Itaconic Acid Production by *Aspergillus terreus* Using Pod of *Moringa oleifera* as Substrate in Solid State Fermentation

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Abstract: Itaconic acid was produced by *Aspergillus terreus* under solid-state fermentation using the pod of *Moringa oleifera* as substrate. Grinded *Moringa* pod was weighed into separate shake flasks containing salts and *Aspergillus terreus* (3.2x 10^4spores/ml) was added. The substrate was left to ferment for 5 days. Carboxylmethylcellulose (CMC) was used as a control substrate. Itaconic acid was assayed using standard procedures. Different fermentation parameters such as substrate concentration, inoculum size and incubation period were varied to determine the maximum production of Itaconic acid. Light spectrophotometer was used to measure the absorbance of the Itaconic acid concentration produced after each day of fermentation at 385nm. The maximum yield of Itaconic acid of 18.39 mg/ml was obtained with a substrate concentration of lOg, while 17.2mg/ml was produced with an inoculum size of 6ml (3.2x $I0^4$cfu/ml) and 13.91 mg/ml on day 5. It is concluded that the pod of *Moringa oleifera* can be used as a substrate for Itaconic acid production using *A. terreus* in solid state fermentation

Key words: Itaconic acid, *Moringa oleifera, Aspergillus terreus,* Fermentation

Introduction

Itaconic acid, also known as methyl succinic acid, is an unsaturated di-carboxylic organic acid (Wilke and Vorlop, 2001) with the formula $C_5O_4H_4$. Its primary application is in the polymer industry where it is employed as a co-monomer for the industrial synthesis of polyesters, plastics, and artificial glass; in the preparation of bioactive compounds in the agriculture, pharmacy, and medicine sectors; coatings, acrylic fibres, detergents, adhesives, thickeners, and binders, and a range of other products. The first reported biological source of Itaconic acid was *Aspergillus Itaconicus.* Shortly thereafter, it was discovered that *Aspergillus terreus* also produced Itaconic acid in higher quantities (Meena *et al.,* 2010). *Ustilago maydis* has also been reported to be used in the commercial production of Itaconic acid (Chandragiri and Sastry, 2011). Itaconic acid is a colourless crystalline carboxylic acid obtained by the fermentation of carbohydrates. The primary application of Itaconic acid is in the polymer industry, where it is employed as a co-monomer at a level of 1–5% for certain products. Its derivatives are used in medicine and cosmetic preparation. The market volume of Itaconic acid has been estimated to be about 15,000 metric tons per year and is expected to grow further, if the selling price is reduced (Willke and Vorlop 2001). A large variety of carbohydrate materials have been used for fermentative production of Itaconic acid. The best sources of carbon are sugars and to a lesser extent sugar alcohols, sucrose from cane and beet sugar. containing glucose, sucrose and maltose from hydrolysed starch are presently used in commercial production. In general, glucose, sucrose and xylose are preferred raw materials for Itaconic acid fermentation, which are known to be utilized efficiently by most *Aspergillus* spp. (Kautola *et al.*, 1990, Yahiro *et al.*, 1995; Zhaoling and Shu, 2000). Fungi such as the genus *Aspergillus* are mostly used for industrial production of organic acids like Itaconic acid (Billington, 1991). *Aspergilus terreus* is frequently applied for Itaconic acid production that's grown under phosphate-limited conditions (Roehr and Kubicek, 1996; Willke and Vorlop, 2001). There are reports of Itaconic acid production by the *Candida* mutant strain and *Rhodotorula* species. Tabuchi *et al.* (1981) isolated a strain, putatively recognized as a *Candida* that produced Itaconic acid at the 35% yield when grown under phosphate-limited conditions. William *et al.*(2006) reported the ability of *Pseudozyma Antarctica* to produce Itaconic acid from glucose and other sugars under nitrogen limited growth conditions. However some species of the plant pathogenic fungal genus *Ustilago* are known to produce Itaconic acid during fermentation (Willke and Vorlop, 2001).

Moringa oleifera is the most widely cultivated species and it is a native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan. It is commonly grown for human food, medicine, dye, fodder and water clarification. It has an impressive range of medicinal uses with high nutritional value. In addition to its compelling water purifying powers and high nutritive value. *Moringa oleifera* is very important for medicinal value. All parts of *Moringa* tree are edible and have long been consumed by human. Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act on cardiac and circulatory stimulants, possess antitumor

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antipyretic, antiepileptic, anti-inflammatory (Kumar and Lonsane,2001), anti-ulcer, anti-spasmodic, diuretic, anti-hypertensive, cholesterol lowering, antioxidant, antidiabetic, hepato protective, antibacterial and antifungal activities and are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in south Asia (Anwar *et al.*, 2007; Paliwal *et al.*, 2011).

