The Isolation and Improvement of *Aspergillus niger* by Radiation for Higher Production of Citric Acid (03-01-01-T001)

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ABSTRACT

Local citric acid producer of fungal strain *Aspergillus niger* have been successfully isolated from stale bread and onion. The isolates, designated as SB 1 and NN I showed a potential performance for citric acid production of 49% and 52% yield respectively, in shake flask studies. The strain improvement on NN I was carried out by radiation induced mutation by gamma rays at LD_{50} of 1.28 kGy.

Keywords: *Aspergillus niger*, Citric Acid, Radiation, Mutation.

Introduction

Citric acid is normally catabolised in living cells via the citric acid (Krebs) cycle whereby carbohydrates are oxidized to energy and CO2. It is accumulated in significant quantities by certain fungi, yeast and some bacteria. For citric acid fermentation industry, the traditional organisms used are selected strains of *Aspergillus niger*. This fungi can utilize cheap raw materials and it can produce high and consistent yields, thereby making the process more economical. The feedstock for the fermentation process being glucose supplemented with limited concentration of phosphate, ammonium and trace elements. Submerged fermentation occurred in two stages; biomass generation followed by citric acid production and excretion (Roehr etal., 1981)[1].

However as a primary metabolic product citric acid is not likely to be excreted under natural conditions in noticeable amount. In all fermentation process, the use of carefully selected strains is essential. In order for the strain to be viably used as industrial strain, it has to be improved physically or chemically. Mutagenesis and screening has been the method of choice for improvement of industrial citric acid production since *Aspergillus niger* lacks a sexual cycle and is refractory to classical genetic approaches (Bigelis,1989)[2]. Banik (1975)[3] has reported the derivation of mutants of *Aspegillus niger* by sequential mutagenesis using UV, which has the ability to produce about 70.5 mg/ml of citric acid in a sucrose medium while the wild type produced only 11.6 mg/ml. Similarly Rugsaseel et al. (1993)[4] has reported the isolation of a mutant of *Aspergillus niger* by sequential mutagenesis of the spores using nitrous acid, nitrosoquanidine and UV light. This mutant has been found to produce more citric acid than the parent when cultured in starch medium.

Strains improvement by selection for specific biochemical blocks or via recombinant DNA techniques have never been reported for *Aspergillus niger*. The reason for the lack of progress in the application of genetics is primarily the incompleteness of information on the genes, enzymes, limiting steps, regulatory factors and specific mechanisms related to citric acid overproduction.

The aim of this study is to improve the isolates of *Aspergillus niger* by radiation induced mutation for higher production of citric acid at a commercial production yield of between 55-75% by submerged fermentation process.

Materials and Methods

Fungal isolation

Rotten bread and onion were collected for potential source of *Aspergillus niger*. One gram of bread sample was suspended in 100 ml maximum recovery diluent. For onion sample, the swab technique using sterile cotton ball was applied on one gram of the outer layer of the onion. The cotton ball was then suspended in 100 ml maximum recovery diluent. The suspension were shaken vigorously before plating on malt extract agar with 20 %sucrose. The plates were incubated at 30°C for 48 hours. Different colonies were isolated and subcultured onto fresh agar plates and the process was repeated several times by the 16 streak method (streak dilution) in order to obtain pure cultures. Single colonies were stocked on agar slants and kept at 30°C for identification.

Identification of *Aspergillus niger*

Morphological studies of the fungal isolates were conducted using light microscope. Taxonomic study was done based on the The Genus *Aspergillus* (Raper and Fennel[5], 1977). The identification was confirmed by Plant Pathology Department, Universiti Putra Malaysia.

Fermentation Broth

The minimal media of the following composition (Othman et.al[6].,1999) : NH_4NO_3 (3.10g/l), $K_2H_2PO_4$ (0.15g/l), $MgSO_4$ (1.10g/l), Fe_2SO_4 (0.10g/l) $ZnSO_4$ (6.60g/l) and NaCl (0.15g/l) was prepared for the shake flasks studies. The concentrations of 20% decationised glucose syrup were supplemented as a sole carbon and energy source. The pH of the fermentation medium was adjusted to 2.5 with H_2SO_4. 150 ml media was dispensed into 500ml conical flask and sterilized by autoclaving at 121°C, 15 psi for 15 minute.

