Influence of Different Nitrogen Sources on Citric Acid Production from Surface Culture of *Aspergillus niger*

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Abstract

The effect of peptone on citric acid production was evaluated in surface culture of *Aspergillus niger*. The maximum citric acid concentration produced in production medium and peptone free production medium was 47.1 and 19.5 g/l respectively on the 15th day. Supplementation of the production medium, or peptone - free production medium, with either soya bean flour or soya meat powder, decreased citric acid production while increasing fungal growth. An increase in the ammonium nitrate concentration in the production medium of 0.25 g/l (from 0.5 to 0.75 g/l) improved citric acid production by 4.9 g/l, with no change in growth. When the concentration of peptone in the production medium with increased concentration of ammonium nitrate (0.75 g/l) was doubled (14 g/l), citric acid production increased from 52 to 58 g/l, with a decrease in production time to 8 days.

Key words: Citric acid, ammonium nitrate, peptone, soya bean flour, soya meat powder, surface culture.

Introduction

Citric acid is produced by fungus as a primary metabolite in the acid producing phase [1,2], which parallels the growth phase. Hence growth phase is important for citric acid production [1]. The concentrations and types of nitrogen and carbon sources in a medium are the deciding factors of the fungal growth [3]. Under solid state fermentation conditions, soya meat powder was preferred for glucoamylase production and protease production by *Aspergillus niger* [4,5] and soya bean flour was preferred by *Bacillus licheniformis* for α-amylase production [6]. The presence of ammonium phosphate reduced glucoamylase production by *Botryodiplodia theobromae* while soya bean flour improved the enzyme production in submerged fermentation [7]. *Lactobacillus delbrueckii* produced higher amounts of lactic acid in medium containing yeast extract than in media with peptone or ammonium sulphate [8]. These studies implied the importance of nitrogen sources and their preference by different organisms under various cultivation conditions to give different levels of products. In this paper we report the improvement of citric acid production in surface culture from a locally isolated and mutated *Aspergillus niger*, by altering the nitrogen sources in the medium.

Materials and Methods

Materials

Soya bean and soya meat (Delmage Forsyth & Co Ltd., Sri Lanka) available in the local market were milled and sieved using a domestic grinder and sieve respectively. All the other chemicals used were of analytical grade.

Organism

A locally isolated *Aspergillus niger* was used. This fungus exhibited branched and septate hyphae with asexual reproductive structure and conidia on conidiophore which is unbranched and each had a single conidial head. Matured conidial heads showed split loose conidial columns. During maturation, colour of conidia changed from lighi brown through red to black. The organism was subcultured every two weeks on Potato Dextrose Agar (PDA) slants and stored at 4ºC [7]. Whenever the spores were required, they were suspended in 1% (v / v) Tween - 80 and the number of spores were counted using a haemocytometer.

Analytical methods

Citric acid [9] and reducing sugar [10] in the medium were estimated spectrophotometrically. To determine the dry weight of the mycelium, the lower surface of the mycelial mat was washed with distilled water and dried at 80°C to constant weight.

Basal medium

Basal medium contained (%, w/v) glucose, 5.0; NH_4NO_3, 0.05; KH_2PO_4, 0.05; $MgSO_4.7H_20$, 0.01; peptone, 0.7; $ZnSO_4$, 0.1 x 10^{-4}, ferrous ammonium sulphate, 0.1 x 10^{-4} and $CuSO_4.5H_2O$, 0.06 x 10^{-4}.

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Production medium

The production medium was prepared by modifying the basal medium. The glucose content was changed to 14.0 % (w/v) and in methanol, 3.0 % (v/v) and gingilly oil (Indian sesame oil, an edible oil), 2.0 % (v/v) were included.

Preparation of mycelial inoculum

The basal medium (10ml) was inoculated with 1x 10^8 spore suspension (6 days old) to bring the total spore concentration to 1 x 10^7 spores ml^{-1} and incubated at 30°C. The mycelium (65h old) was split into fragments (1.3 x 1.3 x 0.1 cm^3) and used as inoculum (1piece / 5ml).

Effect of peptone, soya bean flour and soya meat powder

Production medium (Medium 1), production medium supplemented with either 20 g/l soya bean flour (Medium 2) or soya meat powder (Medium 3), peptone free production medium (Medium 4), peptone free production medium supplemented with either 20 g/l soya bean flour (Medium 5) or soya meat powder (Medium 6) were inoculated with mycelial inoculum and incubated at 30°C under diffused light. Citric acid and reducing sugar in the media were monitored, and dry weight of the mycelium was determined at the end of fermentation.