Materials and Methods

Sample Collection and Identification

The pod of *Moringa oleifera* was collected at Shao, Kwara State and was identified at University of Ilorin, Department of Botany with voucher number UITH001/1011.Itwas taken to the laboratory immediately, the seeds were removed and discarded and was dried at 60 ^{0}C for 2hours in the oven. The pods were blended (Mikacchi, MK-999) into smooth powder and stored in a closed jar for use.

Collectionof Test Organism

Aspergillus terreus was obtained from Federal Institute of Industrial Research Oshodi (FIIRO) Lagos State, Nigeria. This culture was maintained on potato dextrose agar (PDA) slants and the pure culture was sub-cultured into PDA slant. The slants were labeled and kept at temperature of about 4 ^{0}C in the refrigerator for use.

Preparation of Spores Suspension

Aspergillus terreus was maintained on potato dextrose agar (PDA) slant for 7 days and stored at 4 ^{0}C in the refrigerator. For spore suspension preparation, 10ml of sterilized water was added to the 7days old culture slant of the fungi. At the end of the incubation period, the surface of the culture was scratched with sterilized loop and agitated thoroughly on a shaker to suspend the spores. The spores were counted using the Neubauer Haemocyto meter which gives 3.2 $\times 10^4$ spores per ml of *Aspergillus terreus* was used as innocula throughout the study.

Preparation of Fermentation Media

The powdered sample of varying concentrations of Moringa pod was weighed into the conical flasks. One millilitre (1ml) each of 0.004% ($CuSO_4$.) 0.0088% (KH_2PO_2.) 0.095% ($MgSO_4$.) and 0.25% (NH_4Cl) salts were measured into the flasks and were autoclaved at 121 ^{0}C for 15minutes. After cooling, 1ml (3.2 $\times 10^4$ spores per ml)of the spore suspension was added, it was incubated at 28±2^{0}C for 5days. Samples were taken every day for assay under sterile condition. Readings were taken in triplicates.

Preparation of Bromine Water

The bromine reagent used for assay of Itaconic acid was prepared according to Friedkin (1945). The bromine reagent was stored in a brown bottle in the refrigerator to slow the deterioration of bromine considerably.

Preparation of Salts

The mineral salts medium for Itaconic acid production were prepared according toMeena*et al.*(2010)by dissolving 0.004% ($CuSO_4$.) 0.0088% (KH_2PO_2.) 0.095% ($MgSO_4$.) and 0.25% (NH_4Cl)s in 100ml of distilled water and kept for use.

Solid-State Fermentation Process

Five grams of dried *Moringa oleifera* powder was dispensed into a 250ml Erlermeyer flask and moistened with 30ml of distilled water. The mouth of the flask was plugged with a cotton wool covered with aluminium foil and was sterilized at 121^{0}C for 15minutes in an autoclave, and then cooled, after which 1ml of fungal spore suspension was inoculated and was incubated at 28±2 ^{0}C for 5 days.

Effect of Varying Incubation Period

Ten grams (10g) of Moringa pod powder was weighed and 1ml of each of 0.004% ($CuSO_4$), 0.0088% (KH_2PO_2), 0.095% ($MgSO_4$), and 0.25% (NH_4Cl) salts were added each and was autoclaved for 15minutes at 121^{0}C. One millilitre (1ml) of the spore suspension was added and was incubated at 28±2^{0}C for 10days. After fermentation, each of the substrates was filtered and the absorbances of the filtrate were read at 385nm of a spectrophotometer (Visible spec LI-722). The absorbance was translated using the Itaconic standard curve. Readings were taken in triplicates.

Effect of Varying Substrate Concentration

The substrate concentrations were varied at 5g, 10g, 15g, 20g, and 25g of Moringa pod. One millilitre (1ml) of each of 0.004% ($CuSO_4$), 0.0088% (KH_2PO_2), 0.095% ($MgSO_4$), and 0.25% (NH_4Cl) salts were added each and was autoclaved for 15minutes at 121^{0}C. One millilitre (1ml) of the spore suspension was added and was incubated at 28±2^{0}C for 5days. After fermentation, each of the substrates was filtered and the absorbances of the filtrate were read at 385nm of a spectrophotometer (Visible spec LI-722). The absorbance was translated using the Itaconic standard curve. Readings were taken in triplicates.

