

# Screening of Different Substrates for Improved Production of GA3 by Solid State Fermentation Using *Fusarium moniliforme*

Babli Haldar <sup>1</sup>, Rashmi Pal <sup>2</sup>, Amrita Sengar <sup>3</sup>, Dr. Vivek Mishra <sup>4</sup>

Research Associate, Department of R&D, Kilpest India Limited, Bhopal, India<sup>1</sup>

Senior Research Scientist, Department of R&D, Kilpest India Limited, Bhopal, India<sup>2</sup>

Research Associate, Department of R&D, Kilpest India Limited, Bhopal, India<sup>3</sup>

Head, Department of R&D, Kilpest India Limited, Bhopal, India<sup>4</sup>

**ABSTRACT:** 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. A study was carried out using *F. moniliforme* for production of plant PGPR GA3 using a variety of substrates. Our study focused on low cost easily available substrates which were used for preparation of novel substrate for SSF. A series of experiments were designed with low cost substrates like Citric peel with ions supplement (CPIS), Wheat bran, Sugarcane bagasse, and Mixed substrate (citric peel & sugarcane bagasse). Upon HPLC (High Performance Liquid Chromatography); 3.9% GA3 concentration was obtained in CPIS, hence CPIS was detected as best substrate. The research assumes critical importance as demands for plant PGPR exceeds the production capacity and our low cost GA3 can fulfil the demand gap helping to increase agricultural output and provide better economic return.

**KEYWORDS:** Solid State fermentation, Gibberellic acid, High Performance Liquid Chromatography

## I. INTRODUCTION

Gibberellic acid (also called Gibberellin A3, GA, and GA3) is a hormone found in plants and fungi. Gibberellic acid (GA3), the most important gibberellin, is a class of diterpenoid that functions as plant growth regulator. Its chemical formula is C<sub>19</sub>H<sub>22</sub>O<sub>6</sub>. It affects stem elongation, elimination of dormancy, flowering, sex expression, enzyme induction and leaf and fruit senescence [10]. It is used on a large scale for growth of seedless varieties of grapes in India. It is also used for breaking dormancy of several seeds germination of barley for malt preparation [3]. Gibberellins (GAs) play a role in the growth, development and sexual differentiation processes of different plant species. Gibberellic acid can affect growth by its effect on cell growth and cell elongation. Such effects are often seen in stem growth as well as root growth. To date, 136 GAs have been identified in plants, fungi and bacteria but only a few of them possess biological activity. Industrially GA3 is produced by solid state fermentation (SSF) using the ascomycetous fungus *F. moniliforme* [12]. Gibberellic acid is a secondary metabolite of *F. moniliforme* in its stationary phase [6]. The most important bioactive gibberellins are GA1, GA3, GA4 and GA7. Many of the other GAs are biosynthetic intermediates or catabolites of bioactive GAs which belong to the group of "C<sub>19</sub> gibberellins". Gibberellins have been quantitatively analysed in several species. Many chromatography techniques have been developed for the analysis of gibberellins with instruments, such as high performance liquid chromatography [8] and gas chromatography-mass spectroscopy (GC-MS).

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## II. MATERIALS AND METHOD

### Microorganism

*Fusarium moniliforme* 1100 was procured from NCIM. It was cultured in PDA (Potato Dextrose Agar) plates and incubated in BOD incubator at 25°C for 5 days. *Fusarium moniliforme* 1100 was conserved in PDA slants and kept at 4°C for up to three months.

### Substrates

Low cost substrates like Citric peel with ions supplement (CPIS), wheat bran and sugarcane bagasse (SCB) were used. These substrates were previously dried, and ground in grinding machine.

### Development of Inoculum Production

Potato Dextrose Broth medium was used for inoculum production and was tested in relation to cells viability of *Fusarium moniliforme* 1100 strain and GA3 production by SSF.

### PDB (Potato Dextrose Broth) Medium

Loop full of *F. moniliforme* mycelia was inoculated in 1 L Erlenmeyer flask containing 500 ml PDB medium and kept in BOD incubator at 25 °C for 5 days with intermittent shaking.

### Substrate optimization for solid state fermentation of *F. moniliforme*.

#### I) Citric peel with ions supplements substrate medium

Media for SSF was prepared by using 250 gm dry citric peel, 50 gm of soluble starch, 7.5 gm glucose and 550 ml of mineral salt solution (MMS). MMS was used to make thick slurry and was prepared by MgSO<sub>4</sub> (0.150 gm/L), CuSO<sub>4</sub> (0.150 gm/L), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1 gm/L) and Urea (0.150 gm/L). The initial moisture content of the medium was adjusted to 65%.

