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# Production of Citric Acid by *Aspergillus niger* MTCC 282 in Submerged Fermentation Using *Colocassia antiquorum*

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**Abstract:** The filamentous fungus, *Aspergillus niger* MTCC 282, was used to produce citric acid in submerged fermentation with *Colocassia antiquorum* (10% w/v) as the substrate. The maximum yield of citric acid (46.5 mg $mL^{-1}$) was obtained with inoculum age (7 days), inoculum level (2% v/v), temperature (30°C), pH (4.0), sucrose (2.0% w/v) and ammonium nitrate (1.2% w/v). The addition of methanol to the fermentation medium resulted in substantial increase in the production of citric acid.

**Key words:** Citric acid, *Aspergillus niger* MTCC 282, *Colocassia antiquorum*, submerged fermentation, optimization

## INTRODUCTION

Citric acid is an intermediate in the Tricarboxylic acid cycle when carbohydrates are oxidized to carbon dioxide. It is responsible for the tart taste of various fruits in which it occur, i.e., lemons, limes, figs, oranges, pineapples, pears and goose berries. Because of its high solubility and low toxicity, citric acid can be used in food and pharmaceutical industry (Rohr, 1998). It is estimated that over 65% of the citric acid produced is consumed for food and beverages. At present about 99% of world's citric acid is produced by fermentation with some bacteria and fungi (Micheal *et al*., 2006).

Filamentous fungus *Aspergillus niger* belongs to microorganisms of extreme biotechnological importance since it is used for the production of various primary metabolites (organic acids) and enzymes. In fact, citric acid production by this fungus is one of the most efficient bioprocess in terms of productivity, since *Aspergillus niger* can convert up to 80% of the substrate to the final product. Because citric acid is a commodity chemical, it is necessary to utilize inexpensive and readily available raw materials for commercial production. Starchy materials are more suitable substrates for the citric acid production since they are renewable and available in large quantities at cheaper rates. There are several reports on different strains and effects of nutritional factors on their growth for citric acid production (Kim *et al*., 2006; Demirel *et al*., 2005; Kumar *et al*., 2003; Ali *et al*., 2001).

The present study reports the optimization of process variables: temperature, pH, inoculum age, inoculum level, substrate concentration, carbon source, nitrogen source and methanol for the production of citric acid using *Aspergillus niger* MTCC282 under submerged fermentation.

## MATERIALS AND METHODS

### Microorganism

Pure cultures of fungus *Aspergillus niger* MTCC 282 procured from Institute of Microbial Technology, Chandigarh, was raised on potato-dextrose agar medium. It is incubated aerobically at 37°C for 7 days.

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Table 1: The chemical composition of *Colocassia antiquorum* (Diop and Calverly, 1998)

| Constituents | Composition (%) |
|---|---|
| Moisture | 63-85 |
| Carbohydrates | 13-29 |
| Proteins | 1.2-3.0 |
| Fats | 0.16-1.18 |
| Crude fiber | 6.6-11.8 |
| Ash | 0.6-1.3 |

### Substrate

Because of its rich carbohydrate content, *Colocassia antiquorum* was used as a potential substrate for the production of citric acid. The chemical constituents of *Colocassia antiquorum* were given in Table 1.

### Fermentation

Two hundred fifty five milliliter conical flasks with 50 mL of *Colocassia antiquorum* medium (5 g in 50 mL of distilled water) containing different concentrations of nutrients were inoculated with 1 mL of homogenous spore suspension ($10^7$-$10^8$ spores $mL^{-1}$). The flasks were incubated at 30°C on a shaker (120 rpm) for a maximum fermentation period of 7 days. The fermented biomass in each case was filtered and centrifuged. The supernatant was ultra-filtered through filter paper and the filtrate was analyzed for citric acid.

### Optimization of Culture Conditions

The growth medium of *Colocassia antiquorum* was fermented with *Aspergillus niger* MTCC 282 for different fermentation periods with varying levels of substrate, pH, temperature, inoculum age, inoculum level, sucrose as the carbon source, ammonium nitrate and sodium nitrate as nitrogen sources and methanol in shake flasks. The experiments were carried out in such away that the parameter optimized in one experiment was maintained in the subsequent investigation.

### Citric Acid Analysis

Citric acid was estimated by the acetic anhydride and pyridine method of Marrier and Boulet (1958).

