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Isolation and Characterization of *Actinomycets* Producing Gibberellic Acid

Patil N. G[1], Patil V. R.[2]

[1]Assistant Professor, Loksevak Madhukararao Chaudhari, College of Pharmacy, Faizpur, Dist.Jalgaon-425 001, Maharashtra, India

[2]Principal, Loksevak Madhukararao Chaudhari, College of Pharmacy, Faizpur, Dist.Jalgaon-425 001, Maharashtra, India

Abstract: ***Actinomycetes are broadly studied concerned with only the production of antibiotics and production of plant growth hormone, GAs, remained the thrust area. As this is tropical region having high temperature(25-45^{0}C), the soil rich in thermo tolerant microorganisms, especially actinomycetes. To compete with the world market, the farmers have to use costly gibberellins, to get quality as well as the quantity of agricultural products. Here our aim was to perceive such actinomycetes having the higher capabilities to produce gibberellins. One on spreading's such isolates actinomycetes in the fields eventually may result in the higher agricultural yields. Considering the facts actinomycetes were isolated from these temperate regions and four strains of actinomycetes were detected for their ability to produce GAs. Among these isolates strain no. four was able to produce the maximum yield of GA3.Using casein starch broth as the fermentation medium. The detection of GA3 was done using folin-wu method (Grahm and Handerson 1961) and UV spectrophotometer.***

Keywords: GA3, casein, actinomycetes, gibberellins

1. Introduction

Gibberellic acid (GA3) is an important member of the gibberellins family and acts as a natural plant growth hormone, controlling many development processes, which is gaining great attention all over the world due to its effective use in agriculture, nurseries, tissue culture, tea gardens, etc. (Davies,2004; Shukla et al., 2005). GA3 is a secondary metabolite, a class of diterpenoid that functions as plant growth regulator having an empirical formula of $C_{19}H_{22}O_6$ [3].

Industrially GA3 is produced by submerged fermentation (SmF) using the ascomycetous fungus *Gibberella fujikuroi*, renamed *Fusarium fujikuroi* [4]. Other bacteria that belong to the genus *Azotobacter* and *Azospirillum* [5] also synthesize GA3. Recently, a *Pseudomonas* sp. isolated from wastes of processed olive has also been shown to produce GA3 (285 mg/L) [6]. The cost of GA3 has restricted its use to preclude application for plant growth promotion, except for certain high value plants. Reduction in its production costs could lead to wider applications for a variety of crops [7, 8].

The cost of GA3 production using SmF is very high, mainly due to extremely low yield and expensive downstream processing. Therefore in recent years the possibility of using solid state fermentation (SSF) has attracted a great deal of attention [9, 11]. In fact, the SSF technique has shown a number of economic advantages over SmF process in the production of microbial biomass and metabolites and the valorization of agro-industrial by-products [12, 13, 14]. The main objective of the present investigation was isolation, selection and characterization of strains for GA3 production by SSF.

Table 1: List of organisms produces GAs

S. No	Strains	References
1.	*Fungi*	
	Gibberella fujikuroi	Muromstsev and Globus (1976)
	Aspergillus flavus	Nair and Subba Rao (1977)
	Neurospora crassa	Kawanabe et al (1983)
	Penicillium species	Aube and Sackston (1965)
2.	*Bacteria*	
	Azotobacter chrococcum	Vancura (1961)
	Bacillus cereus	Montuelle (1966)
	Pseudomonas fluorescents	Panosyan and Babayan (1966)
3.	*Yeasts*	
	Candida pulcherrima	Aseeva and Barmenkove (1967)
	Torula pulcherrima	Krasilnikov et al (1958)
4.	*Actinomycetes*	
	Actinomycetes (unidentified)	Krasinikov et al (1956)
	Actinomycetes sp.	Katznelson and Cole (1965)
	Nocardia sp.	Brown (1972)

2. Materials and Methods

2.1 Isolation and Characterization of Actinomycetes

Surface soil sample was collected from local area of market, chopda, Jalgaon District, Maharashtra. Casein starch agar medium was used for isolation of actinomycetes having composition in (g/l) of casein 0.3, starch 10, NaCl 2.0, KnO_3 2.0, $Mgso_4.7H_2o$ 0.05, $CaCO_3$ 0.02, Agar-Agar 25, Distilled water 1000ml with PH 7.2. Casein starch broth was used as the fermentation medium for the production of gibberellic acid having composition in (g/l) of casein 0.3, starch 10, NaCl 2.0, KnO_3 2.0, K_2HPO_4 2.0, $MgSo_4$ $7H_2O$ -0.05, $Caco_3$ -0.02, Distilled water 1000ml with pH 7.2.

