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Research paper

Itaconic Acid Production by Novel Aspergillus Niveus in Solid State Fermentation Using Agrowastes

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Abstract

Itaconic acid (IA) is an organic compound. This dicarboxylicacid is a white solid that is soluble in water, ethanol, and acetone. Historically, itaconic acid was obtained by the distillation of citricacid, but currently it is produced by fermentation. Itaconic acid is essentially utilized as a co-monomer in the generation of polymers, for example, tar, plastic, elastic, paints, surfactant with applications in the paper and structural covering industry.. The fermentative production of Itaconic acid was probably achieved by the filamentous fungus *Aspergillus terrus, Aspergillus itaconicus* and mainly by *Aspergillus niger*. In the present work, an attempt was made to produce IA by Aspergillus niveus employing Solid State Fermentation (SSF) from various agro wastes like rice bran, rice husk,tamarind seed,wheat stuff and sugarcane bagasse as carbon substrates, which was pretreated in order to soften it.10 g of each substrate was taken in a 500 ml conical flasks separately and supplemented with 40 mL nutrient solution containing glucose, at pH 3.5. One milliliter inoculum containing 1×10^7 spores was added and moisture was maintained at 70%. After incubation at 32°C for 14 days, the acid production was estimated by spectrophotometric method and by HPLC analysis. Interestingly, the yield of itaconic acid was promising with all the above substrates, where tamarind seed, sugarcane bagasse and rice bran supported higher yields.

Keywords: *Itaconic acid, TCA, Aspergillus ,niveus, innoculum*

1. Introduction

With another enthusiasm for economical advancement, the compound business is making numerous endeavors to supplant petrochemical-based monomers with regular ones. Itaconic corrosive (IA, OECD name: methylene butanedioic corrosive; regular equivalent words: methylene succinic corrosive, 3-carboxy-3-butanoic corrosive, propylenedicarboxylic corrosive) is one of the promising substances inside the gathering of natural acids [1, 2, 3]. It is a white crystalline unsaturated dicarbonic acid with one carboxyl group conjugated to the methylene group. IA can be regarded as an α-substituted acrylic or methacrylic acid and is isomeric with citraconic and mesaconic acid. It is steady at acidic, impartial and center essential conditions at direct temperatures. Itaconic acid is a naturally occurring unsaturated 5C dicarboxylic acid which is also known as methylenesuccinic acid or methylenebutanedioic acid. Itaconic acid was first described by Baup in 1837[4] when he discovered it as a product of citric acid distillation. Itaconic acid has the stoichiometric formula C5H6O4 and a molar weight of 130.1 g/mol. It exists as white to light beige crystals with a density of 1.573 g/mL at 25°C, a melting point of 165-168°C and a flash point of 268°C. It dissolves in water up to 80.1 g/L at 20°C which makes it quite easy to purify by crystallization [5, 6]. In an recent report IA was additionally found to break down well in a few alcohols including methanol, 2-propanol and ethanol with the dissolvability expanding with temperature. Itaconic corrosive is likewise promptly biodegradable in nature. Itaconic corrosive is profitable as a monomer due to its extraordinary synthetic properties, which get principally from its methylene gathering and its ownership of two carboxylic corrosive gatherings. Itaconic corrosive can partake likewise polymerization, giving polymers with numerous free carboxyl gatherings that give beneficial properties on the poly-mer [7]. It can either be self-polymerised or can act as a co-monomer with other monomers to form heteropolymers. It has two protonation states with pKa values of 3.85 - 5.45 and with a degree of re-duction of 3.6, it is just a little more oxidised than glucose with a value of 4.0. Itaconic acid is about twice as acidic as acrylic acid and more reactive than maleic and fumaric acids which are potential monomeric substitutes. Itaconic acid readily forms a range of metallic salts and dieters such as dimethyl itaconate and di-n-butyl itaconate both of which are available commercially [8]. Itaconic anhydride might be utilized for the readiness of mono esters, for example, monomethyl itaconate or respond with amines to yield N-substituted pyrrolidones with genuine or proposed utilizes as a part of oils, cleansers, shampoos, herbicides and pharmaceuticals [9, 10]. A condensate of lauric acid and aminoethylethanolamine responds with IA to give an imidazoline subordinate which is a functioning fixing in shampoos. As of late two new itaconic corrosive deriva-tives (-)- 9-hydroxyhexylitaconic corrosive, and (-)- 9-hydroxyhexylitaconic corrosive 4-methyl ester were found as metabolites of Aspergillus aculeatus CRI322-03 [11, 12].

