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ITACONIC ACID PRODUCTION FROM BANANA PEEL WASTE BY SUBMERGED FERMENTATION USING *Aspergillus niger* and *Aspergillus terreus*

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ABSTRACT

Itaconic acid (IA) a promising organic acid with a wide range of industrial and biomedical applications was produced in this study by submerged fermentation using two fungi; *Aspergillus terreus* ATCC 20542 and *Aspergillus niger* CBS 513.88. An agro-based waste, banana peel (BP) (*Musa sapientum*) served as substrate. The proximate analysis of the BP was also determined. The BP substrate was pretreated by alkali hydrolysis and used as substrate in a mineral salts medium in submerged fermentation. Fermentation conditions were: pH 5.0; 1% inoculum; 10% substrate concentration; temperature 25±2°C for 7 days. Carboxylmethylcellulose (CMC) was used as control. Fermentation conditions were varied to optimize the yield of IA. The parameters which gave the highest yields were thereafter combined in a single fermentation. Results of the proximate analysis of the BP showed carbohydrate 57.80%, crude protein 12.72%, crude fibre 8.37%, crude fat 3.80%, ash 4.02%, and moisture 13.29%. The yields of IA produced by *A. terreus* and *A. niger* were 65.3±0.20 g/L and 72.5±0.17 g/L respectively on Day 5 of fermentation. After optimization, IA yield by *A. terreus* and *A. niger* was 109.43 ±0.24 g/L and 137.2 ±0.37 g/L respectively on Day 4. These results support the use of BP as a suitable substrate for IA production.
Keywords: Itaconic acid, Banana peel, *Aspergillus terreus*, *Aspergillus niger*, fermentation

INTRODUCTION

Itaconic acid (IUPAC: 2-Methylenebutanedioic acid) (synonyms: 2-Methylenesuccinic acid, 2-propene-1,2.dicaboxylic acid, Propylenedicarboxylic acid) has the chemical formula $C_5H_6O_4$; molecular weight 130.1; melting point of 167-168^o C; density of 1.633 g/L at 20°C; is stable at acidic, neutral and middle basic conditions at moderate temperature (Tate, 1970; Chandragiri and Sastry, 2011; Johann, 2012). IA is a dicarboxylic acid, while crystalline, soluble in water, ethanol and acetone and is one of the "top-12" promising organic acids assigned by the US Department of Energy which can be used for several specialties and 'green plastic' (Johann, 2012; Hajian and Yusoff, 2015). IA is about twice as acidic as acrylic acid, more reactive than maleic and fumaric acids which are potential monomeric substitutes (Fink, 2013). It also readily forms a range of metallic salts and diesters such as dimethyl itaconate and di-n-butyl itaconate, both of which are available commercially (Antia *et al*., 2011).

IA was reportedly produced first by *Aspergillus itaconicus*. It was discovered later that other species of *Aspergillus* such as *Aspergillus terreus* and *Aspergillus niger* also can produce IA (Meena *et al*., 2010). It is asserted that the genetic manipulation of *A. niger* involving the insertion of *CadA* gene could even improve IA production

further (Li *et al.*, 2012). Apart from the *Aspergillus*, *Ustilago maydis* and *Pseudozyma antartica* have also been discovered to produce IA (Levinson *et al*., 2006). Various agro-based wastes have been employed in the fermentative production of IA with varying degrees of success. These include corn starch (Yahiro *et al*., 1997); cane molasses (Meena *et al*., 2010); *Jatropha* seedcake (El-Iman *et al*., 2013); sweet potato peel (Omojasola and Adeniran, 2014); groundnut shell, rice bran, orange pulp, sugarcane bagasse (Rafi *et al*., 2014).

Driven by global concerns over diminishing stocks of fossil fuels and the need to step up the production of 'environmentally friendly green chemicals,' the global market for IA is forecast to reach over US$398.7 million by 2017. (Hajian and Yusoff, 2015). USA represents the largest market for IA globally, and IA one of the renewable chemicals are expected to replace about 5% of all petrochemicals by 2025 (GIA, 2011). Despite its enormous potential, the high cost of producing IA has restricted the use of IA to a few application areas.

