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NEW SPECTROPHOTOMETRIC METHODS FOR STUDIES ON OPTIMIZATION, IMMOBILIZATION AND KINETIC PARAMETERS FOR THE PRODUCTION OF ITACONIC ACID BY *Aspergillus terreus* MTCC 479

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ABSTRACT

Itaconic acid is commercially produced by the cultivation of *Aspergillus terreus* with different carbon sources. Molasses is one of the best carbon sources among various carbohydrates, because it is pure, inexpensive and available in a mass supply. The reaction was carried out at various molasses concentrations, temperatures, incubation time intervals, agitation speed and pH. The present study reveals that the maximum itaconic acid was produced at 35^{o}C, molasses concentration of 10 %, agitation speed of 200 rpm, at 3.5 pH and incubation time of 120 hrs with a yield of 0.4976 g/L, specific growth rate of 0.04199 hr^{-1} and minimum doubling time of 14.84 hrs. The maximum itaconic acid concentration was obtained for 0.5 cm cube and the concentration doubled after 14 days with immobilized mycelium than free mycelium.

Key words: Itaconic acid, *Aspergillus terreus*, Immobilization, Kinetic parameters.

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.

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Itaconic acid is a colorless crystalline carboxylic acid obtained by the fermentation of carbohydrates. Itaconic acid was discovered by Baup (1837)[1] as a thermal decomposition product of citric acid. The biosynthesis by fungi from carbohydrates was first reported by Kinoshita[2], 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.[3] Attempts were also made to develop a biotechnical process for itaconic acid production.[4,5] 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.[5] 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 comonomer at a level of 1-5 % for certain products[6]. Its derivatives are used in medicine and cosmetic preparation. Itaconic acid can react with acrylic and methacrylic acid or their esters, which is widely employed to prepare resins used in emulsions coating, leather coating, coatings for car, refrigerators and other electrical appliances to improve adhesion, color and weather resistance.[6] 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.[7]

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.[8-10] Though several raw materials are used; molasses, a by-product of sugar industry is a very convenient raw material for itaconic acid production. *Aspergillus terreus* is one of the patented microorganisms reported to utilize molasses as a good source of carbon.[11] The present study was designed for increasing the production of itaconic acid feasible at commercial level and an attempt has been made to optimize the different physico-chemical parameters and kinetic parameters required for obtaining the maximum production of

itaconic acid using selected *Aspergillus terreus*.

EXPERIMENTAL

Materials and methods

The microbial strain used in the present study was procured from Microbial type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Czapek Dox medium was used for cultivation of the strain and incubated at 25°C for 5 days.[12] 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 by *A. terreus*.

Czapek dox medium pre-cultural medium

3 g of sucrose was dissolved in 100 mL of distilled water. 0.2 g of sodium nitrate, 0.05 g of magnesium sulphate, 0.05 g of potassium chloride, 0.001 g of ferrous sulphate and 0.1 g of dipotassium hydrogen phosphate was added to the above solution. The pH was adjusted to 7.3 and sterilization was done at 121^{o}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. Sterilized 0.25 % (w/v) NH_4Cl, 0.095 % (w/v) $MgSO_4$, 0.0088 % (w/v) KH_2PO4 and 0.0004 % (w/v) $CuSO_4$ were added to it and the pH was adjusted to 5.0.[13,14] The amount of sucrose present in the molasses was estimated by dinitrosalicylic acid (DNS) method.[15] For shake flask fermentation, a different innoculum 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 DISCUSSION

Effect of various percentages of molasses on itaconic acid production

The study on variation of percentage of molasses on itaconic acid production has shown significant variation on growth and metabolism and in turn, in production of itaconic acid. As it was well documented that *Aspergillus terreus* was reported to grow well in percentage range of 3-11%, 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 from Fig. 1 shows that optimum itaconic acid production was found to be at 10% of molasses for *Aspergillus terreus* in fermentation medium at 120 hours of incubation, which is 22.5 g/L. In agreement with our present findings, the observations of Butterworth[16], a similar high production of itaconic acid was observed using high concentration of molasses.

