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Production of itaconic acid by *Ustilago maydis* from agro wastes in solid state fermentation

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ABSTRACT

Itaconic acid (IA) is one of the hopeful substances within the cluster of organic acids. IA is used in artificial glass, bioactive compounds in pharmacy, medicine, agriculture, for the synthesis of fiber, resin, plastic, rubber, paints, surfactant, ion-exchange resins and lubricant. Most recurrently used microorganism for commercial production of IA is *Aspergillus terreus*. Some filamentous fungi belonging to *Ustilaginales* also produce IA. In the present work, an attempt was made to produce IA by *Ustilago maydis* employing Solid State Fermentation (SSF) from various agro wastes like ground nut shells, rice bran, rice husk, orange pulp, ground nut oil cake, orange pulp and sugarcane bagasse as carbon substrates, which were used after pretreatment. 10 g of each substrate was taken in a 500 ml conical flasks separately and supplemented with 20 mL nutrient solution containing glucose, at pH 3. One milliliter inoculum containing 1×10^7 spores was added and moisture was maintained at 60%. After incubation at 32°C for 5 days, the acid production was estimated by spectrophotometric method and by HPLC analysis. Interestingly, the yield of itaconic acid was promising with all the above substrates, where orange pulp, sugarcane bagasse and rice bran supported higher yields.

Key words: itaconic acid, SSF, agro wastes, *Ustilago maydis*

Introduction

Itaconic acid (IA), also known as methylene butanedioic acid, methylene succinic acid, 3-carboxy-3-butanoic acid, propylene dicarboxylic acid is one of the promising substances within the cluster of organic acids. It is a white crystalline unsaturated dicarbonic acid with one carboxyl group conjugated to the methylene group. Characteristics are molecular weight 130.1, melting point 167-168°C, boiling point 268°C, solubility in water 83.103 g/l, density 1.632 g/l at 20°C, pH is 2 in aqueous solution of 80 mg/l, (Tate, 1970). IA can be regarded as an α-substituted acrylic or methacrylic acid and is isomeric with citraconic and mesaconic acid. It is stable at acidic, neutral and middle basic conditions at moderate temperatures (Tate & Othmer, 1981). A special new market has opened for the use of IA in artificial glass (Kin et al., 1998) and in bioactive compounds in agriculture, pharmacy and medicine (Bagavant et al., 1994). Additionally, as a multipurpose starting material, IA is used in many selective enzymatic transformations to form useful polyfunctional building-blocks (Ferraboschi et al., 1994). Itaconic acid was discovered by Baup in 1837 (Willke & Vorloop, 2001), as a thermal decomposition product of citric acid. Itaconic acid was first reported as a product of mold *Aspergillus itaconicus* metabolism, which was isolated from the juice of salted plums. This green species grows well only on media of high osmotic pressures such as concentrated sugar solutions and produces itaconic acid on media containing KNO_3 as nitrogen source and 25% sucrose (Kinoshita, 1932). Calam et al., (1939) reported on obtaining small quantities of itaconic acid from a strain of *Aspergillus terreus* and preliminary investigations conducted by Moyer & Coghill (1945) confirmed the suitability of *Aspergillus terreus* for bringing about this reaction. Over 300 strains of *A. terreus* were screened and found eleven as efficient producers of itaconic acid from glucose (Lockwood & Reeves, 1945). Industrial production of IA by submerged fermentation was initiated by Pfizer Co. Inc (Pfeifer et al.,