Spore Suspension and Inoculation

The *Aspergillus niger* isolates from the stale bread and onion were grown on agar slants and incubated at 30°C . The spore was harvested at day eight by adding 200 µL sterile 0.1% (w/v) tween 80 followed by 5ml maximum recovery diluent. The spore was gently scrapped off with inoculating loop and transferred into a McCartney bottle. The stock spore was serially diluted with maximum recovery diluent and final concentration of $1x10^6$ /ml were used to inoculate the fermentation broth. The flasks were then incubated in a rotary shaker set at 280 rpm,30°C.

3 ml of broth sampled every two days from the flasks were dispensed in 1.5 ml Eppendorf tubes and centrifuge using microcentrifuge for 5 minutes at 13,200 x g, room temperature. The supernatant was filtered through a Gelman nylon acrodisc of 0.45 m pore size. The filtered samples were stored in a freezer prior to use for citric acid and residual glucose contents.

Sample Analyses

The filtrate was analysed for citric acid and glucose concentration using HPLC (Waters). The citric acid was determined using an Inertsil ODS2 column, 0.05% diammonium hydrogen phosphate buffer at pH 2.8 as mobile phase at a flowrate of 1ml/min and analysed by photodiode array detector (PDA) at 214 nm. The residual glucose was determined using Supelco NH2 column with acetonitrile:water (75:25) as mobile phase at a flowrate of 1 ml/min detected with refractive index detector (RID) at 560 nm.

Radiation Survival Test

Radiation sensitivity test was carried out for isolate NN1 which showed slightly higher level of citric acid compared to isolate SB1. The eight day spore were harvested, and the suspension in cold saline were centrifuged twice at 5000 rpm 20 min. , 4°C, washed with 25 ml cold saline and filtered through glass

wool. The pellet was resuspended in sufficient cold saline to give a spore concentration of 10^7-10^8/ml. The suspension was then dispensed in 10 ml volumes into screw cap test tube and irradiated on ice at the CO^{60} gamma cell with attached gammachrome dosimeters. The dose rate was 13 kGy/hour. The suspension was irradiated within the dose range of 0 to 2.5 kGy. The survivors were serially diluted in maximum recovery diluent, surface plated on malt extract agar and incubated at 30°C for 48 hours. The logarithm of the survivors was plotted against radiation dose to determine the survival rate. The LD_{50} (the radiation dose that reduced the survivors by 50 %) were determine through linear estimation of the survival curve.

Radiation Induced Mutation

The spore suspension of isolate NN 1 was freshly prepared as above and irradiated at LD_{50} dose, serially diluted with maximum recovery diluent, surface plated on malt extract agar and incubated at 30°C. After 2 days colonies were transferred using inoculation loop onto malt extract agar to be kept as master plate. Colonies with the ability to produce citric acid were screened on Tricarboxylic acid medium : NH_4Cl 1.0%,$(NH_4)_2NO_4$ 1.0%, KH_2PO_4 0. 5%, thiamine HCl 300 µg/l, agar 2%, sodium acetate 0.7%. Sodium acetate was substituted with either three of the following: 0.1% sodium monofluoroacetate, 0.7% sodium succinate, or 0.7% sodium citrate. The plates were incubated at 30°C for 2 days. Based on Akiyama et al[7].,1973, a higher producer of citric acid was a colony which was able to grow in media containing acetate but unable to grow in media containing citrate or monofluoroacetate. Selected over producer strains were then tested quantitatively for citric acid production in shake flask fermentation.

Result and Discussion

Characterization of *Aspergillus niger* Isolates

Isolates of *Aspergillus niger* have been successfully isolated from the onion and stale bread sample, designated as NN1 and SB1 respectively. Microscopic examination showed that colonies on malt extract agar grow rapidly at 25-30 °C with filamentous, white, basal mycelium. After 24-36 hours, the colonies started to sporulate with black, velvety conidia. Both cultures of NN1 and SB1 produced heavy sporulation with short mycelium on agar slant.

