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Submerged Fermentation of *Jatropha curcas* Seedcake in Production of Itaconic Acid by *Aspergillus niger* and *Aspergillus terreus*

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ABSTRACT

In this study, two fungi: *Aspergillus niger* (ATCC 16404) and *Aspergillus terreus* (ATCC 20542) were used to ferment *Jatropha* seed cake (JSC) by submerged fermentation for the production of itaconic acid. The physico-chemical analysis of JSC was determined. JSC was shelled, defatted and used as substrate in mineral salts media and the inocula of *A. niger* and *A. terreus* for eleven days at pH 5.42, 29±2°C, 10% substrate and 2 ml inocula (2.6 x 10^8 spores/ml *A. niger*) (3.7 x 10^8 spores/ml *A. terreus*). Carboxylmethylcellulose (CMC) was used as control. Optimization experiments were conducted by varying fermentation parameters. Results of the physico-chemical analysis revealed carbohydrate 16.23%; protein 29.3%; fibre 10.42%; fat 32.13%; ash 5.75% and moisture 6.15%. Itaconic acid yields of 154.0 g L^{-1} and 208.0 g L^{-1} were produced by *A. niger* and *A. terreus* respectively. Results of the optimization showed higher yields of itaconic acid by *A. niger* to 209.0 g L^{-1} at pH 2.5, 3 ml inocula, 25% substrate, at 26±2°C on Day 8 and *A. terreus* yielded of 218.0 g L^{-1} at pH 3.5, 4ml inocula, 25% substrate at 29±2° C on Day 8. These results support potential of JSC for industrial production of itaconic acid.

INTRODUCTION

Large amount of wastes are generated every year from the processing of agricultural raw materials. Most of these wastes are used as animal feed or burned as alternative for elimination. However, such wastes usually have a composition rich in sugars, minerals and proteins, and therefore, they should not be considered “wastes” but raw materials for other industrial processes. Agro-industrial wastes have been demonstrated to be suitable substrates via fermentation in the production of organic acids and enzymes (Couto and Sanroman, 2006; Ncube *et al.* 2012). The presence of other nutrients aside the lignocellulosic residues in these wastes make them suitable for the rapid growth of microorganisms, especially fungi (Mussato *et al.*, 2012). Filamentous fungi are well known for their ability to degrade lignocellulosic biomass. In addition, they have the ability to convert the products of the biomass degradation into value added products such as various organic acids and biofuels (Liaud *et al.*, 2014).

Jatropha curcas Linn., a small shrub belonging to the family Euphorbiaceae, is known as 'physic nut' and 'purging nut' in English; 'purgeernoot' and 'schijtnoot' in Dutch; and 'hab el melluk' in Arabic (Sharma *et al.*, 2012). Native to Central and South America, it is also found in parts of Africa, South East Asia and India and is used traditionally as a hedge to protect fields and farmlands as it is not browsed by cattle or other animals (Belewu and Sam, 2010). The plant has various industrial and medicinal uses (Agbogidi *et al.,* 2013) and is gaining increasing importance as an emerging source of biodiesel which is leading to a rapid accumulation of the cake. Despite being nutrient-rich, *Jatropha* cake contains toxic and anti-nutrient components such as phorbol esters, trypsin inhibitors, lecithin, saponin and phytate and cannot be consumed by humans or animals so it is cheap and readily available (Phenugnuam and Suntornsuk, 2013).

Itaconic acid (IA) is an unsaturated dicarbonic acid with high potential as a chemical building block and can be used for a plethora of industrial products including resins, plastics, elastomers, carpet and book cover coatings, adhesives, high-strength enhanced fiberglass, artificial gems, synthetic glass with non linear characteristics and paints (Steiger *et al.,* 2013; Hajian and Yusoff, 2015). IA (IUPAC: 2-methylenebutanedioic acid) has the chemical formula $C_5H_6O_4$; has a melting point of 167-168^o C; density of 1.632; is stable at acidic, neutral and middle basic conditions at moderate temperature (Ramesh and Sastry, 2011; Johann, 2012). With a market price of about $2 per kg (Van der Straat *et al.,* 2014), IA is currently expensive, so alternative or cheaper substrates may make the production process more profitable. The global IA market was valued at $126.4m in 2014; however, driven by concerns over diminishing world stocks of fossil fuels, global warming issues and the need to step up manufacture of 'green chemicals', the IA market is projected to reach $204.6m by 2023 (TMR, 2015).