IA has been applied in the manufacture a wide range of products including industrial adhesives, detergents, high-strength plastic fiberglass, coatings, artificial jewellery, specialty lenses, synthetic rubber, chemical fibres, pesticides and shampoos (Kin *et al*., 1998; Hajian and Yusoff, 2015). Applications of IA have also been extended to the biomedical fields, including the preparation of Glass Ionomer Cement (GIC) used in restorative dentistry (Nagaraja and Kishore, 2005) and N-vinylcaprolactam, a co-polymer of IA also used in clinical dentistry (Okabe *et al*., 2009).

Agricultural wastes are generated massively from various agricultural operations. This agro-waste constitutes a significant proportion of worldwide agricultural productivity. Although, the accumulation of these agro-wastes over the years results in deterioration of the environment and causes various hazards, it can be bioconverted to yield value-added products such as important chemicals and biofuels. Most of these agro-wastes are made up of complex carbohydrates which could be broken down by microorganisms into fermentable simple sugars for the production of various organic acids.

Generally, agro-residues and forest products are considered the best sources of cheap substrates (Nandini *et al*., 2014). Banana (*Musa sapientum*) is one of the most popular fruits in the world. A member of the genus *Musa* (parts of the family Musaceae), it is considered to be derived from the wild species of *Musa acuminata* and *Musa balbisiana* (Rungnapa *et al*., 2007). Bananas are rich sources of carbohydrates and potassium while they are low in protein (Happi *et al*., 2007). The fruit selected for use in this study produces wastes that are available in bulk. The world production of banana was estimated at 99 million tonnes, although this figure is an approximation because a large percentage of the world's banana production comes from subsistence farming on relatively small plots of land and gardens where statistics are lacking (FAO, 2008).With the large production of the fruit comes the waste disposal problem. The disposal of banana peel is a matter of concern for fruit-processing industries. Emphasis is laid only on banana fruits harnessed and marketed fresh or as processed juice, while fruit peel produced in great quantities during the process are mainly discarded as waste (Ezejiofor *et al*., 2011). The method of disposal can constitute an environmental or ecological hazard. Instead of discarding these fruit wastes, they can be utilized for as cheap substrates for fermentation purposes, where they will serve for the production of other value added products.

The objectives of this study were to determine the suitability of banana peel as a cellulosic substrate for the fermentative production of IA; to determine the ability of *Aspergillus niger* and *Aspergillus terreus* to utilize banana peel as

substrate and to determine the optimal conditions for IA production.

MATERIALS AND METHODS

Sample Collection and Identification

Bananas (*Musa sapientum*) samples were purchased from Ipata Market, Ilorin, Kwara State. Nigeria. They were identified at the Herbarium of the Department of Plant Biology, University of Ilorin.

Test Organisms and Preparation of Spore Suspensions

The organisms used in this study were *Aspergillus niger* (CBS 513.88) *Aspergillus terreus* (ATCC 20542) collected from the Microbial Culture Collection of the Department of Microbiology, University of Ilorin, Nigeria The organisms were maintained on Potato Dextrose Agar (PDA) and stored at 4°C until use. For the preparation of the spore suspension, 10 ml of sterile water was added to 5-day old culture slants of the fungi, the surface of the culture was scratched with a sterilized loop and agitated thoroughly at 250 rpm on a shaker to suspend the spores (Omojasola and Jilani, 2009). The number of the spores were counted by using the improved Neubauer Haemocytometer (Weber England B.S 748) and adjusted to approximately 2.4 x 10^6 CFU/ml and 3.1 x10^6 CFU/ml of *Aspergillus niger* and *Aspergillus terreus* respectively which were used as inocula throughout the study.