## RESULTS AND DISCUSSION

### Fermentation Periods

For the optimization of fermentation period, growth medium containing 10% (w/v) *Colocassia antiquorum* was autoclaved, inoculated (2% v/v) and incubated for 24, 48, 72, 96, 120 and 144 h, respectively, at pH 5.0 and 30°C. The maximum citric acid yield (28.5 mg $mL^{-1}$) was observed in solution harvested after 120 h. It was observed that production of citric acid increased with an increase in fermentation period from 96-120 h, reached its maximum after 120 h and decreased thereafter (Fig. 1). Azad *et al* reported a maximum citric acid yield after 144 h of fermentation by *Aspergillus niger* GCB-14 using cane molasses as the substrate.

### Substrate Level

The maximum yield of citric acid (28.5 mg $mL^{-1}$) was observed with 10% *Colocassia antiquorum* in continuous shaking culture medium (Fig. 1).

### Effect of pH

Media of *Colocassia antiquorum* (10%) were adjusted at different pH values i.e., 2, 3, 4, 5 and 6 and the fermentation was carried out for 7 days. The results showed a maximum yield of citric acid (31.5 mg $mL^{-1}$) at pH 4.0 (Fig. 2). The optimal production of citric acid by *Aspergillus niger* GCMC-7

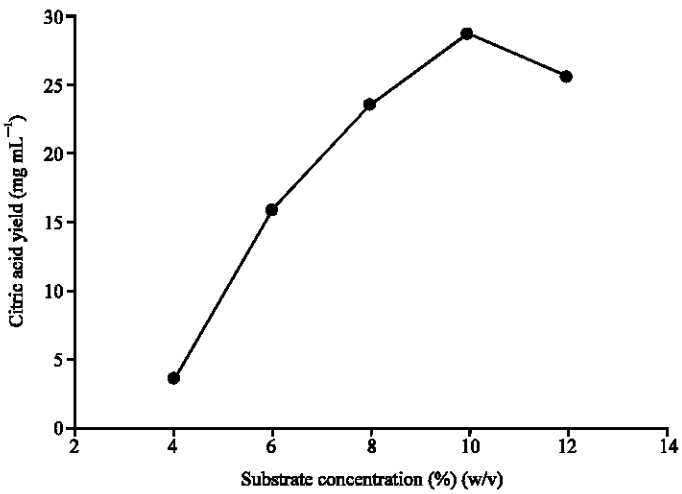


Fig. 1: Effect of substrate concentration on the production of citric acid by *A. niger* MTCC 282 at 30°C and 180 rpm

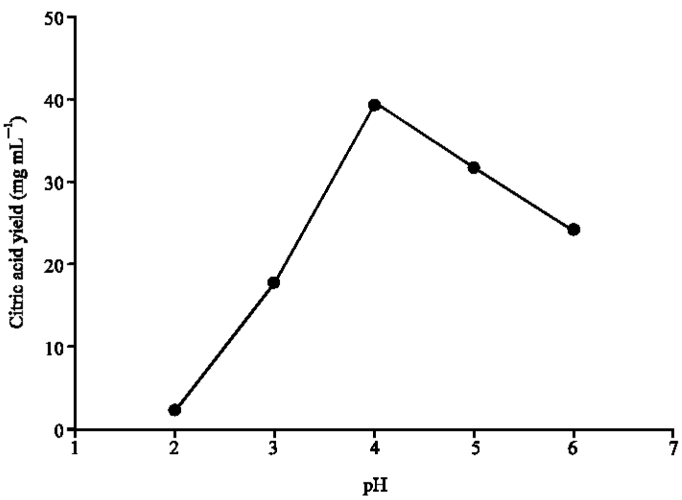


Fig. 2: Effect of initial pH on citric acid yield by *A. niger* MTCC 282 at 30°C (180 rpm)

was reported at pH 5.4 (Haq *et al*., 2002). This might be because a high pH results in the deactivation of the enzymes necessary for the citric acid production. At high pH, oxalic acid and other by-products may be formed rather than citric acid; also glucose oxidase was activated reducing citric acid production (Kubicek and Rohr, 1989).

