ISSN: 2277-5536 (Print); 2277-5641 (Online)

RANDOM MUTAGENEISIS STIMULATED OVERPRODUCTION OF CITRIC ACID BY *ASPERGILLUS NIGER*

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ABSTRACT
The *Aspergillus niger* was subjected to random mutagenesis by ethidium bromide, sodium bromide (chemical mutagens) and UV radiations (physical mutagen) for production mutant strain capable of overproducing citric acid. Mutant strains and wild strain was compared for their citric acid production ability, substrate (glucose) utilization and yield (Y_{PS}). All the mutants produced more citric acid with lesser consumption of glucose compared to parental strain. Of all mutant generated by UV exposure for 65 min. generated a strain the produced highest concentration of citric acid. The values of product yield with respect to substrate consumed showed that sodium azide and ethidium bromide treatment improved citric acid yield compared to wild type and UV mutant.

KEY WORDS: *Aspergillus niger*, ethidium bromide, sodium bromide, UV radiations, strain, citric acid.

INTRODUCTION

Citric acid is the most important organic acid produced in large amount preferably by fungal fermentation (Soccol1 *et al*., 2006). Many microorganisms are known to accumulate citric acid but *Aspergillus niger* remains the favorite employ for industries. Most of them, however, are not able to produce commercially acceptable yields. This fact could be explained by the fact that citric acid is a metabolite of energy metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances (Vandenberghe *et al*., 1999). Only few specific strains of *A. niger* are capable of overproducing citric acid have been developed for various types of fermentation processes. The yield of citric acid from these strains often exceeds 70% of the theoretical yield on the carbon source (Papagianni, 2007).
The improvement of citric acid producing strains has been carried out by mutagenesis and selection. The most employed technique has been by inducing mutations in parental strains using mutagens (Haq *et al*., 2001; Pandey *et al*., 2001; Vandenberghe *et al*., 1999). Such mutants of *A. niger* are used for commercial production (Jianlong *et al.*, 2000). Among physical mutagens, γ-radiation (Bonatelli and Azevedo, 1983; Gunde-Cimerman *et al.*, 1986; Islam *et al.*, 1986) and UV-radiation (Pelechova *et al*., 1990) have often used. To obtain hyperproducer strains, frequently UV treatment could be combined with some chemical mutagens, e.g. aziridine, N-nitroso-N-methylurea or ethyl methane-sulfonate (Musilkova *et al.*, 1983). By using a suitable selection technique on model medium with non-specific carbon sources, a strain yielding high amounts of citric acid from unusual substrates can be obtained from the mutants produced (Vandenberghe *et al*., 1999). Comparative genomics studies of citric acid producing *A. niger* ATCC 1015 versus enzyme-producing CBS 513.88 revealed that citric acid over producing strains had mutations in genes related to plasma membrane-bound ATPase, in the enzymes of the GABA shunt of the TCA cycle and in components of all steps of the electron transport chain, which are thought to be relevant for the production of citric acid (Andersen *et al.*, 2011).

In the current communication we used three mutagens; UV radiations (254nm), Sodium azide and Ethidium bromide. The mutants were randomly selected and tested for their citric acid production ability. The citric acid production was correlated with glucose utilization the energy source added to the medium. Furthermore, the yield of all strains was compared for their superiority of citric acid production over others. Yield is the ratio of mass or moles of product formed to the mass or moles of reactant consumed. Because of the complexity of metabolism and the frequent occurrence of side reactions, yield is an important term in bioprocess analysis (Doran, 1995).

MATERIALS AND METHODS

Fungal culture: *Aspergillus niger* culture, the soil isolate was used for current studies. The fungus was identified from its typical microscopic structure and black sporulation on potato dextrose agar (Fig 1). The organic acid production ability was confirmed by growing culture on Sabroud dextrose agar (Peptone 10g, Dextrose 40g, Agar 20g, Distilled water- 1.0L, pH- 5.6) incorporated with bromophenol blue. Within three days the media beneath *A. niger* colony turned from blue to yellow (Fig 2). Production of citric acid was qualitatively confirmed TLC of cell free Sabroud dextrose after growth of *A. niger* for three days.

Detection of citric acid: Was done by thin layer chromatography. TLC plates of silica gel G were used. The chromatogram was developed in solvent system n- Butanol: Formic acid: Water (40: 20: 40). 0.4% aqueous solution of bromophenol blue was used as locating agent.

Estimation of citric acid: Citric acid concentration was titrimetrically estimated. The fermented broth was titrated against 0.1N NaOH using phenolphthalein indicator. The amount of NaOH required for complete neutralization is determined. The amount of citric acid is calculated from the relation: 1mL of 0.1N NaOH=7mg of citric acid/mL.