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1952). IA was also produced from corn starch (Reddy & Singh, 2002), sago starch hydrolysate, (Lies et al., 2007) and Jatropha seed cake (Rao et al., 2007) .There is meager information on the use of *Ustilago* species for the synthesis of IA (Tabuchi et al., 1981). Haskins et al. (1955) found IA in the fermentation broth of *U. zeae* strain while screening several *Ustilago* strains for production of ustilagic acid and other metabolic products. One strain among these species produced about 15 g/L (Okabe et al., 2009). Panakova et al. (2009) isolated a strain, putatively identified as a *Candida* that produced IA at a 35% yield when grown under phosphate-limited conditions. *U. maydis* appears to be a candidate for an alternative producer of IA. This basidiomycetes fungus, a free living yeast-like nonpathogenic and filamentous pathogenic forms, may produce high amounts of IA. Most of the work was done by submerged fermentation conditions. Little attention was given on production of IA by solid state fermentation (SSF) (Tsai et al., 2001). In China, several groups have been working on the improvement of itaconic acid fermentation, including solid-state fermentation (Yu et al., 2009). Qingdao Kehai Biochemistry Company produces 10,000 Mt/year of itaconic acid, which is about 50% of the total production capability in China or 18% of worldwide production (Jin et al., 2010). In the present study, efforts were made on production of itaconic acid using *U. maydis* with cheap and abundantly available substrates (agro wastes) such as ground nut shells, rice bran, rice husk, orange pulp, ground nut oil cake, orange pulp and sugarcane bagasse to reduce the substrate costs employing solid state fermentation (SSF).

Materials and Methods

Solid state fermentation was carried out in 500 ml of conical flask containing 10 g of solid substrate. The fungal strain *Ustilago maydis* (MTCC No-1474) used in the present study was procured from Microbial type culture collection, Institute of Microbial Technology, Chandigarh, India and was maintained on Czapek Dox agar medium at 25°C.

Substrates

The substrates used in the present study were agro wastes like ground nut shells, rice bran, rice husk and ground nut oil cake procured from local mills in and around Anantapur. The sugar cane bagasse and fresh orange fruit wastes (with peel and pulp obtained after removal of the external part of the skin) were obtained from local fruit stalls. The agro wastes were sun dried for few days. The dried substrates were ground to small particles of 0.5 mm in size using an electrical grinder stored in polythene bags at room temperature for further use.

Pretreatment of the substrates

The substrates used were mostly cellulosic in nature. The fungal strains cannot be cultivated in a relatively short time by establishing the method of solid state fermentation. Generally solid state fermentation involves the growth of microorganisms on moist substrates. So before fermentation the substrate need pretreatment, so that the substrate becomes soft and can easily be utilized by the microorganisms. Pretreatment can be achieved either physically or chemically. For physical treatment 100 g of substrate on dry weight basis was taken in 500 ml conical flask and washed twice with distilled water and sterilized at 121ºC and at 15 psi for 20 minutes in an autoclave. The samples were dried at 80ºC in a hot air oven for 12 h and used as substrate for fermentation. This steam treatment enables the moist air to pass through the substrate and makes it soft. Chemical treatment was done with 1N NaOH and 1N H_2SO_4 for which 200 ml of pretreatment solutions 1N H_2SO_4 and 1N NaOH were added to 100 g of substrates separately and autoclaved at 121ºC for 15 min. After treatment, the samples were washed thoroughly with distilled water to neutralize the effect of pretreatment solutions and dried in an oven at 80ºC for 12 h (Pandey et al., 2000). Fermentation was carried out by taking 10 g of substrates in 500 ml conical flasks, to which 20-40 ml of the nutrient solution of the following composition (g/L), glucose 40.0 g/L; $(NH_4)_2SO_4$, 2.36 g/L; KH_2PO_4, 0.1 g/L; $MgSO_4$, 2.1 g/L; $CaCl_2$, 0.13 g/L; NaCl, 0.074 mg/L; $CuSO_4.5H_2O$, 0.2 mg/L; $FeSO_4.7H_2O$; 5.5 mg/L, $ZnSO_4.7H_2O$, 1.3 mg/L in distilled water (Petruccioli et al., 1999) was added. The contents were sterilized by autoclaving at 121ºC for 20 min. After sterilization the spore suspension containing $1x10^7$ spores/ml of *U. maydis* was added as inoculum in solid state fermentation process. The fermentation was carried out at different conditions like temperature, pH, moisture (%) and incubation periods, changing one variable and keeping all others constant. All the experiments were conducted in triplicates and the average values were represented. After fermentation substrates were washed with buffer of pH 7.0 and filtered. The filtrate served as a source of the product.

