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Submerged Fermentation of Orange Albedo to Produce Gibberellic Acid Using *Fusarium moniliforme* and *Aspergillus niger*

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Abstract

This study investigated the potential of orange albedo, an agro-industrial waste, as a suitable substrate for the production of gibberellic acid (GA) through submerged fermentation using *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 due to the high cost of synthetic and plant-extracted GA. The orange fruits were washed and the albedo removed. The albedo was dried, ground, and its proximate composition was determined. The ground orange albedo was incorporated into a modified CzapekDox medium and was fermented using the test fungi. Carboxymethyl cellulose (CMC) served as control. Fermentation conditions were: pH 5.5; inoculum size, 1 mL (5 x 10^5 CFU/mL *F. moniliforme*) (2 x 10^6 CFU/mL *A. niger*); substrate concentration 2 g; temperature 25±2 $^{\circ}$C for seven days. Fermentation was optimized by supplementation with copper sulphate and variation of fermentation conditions. Results of proximate analysis were: moisture 7.46%; crude protein 4.69%; lipids 0.62%; ash 2.41%; crude fibre 27.67%; and carbohydrate 57.15%. GA yield by *F. moniliforme* and *A. niger* on the orange albedo substrate was 5.53 g/L and 6.33 g/L respectively. This increased to 9.39 g/L by *F. moniliforme* and 7.42 g/L by *A. niger* after optimization. These results support the suitability of orange albedo as a promising cheap substrate production of GA.

Keywords*:* Orange albedo, gibberellic acid, fermentation, *Aspergillus niger*, *Fusarium moniliforme*, fruit waste

1. Introduction

Gibberellins are isoprenoid phytohormones which play important roles in early germination processes of plants by activating enzyme production and mobilizing storage reserves (Rademacher, 2016). Gibberellic acid (GA) is one of the most important members of the gibberellins due to its industrial and agricultural applications (Rodrigues *et al.*, 2009). Over 120 members of this group of phytohormones have been identified and structurally characterized using chemical and spectroscopic methods (Macmillan, 2002). Among the gibberellins, the ones that have been reported as bioactive are GA_1, GA_3, GA_4, and GA_7. The biologically-inactive gibberellins occur in plants as precursors for the synthesis of the bioactive ones (Yamaguchi, 2008). Gibberellic acid (gibberellin A_3 or GA_3) is one of the most important members of the bioactive gibberellins due to its industrial and agricultural applications (Ates *et al*., 2006).

Gibberellic and abscisic acids are endogenous growth-regulating hormones which control the breaking of seed dormancy to germination alongside other factors such as light, temperature, moisture, and nutrients (Gupta and Chakrabarty, 2013). While GA stimulates seed germination, abscisic acid on the other hand, is concerned with the establishment and maintenance of dormancy. GA has been used extensively for the promotion of crop yields, resistance to pest, alleviation of plant stress, reduction in fruit spoilage and the reduction in flowering times of ornamental plants (Barani *et al*., 2013; Akter *et al*., 2014; Alrashdi *et al*., 2017; Alvarenga *et al*., 2017). However, its high cost has restricted its application to the growth-promotion of plants with high economic values. While GA can be isolated from some tissues in plants, it is a difficult process often marked by poor yields which may be as low as 38 mg/tonne of plant tissue (Mander, 2003). In a similar manner, the production of gibberellins through chemical synthesis is very complicated and unprofitable for industrial applications (Rademacher, 2016). Research has been geared to finding wider GA applications in agriculture and plant biotechnology (Shukla *et al*., 2005; Da Silva *et al*., 2013). Hence, there is a need to utilize cheap substrates for the production of GA.

