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Production of Citric Acid by *Aspergillus Niger* Using Oat Bran as Substrate

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Abstract

Aspergillus niger strains were isolated from various sources like soil, floor, window glass and Microbiology lab. These strains were screened for the production of citric acid and the strain that produced maximum amount of citric acid was selected and identified based on its cultural characteristics, employing slide culture method. A basal medium made of Oat bran was employed for the production of citric acid (using SSF). Optimization studies were conducted by varying some nutritional and physiological parameters. Fructose concentration of 20% in the basal media enhanced citric acid production. A concentration of 0.25 (%w/v) NH_4NO_3 was found optimum. Likewise, an incubation temperature of 28^0C, pH 4.5 and incubation period of 72h were found optimum for the production of citric acid using oat bran as substrate. Maximum amount of citric acid produced was 62g/kg of oat bran.

Keywords: Oat bran, Citric acid production, Aspergillus niger

1. Introduction

Among the organic acids industrially produced, citric acid is the most important in quantitative terms. Citric acid is used in the food, beverage, pharmaceutical, chemical, cosmetic and other industries. The worldwide demand of citric acid is about 6.0×10^5 tons per year and it is bound to increase day by day. With an estimated annual production of 1,000,000 tons, citric acid is one of the products of fermentation with the highest production level worldwide. Commercial production of citric acid is generally by submerged fermentation of sucrose or molasses using the filamentous fungus *A. niger* or synthetically from acetone or glycerol (Torres et al., 1998; Fernando et al., 2000; Adachi et al., 2003; Haq et al., 2004). In the recent times solid state fermentation (SSF) is an alternative to submerged fermentation in the production of microbial

metabolites. Solid-state fermentations refer to the cultivation of microorganisms in a low-water-activity environment on a non-soluble material acting as both nutrient source and physical support (Pandey**,** 2003). The major advantages of solid-state fermentation over submerged fermentation include higher yields, low water requirement and lower operating costs.

The present study is based on the usage of oat bran as substrate for the production of citric acid. An oat grain is made up of two parts: the inedible outer hull (the husk) and the oat kernel (groat). Within the groat are found the oat germ, the starch cell and the outer layer. Oat bran is derived from the outer layer of the oat groat (hulled oat kernel).

Many microorganisms have been evaluated for the production of citric acid including bacteria such as *Bacillus licheniformis, B. subtilis, Corynebacterium* spp. (Kapoor *et al.*, 1983), fungi such as *A. niger, A. awamori, A. foetidus, Penicillium restrictum* (Mattey and Allan, 1990; Kubicek, 1998). Yeast such as *Candida lipolytica, C. intermedia* and *Saccharomyces cerevisiae* (Crolla and Kennedy, 2001; Archer *et al.*, 2001; Kamzolova *et al.*, 2003). However, *A. niger* a filamentous fungus remained the organism of choice for citric acid production due to ease of handling, its ability to ferment a variety of cheap raw materials, and high yields (Schuster *et al.*, 2002).

There are three different separation methods that can be employed for recovering citric acid from the fermentation broth. These are Precipitation, Ion Exchange and Solvent Extraction. Precipitation is the most commonly used method. It is more economical to remove citric acid as calcium citrate by lime precipitation than by ion-exchange treatment. The ion exchange method may be used for treating lime juice rather than filtered acid juice. Solvent extraction is a possible alternative to the classical method but because the available solvents tend to extract some of the impurities too, it is easier to apply to products from glucose or alkane based substrates. The advantage of this process is that it avoids the use of lime and sulfuric acid and the concomitant problem of gypsum (calcium sulphate) disposal. Gypsum is produced as a waste product during the purification process of citric acid and its disposal is a complicated issue.

2. Materials and Methods

2.1 Isolation of Aspergillus niger

Aspergillus niger strains were isolated from the following samples: Soil, Window glass, corridor floor and Microbiology Lab. To isolate *Aspergillus niger* strain from the collected samples, Potato Dextrose Agar (PDA) was used. Composition of Potato Dextrose Agar (PDA) is Potato, 100g; Dextrose, 10g; Agar, 10g and distilled water, 500ml. pH: 5.6 ± 0.2 at 25^0C.

