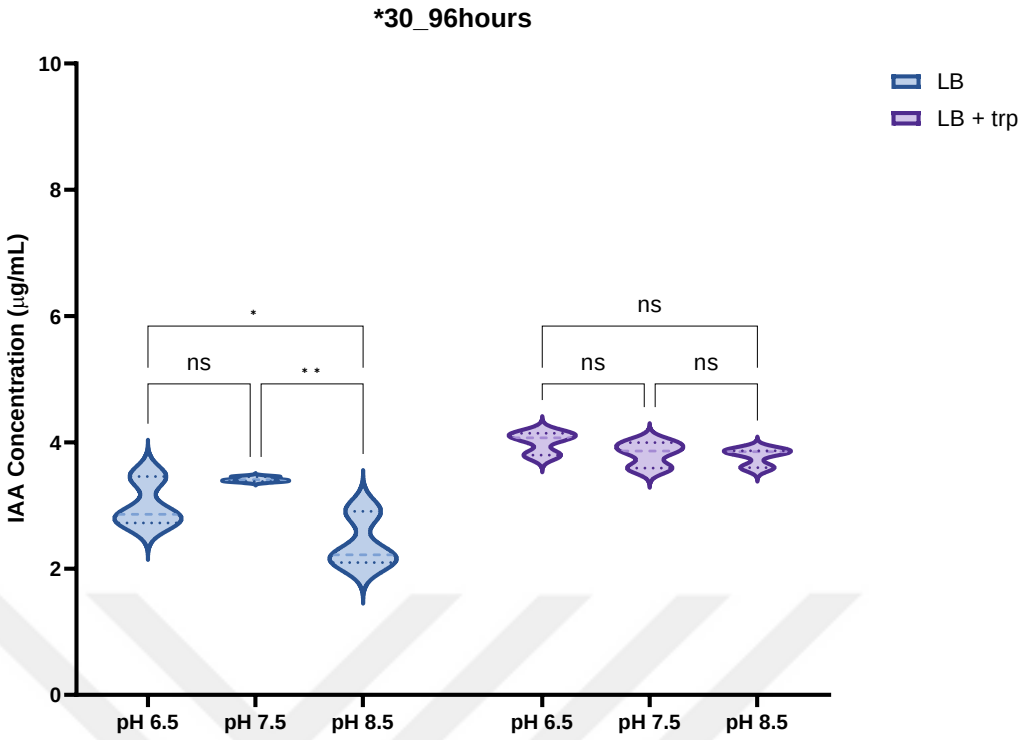

Figure 4.4. IAA biosynthesis at 30 °C with 96 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

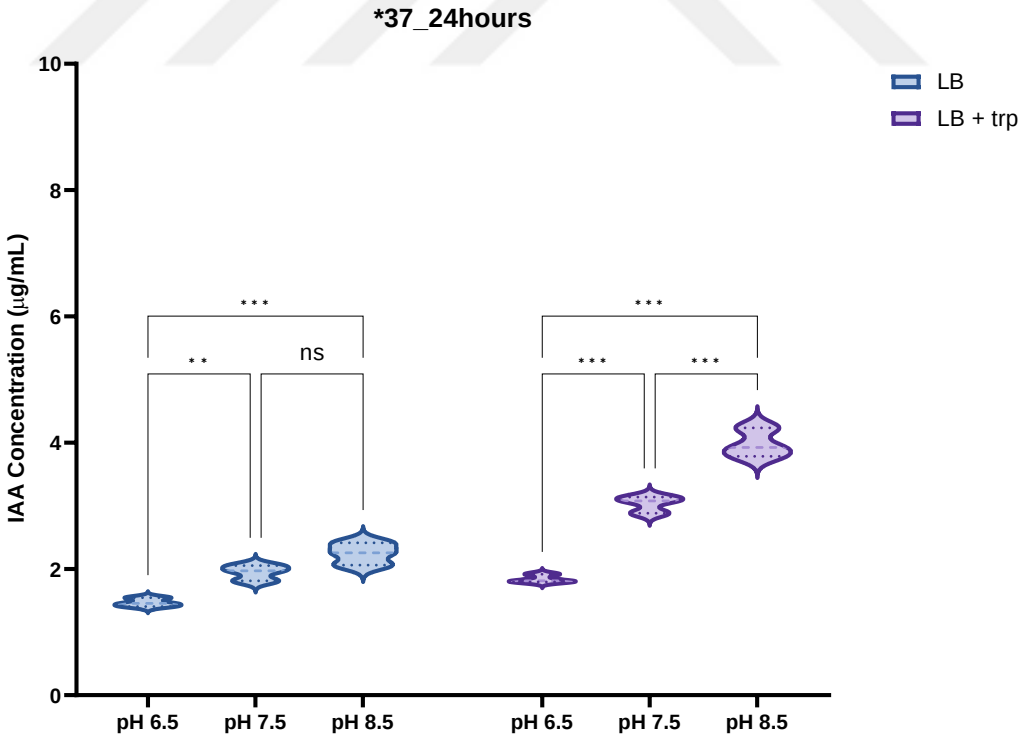

Figure 4.5. IAA biosynthesis at 37 °C with 24 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

