PRODUCTION OF ITACONIC ACID THROUGH SUBMERGED FERMENTATION EMPLOYING DIFFERENT SPECIES OF ASPERGILLUS

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ABSTRACT
Itaconic acid was best produced by fungal species than bacterial species. The 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 itaconic 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. For increasing the productivity, the influence of starting substrate concentration, the optimum timing of additional substrate and the possibility of semi-continuous fermentation were analyzed in a series of bench-top and pilot-plant fermentations. Basing on the results of this systematic study, optimal inoculation and fermentation methodology can be developed for commercial application. The present 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 growth studies and kinetics in batch cultures using A. niger, A. terreus, A. nidulans and A. flavus was investigated for itaconic acid production. The specific growth rate (µmax) is maximum for A. terreus (0.04199 hr⁻¹). The yield factor (Yx/s) for cell mass is maximum for A. terreus (0.4976 g/g). The doubling time is minimum for A. terreus (14.84 h), The yield factor (Yp/x) is maximum for A. terreus (0.4387 g/g).

Key words: Itaconic acid, specific growth rate, doubling time and fungal species.

INTRODUCTION
Organic acids with wide applications in various fields are made from living cells commercially. Organic acids like citric acid, gluconic acid, itaconic acid and lactic acids are manufactured by means of such large-scale bioprocesses. Among them, the itaconic acid (Methelene Butanedioic acid common synonyms: Methylene succinic acid, 3-carboxy-3-butanoic acid, propylenedicarboxylic acid) is the most promising one.

Itaconic acid is a colorless crystalline carboxylic acid obtained by the fermentation of carbohydrates. The biosynthesis by fungi from carbohydrates was first reported by Kinoshita, who isolated itaconic acid from the growth medium of an osmophilic fungus, Aspergillus itaconicus. Later, other fungal strains, mainly of the species Aspergillus terreus, were found to be more suitable. At the Northern Regional Research Laboratory (NRRL) of the U.S. Department of Agriculture in Peoria, Illinois, a screening programme of more than 300 strains identified the most published strain, A. terreus NRRL 1960. Attempts were also made to develop a biotechnical process for itaconic acid production. Later, optimized industrial processes were established providing the limited market with itaconic acid. The prominent developments in itaconic acid production (batch fermentation, free suspended biomass) took place before 1966. Over the next 15 years, the interest in itaconic acid production declined, as indicated by the few publications during this
time. Since the early 1980s, there has been increasing concern regarding sustainability, environmental conservation, renewable resources and rising energy costs. 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 1. Its derivatives are used in medicine and cosmetic preparation. Itaconic acid can react with acrylic and methacrylic acid or their esters which is widely employing to prepare resins used in emulsions coating, leather coating, coatings for car, refrigerators and other electrical appliances to improve adhesion, color and weather resistance 3. The market volume of itaconic acid has been estimated to be about 15,000 metric tons per year and is expected to grow if the selling price is reduced.

In general, glucose, sucrose and xylose are preferred raw materials for itaconic acid fermentation, which are known to be utilized efficiently by most of the Aspergillus sp. Though several raw materials are used Molasses, a by-product of sugar industry is a very convenient raw material for itaconic acid production 6. Aspergillus terreus is one of the patented microorganisms reported to utilize molasses as a good source of carbon. The present study was designed for study on increasing the production of itaconic 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.

EXPERIMENTAL

The microbial Strains used in the present study were procured from Microbial type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Czapek Dox medium was used for cultivation of the strains and incubated at 25°C for 5 days. Spores formed were washed out twice with 10 ml distilled sterilized water each time. Spore suspensions containing about log 8/ml were prepared and used as inoculums for the fermentation process. Submerged fermentation process was carried out in a 250 ml Erlenmeyer flask containing 100 ml media. Each flask was inoculated with the given spore suspension and incubated at different temperatures and different time intervals. The effect of molasses concentration on itaconic acid production was investigated.

Czapek Dox medium precultural medium

3 g of sucrose was dissolved in 100 ml of distilled water. 0.2 gr of sodium nitrate, 0.05 gr of Magnesium sulphate, 0.001 gr of ferrous sulphate and 0.1 gr of di potassium hydrogen phosphate was added to the above solution. The pH was adjusted to 7.3 and sterilization was done at 121°C pressure of 15 psi for 20 min in an autoclave.

