

Process parameters optimization for biosynthesis of gibberellic acid using orange waste as an alternative substrate

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Abstract

Gibberellic acid (GA₃) is the most important gibberellin that affects various plant developmental processes. As such, it has an international market value of \$106 million dollars. However, it is costly in the market because of high downstream processing cost that is linked to its current mode of production via submerged fermentation. Solid-state fermentation, in contrast, minimizes this limitation of cost when optimal process parameters are used. Hence, this study utilized orange waste as a cheap substrate in the bio-production of gibberellic acid through the optimization of three process parameters (incubation temperature, pH and substrate concentration). The study also investigated the interactive effects of these parameters on the yield of gibberellic acid. Using Design Expert® software, a 19-run experiment, varied at five levels, was generated. Accordingly, each fermentation reaction that had been supplemented with 0.03% FeSO₄·7H₂O and 0.01% (NH₄)₂SO₄ was inoculated with a constant fungal inoculum. The highest yield of gibberellic acid was with a combination of 19.5 g of substrate set at 30 °C incubation temperature with a pH of 5.5. The interaction between the three factors was a linear relationship. The bio-production of gibberellic acid using orange waste as an alternative substrate suggests the possibility of a further reduction in cost of production of high-end value metabolites when proper optimization is carried out.

Keywords: Plant hormone, solid-state fermentation, agro-residue, fungus, gibberellic acid.

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INTRODUCTION

Plant hormones, are small signaling molecules that affect plant growth and development (Bilkay *et al.*, 2010; Shani *et al.*, 2013; Sleem, 2013), and include auxins, cytokinins, gibberellins, abscisic acid, and ethylene. Gibberellins are a large family of structurally related diterpenoid carboxylic acids that occur in green plants and some microorganisms, which serve as plant growth regulators (Machado *et al.*, 2002; Urbanová *et al.*, 2011; Rangaswamy, 2012). Gibberellic acid (GA₃) is the most important gibberellin. It has an impact on various plant developmental processes, including stem elongation, seed germination, dormancy break and mobilization of endosperm reserves, enzyme induction, flowering, crop yield, sex expression, leaf and fruit senescence (Rodrigues *et al.*, 2009; Luís *et al.*, 2013; da Silva *et al.*, 2013; Camara *et al.*, 2018). Similarly, GA₃ has diverse applications in agriculture, brewery industry, biotechnology, tissue culture and viticulture (Ohlsson and Berglund, 2001; Marzouk and Kassem, 2011; Ferrara *et al.*, 2014; Archana and Sivachandiran, 2015; Beerappa *et al.*, 2019; Demes *et al.*, 2021; Ghimire *et al.*, 2021; Yadav *et al.*, 2022) and is gaining attention all over the world with an international market value of 106.53 million USD in 2021 (HNY Research Limited, 2022).

Furthermore, GA₃ can be obtained by either microbial fermentation as secondary metabolite, extraction from plants or chemical synthesis, though economically not feasible by plant extraction and chemical synthesis (Pandey *et al.*, 2008; Luís *et al.*, 2013). GA₃ has been synthesized using *Gibberella fujikuroi*, *Aspergillus niger*, *Rhizobium phaseoli*, *Azospirillum brasilense*, *Pseudomonas spp.*, and *Phaeosphaeria spp.* (Rademacher, 1994; Escamilla *et al.*, 2000; Karakoc and Aksoz, 2006; Bilkay *et al.*, 2010; Sleem, 2013; Li *et al.*, 2022). GA₃ production by submerged fermentation (SmF) is affected by different physical factors such as pH, incubation time and temperature and also gives a low yield with high production costs, which result into a higher sales price (Qian *et al.*, 1994; Camara *et al.*, 2018; Ben Rhouma *et al.*, 2020). This high cost is due to the poor yield generated and its presence in dilute form, which results in higher downstream processing and waste water disposal expenses, as well as a high cost of importation for developing countries (Machado *et al.*, 2002; Rangaswamy, 2012). These have restricted its use to preclude application for plant growth promotion, except for certain high value plants (Machado *et al.*, 2002).