## Effect of Temperature

The effect of different incubation temperatures (25, 30, 35 and 40°C) on the production of citric acid was studied and results (Fig. 3) indicate that 30°C was the optimum for maximum yield of citric acid (36.25 mg mL$^{-1}$). Temperature between 25-30°C was usually employed for culturing of *Aspergillus niger* GCMC-7, but temperature above 35°C was inhibitory to citric acid production (Haq *et al*., 2002). The decrease in citric acid production could be because due to the increase in the denaturation of enzymes because of high temperature and oxalic acid production increases above 30°C (Doelger and Prescott, 1934).

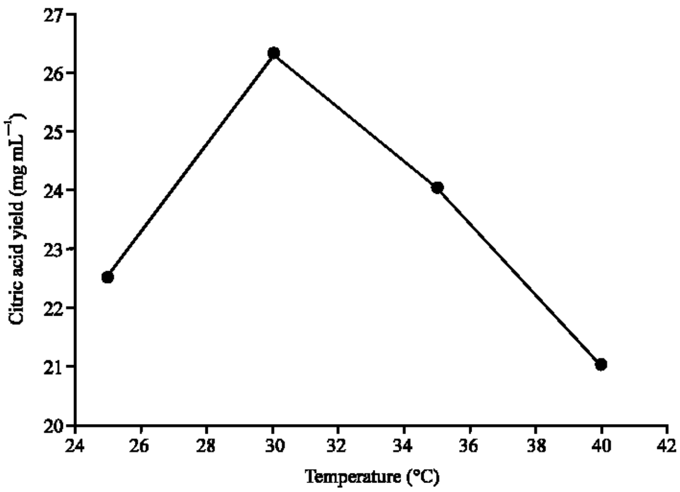


Fig. 3: Effect of temperature on the production of citric acid by *A. niger* MTCC 282 at pH 4.0 (180 rpm)

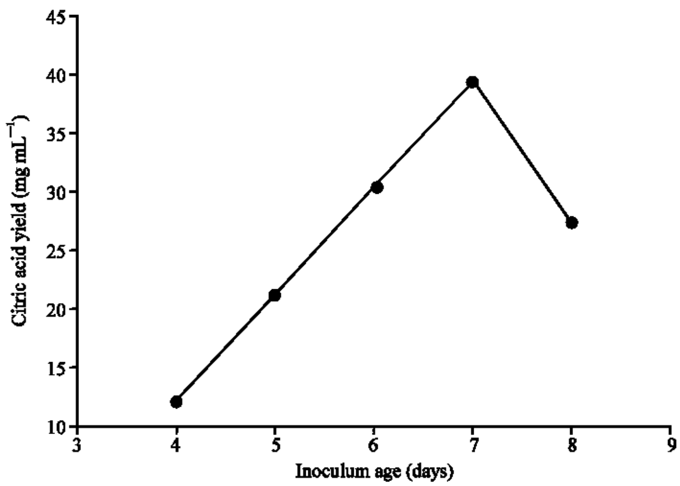


Fig. 4: Effect of inoculum age on the production of citric acid using *A. niger* MTCC 282 at pH 4.0 and 30°C (180 rpm)

**Effect of Inoculum Age**

The effect of different inoculum ages (4, 5, 6, 7 and 8 days) on the production of citric acid was studied. *Aspergillus niger* MTCC 282 of 7 days old culture was observed to be optimum for the production of citric acid (39.0 mg mL$^{-1}$) after which the citric acid yield decreased with increase in age of inoculum (Fig. 4). Prado *et al*. (2005) reported 7 days old inoculum was the optimum for the production citric acid by *Aspergillus niger* LPB-21 using cassava bagasse. The decrease in the citric acid production with increase in the age of inoculum after 7 days may be due to the starting of death phase of the slant.

**Effect of Inoculum Level**

Various inoculum levels (1, 2, 3, 4 and 5%) were tried to study their effect on the production of citric acid. High citric acid yield (40.5 mg mL$^{-1}$) was obtained at 2% (v/v) inoculum level (Fig. 5). The