Oranges (*Citrus sinensis*) are grown in more than 125 countries, and the worldwide production for 2016/17 was estimated at 50.2 million tonnes (USDA, 2017). Apart from fruit-processing industries, oranges are also consumed for their fleshy fruit and juice, after which the peel and albedo are discarded into the environment. Citrus

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** Abbreviations: CMC (Carboxymethyl cellulose), GA (Gibberellic acid), OA (Orange albedo).

peel waste comprises about 50% of the fresh weight of the fruit (Rodriguez-Fernandez *et al*., 2011). The orange -juice processing industries produce significant volumes of wastes made up of soluble and insoluble carbohydrates (Zhou *et al*., 2011). The disposal of fruit wastes poses considerable environmental and economic problems (Bezalwar *et al*., 2013). However, the utilization of these wastes for fermentation purposes will not only reduce their potentially deleterious effects on the environment, but will also serve as cheap carbon sources for the industrial production of value-added products (Rivas *et al*., 2008, Torrado *et al.*, 2011; Omojasola and Benu, 2016).

Industrially, GA is produced largely by a submerged fermentation technique using *Gibberella fujikuroi* (renamed *Fusarium fujikuroi*), the perfect stage of *Fusarium moniliforme* (Bruckner and Blechschmidt, 1991; O'Donnell *et al*., 1998; Santos *et al*., 2003). Other methods of GA production, which include the chemical synthesis and extraction from plants, are not economically feasible (Sleem, 2013). While solid-state fermentation has been reported to have a potential for higher yields, lower energy consumption, reduced risk of bacterial contamination, lower catabolic repression, and lesser environmental impacts (Viniegra-Gonalez *et al*., 2003; Torrado *et* al., 2011; Rangaswamy, 2012; Zhang *et al*., 2015), however, it is difficult to monitor the fermentation parameters such as pH, inoculum concentration, nutrient composition, dissolved oxygen composition and fermentation time, and to optimize them using solid-state fermentation (Kumar *et al.*, 2011). In addition, submerged fermentation allows an easier purification of the product (Subramaniyam and Vimala, 2012). Some other microorganisms that have been found to produce GA include: *Aspergillus niger*, *Azospirillum*, *Azotobacter*, *Bacillus* spp. and *Pseudomonas* spp. (Rademacher, 1994; Cihangir, 2002; Ates *et al*., 2006; Karacoç and Aksöz, 2006; Ambawade and Pathade, 2015). A variety of agro and fruit wastes have been utilized in the production of organic acids using submerged and solid-state fermentation such as pineapple peel, sugarcane baggasse, banana peel to produce citric acid (Kareem and Rahman, 2013; Omojasola *et al*., 2014), cashew apple juice and corn cob to produce oxalic acid (Betiku *et al*., 2016; Mai *et al.*, 2016), *Jatropha* seedcake, sweet potato peel to produce itaconic acid (El Imam *et al*., 2013; Omojasola and Adeniran, 2014) Shea nut shell, citric pulp, soy bran, soy husk, cassava bagasse and coffee husk to produce GA (Rodrigues *et al.*, 2009; Kobomoje *et al*., 2013).

To our knowledge, there is a dearth of data on the suitability of orange peel wastes for the production of GA. Hence, the primary aim of the current work was to study the suitability of orange albedo as a substrate for the production of GA by *F. moniliforme* and *A. niger*.

2. Materials and Methods

2.1. Collection of Samples and Test Organisms

The oranges (*Citrus sinensis*) were procured from the Ipata Market in Ilorin in Kwara State, Nigeria (with coordinates 8.99897 N, 4.561369 E) in November of 2016. The orange fruits were authenticated at the Herbarium Unit of the Department of Plant Biology at the University of Ilorin, with voucher specimen number UILH/001/996. The microorganisms used for the fermentation were *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 obtained from the Microbial Culture Collection of the Department of Microbiology at the University of Ilorin in Nigeria. They were maintained on PDA slants at 4°C to be used later.

2.2. Substrate Preparation

The orange fruits were washed with clean water to remove dirt; after which they were peeled, taking care while separating the peel from the albedo. The albedo was then air-dried for seven days. It was thereafter ground into fine particles (1 mm particle size) by an electric blender (Binatone BLG 699). Then it was stored in a cool and dry place to avoid moisture uptake (Nandini *et al*., 2014).

2.3. Proximate Analysis

The proximate analysis of the substrate was carried out using standard procedures. The parameters investigated were moisture content (Bradley, 2010), lipid, crude fibre, ash, crude protein and carbohydrate contents (AOAC, 1990, 2002).