Production medium

10 % (v/v) of molasses was mixed in 100 ml of distilled water and sterilization was done at 121 °C pressure of 15 psi for 20 min in an autoclave. To it add sterilized 0.25 % (w/v) NH₄Cl, 0.095 % (w/v) MgSO₄, 0.0088 % (w/v) KH₂PO₄ and 0.0004 % (w/v) CuSO₄ and the pH was adjusted to 5.0. The amount of sucrose present in the molasses was estimated by Dinitro salicylic acid (DNS) method 7. For shake flask fermentation, a different inoculum percentage of A. terreus was inoculated to the production medium in a 250 ml shaking flask and cultured on a rotary shaker. The sample was collected at an interval of 24 h. The collected sample was used for the determination of itaconic acid and sucrose consumed was estimated. The qualitative analysis of itaconic acid was measured by UV spectrophotometer at 385 nm.

RESULTS AND DISCUSSIONS

Effect of different physicochemical parameters like temperature, agitation speed, pH and inoculum size, maximum yield, specific growth rate and minimum doubling time were determined. In general, the itaconic acid was best produced by fungal species than bacterial species. The 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 itaconic 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. For increasing the productivity, the influence of starting substrate concentration, the optimum timing of additional substrate and the possibility of semi-continuous fermentation were analyzed in a series of bench-top and pilot-plant fermentations. Basing on the results of this systematic study, optimal inoculation and fermentation methodology can be developed for commercial application. The present 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. And the study was further aimed at studying the influence of various factors which are affecting the production of itaconic acid optimizing these factors to obtain maximum production.

**Optimization of media**

In general, any change in culture conditions greatly influences the production ability of a microbial strain. A complex and variable set of culture conditions which are necessary for obtaining high synthesis of itaconic acid were selected and studied in the present study.

**Effect of Substrate concentration**

A comparative account of production of itaconic acid using different substrate sources at a concentration of 5% (Cane molasses, Potato peel, Banana peel and Rice bran) using *A. niger*, *A. terreus*, *A. nidulans* and *A. flavus* was studied at different time intervals from zero to 120 hours and it was observed that there was a steep increase in itaconic acid production with an increase in time of incubation. The maximum production of itaconic was obtained at 120 hours of incubation with all the selected species and the production was more with the substrate of molasses than other sources. Among all the selected species *A. terreus* has exhibited maximum yield of 8.10 g/lt followed by *A. niger*, *A. nidulans* and *A. flavus* (6.3, 5.6 and 4.8 g/lt respectively). From the results it is apparent that the maximum concentration of itaconic acid was observed with cane molasses and the present findings were in consonance with the observations.

![Fig.-1: Effect of Substrate concentration](image)

**Influence of percentage of Molasses**

The study on variation percentage of molasses in itaconic acid production has shown significant variation on growth and metabolism in turn, in production of itaconic acid. As it was well documented that the
different species of Aspergillus were reported to grow well in percentage range of 3-11% (Bressler and Braun, 2000), in the present study an attempt has been made to determine the optimum percentage of molasses, the varying concentrations of molasses was added to the crude medium (3 to 11%). The results envisaged that optimum itaconic acid production was found to be at 10% of molasses for all the selected species of Aspergillus in fermentation medium at 120 hours of incubation which is 22.5 g/lt for A. terreus and 20.05 , 16.40 and 14.13 g/lt with the A. niger, A. nidulans and A. flavus respectively. A high production of itaconic acid was observed using high concentration of molasses.

**Effect of incubation time**
The cultures were incubated under proper conditions at different time intervals viz. 0, 24, 48, 72, 96, 120 and 144 hrs were used to investigate the influence on itaconic acid production. It was observed that there is a steep increase in the itaconic acid production with an increase in time of incubation showing maximum at 120 hrs, with continuous increase in biomass concentration and simultaneous decrease in the substrate level with the four selected Aspergillus species. A maximum yield of 22.50 g/lt was given by A. terreus followed by A. niger, A. flavus and A. nidulans as 20.05, 16.40 and 14.13 g/lt respectively (Tables 9-12; Figures 9-12). The present results were in agreement with the studies conducted with a species of Aspergillus nidulans wherein a steep increase in itaconic acid production was observed at 120 hours10.