Several studies aimed at decreasing the production costs of GA₃ through different approaches have been reported. Some evaluated different fermentation processes (Machado *et al.*, 2002; Pandey *et al.*, 2008; Rodrigues *et al.*, 2009) and optimized culture conditions (Escamilla *et al.*, 2000; Karakoc and Aksoz, 2006; Siddikee *et al.*, 2010; Ben Rhouma *et al.*, 2020). Others studied the potential of genetic modification of fermentation microbe (Li *et al.*, 2022), and utilized cheaper agro-wastes (Machado *et al.*, 2002; Rangaswamy, 2012; da Silva *et al.*, 2013; Kobomoje *et al.*, 2013). In their review, Camara *et al.* (2018) summarised different substrates and fermentation strategies used for the production of GA₃ using *Fusarium spp* such as Wheat bran (1.2g/kg; 6.8g/kg; 3g/kg (Kumar and Lonsane (1990); Agosin *et al.* (1997); Bandelier *et al.* (1997), respectively) and Cassava (250mg/kg (Tomasini and Fajardo, 1997)) for solid-state fermentation (SSF). For SmF, Glucose and lactose yielded 25mg/L (Tomasini and Fajardo, 1997); Glucose and rice flour yielded 3,900mg/L and 1,175mg/L (Escamilla *et al.*, 2000 and Uthandi *et al.*, 2010, respectively) and Glucose alone yielded 15g/L and 216mg/L (Rangaswamy, 2012 and Albermann *et al.*, 2013, respectively). Similarly, de Oliveira *et al.* (2017) compared the effect of different fermentation systems with citric pulp as the substrate and recorded highest GA₃ production via SSF (7.6g/kg; 946mg/L) than via SmF (2.74g/kg; 236mg/L). The authors also tried a semi solid-state fermentation (SSSF) with the citric pulp and recorded 7.69g/kg; 331mg/L of GA₃ suggesting a possible paradigm shift to the system in the future.

In this locality, orange waste is a cheap and readily available agro residue that is locally generated throughout the season and is relatively not utilized for any process, but thrown into dump areas. SSF as an alternative cost effective method could make use of agro-industrial residues as substrates towards GA₃ production, and for the production of high-value products such as secondary metabolites (Rangaswamy, 2012; Kobomoje *et al.*, 2013; Luís *et al.*, 2013; Ja'afar and Shitu, 2022). SSF system not only minimizes production and extraction costs, but also increases yield of the required end product (Pandey *et al.*, 2008; Admassu *et al.*, 2015; Sindhu *et al.*, 2015). However, its scale-up is difficult due to variances in fermentation process parameters (Camara *et al.*, 2018) amongst which are temperature, pH, agitation, aeration, humidity and moisture (Karakoc and Aksoz, 2006; Singhanian *et al.*, 2009).

Therefore, this study investigated the potential of orange waste as an alternative substrate, and identified the optimal process parameters conditions (pH of media, incubation temperature and substrate concentration) for GA₃ biosynthesis by employing a statistical approach.

MATERIALS AND METHODS

Aspergillus niger strain

Aspergillus niger (*A. niger*) was a kind donation from the Department of Microbiology, Modibbo Adama University, Yola. It was stored on PDA slants until the time of use.

Substrate collection and processing

Orange waste (orange peel and left over pulp) was obtained from local orange sellers. It was washed with clean water and then air-dried. Finely ground powder was stored in airtight container for further use.

Inoculum preparation

A. niger was grown in 250 ml Erlenmeyer flasks containing 100 ml PDA broth incubated at 30 °C for 4 days (Rangaswamy, 2012).

Solid-state fermentation optimization

To determine the optimum process parameters for GA₃ production, a mathematical approach using Design Expert® (version 6) was employed to design the experiment. Independent factors; pH of media, incubation temperature and substrate concentrations, varied at five levels generated 19 experiments (Table 1). To each experiment, 3.5 ml of the inoculum was added.