Performance of citric acid production

About $1x10^6$spore/ml of NN1 and SB1 were inoculated into the fermentation media . The fermentation broth became more viscous as the mycelium grow heavily. However, the formation of biomass (small globules) by aggregation of germinating conidia appeared visually after two days fermentation which significantly reduced the viscosity of the broth. The age and the quantity of spore inoculated contribute to the yield of citric acid (Othman et al[6].,1999). Younger spore, less than five days old has a long lag time of 10 days while older spore, more than 15 days old, produced biomass after 3 days consisting mainly of mycelia growth , with very low citric acid level. The optimum age was spores harvested between day eight and ten. Less (< $1x10^5$ spore/ml) spore inoculated gave a longer lag time of 7 to 10 days before any biomass is visible in the flasks while excessive (>$1x10^{10}$ spore/ml) spore inoculation led to a heavy mycelia formation (unpublished report).

Performance of citric acid production of *Aspergillus niger* strain SB1 and NN1 in shake flasks fementation was shown in figure 1 and 2 respectively. The highest yield of citric acid for SB1 was 49% and 52% for NN1,both at day twenty after which the citric acid level declined gradually indicating that the acid was being degraded or utilized by the fungi. Initial citric acid production of 0.5% was observed eight days of fermentation for strain SB1 while for NN1 the citric acid was detected two days earlier. During the fermentation period the pH of the broth for SB1 and NN1 were between 1.5 to 2.5. pH value higher than 3.0 led to substantial amount of oxalic and gluconic acid (Rohr et al., 1983).

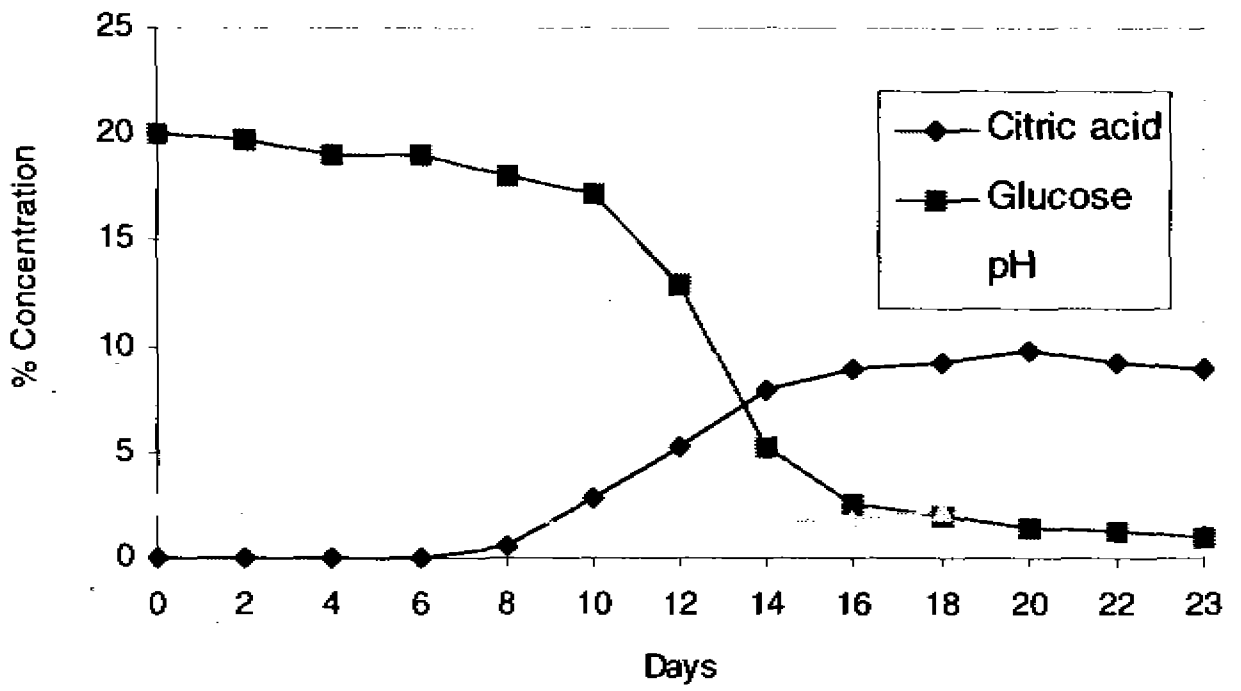

Figure 1: Citric Acid Fermentation by Aspergillus niger Strain SB1 in Minimal Medium with 20 % Glucose @ pH 2.5

