Improved Production of Gibberellic Acid by *Fusarium moniliforme*

Vidhya Rangaswamy

Industrial Biotechnology Group, Reliance Life Sciences Pvt. Ltd.
vidhya_rangaswamy@relbio.com, vhrang@yahoo.com

**Abstract**  Optimization studies for improvement in the yield of gibberellic acid production by *Fusarium moniliforme* in submerged and solid-state fermentation is the focus of this paper. In the current study, use of jatropha seed cake as substrate for solid-state fermentation resulted in an unprecedented gibberellic acid yield of 105 mg/g of moldy bran. A 2.5 fold increase in the titre resulting in 15 g gibberellic acid /L could also be obtained by optimization of physiological parameters in submerged fermentation. This is the first study reporting such high yield of gibberellic acid and presenting a commercially viable production process using cheap substrates.

**Keywords**  Process Optimization, Submerged Fermentation, Solid-State Fermentation

1. **Introduction**

Gibberellic acid (GA₃), the most important gibberellin, is a class of diterpenoid that functions as plant growth regulator [1]. It affects stem elongation, elimination of dormancy, flowering, sex expression, enzyme induction and leaf and fruit senescence. GA₃ is a high valued industrially important biochemical with various applications in agriculture with price ranging around $ 25/g in the international market[1-3].

GA₃ is presently produced largely by submerged fermentation techniques using *Fusarium moniliforme* or *Gibberella 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 factors that account for high cost of GA₃ in present market scenario are the low yield of GA₃ produced and its presence in dilute form in submerged fermentation; leading to higher costs of downstream processing and disposal of waste water.

GA₃ can also be produced by the solid-state fermentation (SSF), which has got a tremendous potential for production of secondary metabolites. There are many advantages that make the SSF process commercially viable such as greater yields, lower energy consumption, a lesser environmental impact of the process, and differential expression of metabolites. The yields obtained from SSF are decent enough to offset the higher costs of downstream processing, thereby lowering the cost of gibberellic acid.

In the present study, optimization of production of GA₃ by SSF and submerged fermentation, using *Fusarium moniliforme* has been investigated. An economically viable process for commercial production of GA₃ is described.

2. **Materials and Methods**

2.1. **Organism and Growth Conditions**

*Fusarium moniliforme* NCIM 1100 was obtained from National Collection of Industrial Microorganisms, Pune, India. The strain was cultured and maintained on Potato dextrose agar (PDA) slants.

2.2. **Submerged Fermentation**

*F. moniliforme* culture was inoculated from the PDA slants into 250 ml of CD broth (composed of (g/L) sucrose, 30; NaNO₃, 3; K₂HPO₄, 1; MgSO₄.7H₂O, 0.5 ; KCl, 0.5 and FeSO₄, 0.01, pH 6.0) and incubated at 30°C for 10 days at 150 rpm. Cell growth was monitored every 24 h and GA₃ was estimated in the supernatant. All experiments were carried out at least in triplicates to ensure reproducibility.

2.3. **Solid Substrate Fermentation**

For preparation of inoculum, the fungus was grown in 100-ml Erlenmeyer flasks containing 25 ml CD broth at 150 rpm at 30°C for 4 days. Jatropha seed cake was obtained after
extraction of oil from *Jatropha curcas* seeds. For SSF using jatropha seed cake as substrate, 5 g of the cake was mixed with 8 ml of mineral salt solution (CuSO₄, 0.007g; FeCl₃, 0.007g; and ZnSO₄, 0.007g dissolved in 1 liter of 0.2 mol/L HCl). The initial moisture content of the medium was adjusted to 60%. The sterile production medium was inoculated with 3.5 ml of 4-day old inoculum of *F. moniliforme*, mixed thoroughly and incubated at 30°C for 10 days at 45° angle. The production of GA₃ was monitored every 2 days up to 10 days.