#### II) Wheat bran substrate medium

Media for SSF was prepared by using 250 gm wheat bran, 50 gm of soluble starch, 7.5 gm glucose and 450 ml of MMS. MMS was used to make thick slurry. The initial moisture content of the medium was adjusted to 65%.

#### III) Sugarcane bagasse substrate medium

Media for SSF was prepared by using 310 gm sugarcane bagasse, 8 gm of soluble starch and 1 L of MMS to make thick slurry. The initial moisture content of the medium was adjusted to 65%.

#### IV) Dry citric peel and Sugarcane bagasse medium (1:1) substrate medium

Media for SSF was prepared by using 75 gm dry citric peel, 75 gm sugarcane bagasse, 8.33 gm of soluble starch, 15 gm glucose and 300 ml of MMS to make thick slurry. The initial moisture content of the medium was adjusted to 65%.

### Sterilization of substrate medium

After medium preparation by using CPIS, wheat bran, SCB; the substrate medium was transferred into the culturing plates having 10 cm diameter. Plates were covered with autoclaveable poly bag and then sterilized in an autoclave maintained at 121°C under 15 psi pressure for 20 minutes. After autoclaving, sterile medium was inoculated with 35 ml of a 5 days old inoculum of *F. moniliforme* 1100 in each plate. Culture plates were kept in BOD incubator at 25 °C for 10 days.

### Harvesting of crude GA3 (Gibberellic Acid)

#### Cycle 1

SSF of *F. moniliforme* was done by using CPIS, wheat bran, SCB substrate medium and after incubation of 10 days harvesting of *F. moniliforme* was done. Culture recovered from SSF was divided into three parts. 1<sup>st</sup> part of the culture was crushed and butanol was added to it in 1:2 ratios in 5 L conical flask. The conical flask was kept on rotatory shaker for 8 hours at room temperature. After 8 hours conical flask containing *F. moniliforme* culture with butanol was taken

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and liquid slurry was filtered with the help of muslin cloth. Filtrate was used in 2<sup>nd</sup> cycle with addition of 2<sup>nd</sup> part of the *F. moniliforme* culture. Again the conical flask was kept on rotatory shaker for 8 hours at room temperature. After filtration filtrate was used in 3<sup>rd</sup> cycle with addition of 3<sup>rd</sup> part of the *F. moniliforme* culture. The conical flask was again kept on rotatory shaker for 8 hours at room temperature. After 3<sup>rd</sup> cycle, filtrate was used as butanol extract of crude GA3.

### High Performance Liquid Chromatography (HPLC) of crude GA3

Butanol extract of crude GA3 which was obtained after 3<sup>rd</sup> cycle of harvesting was subjected to HPLC for quantitative analysis. 2 % standard was prepared by dissolving 2 gm pure GA3 powder in 100 ml HPLC methanol. Sample was prepared in 1:1 ratio with HPLC methanol. 20µl sample was injected by using a Rheodyne injector. Crude GA3 was estimated by using Shimadzu HPLC at 206 nm on a C18 column using HPLC Methanol as mobile phase at 1 ml/minute flow rate.

### Calculations to determine the % concentration of GA3

$$GA3 \text{ Concentration}(\%) = \frac{\text{Peak of sample} \times \text{weight of STD} \times 93}{\text{Peak of STD} \times \text{weight of sample}}$$

### Formulation Preparation

GA3 formulations were prepared by using CPIS and SCB butanol extract of crude GA3. 25 ml of butanol layer extract of crude GA3, 22.5 ml solvent C9 and 2.5 emulsifiers were mixed to prepared formulation and were used for plant experiments.

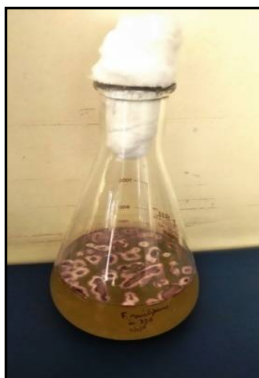
### Plant Experiment

Plant experiments were performed using chickpea seed with two sets of 4 pots. In each pot 40 seeds of chickpea were sowed. The trail was designed to have negative control, positive control and the experimental pot (test Control) which was sprayed after 8 days of sowing with CPIS and SCB formulations of butanol extract of crude GA3. Pure GA3 was used as positive control. 1ml of formulation was added in 1 L of water and 100 ml of this solution was sprayed over chickpea plant in each pot. The experiment was conducted for 60 days and the pots were sprayed with CPIS and SCB formulations in intervals of every 8 days.

