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Effect of growth media components and growth condition on indole – 3 – acetic acid (IAA) production by *Pseudomonas putida* isolated from soil

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Abstract

Pseudomonas putidaPST-1 isolate isolated from soil of plant root was used for high production of indole acetic acid. Indole acetic acid (IAA) production is a major property of rhizosphere bacteria that stimulate and facilitate plant growth. Optimization of indole acetic acid production was carried out at different cultural conditions of pH temperature, incubation period, and the amount of inoculum of bacteria. The best chemical medium for high IAA production (82 Mg/ml) was Luria-Bertani broth medium consisted of 1.2gm tryptophan and 10gm peptone in their components, while the cheese whey medium was the best natural medium for IAA production was (66 Mg/ml). IAA production byPseudomonas putida PST-1 was optimized by studying some factors the results revealed that the maximum IAA value was obtained when the isolate cultivated in Luria-Bertani broth medium supplemented by tryptophan 1.2gm / 1L and peptone 10gm/1L, adjusted at pH 7 and incubated at 30 c for 4 days with the viable count of bacteria was ($19,8 \times 10^{3)}$. These results suggest that IAA producingPseudomonas putida PST-1 could be promising candidate for utilization in growth improvement of plants of economic and agricultural value.

Keywords: indole -3- acetic acid;Pseudomonas putida;optimization; cheese whey.

تأثير مكونات الوسط الزرعي وظروف النمو على انتاج الأندول اسيتك اسد (IAA) في بكتريا *Pseudomonas putida* المعزوله من الترب

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الخلاصة

تم الحصول على عزلة بكتريا (PST – 1) *Pseudomonas Putida* من ترب جذور النباتات وتم انتقاءها بموجب فعاليتها على انتاج الاندول اسيتك اسد. انتتاج الاندول اسيتك اسد هو الميزة الرئيسية في بكتريا الرايزوسفير الذي يسهل وينشط نمو النباتات. اختبرت الظروف المثلى لانتاج الاندول اسيتك اسد تحت تأثير مكونات الوسط الزرعي وظروف المزرعة من حرارة

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وpH وفترةالحضانة وكمية اللقاح البكتيري. كان افضل وسط الإنتاج اعلا تركيز من الاندول اسيتك اسد (82 مايكرو غرام / مل) هو وسط اللوريا بيرتاني الحاوي على التربتوفان بنسبة (1,2 غرام) وعلى البيبتون بنسبة (10 غرام) لكل لتر وبينما كان وسط (شرش الجبن) الطبيعي من افضل الأوساط الطبيعية لإنتاج الاندول اسيتك اسد بتركيز (66 مايكرو غرام / مل) .

اظهرت النتائج انه اعلى قيمة لإنتاج الاندول اسيتك اسيد تم الحصول عليها عندما نميت العزلةالبكتيرية (PST−1)*Pseudomonas Putida*في وسط اللوريا بيرتاني السائل الحاوي على التربتوفان والبيبتون تحت pH قيمة 7 وحضنت بدرجة حرارة 30 درجه مئويه لمدة(4 أيام) مع وجود عدد حي بكتري بلغت قيمته 19.8×10^3.

اظهرت النتائج ان عزلة بكتريا (PST−1)*Pseudomonas Putida* استطاعت ان تكون مؤثره بشكل موجب للاستعمال في تطوير وتحسين النباتات للقيمة الاقتصادية والزراعية .

Introduction

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism produced by several microorganisms including plant growth promoting Rhizobacteria [1]. The microorganisms isolated from rhizosphere region of various crop have an ability to produce indole acetic acid as secondary metabolites due to rich supply of substrate [2]. The (IAA) is the best characterized and the most abundant member of the auxins family [3]. Bacteria synthesize auxins in order to perturb host physiological process for their own benefit [2].

IAA is a heterocyclic compound containing a carboxymethyl group (aceticacid). It is the most studied phytohormone and involved in numerous mechanisms in plant physiology[5].

Auxins are responsible for division, extention, and differentiation of plant cells and tissue. Phytohormones of this group increase the rate of xylum and root formation, control process of vegetative growth, tropism, fluorescence, and fractionation plants and also affect photosynthesis, pigment formation, biosynthesis of various metabolites and resistance to a biotic stress factors [6]. IAA helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake [7].

