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Industrially Important Enzyme and Plant Growth Promoter Potential of Soil Actinomycetes

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ABSTRACT

Enzymes and plant growth promoters have been found out to be the most widely used bacterial secondary metabolites, in industrial processes. Approximately, 10,000 bioactive metabolites which is 45% of the all bioactive microbial metabolites discovered, are produced by Actinobacteria. Therefore, they are considered as one of the most valuable microbial groups in biotechnology. In this study, the enzymes and Indole Acetic Acid (IAA) production capability and efficiency of soil Actinomycetes were explored. The enzymes production capability of 16 soil Actinomycetes isolates was tested. The number of isolates produced amylase, protease, L-asparaginase, cellulase, and lipase were 16, 8, 16, 14, and onerespectively. IAA production capability of the 16 soil-Actinomycetes isolates wasdetermined both qualitatively and quantitatively. Ten isolates out of 16 produced IAA at the concentration range of 5-66.15 µg/mL. Conditions, such as L-tryptophan concentration, pH ofthe medium of IAA production, and time duration of the fermentation were optimized in order to enhance the IAA production performance of Actinomycetes.

Keywords- Soil, Actinobacteria, Enzyme, IAA

I. INTRODUCTION

Actinomycetes is a group of prokaryotes that exhibits special morphological, cultural, biochemical, and physiological characteristics. Their potential of producing antimicrobial compounds, enzyme inhibitors, immune modifiers, enzymes, and growth promoting substances both in plants and animals have made them a good source for industrial production of such compounds. At present, throughout the world, many novel types of research are being carried out on them to explore the above potentials (Chavan, Mulaje and Mohalkar, 2013). Actinomycetes are dominant in soil and responsible for the degradation of resistant carbohydrates such as chitin, cellulose. Some of them can breakdown toxic materials. Therefore Actinomycetes can be used in bioremediation at contaminant sites as well (Sharma, Dangi and Choudhary, 2014).

Enzymes is one of the most important category of products, industrially produced using bacteria. Because bacteria can be simply cultured on the cheapest nutritional media and also easy for the downstream processing. Actinomycetes are often used for the industrial production of enzymes such as protease, cellulase, amylase, xylanase, lipase, pectinase, and peroxidase. So existing enzymes are engineered for new physical and physiological characteristics and it make sure the better performance of the enzyme like high productivity, specificity, and stability at extreme temperature, pH, and tolerance to inhibitors (Prakash *et al*., 2013).

The starch scarification ability of the enzyme is also most employed the above industries. As an example, amylase is the most applicable enzyme in the medical, analytical and chemical, textile, food, and brewing industries which is industrially produced using Actinomycetes as their extracellular enzyme. Thermophilic and acidophilic amylases from *Streptomyces erumpens* havespecial applications in bakery, brewing, and alcohol industries (Selvam, Vishnupriya and Bose, 2011). Cellulases can break down cellulose into soluble sugars; these biodegradation capabilities are very important in the carbon cycle and also nowadays cellulases are used for bio fuel and industrial ethanol productions (Barati and Sadegh Amiri, 2015). Except that Actinobacteria like *Streptomyces spp*, *Thermobifida halotolerans*, *Thermomonospora spp*, and *Streptomyces ruber* are used for industrial cellulase production and those are used as detergent for removing stains, finishing, and softening of cotton in the textile industry, deinking and modification of fibers in the paper and pulp production (Prakash *et al.*, 2013).

Lipases are hydrolytic enzymes that catalyze the hydrolysis of the ester bonds of triglycerides into glycerol and fatty acids, or in transesterification (Thakur, 2012). Actinobacteria like *Strptomyces griseus* is the major source for the industrial production of lipase enzymes. In industries, lipases are used for removal of stains as a detergent, flavoring cheese in the food industry, for deinking and staining in the textile industry, stabilize the dough and conditioning in the bakery industry (Ranjani, Dhanasekaran and Gopinath, 2016). L-Aparaginase enzymehydrolyzes L-asparagine into aspartic acid and ammonia. L-Asparagine is an essential amino acid that is required for the growth of tumor cells, however, normal cell-growth does not depend on L-asparagine (Senthil Kumar and Selvam, 2011). Therefore it's widely used as a therapeutic agent for treating acute lymphoblastic leukemia in children and lymphosarcoma (Khamna, Yokota and Lumyong, 2009). Many Actinobacterial

species are using as a good source of industrial L-asparaginase production eg. *Streptomyces spp* (Savitri, Asthana and Azmi, 2003).

