Chapter 7
7.0 STRAIN IMPROVEMENT STUDIES FOR MAXIMUM PRODUCTION OF CITRIC ACID

Many factors need to be considered by citric acid production to obtain the economically most favorable process. Strain breeding is one of these factors. In this chapter ways to improve the fungal strains genetically are summarized as well ad Commercial production of citric acid is performed mainly with *Aspergillus niger* and to some extent with *Candida lipolytica*. As the existing fermentation process usually give high yields, the main objective of strain breeding now a days is direct shortening of fermentation time. However, other factors may also be relevant for strain improvement. For example, accumulation of a high concentration of citric acid by *A. niger* results from quite extreme culture conditions and strain breeding may decrease the sensitivity of the process to these conditions.

The number of reports considering strain improvement that have appeared in literature is limited. Rohr *et al.*, (1983); Kubicek and Rohr (1986); Mattery (1992) have reviewed much of the earlier literature. However, some research and screening activities are ‘hidden’, i.e. performed by industry and not published for obvious reasons. Generally methods used for strain improvement of *A. niger* are follows.


7.1 Mutation (UV radiation):

The selected strains *A. niger* isolate was maintained on potato dextrose agar (PDA) slants. Five steps were employed for mutagenizing the parent strain: (1) UV irradiation was performed as follows: the spores of the parent strain grown on a PDA slant were transferred into the rich medium to induce germination. The culture broth was centrifuged and the resulting spores were washed with sterile physiological saline twice and then suspended in sterile physiological saline. Spore suspension was filtered through sterile glass wool to obtain single spores suspension and 10 ml single spore suspension was exposed to UV irradiation at 254 nm for 0–40 min at a distance of 20 cm (Flow sheet).
**Flow sheet: Mutagenesis by UV irradiation**

Cells grown in potato dextrose agar (PDA) for 48 h

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Cell suspension centrifuged at 4000 rpm for 5 min

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Pellet washed twice using sterile distilled water

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Re-suspended in 0.1M phosphate buffer pH 7.0 Cell density (10^8 cells/ml)

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10 ml of the above cell suspension taken in petri plates (10 cm diameter)

↓

Exposed to UV (254 nm) for different time intervals (5, 10, 15, 20, 25, 30 min)

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Samples plated on to PDA medium

↓

Incubated at 30°C for 3-4 days

↓

Survivors (mutants) isolated and tested for thermo-tolerance and citric acid production.

↓

UV mutation was carried out with the *A. niger* and a survival curve was plotted.

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7.1.1 Isolation of UV mutagenized cells:

In mutagenesis induced using UV, 100% killing of cells was observed at 30 min and LD₅₀ was found to be at 15 min (Fig. 39). About 10 single colonies from different plates (LD₁₀-LD₉₀) exposed to different time intervals were selected based on colony morphology.

![Fig. 39: Effect of UV light (λ=254 nm) exposure time on survival of A. niger.](image)

7.1.2 Conclusions:

*A. niger*, thermotolerance was improved in cells subjected to both UV radiation mutagenesis. Mutant strains, have shown increased growth rate and survival rate at 40 °C temperature compared to the parent strain. Citric acid yield of 12.0g/100 ml was observed with the mutant strain which is slightly higher than the original parental strain.

7.2 Other mutagenic techniques that are frequently employed:

1) Co⁶⁰ irradiation was performed as follows: 10 ml suspension single spore obtained according to the procedure described above was transferred into a tube, and then was exposed to Co⁶⁰ irradiation with a dose of 0–120,000 rad.

2) Diethyl sulfate (DES) treatment procedure was as follows: potassium phosphate buffer was used instead of physiological saline in the procedure.
described in UV irradiation (1). Four milliliter of the single spore suspension
was treated with 0.2 ml DES in 16 ml of sterile 0.01 mol/l potassium
phosphate buffer (pH 7.0) at 30°C for 0–40 min.

3) Combined UV and DES treatment. It was carried out by treating the spore
suspension with DES for 8 min followed by the UV procedure with an
irradiation time of 4 min.

7.3 Citric acid yield obtained from mutant strain of *Aspergillus niger*:
Varied yields are obtained from different strains of *Aspergillus niger*. The
results are represented graphically (Fig. 40-44). From the results obtained it can be
concluded that the mutant strains have produced more yield than normal wild strain
cultures.