Substrate Pretreatment

The banana fruits were washed with clean water to remove dirt; after which they were peeled. The peel was then air-dried for 7 days and then pretreated using the alkali hydrolysis method (Omojasola and Jilani, 2009). The BP was pounded into small bits with a porcelain mortar and pestle; and then autoclaved for 1 h at 121°C with 5% (w/v) NaOH for delignification. The autoclaved samples was filtered, washed thoroughly with distilled water and neutralized with 1 M HCl. It was washed again with distilled water and dried at 70°C for 1 h. The treated sample was thereafter ground with an electric blender (Binatone BLG 699) to form a fine powder stored in a cool and dry place to avoid uptake of moisture (Nandini *et al*., 2014). Carboxymethyl cellulose (CMC) was used as control.

Proximate analysis of banana peel

The proximate analysis of the banana peel was determined. The parameters analyzed were dry matter content; moisture content; ash; crude protein; total sugar; fat content; total carbohydrate and crude fibre (AOAC, 2002).

Submerged Fermentation

Mary Mandel's Mineral Salts Medium was used for fermentation (Li *et al.*, 2011). The medium was composed of 3 g $NaNO_3$, 0.8 g $MgSO_4$, 1 g KH_2PO_4, 0.01g $FeSO_4$ and 0.5g KCl in 1 litre distilled water. Ten grammes of the BP substrate were mixed with 100 ml of the prepared medium in 250 ml Erlenmeyer flask. These flasks were sterilized in the autoclave at 121°C for 15 minutes at 15 psi. It was then inoculated with 2.4×10^6 and 3.1×10^6 spores/ml for *A. niger* and *A. terreus* separately. These were incubated at 25 ±2°C on a rotary shaker at 400 rpm (Meena *et al*., 2010). IA yield was estimated for at 24 hour intervals using bromination method (Tsai *et al.*, 2001, Lies, 2007).

Optimization for IA Production

Different fermentation parameters were varied in order to increase the yield efficiency of BP under optimal conditions for IA production. Fermentation conditions varied were: pH (3.0-6.0); Substrate concentration (4.0-10.0 g); inoculum size (1-4 %). These conditions were varied by changing one variable while keeping the others constant. Optimal conditions were later combined in a single fermentation.

Data Analysis

The statistical analysis of the data was done using the Statistical Package for Social Sciences for Windows version 15.0 (SPSS, 2004). All data are expressed as means of triplicates ± SEM or SD and values of $p < 0.05$ were considered significant, where 'n' represented independent experiments.

RESULTS

Proximate Analysis

The data from this study confirm the presence of nutrients which would serve as suitable substrate for the fermentative production of IA using *A. terreus* ATCC 20542 and *A. niger* CBS 513.88 The proximate analysis of the banana peel revealed that it contained carbohydrate 57.80%, crude protein 12.72%, crude fibre 8.37%, crude fat 3.80%, ash 4.02% and 13.29% moisture. The sugar content in the banana sample was analysed as glucose 7.86 mg/L and sucrose 9.40 mg/L. These nutrients serve as carbon, nitrogen and energy sources for IA production.

Fermentation

The pre-optimization fermentation of BP yielded 65.3±0.20 and 72.5±0.17 g/L by *Aspergillus niger* and *Aspergillus terreus* respectively after 5 days of fermentation (Table 1). While the IA yield by *A. terreus* was higher than that of *A. niger*, it was not significantly different at ($p<0.05$). These yields were significantly higher ($p<0.05$) than 15.7 ± 0.24 and 24.2 ± 0.26 g/L produced by the fungi respectively by from the CMC control. It was observed that IA yields increased steadily up to Day 5, after which the yield decreased sharply until the end of the fermentation. It was also observed that IA yields of *A. terreus* were higher than *A. niger* (Table 1) but not significantly higher ($p<0.05$).