Plant growth hormones are organic compounds that make positive growth responses on plants even in very low concentrations. There are five major groups of plant hormones; auxins, gibberellins, ethylene, cytokinins, and abscisic acid. These hormones can either stimulate or inhibit plant growth (Saharan and Nehra, 2011). Indole-3-acetic acid (IAA) is generally considered as the most important native auxin. It regulates tropic responses, organogenesis, and cellular responses like cell expansion, division, and differentiation (Ameur and Ghoul, 2012). *Streptomyces sp* have been isolated as a good source for IAA production by treating wheat plants with the culture of *Streptomyces* and observing plant growth in terms of shoot fresh mass, dry mass, length, and diameter (Ranjani, Dhanasekaran and Gopinath, 2016) ex: *Streptomyces sp* including *S.scabies, S.violaceus, S.griseus, S.exfoliatus,* and *S.coelicolor* (Manulis and Lichterl, 1992). Now it's clear that bacterial secondary metabolites are widely used in industrial processes and Actinomycetes species are the major sources of these compounds.

II. METHODOLOGY

Unpolluted garden soil samples were collected. Actinomycetes were isolated, identified as described in "Soil Actinomycetes : potential source of novel antimicrobial substances," 2019. Their enzymatic activity and IAA production capability were studied as given below.

2.1 Enzyme activity of soil Actinomycetes

2.1.1 Cellulase activity

A loopful from isolated Actinobacteria culture was spot inoculated on Carboxymethyl cellulose plates and incubated at 30ºC for 7 days. In order to visualize hydrolyzed zones, plates were flooded with 0.1% Congo red solution and thereafter washed with 1M NaCl (Sethi *et al.*, 2013).

2.1.2 Protease activity

Actinomycetes isolates were spot inoculated on Skim milk Agar plates and incubated at 30ºC for 5 days. Clear zones around colonies after incubation indicated the protease activity (Kannikan and Rebecca, 2018)

2.1.3 Amylase activity

Starch agar plates were spot inoculated with isolates and incubated at 28ºC for 72 hours. After incubation, the plates were flooded with Gram's iodine and kept for 5 minutes. Then plates were observed for colonies with clear zones around, which indicate the amylase activity (Selvam, Vishnupriya and Bose, 2011).

2.1.4 Asparaginase activity

Actinomycetes isolates were first grown on YM (Yeast and Molt extract agar medium) agar plates at 28±2ºC for 5 days, then Asparaginase Dextrose Salt agar plates were spot inoculated with cultures from YM agar plates and incubated at 28±2ºC for 7 days. Pink color colonies were considered as L-Asparaginase producing strains (Saxena and Sinha, 1981).

2.1.5 Lipase activity

Tween containing peptone agar plates were spot inoculated with actinomycetes isolates and incubated at 28±2ºC for 7 days (Sierra, 1957).

2.2. Indole Acetic Acid production.

Characterization of isolates for the Indole Acetic Acid production was done according to the method described by Bano and Musarrat (Bano and Musarrat, 2003); Actinomycetes isolates were grown on Yeast and Malt agar plates at 28±2ºC for 5 days. Yeast and Malt broth 5 mL, contain 0.2% L-Tryptophan were inoculated by actinomycetes isolates grown on YM agar and incubated at 28±2ºC for 7 days with shaking at 125 rpm. After incubation, cultures were centrifuged at 11000 rpm for 15 minutes. Supernatant 1 mL was mixed with 2 mL of Salkowski reagent ($FeCl_3$ (0.5 M) 1 mL, Perchloric acid (35%) 50 mL). The appearance of pink color indicates the presence of IAA. After 20-30 minutes, the concentration of IAA was determined colorimetrically measuring the absorption of light of which wavelength is 535 nm.

2.2.1 Optimization of conditions for IAA production pH

Actinomycetes isolates grown on Yeast and Malt agar at 28±2^{0}C for 5 days and inoculum were transferred to Yeast and Malt broth (pH= 4,5,6,7,8,9) and incubated at 28±2^{0}C for 7 days shaking at the rpm of 125. At the end of the incubation, cultures were centrifuged at 11000 rpm for 15 minutes. The supernatant (1 mL) was mixed with 2 mL of Salkowski regent. The appearance of a pink color indicates the presence of IAA. After 20-30 minutes the concentration IAA was determined colorimetrically using the light of which the wavelength was 535 nm.