2.4. Spore Suspension

Fungal spore inoculum was produced by washing spores of a fully-sporulated (7-day old) Potato Dextrose Agar (Difco) slant of each test fungus with 10 mL of sterile distilled water in sterile 250 ml Erlenmeyer flasks. The flasks were then agitated at 150 rpm for thirty minutes for uniform dispersal of spores (Omojasola and Benu, 2016) and adjusted approximately to 5.0×10^5 CFU/mL and 2.0×10^6 CFU/mL for *F. moniliforme* and *A. niger* respectively. The size of the inoculum was determined by counting using the improved Neubauer haemocytometer.

2.5. Fermentation Media

The fermentation medium was a modified CzapekDox broth using the method of Rangaswamy (2012) with replacement of sucrose with orange albedo substrate. The fermentation medium was compounded by adding 2 g of substrate to 100 mL of mineral salts medium. The composition of the mineral salts in 1 litre of water was $NaNO_3$ (3g), K_2HPO_4 (1g), $MgSO_4.7H_2O$ (0.5g), KCl (0.5g), and $FeSO_4$ (0.01g). The fermentation medium was sterilized by autoclaving at 121 °C before use.

2.6. Submerged Fermentation

The test organisms were drawn separately from the spore suspension, and each was inoculated into 100 mL of sterile fermenting medium. The fermentation was carried out at 25±2 °C on a rotary shaker (LH Fermentation, Model Mk V orbital shaker) at 150 rpm for seven days. The final pH was adjusted using 2M NaOH or 1M HCl. The GA production was monitored every twenty-four hours.

2.6.1. Optimization of GA Production

The optimization experiments were conducted varying the following parameters: fermentation period (fermentation was allowed to continue till GA yield began to drop); pH (4.5 - 5.5); inoculum size (1.0 – 2.0%); substrate concentration (1.0 – 3.0g).

2.6.2. Media Supplementation

The effect of copper sulphate ($CuSO_4$) supplementation on GA production was evaluated. Three concentrations of $CuSO_4$ (0.02% w/v, 0.05% w/v, and 0.08% w/v) were added to different fermentation media (Chinedu *et al*., 2011), and the fermentation proceeded under the same conditions as the non-supplemented cultures.

2.6.3. Assay of GA

This was estimated in the supernatant of fermentation media by spectrophotometrically (Searchtech 752N UV-VIS) using a modified method described by Berrios *et al.* (2004) at 254 nm. The amount of gibberellic acid was calculated from the standard curve obtained by dissolving 0.4 g in absolute alcohol, and diluted to 100 ml in a volumetric flask with absolute alcohol. Each series of data obtained from the spectrophotometric measurement was fitted by linear regression analysis using GraphPad Prism software. The calibration graph obtained was used for the determination of the concentration of gibberellic acid with interpolated values after entering the obtained figures of absorbance.

2.6.4. Recovery of GA

GA was recovered from the fermentation media using methods described by Rachev *et al*. (1993), and Ates *et al*. (2006). The fermentation broth was filtered to separate the mycelia from the media. The filtrate was then adjusted to pH 2 - 2.5 with 2 N HCl, and extracted with ethyl acetate (ratio 1:3, filtrate to solvent). The ethyl acetate phase was treated with activated charcoal 1:1.33% (w/v), and re-filtered to remove the activated charcoal. The ethyl acetate phase was extracted with equal volume of saturated $NaHCO_3$ to separate the GA from other organic impurities. This was further acidified to pH 2.5 with 2 N HCl; re-extracted, dried over anhydrous Na_2SO_4 and concentrated to about 2% of its initial volume using a rotary evaporator. The concentrate was kept at 8^oC for crystallisation.

2.7. Data Analysis

Statistical significance was determined using the one-way analysis of variance (ANOVA) and two-way ANOVA, while multiple comparisons between means were determined by Tukey's or Sidak's multiple comparisons test. Analysis was performed using GraphPad Prism software (GraphPad Software Inc. La Jolla, CA, USA), and SigmaPlot for Windows (version 10.0) (SysStatSoftwares Inc.). All data are expressed as means of triplicates ± SEM or SD, and values of (p<0.05) were considered significant, and 'n' represented independent experiments.