2.4. Analytical procedures

GA₃ was estimated spectrophotometrically by the method described by Berriso et al[7] at 254 nm. GA₃ was also detected by HPLC method at 206 nm on a C18 column using methanol: water (3:1) as the mobile phase at 1 ml/min flow rate[8]. The GA₃ elutes in 3 min under these conditions. Qualitative determination of GA₃ was done by TLC as described by Puchooa et al[9]. 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) H₂SO₄ in methanol containing 50 mg FeCl₃ and heated in oven at 80°C for 10 min. GAs fluoresce and appear as greenish spot under UV light.

2.5. Extraction of GA₃ from the SSF

Gibberellins were extracted from SSF by adding 100ml of distilled water to moldy bran in each flask. The mixture was kept on shaking incubator at 150 rpm for 2 h. The slurry from each flask was filtered through muslin cloth and the volume of the filtrate was made to 100 ml. Filtrate was centrifuged at 10,000 rpm for 10 min at 28°C. Supernatant was collected and analyzed for GA₃ concentration spectrophotometrically. All experiments were performed in triplicate.

2.6. Purification of GA₃ from the SSF extract

Isolation of GA₃ from the SSF extract was done by the method described by Ergun et al[10]. Briefly, to 5 ml of extract, 60 ml of solvent consisting of methanol, chloroform, and 2 N ammonium hydroxide (12:5:3 v/v) and 25 ml of distilled water was added. The mixture was shaken well in a separating funnel. After removal of the bottom chloroform layer, the methanol in the upper aqueous layer was evaporated. The pH of the remaining solution was adjusted to 2.5 and extracted thrice with 15 ml of ethyl acetate per cycle. The ethyl acetate phase was collected and evaporated to dryness. The dried material was dissolved in 5 ml of ethanol and GA₃ was estimated.

3. Results and Discussion

The present study is aimed at improvising the production of the agriculturally important growth hormone, GA₃, using submerged and solid-state fermentation strategies.

3.1. Submerged fermentation in Czapek – Dox broth

The growth and GA₃ production of *F. moniliforme* culture in Czapek – Dox (CD) broth was monitored. After an initial lag of 2 days, there was an exponential increase in growth (data not shown). The log phase continued up to 4 days before reaching the stationary phase. Production of GA₃ started from 6th day and peaked on the 8th day reaching a concentration of about 5.8 g/L. The production remained constant thereafter.

3.2. Physiological optimization

It is known that physiological factors considerably influence the GA₃ production in submerged fermentation[3,6]. To improve the yield of GA₃ in the production medium, growth parameters including pH, temperature, incubation time and media were optimized.

3.2.1. Optimization of pH in Submerged Fermentation

Effect of initial pH of the medium on GA₃ production was investigated (Figure. 1A). It was noted that initial pH of the medium did not greatly influence the production of GA₃ although highest yield of 6.5 g/L was obtained on the 8th day when the initial pH was adjusted to 7.0. Similar profile was reported for GA₃ production in *Pseudomonas* wherein a maximum yield of 0.3 g/L was obtained at pH 7.0[6]. However, Borrow et al[11] reported that GA₃ production decreases when the pH was outside the range of 3.0-5.5 in a stirred culture. The growth of the fungi was however better at pH 8.0 (data not shown).

3.2.2. Optimization of Temperature in Submerged Fermentation

![Figure 1A](image-url). Effect of pH on GA₃ production in submerged fermentation at pH 5 (- - -), pH 7 (- - -) and pH 8 (- - -)

Role of temperature on growth of *F. moniliforme* and production of GA₃ was evaluated. Incubation at 30°C was optimum for GA₃ as the yield increased to 5.8 g/L which corroborates well with the production profile in published reports[1,6] (Figure. 1B). The growth was however better at 23 and 25°C as compared to 30°C indicating a distinct difference in conditions for growth and production of GA₃.
Figure 1B. Effect of temperature on GA3 production by *F. moniliforme* in submerged fermentation at 23°C (-X-), 25°C (-♦-), 30°C (-▲-) and 37°C (-■-)

3.2.3. Optimization of Carbon Source in Submerged Fermentation

To determine the role of individual carbon source favoring high yields of GA3 production, sucrose in the CD medium was replaced with glucose, galactose, xylose, glacial acetic acid or methanol at a final concentration of 20 g/L. Of all the carbon sources, sucrose was the best giving a yield of 15 g/L under optimized conditions. Glucose was found to be equally effective whereas all other carbon sources gave lower or no GA3 production (Figure 2) in 10 days. This is by far the highest yield reported through submerged fermentation.

