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FERMENTATIVE PRODUCTION OF ITACONIC ACID BY USING *ASPERGILLUS TERREUS*

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ABSTRACT

Aspergillus species were known to be the best species for the production of Itaconic acid. However very little is known about the industrial production of Itaconic acid in India and virtually nothing is known about its production cost analysis. In view of the extensive industrial use of Itaconicacid, the present study was designed to increase the production of Itaconic acid and an attempt was made to optimize the nutrient medium using *Aspergillus terreus*. Reducing sugars and phosphorus were measured by the DNS method of Miller (1959) as described by Strickland and Parson (1972) respectively. The cost-effective substrates like Jatropha seed cake, ground nut cake, coconut oil cake, glucose, oat meal and corn flour were used as carbon source for the production of Itaconic acid by using locally isolated *Aspergillus terreus* strain. Gene Bank accession number of submitted nucleotide sequence is JF495477**.** The highest yield was observed in Jatropha seed cake (13.60 g/lit),followed by Ground nut cake (10.08g/lit), Coconut oil cake (9.17g/lit), Glucose (7.55 g/lit), Oat meal (4.32 g/lit) and Corn flour (3.27 g/lit).

Keywords: *Aspergillus terreus*, Itaconic acid, Fermentation, Methylene succinic acid, Secondary

INTRODUCTION

Itaconic acid is one of the most important organic acid being produced from several fungal species and widely used as a substitute for acrylic acid in making polyester resins or plastics **[1]**. *Aspergillus* **[2, 3]** and *Candida* **[4]** species produce itaconic acid as their secondary metabolite. *Aspergillus terreus*is commonly used for commercial production of itaconic acid because it is reported as high yield producer **[5]**. It is also called as Methylene succinic acid, an unsaturated acid with two carbonyl groups and conjugated double bonds. Because of uncomparable characteristics, itaconic acid is used as a co monomer for certain products **[6, 7]** and raw material for latex, synthetic resins, adhesives, paints and additive for acrylic resins, fibers and paper etc., **[8]**. It is also used as acidulant for pH adjustment in food industry. Though it has got immense industrial applications, the usage of itaconic acid is restricted because of high price and low productivity from the industries. This prompted theinvestigator to look for alternatives and to increase the production with low cost by using wild type fungal strains, which are isolated locally from soil collected from Seshachalam forest area. At present, commercial production of itaconic acid is from molasses and starchy biomass which is of high price carbon source for microbial production **[9, 10]**. Studies on fermentative production of Itaconic acid are meager on oil seed cakes like ground nut seed cake, coconut seed cake, Jatropha seed cake for the production of itaconic acid. These are considered as cost effective carbon sources for the biological production. However previous reports indicate that the production is high with starchy biomass **[11]**. Hence an attempt has been made to investigate the production of Itaconic acid using low-cost carbon source and wild type *Aspergillus terreus* isolated from soil.

MATERIALS AND METHODS

Soil collection and Characterization:

The soil sample was collected from Seshachalam hill forest area. The collection was carried outrandomly at 6 different places. Sterile bags and scalpel were used to collect soil from the field and then all the six samples were mixed thoroughly to increase homogeneity and brought to the lab. Physico chemical parameters like water holding capacity, pH, organic Carbon content, Electricalconductivity, Carbon, Phosphorus and Nitrogen were studied.

Enumeration of fungi

The fungal populations present in the collected soil were enumerated by serial dilution method andspread plate method. The fungal population was cultivated on Sabouraud's agar medium with following composition. Peptone 10.0 g/lit, Dextrose 40.0 g, Agar agar 18 g and pH was adjusted

to5.6. After preparation of medium 20ml of sterile medium was transferred to sterile Petri plate andallowed for solidification. After solidification of the medium 0.1ml of soil suspension was spread bythe help of spreader & incubated at 37°C for 7 days. After the incubation of plates the fungalcolonies formed on the medium was counted by cube colony counter. The dominant fungal specieswere again sub cultured on the PDA slants for further studies. Specific medium was used to identify *Aspergillus terreus*. Colony morphological characters were studied and confirmed it as *Aspergillus terreus* by using molecular markers.