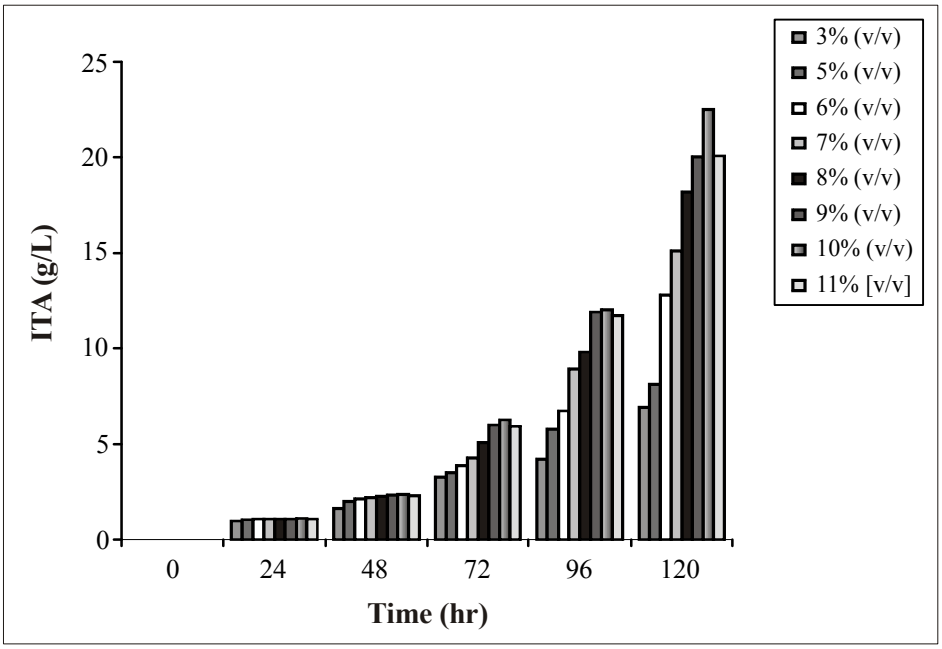

Fig. 1: Effect of various percentages of molasses on itaconic acid production

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. A maximum yield of 22.50 g/L was given by A. terreus. The present results from shown in Fig. 2 are agreement with the studies conducted with a species of *A. terreus* wherein a steep increase in itaconic acid production was observed at 120 hours.[17]

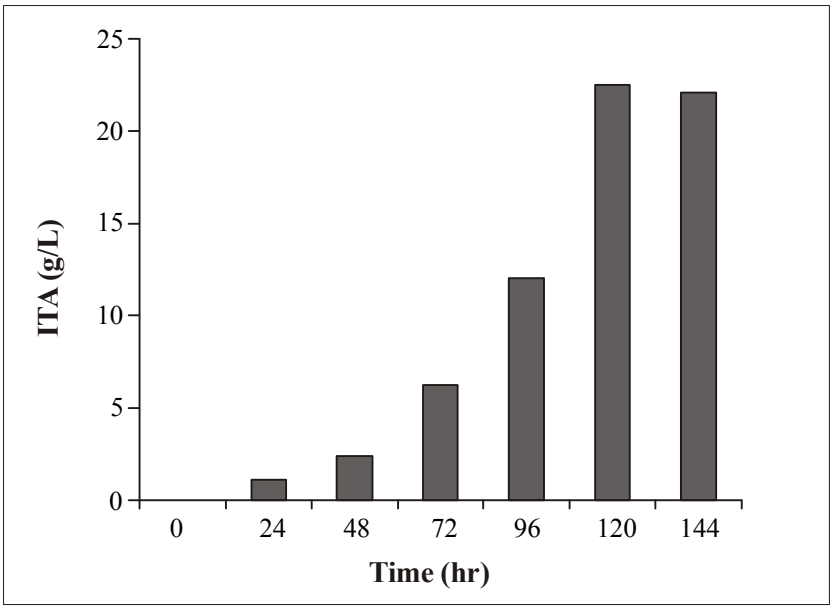

Fig. 2: Effect of incubation time

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 *A. terreus* and it was observed that the itaconic acid production was found to be maximum at pH 3.5. 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/L was obtained with *A. terreus* The observed results from shown in Fig. 3 indicate that the present study are in corroboration with the report by Friedkin[18], wherein 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[19].