Media preparation

Media was prepared according to the method described by Machado *et al.* (2002). Briefly, to a specific gram of the processed substrate as designed by the software, it was dissolved in an equivalent mineral solution containing 0.03% FeSO₄.7H₂O and 0.01% (NH₄)₂SO₄ in a ratio of 1:4 v/w (mineral solution: substrate concentration). The pH of the final media was adjusted as provided (Table 1) and it was sterilized by autoclaving at 121 °C for 15 mins. This was subsequently inoculated with 3.5 ml of the inoculum. Media was mixed thoroughly and

incubated at specific temperatures generated by the software (Table 1) for 5 days.

Extraction of GA₃

The extraction of GA₃ was done as described by Rangaswamy (2012). Briefly, distilled water was added to the fermented product in each flask in a ratio of 10:1 v/w (distilled water: initial substrate concentration) of the substrate initial concentration. The mixture was shaken vigorously. The slurry from each flask was filtered through muslin cloth and then through a filter paper. The filtrate was centrifuged at 10,000 rpm for 10 min and supernatant was collected.

Purification and quantification of GA₃

Purification of GA₃ was done as described by Ergün *et al.* (2002) with little modifications of solvent final volumes. Briefly, to 5 ml of the extracted supernatant, 20 ml of solvent consisting of methanol, chloroform and ammonium hydroxide (12:5:3 v/v) was added. Additional 10 ml of distilled water was added to the mixture and shaken well. In a separating funnel, the bottom layer chloroform was removed and the methanol in the upper aqueous phase was evaporated. The pH of the remaining solution was adjusted to 2.5 and extracted with ethyl acetate. The ethyl acetate phase was collected and evaporated to dryness in a water bath at 50 °C. The dried material was dissolved with ethanol and GA₃ concentration was estimated spectrophotometrically by comparing with a standard of pure GA₃ at 254 nm.

RESULT AND DISCUSSION

In an effort to determine the optimum process parameters for the production of GA₃ by the fungus *A. niger*, three process parameters (incubation temperature, pH of media and substrate concentration) were investigated through response surface methodology. For this purpose, a 19-run experiment based on face-centered central composite design (FCCCD) was generated by Design Expert®, and the result is presented in Table 1 while Table 2 shows the statistical variance analysis. Figure 1 shows pictorial representation of extraction and purification of GA₃.

Table 1: Solid-state fermentation experimental runs and yield of GA₃

Process parameters				Response
Run	Temp (°C)	pH	Substrate (g)	GA ₃ concentration (g)
1	30	5.5	8.5	1.09
2	20	3	2	0.27
3	30	5.5	8.5	1.11
4	30	9.71	8.5	0.71
5	46.8	5.5	8.5	1.02
6	30	1.3	8.5	0.98
7	20	8	15	1.12
8	40	8	15	1.44
9	30	5.5	19.5	2.74
10	40	3	15	1.32
11	30	5.5	2	0.54
12	20	8	2	0.54
13	30	5.5	8.5	1.50
14	30	5.5	8.5	1.26
15	20	3	15	2.44
16	40	3	2	0.45
17	13.2	5.5	8.5	0.60
18	30	5.5	8.5	0.98
19	40	8	2	0.36