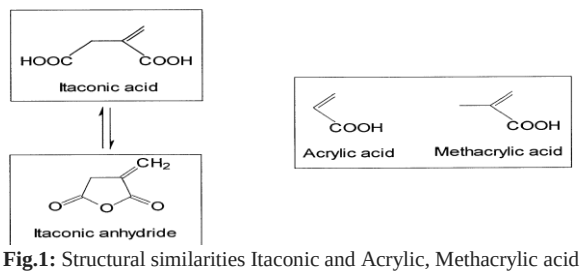


Fig.1: Structural similarities Itaconic and Acrylic, Methacrylic acid

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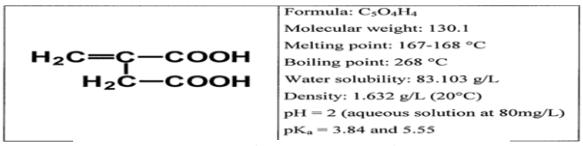


Fig. 2: Formula and few properties of itaconic acid

For several decades, the metabolic pathway for IA production was a point of discussion. In 1931, Kinoshita discovered the IA production by Aspergillus itaconicus (supposedly an A. terreus strain or a close relative) and suggested a decarboxylation of aconitate as key reaction [13, 14]. In their studies of A. terreus, Calam et al. objected Kinoshita since they could not identify citrate as intermediate product. In 1957, three studies of Bentley and Thiessen [15] showed strong indications for the presenceof a cis-aconitate decarboxylase (CAD) in cell extracts of A. terreus which might catalyse the reaction to IA [16,17]. Notwithstanding, they watched that citrus extract was an awful antecedent for the creation of IA. In this way, a few investigations questioned an association of the tricarboxylic corrosive (TCA) cycle [18, 19] and tried to describe an alternative pathway from pyruvate – which had been definitely identified as intermediate – to IA. In the 1990s, Jaklitsch and Bonnarme investigated the three suggested pathways from pyruvate to IA that had been discussed over the previous decades:

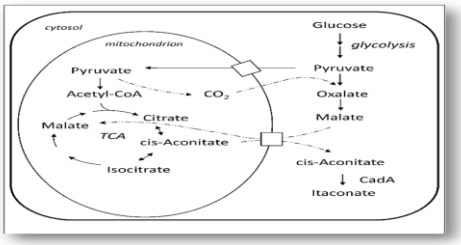


Fig. 3: Biosynthesis pathway of itaconate and its compartmentalization between cytosol and mitochondria

Although A. terreus is considered to be a popular IA-producing microorganism, several attempts have been made by researchers to investigate alternative producers of IA. Organisms such as Pseudozyma Antarctica and Ustilago maydis produce about 30 and 53 g/L of IA, respectively, in the shake flask level, using glucose as a carbon source [20, 21, 22]. Despite the fact that these living beings can create IA by its digestion, the generation in these life forms is less when contrasted with A. terreus [23, 24]. Tkacz and Langein developed a strategy to incorporate the cis-aconitate decarboxylase gene into a citric acid-producing strain Aspergillus niger for enhancing IA production [25, 26]. And developed a successful transformant of *A. niger* and an optimized medium that can produce about 0.13 g/L IA. Blumhoff focused on the key compound aconitase and cis-aconitic corrosive decarboxylase (Cad) in A. niger, which prompt a 14-24 overlay increment in the IA yield And built up a hereditarily adjusted Escherichia coli for the creation of IA.