7.3.1 Effect of mixed substrates on citric acid production with mutant
strain:
The yield of citric acid from mutant strain at different combinations of paddy
and sorghum is observed and the maximum yield is obtained at higher concentration
of sorghum and lower concentrations of paddy (Fig. 40). The optimum yield is
obtained at the combination of 20: 80 % of paddy and sorghum for mutant strain. The
yield obtained with mutant strain is higher than the isolated strain. Ikram-ul-Haq *et
al.*, (2002) study shows that cultural conditions for citric acid production by fungi
vary from strain to strain and also depend on the type of process. The optimization of
cultural conditions is the key for high and consistent yields of metabolites like citric
acid. In the present study, the mutant strain of *Aspergillus niger* (GCMC-7) supported
maximum production of citric acid (106.65 g/l) without supplements which is
substantial. The addition of nitrogen sources and minerals like calcium and phosphate
may further increase the production of citric acid, as required for an industrial
process.

7.3.2 Citric acid yield obtained from different concentrations of
paddy substrate (mutant strains):
The experiment was performed with 10%, 15%, 20%, 25% paddy malts with
mutant strain and a maximum yield of 11.6g/100ml was obtained Due to the strain
improvement process of citric acid in the mutant strain is increased tremendously than the original parent strain (Fig. 41). Sikander Ali (2004) works shows that the fed-batch culture study was also carried out and found to give consistent citric acid yields although net productivity was lower than the control. In the present investigation, the maximum amount of citric acid (94.02 g/l) was obtained 24 h after incubation when the sampling vs. fed-batch level was maintained at 2.0 L (v/v). The mutant No. 3 gave maximum citric acid production (37.83+1.61 g/l), which is 1.37 fold lower than its parental strain. So, the mutant strain A. niger GCNG-7, a hyper citric acid producer was selected for its exploitation for the citric acid production in shake flask and stirred Fermentor.

7.3.3 Citric acid yield from mutant strain with different concentrations of sorghum malt substrate:

The experiment was performed with 10%, 15%, 20%, 25% sorghum malts with mutant strain and a maximum yield of 12.0g/100ml was obtained. The mutant yielded significantly more citric acid than the original parent strain (Fig. 42).

![Graph showing citric acid yield from mixed substrates](image)

**Fig. 40:** Effect of mixed substrates on citric acid production with mutant strain.
Fig. 41: Citric acid yield obtained from different concentrations of paddy malt substrate (mutant strain).

Fig. 42: Citric acid yield from mutant strain on various sorghum malts.
7.3.4 Effect of temperature on citric acid production:

The optimum yield of citric acid from mutant strain at different ranges of temperatures is studied and the optimum temperature is found at 33°C. The temperature of fermentation medium is one of the critical factors that have a profound effect on the production of citric acid (Fig. 43). A temperature of 33°C was found to be the best for citric acid fermentation. When the temperature of medium was low, the enzyme activity was also low, giving no impact on the citric acid production. But when the temperature of medium was increased above 30°C, the biosynthesis of citric acid was decreased. It might be due to the accumulation of by-products such as oxalic acid. Different workers have also used 30°C as the cultivation temperatures and obtained higher values of actual product (Vergano et al., 1996; Arzumnov et al., 2000). But when values were divided by the time of fermentation, all values were lower than the one supported by the isolate used in these studies. A temperature of 40°C was the most favorable for oxalic acid production while the citric acid accumulation was completely inhibited at this temperature as reported by Srivasta and Kamal (1979). When the temperature of the medium was increased above 30°C, the biosynthesis of the citric acid decreased, it might be due to that high temperature can cause denaturation of the enzyme citrate synthase. An increase in the temperature of incubation beyond 30°C has been found to decrease the citric acid yield due to catabolite repression and increase in the oxalic acid accumulation (Doelger and Prescott, 1934). Thus it was concluded that 33°C is the most suitable temperature for mycelial growth and fungal morphology and subsequently citric acid production.

7.3.5 Effect of pH on citric acid production with mutant strain:

The optimum yield of citric acid from mutant strain at different ranges of pH is studied and the optimum pH is found to be 4.8 the maintenance of a favorable pH is very essential for the successful fermentation of citric acid (Fig. 44). Decrease in pH caused reduction in citric acid production. The mutant grows at lower pH and yield was more than that of isolated strain. The pH of a culture may change in response to microbial metabolic activities. The most obvious reason is the secretion of organic acids, such as citric acid, which will cause the pH decrease. Changes in pH kinetics also depend highly on the microorganism. With Aspergillus sps, Penicillium sps and Rhizopus sps. pH can drop very quickly to less than 3.0. For other groups of on fungi
such as *Trichoderma, Sporotrichum, Pleurotus sps.* pH is more stable between 4.0 and 5.0. The nature of the substrate and production technique also influences pH kinetics. In this way initial pH must be very well defined and optimized depending on the microorganism, substrate and production technique.

**Fig. 43:** Effect of temperature on citric acid production with mutant strain.

**Fig. 44:** Effect of pH on citric acid production with mutant strain.