The objectives of this study were to determine the suitability of JSC for the fermentative production of IA using *A. niger* and *A. terreus*; and to determine the optimal conditions for its production.

MATERIALS AND METHODS

Plant material and Microorganisms

Jatropha curcas seeds were collected from the Department of Crop Production, Faculty of Agriculture, University of Ilorin, Kwara State, Nigeria. The test organisms *Aspergillus niger* (ATCC 16404) and *Aspergillus terreus* (ATCC 20542) were obtained from the Federal Institute of Industrial Research Oshodi (FIIRO) Nigeria. The cultures were maintained on potato dextrose agar slants and kept at 4°C prior to use. Fungal spore inocula were produced by suspending spores in sterile distilled water and adjusting approximately to 2.6 x 10^8 spores/ml for *A. niger* and 3.7 x 10^8 spores/ml for *A. terreus* by counting with the improved Neubauer Haemocytometer (Petruccioli *et al.,* 1999).

Substrate Pre-treatment

The JSC substrate was pre-treated using the method of Omojasola and Jilani (2009). The seeds were dried, crushed and defatted with petroleum ether and autoclaved for 1 h at 121° C with 5% (w/v) NaOH (20 ml g^{-1} of substrate) in separate flasks. The autoclaved materials were filtered through a sterile muslin cloth. Sample was thoroughly washed with water and neutralized with 1 M HCl, washed again with distilled water and dried at 70° C.

Physico-chemical analysis of JSC

The parameters analyzed were pH, total carbohydrates, crude fibre, crude protein, ash, fat, moisture content (AOAC, 1990).

Fermentation

The fermentation medium was Mary Mandel's Mineral Salts Medium (MSM) consisting of 3g $NaNO_3$, 1g KH_2PO_4, 0.8g $MgSO_4$, 0.01g $FeSO_4$, 0.5g KCl, were added to distilled water. The pH was adjusted using

a digital pH meter (Denver Model 20 pH/Conductivity meter) to 5.8 with 0.1N NaOH or HCl. Ten grams of JSC were added. The sterilized media were inoculated with 2 ml of 2.6 x 10^8 and 3.7 x 10^8 spores/ml of *A. niger* and *A. terreus* separately. Each flask was cultured on a rotary shaker (Gallenkamp, England) at 200 rpm; temperature 28±2°C. The samples were assayed for IA at 24 hour intervals using the UV spectrophotometer (Thermo Fisher Scientific. GENESYS 20 Model 4001-4) at 385 nm (Meena *et al.,* 2010).

Optimization for Itaconic Acid Production

The fermentation conditions were varied with a view to determine yield efficiency of JSC under optimal conditions for IA production. Fermentation conditions varied were: substrate concentration (5-25%); pH (0.5-3.5); time (1-12 days); and inocula size (3-6 ml). These conditions were varied changing one variable and keeping all others constant. Optimal conditions were later combined in a single fermentation to optimize the yield of the acids.

Data Analysis

Statistical significance was determined using 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.

RESULTS

Proximate analysis

The results of the physico-chemical analysis of JSC substrate were carbohydrate 26.23±0.62%; crude protein 49.3±0.02%; crude fibre 10.42±0.1%; ash 5.75±0.03%; fat 2.13±0.05% and moisture 6.15±0.13%.

Fermentation

The pre-optimization fermentation of JSC yielded the maximum amount of IA on Day 9 of fermentation. *A. niger* yielded 154 g L^{-1} while *A. terreus* yielded 208 g L^{-1} (Table 1). The yields from the JSC substrate were significantly higher ($p<0.05$) than the CMC control. The yield declined sharply by Day 10. It was also observed that the yield from *A. terreus* was higher than *A. niger*.

Optimization of fermentation

When the fermentation time was varied, IA production rose steadily and peaked at 110 g L^{-1} and 122 g L^{-1} on Day 8 by *A. niger* and *A. terreus* respectively after which it declined (Fig. 1). For substrate concentration, 25% w/v gave the maximum IA yield of 186 g L^{-1} and 189 g L^{-1} by *A. niger* and *A. terreus* respectively on Day 9 of fermentation (Fig. 2). pH 3.5 yielded 195 g L^{-1} by *A. niger* on Day 9, while pH 2.5 yielded 204 g L^{-1} by *A. terreus* on Day 5 (Fig. 3); inocula size variation recorded maximum IA yields at 3 ml with 146 g L^{-1} by *A. niger*, and 4 ml with 153 g L^{-1} by *A. terreus* (Fig. 4).