2.2 Screening of the fungal cultures

Production of citric acid was analyzed based on the pH values and O.D (Optical Density) values at the end of fermentation. All four isolates produced citric acid. Among all, the isolates from sources C (microbiology lab) and B (soil) produced

appreciable amounts of citric acid. Only the isolate from source C was selected for further study.

2.3 Slide Culture Preparation

Slide culture technique was employed to study the cultural characteristics of Isolate C. In a small Petridish, after placing a sterile filter paper and some sterile distilled water, a few centimeters apart, two pieces of glass rods were placed on the filter paper. Clean, alcohol flamed and heat sterilized glass slide was placed on the rods. Using a sterile spatula, a small square (about 1 x 1 cm) of agar block was cut from the Potato Dextrose Agar (PDA) and placed on the centre of the slide. Using a needle, the agar was inoculated with a small amount of *A. niger* strain under test on each of the four sides of the block. A heat-sterilized cover slip was placed over the block and pressed down gently. It was incubated at 28^0C and examined daily for sufficient growth. When good growth was appeared, a drop of Lacto Phenol Aniline Blue (LPAB) was placed on a clean slide, the cover slip was removed using forceps and passed the top side of the cover slip in front of the incinerator opening to fix and placed it over the LPAB on the slide. The preparation was examined under the light microscope.

2.4 Preparation of spore suspension

Spores were harvested by flooding the plates with sterile distilled water containing 0.05% Tween 80 as a wetting agent, after which spores were scraped from the surface of the colonies with a sterile spatula. The resulting suspension was shaken in a 100ml Erlenmeyer flask to break up the spore chains. The concentrations of spores were determined using a haemocytometer (under 400x magnification), after which the suspension was further diluted in sterile Tween 80 solution to achieve the desired concentration (10^7 spores per ml).

2.5 Preparation of substrate (oat bran)

After drying at 105ºC to constant weight, the substrate (oat bran) was moistened with 40ml salt solutions containing (g/l): $(NH_4)_2SO_4$, 5; KH_2PO_4, 5; $MgSO_4$.$7H_2O$, 1; NaCl, 1; $FeSO_4$.$7H_2O$, 1 and sucrose, 100g/kg. Methanol 3% (volume/weight) was added to the substrate before inoculation. The initial pH of the substrate was adjusted to about 4.5 with NaOH or HCl. Before inoculating with the isolates, the substrate was autoclaved twice at 121ºC for 20 minutes. The flasks were cooled to ambient temperature and inoculated with 1ml of spore suspension containing about 10^7spores per ml and incubated at 30ºC in an incubator for 3 days by agitating the flasks at 12hrs intervals (Dhandayuthapani, 2009).

2.6 Fermentation

Solid state fermentation method was used for the production of citric acid in the laboratory using 250ml Erlenmeyer flask as the small scale laboratory fermentor. After sterilization by double autoclaving, the flasks containing the pretreated substrate were cooled and inoculated with 2 ml inoculums culture and incubated at 28ºC for 72 hr.

2.7 Recovery of citric acid from fermentation broth

Recovery of citric acid was done after 3 days of incubation. Flasks were taken out from incubators. To 10g of fermented product, 100ml DW was added. After loosening the impregnated mycelium with the substrate using a glass rod, the flask containing the broth was shaken on an Orbital Shaker at 121rpm for 1hr. The broth was filtered using Whatman No.1 filter paper. The supernatant was tested for the pH and stored at 4^0C for citrate analysis (Xu *et al*., 1989).

2.8 Quantitative analysis of citric acid

The filtrate (containing citric acid) was taken and the citric acid content was determined by spectrophotometer at 420nm using Acumen UV/Vis Spectrophotometer after adding pyridine and acetic anhydride. For each 1ml of sample, 1.3 ml of pyridine and 5.7ml of acetic anhydride were added to develop color (Marrier and Boulet, 1958).