**Effect of pH**
The fermentation medium with adjusted pH of 3.0, 3.5, 4.0, 5.0, 6.0 and 7.0 were used for the determining the influence of pH on itaconic acid production by Aspergillus species and it was observed that the itaconic acid production was found to be maximum at pH 3.5 for all the selected fungal species. The levels of itaconic acid was found to increase with the pH from 3.0 to 3.5 and observed to decrease with further increase in pH from 3.5. A maximum production of 24 g/lt was obtained with A. terreus and effective production was also seen with A. niger, A. flavus and A. nidulans which are 22, 17.50 and 16.10 g/lt respectively. The observed results in the present study are the optimum pH was found to be 3.5.

It is well established that both the internal and external proton concentration play a significant role on the growth and metabolism of a microbe. The microorganisms have the mechanism to maintain the intracellular pH at a relatively constant value, though the pH varies in the external environment. When pH differs from the optimal value, there will be an increase in maintenance energy requirement. The optimum pH of the medium often affects growth and product formation by influencing the uptake of
nutrients, metabolic pathway in itaconic acid biosynthesis and other physiological activities. It was reported that the lack of pH control during the fermentation process may result in strong adverse effects on itaconic acid production and might yield in low levels of itaconic acid.

The results envisage that the extent of itaconic acid production in the present study, indicate that the optimum pH was at 3.5 for all the selected organisms (A. niger, A. terreus, A. nidulans and A. flavus). Thus, this condition not only facilitates cell growth but also act to prevent proliferation of other potential contaminating microorganisms.

Effect of NH₄Cl

Different percentages of ammonium chloride were used to investigate its influence on itaconic acid production. The varying concentrations (0.2 to 0.4%) of ammonium chloride was supplemented to the growth medium of four Aspergillus species in view of the literature support, where ammonium chloride greatly influenced the itaconic acid production by A. flavus. At a concentration of 0.35% NH₄Cl, the maximum itaconic acid production was investigated in all four selected species.
The results were more conspicuous with *A. flavus* compared to other three species

**Effect of temperature**

The external temperature shows a significant effect on the cell growth, metabolism and thereby the production of itaconic acid. All the four species of *Aspergillus* were found to grow in the range of 15 to 45°C. These four species of *Aspergillus* were used to produce itaconic acid at different temperatures mentioned above and production of itaconic acid was observed with all the temperatures studied. A maximum production of 26.10 g/lt itaconic acid was obtained with *A. terreus* and 23.5, 17.90 and 16.90 g/lt of production with *A. niger*, *A. flavus* and *A. nidulans* respectively at 35°C. The production of itaconic acid was found to increase with temperature up to 35°C and a slight decrease was observed with further increase in temperature from 35-38°C. The results that were obtained envisage that the observed results are significant as the calculated value is less than the table value and accept the null hypothesis.

Temperature is one of the important physical factors influencing the growth of the fungal species. Due to high fermentation temperature, it is relatively easy to collect the products and thereby avoid the decrease of yields, often associated with product inhibition. The present investigation suggests that the itaconic acid production at different temperatures of operation, indicating that the optimum temperature is 35°C for obtaining maximum itaconic acid yield. This is in agreement with the observation of Kautola et al. (1985), using *A. terreus* maximum itaconic acid production was achieved at 35°C. After the optimum temperature the over all growth rate began to fall due to increase in rate of microbial death, as the death rate is also a function of temperature. This high value of cell death increases with increase in temperature, than the growth rate. Hence the over all growth rates rapidly decline above the optimal temperature. Apart from this, the product inhibition effect is also more at higher temperatures than at lower temperatures.

When temperature is increased above the optimum, the requirements for cellular maintenance also increase i.e. the maintenance coefficient of cells increase with increasing temperature and with activation energy of 15 to 20 K cal/mol, resulting in a decrease in the yield coefficient.
Effect of agitation speed
To study the effect of agitation i.e. rotation speed on the production of itaconic acid the revolutions used for the production itaconic acid using selected *Aspergillus* species were adjusted to different speeds viz., 150, 200 and 250 rpm. These different agitation speeds show a significant effect on the growth, metabolism and production of itaconic acid. All the four selected species of *Aspergillus* were found to grow well in the range of 150 to 250 rpm. The maximum itaconic acid production was achieved at 200 rpm in all the four selected species. Similar to our earlier observations the *A. terreus* dominated the remaining species giving a maximum production of 27g/lt of itaconic acid at an incubation period of 120 hrs and agitation speed 200 rpm, followed by *A. niger*, *A. flavus* and *A. nidulans* whose production was found to be 24, 18 and 17g/lt respectively. Interestingly a slight decrease in the production of itaconic acid was observed with the increase in rpm from 200 to 250 rpm.