When the conditions that gave maximum IA yields were combined in a single fermentation, the highest yields were 209 g L^{-1} by *A. niger* and 218 g L^{-1} by *A. terreus* on Day 8 of fermentation.

Table 1: The fermentation of *Jatropha* seedcake by *Aspergillus niger* and *Aspergillus terreus* for the production of itaconic acid

Days	Quantity of Itaconic Acid produced (g L^{-1})			
	Aspergillus niger		*Aspergillus terreus*	
	JSC	CMC	JSC	CMC
0	0.0	0.0	0.0	0.0
1	44.5 ± 0.21^{b}	43.0 ± 0.49^{b}	47.0 ± 0.09^{b}	33.0 ± 0.10^{ab}
2	66.0 ± 0.31^{bc}	50.0 ± 0.43^{b}	98.0 ± 0.59^{cd}	56.0 ± 0.11^{bc}
3	71.0 ± 0.92^{c}	57.0 ± 0.47^{bc}	105.0 ± 0.40^{d}	68.0 ± 0.11^{bc}
4	78.0 ± 0.14^{c}	60.0 ± 0.14^{bc}	100.0 ± 0.59^{cd}	55.0 ± 0.11^{b}
5	82.0 ± 0.18^{c}	63.0 ± 0.08^{bc}	110.0 ± 0.17^{d}	57.0 ± 0.24^{bc}
6	124.0 ± 0.10^{de}	65.0 ± 0.50^{bc}	121.0 ± 0.32^{d}	46.0 ± 0.21^{b}
7	134.0 ± 0.89^{e}	73.0 ± 0.11^{c}	134.0 ± 0.17^{e}	55.0 ± 0.24^{b}
8	152.0 ± 0.34^{ef}	86.0 ± 0.31^{cd}	123.0 ± 0.33^{de}	63.0 ± 0.65^{bc}
9	154.0 ± 0.05^{ef}	111.0 ± 0.82^{d}	208.0 ± 0.14^{gh}	80.0 ± 0.20^{c}
10	109.0 ± 0.15^{d}	92.0 ± 0.22^{cd}	143.5 ± 0.02^{e}	43.0 ± 0.20^{b}

Key: JSC- Jatropha Seed Cake; CMC- Carboxy methylcellulose; Fermentation parameters: Substrate concentration 10%; pH 5.42; inocula size 2 ml; Temperature 29±2° C. Values presented are Mean±SD; Values with different superscripts are significantly different at $p<0.05$

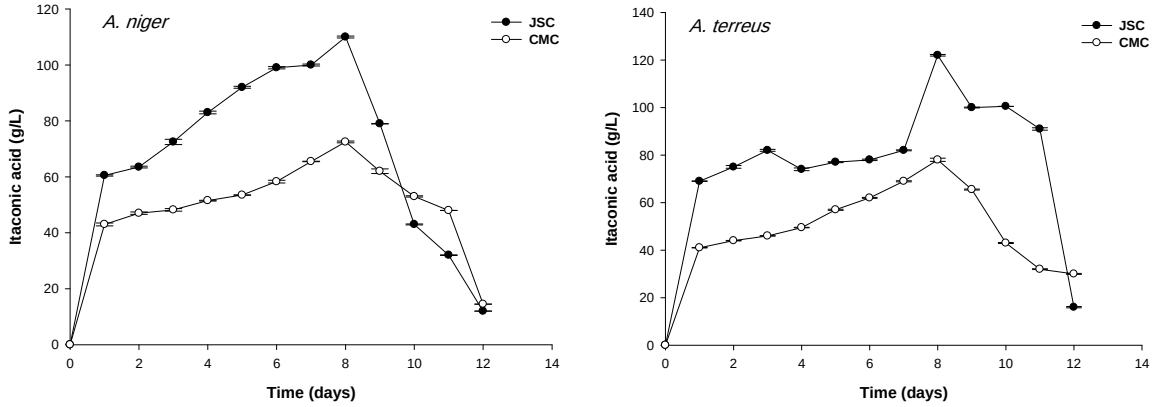

Fig. 1: Effect of varying fermentation time on itaconic acid production by *A. niger* and *A. terreus* using *Jatropha* seedcake as substrate