2. Materials and Methods

2.1 Sample Collection:

The soil sample was collected from the college premesis. The sample was harvested from the domestic waste sewage near the mess.The sample was collected as a wet mass which serves as a wide space of bacterial and fungal colonies.

2.1.1 Isolation of Fungi:

1gm of soil sample were mixed 10ml of sterile distilled water and marked as 10^{-1}. 1ml was pipette out and mixed with 9ml of distilled water and marked as 10^{-2} from 10^{-9} dilution. 0.1ml was pipette out and poured on PDA (Potato Dextrose Agar) medium plate. The PDA medium was prepared. To avoid the bacterial contamination streptomycin antibiotic was added to the sterile medium. The medium was poured into the sterile petridish for the dilution of 10^{-3} to 10^{-7}. 0.1ml of samples inoculated into each plate and have spreaded over with L-rod. The plate were incubated at 28˚C for 3 days and considered as mother culture [27].

2.1.2 Identification of Fungus by Using Lactophenol Cotton Blue Staining:

Lactophenol cotton blue stains the fungal cytoplasm and provides a light blue background against which the walls of hyphae can readily be seen. It contained four constituents phenol. Which serve as fungicide, Lactic acid, which act as clearing agent; cotton blue; which stains the cytoplasm of the fungus; and glycerine which gives a semipermanant slide preparation. A loopful of culture was placed on the clean glass slide containing few drops of lactophenol cotton blue stain[9]. Mix gently with sterile needle. A clean coverslip was placed over the stain care was taken to avoid the formation of gas bubbles. The slide was observed under the microscope (400x) and the image was photographed. The morphological identification has been done by referring the standard manual [28].

2.1.3 Secondary Screening of Itaconic Acid:

After primary screening ,secondary screening was done by sub culturing the individual fungal colonies from the plate 10^4 - 10^6.Secondary screening for organic acid production under nitrogen-limited growth conditions was carried in a medium with the following composition (g/l): Glucose or glycerol 80, (NH4)2SO4 0.5g, KH2PO4 1.7, NA2HPO4 12, MgSO4.7H2O 1.4, CaCl2 0.02, ZnSO4.7H2O 0.02, FeSO4.7H2O 0.05, MnSO4.H2O 0.02, thiamine hydrochloride 0.006, yeast extract 0.5, initial pH was 6.0 and sterile bromoceresol purple (32 mg/L) was added post autoclave as a pH indicator.Incorporation of calcium carbonate in the medium is also used to screen organic acid producing microbes on the basis cleared zone of dissolved calcium carbonate around the colony [29,30].These screening approaches do not give idea that which organic acid has been produced.The clear zone was observed and incubated at 28°C for 4-5 days and maximum fungal colonies of individual species was observed.Then the sample was subjected for species identification by means of 18 S rRNA sequencing.

2.1.4 Fungal Colonies:

These are the different fungal colonies obtained after sub culturing the individual colonies.

- Penicilium funiculosum
- Penicillium vinaceum
- Aspergillus niveus

2.1.5 Fungal Identification:

rDNA sequences of isolated fungal colonies has been deposited in the GenBank database under accession no.MG183809. Thus the identification of species is done by 18 S rRNA sequencing. And got registered as Aspergilllus niveus atlast phylogeny tree obtained of the novel species is given the Fig.6

2.2 Itaconic Acid Productions by *Aspergillus Niveus*:

2.2.1 Submerged Fermentation:

Czapek Dox medium is used for fermentation process.

2.2.2 Itaconic Acid Production from Asperigillus Niveus:

To the 500ml Erlen Meyer flask 200ml of Czapek- Dox broth was prepared [31,32]. The medium was autoclaved for 20 min at

121°C and to it fungal culture was inoculated.And incubated at 30˚C for 10-14 days in rotatory shaker for maximum fungal growth [33].Then the sample (broth) was subjected to HPLC analysis.

