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OPTIMIZATION OF FERMENTATION CONDITION FOR INDOLE ACETIC ACID PRODUCTION BY *PSEUDOMONAS* SPECIES.

N.Balaji[1], S.S.Lavanya[1], S.Muthamizhselvi[2] and K.Tamilarasan[1]
[1]Department of Biotechnology, Madha Engineering College, Chennai -600069, India
[2]Department of plant Biology and plant Biotechnology,
Quaid-E-millath government college for women, Chennai, India.

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ABSTRACT:
Production of Indole Acetic acid (IAA) by *Pseudomonas* sp. using basal media with effect of various carbon and nitrogen sources and environmental conditions was studied. The effect of L-tryptophan concentration was studied and the highest yield of 170 µg/ml on 48 hr was obtained using 0.5% concentration. The effect of various carbon sources such as glucose, starch, lactose, cellulose and glycerol was studied. The best yield of 190 µg/ml was obtained with the medium supplemented 2% of glycerol. The effect of nitrogen source was studied by adding peptone, soybean meal, yeast extract, beef extract and tryptone. The maximum yield of 210 µg/ml was obtained with supplemented yeast extract as a organic nitrogen source. The most significant factors in the production media identified using Plackett-Burman design. Glycerol was found to be most significance factor IAA production. Central Composite Design (CCD) employed to optimize condition for IAA production were found to glycerol 2.5% (v/v); temperature 32°C and 7 pH. The various effects of the factors were studied by student's t-test and Fisher's F-test for Analysis of variance (ANOVA) and second order polynomial model was developed. The higher value coefficient of determination (R^2=0.904) justified an excellent correlation between factors and IAA production and the model fitted well with high statistical reliability and significance.

Keywords: Indole acetic acid, basal media, glycerol, submerged fermentation, optimal media.

[I]INTRODUCTION

New and novel solutions for plant growth enhancements are required to ease the burden imposed on our environment and other resources. Here we look at potential solutions to these issues by examining some of the research conducted regarding the biological applications of free-living plant growth promoting *rhizobacteria* (PGPR). PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (auxin, gibberellin, ethylene etc.), siderophores, HCN and antibiotics [1]. There is sample evidence that the mode of action of many PGPR is by increasing the availability of nutrients for the plant in the *rhizosphere* [6,11]. Indole acetic acid is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism by several microorganisms including PGPR [4,9]. Microorganisms inhabiting *rhizospheres* of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils [8,12]. Plant morphogenic effects may also be a result of different ratios of plant hormones produced by roots as well as by *rhizosphere* bacteria. Diverse soil microorganisms including bacteria [10], fungi [13] and algae [3] are capable of producing physiologically active quantities of auxins, which may exert pronounced effects on plant growth and establishment. This work for media and environmental condition were optimized for maximum growth and IAA production was studied.

[II] MATERIALS AND METHODS

2.1 Isolation of Plant Growth Promoting Rhizobacteria from Soil

Rhizospheric soils of different crops (Patty, sugar cane, cauliflower) in the vicinity of Chennai city, India. The *Pseudomonas* sp. was isolated from the soil using King's B media. The isolated colonies were sub-cultured in nutrient agar slants.

2.2 Indole acetic acid (IAA) production

The isolated *Pseudomonas* sp. strain was used for IAA production. The production media containing (w/v): 0.5% L-tryptophan, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.01% $MgSO_4.7H2O$, 0.2% $CaCO_3$ and 1% glucose. The cultivation was then performed at 30°C for 72 hr. Sample of 5 mL culture was collected at 12 hr intervals, and the biomass was separated by centrifugation at 10,000 rpm for 15 min. The cell free supernatant was used for determination of IAA production under standard assay conditions.

2.3 Estimation of IAA

The cell-free supernatant was used for IAA estimation. 10 ml of supernatant, 2 ml of Salkowsky's reagent was added and incubated for 30 min under darkness. The amount of IAA contant was determined colorimetrically at 540 nm [7].

2.4 Nutritional factors affecting IAA production using Classical method of optimization

The effect of varied L-tryptophan concentration (0 to 1.0%) on IAA production was studied. Other media components maintained as (w/v): 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.01% $MgSO_4.7H2O$, 0.2% $CaCO_3$ and 1% glucose. The effect of various carbon (glucose, starch, lactose, cellulose and glycerol) and organic nitrogen (peptone, soybean meal yeast extract, beef extract and tryptone) sources on IAA production was investigated by using the basic medium.

