Chapter 3
3.0 MATERIALS AND METHODS

3.1 Materials:

3.1.1 Chemicals:

The chemicals that are used in the production of citric acid were of analytical grade. Potato dextrose agar medium was procured from Hi-media, Bombay (India), salts like ammonium sulphate, sodium hydroxide, potassium hydroxide were procured from Qualigens (India). Acetic acid, pyridine, acetic anhydrides were procured from Sigma chemical co. (USA). Hydrochloric acid and Dinitrosalicylic acid were procured from Merck (India). Bioglucanase enzyme were obtained from Biocon India, Bangalore (India), absolute alcohol and methanol were procured from SD Fine Chemicals, Bombay (India).

3.1.2 Equipment:

The equipment that are used in the production of citric acid are Incubator, Autoclave, Laminar air flow chamber, Microwave oven, Electronic weighing balance, UV, Visible- Spectrophotometer, Centrifuge, Shaker, Spirit lamp, Inoculation needle and Fermentor.

3.2 Methods:

3.2.1 Organic nitrogen sources preparation: The seeds and other various plant parts which are excess in nitrogen sources were taken. These sources are boiled for half an hour, the resulting juice obtained is taken as the source of nitrogen; these sources are added in different concentration of media to study the effect on the citric acid product.

3.2.2 Malting and mashing of grains: Good quality grains of paddy and sorghum from the local market was used as the fermentation media. The grains that are used washed with tap water, followed by distilled water to discard the unnecessary surface containments. These grains are now kept steeping for 18 h distilled water, 0.2% KOH, 0.2% NaOH. After steeping the grains are allowed to germinate for 3 days in a humidity chamber under favorable conditions of temperature. The grains so germinated are allowed to sundry for another 3 days so that all the moisture will be totally exhausted from the grains. These germinated and dry grains are powdered in to fine particles of uniform size using apex mill. During the germination process, the
enzyme will become activated and majority of the starch will be broken into simple sugars. The whole process is called as malting. The resulted malted grains reducing sugar content is further enhanced by mashing the grains by incubating the powder dissolved in distilled water at 55°–60°C for 24 h. At this temperature of mashing the enzyme activity is further enhanced and reducing sugar yield will be increased.

3.2.3 Citric acid production: It is produced by submerged fermentation. The mashed malted grains of sorghum and paddy are taken in a 250 ml Erylen-Meyer flask (total volume of fermentable media adjusted to 100 ml) at different concentrations (10, 15, and 20%). The contents are then autoclaved at 121°C, 15 lbs pressure for 15 min. The mashing process not only increases the reducing sugar content but also the protein quality. So extra-nutrients need not be added to the fermentation medium, which makes this process highly economically feasible. 5-8% of inoculum from the pre inoculum is added to the fermentation media and incubated in orbital shaker at 500 rpm, 30°C for a period of 5-8 days.

3.2.4 Glucose estimation:
Residual glucose concentration in the fermenting medium was measured using the DNS method (Miller, 1959).

DNS reagent preparation (100 ml)

1 g of 3, 5-DNSA is dissolved in a beaker containing 20 ml of 2 N NaOH under stirring and slight warming conditions. Simultaneously, 30 g of Sodium potassium tartarate is separately dissolved in 80 ml of distilled water. Both the solutions were mixed, stored in brown coloured bottle for usage as reagent.

Procedure

2.0 ml of DNS reagent is added to sample (0.2 ml), containing 0-2 mg reducing sugar (i.e. 0-10 mg/ml). The tube is placed in a boiling water bath and the solution heated at 100°C for 5 min, rapidly cooled in ice to room temperature. Use 0.2 ml distilled water plus 2.0 ml DNS reagent, heated as above, as blank to zero the spectrophotometer. Absorbency is read at 570 nm.
3.2.5 Paper chromatography:

The reducing sugars in the malted and mashed grains are qualitatively estimated by paper chromatography. 10 μl of the sample were spotted along with the standard reducing sugars on Whatman No.1 filter paper and run in the ascending order with a solvent system containing acetone:butanol:water in 4:1:1 ratio at 30°C for 36 h (3 successive runs of 12 h each). Sugars spots were detected with alkaline silver nitrate reagent.

