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RESEARCH ARTICLE

Optimization and Production of Itaconic Acid from Estuarine *Aspergillus terreus* using Economically Cheaper Substrate

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ABSTRACT

Itaconic acid (IA) is an organic acid. It is used in medicine, resins, agriculture, and polymer production. In the present study, sediment sample was collected aseptically from Vellar estuary, Parangipettai, Tamil Nadu, India. About 1.6×10^2 to 6.1×10^3 colony forming units/g density of fungal strains were isolated and screened for IA production. As a result of the tested strains *Aspergillus terreus* was observed as the most potential strain. Optimization was done at different temperatures (25–45°C), in different pH (5.0–7.0). The impact of salinity on IA production was evaluated using various salinity (5–25 ppt), carbon sources (1% w/v of glucose, sucrose, dextrose, and maltose), nitrogen sources (0.5% sodium nitrate, ammonium nitrate, and potassium nitrate), and cheaper sources (1% w/v molasses, jackfruit waste, wheat bran, and coconut oil cake). As a result optimized culture condition for IA production was 1% w/v of glucose - best carbon source, 1% w/v molasses - best cheaper carbon source, 0.5% of sodium nitrate - best nitrogen source, salinity - 20 ppt, temperature - 40°C, and pH - 5.5 and incubation time – 96 h. Compared to glucose (0.41 mg/ml) production of IA was high when molasses (0.61 mg/ml) was used as carbon source, it is also economically good. Mass scale culture was done using molasses instead of glucose with an optimized parameter. After mass scale culture, IA production was 6.3g/l.

Key words: Cheaper substrates, estuarine *Aspergillus terreus*, itaconic acid, optimization

INTRODUCTION

Marine-derived fungi are rich sources of metabolites. Fungi produce a variety of lipids including fatty acids in free or esterified form, for example, glycerides, phospholipids, glycolipids, sterol esters sphingolipids, or simple esters as well as other lipids, for example, free sterols, sterol glycosides, and hydrocarbons. Organic acids such as citric acid, gluconic acid, itaconic acid (IA), and lactic acids are manufactured by means of such large-scale bioprocesses. Among them, the IA (methylene butanedioic acid common synonyms: Methylene succinic acid, 3-carboxy-3-butanoic acid, propylenedicarboxylic acid) is the most promising one.[1]

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It is a white crystalline unsaturated dicarbonic acid in which one carboxyl group is conjugated to the methylene group. There is a 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.[2]

In general, glucose, sucrose, and xylose are preferred raw materials for IA fermentation, which are known to be utilized efficiently by most of the *Aspergillus sp.*[3] The present study was designed for study on increasing the production of IA feasible at commercial level and an attempt has been made to optimize the different physico-chemical parameters required for obtaining the maximum production of IA using *Aspergillus terreus*.

MATERIALS AND METHODS

Isolation of fungi

Sediment sample was collected aseptically from Vellar estuary, Parangipettai, Tamil Nadu, India. 1 g of sediment sample was mixed in 9 ml and 99 ml sterile water blank, respectively. This suspension was serially diluted up to 10^{-4}. 1 ml of the diluted sample was taken from 10^{-3} to 10^{-4} dilutions and was pour plated using 15–20 ml potato dextrose agar prepared in 50% sea water and incubated at 30°C for 5 days.

Screening of potential strain

Screening for high IA producers was done by the gradient plating method.[4]

Identification of strain

Isolated potential strain was stained using lactophenol cotton blue and visualized under the microscope.

IA Production

Czapek Dox medium (sucrose 2.25 g, sodium nitrate 0.15 g, magnesium sulfate 0.037 g potassium chloride 0.037 g, ferrous sulfate 0.007 g, dipotassium hydrogen phosphate 0.07 g 50% sea water 100 ml, and pH 7–7.2) is used for the fermentation process.

Optimization of media for IA production

For optimization of IA production to find the optimum culture conditions for its production, the strains were cultured at different temperatures (25–45°C), in different pH (5.0–7.0). The impact of salinity on IA production was evaluated using various salinity (5–25 ppt). Carbon sources (1% w/v of glucose, sucrose, dextrose, and maltose), nitrogen sources (0.5% sodium nitrate, ammonium nitrate, and potassium nitrate), and cheaper sources (1% w/v molasses, jackfruit waste, wheat bran, and coconut oil cake). All the experiments were carried out in 500 ml conical flasks containing 100 ml of production medium in a shaker at 150 rpm at room temperature.

Mass scale culture of IA production

At optimized parameters, 1 ml of culture was inoculated 1000 ml of production media in a shaker at 150 rpm at room temperature.

Estimation of IA

Estimations were conveniently carried out by modifying the permanganate method.[5] The assay has a range of 0–200 g of IA. Stock solutions were prepared as follows: Metaphosphoric acid pellets (8.5 g) were dissolved in 20 ml of matter; the filtered solution could be kept 3–4 day. Potassium permanganate, 5 ml of 0.1 N solution, was diluted to 100 ml immediately before use. A standard solution of 1 g of pure itaconic acid in 1 L 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 tube. The tubes were chilled in ice for 10 min, 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 min. The optical density was determined immediately by ultraviolet spectrophotometer at 540 nm.