3. Results

3.1. Proximate Analysis

The proximate analysis of the orange albedo substrate showed that it contained 7.46% moisture, 4.69% crude protein, 0.62% lipid, 2.41% ash, 27.67% crude fibre and 57.15% carbohydrate (Table 1).

Table 1. Proximate composition of orange albedo.

Moisture Content (%)	Crude Protein (%)	Lipid Content (%)	Ash Content (%)	Crude Fibre (%)	Carbohydrate (%)
7.46±0.02	4.69± 0.18	0.62±0.01	2.41±0.14	27.67±0.45	57.15±0.98

Values represented are means of triplicates ±SEM

3.2. Pre-optimization of GA Production

The GA production by *F. moniliforme* peaked at 5.53 ±0.02 g/L on Day 6, while the maximum yield by *A. niger* 6.30 ±0.01 g/L was on day five (Table 2). The CMC control produced significantly lower (p<0.05) yield than the OA substrate. *F. moniliforme* and *A. niger* produced 3.62 ±0.01 g/L and 2.61 ±0.07 g/L of GA respectively when CMC was used as substrate.

Table 2. Production of gibberellic acid by submerged fermentation of orange albedo using *Fusarium moniliforme* and *Aspergillus niger*..

Time (Days)	Gibberellic acid (g/L)			
	Fusarium moniliforme		*Aspergillus niger*	
	Orange albedo	CMC (Control)	Orange albedo	CMC (Control)
1	0.48±0.05^a	0.14±0.01^b	2.42±0.01^a	0.03±0.01^b
2	0.90±0.12^b	0.91±0.02^a	2.80±0.01^a	0.15±0.01^b
3	2.50±0.61^a	1.73±0.01^a	4.15±0.02^a	0.82±0.02^b
4	1.67±0.80^b	3.62±0.01^a	4.10±0.16^a	1.24±0.11^b
5	2.22±0.01^a	1.88±0.04^b	6.30±0.01^a	2.61±0.07^b
6	5.53±0.02^a	1.78±0.02^b	3.73±0.01^a	2.54±0.01^b
7	5.25±0.13^a	2.63±0.03^b	4.07±0.17^a	1.46±0.03^b

Values represented are means of triplicates ±SEM of amount of gibberellic acid. Means with the same superscript in a column are not statistically different from each other (*F. moniliforme* and *A. niger* were compared separately)

3.3. Optimization of GA Production

To optimize the GA yield, fermentation parameters such as time, pH, inoculum size and substrate concentration were varied.

3.3.1. Effect of Varying Fermentation Time

The GA yield by *F. moniliforme* peaked on the day six of fermentation (5.5 ±0.03 g/L); however, there was no significant difference (p<0.05) in the yields on day six and seven. The highest GA yield of 6.3 ±0.09 g/L by *A. niger* was recorded on day five (Figure 1). Generally, the yields from the OA substrate were higher than the CMC control.

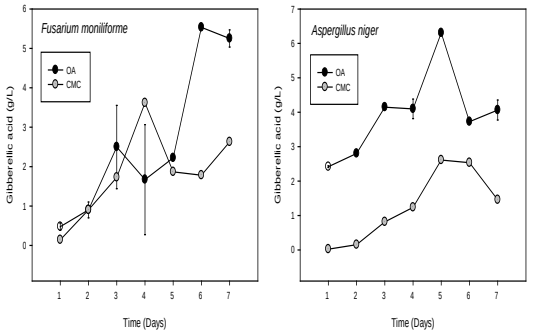

Figure 1. Effect of varying fermentation time on gibberellic acid production by *F. moniliforme* and *A. niger* using orange albedo.

3.3.2. Effect of Varying pH

F. moniliforme produced the highest GA yields at pH 5.0 (12.96 ±0.03 g/L) significantly higher than pre-optimization yields (Figure 1). The lowest peak yield of 5.53 ±0.03 g/L was at pH 5.5. For *A. niger*, pH 5.5 recorded the highest yield of 6.30 ±0.98g/L, while the lowest peak yield of 2.62 ±0.02 g/L was recorded at pH 4.5 (Figure 2).