Citric acid standard was prepared as follows: Citric acid standard was prepared from citric acid monohydrate by dehydrating it for three days at 90ºC to get anhydrous citric acid. Anhydrous citric acid obtained by heating the monohydrate at 90ºC to constant weight about 72hours was used to prepare a stock solution (50mg/ml) which is stable for at least one year at 0ºC. Standards prepared from the stock solution are stable for one month at 0ºC. Using dilution rule, $M_1V_1=M_2V_2$. To make 0.3mg/lm, 0.25mg/ml, 0.2gm/ml, 0.15gm/ml, 0.1mg/ml and 0.05gm/ml citrate solution, 60µl,50µl, 40µl,30µl,20µl and 10µl per 10ml distilled water was used respectively. (**Hailemariam Feleke, 2010**)

2.9 Optimization Studies

Optimization studies were conducted to study the effect of some nutritional (Fructose and NH_4NO_3) and physiological parameters (pH, incubation temperature and incubation time) on citric acid production.

3. Results & Discussion

3.1. Screening of Aspergillus niger strains and Cultural characteristics of Isolate C

All the four isolates produced citric acid but the strain procured from Microbiology Lab (Isolate C) produced maximum amount of citric acid. Hence, isolate C was employed for further study. Isolate C was grown on PDA agar medium and identified. Colonies on potato dextrose agar at 28°C are initially white and wooly. Later it turned black in color due to conidial production. Growth produced radial fissures in the agar.

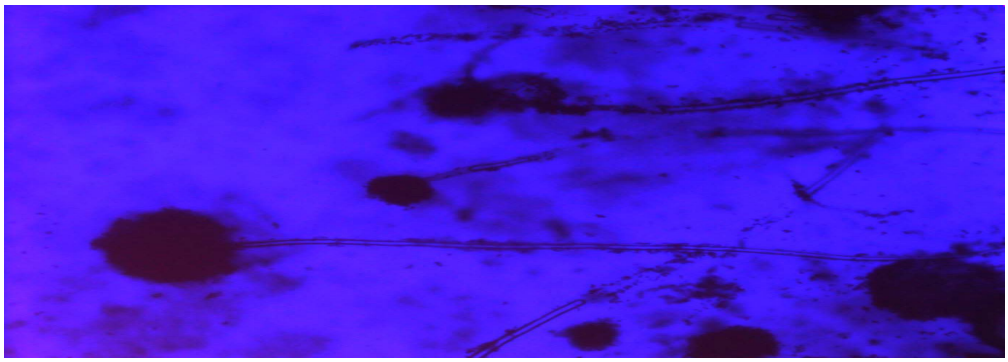

Fig. 1: Microscopic picture of *A.niger* treated with LPAB.

3.2 Optimization Studies

The effect of fructose concentration on citric acid production is shown in Fig.2 and the values in Table 1.

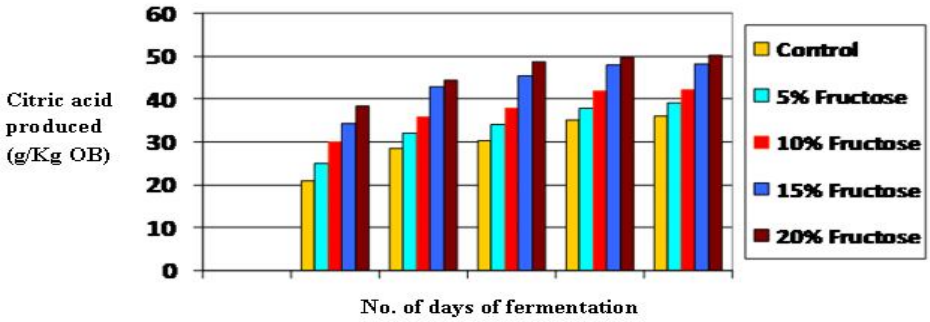

Fig. 2: Effect of Fructose concentration on CA production.

Table 1: Citric acid production- Variation of Fructose concentration.