2.2 Analytical Practice

GA3 was estimated spectrophotometrically by the method described by Berriso et al (Hanson J.R., 1968) at 254 nm. Qualitative determination of GA3 was done by TLC as described by Puchooa et al (Karakoc S. and Aksoz N., 2006).

The GA3 extracted from the fermentation was dissolved in ethanol and separated by TLC using isopropanol – ammonia - water (10:1:1, v/v/v) as mobile phase. The plates were sprayed with 3 % (v/v) H_2SO_4 in methanol containing 50 mg $FeCl_3$ and heated in oven at 80^0C for 10 min. GAs fluoresce and appear as greenish spot under UV light.

2.3 Fermentation

The medium casein starch broth was inoculated with four isolates of actinomycetes (3 loop full) in 250ml shake flask containing 100ml medium and flask were incubated in incubator shaker at 28^0C with rotation speed 200rpm. After 10-14 days incubation the fermented broth was filtered and subjected for the detection of GA3 using Folin-wu method (Graham and Henderson 1961).

2.4 Detection of GA3 in Fermented Broth

From each fermented broth 1ml filtered solution was taken in volumetric flask of 25ml.Then folin-wu procedure was followed, which was used for detection of gibberellic acid solution. Then the absorbance was measured at 780nm on spectrophotometer. On comparing this absorbance with the standard graph of gibberellic acid, the quantity of gibberellic acid from fermented broth was obtained.

3. Result and Discussion

3.1 Isolation of Actinomycetes

The soil sample were exposed to heat 80^0C for 1hr, so is to reduce microflora other than actinomycetes and streaked on casein starch agar plates. The plates were incubated at room temperature for 4 to 5 days to get isolated colonies of actinomycetes.
Medium- Casein Starch Agar
Incubation Temperature- 28^0C
Incubation period-96hrs.

Table 2: Colony characteristics of four different strains of actinomycetes

Sr. No.	Strain Number				
		1	2	3	4
1	Size	1.5mm	1.5mm	2mm	2mm
2	Shape	circular	circular	circular	Circular
3	Color	white	white	off white with white periphery	White
4	Elevation	convex	umbonate	convex	Umbonate
5	Margin	entire	entire	entire	Entire
6	Consistency	leathery	leathery	leathery	Leathery
7	Opacity	opaque	opaque	opaque	Opaque
8	Gram character	Gram +ve	Gram +ve	Gram +ve	Gram +ve
9	Pigmentation from (after 192 hrs)	Yellow fain brown	yellow	Brown	Brown

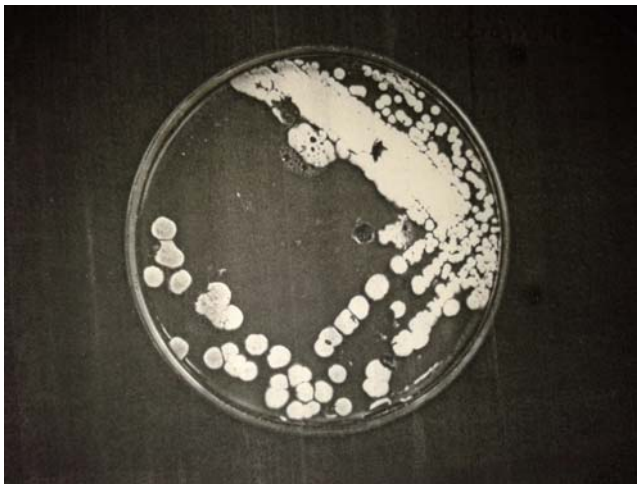

Figure 1: Isolation of Actinomycete from soil sample

Table 3: Biochemical characteristics of four strains of actinomycetes
Degradation Test