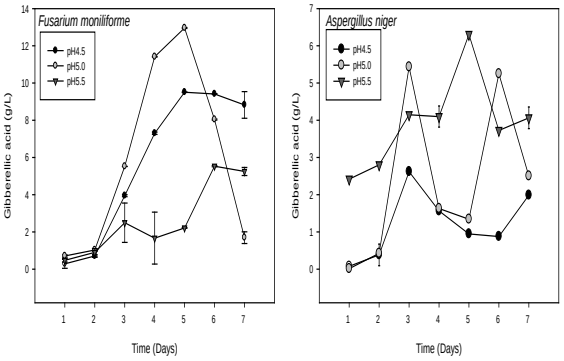

Figure 2. Effect of varying pH on gibberellic acid yield using *F. moniliforme* and *A. niger* grown on orange albedo

3.3.3. Effect of Varying Inoculum Size

The highest GA yield was recorded using 2% inoculum for both fermenting organisms. The yield of *F. moniliforme* was 15.96 ±0.04 g/L and *A. niger* 10.74 ±0.04 g/L (Figure 3). The yield by *F. moniliforme* was the highest yield in this study and was significantly higher ($p<0.05$) than pre-optimized yields.

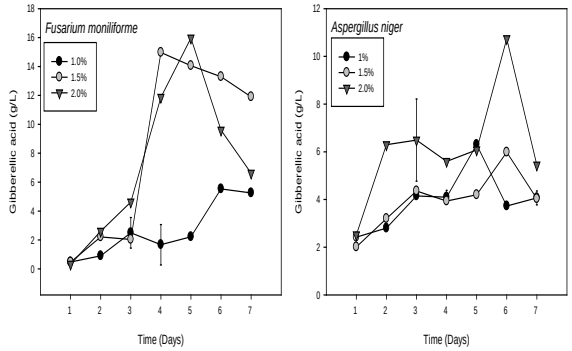

Figure 3. Effect of varying inoculum size on gibberellic acid yield using *F. moniliforme* and *A. niger* grown on orange albedo.

3.3.4. Effect of Varying Substrate Concentration

Peak yields of GA were obtained at substrate concentration of 3 g for both *F. moniliforme* and *A. niger*. *F. moniliforme* yielded 9.47 ±0.09g/L, while *A. niger* yielded 10.78 ±0.08 g/L (Figure 4). These yields were also significantly higher than pre-optimized yields.

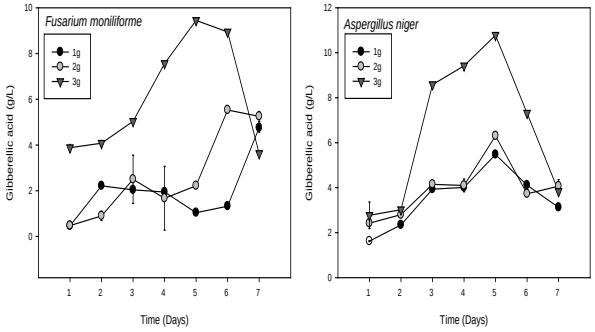

Figure 4. Effect of varying substrate concentration on gibberellic acid yield using *F. moniliforme* and *A. niger* grown on orange albedo.

3.3.5. *Effect of Medium Supplementation*

The yields of gibberellic acid obtained after supplementation with different concentrations of $CuSO_4$ recorded highest GA yields of 5.86 g/L on day seven by *F. moniliforme* using 0.08% $CuSO_4$ and 6.33 g/L on day five by *A. niger* also with 0.08% $CuSO_4$ supplementation (Table 3). It was also observed that there were no significant differences in the yields obtained at 0.02% and 0.08% on day seven for *F. moniliforme* and all the concentrations used on day five for *A. niger* (Table 3).

Table 3. Gibberellic acid production by *F. moniliforme* and *A. niger* grown on orange albedo supplemented with different concentrations of copper sulphate.