IAA stimulates cell elongation modificating certain conditions like increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall and protein synthesis, inhibit or delay abscission of leaves, induce flowering and fruiting and increase the dry weight of leaves and roots[5].

Materials and methods:

Samples collection

Fifteen soil samples were collected from three different regions in Baghdad city, the samples were collected randomly from rhizospheric soils (5-10 cm in depth). Using sterile containers and transported to the laboratory and preserved at 4°c until using.

Isolation of *Pseudomonas*

For soil samples, suspension was prepared by adding 1g (dry weight) of each soil sample in 10 ml of sterile distilled water, and mixed well. Flask containing 100 ml of selective liquid mineral salts medium, consisted of 1gm KH_2PO_4, 1gm K_2HPO_4, 1gm NH_4NO_3, 1gm $(NH_4)_2SO_4$, 0.2gm $MgSO_4$. $7H_2O$, 0.5gm Nacl, and 0.5gm $FeSO_4$. $7H_2O$, all these components were dissolved in 900ml of distilledwaterand 1ml from trace element solution consisted of 0.23 gm $ZnSO_4.7H_2O$, 0.18 gm $MnSO_4.5H_2O$, 0.1 gm $CuSO_4.5H_2O$, all these components were dissolved in 100 ml of distilled water was added [8].

The culture was incubated in a shaker incubator at 120 rpm at 35°c for seven days, thenin order to get separated colonies, a loop full of the broth culture mentioned above was transferred to inoculate the nutrient agar (dispensed in sterile petri dishes) using ABC streaking method. Then incubated at 35°c for seven days. The process was repeated several times to get pure culture [9].

The isolated bacteria were purified by inoculating them on plates congaing nutrients medium. The bacteria were purified by repeated inoculation. After ensuring purity, the

cultures were sub-cultured on nutrient slants and allowed to grow for a period of 24 hrs and subsequently stored at 4 °C as stock cultures were transferred to fresh nutrients slants at regular intervals of 3 months.

Identification of *pseudomonas*

Pseudomonas isolates were identified by morphological features, microscopic examination biochemical tests, and by VITEK 2 compact system.

Determination of indole acetic acid production

1- Qualitative method [10].

Two methods were used for detecting of IAA

a- Culturing on solid medium Luria – Bertani (LBagar) medium.

The bacterial isolates were cultured on plates of Luria_Bertani(LB) agar medium consisted of 10 gm peptone, 5 gm yeast extract, 5 gm NaCl, 1.2 gm tryptophan,15 gm agar, and 1 litter D.W. pH was adjusted to 7.

Tryptophan was sterilized by filtration and then added to the peptone- yeast extract sterilized medium,(this medium was sterilized by autoclave at 121 °C for 15 minutes). After an appropriate incubation period (1 – 3) days at 32 °C the plates were treated directly with Salkowski reagent consisted of 2 ml $FeCl_3$ (0.5M), 49 ml per chloric acid (70%), and 49 ml D.W. [11]. The reaction was allowed to procced until adequate color developed. Bacteria producing IAA were distinguished by the formation of a characteristic red color.

b- Culturing in liquid medium Luria-Bertani(LBbroth) medium.

The bacterialisolates were cultured in (LB) broth medium consisted of same components in the LB agar medium but without agar. After an appropriate incubation period (1-4) days at 30°C, the broth culture was treated with few drops of Salkowskireagent, the reaction was allowed to proceed until adequate red color developed.

2- Quantitative assay of IAA [10].

a- IAA production.

The bacteria isolates were cultured in Luria-Bertani broth medium and incubated at 30 °C for (2 – 7) days on rotary shaker. The cultures were centrifuged at 500 rpm for 25 minutes and the IAA activity was measured in the supernatant.

b- Estimate of indole acetic acid concentration.

A standard curve of indole acetic acid was established by preparing serial concentration of (IAA)(10 – 300 Mg/ml). Two ml of salkowski reagent was added to 1ml of each concentration and incubated in the dark at room temperature for 25 minutes and then optical density of the solutions were measured at 540 nm [12].

IAA was estimated in the supernatant following the same steps of standard curve and the concentration of IAA was obtained depending on the standard curve.

- **Production of indole acetic acid by using different media**

a. chemical media

1- Luria-Bertani broth medium but the tryptophan was instead of Lysine,tyrosine, and phenyl alanine respectively.