2.5 Statistical Method of Optimization

This design is extremely useful in finding the importance of the factors affecting the IAA production. The PBD experimental runs were carried out to evaluate the effect of six medium components for IAA production. The media components such as glycerol, yeast extract, tryptophan, KH_2PO_4, $MgSO_4$ and NaCl which is considered to be important for IAA production. Each component was tested at two concentration levels, low and high. Table 1 shows the factors considered for investigation, the PB design in 12 experimental run for IAA production. All experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml media at 200 rpm. Sample of 5 mL culture was collected at 48 h, and the biomass was separated by centrifugation at 10,000 rpm for 15 min. The cell free supernatant was used for determination of IAA production under standard assay conditions.

Central Composite Design (CCD) developed by the Minitab 14 software was used to optimize the condition for IAA production yielding a 20 set of experiments. The remaining factors were maintained at their medial values. Each factor in the design was studied at three different levels (−1, 0, +1). All the variables were taken at a central coded value considered as zero. The minimum and maximum range of variables investigated and their values in actual and coded form are listed in Table 3. The experimental design includes 20 runs; fermentation was carried out separately for each run with replicates. Upon completion of the experiments, IAA production was taken as a dependent variable or response. The average maximum IAA production was taken as the dependent variable or response (Y).

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_i X_i^2 + \Sigma \beta_{ij} X_i X_j$$

Where Y is the predicted response, β_0 the intercept, β_i the linear coefficient and β_{ij} is the interaction coefficient. The statistical significance of the regression coefficients was determined by student's t-test. The second order model equation was determined by Fischer's test, and the proportion of variance explained. An analysis of variance (ANOVA) was performed, and three dimensional response surface curves were plotted to study the interaction among these factors.

[III] RESULTS AND DISCUSSION

3.1 Effect of L-tryptophan Concentration on IAA production

The concentration of L-tryptophan was varied from 0% to 1.0% in the production medium to study its effect on IAA production. L-tryptophan concentration has a strong influence on the production of IAA production, as shown in Figure 1. The maximum IAA production of 170 µg/mL was obtained when 0.5% of L-tryptophan was used. An increase in L-tryptophan concentration significantly decreased the IAA production. The lowest IAA production of 60 µg/mL was found for 0% concentration of L-tryptophan.

3.2 Effect of Carbon Source on IAA production

The effect of various carbon sources such as glucose, glycerol, lactose, starch and cellulose, was studied for IAA production in submerged fermentation using 0.5% of L-tryptophan. Glycerol used as carbon source, gave the maximum IAA production 190 µg/mL (Figure 2). However, the addition of glucose, lactose, starch and cellulose totally inhibited the IAA production.

3.3 Effect of Nitrogen Source on IAA production

The effect of various organic nitrogen sources such as peptone, soya bean, yeast extract, beef extract and tryptone was studied for IAA production in submerged fermentation. IAA production 210 µg/mL was the highest with yeast extract as the nitrogen source followed by beef extract 195 µg/mL(Figure 3).

3.4 Placket-Burman Method

IAA production using PB experiments showed a wide variation from 158 to 244 µg/ml was obtained. Estimated t-value, p-value and confidence level giving the effect of variables on IAA production is shown in Table 4. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. In this work, variables with confidence levels greater than 90% were considered as significant. On analysis of student's t-test for calculated t-values, p-values and confidence level of the variables, glycerol showed positive effects and yeast extract, L-tryptophan, KH_2PO_4, $MgSO_4$ and NaCl have shown negative effects on IAA production. On the basis of the calculated p-values at 90% confidence level, glycerol (confidence level = 95.5%) were identified as the significant media components on IAA production. Hence, the main effect of media components on IAA production was also studied graphically using Pareto chart as shown in Figure.4. Pareto chart given that the glycerol is the significant factors (90% confidence level) for IAA production using *Pseudomonas* sp.

3.5 Central Composite Design

CCD has been applied in the search for the optimum conditions and in the analysis of the interaction of variables for IAA production. All the experiments were carried out in duplicate and average IAA production given in Table 4 was subjected to multiple linear regression analysis using MINITAB 14 software. The effect of glycerol, temperature and pH on IAA production was described in the form second order polynomial model in coded units (equation 2).

$$\text{IAA production } (\mu g/ml) = -1294 + 26.11X_1 + 27.78X_2 + 289.22X_3 - 8.92X_1^2 - 0.44X_2^2 - 20.26X_3^2 + 0.82X_1X_2 - 1.50X_1X_3 + 0.06X_2X_3 \quad \ldots (2)$$