Sr. No.	Test	1	2	3	4
1.	Glucose	- ve	- ve	+ ve	+ ve
2.	Arabinose	-ve	+ ve	+ ve	+ ve
3.	Fructose	+ve	-ve	+ve	+ve
4.	Sucrose	+ve	+ve	+ve	-ve
5.	Citrate utilization	-ve	-ve	+ve	-ve
6.	Catalase	+ve	+ve	+ve	+ve
7.	Oxidase	+ve	+ve	+ve	+ve

Table 4: The results of degradation of complex substances by four strains of Actinomycetes
Tolerance Test

Sr.	Test	Strain1	Strain 2	Strain	Strain 4
1	Casein	+ve	+ve	+ve	+ve
2	Tyrosine	+ve	+ve	+ve	+ve
3	Gelatin	+ve	+ve	+ve	+ve
4	Starch	+ve	+ve	+ve	+ve

Table 5: The results of degradation of complex substances by four strains of Actinomycetes.
Tolerance Test

Sr. No.	Growth at				
1.	a. 45^0C	+ve	+ve	+ve	+ve
	b. 50^0C	+ve	+ve	+ve	+ve
	c. 80^0C	-ve	-ve	-ve	-ve
2.	Sodium chloride				
	a. 5 % w/v	+ ve	+ ve	+ ve	+ ve
	b. 10 % w/v	+ ve	+ ve	+ ve	+ ve

3.2 Solid State Fermentation

The medium casein starch broth was inoculated with four isolates of actinomycetes (3 loop full) in 250ml shake flask containing 100ml medium and flask were incubated in incubator shaker at 28^0C with rotation speed 200rpm. After 10-14 days incubation the fermented broth was filtered and subjected for the detection of GA3 using Folin-wu method (Graham and Henderson 1961).

3.3 Detection of GA3 in Fermented Broth

From each fermented broth 1ml filtered solution was taken in volumetric flask of 25ml. Then folin-wu procedure was allowed, which was used for detection of gibberellic acid solution. Then absorbance was measured at 780nm on UV spectrophotometer. On comparing this absorbance with the standard graph of gibberellic acid, the quantity of gibberellic acid from fermented broth was obtained.

3.4 For preparation of Standard Gibberellic acid curve

Sr. No.	1ml sample with concentration	Optical density at 780nm
1	10 ug/ml	0.012
2	20 ug/ml	0.051
3	40 ug/ml	0.091
4	60 ug/ml	0.13
5	80 ug/ml	0.175

Table 6: Different concentrations of GA3 and its absorbance

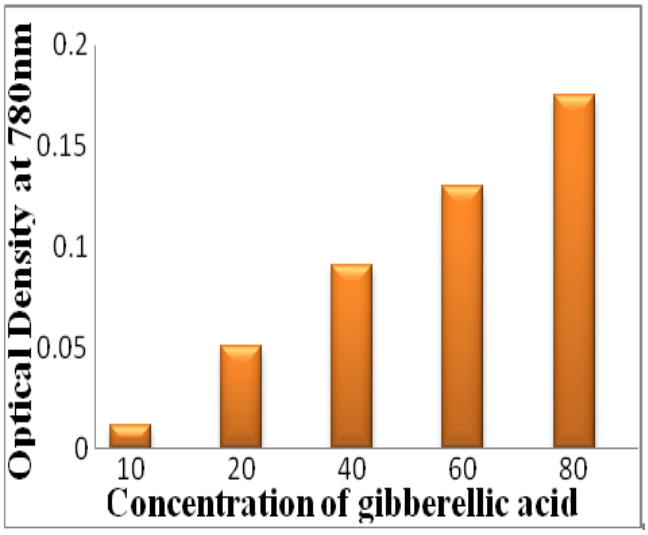

Figure 1: Concentration of GA and its absorbance

Folin-wu method was used to determine absorbance. The absorbance was plotted on graph against the concentration of GA3 to get standard curve, straight line. It was used to determine the quantity of GA3 produced in the fermented broth. The Fig. 1 shows the standard curve of GA3 concentration vs. its optical density. By using different concentration of GA3, its absorbance was detected on spectrophotometer and standard curve was obtained which was straight line. This was used to determine the concentration of GA3 produced in the fermentation medium by four different strains of actinomycetes.

Table 7: The pH change by the four different strains of actinomycetes in the fermentation medium during fermentation.