Effect of Varying Inoculum Size

Spore suspension containing about 3.2 $\times 10^4$ spores per ml suspension of *Aspergillus terreus* was prepared according to the method of Pertuccioli *et al.* (1989) and used as inoculum for the fermentation process. Different inoculum sizes ranging from 2-10ml was used to inoculate the substrate. Assay for Itaconic acid was done as described above. Readings were in triplicates.

Estimation of Itaconic Acid Production

According to Friedkin (1945), a 3ml Becknan curvette was added 0.3ml of bromine reagent from a micro-pipette up to 1.0ml of sample and HCL at pH 1.2 to a volume of 3.0ml. After 15minutes at room temperature, the change in optical density was read at 385nm from a spectrophotometer.

Statistical Analysis

Results obtained were analysed using the SPSS version 16 software (IBM SPSS 16.0) and reported as Mean and Standard Deviation, samples were also analysed using one way ANOVA (Analysis of Variance).

Results

Effect of Varying Incubation Period on the Production of Itaconic Acid Using *Aspergillus terreus*

Ten grams (10g) of the substrate was fermented for 10days at28±2 ^{0}C, the maximum yield of 13.91mg/ml Itaconic acid was obtained on day 5.The minimum yield of 7.86 mg/ml Itaconic acid was obtained on day 10.

Effect of Varying Substrate Concentration on the Production of Itaconic Acid Using *Aspergillus terreus*

The maximum production of Itaconic acid obtained was 18.4mg/ml at 24 hours of fermentation with 10g of *Moringa* pod. The lowest production of Itaconic acid is 8.0mg/ml with 5g at 120 hours. There was a decline in the production of Itaconic acid on day 4 and 5.

Effects of Varying Inoculum Size on the Production of Itaconic Acid Using *Aspergillus terreus*

Maximum production of Itaconic acid obtained was 17.20mg/ml with 6ml of spore suspension of *Aspergillus terreus* on Day 3. The lowest production of Itaconic acid 7.0 mg/ml was obtained with 2ml of spore suspension on Day 1 after 24hours of fermentation.

All values are means±SD of amount of Itaconic acid produced in milligram released from 1 millitre of the substrate as derived from translation of absorbance values using the Itaconic standard curve. Values on the same row/column at different subscript are significantly different at p□0.05.

Table 1: Effect of Varying Incubation Period on Itaconic Acid Production by *Aspergillus terreus* Using *Moringa oleifera* pod

Fermentation(days)	Substrate	CMC
1	11.00±0.8^a	6.34±0.2^a
2	11.58±0.1^a	6.35±0.1^a
3	11.78±0.1^a	6.53±0.1^a
4	12.38±0.8^a	6.41±0.2^a
5	13.91±2.7^b	7.42±0.1^b
6	13.36±1.3^b	7.89±0.2^c
7	12.18±1.8^b	7.17±0.1^b
8	11.31±1,0^b	6,93±0.2^a
9	10.60±0.4^b	6.93±0.1^a
10	7.86±1.2^b	6.51±0.3^a

Note: All values are means±SD of amount of Itaconic acid produced in milligram released from 1 millitre of the substrate as derived from translation of absorbance values using the Itaconic standard curve. Values on the same row/column at different subscript are significantly different at p 0.05.

Table 2: Effect of Varying Substrate Concentration on Itaconic Acid Production by *Aspergillus terreus* using *Moringa oleifera* pod .

	Quantity of Itaconic acid (mg/ml) / Substrate concentration (g)				
Fermentation period(days)	***5***	***10***	***15***	***20***	***25***
1	11.33±0.2[a]	18.39±1.9[b]	9.67±1.2[a]	9.23±1.2[a]	9.86±0.2[a]
2	12.7±0.6[b]	13.8±1.6[b]	12.3±0.6[b]	11.2±0.2[a]	11.9±0.9[a]
3	12. 25±0.4[b]	10.69±0.9[a]	11.11±0.5[a]	10.71±0.3[a]	11.14±0.5[a]
4	9.83±1.6[a]	10.15±0.6[b]	9.59±1.4[a]	9.46±1.8[a]	10.031±0.6[a]
5	8.04±0.1[a]	9.05±0.1[b]	8.45±0.2[a]	8.75±0.1[a]	10.57±0.1[a]
Fermentation period(days)		**CMC(control)**			
1	10.86±0.1[b]	10.33±0.4[a]	10.18±0.5[a]	10.42±0.6[a]	10.34±0.6[a]
2	11.1±0.9[b]	9.2±0.9[a]	10.2±1.0[b]	9.7±0.7[b]	11.0±0.5[a]
3	6.30±0.1[a]	6.55±0.1[b]	6.19±0.1[a]	6.44±0.5[c]	6.68±1.3[b]
4	6.22±0.1[a]	6.22±0.1[a]	6.63±0.1[b]	7.02±0.1[c]	6.59±0.4[b]
5	7.46±0.7[a]	7.74±0.1[b]	7.95±0.1[a]	7.58±0.1[a]	6.69±0.5[a]

Note: All values are means±SD of amount of Itaconic acid produced in milligram released from 1 millitre of the substrate as derived from translation of absorbance values using the Itaconic standard curve. Values on the same row/column at different subscript are significantly different at p□0.05.

