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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(2): 373-376
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www.thepharmajournal.com
Received: 10-12-2022
Accepted: 23-01-2023

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Itaconic acid production by fungal isolate ITA27

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DOI: https://doi.org/10.22271/tpi.2023.v12.i2e.18453

Abstract
Itaconic acid is one of industrially important organic acid produced by fungi. It is an unsaturated dicarboxylic acid, which can be used as a replacement for petroleum base chemicals. It can be used as monomer in synthesis of biodegradable polymer such as synthesis of fibre, plastics, detergents, rubber, lubricants and bioactive compounds. In this study, thirty five fungal isolates were retrieved from soil samples collected from different sites of Hisar (Agri-tourism Centre, HAU fields CCSHAU Hisar and field of Khanda Kheri, Hisar). Six isolates were selected on the basis of primary screening for organic acid production done on Czapek Dox agar/Potato dextrose agar using bromocresol green as pH indicator. Selected fungal isolates were observed under microscope for morphological characteristics. Fungal isolate ITA27 which was *Aspergillus* sp. on basis of morphological characteristics was selected for production of itaconic acid under submerged fermentation. HPLC analysis of itaconic acid produced by fungal ITA27 showed peak having similarity to standard itaconic acid. Fungal isolates ITA27 showed production of 1.28g/l itaconic acid after incubation period of 8 days.

Keywords: Itaconic acid, Fungi, Polymer, HPLC, Fermentation

Introduction
Microbial fermentation is a process for value-addition of products. Microbes can be utilized to produce valuable metabolites, which can be used as an alternative for fossil based chemicals. These products have various advantages such as low cost, reusability and also lead to pollution free production [1]. Organic acid is one of such product produced by micro-organisms. Some of example of organic acid produced by microbes are citric acid, itaconic acid, succinic acid and gluconic acid. Microorganisms also produce organic acids for solubilisation of minerals such as phosphorus, potassium and zinc which help in improving agricultural productivity [2].
Organic acids can be produced by bacteria, fungi and yeast, but most commonly fungi are exploited as they can produce it in higher amount using low cost organic wastes [3]. Most common method used for microbial organic acids is submerged fermentation. Almost 80% of organic acids in industries are produced using submerged fermentation as it give higher productivity and is less costly. Now a days, solid state fermentation is used in which organic wastes can be used as substrate for organic acid production, which provide both carbon source and energy to fungi [4].
Itaconic Acid (IA), one of the important organic acid has various applications. It has ability to make polymers because of presence of free carboxyl groups [5]. It has application in replacing petroleum based chemicals which will reduce negative impact on environment [6]. Itaconic acid is mainly produced by fungi using submerged or solid state fermentation [7]. It is used in manufacturing of resins, Itaconic acid (IA) (2-methylidenebutanedioic acid), an unsaturated dicarboxylic acid, is a highly desired platform chemical, which is used as a building block or additive in manufacturing fiber, resins, lattices, plastic, detergents, rubber, paint, surfactants, lubricants and bioactive compounds [8, 9] (Bafana and Pandey, 2018; Komáromy *et al*., 2019).

Materials and Methods
Fungal isolation
Fugal cultures were isolated from soil samples collected from different sites of Hisar (Agri-tourism Centre, HAU fields CCSHAU Hisar and field of Khanda Kheri, Hisar) using serial dilution agar plating method on potato dextrose agar media. Isolates were purified and maintained at 4 °C for further use.

Screening for organic acid production

Qualitative screening for production of organic acid was carried out by plate assay on potato dextrose agar amended using bromocresol green. Fungal cultures were spotted on agar plates and incubated at 30 °C for 4-7 days. Production of yellow zone around fungal culture was observed as it indicates organic acid production by fungi. Fungal culture producing organic acid were characterized on basis of morphological observation [10]. Quantitative screening for organic acid production was carried out using potassium permanganate method.

Submerged fermentation

Production of organic acid from fungal isolate was carried out using submerged fermentation. The production was carried out in 500 mL Erlenmeyer flasks containing 250 mL production media (Glucose 80G; $(NH_4)_2So_4$- 0.5g; KH_2PO_4- 1.7g; Na_2HPO_4- 12.0g; $CaCl_2$- 0.02g; $ZNSO_4.7H_2O$-0.02g; $FeSO_4.7H_2O$-0.02g; $MnSO_4.7H_2O$-0.02g; Thiamine HCl-0.006; Yeast extract-0.5 g having initial pH 6.0 amended with bromocresol green: 32 mg/L. The concentration of organic acid was determined at different time intervals up to 14 days [11].