Figure 2. Effect of various carbon sources on GA3 production in submerged fermentation by *Fusarium moniliforme*. Glucose (-●-), Sucrose (-○-), Galactose (-■-), Xylose (-▲-), and Methanol (-X-) were supplied as carbon sources for the fermentation. Glacial acetic acid did not support any growth.

GA3 levels reported in literature are significantly lower as reviewed by Kumar and Lonsane[12]. A recent study on morphological mutants of *G. fujikuroi* has reported only 0.7 g/L of GA3[13]. Highest yield reported in the literature is 5 g/L by submerged fermentation[14] using a fed-batch cultivation mode under conditions of nitrogen limitation using genetically improved strains. The yields have been significantly lower in all other studies. Duran-Paramo et al[15] have reported a yield of 0.12 g/L whereas another report wherein dairy waste has been used as basal medium, a yield of 0.7 g/L was obtained. Eleazar et al[16] reported a yield of 2.862 g/L using immobilized *G. fujikuroi* mycelium in fluidized bioreactors.

3.3. Solid-State Fermentation

Kumar and Lonsane[12] have comprehensively reviewed the potential of the SSF technique for GA3 production and have carried out various investigations. GA3 fermentation in the present study was carried out by solid-state fermentation using jatropha seed cake as a substrate. Jatropha seed cake is a readily available waste product from biodiesel plant wherein oil extracted from seeds of *Jatropha curcas* is transesterified into biodiesel. Jatropha seed cake is a relatively recalcitrant lignocellulosic substrate having a cellulose content of 15 % and lignin content of 30 %.

In the present report, jatropha seed cake was used as a substrate for SSF. Interestingly, an unprecedented yield of 105 mg GA3/g of substrate was obtained by 4th day and remained constant thereafter (Figure 3). This is so far the best reported yield of GA3 obtained by SSF. This is the first report where the feasibility of using jatropha seed cake as a substrate for SSF has been investigated. The 5-fold improvement in the yield in our studies compared to that reported in the literature is unarguably contributed by the substrate jatropha seed cake. The seed cake may be providing the right combination of carbon and nitrogen to the fungus for production of GA3. Several fungi are known to be cellulolytic in nature and most commonly used cellulases that have been used in biomass pretreatment process are from *Trichoderma reesei* and *Aspergillus niger*[17]. The GA3 producer, *F. moniliforme* is also known to be cellulolytic in nature[18]. This attribute of the fungi may be enabling it to utilize the cellulogenic sugars from the substrate more effectively thereby resulting in high yield of GA3. Detailed analysis of the factor(s) responsible for promoting such high yields of GA3 is warranted. The jatropha seed cake is a waste from the biomass industry is toxic due to the presence of phorbol esters and may need detoxification prior to being used as land feed or animal feed. However, they can be used ‘as is’ as substrate for production of this valuable phytohormone.

Figure 3. Solid-state fermentation for production of GA3 using jatropha seed cake as substrate

Use of variety of substrates for SSF has been cited in the literature. Machado et al[19], have reported a yield of 0.925 mg of GA3/g of biomass using coffee husk and, cassava bagasse as a medium substrate whereas, after 18 days of cultivation, Qian et al[20], reported a yield of 19.3 mg GA3/g
of dry fermented substrate. However, this was partly due to high degradation of the corn flour substrate. Agosin et al[21] achieved yields of 3.8 mg GA3/g vermiculite and of 6.8 mg GA3/g initial dry mass over 190 (h), using a wheat bran culture medium. A yield of 8 g/kg was obtained on rice by Reuter et al[22]. Other literature wherein wheat bran have been used as medium, have reported very low yields of GA3 such as 1.14 g/kg[23], and 1.2 g/kg [24].