Concn. of Fructose	No. of Samples				
	1	2	3	4	5
Control (0% fructose)	21	28.6	30.3	35	36
5% fructose	25	32	34.1	37.7	39
10% fructose	30.1	35.8	37.9	41.8	42.1
15% fructose	34.2	42.9	45.5	47.8	48.1
20% fructose	38.2	44.4	48.7	49.7	50.1

Effect of NH_4NO_3 on production of citric acid: Effect of NH_4NO_3 on citric acid production by *A. niger* is shown in Fig.3 and Table 2. An NH_4NO_3 concentration of 0.25% (w/v) gave maximum production of citric acid. A gradual increase in its concentration in the fermentation medium led to a fall in citric acid levels. Supplementing less than 0.25% (w/v) of NH_4NO_3 also led to a decline in citric acid production, which finally reached the control value (30g CA per Kg of Oat bran).

Nitrogen constituent has a profound effect on citric acid production because nitrogen is not only important for metabolic rates in the cells but it is also basic part of cell proteins.

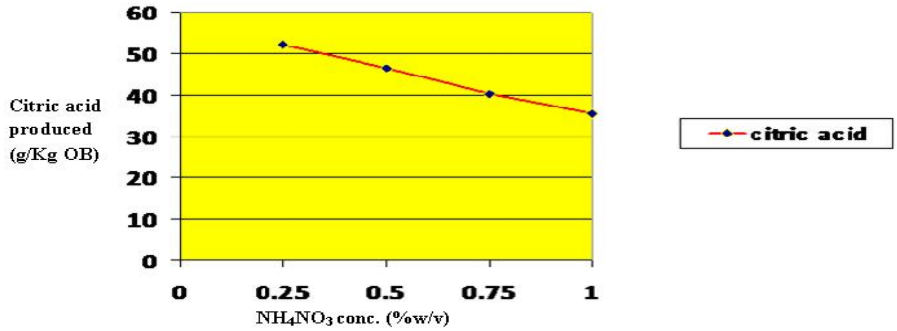

Fig. 3: Effect of NH_4NO_3 concentration on CA production.

Table 2: Effect of NH_4NO_3 on citric acid production.

Conc. of NH4NO3 (% w/v)	No. of samples				
	1	**2**	**3**	**4**	**5**
0.25	52.2	50.3	51.3	45.5	60.9
0.5	41.3	52.2	44.7	44.4	46.6
0.75	44.6	40.3	34	35.7	48.4
1	29.2	34.8	35.6	35.7	40.8

Effect of pH on citric acid production: The effect of pH on citric acid production is shown in Fig. 4 and Table 3. A pH value of 4 was found optimum for producing maximum amounts of citric acid i.e. 72g/Kg oat bran. Above as well as below this optimum value (pH=4) citric acid amounts were found to decline in the fermentation broth.

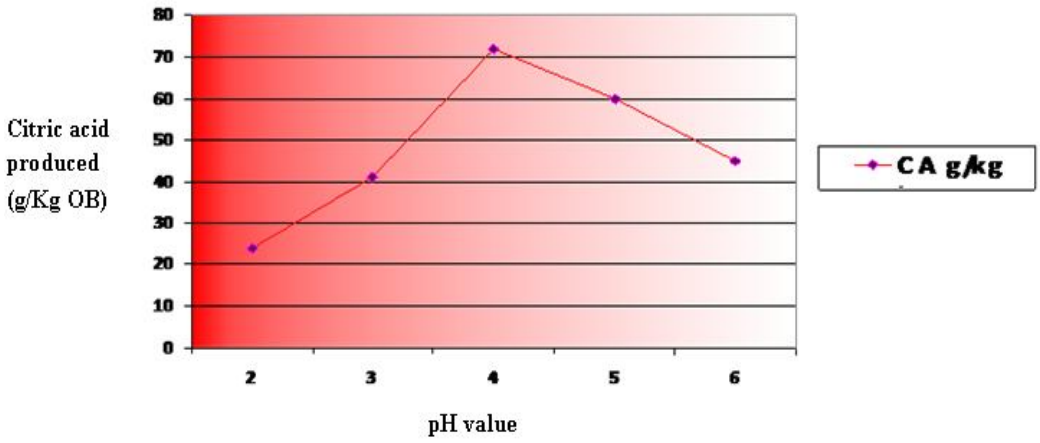

Fig. 4: Effect of incubation temperature on Citric acid production.