Estimation of residual substrate: The residual substrate (glucose) was estimated by 3,5-di-nitro salicylic acid method (Jayraman, 1981).

Treatment of *A. niger* with mutagenic agents: Three mutagenic agents were used UV radiations (physical mutagen), Ethidium bromide and Sodium azide (chemical mutagens). The homogeneous spore suspension of *A. niger* prepared in saline + Tween 80 was exposed to mutagenic agents. The concentration of spores was determined by microscopic counting on hemocytometer. Number of spores in the central 25 chamber was counted. The solution was diluted to 100 spores/ml. For treatment with UV radiations 0.1ml of *A. niger* spore suspension was spread on surface of Sabraud dextrose agar incorporated with bromophenol blue. Plates were then exposed to UV for different time interval (5 to 100 sec). Followed by incubation at 30ºC for 3 days. Treatment of ethidium bromide and sodium azide was given by adding these to spore suspension to get final concentration 1mg/ml. After every 15min 0.1mL of spore suspension was taken out and spread on agar medium and incubated at 30ºC for 3 hours.

Screening for Increased Citric Acid Productivity: Randomly, colonies were selected from survivors of mutagen treatment and tested for increased productivity. For citric acid production, spore from selected colony were inoculated into Sabraud dextrose broth and incubated at 30ºC for 3 days. After incubation, the mycelium was separated by filtration. The filtrate was assessed in presence of citric acid by TLC. Citric acid concentration was estimated by titration.

Calculation of Product Yield (Y_{PS}): The product yield from substrate (Y_{PS}) is the ratio of mass or moles of product formed to the mass or moles of reactant consumed. The media used for citric acid production contained glucose from which *A. niger* can derive energy and produce citric acid as well. The Y_{PS} calculated by formulae,

$$Y_{PS} = \frac{\text{g product formed}}{\text{g substrate consumed}}$$

For calculation of yield as per progress of fermentation the value of difference of product formed ($[P]_{t2}$ - $[P]_{t1}$) was divided by value of difference of substrate (glucose) consumed ($[S]_{t2}$ - $[S]_{t1}$)

RESULTS AND DISCUSSION

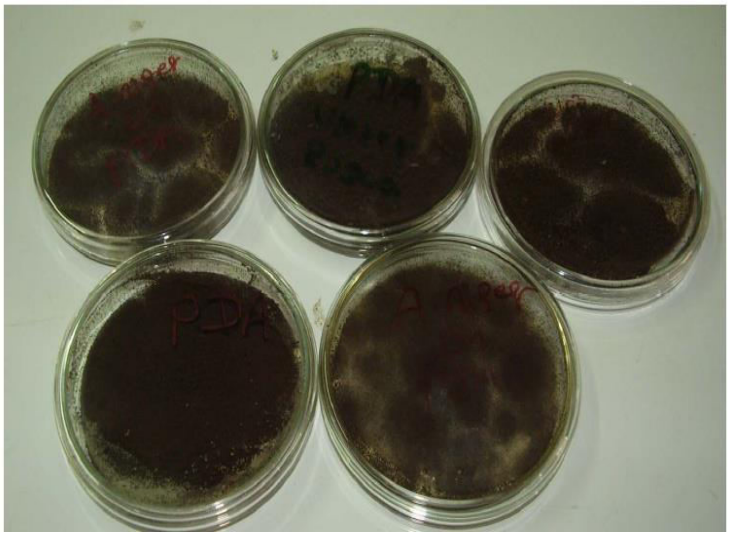

Figure 1: Black sporulation of *A. niger*.

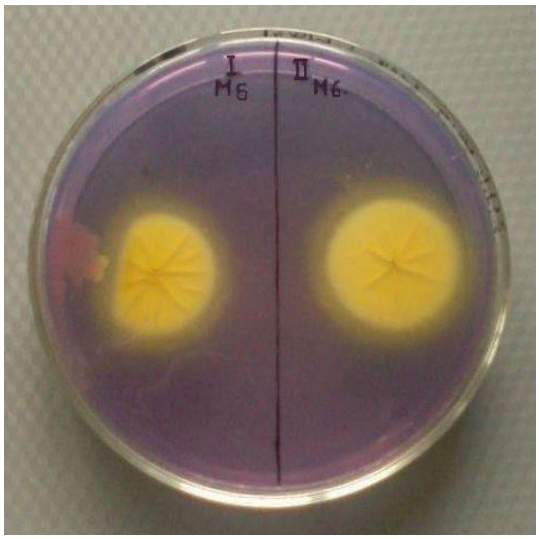

Figure 2: Organic acid production.