Lower incubation temperature of 23°C as compared to 28°C or 30°C reported in literature could be another factor for the high GA3 yields in our studies[23,25,26]. Only one report describes a temperature of 22°C for fermentation wherein the yield of GA3 was only 3 g/kg[27].

The GA3 extracted from the SSF migrated on TLC as a single spot similar to the standard and showed an Rf value of 0.74 thereby indicating its purity (data not shown).

The focus of the present study was on optimization for improved production of GA3 by F. moniliforme using submerged and solid-state fermentation techniques. The most significant observation was the GA3 yield of 105 mg/g of moldy bran when jatropha seed cake was used as a substrate in SSF. This is the first report on obtaining such high yields of GA3 by any mode of fermentation. Besides, SmF also gave a yield of 15 g/L fermentation broth which is about 3-fold higher than the highest yield reported in the literature by this mode. Thus, irrespective of the fermentation process employed, the yields obtained using our strain was consistently higher. Needless to say, our process is a potent candidate for commercially viable production of GA3.

4. Conclusions

A process for production of very high titres of gibberellic acid production by solid-state fermentation is described. The process is easily scalable and employs cheap raw materials rendering it economical.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the encouragement and support of Reliance Life Sciences Pvt. Ltd., in carrying out the research work.

REFERENCES

[1] Jeffery’s, E.G., 1970, The gibberellin fermentation. Adv. Appl. Biol., 13, 283-316.
[2] Avinash, C.S., Shahid, A., Agarwal, D.K., Sarbhyo, A.K., 2003, Screening of potential gibberellin producing Fusarium strains for the hybrid rice production. J. Food and Agri. Environ., 1, 250-253.
[3] Kahlon, S.S., Malhotra, S., 1986, Production of gibberellic acid by fungal mycelium immobilized in sodium alginate.
[4] Santos, E.M.G., Couto, C.M.C.M., Montenegro, M.C.B.S.M., Neves, M.G.P.M.S., Rebelo, S.L.H., Cavaleiro, J.A.S., and Reis, B.F., 2003, Ion-selective electrodes based on metalloporphyrins for gibberellic acid determination in agricultural products. Anal. Bioanal. Chem., 375, 511-516.
[5] Rademacher, W., 1994, Gibberellin formation in microorganisms. Plant Growth Regulation. 15, 303-314.
[6] Karakoc, S., Aksoz, N., 2006, Some optimal cultural parameters for gibberellic acid biosynthesis by Pseudomonas sp. Turk. J. Biol. 30, 81-85.
[7] Berrios, J., Illanes, A., Aroca, G., 2004, Spectrophotometric method for determining Gibberellic acid in fermentation broths. Biotechnol. Letts. 26, 67-70.
[8] Sharma, R., Iyer, J.P., Chakraborti, A.K., Banerjee, U.C., 2004, Determination of gibberellins in fermentation broth produced by Fusarium verticilliodes MTCC 156 by high-performance liquid chromatography tandem mass spectrometry. Biotechnol. Appl. Biochem., 39, 83-88.
[9] Puchhooa, D., Ramburn, R., 2004, A study on the use of Carrot juice in the tissue culture of Daucus carota African J. Biotechnol., 3, 248-252.
[10] Ergun, N., Topcuoglu, F., Yildiz, A., 2002, Auxin (Indole-3-acetic acid), Gibberellic acid (GA3), Abscisic acid (ABA) and Cytokinins (Zeatin) production by some species of mosses and lichens. Turk. J. Bot., 26, 29-32.
[11] Borrow, A., Brown, S., Jefferys, E.G., Kessell, R.H.J., Lloyd, E.C., Lloyd, P.B., Rothwell, A., Rothwell, B., Swait, J.C., 1964, The effect of varied temperature on the kinetics of metabolism of Gibberea fujikuroi in stirred culture. Can. J. Microbiol., 10, 445-466.
[12] Kumar, P.K.P., Lonsane, B.K., 1989, Microbial production of gibberellins: State of the art. Adv. Appl. Microbiol., 34, 29-138.
[13] Lale, G., Jogdand, V.V., and Gadre, R.V., 2006, Morphological mutants of Gibberella fujikuroi for enhanced production of gibberellic acid. J. Appl. Microbiol. 100, 65-72.
[14] Bu’Lock, J.D., 1982, Useful metabolites of Fusarium. in: Moss MO, Smith JE (Eds) The applied mycology of Fusarium. University press, Cambridge, pp 1-2.
[15] Duran-Paramo, E., Molina – Jimenez, H., Brito-Arias, M.A., Robles-Martinez, F., 2004, Gibberellic Acid Production by Free and Immobilized Cells in different culture systems. Appl. Biochem. Biotechnol., 113-116, 381-388.
[16] Eleazar, M., Escamilla, S., Dendooven, L., Magana, J.P., Parra, R., De la Torre, M., 2000, Optimization of gibberellic acid production by immobilized Gibberella fujikuroi mycelium in fluidized bioreactors. J. Biotechnol., 76, 147-155.
[17] Dashiak, M., Schraft, H., Qin, W., 2009, Fungal bioconversion of lignocellulosic residues; Opportunities and perspectives. Int. J. Biol. Sci., 5, 578-595.
[18] Ram, V., 1957, Studies on cellulolytic activity of Fusaria with reference to bacterial and other cellulose substrates. Proc. Natl. Acad. Sci. India 22, 204-211.
[19] Machado, C.M.M., Oishi, B.O., Pandey, A., Soccol, C.R.,
2004, Kinetics of Gibberella fujikuroi growth and Gibberellic acid production by Solid-State fermentation in a packed bed column bioreactor. Biotechnol. Prog., 2, 1449-1453.