## III. RESULT

### Development of Inoculum Production

Inoculum was prepared for SSF of *F. moniliforme*. Cottony white growth and a thin purple layer were observed in the flask when grown in PDB medium.



(A)

Fig. 1 – (A) Growth of *F. Moniliforme* in PDB medium.

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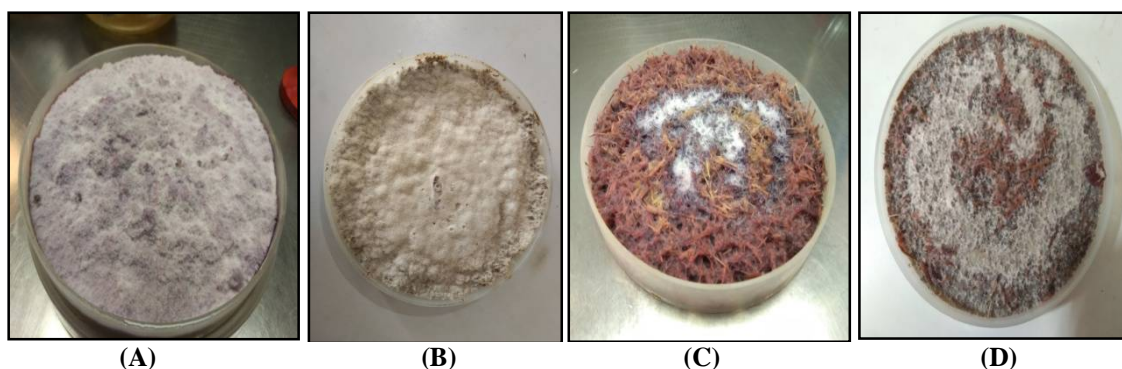
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### Substrate optimization for solid state fermentation of *F. moniliforme*

SSF of *F. Moniliforme* was done using dry citric peel, wheat bran, sugarcane bagasse and CP and SCB (1:1) medium as a substrate. Cottony white growth was observed in the plates which turn purple after incubation, on using wheat bran medium as substrate only cottony white growth was observed in the plates.



**Fig. 2 – (A) Growth of *F. Moniliforme* on dry citric peel medium, (B) Growth on wheat bran medium, (C) Growth on sugarcane bagasse medium and (D) Growth on CP & SCB**

### Result of Harvesting of crude GA3

After 3<sup>rd</sup> cycle of harvesting 100% recovery of butanol extract was obtained on using wheat bran and SCB as substrate, 75 % & 60 % recovery was obtained in CPIS and CP: SCB (1:1) respectively (Table-1).

**Table 1- Recovery of crude GA3 from SSF using different substrate.**

S.No.	Substrate	Total Volume of Butanol	Cycle 1	Cycle 2	Cycle 3	% Recovery of Butanol extract
1)	Wheat Bran	750 ml	850 ml	900 ml	1000 ml	100%
2)	Sugarcane Bagasse	1440 ml	1575 ml	1650 ml	1600 ml	100%
3)	CPIS	800 ml	830 ml	640 ml	600 ml	75%
4)	Citric Peel & SCB	800 ml	800 ml	640 ml	480 ml	60%

### HPLC of crude GA3

On performing HPLC of crude GA3; 3.9 %, 0.965 %, 0.6 % , 0.9 % was obtained in CPIS, wheat bran, SCB and CP: SCB substrate medium respectively (Table-2).