Table 3: Effect of pH on citric acid production.

pH values	No. of samples				
	1	**2**	**3**	**4**	**5**
2	24	29.4	22.9	21.2	23.4
3	35.6	41.7	40.8	50	43
4	71.2	70.6	66.1	72	79.9
5	47.9	61.1	60	70.8	58.2
6	45.1	55.1	32.7	44.5	42.2

Effect of incubation temperature on citric acid production: The effect of incubation temperature on citric acid production is shown in Fig.5 and Table 4. The effect of incubation temperature on production of citric acid was studied by incubating the Erlenmeyer flasks at differing temperatures ranging from 25-35^{0}C. 25^{0}C was found to be the optimum temperature for citric acid production (60g CA/Kg oat bran). An increase as well as a decrease from optimum resulted in poor growth of mycelia, hence, low levels of citric acid. At low temperature, the low citric acid production was attributed to low enzyme activity.

Table 4: Effect of incubation temperature on citric acid production.

Temperature	No. of Samples				
	1	**2**	**3**	**4**	**5**
200C	35.1	35.2	37.2	39.1	29.9
250C	60.4	59.8	55.9	69.3	51.7
300C	56.4	46.2	35.8	46.7	45.2

Effect of incubation period on citric acid production: The effect of incubation period on citric acid production is shown in Fig.6 and Table 5. The effect of incubation period was studied by maintaining fermentation for 1, 2, 3, 4 and 5 days and the resulting amount of citric acid measured. Maximum amount of citric acid was obtained at the end of 72hrs, after which the amount of citric acid declined gradually.

Table 5: Effect of incubation period on citric acid production.

Incubation period	No. of samples				
	1	**2**	**3**	**4**	**5**
0	0	0	0	0	0
24	25.8	20.2	17.8	22.4	18.3
48	41.4	37.3	36.6	44.3	40.9
72	70.9	66.8	70.4	72.6	69.1
96	70.1	65.2	70	70.3	68.5
120	69.1	64.4	69.4	69	67.1

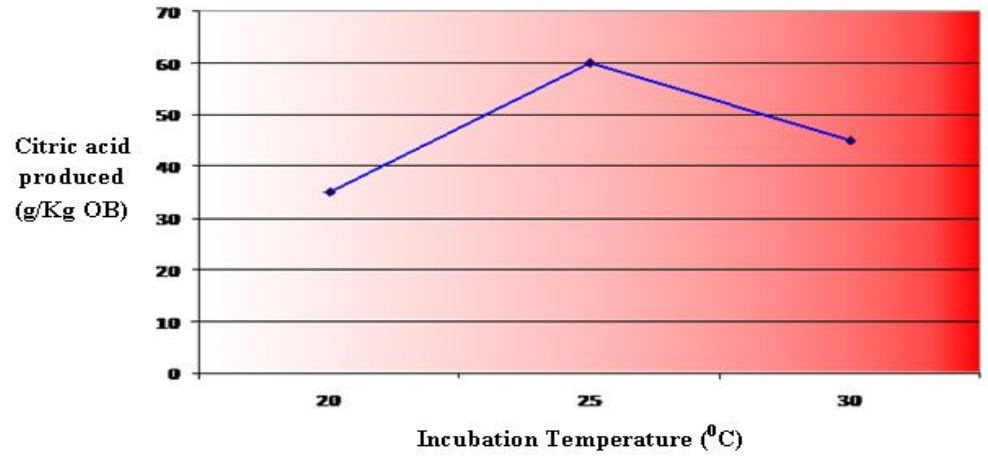

Fig. 5: Effect of Temperature on CA production.