Extraction of IA

The fermented broth was treated with ethyl acetate (V: V) and incubated overnight. The mixture (fermented broth and solvent) was shaken vigorously for 30 min and kept in stationary condition for another 30 min to separate the solvent from the aqueous phase. The organic extract was separated, dried over anhydrous sodium sulfate and concentrated *in vacuo* to yield crude.

RESULTS AND DISCUSSION

IA is used worldwide in the industrial synthesis of resins such as polyesters, plastics, and artificial glass and the preparation of bioactive compounds in the agriculture, pharmacy, and medicine sectors coatings, and other industrial products. The fungal density of sediment sample was varied from 1.6×10^2 to 6.1×10^3 colony forming units/g isolated strains were screened for IA production. Among the screened strains most potential strain was *Aspergillus terreus*. Itaconic acid was first reported

and extracted from *Aspergillus itaconicus*. Later, other fungal strains, mainly of the species *A. terreus*, were found to be more suitable.[6]

Optimization of *A. terreus* among the different temperature maximum production was observed at 40°C of about 0.31 mg/ml [Figure 1]. Another researcher observed 40°C is the optimum temperature for IA production.[2] Temperature is one of the important physical factors influencing the growth of the fungal species.

The temperature shows a significant effect on the cell growth, metabolism and thereby the production of IA.[7] After the optimum temperature, the overall 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 then the growth rate. Hence, the overall growth rates rapidly decline above the optimal temperature.

Maximum IA production recorded at pH 5.5 (0.36 mg/ml) [Figure 2]. Contrarily another researcher obtained that pH 3 is the optimum for IA production.[8] The production was gradually decreased when pH was increased. Salinity is one of the important parameters for marine microbial product development. Different salinity like 5–25 ppt was tested for IA production. Maximum production was observed at 20 ppt (0.39 mg/ml) [Figure 3]. Marine organisms and their products are greatly influenced by the salinity of seawater.

About 1% w/v of glucose, sucrose, dextrose, and maltose were chosen for optimization of IA production. Glucose influence the production of IA compared to other carbon sources. When glucose was used as carbon source, the production was 0.41 mg/g [Figure 4]. *Pseudomonas antarctica* to utilize glucose and produce maximum quantity of IA compared to glycerol.[9] *Ustilago maydis* able to convert the glucose to itaconic acid.[7] Over 300 strains of *A. terreus* were screened and found 11 as efficient producers of IA from glucose.[10]

Among the nitrogen sources optimized maximum production found to be 0.45 mg/ml [Figure 5] when sodium nitrate was used as nitrogen source. In the present observation sodium nitrate influence, the IA production compared to other nitrogen sources. KNO_3 influence the yield of IA production from *A. itaconicus*.[6]

New biotechnological methodologies involving fermentation processes and technologies that use alternative cheap substrates as the carbon source are economically important. A comparative account of