The results envisage that the extent of itaconic acid production in the present study indicate that the optimum pH was at 3.5 for *A. terreus*. Thus, this condition not only facilitates cell growth but also acts to prevent proliferation of other potential contaminating microorganisms.

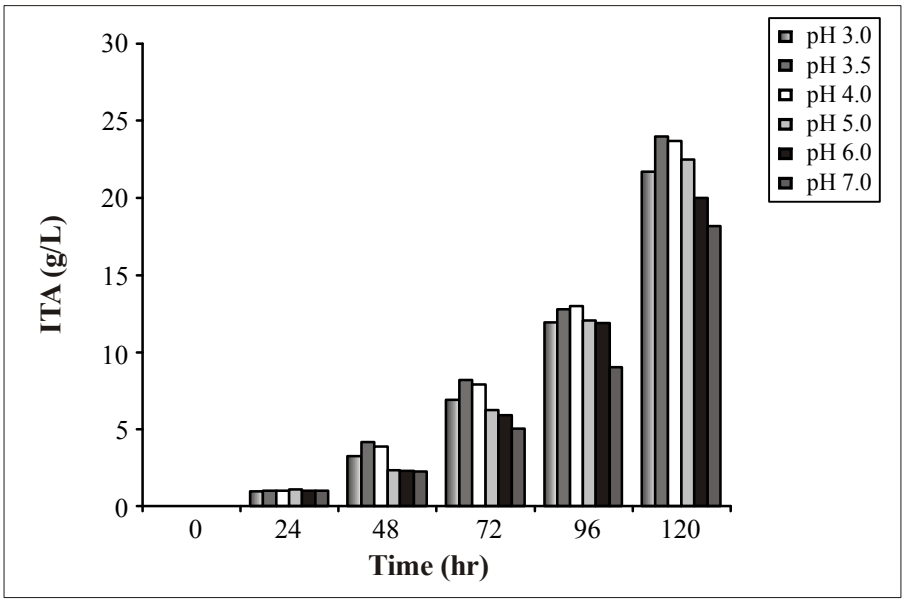

Fig. 3: Effect of pH

Effect of temperature

The external temperature shows a significant effect on the cell growth, metabolism and thereby the production of itaconic acid. *A. terreus* was found to grow in the temperature range of 15 to 45^{o}C. A maximum production of 26.10 g/L itaconic acid was obtained with *A. terreus*. The production of itaconic acid was found to increase with temperature up to 35^{o}C and a slight decrease was observed with further increase in temperature from 35-38^{o}C. The present observed results are found to be in coincidence with the results obtained by Berger.[20]

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. As the growth at high temperature is generally fast and results in large turn over for the fermentation as reported by Ju and Wang[21], this may possibly be true in the present observation also.

The present investigation suggests that the itaconic acid production at different temperatures of operation, indicates that the optimum temperature is 35°C for obtaining maximum itaconic acid yield. This is in agreement with the observation of Kautola *et al*.[8], using *A. terreus*, where 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.[22] 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.[20] 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 increases 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.[23] The results are shown in Fig. 4.