**Table 2 –HPLC values of crude GA3 extracted from different substrates medium.**

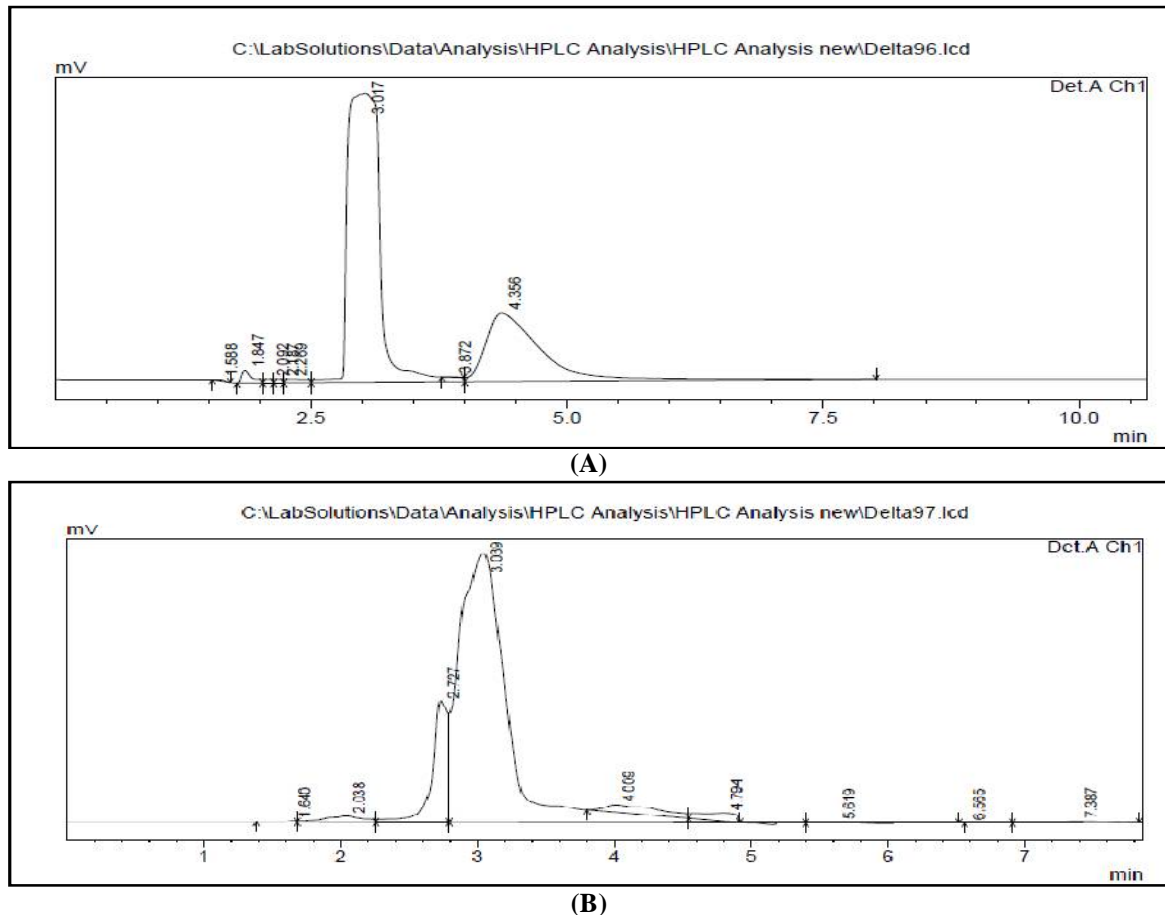
S.No.	Substrate	Retention time	Peak Area	GA3 Concentration in %
1)	2% STD	3.017	70251260	
2)	CPIS	3.039	75228550	3.9%
3)	Wheat Bran	2.879	11777200	0.965%
4)	SCB	2.674	18668207	0.6%
5)	Mixed Substrate	3.00	32698825	0.9%

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Graph 1 – (A) Chromatogram of 2% GA3 standard and (B) Chromatogram of crude GA3 extracted from *F. moniliforme* grown on CPIS medium.

Table 3 –Showing concentration of crude GA3 extracted from different substrates.

S.NO.	Substrate	Crude GA3 Concentration
1)	SCB	0.60 %
2)	Mixed Substrate	0.90 %
3)	Wheat Bran	0.96 %
4)	CPIS	3.90 %