L-tryptophan concentration

Actinomycetes isolates were grown on Yeast and Malt (YM) agar plates at 28±2^{0}C for 5 days. Yeast and Malt broth (5 mL) which contained L-tryptophan was inoculated with Actinomycetes isolates grown on YM agar and incubated at 28±2^{0}C for 7 days shaking at the rpm of 125. After incubation cultures were centrifuged at 11000 rpm for 15 minutes. Supernatant 1 mL was mixed with 2 mL of salkowski regent. The appearance of a pink color indicates the presence of IAA. After 20-30 minutes, the concentration of IAA was measured colorimetrically.

Incubation period

Actinomycetes isolates were grown on Yeast and Malt agar plates at 28±2^{0}C for 5 daysand transferred to Yeast and Malt broths (5 mL, containing 0.2% L-Tryptophan, pH 7).The broth cultures were incubated at 28±2^{0}C shaking at the rpm of 125. Results were observed At the end of the incubation (daily up to 14 days) IAA concentration was determined colorimetrically.

III. RESULTS AND DISCUSSION

Enzymatic activity of soil bacteria

Total of 16 isolates of soil Actinomycetes were subjected to the analysis for their enzyme activity and capability IAA production. Fourteen isolates were positive for the production of cellulase enzyme. These isolates were A3, A4, A9, A11, A13, A14, A16, A20, A26, A27, A29, A30, B3, and B12 (Table 01). The reason for observing significant cellulase activity in most ofthe isolates may be growing them in an environment rich in cellulose for a long period (McCarthy and Broda, 1984; El-Fiky, 2003; Prasad, Bedi and Singh, 2012).

Protease-producing bacteria are the main degraders of organic nitrogen and essential for nitrogen recycling. The qualitative assay on skim milk agar plates revealed that that 8 isolates (A13, A16, A17, A19, A29, A30, B3, and B12) produceprotease by developing a clear zone around the bacterial colonies (Table 01). It proved that actinomycetes can degrade proteinaceous materials in the environment.Qualitative procedure for amylase activity of soil Actinomycetes revealed that all isolates of Actinobacteria, positive for the starch hydrolysis. Although all isolates were positive for the amylase activity literature reveals that most of the Actinomycetes species are positive for amylase activity, but not all (Mohammed *et al.*, 2014; Biosciences *et al.*, 2015; Box and Liverpool, 2019). This 100% amylase activity of soil Actinomycetes isolates may be due to the usage of starch casein agar during the primary screening procedure,The high cellulase, amylase, and protease activity demonstrate the potential of soil actinomycetes in carbon and nitrogen cycles in the biosphere (Figure 01, Table 01).

Isolates of Actinobacteria were tested for the production of L-asparaginase enzyme. All strains were found to be L-asparaginase producers (Figure 01, Table 01). This reveals the suitability of using soil Actinomycetes isolates in pharmaceutical and medical industries (Savitri, Asthana and Azmi, 2003; Khamna, Yokota and Lumyong, 2009; Senthil Kumar and Selvam, 2011).

One isolate, B12 was positive for the lipase activity.However, the literature suggests actinomycetes as a good source of lipase (Cardenas *et al.*, 2001). This low estimate could be due to the error inthe methoddescribed in (Sierra, 1957) used for preliminary screening and testing or maybe low availability of lipids in the sampling environment.