Molecular identification of the isolates by DNA sequencing and analysis:

Aspergillus terreus, isolated from the soil by serial dilution method was maintained on Petri

dishes containing PDA medium (potato dextrose agar). After inoculation from the stock culture slant. The dishes were incubated at room temperature for 5 days and subsequently stored at 5 ˚C. DNAextraction was done from cultured cells using Master Pure TM DNA Purification Kit obtained from Medax Company Chennai, India:25ml of culture was taken and rinsed the culture with equal volume of 0.1 M $MgCl_2$ and transferred the culture pellet to a chilled mortar and made in to powder using liquid nitrogen. It washoroughly mixed to get uniform composition. 300ml of Cell Lysis Solution was added to microcentrifuge tube and cells are suspended, Vortexed and incubated for 15 minutes. 150 ml of Protein Precipitation Reagent was added and Vortex mixed for 10 seconds.Contents were centrifuged for 10 minutes at10,000 rpm. 500ml of isopropanol was used toprecipitate the DNA from supernatant. The DNA was stored at 40C after 70% ethanol wash. RNasetreatment was given for PCR amplified product. The DNA stock sample was quantified usingspectrophotometer at 260 nm and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. the Ultra Violet (UV) absorbance was checked at 260 and 280

nm for determination of concentration and purity. Purity of DNA was judged on the basis of opticaldensity ratio at 260:280nm. The DNA having ratio between 1.8 to 2.0 was considered as goodquality. *Aspergillus terreus* was identified by using ITS1,5.8S ribosomal RNA and ITS4 ,28 SRibosomal RNA gene partial sequence regions. The primers used are

ITS1: TCC GTA GGT GAA CCT GCG G

ITS4: TCC TCC GCT TAT TGA TAT GC

PCR amplification was carried out in a 2.5 µl of 10X assay buffer, 2ml of 25mm MgCl2, 0.5

µl of 10mm dNTP's (Fermetes USA), 0.5 units of Taq DNA polymerase (Fermetes USA), 0.6 µm ofeach primers (ITS-1 and ITS-4) and sterile distilled water (17.8 µl)for all the samples to avoid bandhandling errors. Amplification was performed with a thermal cycles (Ephendroff, Hanburg).The PCR programme constitutes of an initial denaturation at 940C for 2min followed by 30cycles ofdenaturation at 940C for one minute, annealing at 560C for one minute, extension at 720C for oneminute and finally extension at 720C for 15 minutes.

Amplified PCR products were subjected to 1% agarose gel electrophoresis with 1X TBE

buffer as running buffer. The bands were visualized under UV transilluminator with ethidium

bromide (10mg/ml) staining. The DNA bands were documented in gel documentation system (Alphainnotech) and compared with 1KB ladder (Fermetas USA). The PCR amplified product was sent toXcelris Labs Limited, Ahmedabad, India, for sequencing. The sequence was accepted by gene bank.

Raw Materials for medium

Seed cake is a left over material after extraction of oil from seeds. Three different seed cakes

i.e., Jatropha, Coconut, Groundnut were obtained from Nathella Papayya Chetty Oil Company and airdried. Corn flour and oats powder were taken from the grocery stores and these were used inproduction medium.

Medium for mycelium

Initially screening for organic acid production was carried out in a medium with the followingcomposition (Glucose,60.0g; (NH4)2 SO4, 2.36 g; KH2PO4, 0.11g; MgSO4.7H2O, 2.1g; CaCl2.2H2O,0.13g; NaCl,0.074mg; CuSO4.5H2O, 0.2 mg; FeSO4.7H2O, 5.5 mg; MnCl2.4H2O, 0.7mg; ZnSO4.7H2O,1.3mg), and PH 6.5. After initial screening some modifications were made to theproduction medium for optimizing the yield. Then the medium was autoclaved at 125oc at 16lb for 20minutes. Then the medium was used for fermentation with variations in inocolum percentage of *Aspergillus terreus*. For every 24 hours the sample was estimated for total sugars and Itaconic acid.