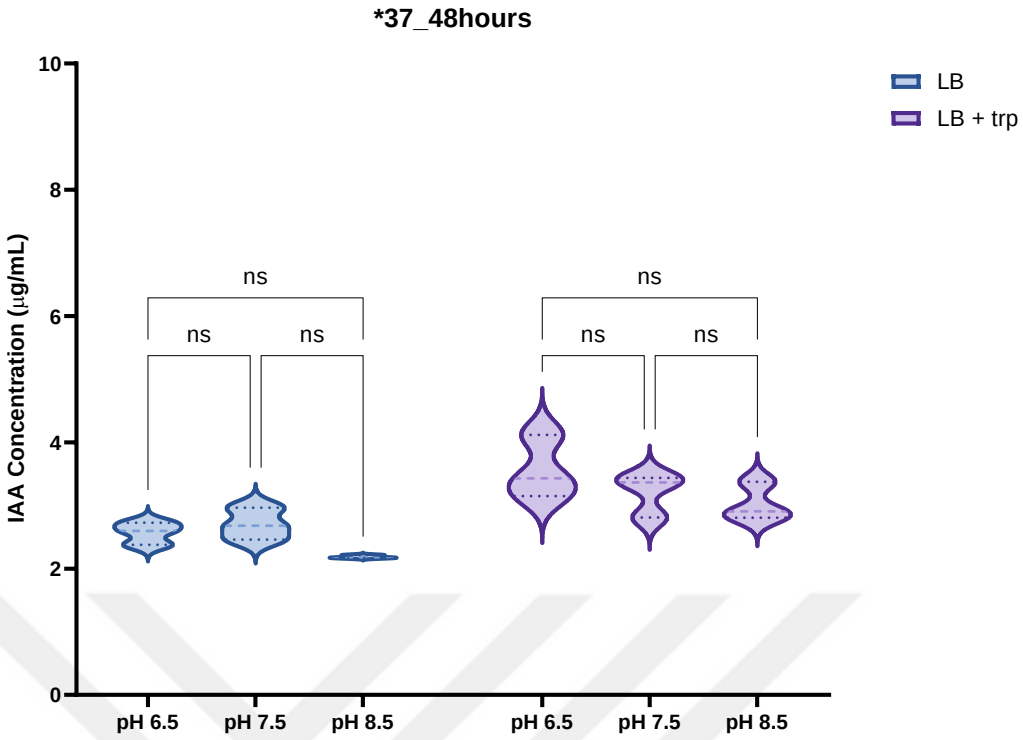

Figure 4.6. IAA biosynthesis at 37 °C with 48 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

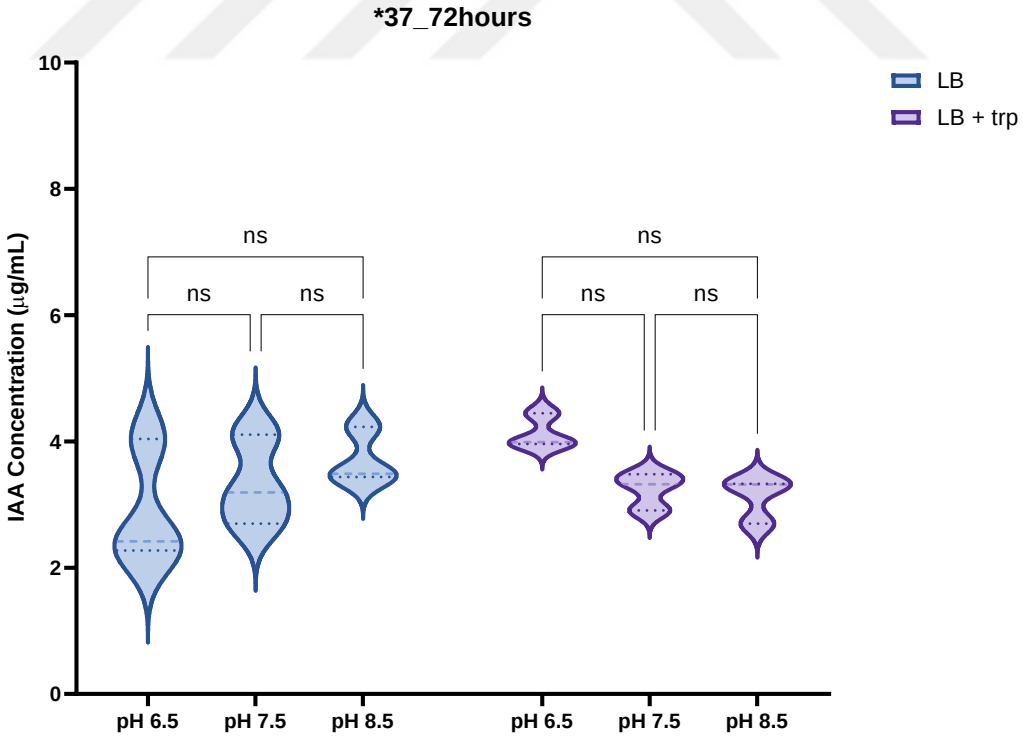

Figure 4.7. IAA biosynthesis at 37 °C with 72 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

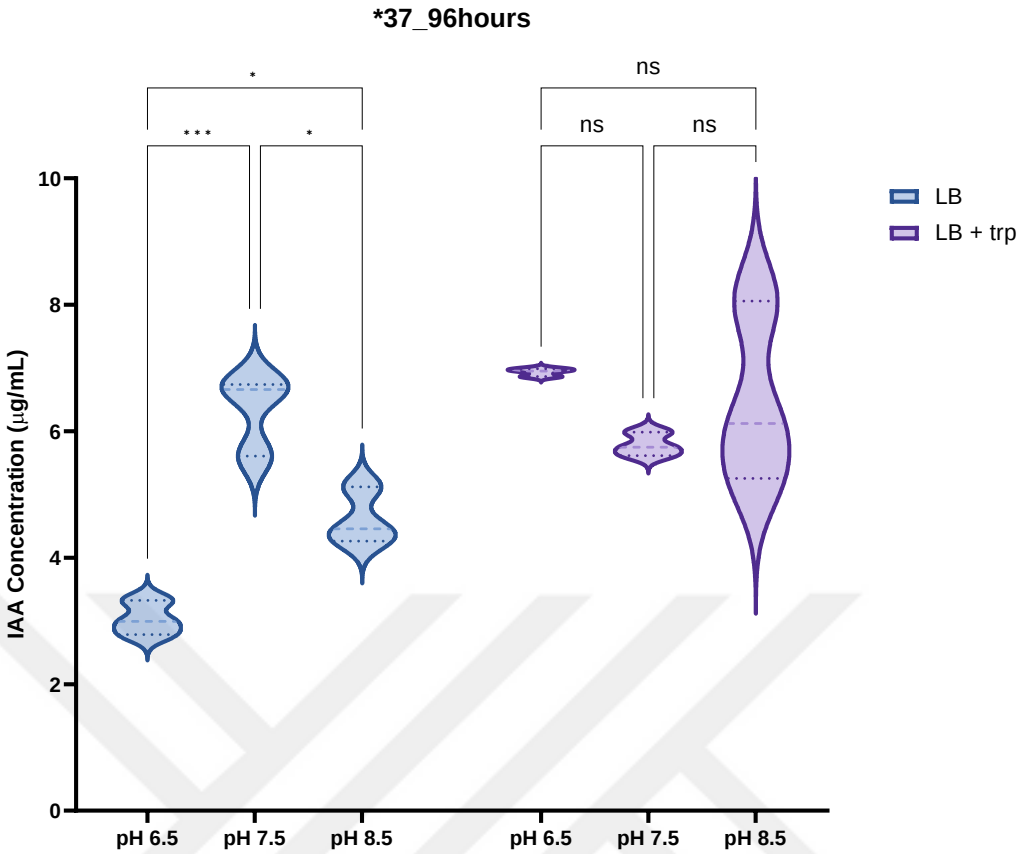

Figure 4.8. IAA biosynthesis at 37 °C with 96 h incubation period.
*p<0.05, **p<0.01, ***p<0.001

In Figures 4.9-11, results of the quantitative IAA analysis were given for the three pH values, considering the effect of incubation times on IAA synthesis at two different temperatures (Figures 4.9-4.11).