The typical black sporulation (Fig 1) and mycelium structure confirmed the used fungus is *Aspergillus niger*. The test strain had the ability to produce organic acid that reduced the pH of media indicated by blue to yellow color change (Figure 2). Thin layer chromatography of fermented broth developed organic acid spots having Rf value (0.70) equal to Rf value of standard citric acid this confirms presence of citric acid in fermented broth. The residual substrate concentration (Table 1) shows that maximum substrate utilization within 24hours. In all the strains the wild type strain utilized more glucose compared all the mutants (Figure 3) this result is in converse to that published by Lotfy et al which suggest mutations improved substrate utilization rate than parental strain. The glucose consumption by SA120 was reduced after 3rd day of incubation. Although of all strains UV65 was found to be slowest in utilization of glucose but it produced maximum citric acid compared to all other strains (Figure 4) this shows maximum conversion of glucose to citric acid. The performance of UV 120 was poor that UV65 suggesting the prolonged exposure to UV might have severely damaged the organisms thereby hampering citric acid production. Both mutants generated by sodium

azide (SA105 and SA120) treatment were found to be more efficient for citric acid production and less glucose utilization compared EB60.

From the yield (Y_{PS}) calculation we found that during initial few days though more than 80% of glucose was used up very less amount of citric acid was produced (Figure 5). This may be because the organism used it satisfy its energy requirement for development of mycelium. On 3rd day of incubation all the strains showed maximum yield. Javed et al also reported that citric acid reached maximum after 72hrs of incubation. Considering the yield SA105 and SA120 were found to be most efficient followed by EB60. This suggested that these chemical mutagens blocked the other paths of glucose utilization and most of it was channeled to citric acid production path. Javed et al also found that ethidium bromide (1mg/mL) treatment for 120min rather than UV treatment yielded higher citric acid producer mutant. Yield of both UV mutants was less than wild strain. And prolonged exposure to UV further reduced the yield (Figure 5).

Table 1: Citric acid production, Substrate utilization and Y_{PS} during fermentation.

Mutant strain	Incubation Time (days)	Conc. Of CA (mg/mL)	Residual glucose concentration (mg/mL)	Y_{PS} $\triangle[P]/\triangle[S]$
Control	1	16.8	3.551	0.46
	3	22.4	3.385	33.73
	5	32.2	2.948	22.43
	7	35.7	2.216	4.78
UV65	1	21.7	3.898	0.60
	3	27.3	3.690	26.92
	5	35.0	3.368	23.91
	7	40.6	2.978	14.36
UV105	1	19.6	3.732	0.54
	3	24.5	3.484	19.76
	5	29.4	3.098	12.69
	7	37.1	2.375	10.65
SA105	1	18.9	3.680	0.52
	3	24.5	3.546	41.79
	5	32.9	3.276	31.11
	7	36.4	2.694	6.01
SA 120	1	17.5	3.620	0.48
	3	23.8	3.477	44.06
	5	31.5	3.098	20.32
	7	35.7	2.444	6.42
EB60	1	18.2	3.601	0.50
	3	23.8	3.444	35.67
	5	30.8	3.011	16.17
	7	35.0	2.510	8.38

Note: *The survivors of mutation treatment were assigned code name that indicated the mutagen used, time of exposure in minutes and strain used. Example: UV65(UV light as mutagen and 65 minute of treatment). Similarly for Ethidium Bromide, EB and for sodium azide, SA was written).*

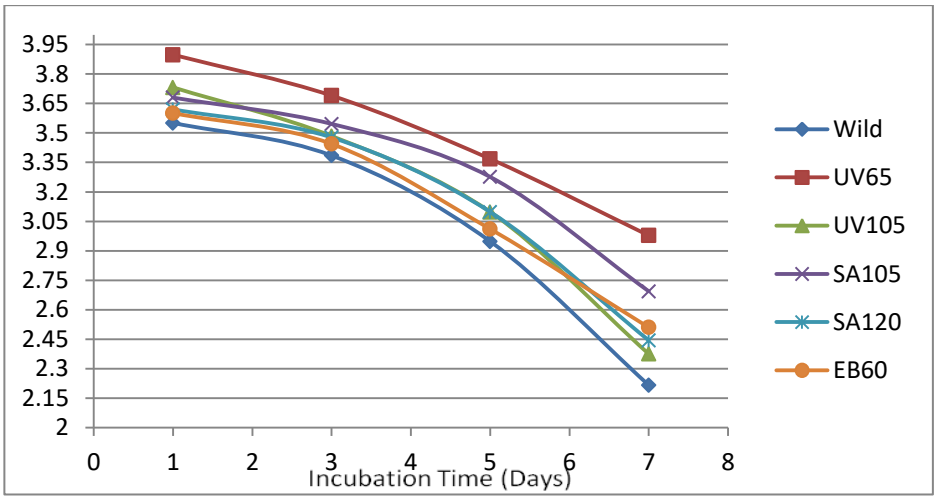

Figure 3. Substrate (Glucose) consumption during fermentation: Wild strain Vs. Mutant strains.