[20] Qian, X-M., Du Preez, J.C., Kilian, S.G., 1994, Factors affecting gibberellic acid production by Fusarium moniliforme in solid-state cultivation on starch. World J. Microbiol. Biotechnol., 10, 93–98.

[21] Agosin, E., Maureira, M., Biffani, V., Perez, F., 1997, Production of gibberellins by solid substrate cultivation of Gibberella fujikuroi. in: Roussos S, Lonsane BK, Raimbault M et al: Advances in Solid State Fermentation. Dordrecht:Kluwer, pp. 355–66.

[22] Reuter, G., Haessler, O., Brueckner, B., et al 1987, Microbial production of gibberellic acid by culturing Fusarium moniliforme strain on solid substrate. GDR Patent DD 252000, 2 Dec 1987.

[23] Prema, P., Thakur, M.S., Prapulla, S.G., Ramakrishna, S.V., Lonsane, B.K., 1988, Production of Gibberellic Acid by Solid state fermentation: Potential and feasibility. Ind. J. Microbiol., 28, 78-81.

[24] Kumar, P.K.R., Lonsane, B.K., 1990, Solid state fermentation: physical and nutritional factors influencing gibberellic acid production. Appl. Microbiol. Biotechnol., 34, 145–148.

[25] Gelmi, C., Pe`rez-Correa, R., Gonza`lez, M., Agosin, E., 2000, Solid substrate cultivation of Gibberella fujikuroi on an inert support. Process Biochem., 35, 1227–1233.

[26] Pastrana, L.M., Gonzalez, M.P., Pintado, J., Murado, M.A., 1995, Interactions affecting gibberellic acid production in solid state culture: A factorial study. Enz. Microbial Technol., 17, 784-790.

[27] Bandelier, S., Renaud, R., Durand, A., 1997, Production of gibberellic acid by fed-batch solid state fermentation in an aseptic pilot-scale reactor. Process Biochem., 32, 141-145.