Effect of NH_4NO_3

Production medium containing varying concentrations (0.25, 0.5, 0.75 and 1.0 g/l) of NH_4NO_3 was prepared, and the experiment proceeded as above.

Effect of peptone

Test media were prepared by taking the optimized amount of NH_4NO_3 with varying concentrations of peptone (0, 3.5, 7, 14 and 17.5 g/l) keeping all other components of the production medium constant.

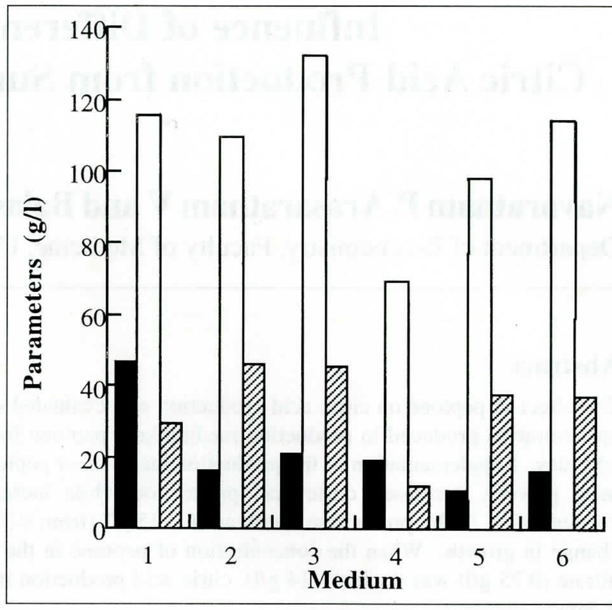

Figure 1: Effect of soya bean flour (20 g/l) and soya meat powder (20 g/l) on *Aspergillus niger* at pH 5.2 and 30°C. (■) Maximum citric acid produced; (□) reducing sugar utilized and (▨) dry weight of the mycelium at the end of fermentation.

Results and Discussions

Effect of peptone, soya bean flour and soya meat powder

Addition of peptone (7 g/l) not only increased the growth of *Aspergillus niger* (2.4 folds) and citric acid production (2.4 fold, Figure 1, Media 1 and 4), but also shortened the fermentation time by one day (from 16 to 15 days, Figure 2a & b). The increase in citric acid