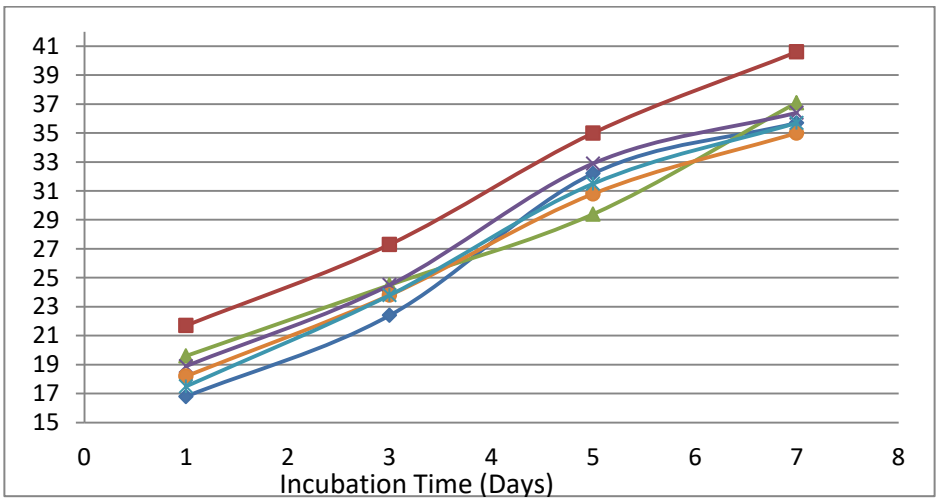

Figure 4. Citric acid production during fermentation: Wild strain Vs. Mutant strains.

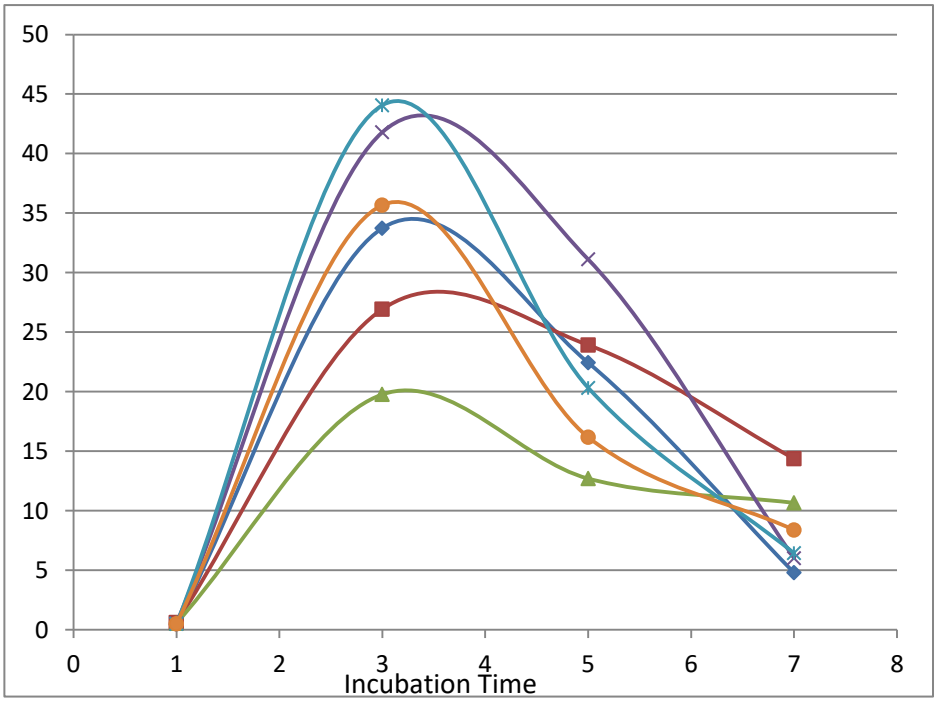

Figure 5. The yield (Y_{PS}) as per progress of fermentation: Wild strain Vs. Mutant strains.

CONCLUSION

All the mutagens somehow reduced the glucose requirements but also improved citric acid accumulation. This resulted either by blockage of other paths of glucose utilization or stopped further degradation of produced citric acid. Chemical mutagens used improved conversion of glucose to citric acid rather than other byproducts. UV mutagenesis reduced such conversion compared to wild type strain. Whereas, according the net citric acid produced the mutant generated by UV treatment for 65 mints was the highest citric acid producer. Thus the random mutagenesis successfully yielded more efficient citric acid producer.

ASKNOWELEDGEMENT

We are thankful to management of MGM's Institute of Biosciences and Technology for allowing us to work in their laboratory and uninterrupted supply of necessary funds. We are grateful for their support for our research.

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