2.3 Analysis:

2.3.1 HPLC-High Performance Liquid Chromatography:

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column [34, 35].
The cultured broth was taken in a 1.5ml eppendoff.And was centrifuged at 10,000 rpm for 10min.From which pellet was discarded and supernatant subjected to HPLC analysis [36,37].
HPLC was done in Agilent Auto sampler and Column used was C18 Waters. 20µl was injected with the flow rate 0.6ml/min.
Stationary phase: ion-exclusive resin for chromatography R (9µm)
Size: l=0.30m,Ø=7.8mm
Data File:D:\HPLC-066\DATA\31-01-201\32 SPL 3
Method:C:\EZChromElite\Enterprise\Projects\Default\Method ITACONIC ACID. Met
Temperature: 37ºC

2.4 Solid State Fermentation:

Solid state fermentation (SSF) is a biomolecule producing process utilized as a part of the nourishment, pharmaceutical, restorative, fuel and material businesses. These biomolecules are generally metabolites created by microorganisms developed on a solid surfaces [4]. This type of fermentation is an alternate to the submerged fermentation and mainly used for industrial purposes.

2.4.1 Substrates:

The most commonly used Agro-wastes are nut shells, rice bran, rice husk and ground nut oil cake, sugarcane bagasse, orange peel etc.,The substrates used in the production of itaconic acid by *Aspergillus niveus* were Tamarind seed and Wheat stuff

2.4.2 Pre-Treatment of Substrates:

The fungal strains cannot be cultivated in a relatively short time by establishing the method of solid state fermentation. Generally solid state fermentation involves the growth of microorganisms on substrates. So before fermentation the substrate need pre-treatment, so that the substrate becomes soft and can easily be utilized by the microorganisms.
Pre-treatment can be done by two ways:

- Physical pre-treatment
- Chemical pre-treatment

2.4.3 Physical Pre-Treatment:

For physical treatment 100 g of substrate on dry weight basis was taken in 500 ml conical flask and washed twice with distilled water and sterilized at 121ºC and at 15 psi for 20 minutes in an autoclave. The samples were dried at 80ºC in a hot air oven for 12 h and used as substrate for fermentation. This steam treatment enables the moist air to pass through the substrate and makes it soft.

2.4.4 Chemical Pre-Treatment:

Chemical treatment was done with 1N NaOH and 1N H_2SO_4 for which 200 ml of pre-treatment solutions 1N H_2SO_4 and 1N NaOH were added to 100 g of substrates separately and autoclaved at 121ºC for 15 min. After treatment, the samples were washed thoroughly with distilled water to neutralize the effect of pre-treatment solutions and dried in an oven at 80ºC for 12 h.

2.4.5 Substrate Preperation:

Fermentation was carried out by taking 10 g of substrates in 500 ml conical flask to which 20-40 ml of the nutrient solution was added.
The contents were sterilized by autoclaving at 121ºC for 20 min.After sterilization the spore suspension of *A.niveus* was added as inoculum in solid state fermentation process. And the medium was incubated at 27°C for 13-15 days.Thus the maximum fungal growth was obtained, which was then harvested for the future hplc analysis. After fermentation substrates were washed with buffer of pH 7.0 and filtered. The filtrate served as a source of the product.

2.5 Analysis:

HPLC
The sample blend to be isolated and investigated is presented, in a discrete little volume (normally microliters), into the surge of portable stage permeating through the section. The parts of the example travel through the segment at various speeds, which are an element of particular physical collaborations with the adsorbent (additionally called stationary stage) [7]. The velocity of every segment relies upon its synthetic nature, on the idea of the stationary phase (segment) and on the composition of the mobile phase. The time at which a particular analyte elutes (rises up out of the segment) is called its maintenance time. The maintenance time estimated under specific conditions is a recognizing normal for a given analyte.Unlike the submerged fermentation solid state fermentation is the cultivation of organisms on a solid substrate. Thus it requires a primary step of converting the sample to liquid texture.
Thus to the 5g of fermented sample 50ml of distilled water was added. And from it 15ml of sample was vortexes in a 15ml eppendoff for 10min in order to disturb the substrate containing cell mass.Then the sample was centrifuged for 10min at 8,000rpm.The supernatant was recovered in a 1.5ml eppendoff and the pellet was discarded. Then the sample was subjected to HPLC analysis.
Serial dilution

