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Optimization and production of itaconic acid using *Aspergillus flavus*

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ABSTRACT

In the present investigation soil samples were collected from Mallipattinum, Thanjavur (Dt). Different species of fungi were isolated from soil sample. Among these fungi Aspergillus flavus were dominantly present in soil. The itaconic acid activity was measured at different pH and different temperature using Aspergillus flavus. It was found to be maximum at pH 4 and temperature at 40˚C in Aspergillus flavus. Itaconic acid is mainly used for stiffening agents made from poly itaconic acid , synthesis of novel biodegradable hydrophilic polymers from itaconic acid derivatives. Itaconic acid is also used in emulsion paints where it improves adhesion of the polymer. It forms copolymers with its esters and other monomers, which are used in the paper industry for wall paper and other paper products. It is also used in the production of adhesives.

Keywords: *Aspergillus flavus*, Itaconic acid, pH, Temperature and Synthesis.

INTRODUCTION

Marine derived fungi are rich sources of bioactive compounds. The marine invertebrates has led to the discovery of many bioactive compounds. In our experience these fungi could grow rapidly in salt containing media (seawater) but most of them hardly grow in media without salt supplements and sometimes those that do grow in non-saline media change their morphology when cultured under such conditions (Parvatkar *et al.*, 2010).

Fungi produce a variety of lipids including fatty acids in free or esterified form (e.g.,) glycerides, phospholipids, glycolipids, sterol esters sphingolipids or simple esters as well as other lipids (e.g.,) free sterols, sterol glycosides and hydrocarbons (Losel *et al.*, 1996).

Itaconic acid (A1) is a promising organic acid. It is a white crystalline unsaturated dicarbonic acid in which one carboxyl group is conjugated to the methylene group. IA is used worldwide in the industrial synthesis of resins such as polyesters, plastics and artificial glass and in the preparation of bioactive compounds in the agriculture, pharmacy and medicine sectors. There is continued interest in developing biological methods to produce compounds with double bonds that are suitable for the manufacture of various polymers. IA also provides possibilities for selective enzymatic transformations to create useful polyfunctional building blocks (Okabe *et al.*, 2009).

The biosynthesis of organic acids in filamentous fungi has been extensively studied. Hence fungi like those of the genus *Aspergillus* are often used for industrial production of organic acids such as itaconic acid (methylene succinic acid). However, the biosynthetic pathway of relatively simple and commercially important compound has still not been unequivocally established (Mattey, 1992). The present study was itaconic acid were produce in *Aspergillus*

flavus to examine the effectiveness of fungi as potent acid source in production medium and optimization of parameter for itaconic acid production.

MATERIALS AND METHODS

Sample Collection

The soil sample was collected from Mallipattinam Thanjavur (Dt).

Isolation of fungi

1gm of soil sample were mixed 10ml of sterile distilled water and marked as 10^{-1} from 10^{-9} dilution. 1ml was pipette out and mixed with 9ml of distilled water and marked as 10^{-3} from 10^{-4} 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^{-4}. 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.

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 glycerin 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. 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 identification has been done by referring the standard manual Ainsworth *et al*., 1973.

Screening for Itaconic Acid

Initial 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, $(NH_4)_2SO_4$ 0.5g, KH_2PO_4 1.7, NA_2HPO_4 12, $MgSO_4.7H_2O$ 1.4, $CaCl_2$ 0.02, $ZnSO_4.7H_2O$ 0.02, $FeSO_4.7H_2O$ 0.05, $MnSO_4.H_2O$ 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. The clear zone was observed.

Itaconic Acid Production in *Aspergillus flavus*

Czapek Dox medium is used for fermentation process. The composition of the Czapek Dox medium used for g/1000ml (weight/volume) is,

- Sucrose 2.25g was dissolved in 75ml distilled water
- Sodium nitrate : 0.15g
- Magnesium sulphate : 0.037g
- Potassium chloride : 0.037g
- Ferrous sulphate : 0.007g
- Dipotassium hydrogen phosphate : 0.07g
- Distilled water : 75ml

Itaconic Acid Production

The fermentation medium was prepared. After medium was inoculated with *Aspergillus flavus*, a suspension containing 10^2 cell/ml.Cultures were grown with 100ml Erlen Meyer flask with 25ml of medium in a rotated shaker (150 rpm) at 30°C. After mycelial growth in fermentation extracellular enzyme were extracted by washing 3 times with 100ml of 60mM sodium phosphate buffer pH 6. The combined extract was filtered assayed of enzyme activity.

Optimization of pH for itaconic acid activity

The activity of itaconic acid was evaluated at different pH values such as 2,3,4,5 and 6 under assay condition. The fermentation media prepared from each pH. The particular pH was adjusted to 2,3,4,5 and 6 by using 1.0 N HCl / 1.0 NaOH. Then the flask was autoclaved at 121°C (15 lbs/15 min). After the sterilization, the flask was inoculated with 0.2ml 2% fungus spore suspension. The flask incubated for room temperature at 160 rpm, 30°C for 7 days.