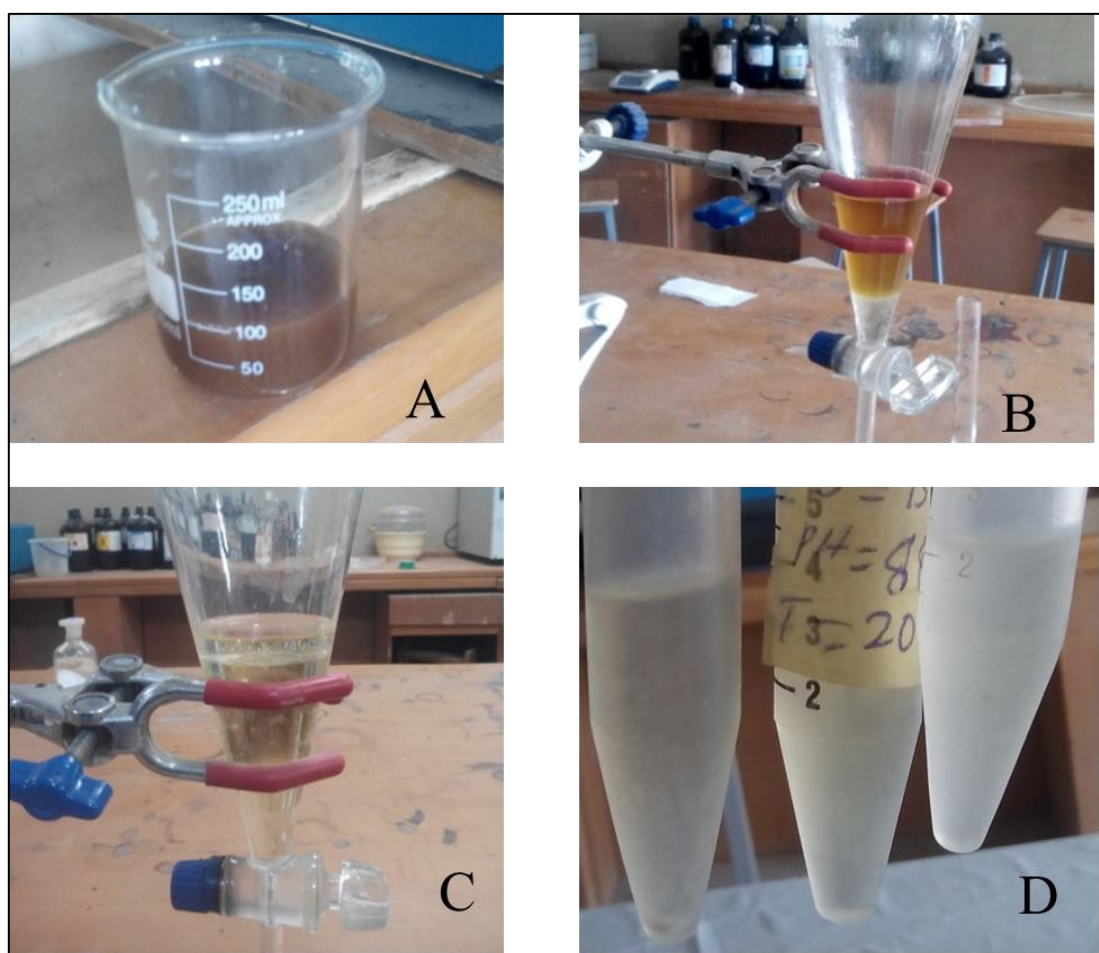


Figure 1. Extraction and purification of GA₃. A: Crude extract of GA₃. B: Solvent extraction. C: Purification with ethyl acetate. D: GA₃ dissolved in ethanol.

Table 2: ANOVA results for response surface linear model of GA₃ production

	Sum of Squares	DF	Mean Square	F Value	*Prob > F
Linear Model	1.18	3	0.39	15.87	>0.0001
Lack of Fit	0.34	11	0.031	3.79	0.1047
R ² = 0.7605		Adjusted R ² = 0.72126		Predicted R ² = 0.5746	
Adequate Precision = 12.085		PRESS = 0.66			