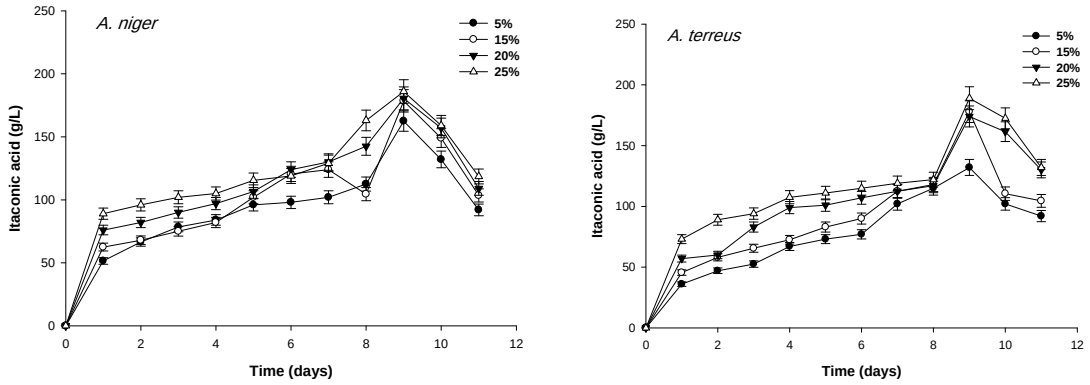

Fig. 2: Effect of varying substrate concentration on itaconic acid production by *A. niger* and *A. terreus* using *Jatropha* seedcake as substrate

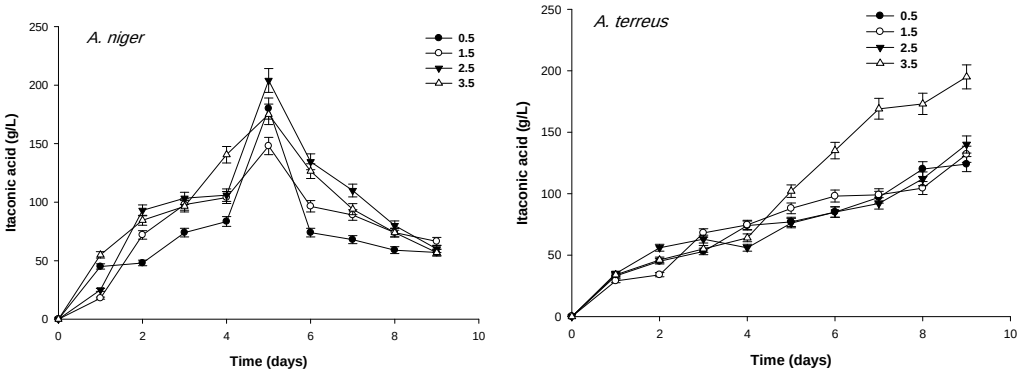

Fig. 3: Effect of varying pH on itaconic acid production by *A. niger* and *A. terreus* using *Jatropha* seedcake as substrate

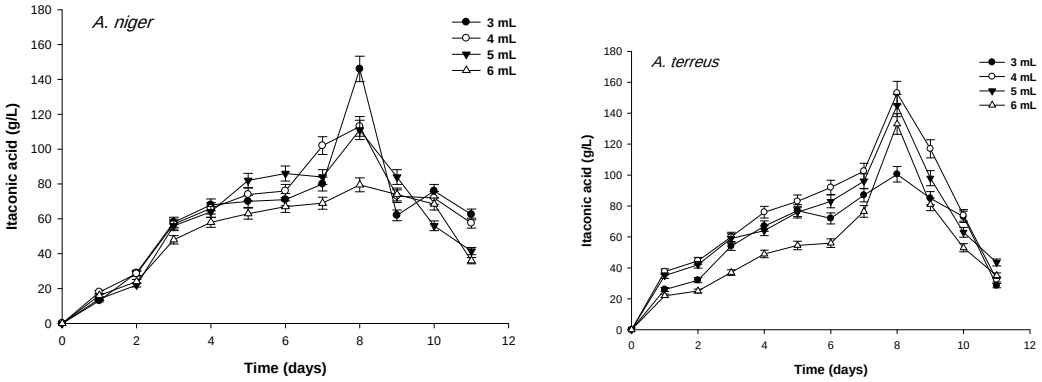

Fig. 4: Effect of varying inoculum size on itaconic acid production by *A. niger* and *A. terreus* using *Jatropha* seedcake as substrate