Time	Gibberellic acid (g/L)							
(Days)	*Fusarium moniliforme*				*Aspergillus niger*			
	0.02% $CuSO_4$	0.05% $CuSO_4$	0.08% $CuSO_4$	Control	0.02% $CuSO_4$	0.05% $CuSO_4$	0.08% $CuSO_4$	Control
1	0.74± 0.11^a	0.70± 0.03^a	0.70± 0.05^a	0.48± 0.05^a	1.75± 0.23^b	1.42± 0.09^c	2.29± 0.08^a	2.42± 0.01 ^{a}
2	1.34± 0.05^a	1.44± 0.06^a	1.48± 0.04^a	0.90± 0.12^a	3.20± 0.10^a	3.28± 0.18^a	2.80± 0.09^b	2.80± 0.01^b
3	1.52± 0.07^b	1.53± 0.08^b	1.45± 0.04^b	2.50± 0.61^a	4.06± 0.09^a	2.83± 0.07^b	4.15± 0.21^a	4.15± 0.02^a
4	1.99± 0.10^a	1.68± 0.09^a	1.74± 0.11^a	1.67± 0.80^a	4.33± 0.25^b	3.65± 0.05^c	5.29± 0.05^a	4.10± 0.16^b
5	3.21± 0.06^a	3.37± 0.06^a	3.79± 0.04^a	2.22± 0.01^b	6.29± 0.12^a	5.99± 0.20^a	6.33± 0.10^a	6.30± 0.01^a
6	5.53± 0.05^a	4.35± 0.16^b	4.04± 0.10^b	5.53± 0.02^a	5.39± 0.09^a	5.56± 0.17^a	3.70± 0.12^b	3.73± 0.01^b
7	5.55± 0.07^a	4.25± 0.12^b	5.86± 0.05^a	5.25± 0.13^a	3.56± 0.08^b	4.22± 0.23^a	3.47± 0.20^b	4.07± 0.17^b

Values represented are means ±SD of amount of Gibberellic acid. Means with the same superscript across a column are not statistically different from each other (*F. moniliforme* and *A. niger* were compared separately)

3.3.6. Optimized Production of GA

The GA yield under optimized conditions recorded 9.39 ±0.16 g/L by *F. moniliforme* and 7.42 ±0.02 g/L by *A. niger* both on day six of fermentation (Table 4). These were significantly higher (p<0.05) than the peak yields obtained from the CMC control under the same optimized conditions. These yields were higher than those from the pre-optimized fermentations which were 5.53±0.02 g/L and 6.30±0.01 g/L by *F. moniliforme* and *A. niger* respectively (Table 2). In addition, *F. moniliforme* produced higher amounts of GA, although not significant (p<0.05) than *A. niger* under optimized conditions.

Table 4. Production of gibberellic acid by submerged fermentation of orange albedo using *Fusarium moniliforme* and *Aspergillus niger* under optimized conditions.

Time (Days)	Gibberellic acid (g/L)			
	Fusarium moniliforme		*Aspergillus niger*	
	Orange albedo	CMC (Control)	Orange albedo	CMC (Control)
1	1.55±0.03^a	0.91±0.03^b	1.72±0.02^a	1.31±0.03^b
2	4.19±0.02^a	2.75±0.03^b	3.81±0.01^a	1.81±0.03^b
3	5.26±0.03^a	5.25±0.02^a	4.35±0.06^a	2.21±0.03^b
4	5.64±0.03^a	5.56±0.06^a	3.84±0.03^a	2.39±0.03^b
5	5.48±0.01^b	6.84±0.06^a	5.60±0.02^a	3.45±0.04^b
6	9.39±0.16^a	7.03±0.04^b	7.42±0.02^a	4.28±0.04^b
7	8.95±0.05^a	3.80±0.09^b	3.80±0.01^a	1.85±0.03^b

Values represented are means ±SEM of gibberellic acid produced. Means with the same superscript in a row are not statistically different from each other (*F. moniliforme* and *A. niger* were compared separately)