2- Luria-Bertani broth medium but the peptone was instead of casein,ammonia, and urea respectively. The bacterial isolate (PST-1) was cultured in all these media above and incubated at 30 °C for (2 – 7) days on rotary shaker. The cultures were centrifuged at 5000 rpm for 25 minutes and the IAA concentration was measured in the supernatant.

b. Natural media

1- Maize medium.

2- Chickpeas medium.

3- Cheese why medium

0.1gm of K_2HPO_4 and $MgSO_4$ was added to each medium for support the growth of bacteria. Thirty gm for each maize, and chickpeas, separately were socked in (100) ml distilled water for 1 hr and then boiled for 15 minutes, the supernatant was taken, the bacterial isolate (PST-1) was cultured in supernatant and incubated at 30 °C for (2 – 7) days on rotary shaker. While cheese whey medium was sterilized by filtration andthen the bacterial isolate (PST-1) was cultured in the medium at 30 °C for (4 – 7) days in rotary shaker. The cultures

were centrifuged at 5000 rpm for 25 minutes and the IAA concentration was measured in the supernatant.

Effect of growthconditions on production of IAA

1. **Temperature**

   The bacterial isolate (PST-1) was cultured in Luria-Bertani(LB) broth medium, consisted of 10 gm casein, 5 gm yeast extract, 5 gm NaCl, 1.2 gm lysine, 15 gm agar, and 1 litter D.W. PH was adjusted to 7 and incubated at different temperature (25 °C, 30 °C, 35 °C, 40 °C, 45 °C) in rotary shocker for (4 – 7) days.

2. **pH**

   The bacterial isolate (PST-1) was cultured inLuia-Bertani(LB) broth medium and incubated at 30 °C pH was adjusted to (4 , 5 , 6 , 7 , 8) in rotary shaker for (4 – 7) days.

3. **The incubation period**

   The bacterial isolate (PST-1) was cultured in (LB) broth medium and incubated at 30 °C for (1 , 2 , 3 , 4) days respectively , pH was adjusted to 7 in rotary shaker.

4. **The amount of bacterial inoculum**

The bacterial isolate (PST-1) was cultured in nutrient broth at 30 °C for 24 h,cerial concentration of bacterial growth (10^{-1} – 10^{-8}) were prepared from the stock. The optical density (O.D). Of the stock was measured at 600 nm. And the viable count of each concentration was measured.

Fifty ml of production (LB) broth medium was prepared and then inoculated with (0.5ml) from each concentration and incubated at 30 °C for (2 – 7) days in rotary shaker, after that the indole acetic acid concentration and the viable count of bacteria were mesured in each concentrations (10^{-1} – 10^{-8}).

Result and Discussions:

Isolation and identification of pseudomonas.

Three isolation of bacteria were obtained from fifteen samples of soil, after culturing on liquid mineral salts selective medium. the growth characteristics of these isolates on ***pseudomonas*** selective medium indicated that the isolates were classified as a member of genus pseudomonas[8]. When these isolate were further identified by morphological and biochemical tests and identified of bacterial isolates by VITEK2 compact system Figure-1 the results showed that they were identified as strains of Pseudomonasputida .

bioMerieux Customer: Microbiology Chart Report

Patient Name: faeza 1 Patient ID: 130-6-1
Location: Physician:
Lab ID: 130-6 Isolate Number: 1

Selected Organism : Pseudomonas putida

Source: 9-11 Collected:

Comments:

Identification Information	Analysis Time:	8.00 hours	Status:	Final
Selected Organism	91% Probability	Pseudomonas putida		
	Bionumber:	1003013101500352		
Organism Quantity:				
ID Analysis Messages				

Biochemical Details

2	APPA	+	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	+	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Figure 1 - VITEK2 compact identification result sheet for *P. putida* isolate.

Qualitative assay of indole acetic acid (IAA) in *P.putida* growth

All*P. putida* isolates were able to produce indole acetic acid. Bacteria producing IAA were identified by the formation of characteristic red color in the(LB) agar and broth medium

Figure- 2 this is because that the formation of chromatic complex between IAA produced by bacteria and Salkowski reagent as the IAA is carboxylic acid in which the carbonyl group bounded with methyl group by C – 3 in the indole ring [13]. IAA produced by*Pseudomonas* spp.can promote plant growth by stimulating root system [14].