Table 3: Effect of varying inoculum size on Itaconic acid production by *Aspergillus terreus* Using *Moringa oleifera* pod

	Quantity of Itaconic acid (mg/ml) / Inoculum size (ml)				
Fermentation periods(days)	**2**	**4**	**6**	**8**	**10**
1	7.01±0.1[a]	9.04±0.2[a]	10.00±0.1[b]	9.67±1.2[a]	8.04±0.1[a]
2	8.10±0.0[a]	9.50±0.2[a]	11.03±0.5[b]	10.18±1.0[a]	9.45±1.0[a]
3	9.45±1.0[b]	9.70±0.3[a]	17.20±0.7[c]	11.08±0.9[b]	12.25±0.4[b]
4	9.04±0.1[a]	10.03±0.6[b]	11.32±0.2[b]	10.18±1.0[a]	10.00±0.9[a]
Fermentation periods(days)			**€MC(control)**		
1	6.00±0.8[a]	6.10±0.8[a]	8.00±0.2[a]	8.50±0.2[a]	9.50±0.2[a]
2	6.35±0.1[a]	7.02±0.1[c]	8.10±0.0[a]	7.10±0.1[a]	8.03±0.1[a]
3	7.42±0.1[b]	7.47±0.1[b]	8.45±0.2[a]	8.50±0.3[a]	8.03±0.1[a]
4	6.30±0.1[a]	7.74±0.1[b]	7.95±0.1[a]	6.69±0.1[a]	6.56±0.4[b]

Discussion

The result illustrated in this study depicts that *Aspergillus terreus* has the ability to produce Itaconic acid using the pod of *Moringa oleifera* in solid state fermentation. Effect of incubation period, substrate concentration and inoculum size period was determined for production of Itaconic acid. The use of *Moringa oleifera* pod and Carboxyl methyl cellulose (CMC) as control showed that increase in time is proportional to acid produced from Day 1-5, thus fermentation after 5 days shows drastic decline in Itaconic acid production by *Aspergillus terreus* (Table 1). This may be due to the reduction of available nitrogen in the fermentation medium, age of fungi, depletion of sugar contents, the pH, Oxygen. This finding is similar to the findings of Rafi *et al.* (2014) who reported an increase in Itaconic acid production as incubation period increased with high yield at day 5.The results is also in agreement with the studies conducted by Meena *et al.* (2010) who obtained maximum production of Itaconic acid using species of *Aspergillus nidulans* wherein a steep increase in Itaconic acid production was observed on the fifth day. The yield of Itaconic acid by *A. terreus* from Carboxyl methl cellulose (CMC), a control substrate was compared with the pod of *Moringa oleifera* (Table 1). There was a higher yield of Itaconic acid fromthe pod of *M. oleifera* than that of the control(CMC).Thus this suggests that it is a good substrate for the production of Itaconic acid using *A. terreus*. This is due to particle size of CMC which is powdery in nature; thus this powdery nature reduced the particle size of CMC, thereby reducing the porosity of the microorganism and clumping of the CMC, this reduce sugar, oxygen and enzyme released by the organisms. A comparative account of production of Itaconic acid using Moringa pod at different concentrations using *Aspergillus terreus* was also studied. The maximum production of Itaconic acid obtained was 10g of substrate. This is in agreement with the work of Meena *et al.* (2010) who reported the optimum yield of Itaconic acid at 10% concentration. This also concurs with the findings of Wilke and Vorlop (2001) who observed that Itaconic acid is achieved by the fermentation with *Aspergillus niger* on a sugar containing media. Inoculum size is a major factor that determines the course of fungal fermentation. Different Inoculum sizes were used for the fermentation of Moringa pod to determine the optimum inoculum size for Itaconic acid production. Maximum Itaconic acid was obtained with 6ml of spore suspension (Table 3) This dispute the findings of Chandragiri and Sastry (2011), who observed that maximum production of Itaconic acid, was obtained with 5 ml of inoculum size of *Ustilago maydis*. This observation implies more of the organism produces more Itaconic acid. It is concluded from the study that the Moringa pod is a good substrate for the production of Itaconic acid using *Aspergillus terreus.*

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