4. Discussion

Fruit wastes constitute part of the most abundant and locally-available agricultural wastes containing high carbohydrate content, which serve as fermentable substrate for microorganisms (Bezalwar *et al*., 2013). The proximate analysis of the OA substrate showed that it contained 57.15% carbohydrate, 27.67% crude fibre, 4.69% crude protein, 0.62% lipids, 7.46% moisture and 2.41% ash (Table 1). This is within the range reported by other workers which have shown OA to contain between 40-64 % carbohydrate, 2-9% protein, 17-35% crude fibre and 0.85 – 13.4% ash (Oikeh *et al.*, 2013; M'Hiri *et* al., 2015; Taha *et al*., 2015; Hassan *et al.*, 2016; Romelle *et al*., 2016). The high carbohydrate content constituted a good carbon source for the growth of the fermenting organisms. In addition, the amount of carbohydrate relative to protein gave a high C/N ratio which is recommended for a good GA production (Kumar and Lonsane, 1989). The production of gibberellins starts during fermentation when nitrogen is depleted in the medium, and continues when enough carbon is available in the substrate (Escamilla *et al*., 2000). Nitrogen repression is a well-known regulatory principle for secondary metabolite formation (Munoz and Agosin, 1993). A good substrate should provide sufficient nutrients for the initial mycelial growth of the fermenting fungi in a nitrogen-limited but balanced medium (Rodrigues *et al*, 2009). This substrate with 57.15% carbohydrate and 4.69% protein fits this criterion. The presence of lipids in the OA is beneficial. Kawanabe *et al*. (1983) and Tudzynski (1999) reported that the biosynthesis of GA is based on acetate and follows the isoprenoid pathway. Therefore, plant oils are inert for catabolite repression. They also provide a pool of acetyl CoA, and may yield precursors for GA biosynthesis.

The highest GA yield by *F. moniliforme* and *A. niger* on OA before optimization was 5.53 g/L and 6.30 g/L respectively with *A. niger* showing higher productivity (Table 2). These differences in yields were statistically significant (p<0.05). This yield was higher than 680 mg/L reported by Muddapur *et al.* (2015) using *Fusarium* sp.; 2.86 g/L by *G. fujikuroi* (Escamilla *et al*., 2000); 0.7 g/L by *G. fujikuroi* (Lale *et al.*, 2006); 1.82 g/L by *F. moniliforme* (Kobomoje *et al*., 2013); 2.8g/L by *F. moniliforme* (Pastrana *et al*., 1993); and 460.06 mg/L by *G. fujikuroi* reported by Cuali-Alvarez *et al*. (2011). However they were lower than 11.3 g/L by *F. moniliforme* (Bilkay *et al*., 2010) and 15 g/L and 32.8 g/L reported by Rangaswamy (2012) and Omojasola and Benu (2016) on *Jatropha* seedcake using *F. moniliforme* and *A. terreus* respectively. The various differences in the GA yields may be attributed to the differences in the conditions of fermentation, substrates and fermenting organisms. *F. moniliforme* and *A. niger* that were used for fermentation in this study are highly cellulolytic (Dashtban *et al*., 2009) and efficient in the utilization of the cellulosic substrate. Physiological factors often determine the outcome of the fermentation process, and may influence the yield of GA (Kahlon and Malhotra, 1986; Karakoc and Aksoz, 2006).

In studying the effect of time on the GA yield, it was observed that GA production commenced on day one of fermentation (Figure 1). This correlates with the observation of Ates *et al*. (2006) and Lale and Gadre (2010) who also recorded GA yields within the first twenty-four hours of fermentation. However, it is contrary to some findings that GA was recorded about forty-six hours after the commencement of fermentation following nitrogen depletion in the medium (Escamilla *et al*., 2000; Rodrigues *et al*., 2009; Rios-Iribe *et al*., 2011). The early onset of GA production may be attributed to the small amounts of protein in the OA substrate leading to its speedy exhaustion (Shukla *et al.*, 2005; Sleem, 2013). GA production peaked on day six for *F. moniliforme* and day five for *A. niger* (Figure 1). Peak GA yields have reported between days 4-8 for *F. moniliforme* (Kumar and Lonsane, 1990; Meleigy and Khalaf, 2009; Rangaswamy, 2012; Omojasola and Benu, 2016) and days 6-12 for *A. niger* (Bilkay *et al*., 2010).