Mutagenesis

Itaconic acid is produced by microorganisms in a secondary pathway and hence not secreted in high concentrations under natural conditions. Hence, strain

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improvement was done by mutagenic treatments to improve the yields of itaconic acid. Mutagenic treatment of *U. maydis* was done by exposing to ultraviolet radiations (UV) and colchicine individually. For UV-treatment spore suspension (1×10^6 spores/ml) was chosen from 6 to 8 day old cultures of *U. maydis*. The spore suspension was spread uniformly on Czapek dox agar plates and were exposed to UV lamp from a distance of 70 cm for periods ranging from 10 to 60 min with 10 min intervals. Plates were wrapped with black paper and incubated at 34ºC for 5 to 7 days. The surviving colonies were isolated and subcultured. The fungal strains thus obtained were assigned identity as UV10, UV20 and UV60. Colchicine treatment was done by suspending spores of *U. maydis* in colchicine solution of concentrations 0.5 µg/ml, 1 µg/ml, 2 µg/ml and incubated for 24 h. After incubation the spore suspension was spread on Czapek dox agar medium separately and incubated at 34ºC for 5 to 7 days. The surviving colonies were isolated and the resulting fungal strains were named as $C_{0.5}$, C_1 and C_2, and subcultured (Reddy & Singh, 2002).

Analysis of itaconic acid

The itaconic acid concentration was measured by colorimetric method at 385 nm (Hartford, 1962) and HPLC with SCL-10AVP system control of Shimadzu company Ltd., Japan, using a C18 column of 4.60 mm diameter and 250 mm length (cat. No. 228-39001-38) with 70% acetonytrile as the mobile phase, at a flow rate of 1 ml/min and a pressure of 90 kgf/cm^2. A UV-detector (SPD-10AVP, CAT. NO. 228-40000-38, Shimadzu company Ltd., Japan) was used at 254 nm. An authenticated itaconic acid standard was used to quantify itaconic acid.

Thin Layer Chromatography (TLC):

Itaconic acid was also identified by chromatography with a standard (100 µg in 100 µl ethanol) prepared from commercially available itaconic acid. With the help of capillary tube a small drop of IA standard and test samples were spotted on silica gel plates and air dried. The plates were developed using diethylether/90% formic acid/water (7:2:1, by vol.) as solvent system in a chromatographic chamber. The plates were allowed to dry and then sprayed with 0.04% bromocresol green in 95% (v/v) ethanol. The plates were dried in hot air oven at 110°C for 20 min (Maloney & Attwood, 1976) development of yellow spots indicated the presence of IA.

Results and Discussion

Efforts were made to produce itaconic acid by solid state fermentation using *Ustilago maydis*. Itaconic acid is generally produced by fungal species of genus *Aspergillus* by fermentation of carbohydrates. *U. maydis* appears to be an alternative producer of itaconic acid. Any change in culture conditions greatly influences the production ability of microbial strains.

Effect of pH

The fermentation media adjusted to pH of 2.0, 3.0, 4.0, 5.0, and 6.0 were used for the determining the influence of pH on IA production by *U. maydis* and it was experiential that the IA production was found to be maximum at pH 3.0 with all the substrates. The amount of IA was found to increase with the pH from 2.0 to 3.0 and thereafter decreased with increase in pH. Maximum production of IA was observed at pH 3 with all the substrates where rice bran (RB), sugarcane bagasse (SCB) and orange pulp (OP) yielded more when compared to the other substrates. It is well-known fact that pH plays important role in metabolism and growth of any microorganism. Microbes maintain intracellular pH at a constant. Any change in the optimum levels my effect the metabolism which in turn influence on rate of product formation. In the present study, the results predict that the extent of IA production was influenced by pH and the optimum pH was 3.0 (Figure 1).