Rhizobium sp. Was a suitable soil microorganism for high level of IAA production [15]. Endophytic fungi ***paecilomcesformosus*** isolated from roots of cucumber plant was able to produce gibberellins and indole acetic and their role in plant growth and stress tolerance under saline conditions [16].

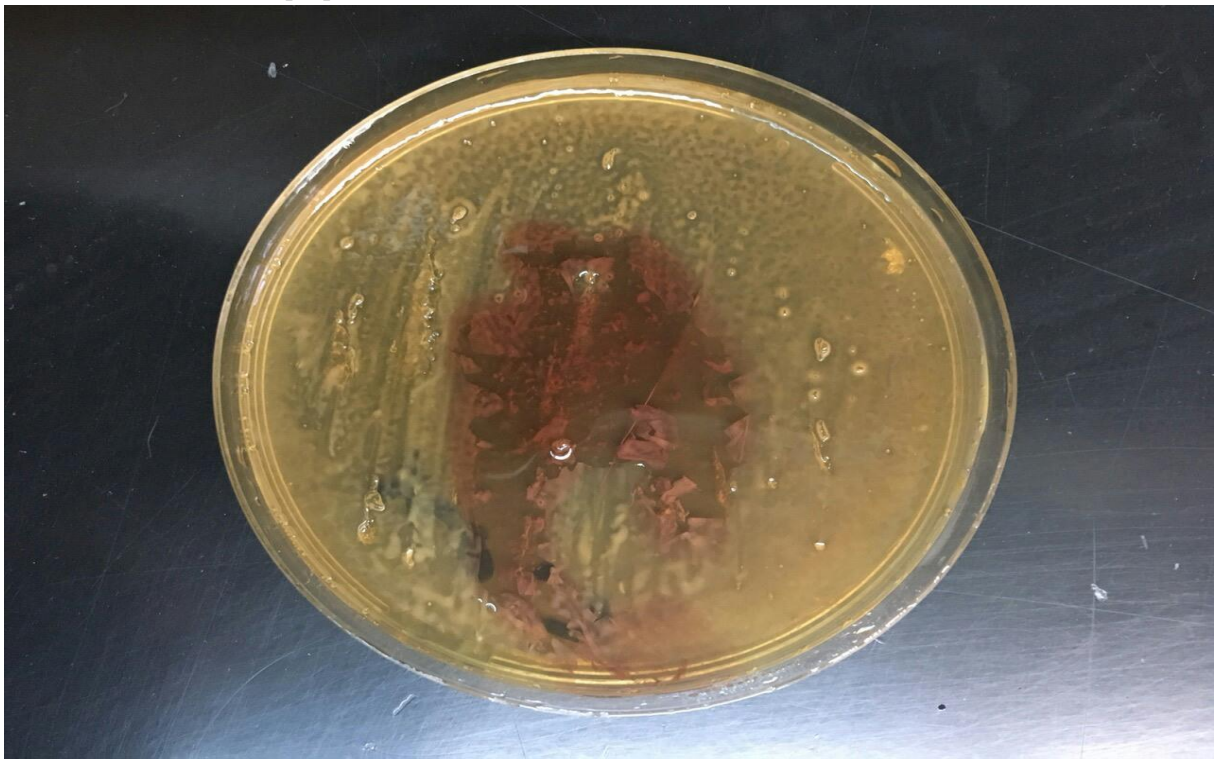

Figure 2- A- production of indole acetic acid (IAA) by*P.putida*in Luria-Bertani agar medium, formation of red color as a positive result