It was observed that the GA yield was highest at pH 5.0 and 5.5 for *F. moniliforme* and *A. niger* respectively (Figure 2). This agrees with the works of other researchers who also observed peaks in GA yields at similar pH ranges (Qian *et al*., 1994; Shukla *et al*., 2005; Bilkay *et al.*, 2010; Kobomoje *et al*., 2013). Borrow *et al*. (1964) observed that GA production decreased when pH was outside the range of 3.0-5.5 in a stirred culture. The pH is considered one of the most important factors on biomass and yield because of its great influence on the physiological activities of the fermenting organisms (Sleem, 2013).

Maximum GA yields were observed when 2% inoculum of both *F. moniliforme* and *A. niger* were used for the fermentations (Figure 3). However, statistical

comparisons of means showed no significant differences ($p<0.05$) between the peak yields obtained when 1.5% and 2% inoculum of *F. moniliforme*; and 1% and 1.5% inoculum of *A. niger* were used. The use of sufficient inoculum for fermentation purposes is important as inadequate inoculum may lead to reduction in biomass and GA production, while excessive inoculum can also lead to low yields resulting from overpopulation and subsequent competition for available nutrients by the fungi (Omojasola and Benu, 2016).

The highest GA yields were recorded at 3 g substrate concentration for both fungi (Figure 4). A balanced amount of substrate is necessary for a good GA production. GA is a secondary metabolite produced in the log/stationary phase of growth. Low glucose concentration (< 4%) is required for GA production and maintenance of biomass in the production phase (Kumar and Lonsane, 1989), meanwhile GA biosynthesis is suppressed by high amounts (>20%) of glucose (Bruckner, 1992).

Supplementation of the fermentation media with different concentrations of copper sulphate appeared to show negligible and no significant difference ($p<0.05$) in GA yield (Table 3). This observation differs from the report of Arakaki *et al.* (2011), who found improved biomass production in yeasts grown under submerged fermentation when $CuSO_4$ was incorporated. The inability of the supplement to increase GA production may be because it is not essential in the normal physiological activities of both *F. moniliforme* and *A. niger*, especially with respect to the production of GA.

After the optimization experiment, the peak production of GA by *F. moniliforme* and *A. niger,* was observed on day six of the fermentation (Table 4). This is in tandem with Meleigy and Khalaf (2009) and Omojasola and Benu (2016) who reported GA production by *F. moniliforme* and *A. niger* respectively to be optimum on day six of the fermentation. In contrast to the pre-optimized fermentations, *F. moniliforme* showed higher productivity. Generally, the optimization of fermentation conditions provided significantly ($p<0.05$) higher yields compared to pre-optimization. The optimized yields obtained on OA by *F. moniliforme* and *A. niger* were 9.39 g/L and 7.42 g/L respectively; corresponding to a 69.8% and 17.78% increase respectively. These results are consistent favorably with those of Ates *et al*. (2006) who reported GA yields 13.0 mg/100 mL and 16.0 mg/100 mL by *G. fujikuroi* and *A. niger* respectively, which increased to 17.5 mg/100 mL and 20.5 mg/100 mL respectively after the optimization using silicone oil. The % GA recovery from the fermentation medium was 5.6% equaling 56.0 mg of GA per g of OA substrate fermented.

5. Conclusion

The production of GA through submerged fermentation of OA by *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 is described in this study. The results indicate that orange albedo (OA) is a cheap and readily available substrate for the production of GA. Yields of GA produced by *F. moniliforme* and *A. niger* were 9.39 g/L and 7.42 g/L respectively. However, in the optimization experiments, yields of 15.97 g/L and 10.74 g/L were produced by *F. moniliforme* and *A. niger* respectively, which are among the highest reported in literature demonstrating that the OA substrate can be used for the efficient production of GA. This indicates that high yields are dependent on the use of appropriate physical and nutritional conditions during fermentation. It can be concluded that *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 can be employed on a large scale in the production of this valuable acid using the agro-waste of OA, which will also help reduce the environmental pollution.

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