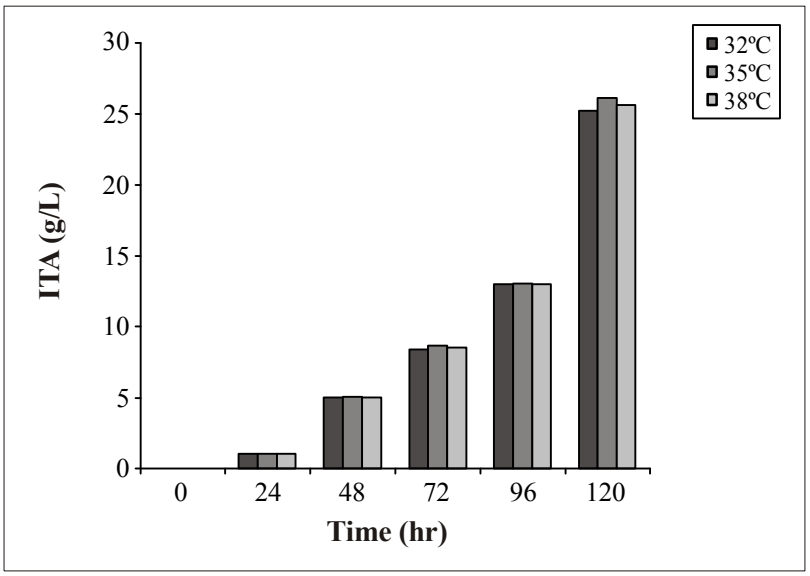

Fig. 4: Effect of temperature

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 *A. terreus* was 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. The maximum itaconic acid production of 27g/L was achieved at an incubation period of 120 hrs and agitation speed 200 rpm. Interestingly, a slight decrease in the production of itaconic acid was observed with the increase in rpm from 200 to 250.

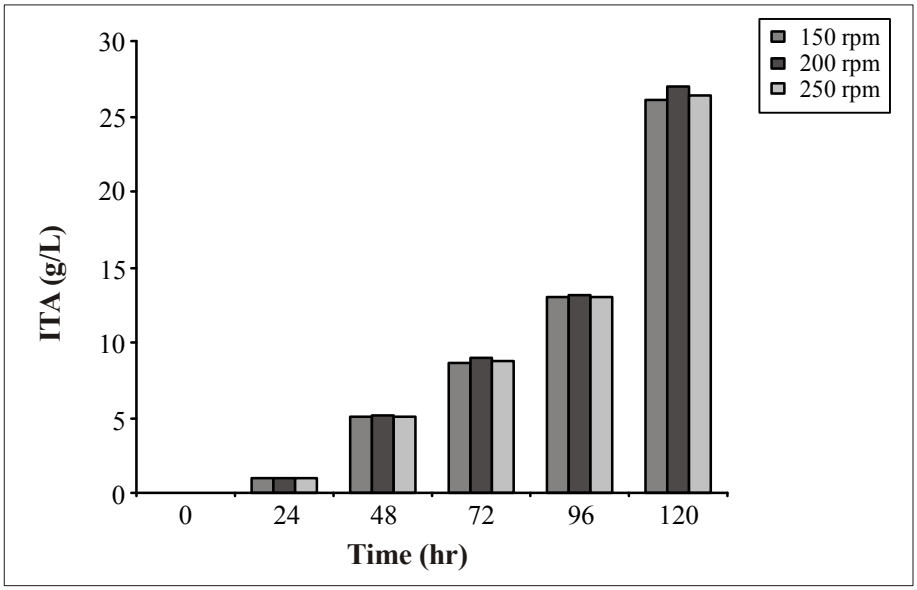

Fig. 5: Effect of agitation speed

Rate of substrate consumption, biomass concentration and product formation

The rate of product formation based on rate of substrate consumption and biomass estimation was studied for *A. terreus*. After 120 hours incubation, the rate of substrate consumption and product formation were analyzed and it was found found that the product formation is inversely related to substrate consumption and maximum concentration of itaconic acid of 27 g/L was seen at 5.5 g/L substrate concentration with *A. terreus*. The results are shown in Fig. 6.

The effect of specific growth rate (μmax) on itaconic acid production

The specific growth rate was investigated and it was noticed that with increase in

time of fermentation, there is a decrease in substrate concentration and increase in biomass concentration. To calculate the specific growth rate, the data points relating to the reciprocal of substrate utilization and biomass concentration are required. The maximum specific growth rate for *A. terreus* was determined as 0.04199 hr^{-1}. The results are shown in Fig. 7.