Biomass estimation

Broth was filtered by using Whatmann No1 filter paper. The filter paper containing Mycelia

was washed with 0.8%saline water and then with the distilled water before keeping it in hot air oven (Thermo Company Pvt Ltd) at 90°C for 2 hours the total sugars concentration was measured by Phenol Sulfuric Method **[12]**. Total reducing sugar concentration was estimated by DNSA method **[13]**. Qualitative assay of Itaconic acid was carried out using UV spectrophotometer at 210 nm **[14]** and the

Itaconic acid was measured by Bromination method **[15]**. The yield was calculated as described by Yahiro *et al* **[16]**.

RESULTS

A wild type, local strain of *Aspergillus terreus* was isolated and characterized form the soil collected from Seshachalam hills. Physicochemical properties of soil were studied **(Table 1)**. Thisfungus was utilized for the production of Itaconic acid to observe the variations in product ion ofItaconic acid. Different substrates like Jatropha seed cake, Coconut seed cake, Ground nut seed cake, Corn flour and Oat meal were used as carbon source for the production of Itaconic acid **(Table 2)**. Highest itaconic acid production was 13.6gm/lt for Jatropha seed cake after 168 hours of incubation. Ground nut seed cake and Coconut seed cake were yielded 10.08 gm/lt and 9.17 gm/lt respectivelyafter 168 hours of incubation time **[17]**. Corn flour and Oat meal showed low productivity whencompared to seed cakes.

The optimization of various parameters like incubation time, temperature, carbon source and

agitation was carried out for media preparation, High yield was observed for all the samples at PH 3 **(Figure 1) [18]** Agitation speed is optimized as 150 RPM for 168 hours of incubation **(Figure 2)**. At 32octemperature after 168 hours of time showed high concentration of Itaconic acid **(Figure 3)**. Inoculum concentration plays an important role. 0.9% inocolum concentration showed highest yield **(Figure 4)**. Similarly glycerol was used as carbon source for production and 6% glycerol concentration in thetotal medium yielded high amount of Itaconic acid **(Figure 5)**. Molecular confirmation of *Aspergillus terreus* was done by sequencing of 5.8S rRNA and 28.S r RNA gene partial sequence. The results areas follows:

1
agtnnggannc gagtcccgacccatcaactattagtaccgagg
tgcttcggcgggcccgc
61
cagagtagctggccgccgggggcgactcgcccccgggcccgt
gcccgccggagacccca
121
acatgaaccctgttctgaaagctngcagtctgagtgtgattcttt
gcaatcagttaaaac
181
tttcaacaatggatctcttggttccggcatcgattaagaacgcag
cgaaatgcgataact
241
aatgtgaattgcagaattcantgaatcatcgagtctttgaacgc
acattgcgccccctgg
301
tattccgggggggcatgcctgtccgagcgtcattgctgccctcaag
cccggcttgtgtgtt
361
gggccctcgtcccccggctcccgggggacgggcccgaaaggca
gcggcggcaccgcgtcc
421
ggtcctcgagcgtatggggcttcgtcttccgctccgtaggcccgg
ccggcgcccgccgac
481
gcatttatttgcaacttgtttttttccaggttgacctcgnatcaggt
agggatacccgct
541 gaacttaagcatatcaataagagaaggaagctatgta

Table 1: Properties of Soil Collected from Seshachalam Hills	
Property	Description
Color	Grey
Odour	Normal
WHC	0.3/gm/ml
pH	7.2
Ec	0.18µg/ml
Carbon	Less
P	5kg/h
N	242kg/h
Texture of soil	
Sand	78.76
Silt	16.12g
Clay	5.2g

Table 2: Fermentative Production of Itaconic acid by using different seed cakes and starchy material

S. No.	Substrate	Itaconic acid (g/lit)	Final Total Sugars (g/100ml)	Dry Mycelium Weight (g/100ml culture medium)	Final pH after one week
1	Jatropha	13.6	0.3	70.36	6.75
2	Ground Nut Oil Cake	10.08	0.2	57.08	9.9
3	Coconut Oil Cake	9.17	0.28	29.89	5.84
4	Oat meal	4.32	0.07	52.94	8.23
5	Corn flour	3.28	0.09	22.73	3.54