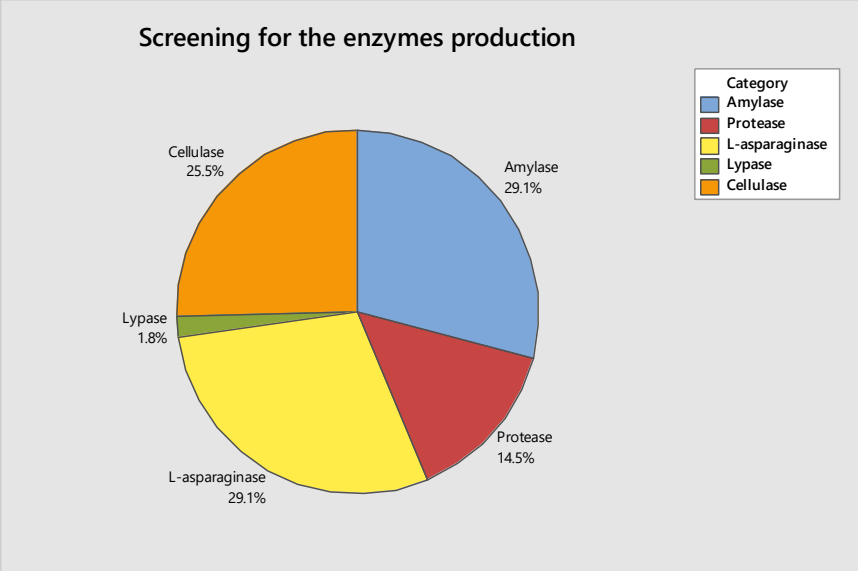

Figure 01: Enzyme activity of soil Actinomycetes

The above results proved that Actinomycetes are the major group of soil microorganisms actively participate in the degradation of environmental pollutants and recycling elements in the biosphere. Apart from these environmental benefits this shows theirindustrial potential as well.

Table 01:- Enzymes activity of soil Actinomycetes

Code of the	Protease	Cellulase	Amylase	Asparaginase	Lipase
A3	-	+	+	+	-
A4	-	+	+	+	-
A9	-	+	+	+	-
A11	-	+	+	+	-

A13	+	+	+	+	-
A14	-	+	+	+	-
A16	+	+	+	+	-
A17	+	-	+	+	-
A19	+	-	+	+	-
A20	-	+	+	+	-
A26	-	+	+	+	-
A27	-	+	+	+	-
A29	+	+	+	+	-
A30	+	+	+	+	-
B3	+	+	+	+	-
B12	+	+	+	+	+

IV. SCREENING OF ACTINOBACTERIAL ISOLATES FOR IAA PRODUCTION

Screening for IAA production.

Indole Acetic Acid is considered as the most active auxin. Many microorganisms produce IAA, in the metabolism of L-tryptophan (Ghosh and Basu, 2006). According to the studies, *Streptomyces sp* are the major IAA producing Actinomycetes (Narayana *et al.*, 2007). IAA functions as an important signaling molecule in the regulation of plant development including organic development, tropic responses, cellular responses such as cell expansion, division, differentiation, and gene regulation (Ryu and Patten, 2008). According to the results, the highest level of IAA production was shown by the isolate A19, and it was 66.150 µg/mL (Table 02). Isolates that produced more than 5 µg/mL of IAA were recognized as positive strains in this study because, IAA stranded curve was plotted, using IAA concentration range of 5 µg/mL 100 µg/mL.

There was a significant difference in IAA production ability of the soil Actinomycetes isolates. According to ANOVA and Tukey's multiple comparison test, the IAA production ability of soil Actinomycetes isolates were grouped. Actinomycetes isolate A19 was in group A and exhibitingthe highestability ofIAA production among the isolates(Figure 02). Good IAA producing isolates were categorized into group B and it contained one isolate(B3).The isolate A26 was categorized under group C which was the group of moderate IAA producing isolates. The isolates withlow IAA producing ability (A9) were categorized under group D. Actinomycetes isolate A4 and A27 hadmoderate to a good level of IAA producing ability. The Actinomycetes isolates that did notproduce IAA were categorized in to the group E (Figure 02).

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

STRAIN	N	Mean	Grouping
A19	3	66.15	A
B3	3	45.68	B
A26	3	29.41	C
A4	3	19.614	C D
A27	3	16.642	C D
A9	3	15.50	D
A13	3	12.07	D E
B12	3	10.831	D E
A17	3	10.259	D E
A14	3	8.749	D E
A30	3	0.000000	E
A3	3	0.000000	E
A29	3	0.000000	E
A20	3	0.000000	E
A16	3	0.000000	E
A11	3	0.000000	E

Means that do not share a letter are significantly different.