The student's t-test was performed to determine the significance of the regression coefficients. The statistical significance of the model was also determined by F-test for analysis of variance (ANOVA) and residuals analysis was performed to validate the model at 95% confidence level. The model fitted well with IAA production and the optimal values from the model was justified ($p = 0.000$).The ANOVA given in Table 5 indicates that the Square term in second order polynomial Model (equation 2) were highly significant ($p<0.005$) and adequate to represent the relationship between IAA production (µg/ml) on glycerol, temperature and pH. The results of response surface methodology obtain 3D surface plot, which represent the effect of each of the four factors and their interactive influence on IAA production. The surface plot (Figure.5) shows that higher and lower level of temperature and pH has no significant effect on the IAA production and increased production was observed at middle level of temperature and pH. From (Figure.6) we can observe that the effect of glycerol and temperature on IAA production, while other factor were fixed as a constant. It was observed that the IAA production was less at lower and high level whereas increases towards middle levels of glycerol. (Figure.7) shows that higher and lower level of glycerol with pH has no significant effect on the IAA production and increased production was observed at middle level of glycerol with pH.

[IV] CONCLUSION

In the present study classical method of optimized media consist 0.5% L-tryptophan, 2% glycerol as carbon source and 0.3% yeast extract as organic nitrogen source. The fermentation media components were further optimized using statistical based PB design and CCD were done for enhancing the production of IAA using *Pseudomonas* sp. The medium components, glycerol is found to be the most significant components that influence the IAA production using PB design. Maximum IAA production of 245 µg/ml were obtained using the optimized culture condition of glycerol, 2.5% ; temperature, 32°C and 7 pH. The comparison of predicted and experimental values showed good correlation between them, implying that the empirical model derived from RSM can be used to adequately describe the relationship between the medium components and the IAA production.

[V] ACKNOWLEDGEMENT

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Tables:

Table1. Plackett–Burman experimental Design for evaluation of six variables with coded values for IAA production

Exp. No.	Glycerol (v/v)%	Yeast extract (w/v)%	KH_2PO_4 (w/v)%	Tryptophan (w/v)%	$MgSO_4$ (w/v)%	NaCl (w/v)%	IAA production (µg/ml)
1	+1	-1	+1	-1	-1	-1	198
2	+1	+1	-1	+1	-1	-1	240
3	-1	+1	+1	-1	+1	-1	180
4	+1	-1	+1	+1	-1	+1	195
5	+1	+1	-1	+1	+1	-1	220
6	+1	+1	+1	-1	+1	+1	244
7	-1	+1	+1	+1	-1	+1	185
8	-1	-1	+1	+1	+1	-1	200
9	-1	-1	-1	+1	+1	+1	222
10	+1	-1	-1	-1	+1	+1	226
11	-1	+1	-1	-1	-1	+1	198
12	-1	-1	-1	-1	-1	-1	158

Table 2. Statistical analysis of Plackett–Burman design of each variable at different level for IAA production

Variable	Lower level(-1)	Higher level (+1)	Main effect	Coefficients	t-value	p-value	Confidence level %
Constant				36448.5	1.97	0.105	100
Glycerol (w/v %)	1	4	30.0	15.0	2.70	0.043	95.5
Yeast extract (w/v %)	1	3	11.3	5.7	1.02	0.355	64.5
KH_2PO_4 (w/v %)	0.01	0.04	-10.3	-5.2	-0.93	0.395	60.5
Tryptophan (w/v %)	0.02	0.08	9.7	4.8	0.87	0.424	57.6
$MgSO_4$ (w/v %)	0.01	0.03	14750.0	7375.0	1.77	0.137	86.3
NaCl (w/v %)	0.01	0.03	1233.3	616.7	1.11	0.318	68.2

Table 3. Experimental range and levels of the three significant variables used in RSM for IAA production

Variables	Range of Levels					
	Actual	Coded	Actual	Coded	Actual	Coded
Glycerol (%v/v), X_1	1.0	-1	2.5	0	4.0	+1
Temperature, X_2	25	-1	32	0	40	+1
pH, X_3	6	-1	7	0	8	+1

Table 4. CCD matrix of independent variables used in RSM with corresponding experimental and predicted values of IAA production

Std Order	X_1	X_2	X_3	IAA production (µg/ml)	
				Experimental	Predicted
1	-1	-1	-1	172	164
2	+1	-1	-1	154	143
3	-1	+1	-1	178	162
4	+1	+1	-1	189	178
5	-1	-1	+1	182	176
6	+1	-1	+1	147	146
7	-1	+1	+1	182	176
8	+1	+1	+1	192	183
9	-2	0	0	167	180
10	+2	0	0	158	169
11	0	-2	0	138	145
12	0	+2	0	158	175
13	0	0	-2	149	168
14	0	0	+2	175	181
15	0	0	0	225	231
16	0	0	0	223	231
17	0	0	0	234	231
18	0	0	0	245	231
19	0	0	0	238	231
20	0	0	0	228	231

Table.5. Analysis of variance (ANOVA) of second order Polynomial model for optimization of IAA production.