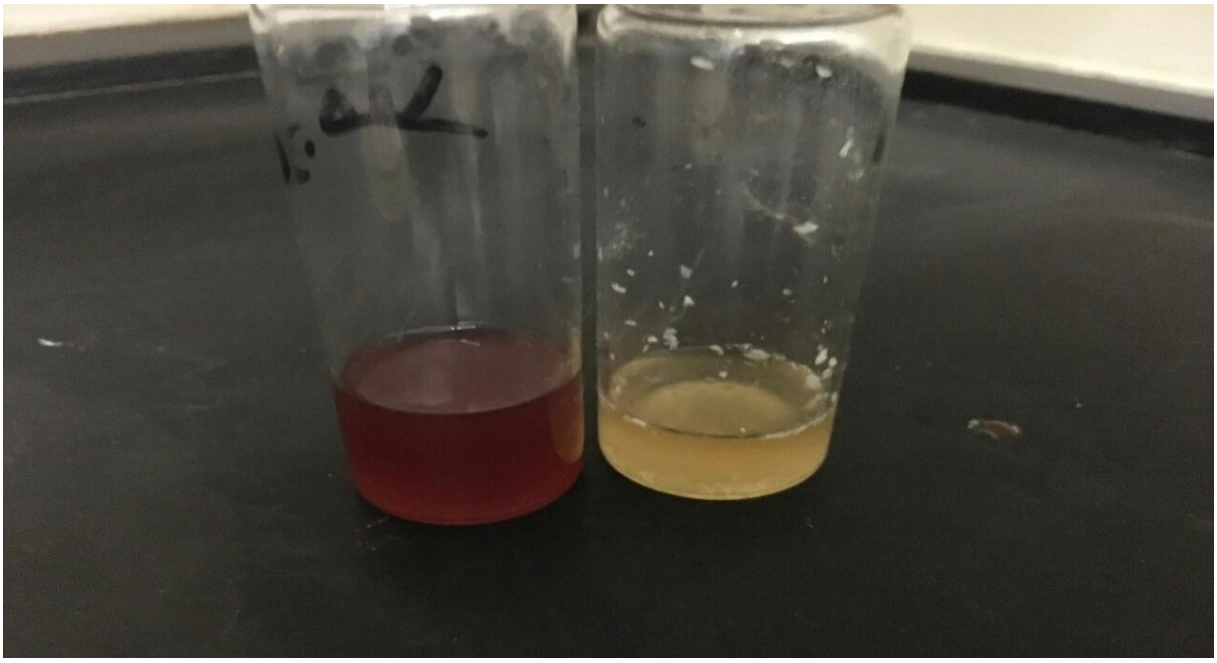

Figure 2- B- production of indole acetic acid(IAA) by *P.putida* in Luria – Bertanibroth medium, formation of red color as a positive result**.**

Quantitative assay of IAA production

Indole acetic acid was estimated for three *P. putida* isolates cultured on (LB) broth medium. the highest concentration was recorded in (PST-1) isolates (82 Mg/ml)while the lowest concentration was recorded in (PST-3) isolate (67 Mg/ml).

The effect of culture media components on the IAA production

One isolate (PST-1) was selected according to their high ability to produce IAA to complete this study. The maximum concentration was recorded in the chemical media (82µg/ml) when using (LB) broth medium consisted of both tryptophan and peptone. This may because of the mineral constituents of this media that favor the production of IAA and may because that the components of (LB) medium (peptone and yeast extract), while the minimum concentration (22 µg/ml) was recorded when using (LB) consisted of tyrosine instead of tryptophan. Table- 1 IAA is one of the most common, naturally occurring plant hormone of the auxin class. IAA is acommon product of L - tryptophan metabolisms produced by several microorganisms including plant growth promoting *Rhizobacteria* (PGPR) [17]. Peptone is the pancreatic digest of proteins that don't completely break down the proteins into amino acids but into peptides and used commonly as several bacteria prefer peptides to amino acids [2].

Table 1- IAA concentration by using different chemical and natural media

IAA concentration (µg/ml)	**Culture media** **Chemical media**
82	(LB) broth medium
67	(LB) broth medium (used casein instead of peptone)
66	(LB) broth medium (used ammonia instead of peptone)
52	(LB) broth medium. (used urea instead of peptone)
51	(LB)broth medium(used lysine instead of tryptophan)
34	(LB) broth medium (used tyrosine instead of tryptophan).
22	(LB) broth medium(used phenylalanine instead of tryptophan)
	Natural media
39	Maize media
40	Chickpeas
66	Cheese whey medium

In the natural media the maximum concentration of IAA (66 Mg/ml) was recorded in cheese whey medium, while the minimum concentration (39 Mg/ml) was recorded in maize medium.

The major proteins in milk are casein and whey. These two milk proteins are both excellent sources of all essential amino acids. Like tryptophan,Leucine, is leucine, and valine. Whey is a fast digesting protein [18].