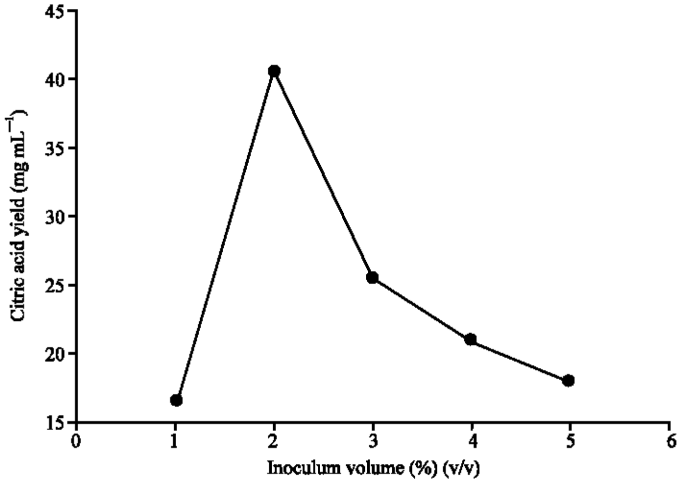


Fig. 5: Effect of inoculum volume fro the production of citric acid using 7 days old culture of *A. niger* MTCC 282 at pH 4.0 and 30°C (180 rpm)

Table 2: Effect of different sugar concentration on the production of citric acid using *niger* MTCC 282

| Sucrose concentration (%) (w/v) | Citric acid yield (mg mL$^{-1}$) |
|---|---|
| 1 | 31.5 |
| 2 | 43.5 |
| 3 | 34.5 |
| 4 | 30.0 |

maximum yield of citric acid was reported by Prado *et al*. (2005) at an optimum inoculum level of 2% (v/v) by *Aspergillus niger* LPB-21 using cassava bagasse. This might be due to a high inoculum level result in the rapid utilization of the substrate to form by-products, which hampers the citric acid production.

**Effect of Carbon Source**

To enhance the rate of fermentation and citric acid production, sucrose with different concentrations was added to the fermentation medium. The results showed that an optimum concentration of 2.0% (w/v) gave maximum concentration of citric acid (43.5 mg mL$^{-1}$) (Table 2). Azad *et al*. (2003) reported that 126 g L$^{-1}$ of initial sugar concentration was optimum for the production of citric acid using *Aspergillus niger* GLB-14.

**Effect of Nitrogen Source**

The concentration of nitrogen source in the growth media has a considerable influence on citric acid production. Nitrogen sources (ammonium nitrate and sodium nitrate) with different concentrations (0.4, 0.8, 1.2 and 1.6% w/v) were studied. The best yield (46.5 mg mL$^{-1}$) was observed in the presence of ammonium nitrate at 1.2% w/v, followed by sodium nitrate (42.0 mg mL$^{-1}$) at 0.8% w/v (Table 3). Haq *et al*. (2005) reported that specific rate of citric acid production by *Aspergillus niger* NG-280 was the highest when ammonium nitrate concentration was between 2.0 and 3.0 mmol g$^{-1}$ cells. At higher concentration of nitrogen source in general causes oxalic acid formation which will ultimately decrease the citric acid yield (Doelgar and Prescott, 1934).

**Effect of Methanol**

Effect of different levels of methanol was studied on the production of citric acid in growth medium containing sucrose 2.0% (w/v), ammonium nitrate 1.2 (w/v) at a pH 4.0 and 30°C. Results indicated that maximum yield of citric acid (52.5 mg mL$^{-1}$) was obtained with 3% methanol. Citric acid

Table 3: Effect of nitrogen source on the production of citric acid using *A. niger* MTCC 282

| Concentration of nitrogen source (%) (w/v) | Citric acid yield in the presence of sodium nitrate (mg $mL^{-1}$) | Citric acid yield in the presence of ammonium nitrate (mg $mL^{-1}$) |
|---|---|---|
| 0.4 | 28.5 | 36.0 |
| 0.8 | 42.0 | 40.5 |
| 1.2 | 24.0 | 46.5 |
| 1.6 | 18.0 | 42.0 |

Table 4: Effect of methanol concentration on the production of citric acid using *A. niger* MTCC

| Volume of methanol (%) (v/v) | Citric acid yield (mg $mL^{-1}$) |
|---|---|
| 1 | 43.5 |
| 2 | 51.0 |
| 3 | 52.5 |
| 4 | 48.0 |

production was found to be enhanced by the addition of methanol up to 3% (v/v) and decreased by its further addition (Table 4). These results accord with Pazouki and Panda (2002) who reported that the addition of methanol significantly enhanced the citric acid yield. The decrease in citric acid production with increase in methanol concentration may be due to the inhibitory effect of methanol on the organism, reduction in mycelial growth and low tolerance to higher concentrations of methanol (Maddox *et al*., 1986).

## ACKNOWLEDGMENTS

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