Factors	Degrees of Freedom	Sum of Squares	Mean Square	F-value	P-value
Regression	9	19649.6	2183.29	10.45	0.001
Linear	3	1422.1	474.03	2.27	0.143
Square	3	17500.5	5833.51	27.93	0.000
Interaction	3	727.0	242.33	1.16	0.373
Residual error	10	2088.6	208.86		
Lack-of-fit	5	1733.8	346.75	4.89	0.053
Pure error	5	354.8	70.97		
Total	19	21738.2			

Figures:

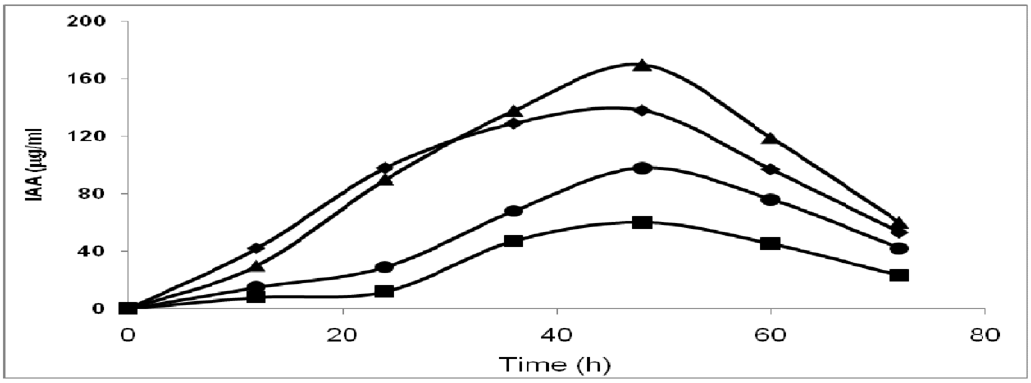

Figure 1. Effect of L-tryptophan Concentrations on IAA production. (0% (■); 0.25% (●); 0.5% (▲); 0.75% (♦)).

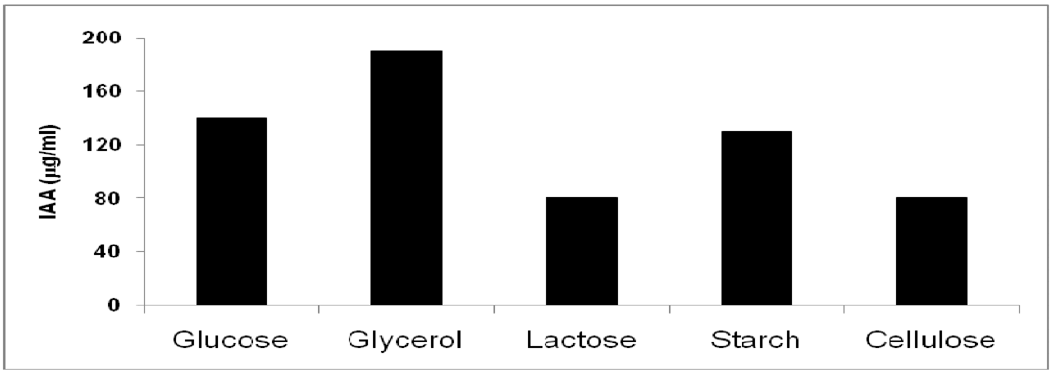

Figure 2. Effect of carbon source on IAA production

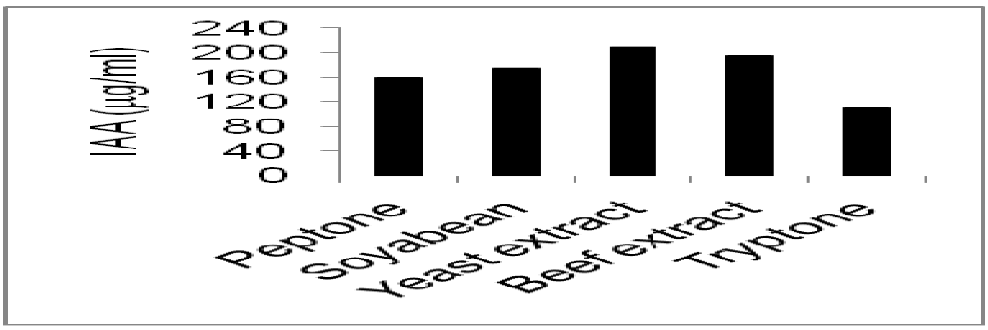

Figure 3. Effect of nitrogen source on IAA production.