Extraction and analysis

After submerged fermentation, cell mass of fungi was separated from broth using filtration followed by centrifugation at 10000 × g for 10 min. Extraction of itaconic acid from fermentation broth was carried out using ethyl acetate as a solvent with the help of separating funnel. Organic solvent layer was separated from aqueous layer and then subjected to analysis using HPLC Analysis was carried out using HPLC at 210 nm using RP-18 column (end cap-ped, 250 × 4.6 mm I.D.; Merck, Darmstadt, Germany) with the mobile phase of 5 mM Na_3PO_4 (pH 3) at 1 ml/min [12]. The peak of IA was identified using standard IA (Sigma-Aldrich, St Louis, MO).

Results and Discussion

Screening of fungal isolates

Thirty five fungal Isolates were retrieved from soil samples collected from various sites. Among all the isolates retrieved, four isolates produced yellow colour zone on the plates amended with bromocresol green. The formation of a yellow zone around the inoculation was used as an indication of the formation of organic acids. For the strains that showed a positive result, the yellow zone diameter was measured. Significant yellow zone was reported in isolate ITA27, which was selected for production of organic acid under submerged fermentation. On the basis of morphological characteristics, isolate ITA 27 showed similarity to *Aspergillus sp*. In the earlier studies, maximum itaconic acid production was reported in *Aspergillus* species, so utilized in production of organic acid at commercial level [13].

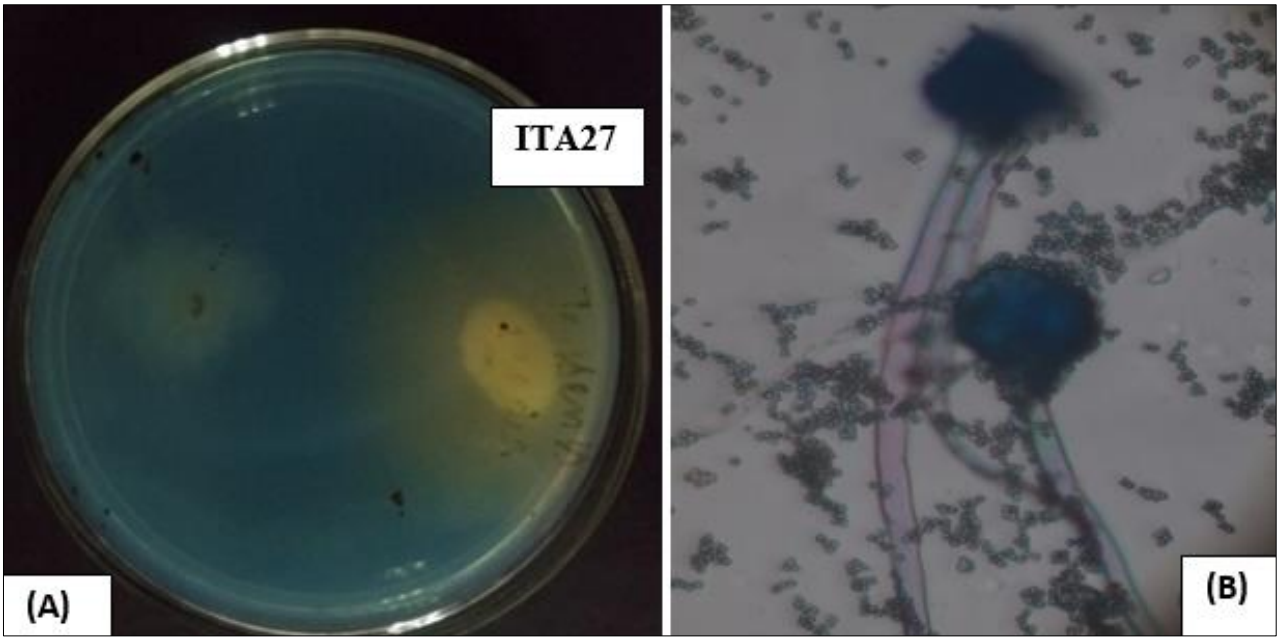

Fig 1(a): Yellowish color zone around fungal isolate ITA27 indicating production of organic acid 1
(b): Microscopic observation of ITA27