- **The effect of growth conditions on the IAA production:**

Effect of temperature on IAA production

One of most important parameter for the growth of IAA producing organisms and their metabolic activity is the temperature of incubation [19]. In our investigation, maximum IAA production was observed at 30 °C Figure-3. *P.putida* grows optimally at 30 °C but can proliferate at temperature as low as 45 °C[20]. For IAA production [21] have reported the

Rhizobium strain VMA30l for elaborated high level of IAA production in a medium having temperature 30 °C. According to[22] 37 °C temperature was optimum for *Bacillus* spp. High temperatures have profound effects on the structural and physiological properties of sporulating and non-sporulating bacteria, with membranes, RNA, DNA, ribosomes, protein and enzymes all affected[25].

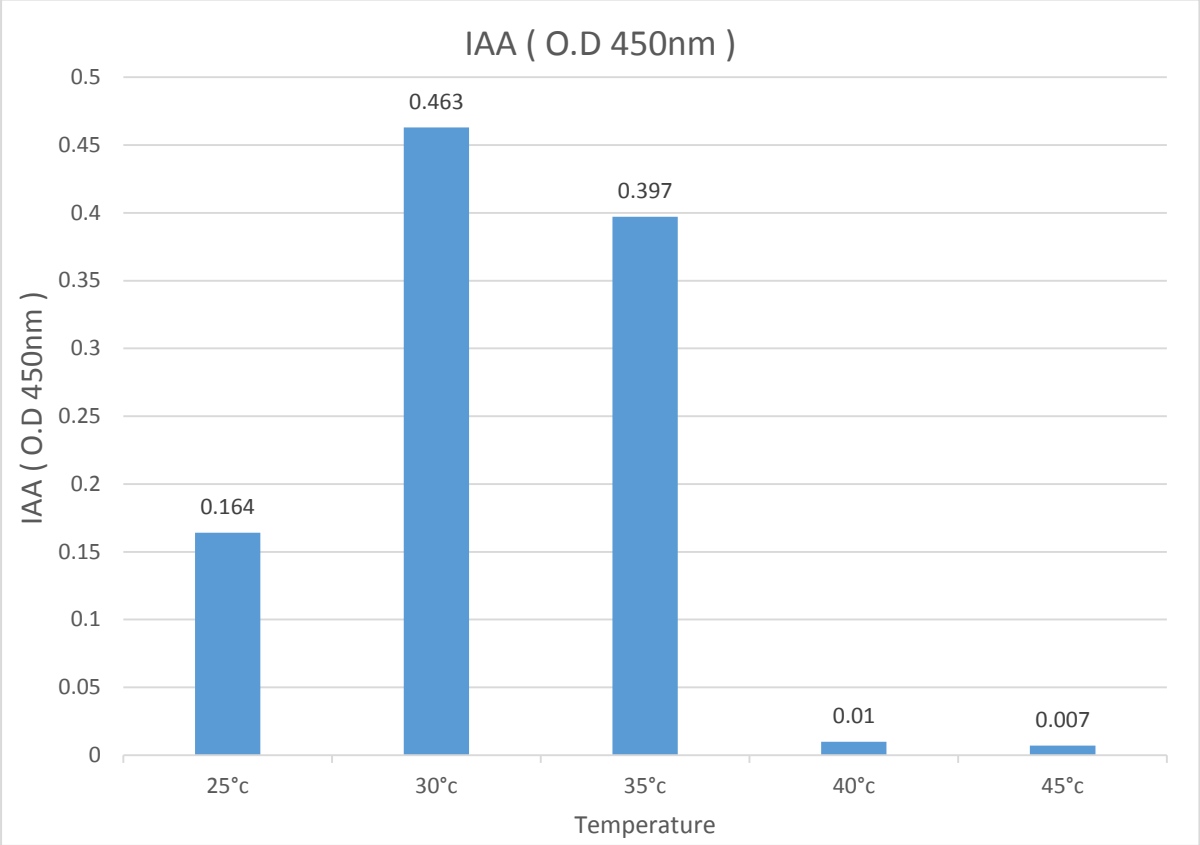

Figure 3- Effect of temperature on (IAA) production.

Effect of pH on IAA production

The second important parameter for the growth of IAA Producing organisms and their metabolic activity is the pH of the IAA production media [23]. The result indicated that the pH 7 was the optimum pH for IAA production Figure- 4. The optimum PH for *p. putida* growth was between 6.5 and 7.0 [24]. For IAA production [23] have reported the Rhizobium strain VMA30l for elaborated high level of IAA production in a medium having pH 7.2. [14] have reported pH 7.0 was suitable for maximum IAA production by Streptomyces sp.