Optimization of Temperature for Itaconic Acid

To determine the effect of temperature on itaconic acid, the reaction was carried out at different temperatures such as 30°C, 40°C, 50°C, 60°C and 70°C. The 30°C flasks were inhubated at room temperature, 40°C, 50°C, 60°C and 70°C flask were incubated in shacking incubator. All the flasks were incubation for 7days. The absorbance is measured at 530nm.

Assay of Itaconic acid

Estimations were conveniently carried out by modifying the permanganate method of Dickman (1952).The assay has a range of 0 to 200g of itaconic acid. Stock solutions were prepared as follows: Metaphosphoric acid pellets (8.5 gm.) were dissolved in 20 ml. of mater; the filtered solution could be kept 3 to 4 days. Potassium permanganate, 5 ml. of 0.1 N solution, was diluted to 100 ml. immediately before use. A standard solution of 1 gm. of pure itaconic acid in 1 liter was diluted 1: 10 before use. 0.3 ml. of metaphosphoric acid was added the solution to be assayed, and the volume made up to 5 ml. in a Klett tube. The tubes were chilled in ice for 10 minutes, and, while still in the ice bath, 2 ml. of potassium permanganate solution (at room temperature) were added. The tubes were removed from the ice bath, shaken to mix the contents, and finally allowed to stand in the dark at room temperature for 10 minutes. The optical density was determined immediately in calorimeter with the 540 nm.

Estimation of Protein

Protein concentrations were determined according to Lowry's Method (1951). To Pipette out 1ml of the working standard solution into a series of test tube. Pipette out 0.1ml of the enzyme extract in to two other test tubes. Made up the volume to 1ml in all the test tubes. 5ml of reagent C was added to each tube. Mixed well and allowed to stand for 10min. Then 0.5ml of reagent D was added. Mixed well and incubated at room temperature in the dark for 30min. After 30min, the blue colour was developed OD values were taken at 660nm. Plotted and standard graph was the amount of itaconic acid calculated.

RESULTS AND DISCUSSION

In the presnt study, isolation and identification of fungi from marine soil. The isolated fungi such as *Aspergillus sparsusi, A. janthinellum, A.ochraceous, A. flavus and A. terreus*. *Aspergillus flavus* were dominantly presented in marine soil sample. So it was selected for further study. The results on the *Aspergillus flavus* was tested for production of itaconic acid. Itaconic acid production and activity was measured by culturing the fungi.

Screening for itaconic acid activity

The result on the *Aspergillus flavus* was tested for production of itaconic acid. Itaconic acid production and activity was measured by culturing the fungi. Initial screening for organic acid under nitrogen limited growth conditions was carried out in a medium. The medium after incubation sterile bromocersor purple added. The clear zone was formed. The previous study was designed to quantify and compare the production of itaconic acid from the four selected microbial fungal species viz., *A. niger, A. terreus, A. nidulans* and *A. flavus* under different physiological conditions using various sources. The influence of various factors which are affecting the production of itaconic acid optimizing these factors to obtain maximum production (Meena *et al*., 2011).

Optimization of pH

Effect of different physicochemical parameters like temperature and pH. In general the itaconic acid was best produced by fungal species. The fermentation medium with adjusted pH of 2, 3, 4, 5 and 6 were used for the determining the influence of pH on itaconic acid production by *Aspergillus flavus*. The observed results in the present study are the optimum pH was found to be 4.The results given in table-1 & fig-1.

Meena *et al*., (2011) reported that *A.niger* and *A. terreus* were known to be the best species for itaconic acid production among the different fungal species studied. However there was no comprehensive study on using latest technologies for increasing the productivity at industrial level and it was not properly established. By keeping this in view, the present study was designed for study on increasing the production of itaocnic acid feasible at commercial level and an attempt has been made to optimize the different physico-chemical parameters required for obtaining the maximum production of itaconic acid using selected *Aspergillus* species.

Optimization of temperature

The external temperature shows a significant effct on the cell growth metabolism and there by the production of itaconic acid. The *Aspergillus flavus* were found to grow in the range 30, 40, 50, 60, 70. The *Aspergillus flavus* were used to produced itaocnic acid at different temperatures mentioned above and production of itaconic acid was observed with all the temperatures studied. A maximum production of 8.766 g/lt. itaconic acid was obtained with *Aspergilluys flavus*. The production of itaconic acid was found to increase with temperature upto 40°C (Table-2&Fig-2)..

Estimation of protein in different pH and temperature of itaconic acid production of *Aspergillus flavus* .

The high amount of protein produced for *Aspergillus flavus* in all pH values and temperature (Table-3,4;Fig-3,4).