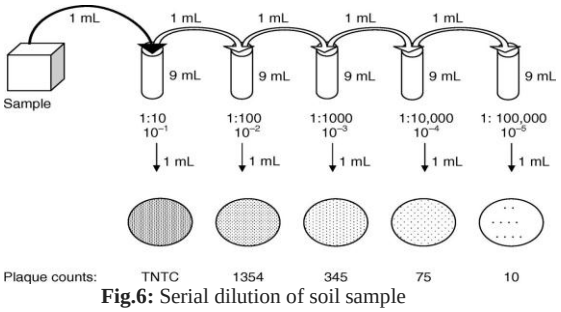


Fig.6: Serial dilution of soil sample

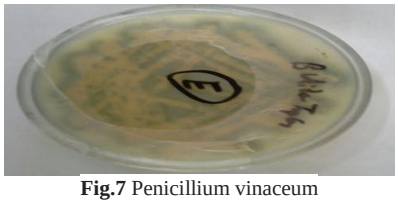


Fig.7 Penicillium vinaceum

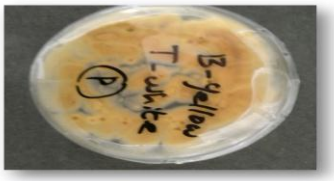


Fig.9: Asperigillus niveus

3. Results and Discussions

Itaconic acid (IA) is one of such high-value platform chemicals that has a wide range of applications and the potential to replace several petroleum-based products. The worldwide annual production of IA is estimated to be 80,000 tons by means of fermentation using different biomass feedstock's, which reflects the high demand for IA as a platform chemical. Thus the production of IA is completely depended on the surface fermentation rather than submerged fermentation. Because surface fermentation is cost efficient unlike the submerged, since the substrate used in Solid state fermentation is cheaper than the broth. Both the substrates Tamarind seed powder and Wheat stuff are highly rich is carbohydrate content, rich carbohydrate substrates are used since the production of itaconic acid is by TCA cycle [38]. The amount of itaconic acid produced was zero when Tamarind seed powder was used, during the incubation period of 7 days. While the HPLC result obtained was positive after increasing the time period but was failed to quantify the amount. Since the area of the crude is large in the graph obtained, the peak obtained was not sharp and accurate [39,40]. Thus the exact amount of itaconic acid present was unpredictable. And the amount of itaconic acid produced when Wheat stuff was used as substrate was 0.533mg during the incubation period of 6 days.

3.1 18S RRNA Sequencing:

Table 3: Species obtained after 18S rRNA sequencing

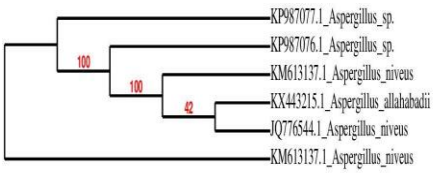
S.No	Sample	Species
1.	E	Penicilium funiculosum
2.	O	Penicilium vinaceum
3.	P	Asperillius niveus

3.2 Blast Result of Aligned Sequence Data:

contigUS CGAAAGAATCATTACCGAG-TGCGGGTCCGCGTGGCCCAACCTCCACCGTGC-TATTGTACCT
GTGTGCTTCGGCGGGCCGCCGCCTTCGGGCTGGCCGCGG
GGGGGCGTCTCTCCCGGCGCC GTGCCGCCGGA-GACCCACATGAACCTGTTTCTGAAAGCTTGTAG-TCTGAATGTGATTTGTTTTGCAATCAG-TTTAAAACTTTTCAACAATGGGATCTCTTGGTTCCGG-CATCGATGAAAAAA AGCAGCGAAATGCGA-TAACTAATGTGAAATTGCA