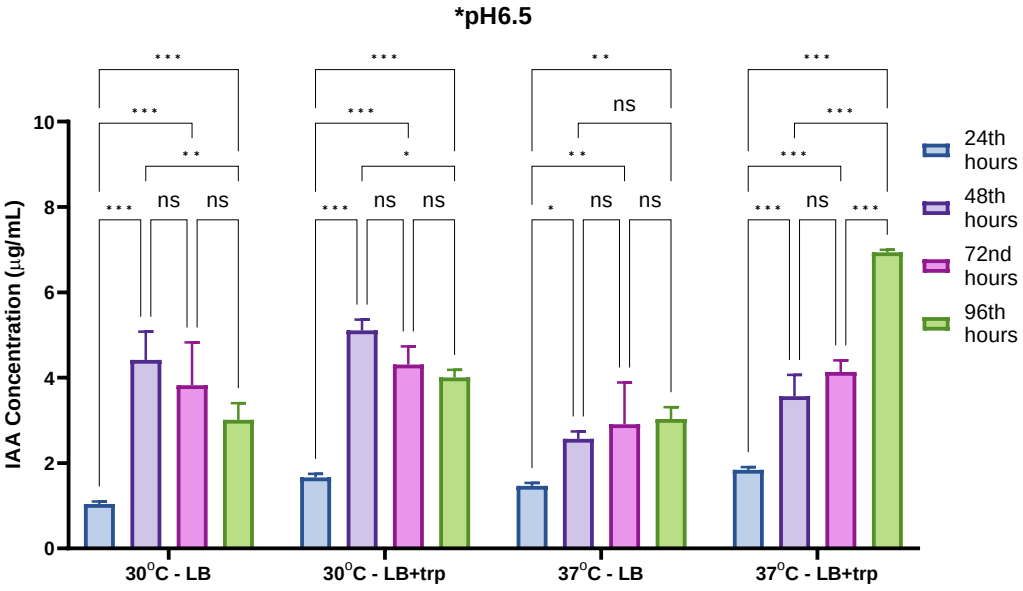

Figure 4.9. IAA biosynthesis at pH 6.5.
*p<0.05, **p<0.01, ***p<0.001

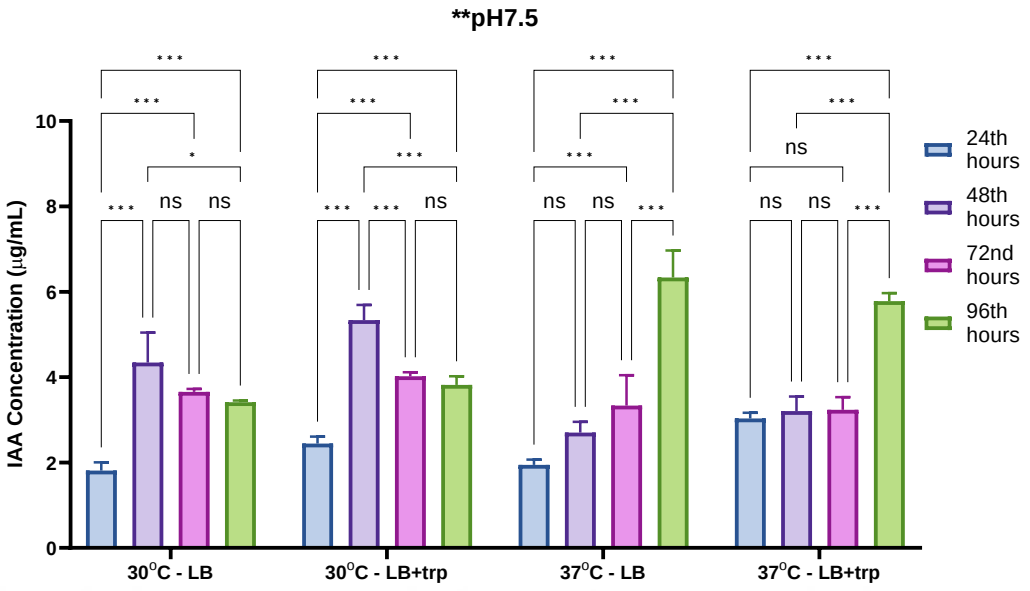

Figure 4.10. IAA biosynthesis at pH 7.5.
*p<0.05, **p<0.01, ***p<0.001

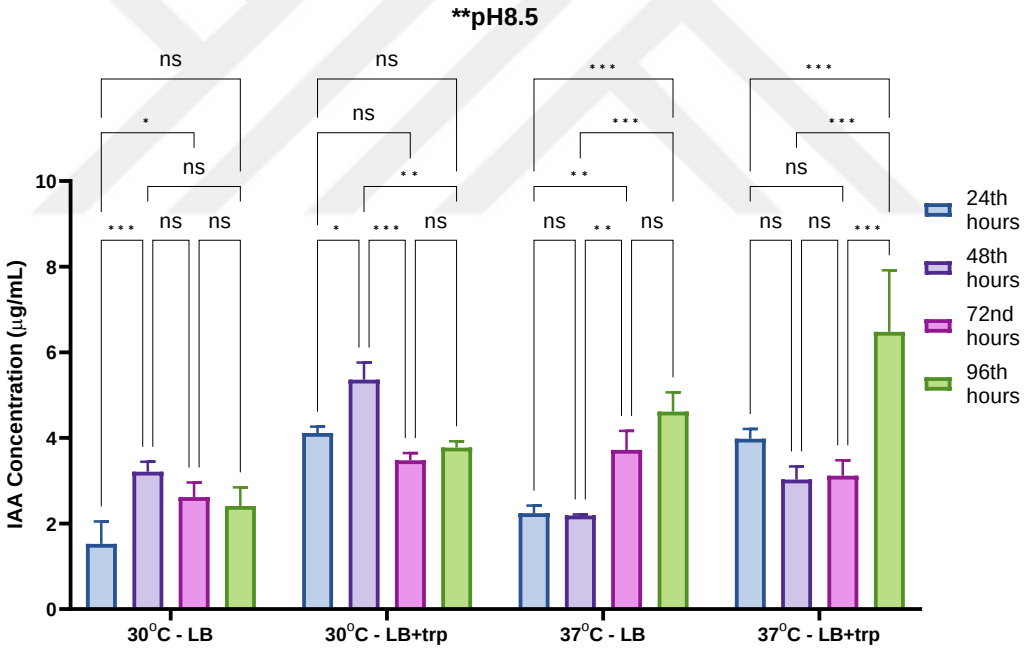

Figure 4.11. IAA biosynthesis at pH 8.5.
*p<0.05, **p<0.01, ***p<0.001

At 30 °C, a significant increase was observed on the 48th hour at all pH values followed by a decreasing profile after the 48th hours. However, at 37 °C, data showed that the IAA biosynthesis increased significantly at the 96th hour along with a fluctuation in between 24th and 96th hours.