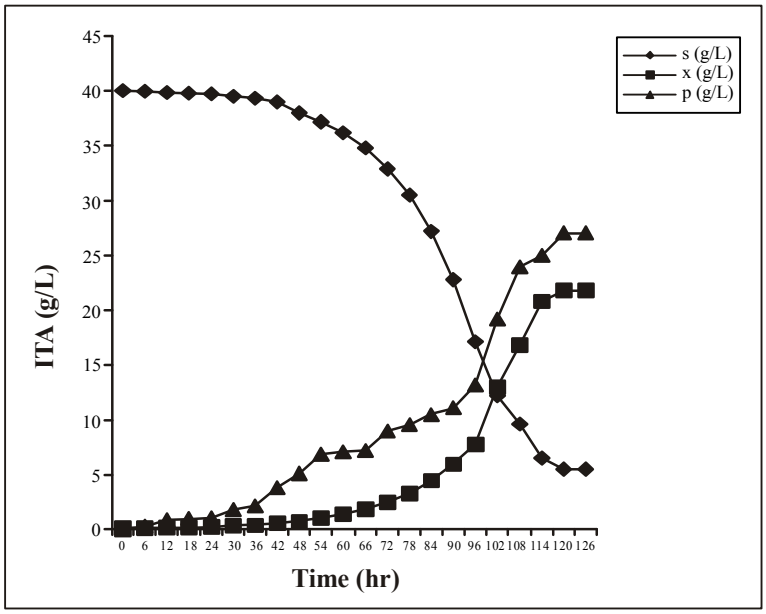

Fig. 6: Rate of substrate consumption (s), biomass concentration (x) and product formation (p)

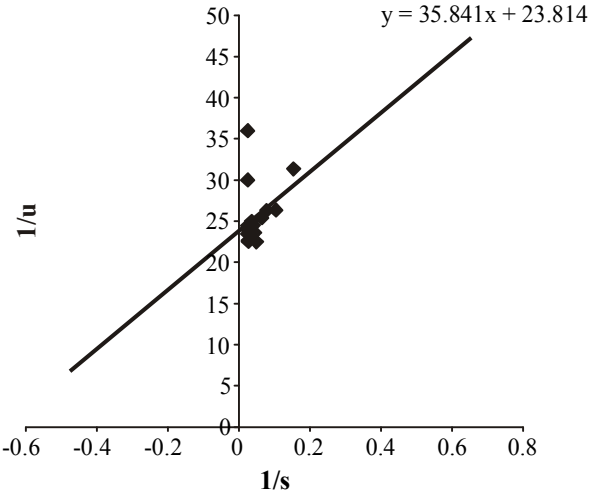

Fig. 7: Effect of specific growth rate (μ_{max}) on itaconic acid production

Effect of the yield factor of cell mass (Yx/s) on itaconic acid production

The yield factor ($Y_{x/s}$) is a ratio of amount of biomass formed per amount of substrate consumed. This was determined from plot of r_s/x vs r_x/x. The slope of the graph gives yield factor. The 6 hours of incubation time interval was selected for determining yield factor ($Y_{x/s}$) for *A. terreus*. The maximum yield factor ($Y_{x/s}$) in *Aspergillus terreus* was observed as 0.4976, g/L. The results are shown in Fig. 8.