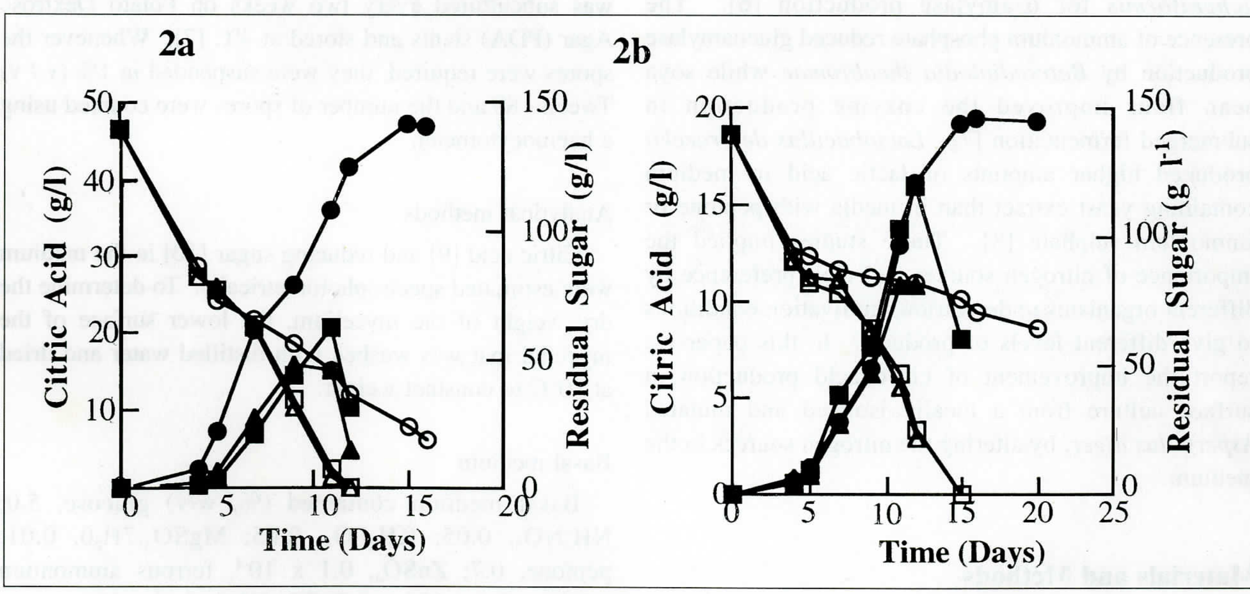

Figure 2: Effect of either soya bean flour (20 g/l) or soya meat powder (20 g/l) supplementation to (a) production or (b) peptone free production medium on citric acid production from *Aspergillus niger* at pH 5.2 and 30°C. (●) Control medium (Either 1 in a or 4 in b), (▲) Medium supplemented with soya bean flour and (■) medium supplemented with soya meat powder. Closed and opened symbols indicate citric acid and glucose respectively.

production was significant ($p < 0.02$, S.E. = 0.48). Therefore the nitrogen content and nitrogen sources in the medium should be optimized. In many instances growth was faster with the addition of organic nitrogen [11]. To enrich the nutrients or substitute for peptone, either soya bean flour or soya meat powder was added. Soya bean flour or soya meat powder increased the growth of the fungus, while decreasing citric acid production (Figures 1 and 2). Citric acid production in Media 5 and 6 was less than that in control medium (Medium 4) and the mycelial growth in Media 5 and 6 was very similar (38 and 37 g/l respectively) roughly 3 times that of the control (12 g/l). When the effects of soya bean flour and soya meat powder in Media 3 and 4 were compared, soya meat powder was less effective in suppressing citric acid production than soya bean flour ($p < 0.1$). This might be due to the removal of the inhibitory compounds during the defatting process of soya bean flour to soya meat powder. The rate of carbon source consumption was faster in media with soya products than in the respective controls (Figure 2). Thus supplementation of peptone free production medium,

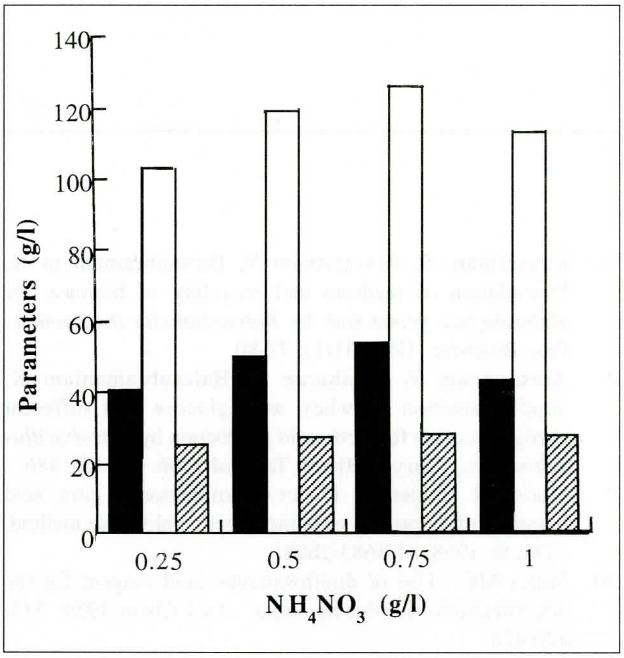

Figure 3: Effect of NH_4NO_3 concentration in production medium on *Aspergillus niger* at pH 5.2 and 30°C. (■) Maximum citric acid produced; (❐) glucose utilized and (▨) dry weight of the mycelium.

and production medium with either soya bean flour or soya meat powder was not useful.

Effect of NH_4NO_3

Maximum citric acid production (53.5 g/l) was observed in the medium containing 0.75 g/l NH_4NO_3 on the 17th day. Slow utilization of sugar paralleled with increase in citric acid production. An average of 60 g/l citric acid was produced by *Aspergillus niger* when 2 g/l of NH_4NO_3 used as the only nitrogen source in a medium containing 140 g/l sucrose [12]. The NH_4NO_3 concentration used in our experiment was only 38.0% of that previously reported [12] and the citric acid yields in this work and earlier reports were 40 and 45% respectively. The concentration of NH_4NO_3 had no effect on growth (Figure 3). Optimized concentrations of soya bean flour and soya meat powder might act better. This experiment shows the necessity for optimum growth in order to achieve maximum citric acid