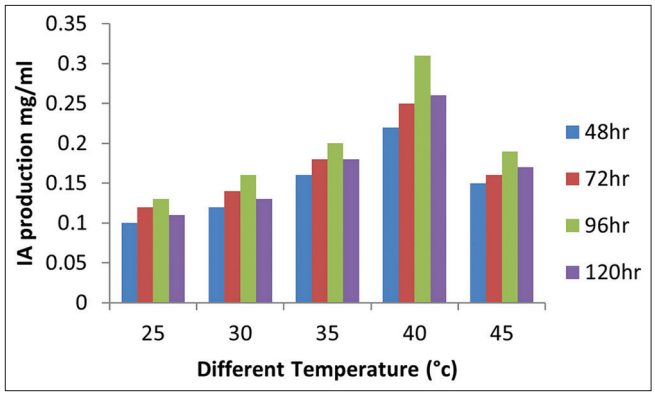

Figure 1: Effect of temperature on itaconic acid production

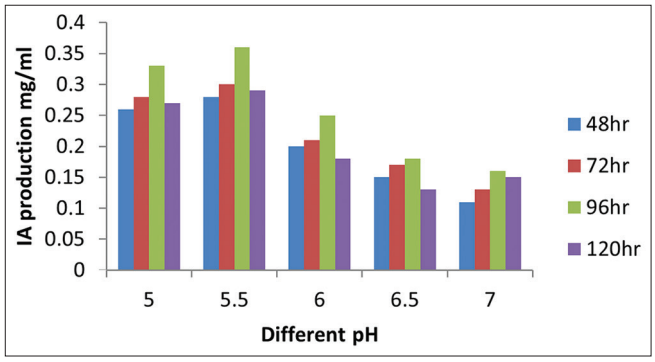

Figure 2: Effect of pH on itaconic acid production

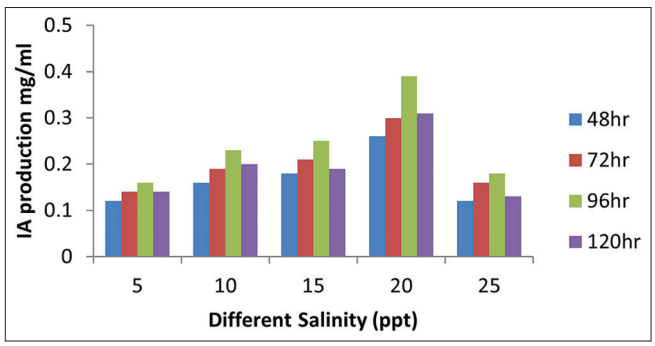

Figure 3: Effect of salinity on itaconic acid production

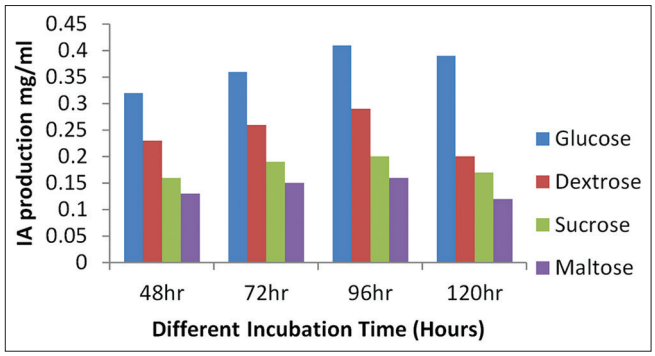

Figure 4: Effect of carbon sources on itaconic acid production

the production of IA using different cheaper substrate sources at a concentration of 1% w/v molasses, jackfruit waste, wheat bran, and coconut oil cake were done. The maximum production of itaconic acid was obtained by 1% w/v molasses of about

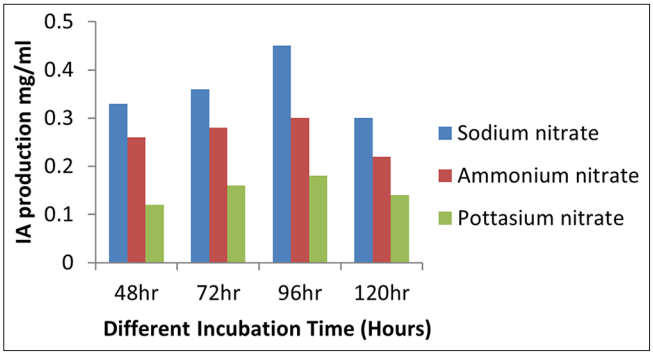

Figure 5: Effect of nitrogen sources on itaconic acid production

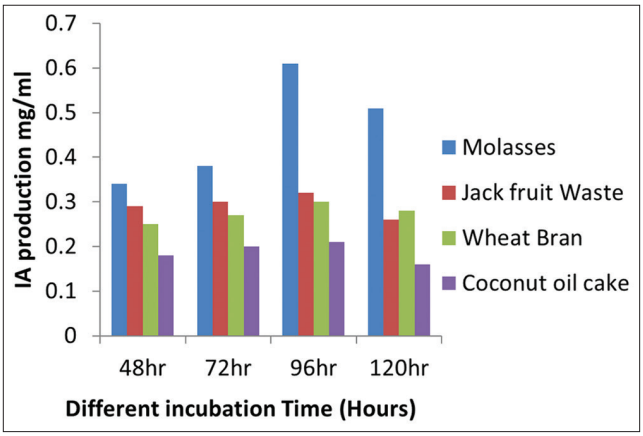

Figure 6: Effect of cheaper sources on itaconic acid production

0.61 mg/ml [Figure 6]. Regarding the incubation time, maximum production was observed at 96 h. Remarkably from the result compared to commercially available carbon source glucose (0.41 mg/g), there is an increased production of IA was observed with molasses (0.61 mg/g). Molasses influence the IA production. Industrial production of IA by submerged fermentation was initiated by Pfeifer Co. Inc.[11] IA was also produced from corn starch[12] sago starch hydrolysate,[13] and Jatropha seed cake.[14] Although several raw materials are used molasses, a by-product of the sugar industry is a very convenient raw material for IA production.[10] *A. terreus* is one of the microorganisms reported to utilize molasses as a good source of carbon.

Mass scale culture was done by optimized parameter, for example, 1% w/v molasses, 0.5% sodium nitrate, salinity - 20 ppt, pH 5.5, Temperature - 40°C, and incubation time - 96 h. After mass scale, IA production was 6.3 g/l.

IA is used in the development of successful organic acids, which is being used in the production of biodegradable plastics. IA is made use as a comonomer 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.

CONCLUSION

A number of marine fungi were screened for IA production, and marine *A. terreus* was found to be a good producer of the IA. With the above confirmation, an attempt was made to optimize the physiochemical parameters as they greatly influence the production levels. From the above study, it was concluded that when the medium was composed with molasses and set at the above conditions maximum yields of IA can be obtained.

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