Alkaline AgNO₃ reagent:

Solution – A: 1 ml of saturated solution of AgNO₃ was diluted to 6 ml with water and the volume was made up to 200 ml with acetone.

Solution – B: Aqueous NaOH (10%) one volume and methanol five volumes.

Solution – C: Na₂S₂O₃ (0.5M).

The paper was dipped in solution – A followed by B and C and washed in tap water and air-dried to see brown colored sugars spots.

3.2.6 Isolation and screening of fungal cultures:

Isolation of fungi was done by diluting the molasses. Fungi were isolated by using the PDA medium. This is specific medium for the required organism. Twenty four strains were isolated from the culturing using the single colony isolation technique. Out of which three different organisms namely (Aspergillus, Penicillium, and Trichoderma) were screened for the suitability of the citric acid production. Apart from the isolates four culture of Aspergillus niger were procured from Microbial Type Collection Centre (MTCC), Chandigarh. One culture is received as gift from Prof. B.Rajesekhar Reddy, Dept of Microbiology, S.K.University, Anantapur (A.P), India.

All the types were subjected to production of citric acid to screen the yield. In all the three species Aspergillus species got the high yield of citric acid. And again three sub types of Aspergillus were screened for the production of citric acid (A. niger, A. elegans, and A. clavatus). In the three subtypes A. niger had produced high yield of citric acid.
3.2.7 Inoculum preparation:

Spore suspension was prepared from a 168 h old slant grown on PDA by adding 10ml of sterile distilled water containing 0.01% tween-80 and scraping the spores with a sterile 0.1ml of spore suspension (about $1\times10^7$ spores/ml) was used to inoculate each flask of SmF (submerged fermentation) medium.

3.2.8 Inoculation medium: The medium used for the inoculation usually contain the following composition.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>30.0</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>3.0</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.01</td>
</tr>
<tr>
<td>Di-Potassium hydrogen phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.3-6.5</td>
</tr>
</tbody>
</table>

The components were taken in conical flask dissolved appropriately in distilled water. In case of viscosity the medium is added with antifoam agents silicone (0.01) was incorporated into the medium. The flasks containing media were plugged with cotton and sterilized at $121^\circ C$ for 20 min.
3.2.9 Mutagenesis:

The selected strains *Aspergillus niger* isolate was maintained on potato dextrose agar (PDA) slants. 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. Spores 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 0–30 min at a distance of 20 cm. In mutagenesis induced using UV, 100% killing of cells was observed at 30 min and LD₅₀ was found to be 15 min. About 10 single colonies from different plates (LD₁₀-LD₉₀) exposed to different time intervals were selected based on colony morphology and size. Further the potency of mutants tested for the production of citric acid using batch fermentation process.

3.2.10 Biomass estimation:

The mycelial biomass formed in submerged fermentation (Smf) was determined by filtering the broth through pre dried and pre weighed Whatmann filter paper. The mycelial mass retained on the filter paper was washed thoroughly with distilled water, blotted well, dried overnight at 100°C to constant weight, cooled in desiccators and weighed to obtain the dry cell weight of the biomass formed.

3.2.11 Extraction and purification of citric acid:

Citric acid from the fermented solids is extracted with hot water in a counter current leaching fashion and concentrated Citric acid is precipitated from the extract as calcium salt by the addition of calcium hydroxide (Rohr et al., 1983). The precipitate is thoroughly washed and free acid is liberated with the addition of dilute sulphuric acid. The liberated acid is treated with activated charcoal to remove coloring matter and other impurities, passed through ion exchange resins and evaporated at 36°C in vacuum evaporators to obtain citric acid mono hydrate (Rohr et al., 1983; Lonsane, 1991).
3.2.12 Estimation of citric acid:

Citric acid from the fermentation broth is quantitatively estimated by acidic anhydride, pyridine using the spectrophotometer at 405nm (Marrier and Boulet, 1953). One ml of the diluted culture filtrate along with 1.30 ml of pyridine was added in the test tube and swirled briskly. Then 5.70 ml of acetic anhydride was added in the test tube. The test tube was placed in a water bath at 32°C for 30 min. The absorbance was measured on a spectrophotometer (405nm) and citric acid contents of the sample were estimated with reference (run parallel, replacing 1.0 ml of the culture filtrate with distilled water) to the standard. The % of citric acid was determined.