Table 1: Pre-optimization production of Itaconic acid by the fermentation of Banana Peel by *Aspergillus niger* and *Aspergillus terreus*

Fermentation time (Days)	Quantity of itaconic acid produced (g/L)			
	Aspergillus niger		*Aspergillus terreus*	
	BP	CMC	BP	CMC
1	18.9 ± 0.17^a	0.3 ± 0.03^b	20.2 ± 0.15^a	0.8 ± 0.03^b
2	26.7 ± 0.29^a	4.4 ± 0.18^b	35.1 ± 0.17^a	9.6 ± 0.03^b
3	31.9 ± 0.20^a	6.2 ± 0.23^b	42.1 ± 0.12^a	11.2 ± 0.17^b
4	42.9 ± 0.23^a	6.7 ± 0.32^b	54.9 ± 0.35^a	15.2 ± 0.32^b
5	65.3 ± 0.20^a	15.7 ± 0.24^b	72.5 ± 0.17^a	24.2 ± 0.26^b
6	43.1 ± 0.23^a	13.7 ± 0.26^b	49.1 ± 0.17^a	19.2 ± 0.40^b
7	29.5 ± 0.20^a	8.7 ± 0.46^b	28.1 ± 0.18^a	12.4 ± 0.20^b

Key: BP: Banana peel, CMC: Carboxymethycellulose, Fermentation conditions: substrate concentration: 10 g; pH: 5.0; Inocula size: 1 %; Time: 7 days.Values presented are Means ± SEM; values with different superscript in a row are significantly different at $p < 0.05$.

Optimization of Fermentation

The results of varying the substrate concentration for IA product optimization revealed maximum IA yield of 163.2 g/L and 165.7 g/L by *A. niger* and *A. terreus* respectively at 4.0 g substrate concentration (Fig. 1a, Fig. 1b). The highest IA yield was observed on Day 5 of fermentation for both organisms after which a decline in IA yield was recorded.

When the pH of the fermentation was varied between 3.0-6.0, maximum IA yields of 64.0±0.74 g/L and 73.1±0.35 g/L by *A. niger* and *A. terreus* respectively were recorded on Day 5 at pH 5.0

after which the IA production declined until the end of the fermentation (Fig. 2a, Fig. 2b).

Inocula size variation recorded maximum IA yield of 71.8±0.27 g/L and 92.4±0.15 g/L by *A. niger* and *A. terreus* respectively at 3% inoculum on Day 5 of fermentation after which decline in yields were observed until the end of fermentation (Fig. 3a, Fig 3b).

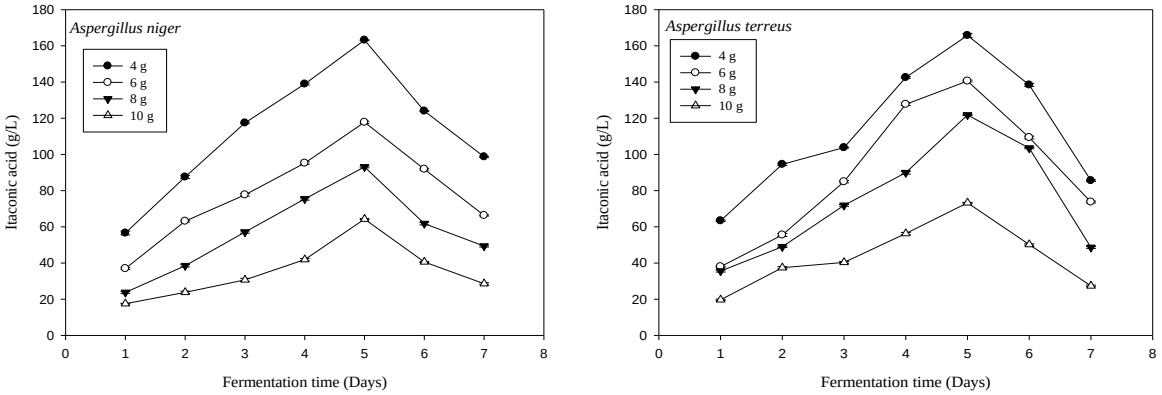

Figure 1a: Effect of varying substrate concentration on itaconic acid production by (a) *Aspergillus niger* and (b) *Aspergillus terreus* using banana peel