Figure 02: Results from the Tukey pairwise comparisons

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Among the actinomycetes isolates, six isolates were negative for IAA production.The reason for thismay be the producing level of IAA not at the detectable sensitivity range of the test used.The range of IAA concentration produced by the isolates was 5-66.15 µg/mL(Table 02). IAA production of Actonomycetes from extream niches in Morocco was 6.70- 75.54 µg/mL (Nafis *et al.*, 2019). Approximately similar range of IAA production also can be seen in soil Actinomycetes. Therefore soil Actinomycetes can be used as the biofertilizer.

Table 02: IAA production of soil Actinomycetes

Identification code of the Isolate	IAA concentration(µg/mL) (Mean ± SE Mean)
A3	**0.000^{E}±0.000**
A4	**$19.614^{C,D}$±0.911**
A9	**15.500^{D}±1.140**
A11	**0.000^{E}±0.000**
A13	**$12.070^{D,E}$±1.450**
A14	**$8.749^{D,E}$±0.591**
A16	**0.000^{E}±0.000**
A17	**$10.259^{D,E}$±0.334**
A19	**66.150^{A}±3.180**
A20	**0.000^{E}±0.000**
A26	**29.410^{C}±9.290**
A27	**$16.642^{C,D}$±0.742**
A29	**0.000^{E}±0.000**
A30	**0.000^{E}±0.000**
B3	**45.680^{B}±1.890**
B12	**$10.831^{D,E}$±0.454**

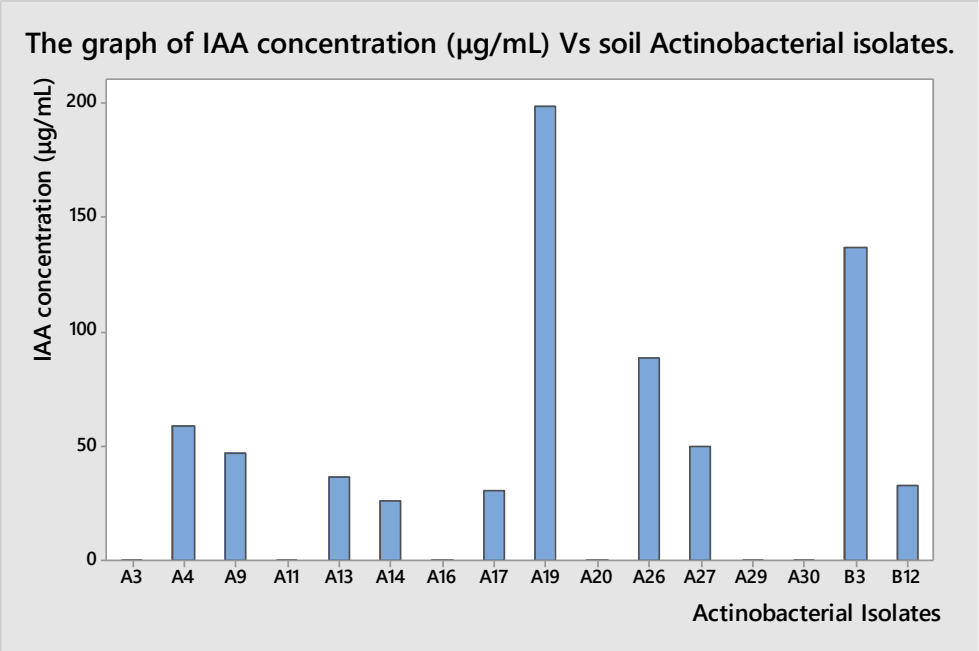

Figure 03: Primary screening of the soil Actinomycetes isolates for the IAA production

Optimization of the conditions for IAA production

Effect of pH on theIAA production

The general pH range of soil is 6 –8 and the average is 7. it provides a good neutrophilic environment to the soil microorganisms (Oskay, Üsame and Cem, 2004).Figure 04showsthat pH 7 is the most suitable pH for the production of IAA and within the range of pH 6 to 8 considerably higher IAA production occurs. It also shows that the IAA production is slightly higher at basic pHsthan the acetic pHs within the range of 6-8.According to Figure 04 Actinomycetes isolates grow within the pH range of 5 – 8. (Shirokikh *et al.*, 2007)have described that the growth of Actinomycetes is slow at acidic and basic pHsaffecting the functions of enzyme systems and the solubility of many substances that are important for bacterial growth (Yurekli, Geckil and Topcuoglu, 2003).