Organism: *Aspergillus niveus*

3.3 Phylogeny Tree:



Result: Aspergillus niveus (MG183809)

3.4 *Aspergillus Niveus* in Submerged Fermentation:

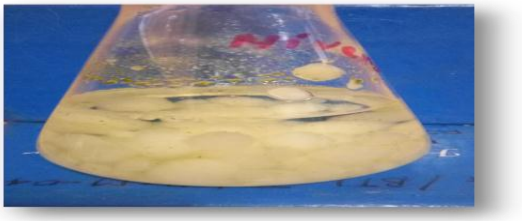
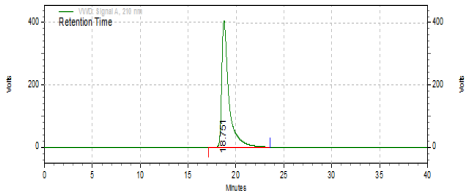


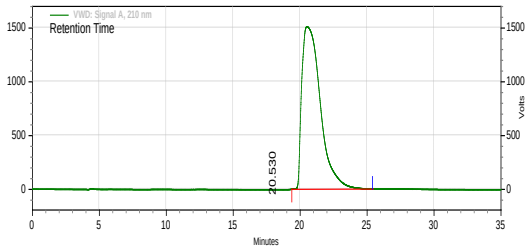
Fig.10: Growth of Aspergillus niveus in Czapek-Dox medium

3.5 HPLC (Submerged Fermentation):

3.5.1 Standard Graph:



3.5.2 Sample graph:



VWD: Signal A, 210 nm Results

Retention time	Area	Area %	Height	Height %
20.530	2449045506	100.00	25353284	100.00
Totals	2449045506	100.00	25353284	100.00

3.5.3 Calculations:

- $\frac{\text{Area of sample}}{\text{Area of standard}} = \frac{2445045506}{1384791211}$
- $\frac{2445045506}{1384791211} \times \frac{25.0}{25} \times \frac{1}{1} \times 1 = 1.815\text{mg}$
- Retention time(t_R)=20.5
- Thus the amount of itaconic acid available in the sample is 1.815mg.

3.6 Solid State Fermentation:

3.6.1 Pretreated Substrates



Fig.11: Tamarind seed



Fig.12: Wheat stuff

3.6.2 Surface Fermentation of a.Niveus:



Fig.13: Growth of Aspergillus niveus in Tamarind seed powder

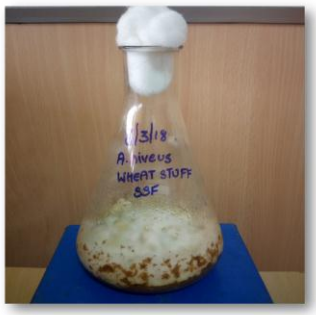
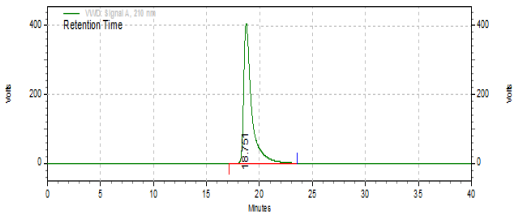


Fig.14: Growth of Aspergillus niveus in Wheat stuff

3.7 HPLC Results:

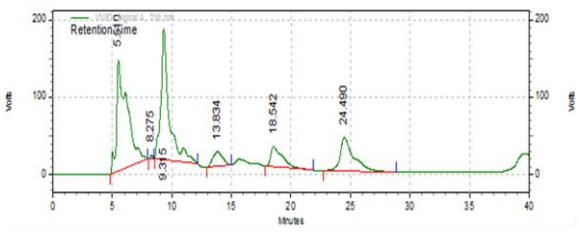
3.7.1 Wheat Stuff (Standard Graph):