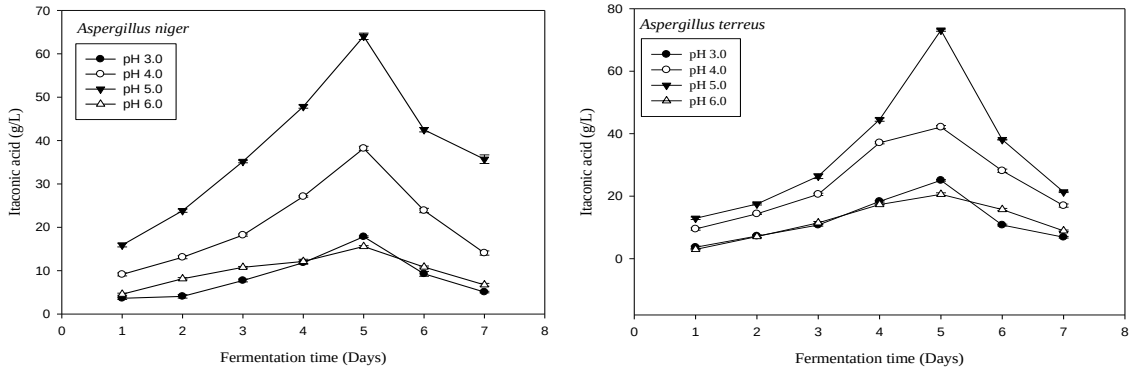

Figure 2a: Effect of varying pH on itaconic acid production by (a) *Aspergillus niger* and (b) *Aspergillus terreus* using banana peel

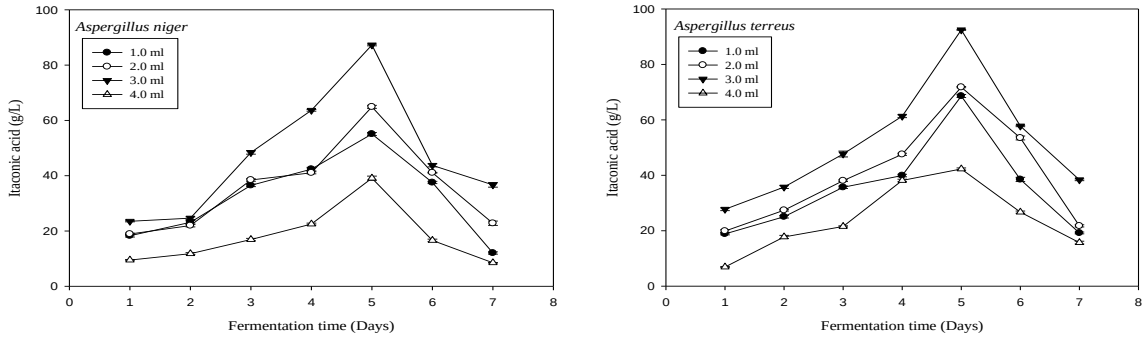

Figure 3a: Effect of varying inoculum size on itaconic acid production by (a) *Aspergillus niger* and (b) *Aspergillus terreus* using banana peel

The optimized parameters (substrate concentration 4g, inoculum size 3%, and pH 5.0) were combined to produce itaconic acid. The results showed increments in the amount of itaconic acid produced to 132.9g/L and 157.5 g/L by *A. niger* and *A. terreus* respectively (Table 2).

This represents 164.4% and 184.3% increase in the final fermentation where all the optimized parameters were combined.