Table-1 Effect of pH for itaconic acid production in *Aspergillus flavus*

S.No	pH	Itaconic acid activity activity(IU/ml)
		Aspergillus flavus
1.	2	0.238
2.	3	0.485
3.	4	1.879
4.	5	0.643
5	6	0.781

Fig-1 Effect of pH for itaconic acid production in *Aspergillus flavus*

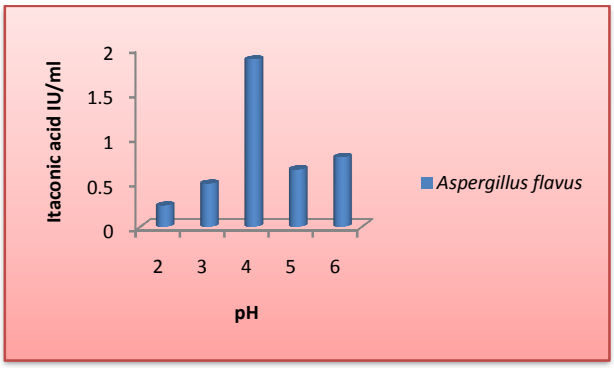


Fig-2 Effect of temperature for itaconic acid production in *Aspergillus flavus*

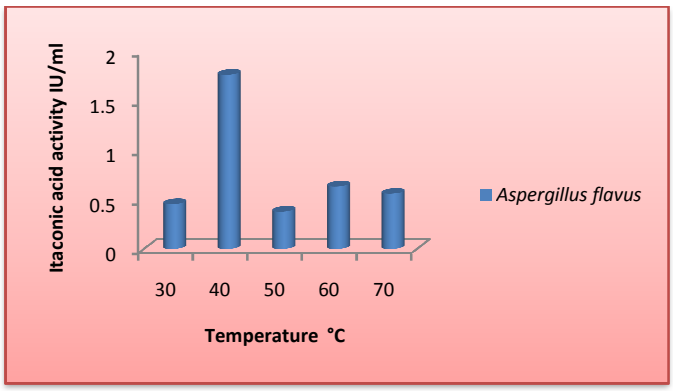


Table-2 Effect of temperature for itaconic acid production in *Aspergillus flavus*

S.No	Temperature(˚C)	Itaconic acid activity (IU/ml)
		Aspergillus flavus
1.	30	0.456
2.	40	1.766
3.	50	0.379
4.	60	0.633
5	70	0.560

Table-3 Estimation of protein in different pH

S.No	pH	Protein activity (IU/ml)
		Aspergillus flavus
1.	2	0.574
2.	3	0.453
3.	4	0.934
4.	5	0.675
5.	6	0.567

Fig-3 Estimation of protein in different pH

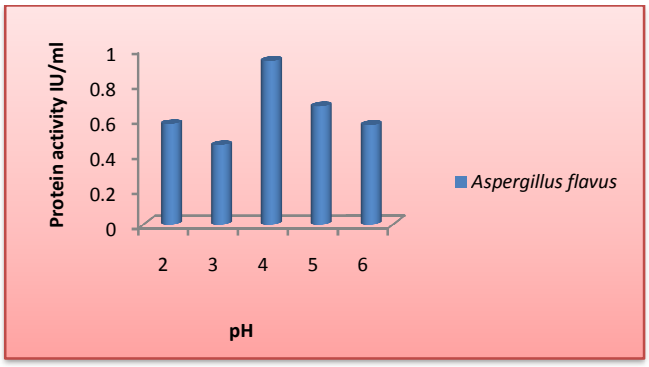
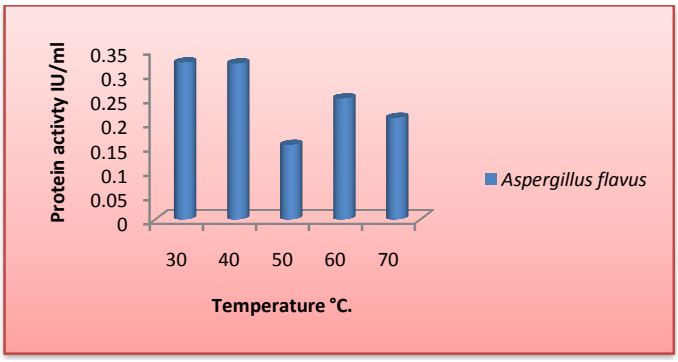


Table-4 Estimation of protein in different Temperatures (°C)

S.No	Temperature(˚C)	Protein activity (IU/ml)
		Aspergillus flavus
1.	30	0.324
2.	40	0.322
3.	50	0.154
4.	60	0.250
5.	70	0.210

Fig-4 Estimation of protein in different Temperatures (°C)



CONCLUSION

In these study concluded that, to date very little research has been directed at the improvement of itaconic acid production. In contrast there has been a larger research effort directed at itaconic acid production to feed the market for biodegradable plastic. Binding material crylic acid emulsion which contains itaconic acid is an excellent non-intertexture fiber products binding material; the bindking agent which contains itaconic acid and polyvinyl chloride monomer also is excellent binding agent for paper and celluloid.

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