4.2 Quantitative Analysis for Intracellular IAA Determination in *Bacillus subtilis* Strain ESU181

Insignificant levels of IAA were detected in the intracellular IAA analysis (Fig. 4.12-15).

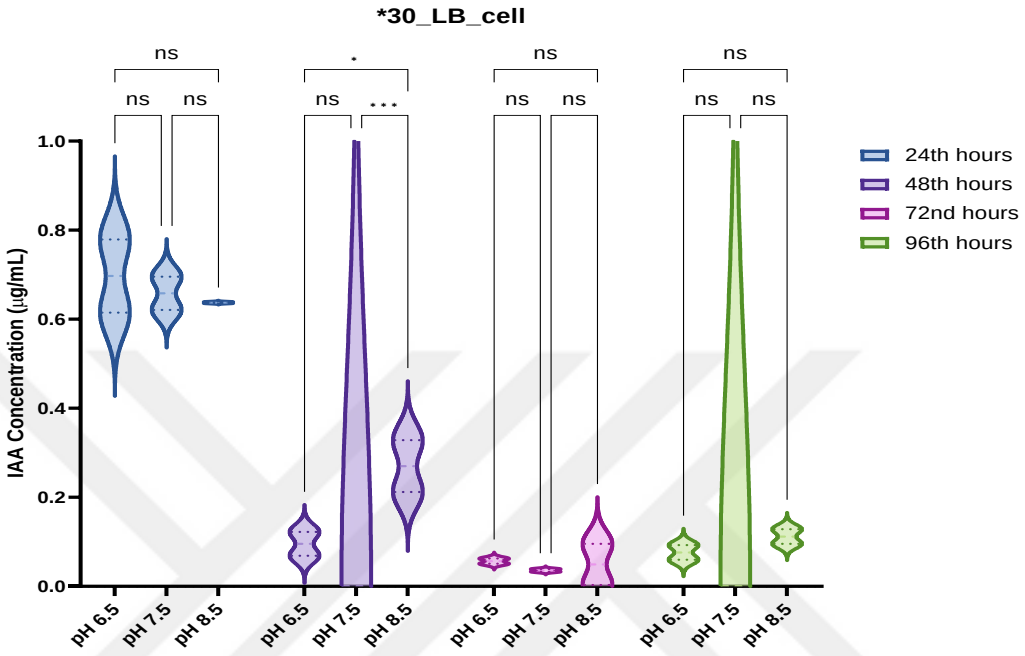

Figure 4.12. Intracellular IAA quantities at 30 °C in LB medium.
*p<0.05, **p<0.01, ***p<0.001

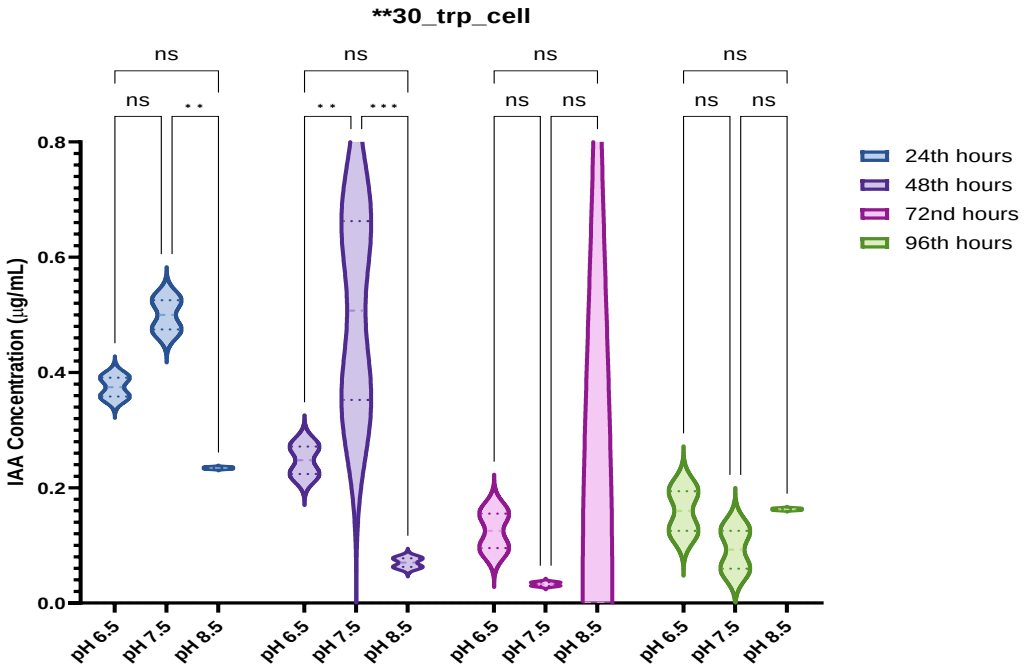

Figure 4.13. Intracellular IAA quantities at 30 °C in LB medium supplemented with 0.1% tryptophan.
*p<0.05, **p<0.01, ***p<0.001