Table 2: The post-optimization fermentation of banana peel by *Aspergillus niger* and *Aspergillus terreus* for the production of itaconic acid

Fermentation time (days)	Quantity of itaconic acid produced (g/L)			
	Aspergillus niger		*Aspergillus terreus*	
	BP	CMC	BP	CMC
1	97.73±0.35[a]	10.5±0.30[b]	104.2±0.23[a]	15.4±0.17[b]
2	102.23±0.24[a]	13.6±0.26[b]	115.8±0.26[a]	16.8±0.31[b]
3	109.43±0.24[a]	14.3±0.37[b]	137.2±0.37[a]	19.2±0.40[b]
4	132.9±0.61[a]	27.7±0.27[b]	157.5±0.17[a]	34.3±0.63[b]
5	117.7±0.27[a]	17.3±0.23[b]	139.1±0.26[a]	22.9±0.40[b]

BP: Banana peel, CMC: Carboxymethycellulose; Fermentation parameters: substrate concentration: 4 g; pH:5.0; Inoculum size: 3.0% values presented are Means ± SEM; values with different superscript in a row are significantly different at pH < 0.05; n=3.

DISCUSSION

The carbohydrate content of 57.80% reported in this study exceeds the observation of Romelle *et al.* (2016) who reported 43.40%. This is an indication that banana peel is high in carbohydrates and provided a good carbon source for IA production. Organic acid production requires relatively high amounts of carbohydrates which not only serve as sources of energy for microbial growth, but also as substrate for the fermentation process (Omojasola and Adeniran, 2014). The BP also contained 12.72% crude protein which served as a source of nitrogen for the growth of the fermenting fungi. Many studies have linked increased organic acid yields with high nitrogen content of the substrate (Betiku *et al*., 2016; Omojasola and Okwechime, 2017). However, some organisms such as *Pseudozyma antartica* can produce IA from glucose and other sugars under nitrogen-limited growth conditions (Hajian and Yusoff, 2015). Banana peel contains appreciable level of lignocellulosic materials and other components such as carbohydrates, vitamins, bioactive compounds and minerals which qualify it for various bioconversion processes (Johann *et al*., 2007; Dzomeku *et al*., 2007).

The results of this study further reinforce earlier observations confirming the suitability of agro-industrial residues as fermentable substrates for organic acid production (Ncube *et al*., 2012; Hajian and Yusoff, 2015). The IA yields in this study are among the highest yields reported in literature. Omojasola and Adeniran (2014) reported yields of 112.67 g/L and 115.67 g/L with sweet potato peel by *A. niger* and *A. terreus* respectively. El-Imam *et al.* (2013) produced 48.70 g/L with *Jatropha curcas* seedcake with *A. terreus*. Rao *et al.* (2007) recorded 24.46 g/L also from *Jatropha* seedcake. The variation in the IA yields from the different substrates maybe due to the differences in the composition of the substrates, fermenting organisms and conditions employed in the fermentation.

Generally, it was observed that *A. terreus* produced higher yields of IA than *A. niger* and this was observed in all the fermentations (Table 1, Table 2, Figs 1-3). *A. terreus* is reported to be a natural producer of IA with yields as high as 115 g/L (Okabe *et al.*, 2009; Kuenz *et al*., 2012; Steiger *et al.*, 2013; Van der Straat *et al*., 2014). *A. niger* is a highly versatile synthetic fungus involved in the production of various organic acids including citric, gluconic, oxalic, malic, acetic, propionic, isobutyric, tartaric, lactic, fumaric and ascorbic acids (Liaud *et al.*, 2014).

The parameters of fermentation were varied to optimize the yield of IA. Most often, changes in fermentation conditions usually have a great influence on the production ability of a microbial strain. With the variation of substrate concentration, 4 g yielded maximum product of 163.2 g/L and 165.7 g/L by *A. niger* and *A. terreus* respectively on Day 5 of fermentation (Figure 1a and 1b). Meena *et al*. (2010) also observed maximum production of IA at 120 h of fermentation although with much lower yields (maximum yield was 8.10 g/L) and this trend was observed when *A. niger, A. nidulans, A. flavus* (6.3, 5.6 and 4.8 g/L respectively) were used as fermenting organisms. Rafi *et al*. (2012) who observed highest IA yield of 28.88 g/L at 4% (w/v) substrate concentration after which a decrease in yield with increase in substrate concentration was observed. This therefore suggests that increase in substrate concentration does not necessarily improve the fermentation ability of the organisms.