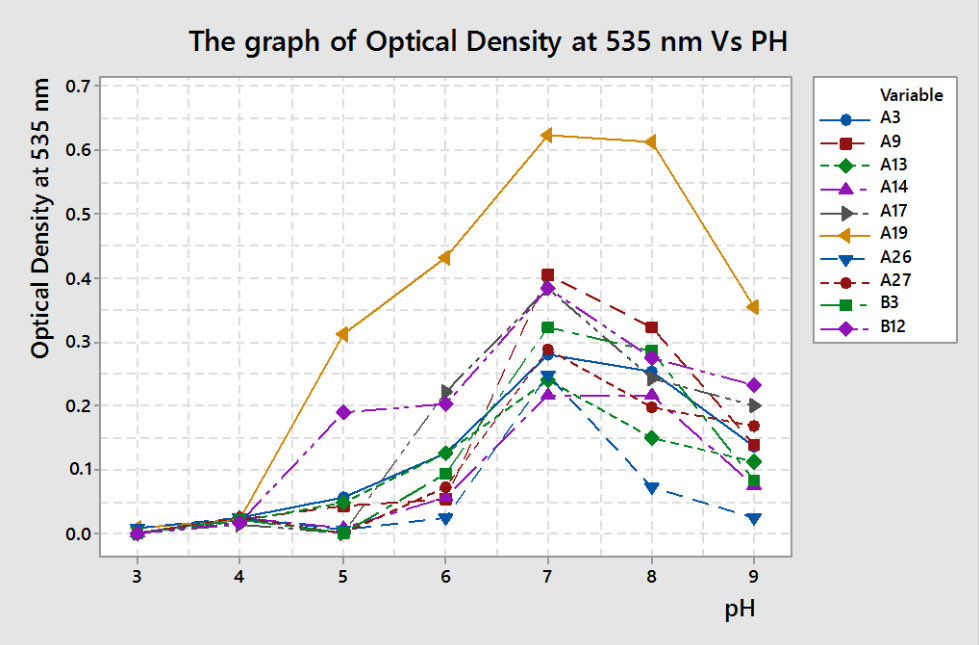

Figure 04: pH optimization for the IAA production

Effect of L-tryptophan level forthe IAA production

L-tryptophan is generally considered as an IAA precursor sincethe additionof L-tryptophan to the bacterial culture medium enhances IAA biosynthesis. No

IAA productionor negligible level of inIAA production is found in media free of L-tryptophan (Costacurta and Vanderleyden, 1995). Most of the Actinomycetes isolates showed the highest IAA production when the medium was supplemented with 0.2% L-tryptophan. The media supplemented with a higher amount of L-tryptophan showed suppression of IAA production. Different microorganisms have significantly different levels of L-tryptophan requirements. According to (Manulis *et al.*, 1994), diffrent*Streptomyces sp* secrete IAA at different L-tryptophan levels.