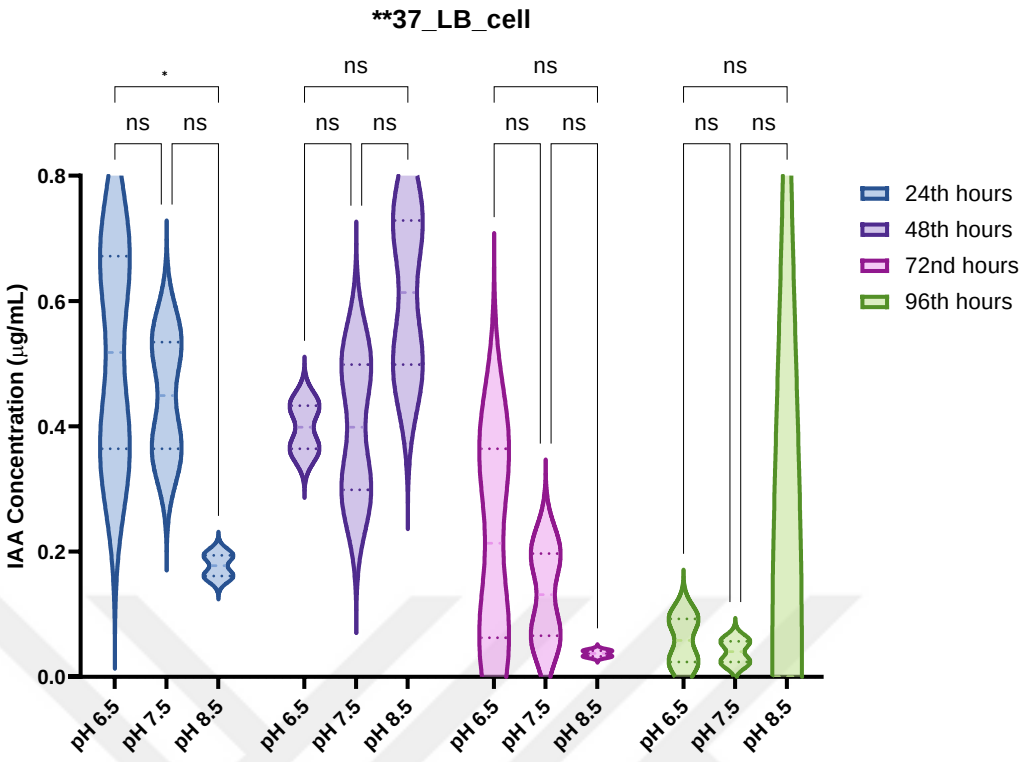

Figure 4.14. Intracellular IAA quantities at 37 °C in LB medium.
*p<0.05, **p<0.01, ***p<0.001

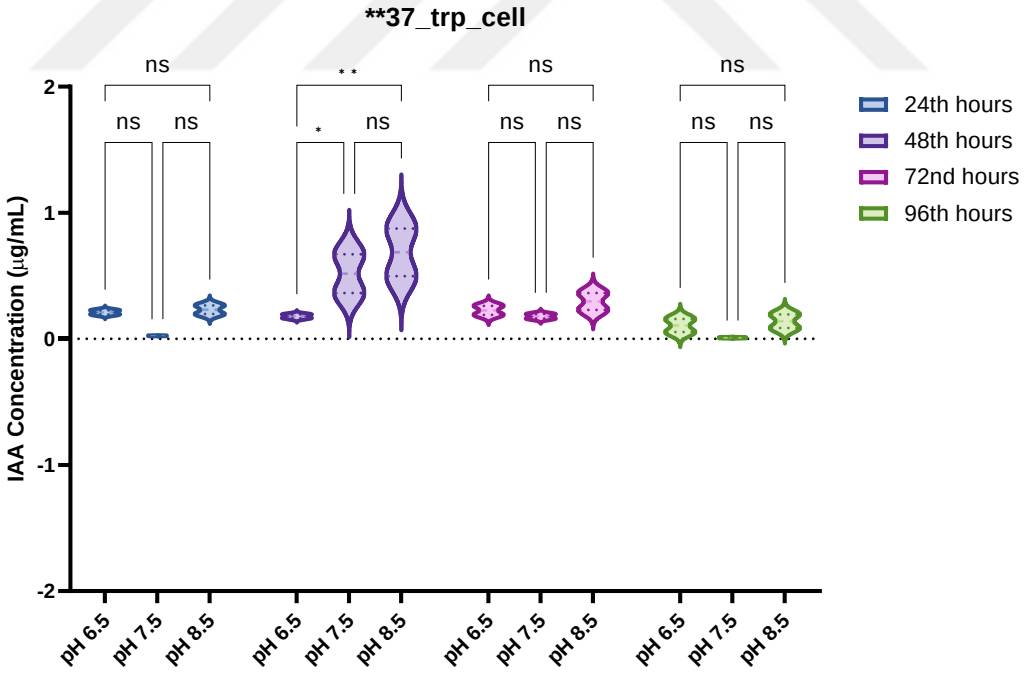

Figure 4.15. Intracellular IAA quantities at 37 °C in LB medium supplemented with 0.1% tryptophan**.**
*p<0.05, **p<0.01, ***p<0.001

4.3 Correlation Between Cell Growth and IAA Biosynthesis

Our data also showed that there is a medium degree of positive correlation between cell growth and IAA biosynthesis with a high significancy till the last 24 hours-period (Figure 4.16).

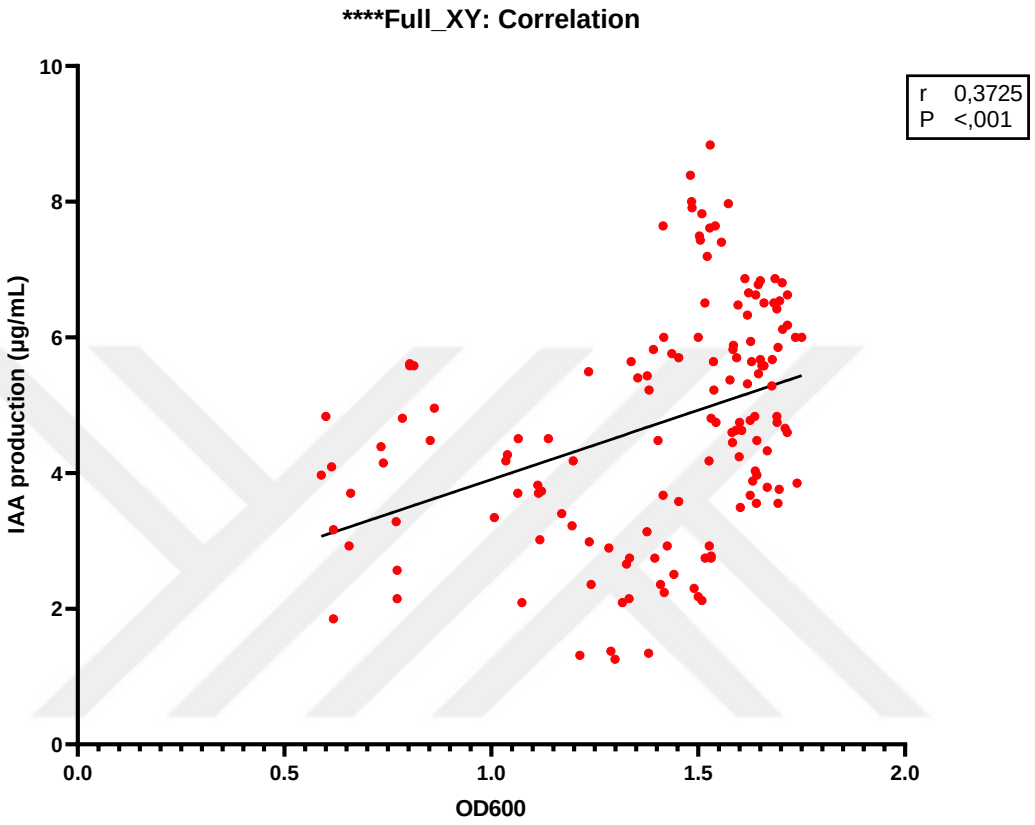

Figure 4.16. Cell growth vs. IAA correlation graph (144 XY pairs).