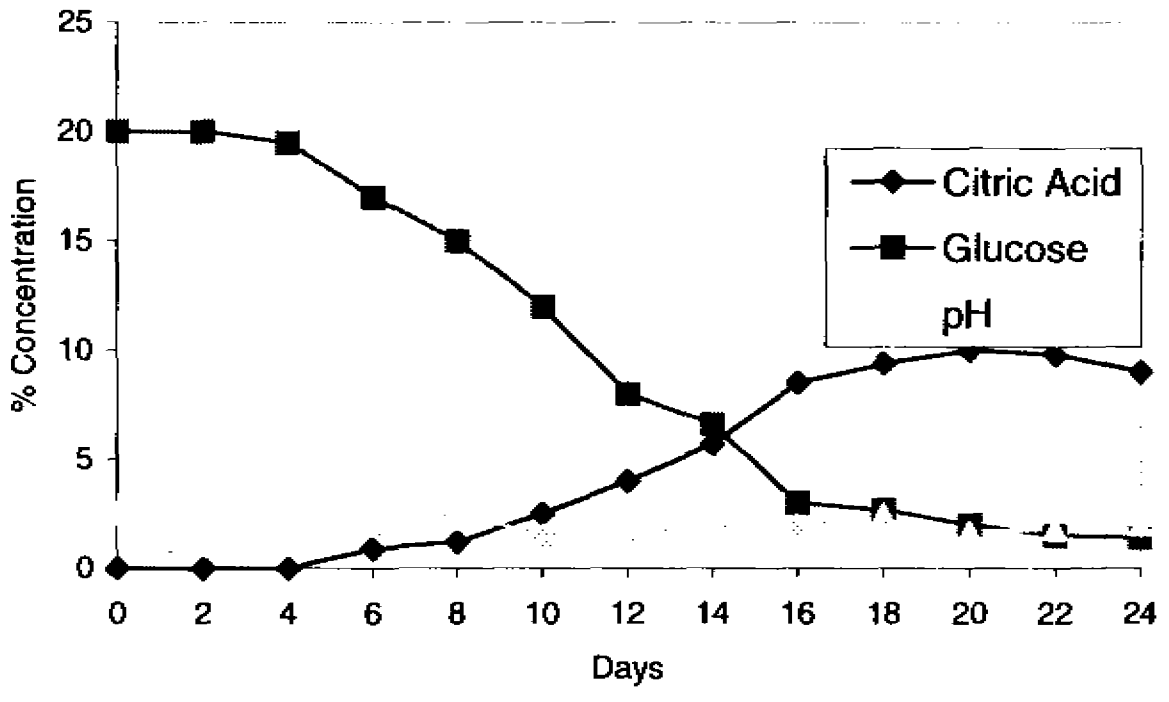

Figure 2: Citric Acid Fermentation by Aspergillus niger Strain NN1 in Minimal Medium with 20% Glucose @ pH 2.5

Radiation induced mutation of *Aspergillus niger*

The only difficulty in screening mutants for better citric acid producers is the lack of a precise and quick method by which citric acid producing strains can be easily selected. Agar plates with nutrient media containing an indicator have been used for preliminary screening of survivors for citric acid producing mutants. This method is, however, not very precise. It is well-known that the type of acid produced by fungi is pH dependent and when agar medium of pH 6-7 containing sucrose is used for detecting acid producers, normally oxalic and gluconic acid producing mutants will be detected.

Isolates of Aspergillus niger strain NN1 was used for radiation induced mutation since its showed a slightly better performance of citric acid production. The radiation sensitivity of the spore irradiated in saline water was shown in Figure 3. The LD_{50} value was 1.28 kGy which was in agreement with Patterson[9], 1988.