**p* < 0.05

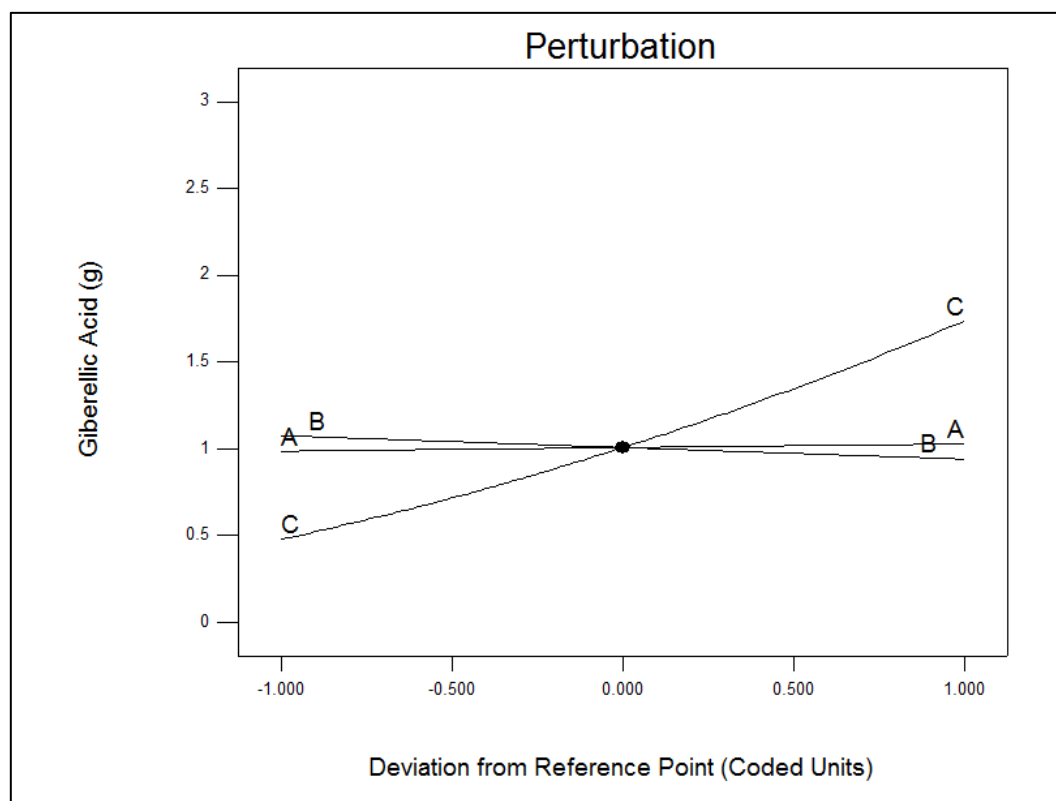


Figure 2. Effect of temperature (A), pH (B) and Substrate concentration (C) on GA₃ yield.

The statistical model representing the predicted concentration of GA₃ as a function of the independent variables within the region under investigation is expressed in coded form (Equation 1):

$$GA_3 \text{ concentration} = (0.6202 + 1.5078 \times 10^{-0.03}A - 0.0137B + 0.0488C) \quad (1)$$

where A, B and C are the coded variables for temperature, pH and substrate concentrations, respectively. Furthermore, the model evaluation was performed through analysis of variance (ANOVA) and coefficient of determination (R²) that measures the goodness of fit of the regression model (Table 2). The good agreement between the adjusted R² and predicted R² showed how fit the model was in predicting the best combination of three process parameters.

The relationship between the response and experimental levels of variables in the study were expressed in the form of a graphical plot (Fig. 2). Figure 2 shows that there is no statistically significant interaction between the variables, rather a linear expression of individual parameter. An increase in substrate concentration resulted to a higher increase in GA₃ concentration, whereas, changes in pH and temperature showed no any significance effect on the yield. Gibberellic acid can also be synthesized from the substrate only without any supplement, but it produces lower result when compared to same treatment with supplement (Ben Rhouma *et al.*, 2020). A preliminary fermentation was performed to ascertain the requirement of supplement in the media. Hence, a result of 186mg and 116mg of GA₃/g of substrate was observed with and without supplement, respectively. As such, all

optimization experiments conducted in this study had 0.01% $(\text{NH}_4)_2\text{SO}_4$ and 0.03% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ as supplement in the final media composition. GA_3 is synthesized when the fungus is brought to stationary phase by nitrogen diminishing with an abundant carbon source (glucose) in the medium (Pandey *et al.*, 2008).

Utilization of substrate was observed to be slow during the first 24 hours, however, the whole substrate was relatively consumed by day five of the fermentation period (media was relatively dark with the fungus covering all over it). This study did not consider inoculation time to production of GA_3 because previous study had shown its minimal effect on GA_3 production (Li *et al.*, 2022). However, Karakoc and Aksoz (2006) reported an initial increase of GA_3 production by 12 hours of incubation and reached its peak by 72 hours. This was speculated to be because of the microbe used since bacterium normally has a shorter doubling time (Pirt 1967; Reischke *et al.*, 2014). In fact, for GA_3 production, the fungi *Fusarium moniliforme* and *Gibberella fujikuroi*, and the bacteria belonging to the genus *Azotobacter* and *Azospirillum* are the microbes of choice (Rademacher, 1994).