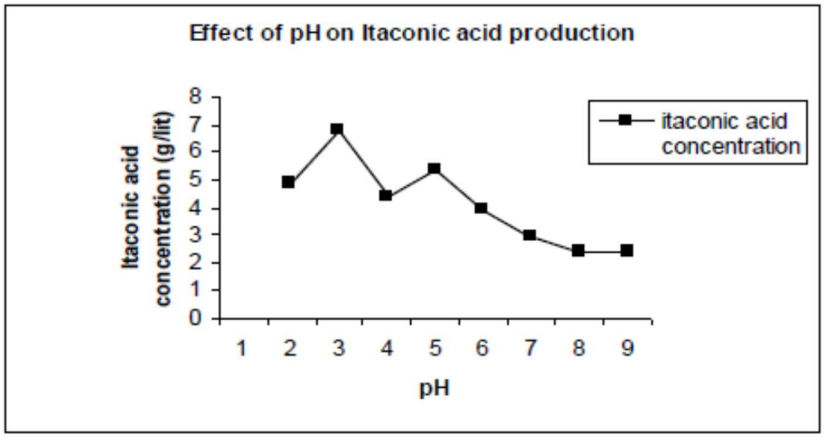

Figure 1

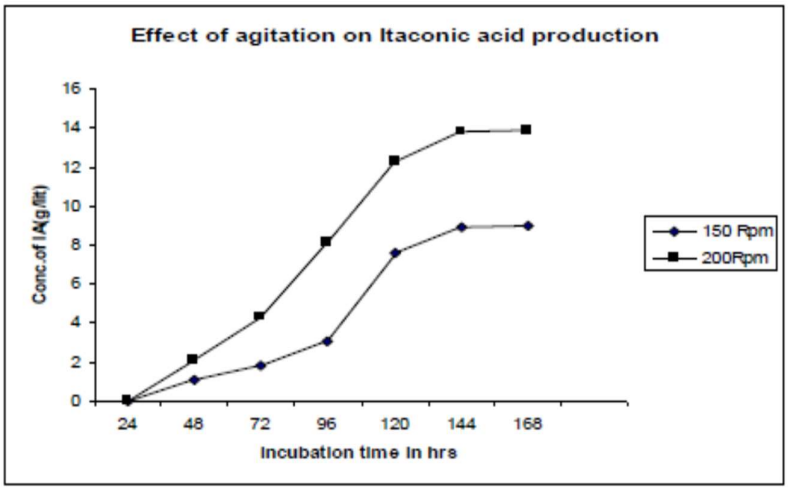

Figure 2

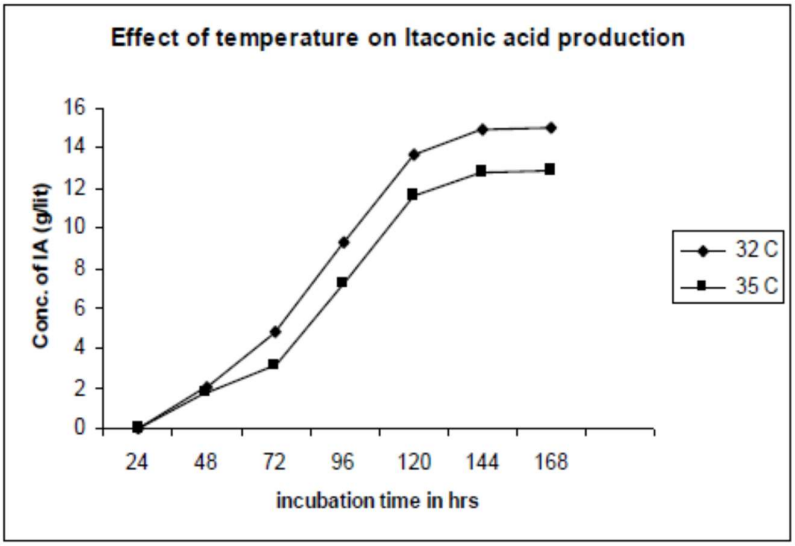

Figure 3

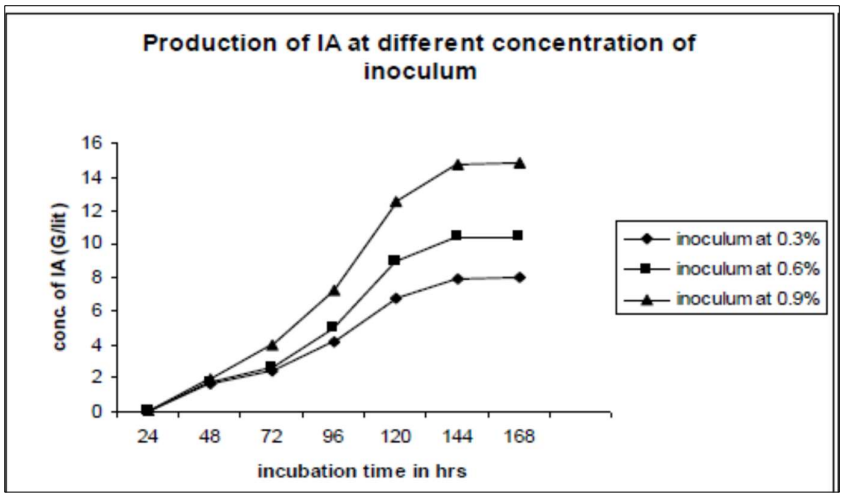

Figure 4

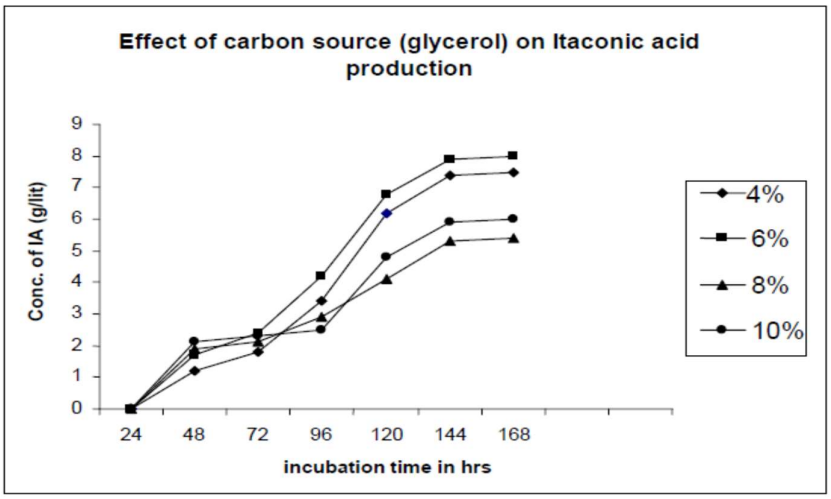

Figure 5

DISCUSSION

In the present study *Aspergillus terreus* local strain was identified and by using Local strain with different substrates in the medium, Itaconic acid production was examined. The effect of glycerol as a substrate showed increased production. The pH, temperature and inoculum concentration were optimized for the increased production of Itaconic acid. In the present study, the evaluation of the effect of different substrates on the culture parameters showed that the strain *Aspergillus terreus* is giving high production for Jatropha seed cake followed by Ground nut seed cake, Coconut seed cake, Glucose, Corn flour and Oat meal. Lowest production was observed for oat meal as it contains rich fiber than Glucose. Corn flour also showed less amount of Itaconic acid production as it is rich in proteins this clearly indicates that the production of Itaconic acid does not depend on the fiber rich and protein rich medium. The Jatropha and Coconut, Ground nut are oil procuring nuts, the leftover seed cake will contain reminiscents of fatty material and carbohydrate rich substrates. Growth yields of itaconic acid is high in Jatropha, this is probably due to the cumulative effect of nitrogen and low phosphorus content which is a positive factor in the fermentative production of Itaconic acid **[19]**. Similarly ground nut oil seed cake and coconut oil seed cake were yielded high amount of Itaconic acid when compared to corn flour and Oat meal as this two are having high phosphorus concentration. The addition of glycerol along with cupric sulphate (8mg/lt) in the medium showed highest yield of Itaconic acid. Hence the present study clearly indicates that the oil giving nuts are the potentialsubstrates for the Itaconic acid fermentative production. It is also clear that fiber rich and protein rich substrates do not yield much Itaconic acid. Further studies to optimize the production process by using mutants and enzyme treated starch varieties are needed to minimize the production cost and to increase the yield of itaconic acid.

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