5. DISCUSSION

IAA is one of the important PGP hormones produced by soil microorganisms. In developing biofertilizers, optimization of PGP activities of the bacterial strains isolated from native species is necessary for obtaining the maximum effectivity.

In literature there are several IAA optimizations in different *Bacillus* species as well as other plant beneficial bacterial genera. Culture medium pH, temperature, energy source, incubation period, aeration, or agitation are factors that make considerable impact on bacterial growth and biosynthesis of metabolites or the other PGP activities of PGPB. Regarding IAA synthesis, the addition of external L-tryptophan is another factor to consider, as tryptophan is the primary precursor of IAA and the influence of exogenous tryptophan on the production of IAA is still being researched. However, there are few studies on *Bacillus subtilis* related with the optimization of IAA biosynthesis. Kumari et al. (2018) is a study in which seven *diazotropic B. subtilis* strains were tested for some of the plant growth promoting activities and the optimization of IAA production on the selected strain. Dat, Cuc, and Cuong (2015) is an IAA optimization study with a statistical approach on *B. subtilis* TIB6 by using response surface methodology. Swain and Ray (2008) is another study on optimization of culture conditions for IAA production by *B. subtilis* strain CM5 in solid state fermentation.

If we consider other studies with different *Bacillus* species, or *B. subtilis* strains which have similar methodology with ours, we can see parallel conclusions along with some contradictory results. Discrepancy in results has also been reported in other studies emphasizing the varying effects of nutritional and environmental conditions on different strains (Elsoud, Hasan, & Elhateir, 2023; Özdal, Gür Özdal, Sezen, & Algur, 2016).

Our results are in line with the studies (Kumari et al., 2018; Wagi & Ahmed, 2019), reporting high auxin production potential by both TRP-dependent and independent pathways in *Bacillus subtilis*.

In Kumari et al. (2018) the optimum pH (4, 5, 6, 7, 8 and 9) was tested and reported as pH 7, the temperature (range: 25, 30, 35, 40, 45 and 50 °C) was reported as 35 °C and the optimum incubation period (range: 0-144 hours) was 96 h, whereas in Wagi and Ahmed (2019) the optimum temperature was 27 °C and 25 °C for *B.*

cereus and *B. subtilis*, respectively, and the maximum IAA concentration was reported at the 24th hour for both. In Suliasih and Widawati (2020), the optimum culture conditions for *B. siamensis* were reported as pH 8 being slightly higher than pH 6 and pH 7, 35 °C, and 96 h of incubation. The study also reported pH 8 to be optimum in refered several other studies. The pH values selected in our study are average levels for Türkiye's soils. In the first 24 hours of incubation, there's an obvious increase in IAA biosynthesis with the increasing pH in both sets. So, to have the maximum IAA yield in 24 h, the medium should be more alkaline.

Our results also confirm that an incubation period of 96 hours is appropriate for observing IAA production according to cell growth and IAA biosynthesis correlation given in 4.3.

The additional TRP had a significant effect at a later stage, on the 3rd and 4th days of incubation (37 ℃), at pH 6.5. However, at pH 7.5, this effect was observed earlier, within the first 24 h and 48 h, at 37 ℃ and 30 ℃, respectively. There is no data that would allow us to reach such a conclusion for pH 8.5. Difference in the additional TRP effects at varying pH levels could be due to the activations of different enzymes which are influenced from specific changes in certain amino acids, in the presence of tryptophan.

Variation of pH and temperature changes the IAA biosynthesis levels because of their effects on the expression of mRNA, and the expression and activity of proteins involved in the IAA biosynthesis.

After having the results for IAA quantities in all samples we observed irregular changes in data at 37 ℃, so decided to investigate the possibility of IAA storage by the cells through the incubation period. Our hypothesis was that the bacterial IAA synthesized was being utilized as an extracellular metabolite and not being stored in the cell. So, in the second part of our study we aimed to find out if any IAA exists inside the bacterial cells, using the pellets from the first part stored at -80 ℃. According to results the cause of decline is another factor, and possibly the IAA degrading enzymes produced by bacteria.

The data of this study provide an opportunity to increase IAA in soil having similar environmental conditions, or by adapting the conditions to get maximum efficiency, also along with bio-inoculant application. Moreover, the data can be adapted to industrial production of IAA.

Regarding the limitations of the study, parameters of pH, temperature and additional tryptophan could have been tested over a wider range, or parameters could be diversified. Lack of expression data related with the TRP-dependent pathway can also be considered as another limitation.

6. CONCLUSIONS AND RECOMMENDATIONS

In this thesis we aimed to investigate the influence of varying culture medium parameters pH, temperature, incubation time and additional tryptophan on IAA biosynthesis capasity in *B. subtilis* strain ESU181 and to optimize IAA biosynthesis within the chosen ranges. The highest IAA concentration was obtained in LB+0.1% (w/v) L-tryptophan culture medium with an initial pH 6.5, at 37 ºC, and by 96 hours of incubation. The additional tryptophan made considerable effect but not as high as expected on this strain. The decline or fluctuation in IAA levels was estimated to be related to reasons other than IAA storage in cells.

Future studies can cover the investigation of IAA biosynthesis pathways in *B. subtilis* ESU181 by focusing on the expressions of a selection of TRP-dependant pathway genes. Proteins that are active on bacterial IAA transport mechanism can be investigated. Also, the TRP-independent biosynthesis of bacterial IAA, which appears to be considerably active in this strain, warrants further investigation and may serve as a research topic. Regarding the industrial production of IAA, detailed optimization studies can be performed for the first 24 hours of incubation period to increase productivity.