Although not statistically significant, it was observed that GA_3 production decreased at low temperatures even at high substrate concentrations. The best results were obtained at 30 °C, which correlates with most reports from literatures (Karakoc and Aksoz, 2006; Rodrigues *et al.*, 2009; Bilkay *et al.*, 2010; Rangaswamy, 2012). However, Li *et al.* (2022) reported a range of 26 – 28 °C, but significantly decreased when it was higher than 32 °C. Similarly, Machado *et al.* (2002) maintained a temperature of 29 °C in their GA_3 production when they noticed insignificant effects between 28 – 30 °C. Furthermore, a slight increase in temperature had no any significant effect on the yield of GA_3 in this study.

Similarly, the pH of the medium has no any significant effect on the yield, which also correlates with results of other literatures (Rodrigues *et al.*, 2009; Rangaswamy, 2012). However, a pH of 5.5 seems to be the optimum as earlier reported (Escamilla *et al.*, 2000; Bilkay *et al.*, 2010). In contrast, Li *et al.* (2022) reported an optimal pH of 4, while Karakoc and Aksoz (2006) and Siddikee *et al.* (2010) reported an optimal pH of 7 for *Pseudomonas* and *Methylobacterium spp.*, respectively. When studying the kinetics of GA_3 production, Machado *et al.* (2002) observed that irrespective of how high the initial pH of media was, it comes down to a range of 5.3 – 5.6 within the first 24 hours of fermentation and

maintains that until the end of the fermentation process.

In this study, the yield of GA_3 was observed to be dependent on the amount of substrate concentration; the yield increased with an increase in substrate concentration (Fig. 2). Similar studies with such claims have been reported (Escamilla *et al.*, 2000; Ben Rhouma *et al.*, 2020). This high utilization of the substrate is, perhaps, associated with the high lignocellulose content of orange peels and the cellulolytic nature of cellulases in *Aspergillus niger*. Cellulases from *A. niger* and *Trichoderma reesei* have been shown to be of choice during biomass pretreatment processes because of their high cellulolytic potential (Rangaswamy, 2012). Thus, with a substrate concentration of 19.5 g and a pH of 5.5 at 30 °C, the highest amount of GA_3 was obtained. The yield of GA_3 (2.74g/19.5g (~140mg/g) of orange waste (w/w)) obtained in this study is somewhat higher than that obtained from previous studies, (105mg/g of Jatropha seed cake, Rangaswamy, 2012; 0.925mg/g of Coffee husk, Machado *et al.*, 2002; 19.3mg/g of Corn flour, Qian *et al.*, 1994).

Optimization studies have traditionally been based on individual parameter variation while keeping other parameters constant (Karakoc and Aksoz, 2006; Siddikee *et al.*, 2010; Camara *et al.*, 2018). Mathematical models that take into account all individual variables have been reported previously (Escamilla *et al.*, 2000; Machado *et al.*, 2002; Ja'afar *et al.*, 2018; Ben Rhouma *et al.*, 2020; Li *et al.*, 2022). Other designs used for GA_3 optimization include Plackett Burman (Li *et al.*, 2022) and Taguchi (Ben Rhouma *et al.*, 2020).

CONCLUSION

Optimization of process parameters in SSF has increased the yield of GA_3 production. Similarly, the bioavailability of orange waste towards the improved synthesis of GA_3 through mathematical optimization suggests the economic significance of this abundant and cheap agricultural residue that has not been harnessed. Utilization of this agro-residue would reduce the cost of GA_3 and other important byproducts hitherto were costly and not readily available and affordable. Nonetheless, further research is required to upscale the production of this hormone.

Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

JNJ designed the experiment, analysed the results and proofread the manuscript while MM conducted the experiment and wrote the draft of the manuscript

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