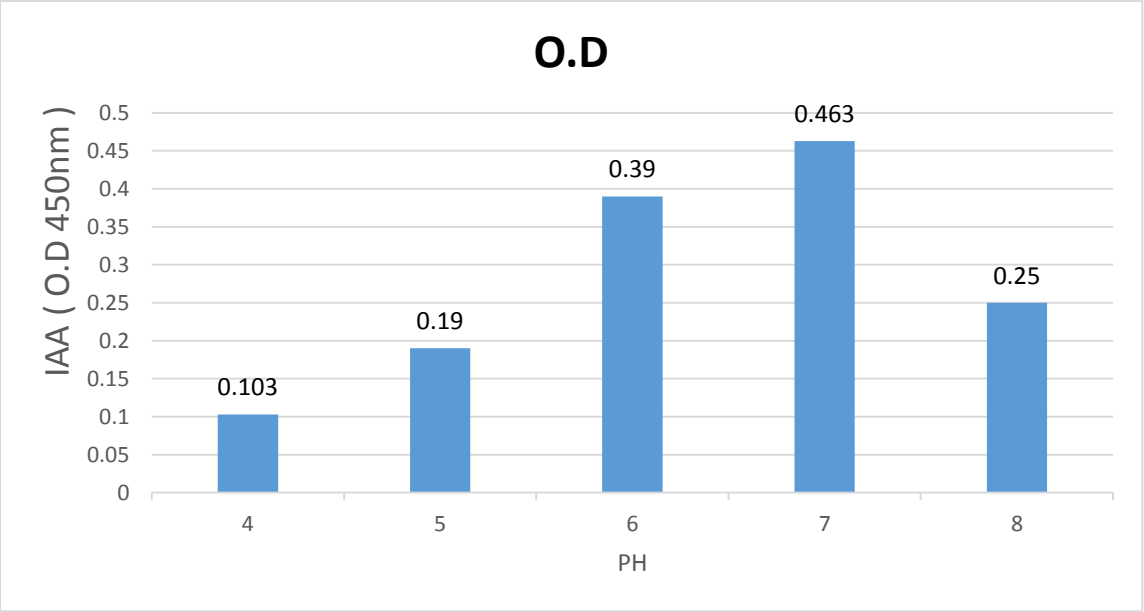

Figure 4 - Effect of pH on(IAA) production.

Effect of incubation period

The effect of incubation period for IAA production by *P. putida* PST – 1 was grown in (LB) broth medium at pH 7.0 and incubated at 37 ˚C in a shaker at 120 rpm for 1 , 2 , 3 , 4 days respectively was estimated. The maximum concentration of IAA was recorded after 4 days as in Figure-5.