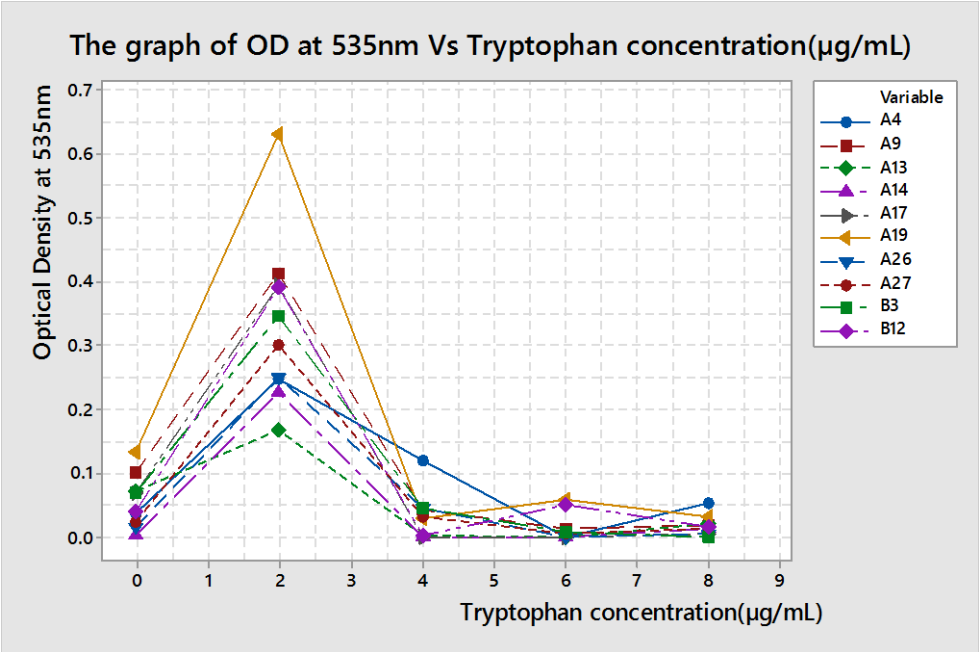

Figure 05: Optimization Tryptophan concentration for IAA production

Effect of the incubation period on the IAA production

According to Figure 06 the highest level of IAA production were observed when cultures were incubated for 6-7 days. After the 7th day, IAA concentration decreased. It may be due to the releaseof IAA degrading enzymes, such as IAA oxidase and IAA peroxidase it had been confirmed using *Rizobium spp.*(Datta and Basu, 2000)

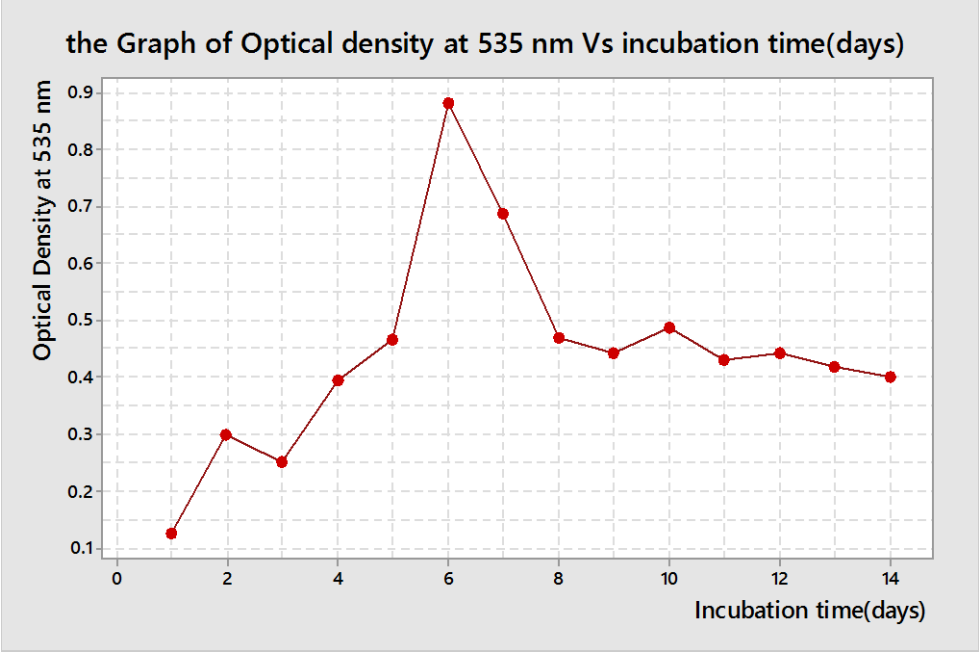

Figure 06: Incubation time optimization using the most effective (A19) isolate

Usually, researchers pay their attention to find special Actinomycetes isolates from the harsh environments because in the industrial processestheir biological productsare exposed to harsh conditions. But, industrial production processes of these enzymes and IAA under ambient conditions may be a good solution for applying them in situations where ambient conditions are available e.g applying IAA producing isolates asa natural fertilizer.

V. CONCLUSION

This study shows that the soil Actinomycetes is a group of bacteria having potential in producing number of industrially and agriculturally important biological compounds such as cellulase, amylase, L-asparaginase, protease, and IAA under ambient conditions. The optimum level of IAA can be obtained in Yeat and Malt brothat pH 7, at the tryptophan concentration of 2 mg/mL within the incubation period of 6-7 days. The development of this type of simple procedure will be an economical and environmentally friendly solution for the current industries in which currently strong conditions are used. These isolates can also be used as a biofertilizer for nonfertile lands and alsothe bioaugmentation of lands without environmental pollution avoiding excessive applications of chemical fertilizers in cultivated fields.

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