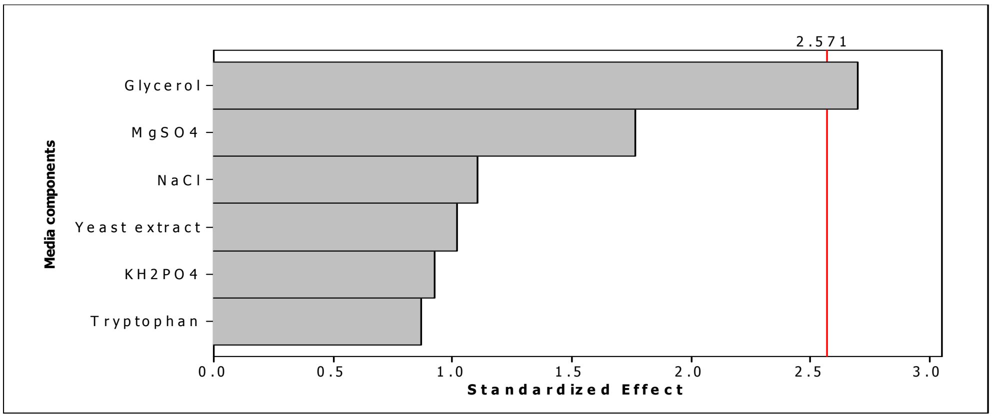

Figure 4. Pareto-plot for Plackett-Burman parameter estimates for six medium components

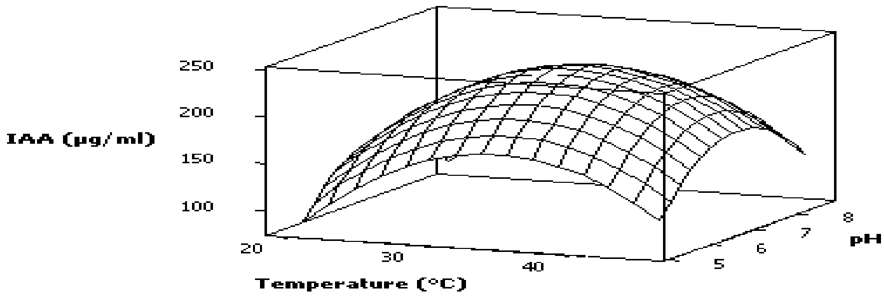

Figure 5. Interaction effect of temperature and pH on IAA production

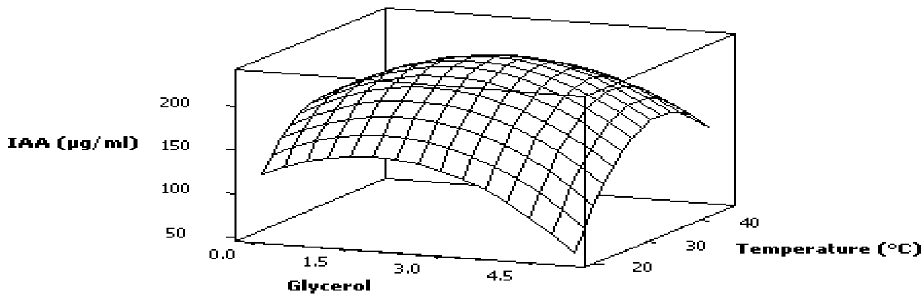

Figure 6. Interaction effect of temperature and glycerol on IAA production

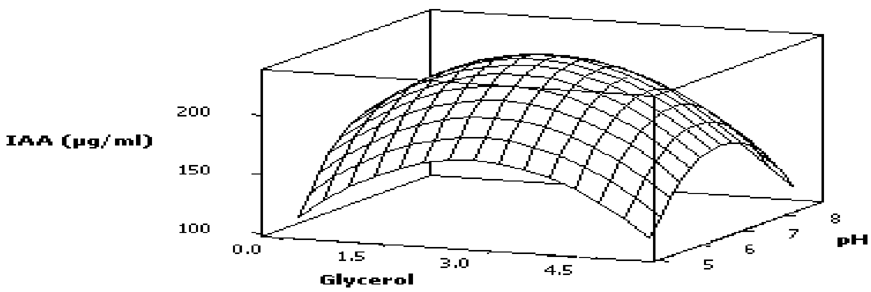

Figure 7. Interaction effect of glycerol and pH on IAA production