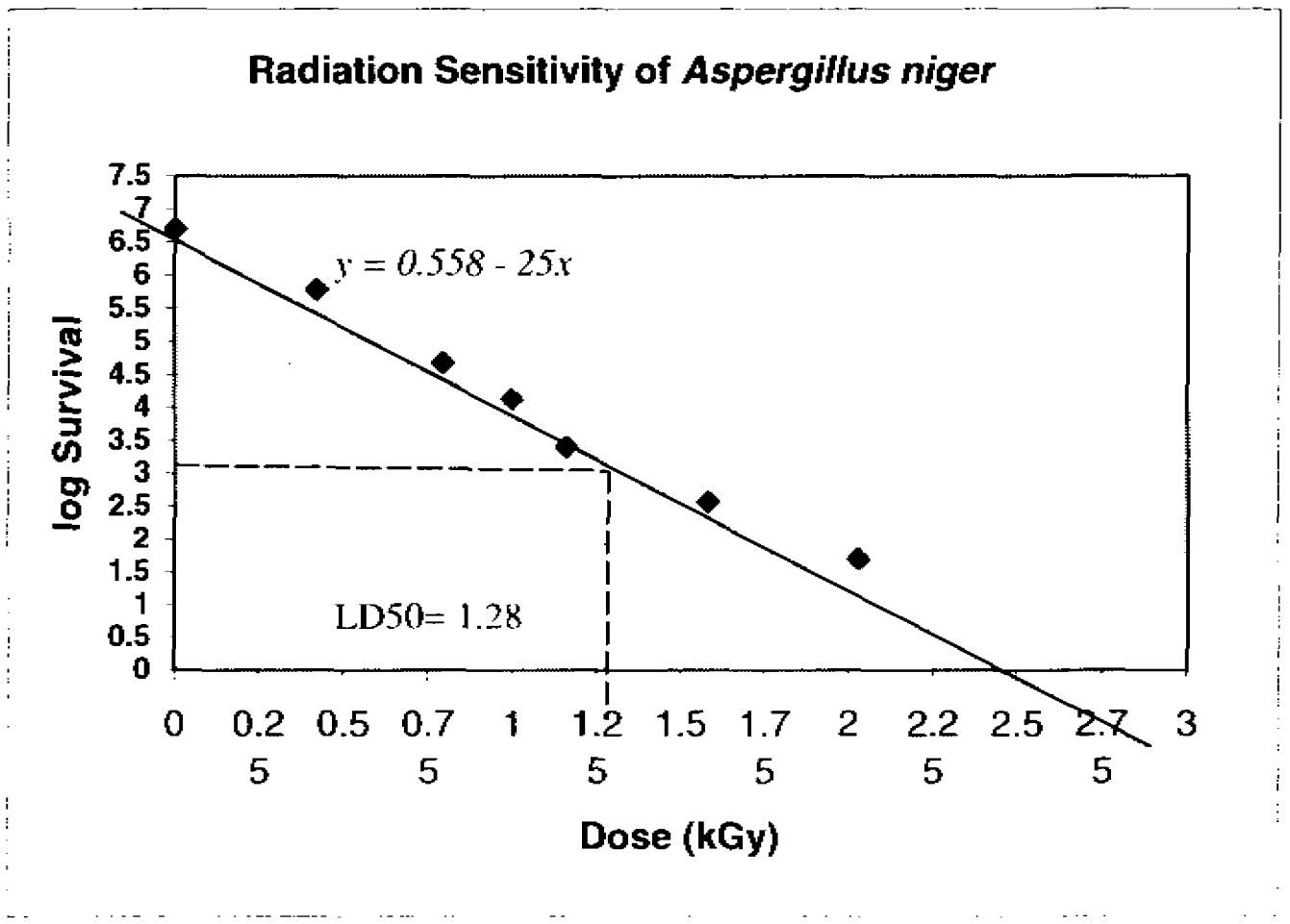

Figure 3: Radiation Survival Curve of *Aspergillus niger* Strains NN1

Akiyama et al[7]..1973 showed that it was possible to screen mutants with improved production of citric acid by testing the ability of the mutant to utilize monofluoroacetate and citrate for growth. The mechanism was not very clear but they made the assumption that low activity of aconitate hydratase (which catalyzed the reversible isomerization of citrate to isocitrate) caused sensitivity of mutants to monofluoroacetate and citrate. Based on this assumptions, colonies which grow well in acetate but showed poor growth on citrate and monofluoroacetate were selected.

About 900 colonies were isolated after irradiation at LD_{50}. 30% of these colonies showed stunted growth in the agar with either colonies grow very slow, produce sparse spore with long thin filamentous mycelium and some colonies unable to sporulate. However, none of the colonies have shown a sensitive growth to monofluoroacetate and citrate containing media.indicating that none of the variants were citric acid overproducer .

A few variants were pick up randomly and tested for its ability to grow in the fermentation media and as expected none was able to form biomass as the wild type. Therefore, the screening and selection of the mutants will be continued.

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