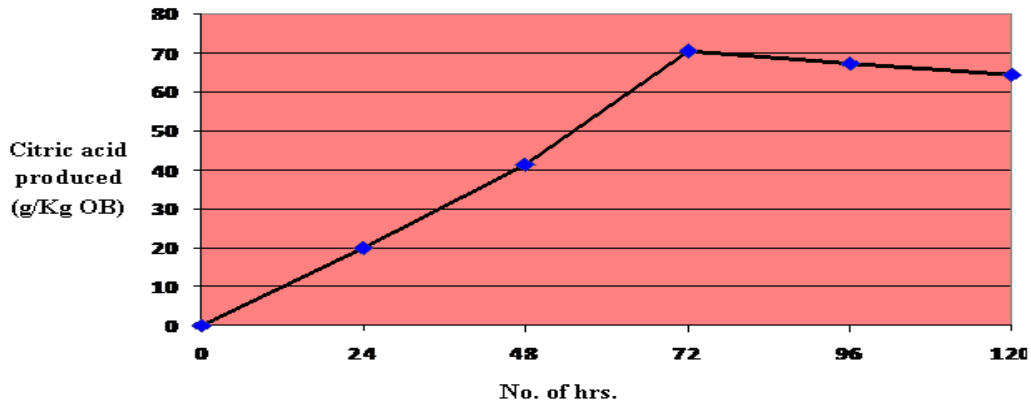

Fig. 6: Effect of incubation time on citric acid production.

References

[1] Adachi, D. M., Toyama, H., Yamada, M., Shingawa, E. and Matsushita, K. (2003). New developments in oxidative fermentation. Applied Microbiology and Biotechnology 60, 643-653.

[2] Archer, D. B., Mackenzie, A. and Jeenes, D. J. (2001). Genetic engineering; Yeasts and filamentous fungi. In: Basic Biotechnology. 2nd edn. Ratledge, C. and Kristiansen, B. (eds.). Cambridge University Press, Cambridge pp. 95-126.

[3] Crolla, A. and Kennedy, K. J. (2001). Optimization of citric acid production from Candida lipolytica Y-1095 using n-paraffin. Journal of Biotechnology 89, 27-40.

[4] Dhandayuthapani K. (2009). Studies on Production of Citric Acid by *Aspergillus niger* in Solid State Fermentation of Peat Mass. *Int. J. Biotech and Biochem.* **5**(3): 223-230.

[5] Fernando, A. V., Carlos, G. A. and Torres, N. V. (2000). Metabolism of citric acid production by *Aspergillus niger*. *Biotechnology and Bioengineering* 70, 82-108.

[6] Haq, I., Ali, S. and Qadeer, M. A. (2001). Fed-batch culture studies during citric acid fermentation by *Aspergillus niger* GCMC-7. Biologia 45, 32-37.

[7] Kamzolova, S. V., Shishkanova, N. V., Morgunov, I. G. and Finogenova, T. V. (2003). Oxygen requirements for growth and citric acid production of Yarrowia lipolytica. Federation of European Microbiological Societies FEMS Yeast Research 3, 217-222.

[8] Kapoor, K.K., Chaudhery, K. and Tauro, P.. Citric acid. In: Prescott and Dunn's Industrial Microbiology, 4th edition, edited by Gerald Reed. AVI Publishing Company Inc.p 709-747 (1982)

[9] Marier, J.R. and Boulet, M. (1958). Direct determination of citric acid in milk with improved Pyridine-acetic anhydride method. *J. Dairy Sci*. 41:1683-1692.

[10] Pandey, A. (2003). Solid-state fermentation. Biochemical Engineering Journal 13, 81-84.

[11] Schuster, E., Dunn-Coleman, N., Frisvad, J. C. and Van Dijek, P. W. (2002). On the safety of *Aspergillus niger*–A review. Applied Microbiology Biotechnology 59, 426-435.

[12] Torres, N. V., Lopez, J. C., Rivero, M. G. and Rojas, M. G. (1998). Kinetics of growth of *Aspergillus niger* during submerged, agar surface and solid-state fermentations. Process Biochemistry 33, 103-107.

[13] Xu, B.D.; Madrit, C.; Röhr, M.; Kubicek, C.P. (1989). The influence of type and concentration of the carbon source on production of citric acid by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 30, 553-558.