Table 3: Optimized production of itaconic acid by *Aspergillus niger* and *Aspergillus terreus* using *Jatropha curcas* seed cake

DAYS	Quantity of Itaconic Acid produced (g L^{-1})			
	Aspergillus niger		*Aspergillus terreus*	
	JSC	CMC	JSC	CMC
0	0	0	0	0
1	65.0 ± 0.01^{bc}	39.0 ± 0.03^{ab}	71.0 ± 0.50^{c}	32.0 ± 0.20^{ab}
2	68.0 ± 0.43^{bc}	42.0 ± 0.48^{b}	74.0 ± 0.43^{c}	44.0 ± 0.40^{b}
3	73.0 ± 0.04^{c}	60.0 ± 0.04^{bc}	78.0 ± 0.01^{c}	52.5 ± 0.15^{b}
4	82.0 ± 0.09^{c}	76.0 ± 0.159^{c}	93.0 ± 0.03^{cd}	65.0 ± 0.06^{bc}
5	96.0 ± 0.54^{cd}	89.0 ± 0.48^{cd}	102.0 ± 0.28^{d}	83.5 ± 0.40^{c}
6	113.0 ± 0.44^{d}	103.0 ± 0.45^{d}	125.5 ± 0.51^{de}	95.0 ± 0.45^{cd}
7	116.0 ± 0.51^{de}	109.0 ± 0.24^{d}	129.0 ± 0.42^{de}	103.0 ± 0.33^{d}
8	209.0 ± 0.09^{gh}	114.0 ± 0.44^{d}	218.0 ± 0.04^{gh}	108.0 ± 0.25^{d}
9	99.5 ± 0.02^{cd}	82.0 ± 0.08^{cd}	108.0 ± 0.37^{d}	65.5 ± 0.03^{bc}
10	78.0 ± 0.02^{c}	78.0 ± 0.003^{c}	74.0 ± 0.03^{c}	44.0 ± 0.06^{b}
11	69.0 ± 0.09^{bc}	74.0 ± 0.004^{c}	43.0 ± 0.03^{b}	19.0 ± 0.03^{a}

DISCUSSION

In this study, JSC was used as substrate for IA production. The proximate composition of the JSC substrate was 26.23% carbohydrate, 49.3% crude protein and 2.13% fat which is within similar range reported by Joshi and Khare (2011); Phenugnuam and Suntornsuk (2013). The protein and fat contents were lower than amounts reported by Belewu and Sam (2010) and Inekwe *et al.* (2012). This may be due to differences in oil extraction techniques and seed variety. The carbohydrate, protein and fat content were high enough to serve as good carbon and energy sources for IA production. The protein content of 49.3% would also serve as a good nitrogen source for microbial metabolism. Nitrogen is one of the most essential constituents of the medium for fungal fermentation and various studies have reported increased organic acid yields with high nitrogen content (Betiku *et al.*, 2016).

The results confirm that JSC is a good substrate for IA production confirming the observations of Couto and Sanroman (2006) and Ncube *et al.* (2012) about the suitability of agro-industrial as fermentable substrates. Many substrates have been used for the fermentative production of itaconic acid employing different organisms. These include corn starch (Yahiro *et al.*, 1997); cane molasses (Meena *et al.* 2010); sweet potato peel (Omojasola and Adeniran, 2014); rice bran, groundnut shell, orange pulp, groundnut oil cake and sugar cane bagasse (Rafi *et al.* 2014); sago starch hydrolysate (Dwiarti *et al.* 2007). JSC has also been used by some earlier workers (Rao *et al.* 2007; El Imam *et al.* 2013). Both workers employed *Aspergillus terreus* as fermenting organism recording peak IA yields of 24.46 g L^{-1} after 120 h and 48.70 g L^{-1} respectively. This study produced higher IA yields than of other workers. This may be due to the rich nutrient composition of the substrate which contained 2.13% fat, 49.3% protein and 26.23% carbohydrate. The IA yield from the JSC substrate was higher than the CMC control in all the fermentations (Table 1). This may also be due to the rich chemical composition of the JSC as compared to CMC a cellulose derivative, an anionic polysaccharide devoid of the other growth stimulating nutrients.