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8. APPENDICES

Appendix 1 Data of IAA quantification in *B. subtilis* Strain ESU181

Table 8.1. Data of Set 1

30 ℃					
24 hours	**LB**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,214	0,044	0,036	1,082	1,040	0,058
1,289	0,046	0,036	1,065		
1,380	0,045	0,033	0,973		
ph 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,074	0,070	0,065	1,946	1,811	0,195
1,317	0,070	0,053	1,587		
1,241	0,079	0,064	1,900		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,299	0,042	0,032	0,965	1,527	0,524
1,327	0,089	0,067	2,002		
1,333	0,072	0,054	1,612		
48 hours	**LB**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,509	0,262	0,174	5,183	4,418	0,663
1,436	0,193	0,134	4,012		
1,596	0,217	0,136	4,059		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,573	0,267	0,170	5,067	4,343	0,699
1,537	0,189	0,123	3,671		
1,516	0,218	0,144	4,293		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,538	0,175	0,114	3,397	3,211	0,230
1,619	0,178	0,110	3,282		
1,637	0,162	0,099	2,954		
72 hours	**LB**				

pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,526	0,140	0,092	2,739	3,822	1,006
1,522	0,241	0,158	4,727		
1,500	0,201	0,134	4,000		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,593	0,191	0,120	3,579	3,655	0,068
1,584	0,195	0,123	3,675		
1,585	0,197	0,124	3,710		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,626	0,123	0,076	2,258	2,615	0,341
1,599	0,142	0,089	2,651		
1,626	0,16	0,098	2,937		
96 hours	**LB**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,735	0,201	0,116	3,458	3,014	0,391
1,690	0,162	0,096	2,861		
1,710	0,156	0,091	2,723		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,679	0,190	0,113	3,378	3,414	0,039
1,693	0,196	0,116	3,456		
1,577	0,180	0,114	3,407		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,693	0,119	0,070	2,098	2,408	0,437
1,591	0,155	0,097	2,908		
1,696	0,126	0,074	2,218		
24 hours	**LB+TRP**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,418	0,075	0,053	1,579	1,664	0,081
1,441	0,084	0,058	1,740		
1,409	0,079	0,056	1,674		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,415	0,123	0,087	2,595	2,446	0,159

1,453	0,120	0,083	2,465		
1,376	0,105	0,076	2,278		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,377	0,182	0,132	3,945	4,115	0,148
1,392	0,195	0,140	4,182		
1,338	0,189	0,141	4,217		
48 hours	**LB+TRP**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,503	0,251	0,167	4,985	5,108	0,254
1,505	0,249	0,165	4,939		
1,415	0,256	0,181	5,401		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,481	0,281	0,190	5,664	5,338	0,355
1,541	0,256	0,166	4,959		
1,484	0,268	0,181	5,391		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,529	0,296	0,194	5,779	5,362	0,400
1,528	0,255	0,167	4,982		
1,485	0,265	0,178	5,327		
72 hours	**LB+TRP**				
pH 6.5					
OD cell	OD IAA	IAA (1 0D)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,619	0,212	0,131	3,909	4,308	0,427
1,613	0,230	0,143	4,256		
1,556	0,248	0,159	4,758		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,639	0,222	0,135	4,043	4,023	0,092
1,622	0,223	0,137	4,104		
1,659	0,218	0,131	3,923		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,646	0,183	0,111	3,319	3,478	0,167
1,629	0,189	0,116	3,463		
1,627	0,199	0,122	3,651		
96 hours	**LB+TRP**				

pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,690	0,215	0,127	3,798	4,004	0,182
1,686	0,230	0,136	4,072		
1,650	0,229	0,139	4,143		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,704	0,205	0,120	3,591	3,816	0,206
1,703	0,228	0,134	3,996		
1,716	0,222	0,129	3,862		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,716	0,207	0,121	3,601	3,773	0,149
1,683	0,218	0,130	3,867		
1,697	0,219	0,129	3,852		

Table 8.2. Data of Set 2

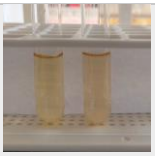
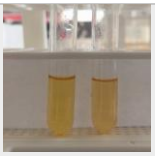
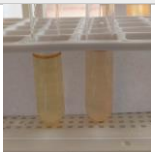
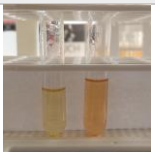
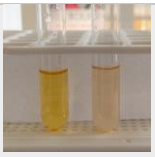
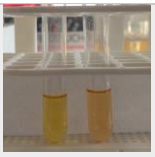
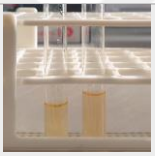
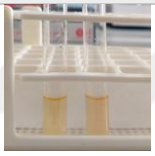
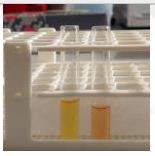
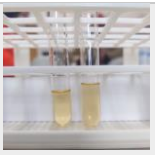
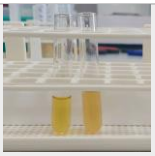
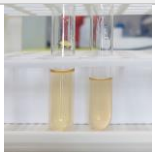
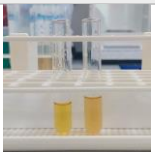
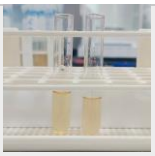
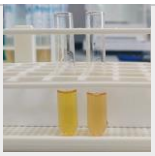
37 ℃					
24 hours	**LB**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,509	0,071	0,047	1,405	1,467	0,070
1,500	0,073	0,049	1,453		
1,490	0,077	0,052	1,543		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,517	0,092	0,061	1,810	1,944	0,123
1,395	0,092	0,066	1,969		
1,425	0,098	0,069	2,053		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,237	0,100	0,081	2,413	2,242	0,178
1,284	0,097	0,076	2,255		
1,334	0,092	0,069	2,059		
48 hours	**LB**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,632	0,130	0,080	2,378	2,567	0,176
1,667	0,145	0,087	2,596		
1,642	0,150	0,091	2,727		

pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,638	0,135	0,082	2,460	2,702	0,254
1,715	0,154	0,090	2,680		
1,600	0,159	0,099	2,966		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,739	0,129	0,074	2,214	2,186	0,025
1,602	0,117	0,073	2,180		
1,641	0,119	0,073	2,165		
72 hours	**LB**				
pH 6.5					
OD cell	OD IAA	IAA (1 0D)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,035	0,140	0,135	4,038	2,910	0,979
1,667	0,127	0,076	2,274		
1,642	0,133	0,081	2,418		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,117	0,101	0,090	2,699	3,333	0,715
1,403	0,15	0,107	3,191		
1,039	0,143	0,138	4,108		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,198	0,140	0,117	3,488	3,719	0,445
1,112	0,128	0,115	3,436		
1,065	0,151	0,142	4,232		
96 hours	**LB**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
0,618	0,062	0,100	2,995	3,035	0,273
0,772	0,086	0,111	3,325		
0,772	0,072	0,093	2,784		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
0,660	0,124	0,188	5,608	6,336	0,632
0,589	0,133	0,226	6,740		
0,614	0,137	0,223	6,661		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
0,770	0,110	0,143	4,264	4,615	0,448
0,618	0,106	0,172	5,120		