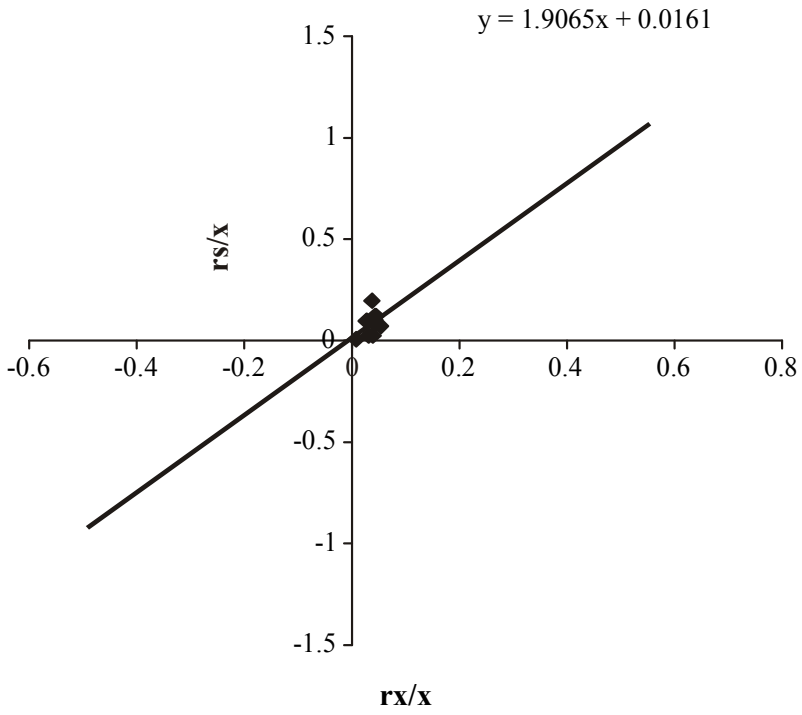

Fig. 8: Effect of the yield factor of cell mass ($Y_{x/s}$) on itaconic acid production

Effect of cell doubling time on itaconic acid production

The time required for the microbial mass to double is called doubling time. The cell doubling time was investigated. It was determined during the exponential growth of the microbe, characterized by straight line on a semi – log plot of ln x vs time. The minimum cell doubling time was observed as 14.84 hrs. The results are shown in Fig. 9.

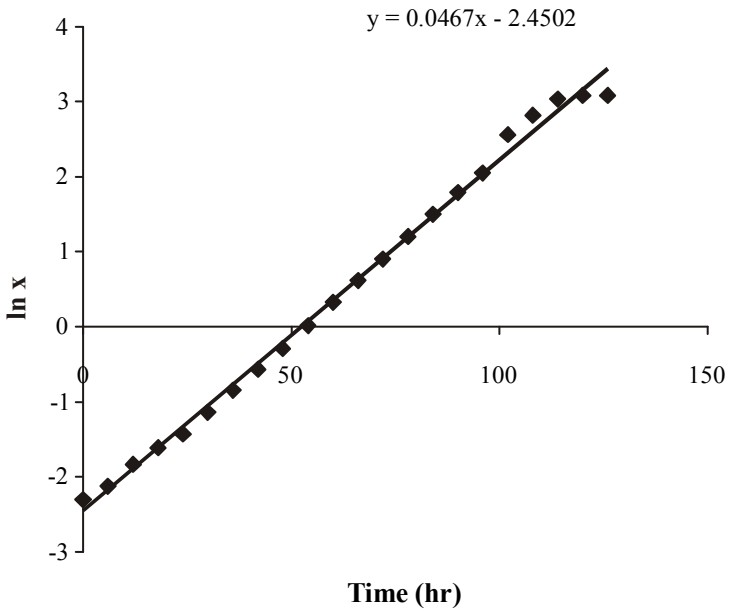

Fig. 9: Effect of cell doubling time on itaconic acid production

Immobilization of Aspergillus terreus

For further improvement and to maximize the itaconic acid production, an attempt was made to develop immobilized cultures with the *A. terreus* using polyurethane foam of size 0.5 and 1.0 cm cube as it was reported to influence the itaconic acid production in earlier studies conducted by Kautola *et al*.[8] The present investigation has shown that 0.5 cm cube, which has almost double the total surface area of 1 cm cubes, gave the highest itaconic acid and also the concentration of itaconic acid was increased several folds after 14 days with immobilized mycelium (42.0 g/L for 1.0 cm cube and 52.0 g/L for 0.5 cm cube) than with free mycelium. The results were in compliance with the observations of Prucssc *et al*.,[24] wherein greater surface area on polyurethane foams gave the highest itaconic acid concentration from *A. terreus*.