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, Figs 1-4). *A. terreus* is reported to be a natural producer of IA with yields as high as 115 g L^{-1} (Okabe *et al.*, 2009; Kuenz *et al.*, 2012; Steiger *et al.*, 2013; Omojasola and Adeniran, 2014; Van der Straat *et al.*, 2014). 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). *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).

Any change in culture conditions potentially alters the production ability of a microbial strain (Meena *et al.*, 2010). In studying the effect of variation of fermentation time, a daily increase in IA yield was observed as fermentation progressed to a maximum on Day 8 by both fermenting organisms (Fig. 1). This time was longer than 120 h reported by Meena *et al.* (2010) using *A. niger*, *A. flavus* and *A. nidulans* on molasses and 5 days by Rafi *et al.* (2009) using *U. maydis* on orange pulp and Omojasola and Adeniran (2014) using *A. niger* and *A. terreus* on sweet potato peel respectively. Simple sugars are the preferred substrates in fermentation; therefore any substrate with large amounts of such sugars will record earlier peaks of product yield than other cellulosic substrates which contain mostly complex carbohydrates.

When the substrate concentration was varied between 5-25%, the maximum IA yield by *A. terreus* 189 g L^{-1} and 186 g L^{-1} by *A niger* at 25%. (Figure 2). Attempts to increase the concentration beyond 25%

altered the consistency of the medium to semi solid. The fermenting organisms were able to efficiently utilize the JSC substrate. *Aspergillus* spp. are reported to possess all the components of the cellulase enzyme system (de Vries and Visser, 2001). In addition, they also produce a number of proteases which will assist in metabolizing the substrate (Castro and Sato, 2014; Sethi and Sahoo, 2016). Chandragiri and Sastry (2011) reported maximum yields at 35% glucose concentration using *U. maydis*; 40% using *Jatropha* seed cake by *A. terreus* (El Imam *et al*. 2013) and 10% using sweet potato peel using *A. niger* and *A. terreus* respectively.

An initial low pH has been suggested to enable the cells develop the biochemical machinery for IA production (Boruta and Bizukojc, 2017). The optimum pH that gave the highest IA yields were pH 2.5 producing 204 g L^{-1} and pH 3.5 producing 195 g L^{-1} by *A. niger* and *A. terreus* respectively (Figure 3). This is similar to the findings of many workers who put the range of optimum pH for IA production between pH 3.0-4.0 (Rao *et al*., 2007; Meena *et al*., 2010; Sudarkodi *et al*., 2012; Chandragiri and Sastry, 2011; El Imam *et al*., 2013; Rafi *et al*., 2014; Omojasola and Adeniran, 2014). Some workers have proposed pH 3.1 as the optimum for IA production (Kuenz *et al*., 2012; Hevekerl *et al*., 2014; Gao *et al*., 2014).

Maximum IA yield was recorded with 3 ml inoculum of *A. niger* producing 146 g L^{-1} and 4 ml inoculum of *A. terreus* which yielded 153 g L^{-1} (Figure 4). 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.*

The yields obtained by *A. niger* and *A. terreus* in the optimized fermentation were 209.0 g/L (35.71% increase) and 218.0 g/L (4.80% increase) respectively (Table 3). 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. niger*. This improvement in yield may be attributed to the highly synthetic nature of *A. niger* (Liaud *et al.* 2014). While *A. terreus* is reported to be the most frequently used commercial producer of IA, it is sensitive to conditions such as substrate impurities which may have a negative effect on the yield (Rao *et al.* 2007).

CONCLUSION

This study was conducted to evaluate the potentials of *Jatropha* seed cake as a substrate for the production of itaconic acid by *Aspergillus niger* and *Aspergillus terreus*. The results obtained have shown the potential of JSC as a good substrate for the production of itaconic acid. *A. terreus* was a better producer of itaconic acid than *A. niger*, with an optimum yield of 218.0 g L^{-1} and 209.0 g L^{-1} respectively. The yields obtained in this study are one of the highest that are available in literature and the data have supported the effective utilization of *Jatropha* seedcake for the fermentative production of itaconic acid. With the growing importance of itaconic acid, this study, therefore, provided a dual importance of utilizing an agro-industrial waste for the bioproduction of IA and a reducing of environmental pollution.

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