0,656	0,098	0,149	4,459		
24 hours	**LB+TRP**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,531	0,092	0,060	1,794	1,841	0,066
1,531	0,093	0,061	1,813		
1,527	0,098	0,064	1,916		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,543	0,159	0,103	3,076	3,033	0,134
1,605	0,155	0,097	2,883		
1,531	0,161	0,105	3,139		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,417	0,201	0,142	4,234	3,980	0,231
1,381	0,175	0,127	3,783		
1,453	0,191	0,131	3,924		
48 hours	**LB+TRP**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,645	0,227	0,138	4,119	3,385	0,500
1,750	0,201	0,115	3,429		
1,678	0,177	0,105	3,149		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,650	0,190	0,115	3,437	3,117	0,343
1,583	0,149	0,094	2,810		
1,659	0,187	0,113	3,365		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,582	0,154	0,097	2,906	3,408	0,303
1,654	0,187	0,113	3,375		
1,691	0,159	0,094	2,807		
72 hours	**LB+TRP**				
pH 6.5					
OD cell	OD IAA	IAA (1 0D)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,138	0,151	0,133	3,961	3,685	0,273
1,354	0,181	0,134	3,990		
1,235	0,184	0,149	4,447		
pH 7.5					

OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,114	0,124	0,111	3,323	3,176	0,295
1,064	0,124	0,117	3,479		
1,170	0,114	0,097	2,909		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
1,007	0,112	0,111	3,320	4,633	0,362
1,121	0,125	0,112	3,329		
1,195	0,108	0,090	2,698		
96 hours	**LB+TRP**				
pH 6.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
0,803	0,187	0,233	6,952	6,935	0,063
0,813	0,187	0,230	6,866		
0,803	0,188	0,234	6,989		
pH 7.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
0,733	0,147	0,201	5,986	5,783	0,188
0,739	0,139	0,188	5,615		
0,862	0,166	0,193	5,749		
pH 8.5					
OD cell	OD IAA	IAA (1 OD)	IAA (µg/ml)	IAA mean (µg/ml)	IAA SD
0,600	0,162	0,270	8,060	6,479	1,436
0,785	0,161	0,205	6,122		
0,852	0,150	0,176	5,255		

Appendix 2 Culture sample views at the end of IAA tests

24 hours	LB	IAA $_{mean}$ (μg/ml)	LB (+TRP)	IAA $_{mean}$ (μg/ml)
pH 6.5		1.040		1.664
ph 7.5		1.811		2.446
pH 8.5		1.527		4.115
48 hours	**LB**		**LB (+TRP)**	
pH 6.5		4.418		5.108
pH 7.5		4.343		5.338
pH 8.5		3.211		5.362
72 hours	**LB**		**LB (+TRP)**	
pH 6.5		3.822		4.308
pH 7.5		3.655		4.023
pH 8.5		2.615		3.478
96 hours	**LB**		**LB (+TRP)**	

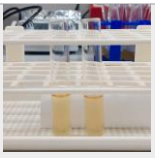
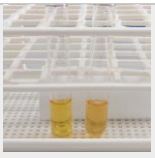
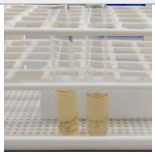
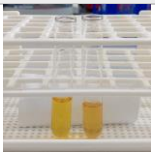
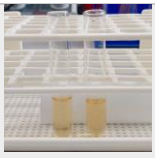
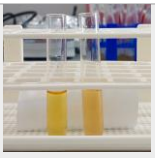
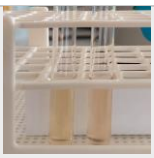
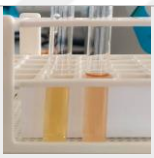
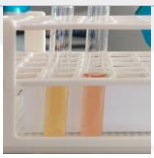
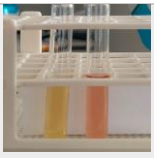
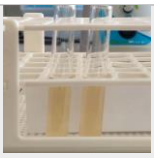
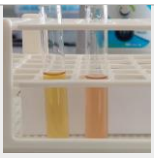
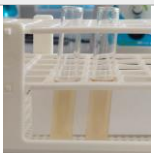
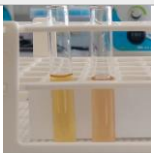
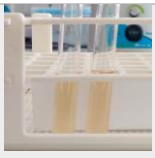
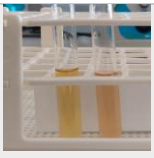
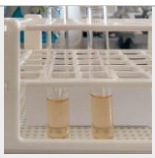
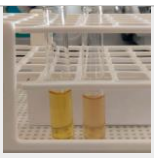
pH 6.5		3.014		4.004
pH 7.5		3.414		3.816
pH 8.5		2.408		3.773

Figure 8.1. Culture sample views at the end of IAA tests in Set 1 - control (left hand sides) vs. inoculated media (right hand sides).

24 hours	**LB**	**IAA $_{mean}$ (µg/ml)**	**LB (+TRP)**	**IAA $_{mean}$ (µg/ml)**
pH 6.5		1.467		1.841
ph 7.5		1.944		3.033
pH 8.5		2.242		3.980
48 hours	**LB**		**LB (+TRP)**	
pH 6.5		2.567		3.385
pH 7.5		2.702		3.117
pH 8.5		2.186		3.408
72 hours	**LB**		**LB (+TRP)**	
pH 6.5		2.910		3.685

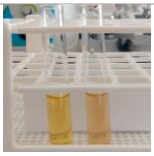
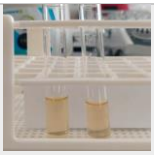
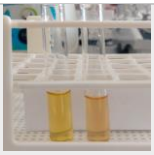
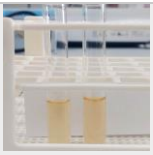
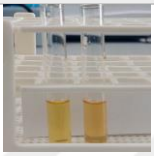
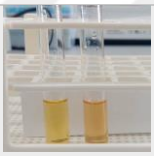
pH 7.5		3.333		3.176
pH 8.5		3.719		4.633
96 hours	**LB**		**LB (+TRP)**	
pH 6.5		3.035		6.935
pH 7.5		6.336		5.783
pH 8.5		4.615		6.479

Figure 8.2. Culture sample views at the end of IAA tests in Set 2 - control (left hand sides) vs. inoculated media (right hand sides).