The studies on optimization of various physico-chemical and nutritional requirements for the production of itaconic acid was found to be very effective. The production of itaconic acid by the different organisms after optimizing the culture parameters revealed to have 3.4 fold increases for *A. terreus*. The results are shown in Fig. 10.

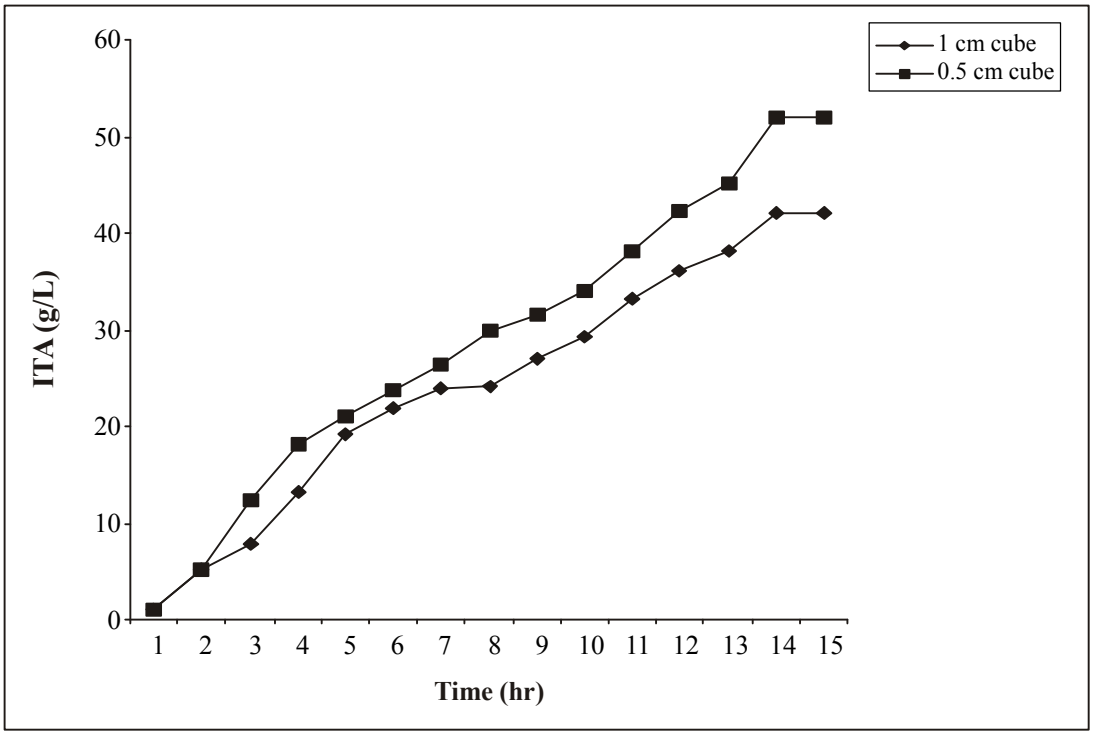

Fig. 10: Immobilization of *Aspergillus terreus*

CONCLUSIONS

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 indigeneous commercial production of itaconic acid so far. The present study was intended to compare the optimal production for indigenous process from *Aspergillus terreus* MTCC 479 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 these above informations, an attempt was made to optimize the physiochemical parameters, which 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^{o}C and pH of 3.5 in crude medium in 120 hrs with 10% inoculum by submerged fermentation.

The growth studies and kinetics in batch cultures using *A. terreus* was investigated for itaconic acid production. The specific growth rate (μ_{max}) for *A. terreus* is (0.04199 $hr^{-1)}$, the yield factor ($Y_{x/s}$) for *A. terreus* is (0.4976 g/g) and the doubling time for *A. terreus* is (14.84 h). The itaconic acid production was observed using immobilized *A. terreus* on polyurethane foam (0.5, 1.0 cm cube). The maximum itaconic acid concentration was obtained for 0.5 cm cube and doubled after 14 days immobilized mycelium than with free mycelium.

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