Submerged fermentation

Change in colour of broth was observed for itaconic acid production during submerged fermentation. Fungal isolate ITA27 showed production of 1.28g/l of organic acid after incubation period of 8 days. Decrease in organic acid production was observed with further increase in incubation time. The reason for this may be deficiency of nutrient with time or increase in accumulation of toxic substances. Further, fungal growth is also inhibited by accumulation of organic acid. Gnanasekaran *et al.*, 2018 [14] observed maximum itaconic acid production after 168h incubation in their studies [14]. Similarly, maximum production of citric acid was observed by *A. Niger*. Using cassava bagasse was achieved after 120 h. Maximum itaconic avid production from *Ustaligo maydis* was observed by Rafi *et al.*, 2012 after incubation period of 120 h [15].

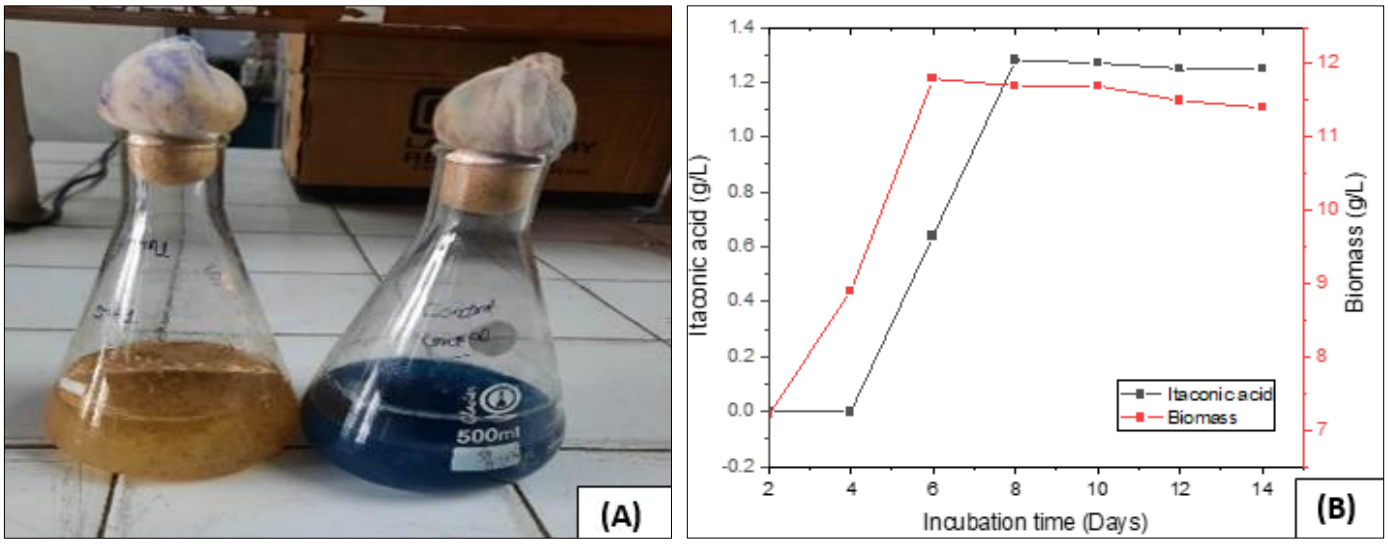

Fig 2: Change in colour of production broth during itaconic acid production by fungal isolate (A) Itaconic acid and biomass production by fungal isolates ITA27 (B)

Extraction

Maximum extraction was achieved at supernatant-solvent ratio of 1:2. After that decrease in extraction was observed with increase in solvent. Gnanasekaran *et al.*, 2018 [14] observed extraction of itaconic acid produced by *Aspergillus niveus* at aqueous to the organic ratio of 1:2 using butanol as solvent [14]. Main problem faced by industries is the extraction of organic acid from fermentation broth. Further analysis was carried out using HPLC. Figure 3 (a) shows the HPLC chromatogram of standard, while Figure 3(b) shows the HPLC chromatogram of acid produced by ITA27.