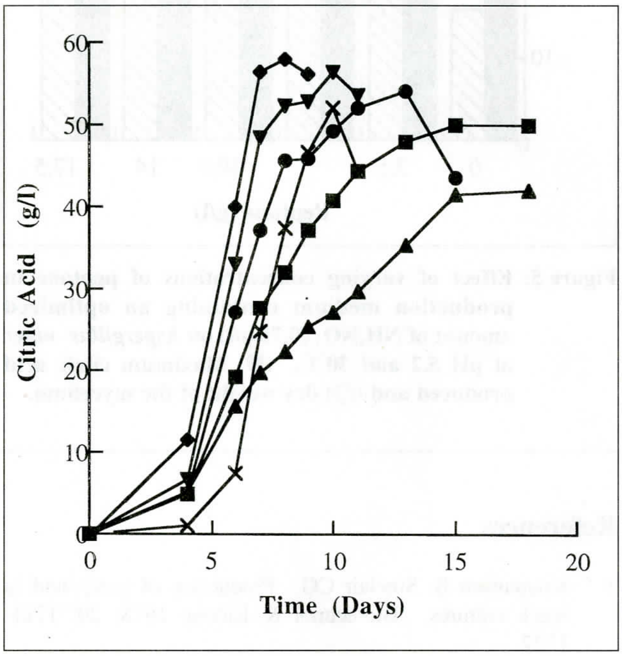

Figure 4: Effect of varying concentrations of peptone in production medium containing optimized amount of NH_4NO_3 (0.75 g/l) on citric acid production from *Aspergillus niger* at pH 5.2 and 30°C. Peptone (▲), 0; (■), 3.5; (●) 7; (▼) 10.5; (◆) 14 and (x) 17.5 g/l. Closed and opened symbols indicate citric acid and glucose respectively.

production. To improve citric acid production, additional NH_4NO_3 (0.25 g/l) should be added to the production medium.

Effect of peptone concentration

The fungus was grown in the production medium with an optimized amount of NH_4NO_3 (0.75 g/l) and varying amounts of peptone. Maximum citric acid (58 g/l) was produced in the medium containing (14 g/l) peptone on the 8th day (Figure 4). Growth of the fungus increased with increasing concentration of peptone (Figure 5). However, maximum citric acid production was obtained when the dry weight of the mycelial mat was 38.3 g/l. Thus, growth of the biofilm is the deciding factor for citric acid production. However, any increase in growth above a certain limit seems to affect the acid production (Figures 4 and 5). In an earlier report, peptone was not included in the

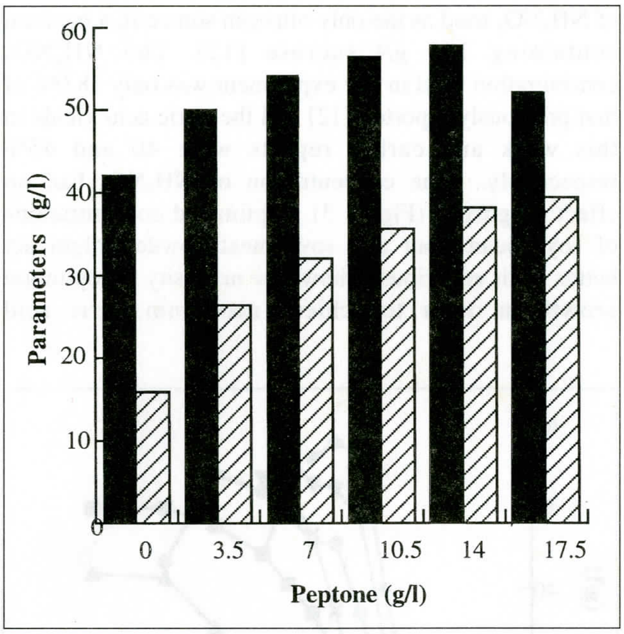

Figure 5: Effect of varying concentrations of peptone in production medium containing an optimized amount of NH_4NO_3 (0.75 g/l) on *Aspergillus niger* at pH 5.2 and 30°C. (■) Maximum citric acid produced and (▨) dry weight of the mycelium.

medium, and therefore the production time was extended to 30 days [12]. These results illustrate the importance of optimizing the type and amount of nitrogen sources for maximum product formation.

In conclusion, among the nitrogen sources used in the production of citric acid by *Aspergillus niger*, the presence of peptone in the medium at 14 g/l yielded highest amount of citric acid (58 g/l) with the reduction in fermentation time (8 day). The use of either soy bean or soy meat (20 g/l) as nitrogen source did not increase the citric acid production. Thus addition of 14 g/l peptone is most suitable for citric acid production.

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