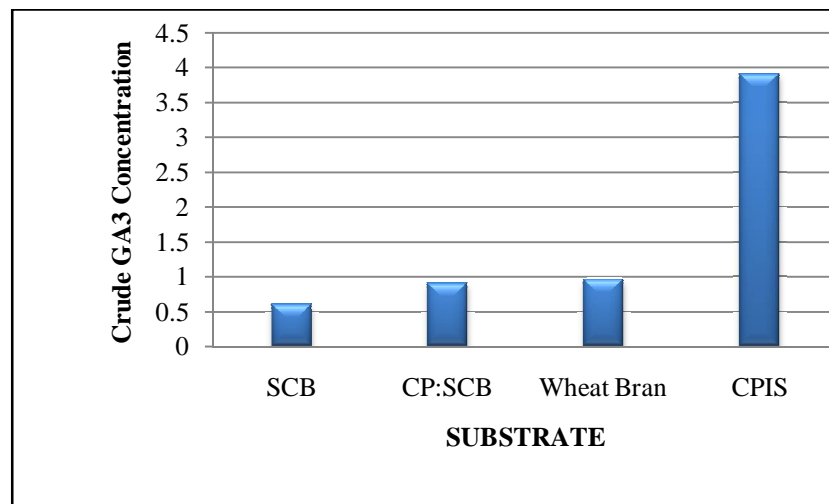


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**Graph 2 - Depicts production of crude GA3 from different substrates.**

### Plant Experiment Results

Plant experiment was carried out using chickpea plant in pots. CPIS and SCB formulations of crude GA3 were sprayed over chickpea plants. Gain in height and number of branches of different chick plants were recorded along with the germination count. After 60 days maximum height gain was observed in plants sprayed with PC. Maximum number of branches was observed in plants sprayed with CPIS formulation. Maximum number of fruits was observed in plants sprayed with CPIS formulation (Table - 4).

**Table 4 - Chickpea plant experiment data after 60 days.**

S.No.	Test	Average Height	Total no. of Branches	Average no. of Fruits	Germination Count
1)	NC	14.13 cm	585	27	37
2)	SCB	18.45 cm	849	37	39
3)	CPIS	16.65 cm	1067	64	38
4)	PC	24.93 cm	1010	35	38



**Fig. 3 - In A; (a) NC, (b) Plant sprayed with CPIS formulation and (d) PC, In B; (a) NC, (b) Plant sprayed with sugarcane bagasse formulation and (d) PC.**

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## IV. DISCUSSION

Gibberellic acid (GA3) is an important hormone, which controls plant's growth and development. Solid state fermentation of *F. moniliforme* was done for production of GA3. Citric peels with ions supplement (CPIS), wheat bran, sugarcane bagasse, and mixed medium (CP & SCB) were used as substrate. Use of variety of substrates for SSF has been cited in the literature. Machado et al, have reported a yield of 0.925 mg of GA3/gm of biomass using coffee husk and, cassava bagasse as a medium substrate whereas [7], after 18 days of cultivation, Qian et al, reported a yield of 19.3 mg GA3/gm of dry fermented substrate [13]. Agosin et al achieved yields of 3.8 mg GA3/gm vermiculite and of 6.8 mg GA3/gm initial dry mass over 190 (h), using a wheat bran culture medium [1]. Other literature wherein wheat bran have been used as medium, have reported very low yields of GA3 such as 1.14 gm/kg, and 1.2 gm/kg. Rangaswamy and Balu reported a yield of 39.4 gm/kg when using wheat bran as a substrate [16]. Rodrigues et al, reported a yield of 5.9 gm/kg of GA3 of biomass using dry CP after 3 days of fermentation [18]. In present study upon HPLC; in wheat bran 0.965% (25.73 gm/Kg of substrate ) crude GA3 concentration was obtained, in sugarcane bagasse medium 0.6% (30.96 gm/Kg of substrate) crude Ga3 concentration was obtained , and in mixed substrate 1:1 (sugarcane bagasse: CPIS) 0.9% (28.8 gm/Kg of substrate) crude GA3 concentration was obtained. **Best substrate was CPIS as GA3 concentration obtained in CPIS was 3.9% (78 gm/Kg of substrate).**

## V. CONCLUSION

GA3 has high economic cost which makes it unavailable for large farming community. We have design a low cost GA3 production strategy utilizing citric peel and other commonly available agricultural waste such as Sugarcane bagasse, Wheat Bran were used for developing a media for growth of *F. moniliforme* using solid state fermentation protocols. CPIS, wheat bran and SCB substrate medium were analysed for GA3 production by *F. moniliforme* using HPLC analysis data. Result concludes that citric peel along with ions supplements gave a high concentration of 3.9% crude GA3 confirming the adaptability of the *F. moniliforme* and its utilization capacity for citric peel. Low cost easily affordable techniques and high concentration viability of GA3 makes our SSF protocol a good alternative to high cost pure GA3 available in markets, the methodology adapted also proved effective in pot trial concluding that crude GA3 of high concentration was present along with large number of other gibberellins promoting plant growth and increased in farm produced yield.

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