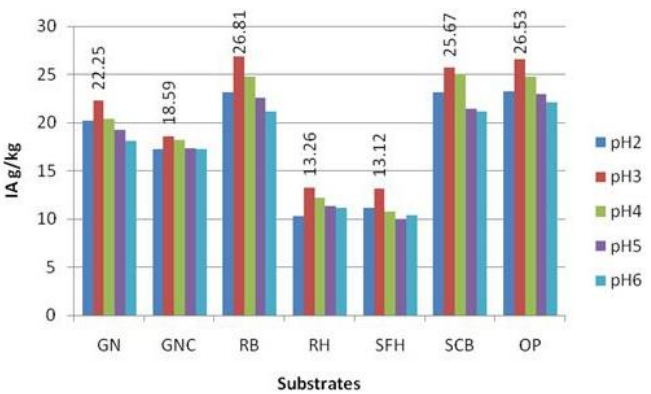

Figure 1. *Effect of pH on IA production by Ustilago maydis. GN: Ground nut shells, GNC: Ground nut cake, RB: Rice bran, SCB: Sugar cane bagasse, OP: Orange pulp, RH: Rice husk, SFH: Sun flower husk.*

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Effect of temperature

The SSF was carried out at different temperatures *viz.*, 25ºC, 30ºC, 32ºC, 34ºC and 37ºC, to determine the effect of temperature on IA production (Figure 2). The environmental temperature one of the important physical factors influence significantly on the cell growth, metabolism and there by the production of metabolites. The production of IA was found to increase with temperature up to 32°C and later decreased with further increase in temperature from 32-37°C. Beyond the optimum temperature growth rate fall due to increase in microbial death rate. Therefore a decrease in IA production could be observed.

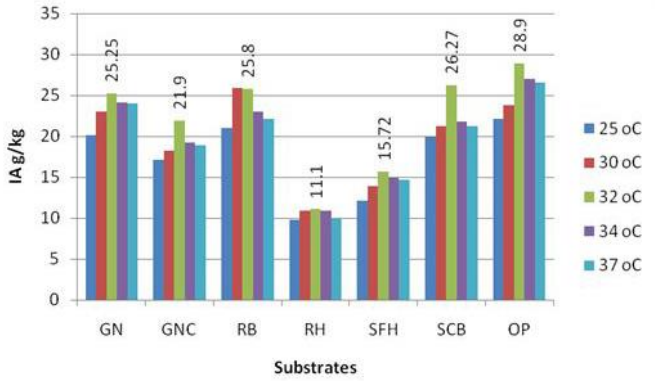

Figure 2. *Effect of temperature (ºC) on IA production by Ustilago maydis. GN: Ground nut shells, GNC: Ground nut cake, RB: Rice bran, SCB: Sugar cane bagasse, OP: Orange pulp, RH: Rice husk, SFH: Sun flower husk.*

Effect of moisture (%)

Experiments were set up to investigate the effect of moisture content of the solid medium at varying moisture levels ranging from 50%, 60%. 70% and 80%, keeping all other parameters constant (Figure 3). Present investigation revealed that at 60% moisture level the fungi produced maximum IA with all the substrates.

Effect of incubation period

The fermentation was setup under other optimum conditions at different time intervals 4, 5, 6 and 7 days to determine the influence on itaconic acid production (Figure 4). It was observed that there is an increase in the itaconic acid production with an increase in time of incubation showing maximum at 5 days where IA production was maximum with orange pulp 28.9 g/kg.

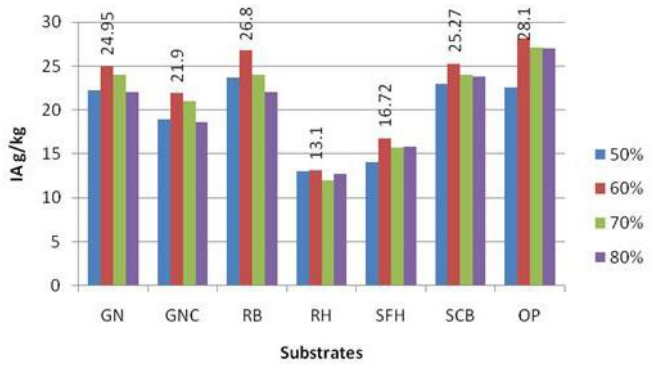

Figure 3. *Effect of moisture (%) on IA production by Ustilago maydis. GN: Ground nut shells, GNC: Ground nut cake, RB: Rice bran, SCB: Sugar cane bagasse, OP: Orange pulp, RH: Rice husk, SFH: Sun flower husk.*