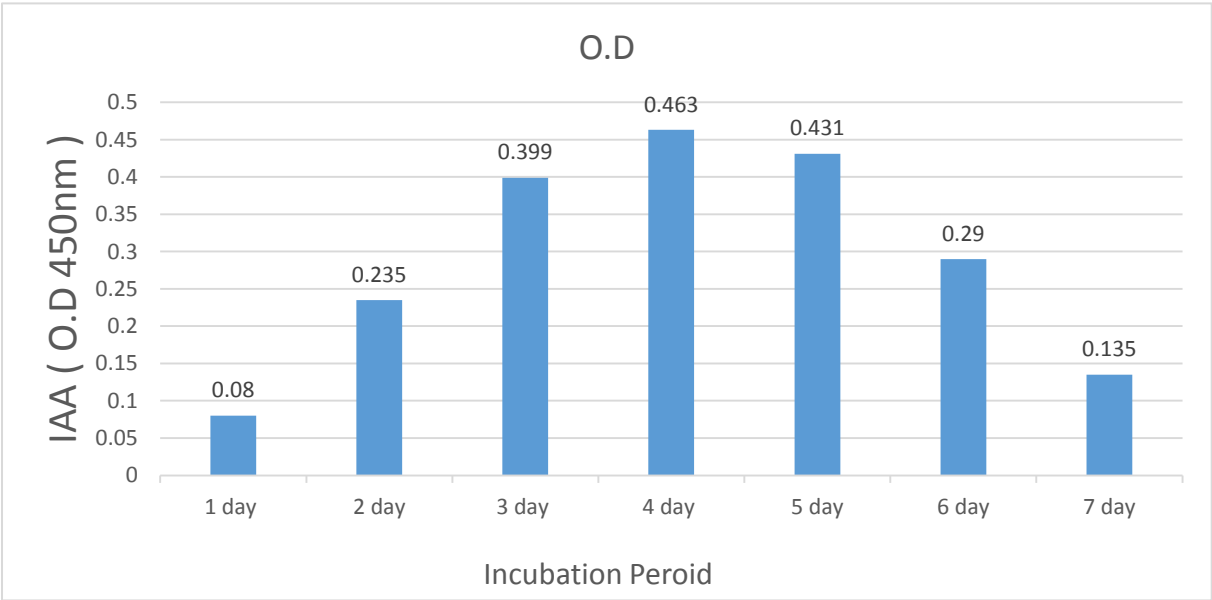

Figure 5- Effect of incubation period on (IAA) production

This because the bacteria was in stationary phase in which all IAA was produced (secondary metabolites), after that period of incubation the bacteria entered the death phase.

Effect of the amount of inoculum

The result was recorded in Figure- 6 it was observed that, the highest concentration of IAA was noted when the viable count of bacteria was (19 .8 × 10^3) while the lowest concentration was noted when the viable count of bacteria was (32 × 10^9). The result indicated that the greater the amount of inoculum increase IAA production and vice versa.

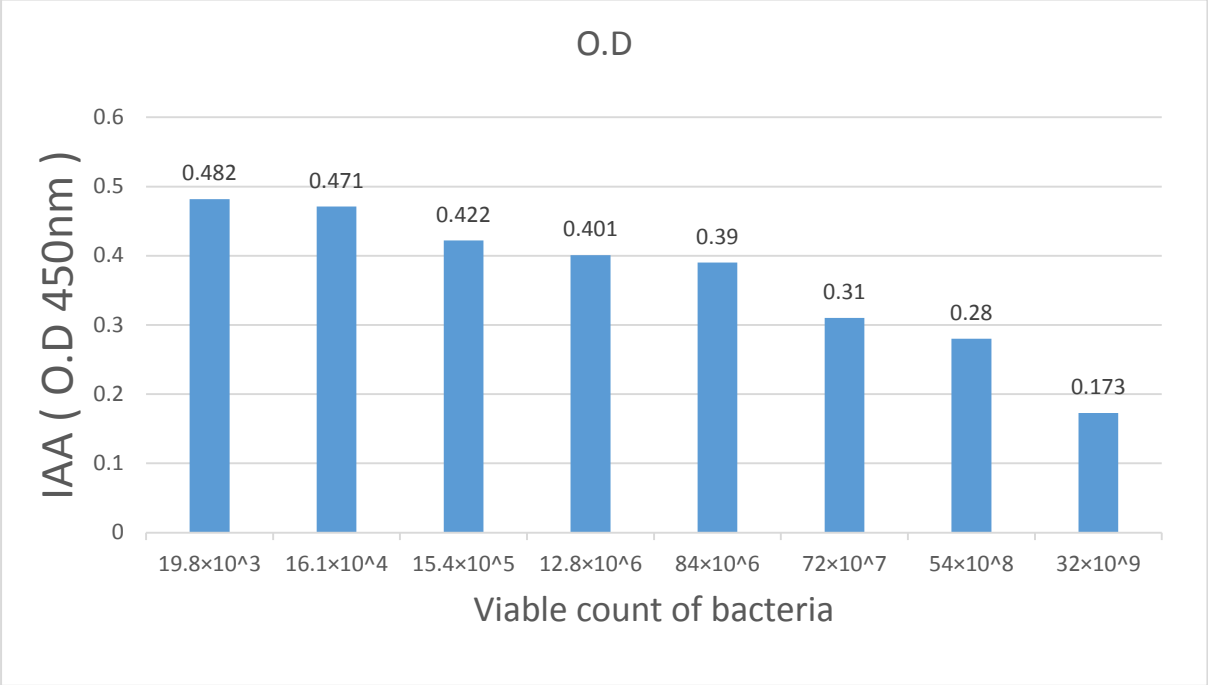

Figure 6- Effect of the amount of inoculum on (IAA) production.

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