When the pH of the medium was varied, pH 5.0 yielded 64.0 g/L and 73.1 g/L by *A. niger* and *A. terreus* respectively (Figure 2a and 2b). The pH is one of the most important parameters that affect IA production by fermentation. The IA yield was observed to be low at pH below 5.0. This contradicts some earlier reports which observed maximum IA yields at pH 3.0-3.5 (Rao *et al.*, 2007; Meena *et al.*, 2010; Rafi *et al.*, 2014). El-Imam *et al.* (2013) and Omojasola and Adeniran (2014) reported slightly higher and lower pH of 2.5 and 4.0 as optimum pH for maximum IA yield. The data in this present study is similar to Vassilev *et al.* (2013) who recorded IA yield of 44.0 g/L at pH 5.5 from olive and beet wastes. The role of the internal and external proton concentration in microbial metabolism is well established. While microorganisms possess the mechanism to maintain intracellular pH at relatively levels, the internal pH may depend on the pH of the external environment. This in turn affects the growth and product formation by influencing nutrient uptake, metabolic pathways and other physiological activities (Meena *et al.*, 2010; Chandragiri and Sastry, 2011). It has been suggested that a lack of pH control may have strong adverse effect on IA leading to low IA yields (Meena *et al*., 2010; Boruta and Bizukojc, 2017).

Maximum IA in this study was recorded at 3% inoculum size yielding 87.3 g/L and 92.4 g/L by *A. niger* and *A. terreus* respectively (Figure 3a and 3b). The amount of inoculum is important because low amounts may give inadequate biomass and lead to a reduction in the IA yield, while excessive inoculum may lead to competition for nutrients (Chandragiri and Sastry, 2011). Although El Imam *et al.* (2013) and Omojasola and Adeniran (2014) reported a higher optimum inoculum size of 5 ml using *U. maydis*; *A. terreus* and *A. niger* respectively. Meena *et al.* (2010) reported a much higher inoculum size of 10% using different species of *Aspergillus.* It was further observed that increase in the inoculum size to 4% led to a decrease in IA yield (Fig. 3a, Fig. 3b). This may be attributed to overcrowding leading to competition among the fermenting organisms for nutrients.

When the conditions which gave optimized yields were combined in a single fermentation, the yields obtained by *A. niger* and *A. terreus* in the optimized fermentation were 132.9 g/L (103.5% increase) and 157.5 g/L (117.2% increase) respectively (Table 2). This represents more than double of the initial pre-optimization IA yields. While the IA yield was highest for *A. terreus*, it was not significantly different ($p<0.05$) from the yield of *A. niger*. In addition, the percentage increase of IA yield was higher in *A. terreus*. *A terreus* is reported to be a natural producer and the most frequently used commercial producer of IA. (Rao *et al.*, 2007).

CONCLUSION

In this study, IA was produced via submerged fermentation using banana peel substrate by two species of *Aspergillus*; *A. niger* (CBS 513.88) and

A. terreus (ATCC 20542). The results show that banana peel is a suitable substrate for the fermentative production of IA. This study has shown that all the fermentation parameters have effect on the IA yield and variation of these parameters during fermentation had a significant influence on the IA production levels. This research was able to provide information on the various physicochemical parameters that can give optimum yield of IA using banana peel waste as substrate when fermented with *A. niger* and *A. terreus*. This was demonstrated when IA yield increased by 103.5% by *A. niger* and 117.2% by *A. terreus* after optimization. The results of this study also confirmed *A. terreus* as the better IA producing organism. Therefore the potential of banana peel can be explored in the industrial production of IA.

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