VWD: Signal A, 210 nm Results

Retention time	Area	Area %	Height	Height %
18.05	1332881523	100.00	15123145	100.00
Totals	1332881523	100.00	15123145	100.00

3.7.2 Wheat Stuff (Sample Graph):

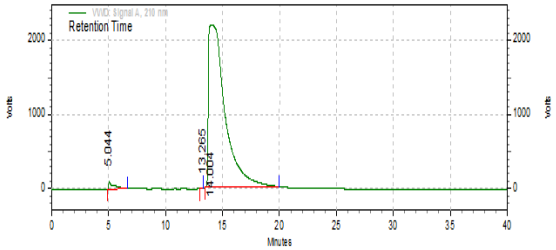


VWD: Signal A, 210 nm Re-sultsRetention time	Area	Area %	Height	Height %
5.044	55319791	1.47	1888413	4.88
13.265	620878	0.02	29335	0.08
14.004	3709786739	98.51	36772228	95.04
Totals	376527407	100.00	38689979	100.00

3.7.3 Calculation:

- $\frac{Area\ of\ Sample}{Area\ of\ Standard} = \frac{30321180}{352218666}$
- $\frac{30321180}{352218666} \times \frac{62}{10} \times \frac{1}{1} \times 1 = 0.533mg$
- Retention Time (t_R) = 18.54
- Thus the amount of itaconic acid available in this sample is 18.54 mg.

3.7.4 Tamarind Seed (Sample Graph):



VWD: Signal A, 210 nm Results

Retention time	Area	Area %	Height	Height %
5.510	148692067	38.26	2415188	35.32
8.275	1188582	0.31	85564	1.25
9.315	132421772	34.08	2845648	41.61
13.834	18258669	4.70	319158	4.67
18.542	30321180	7.80	447239	6.54
24.490	57733850	14.86	725684	10.61
Totals	388616120	100.00	6838481	100.00

3.7.5 Calculations:

The range of the peak lies in the interval 13-20 thus it indicates the presence of Itaconic acid in the sample (tamarind).Since the area of the sample is large, the peak obtained was not sharp and accurate. Thus the amount of acid present cannot be quantified.

3.8 Comparisons:

Table 4: Comparisions of Itaconic acid productivity from A.niveus(MG183809) between Substrates (Tamarind seed and wheat stuff)

SUBSTRATES	INCUBATION PERIOD (days)	AMOUNT IN-JECTED (HPLC) (µl)	ITACONIC ACID PRO-DUCED (mg)
Tamarind seed	23	10	Cannot be quantified
Wheat stuff	6	10	0.533

4. Conclusions

The obtained fungal species from various fungal colonies was tested to be *Aspergillus niveus*, got registered in the Gen Bank and obtained an Accession no. of MG183809.This novel species was further subjected to submerged fermentation to indicate its capa-

bility to produce Itaconic acid. HPLC result of the inoculated czapek-dox broth medium reveals the presence of itaconic acid in the novel species *Aspergillus niveus* (MG183809) of 1.815mg at retention time (t_r) of 20.53.

The further HPLC result of itaconic acid productivity in Solid state fermentation was about 0.533mg at retention time(t_r) of 18.54 in Wheat stuff (substrate).But the HPLC result obtained was negative when the incubation period was about 7days, and then the increase of the time period showed positive result in Tamarind seed powder (substrates), but was not able to quantify. This experiment exhibits the greater Itaconic acid productivity in Wheat stuff than in Tamarind seed powder. Though there is a number of reports regarding the production of itaconic acid by submerged fermentation, only a couple of reports are available on solid state fermentation for itaconic acid production till now, the MG183809 strain achieves a greater itaconic acid yield by solid state fermentation employing Wheat stuff as solid substrate.

- Further increase in the itaconic acid productivity can be achieved by employing Solid state fermentation in various Agro-wastes such Sugarcane bagasse, orange pulp, Watermelon peel, Rice bran, which is cost-effective.

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