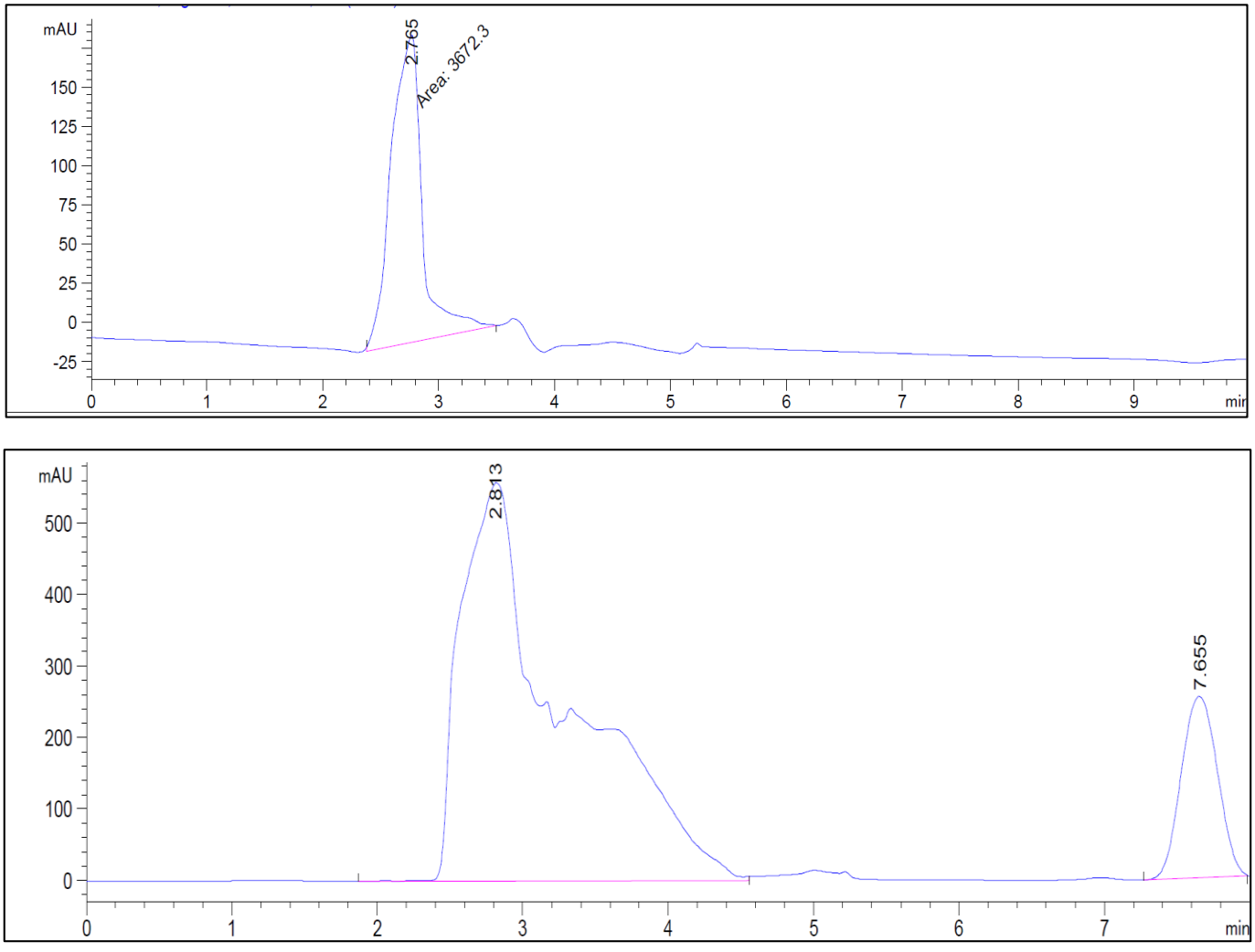

Fig 3: (a) HPLC profile of itaconic acid standard (b): HPLC profile of itaconic acid produced by ITA27

Retention time for standard was found at 2.765 min^{-1}, while retention time for ITA27, it was found 2.813 min^{-1} which shows similar nature of organic acid produced as that of standard itaconic acid. In the standard concentration of itaconic acid used was 50 ppm. On the basis of standard curve amount of itaconic acid produced was found to be 68.77 ppm.

Conclusions

In this work, fungal cultures capable of producing itaconic acid were isolated from soil samples. Production of itaconic acid was carried out using submerged fermentation. Extraction of itaconic acid from fermented broth was carried out using ethyl acetate. Further analysis was confirmed using HPLC.

Acknowledgements

The author acknowledges the authority of Chaudhary Charan Singh Haryana Agricultural University for providing facilities to carry out this research

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