Effect of inoculum size
Varied inoculum size such as 2, 5 and 10% were tried for obtaining the maximum itaconic acid production under optimized conditions such as 35°C temperature, 3.5 pH and 200 rpm. The present investigations have show that 10% (v/v) inoculum size was found to give optimum itaconic acid production by all the selected *Aspergillus* species when incubated for 120 hours. It was observed that the itaconic acid production was increasing between 5 to 10% inoculum. With the further increase in inoculum size and greater than 10%, there was a decrease in itaconic acid production and this phenomenon possibly could be attributed to the competition amongst the cells for nutrients in the medium. Therefore, the fermentation process was carried out with the addition of 10% inoculum in the fermentation medium. A maximum production of itaconic acid of 27g/lt was obtained with *A. terreus* followed by *A. niger*, *A. flavus* and *A. nidulans* whose maximum production at the selected rpm were 24, 18 and 17 g/lt respectively.

The optimized physico-chemical parameters of fermentation medium for itaconic acid production are:

- Cane molasses – 10% (v/v)
- NH₄Cl – 0.35% (w/v)
- pH – 3.5
- Temperature – 35°C
- Incubation time – 120 hrs
- Inoculum size – 10% (v/v)
- Agitation speed – 200 rpm
Identification of high yielding Microbe

Among the four selected species, *A. terreus* has shown maximum itaconic acid production of 27 g/lt, while *A. niger, A. nidulans* and *A. flavus* had shown itaconic acid production of 24, 17 and 18 g/lt respectively, indicating that all the selected species were able to produce itaconic acid. However, the results envisage that the *A. terreus* to be an ideal and good producer of itaconic acid. The results observed in the present study are partly in compliance with those reported by Willke and Vorlop\(^1\). And the level of itaconic acid production also supports their observation, which varies considerably from species to species.
Summary and Conclusions
Recent advancements in the field of industrial biotechnology have led to the development of successful organic acids, which is being used in the production of biodegradable plastics. Itaconic acid is made use as a co-monomer at a level of 1–5% for certain polymer products. It is also important as a constituent for the fabrication of synthetic fibers, coatings, adhesives, thickeners, and binders.

Many of the profitable production processes for organic acids are excellent examples of fungal biotechnology wherein diverse group of microbes are utilized for the efficient production of organic acids. There is no indigenous commercial production of itaconic acid so far. The present study was intended to compare the optimal production for indigenous process from the four sources viz. *Aspergillus niger* MTCC 872, *Aspergillus terreus* MTCC 479, *Aspergillus nidulans* MTCC 818, and *Aspergillus flavus* MTCC 871 and to optimize the crude medium.

The microorganisms were screened for itaconic acid production and *Aspergillus terreus* was found to be a good producer of the itaconic acid. With the above confirmation an attempt was made to optimize the physiochemical parameters as they greatly influence the production levels. One finding in common was that the carbohydrates added to the crude medium had repressive effect. Maximum itaconic acid production was observed at a temperature of 35°C and pH of 3.5 in crude medium in 120 hrs with 10% inoculum by submerged fermentation. In the submerged fermentation the results obtained indicate that the maximum itaconic acid was produced at a temperature of 35°C and pH of 3.5, no significant influence of pH on itaconic acid fermentation between 4.0 and 7.0. Above the temperature of 35°C effect of temperature on the yield of itaconic acid was observed to be very marginal. A crude medium additionally containing 3.5% of ammonium chloride, with an agitation speed of 200 rpm was the best conditions for production of itaconic acid by *A. niger*, *A. terreus*, *A. nidulans* and *A. flavus*.

The studies on the optimization of the process parameters have shown clearly that the *A. terreus* is one of the good producers of the itaconic acid and the kinetic properties were also found to be most dependable for the commercial exploitation. Further, genetic improvement involving mutation studies, identification and up regulation of the necessary genes or turning off the feed back loops will improve the production of itaconic acid making the organism commercially viable.

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