Strain No.	*Initial pH*	*pH after 8 days fermentation*	*pH after 12 days fermentation*
1	7.2	9.03	9.43
2	7.2	8.95	9.42
3	7.2	8.90	9.40
4	7.2	8.55	9.31

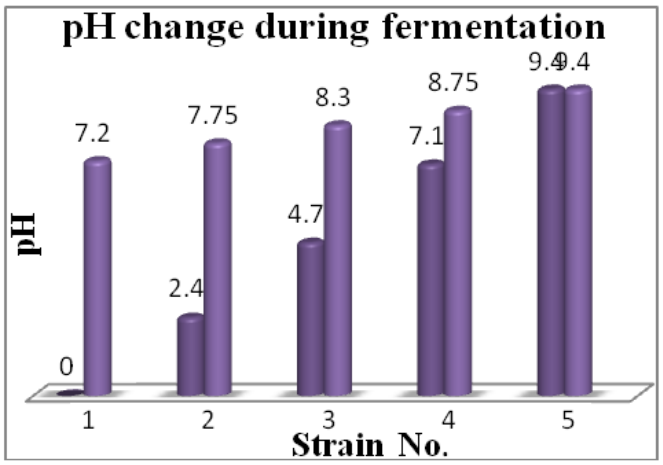

Figure 2: Initially pH was 7.2, but after fermentation it was raised up to 9.3 to 9.4 by all the four strains of actinomycetes.

Table 8: The absorbance of the fermented broths of four different strains of actinomycetes and the respective concentrations of GA3 produced by actinomycetes in fermented broth

Medium	Absorbance at 780 nm of fermented broth			
	Strain 1	Strain 2	Strain 3	Strain 4
Casein starch Broth	0.014	0.016	0.026	0.049
Quantity of GA3 Produced in Ug/ml	10.5	11.5	19.1	25.5

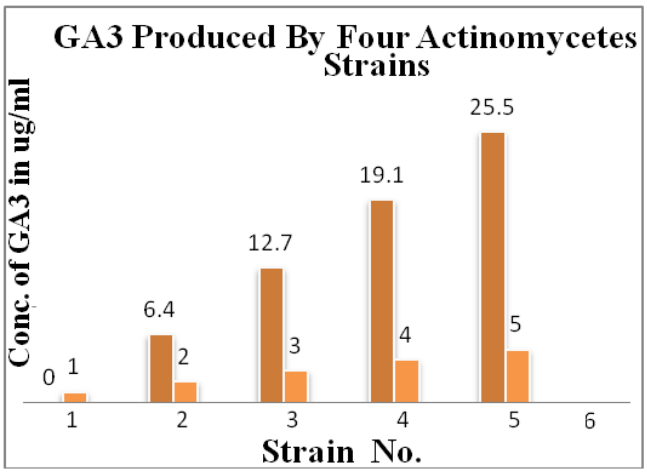

Figure 3: The amount GA3 produced by four different strains of actinomycetes

The GA3 produced by four different strains were quantified using standard GA3 curve. GA3 produced by each strain in ug/ml was: Strain 1 – 10.5, strain 2 – 11.5, strain3- 19.1, strain 4 – 25.5. Among all strains, strain 1 and 2 gave approximately same yield were as strain 3 gave little more strain 4 gave maximum yield of GA3.

4. Discussion

The four isolates of actinomycetes were inoculated in the fermentation medium for gibberellic acid production. The medium was consisting of starch has carbon source, potassium nitrate as nitrogen source for organism growth. The temperature was maintained to 28^0C optimum for growth of actinomycetes and the rotation speed was kept to

200 rpm for aeration and agitation. Lower rotation speed was found to form coagulates of actinomycetes which resist mass transfer. The initial pH of the medium was 7.2, on incubation the pH increased continuously up to 12 days then it remains constant. After stabilization of pH the presence of pH, the presence of gibberellic acid was detected and estimated. All the four strains were able to produced GA3 but the strain number 4 was giving highest yield, in which the pH change was maximum from 8th day up to 12th day.
On providing cheaper medium than casein starch broth as well as on addition of precursor the yield may increase.

5. Conclusion

All the four strains of isolated actinomycetes were found to GA3 producers but strain number 4 was found to be potent one with higher yield, in casein starch broth. For GAs production the cheaper medium should be detected as well as genetic manipulation in actinomycetes should be done so as to get higher yield of GAs.

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