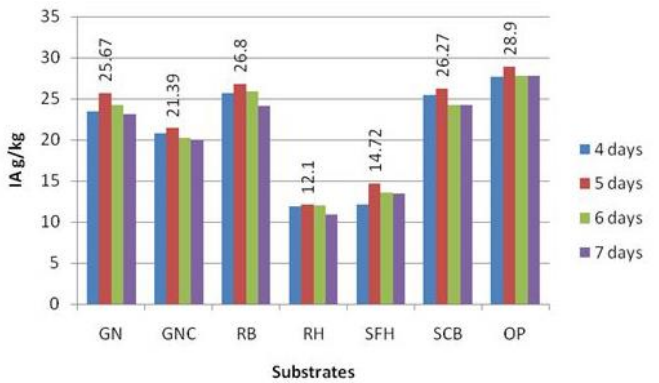

Figure 4. *Effect of incubation period on IA production by Ustilago maydis. GN: Ground nut shells, GNC: Ground nut cake, RB: Rice bran, SCB: Sugar cane bagasse, OP: Orange pulp, RH: Rice husk, SFH: Sun flower husk.*

Production of IA by mutant strains of U. maydis treated with UV and colchicine

The fermentation medium was adjusted to optimum pH 3.0, temperature of 32ºC, moisture level at 60% for production of itaconic acid by selected UV treated and colchicine treated mutant strains of *U. maydis* and incubated for 5 days. After 5 days of incubation the production of itaconic acid by the mutant strains was estimated (Figure 5). The UV treated strain designated as UV 20_3 yielded more amounts with all the substrates when compared with colchicine treated strain $C22_2$.

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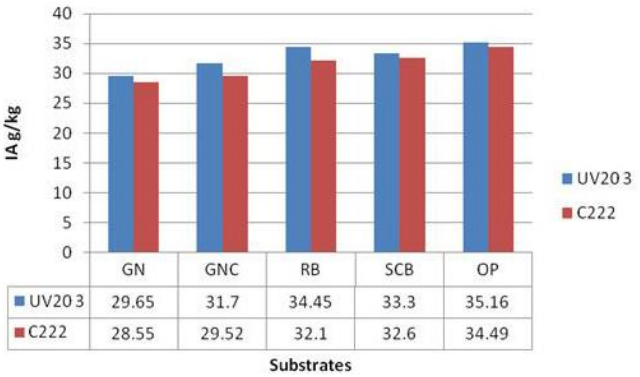

Figure 5. *IA production by UV and colchicine treated mutants*

Though there is a number of reports regarding the production of itaconic acid by submerged fermentation, only a couple of reports are available on solid state fermentation for itaconic acid production till now, the M-8 strain achieves a 55% itaconic acid yield by solid state fermentation employing sugar cane pressmud as solid substrate reported by Tsai et al. (2001) and another report by Yu et al. (2009). Under optimal conditions employing submerged fermentation Petruccioli et al. (1999) reported production of 17.8 g/L IA by *A. terreus*. *Ustilago zeae* produced 15 g/L (Haskin et al., 1955), likewise *U. maydis* produced 53 g/L (Tabuchi, 1991). Chandragiri & Sastri (2011) reported 66.13 g/L IA using *U. maydis* (NCIM-983) under submerged fermentation. A mutant strain of candida produced up to 42 g/L (Hashimoto et al., 1989). Mutant strain of *A. terreus* produced 82 g/L (Yahiro et al., 1995) and 22.5 g/L (Meena et al., 2010). *Aspergillus* strains like NRRL 1960, produced 52 g/L (Yahiro et al., 1997) and RC4 - 67 g/L (Bonnarme et al., 1995). Rao et al. (2007) reported the IA production with Jatropa seed cake and they obtained 24.46 g/L in 120 h.

Conclusion

In the present study, the solid state fermentation method was adapted where the agro wastes rice bran, orange pulp and sugar cane bagasse proved to be good substrates for solid state fermentation over the others for the production of itaconic acid by *U. maydis* (MTCC No-1474) and mutant strains of *U. maydis* at an optimum pH 3, moisture 60%, temperature 32°C and incubation period of 5 days.

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