1. ABSTRACT

Citric acid is mainly of microbial origin. As many strains of fungi such as *Aspergillus niger* secrete only trace amounts of citric acid, mutants are used for its commercial production. In this study a citric acid producing fungal strain was isolated from natural source and improved while optimizing the medium. For easy presentation and understanding, isolation of fungal strain and strain improvement were presented followed by medium optimization and down stream processing of citric acid.

Among the *Aspergillus* strains isolated from decaying lime fruit, laboratory waste and kitchen waste, highest amount of citric acid was produced by the *Aspergillus niger* strain from decaying lime fruit (*Aspergillus niger* P_1, maximum citric acid production = 1.0gl^{-1}) in basal medium [glucose, 50gl^{-1}; peptone, 7.0gl^{-1} and salt mixture consisting of (gl^{-1}) NH_4NO_3, 0.5; KH_2PO_4, 0.5; $MgSO_4$. $7H_2O$, 0.1; $CuSO_4$.$5H_2O$, 0.06 x 10^{-3}; $ZnSO_4$. $7H_2O$, 0.1 x 10^{-3} and ferrous ammonium sulphate 0.1 x 10^{-3}]. By single spore culture of *Aspergillus niger* P_1, *Aspergillus niger* P_2 was selected (maximum citric acid production = 2.4gl^{-1})

After selecting *Aspergillus niger* P_2, strain improvement studies were carried out. Best citric acid producing strains were selected by primary screening for acids in general and secondary screening for citric acid. Acid producers were selected on bromocresol green indicator plates based on Acid Unitage, (A.U.) value (Diameter of acid zone/Diameter of the colony) of a single colony while the best citric acid producer was selected by cultivating the particular strains in liquid surface fermentation (LSF).

To improve citric acid production, *Aspergillus niger* P_2 was subjected to UV - mutation (254nm, 6cm and 10min) and *Aspergillus niger* UV_1

(A.U = 1.35, maximum citric acid production = 4.6gl^{-1}) was obtained. As 5.5% and 4.6% of the spores of *Aspergillus niger* UV_1 survived when they were exposed to UV-irradition for 10 and 20min, in further experiments, UV - irradiation of spore was restricted to 20 min. *Aspergillus niger* UV_1 gave *Aspergillus niger* UV_2 showing 18.1gl^{-1} of maximum citric acid production. Then by chemical mutation (ethyl methane sulphonate 2%, 75 min) of *Aspergillus niger* UV_2, *Aspergillus niger* CM_1 was obtained (maximum citric acid production = 42.1gl^{-1}).

Effects of methanol, gingilly oil and different soluble nitrogen sources on *Aspergillus niger* UV_1 were studied. Supplementation of basal medium with methanol (30mll^{-1}) promoted extracellular and intra cellular citric acid production from 4.6gl^{-1} to 7.8gl^{-1} and from 0.228gl^{-1} to 0.322gl^{-1} respectively without affecting the membrane permeablity of the fungus. Methanol has inhibited the germination of spores in absence of glucose. Increase in glucose concentration from 50gl^{-1} to 100gl^{-1} in presence of methanol (30mll^{-1}) showed improvement in citric acid production (12.1gl^{-1}). Incorporation of gingilly oil (2mll^{-1}) to the basal medium containing 30mll^{-1} methanol increased citric acid production from 7.8 to 12.4gl^{-1}. When NH_4NO_3 in the methanol-gingilly oil basal medium was substituted with either $(NH_4)_2SO_4$ or urea (elemental nitrogen in the medium constant, 0.175gl^{-1}), production of citric acid was unchanged by $(NH_4)_2SO_4$ but reduced in presence of urea (by 14%). From this supplementation studies, basal medium supplemented with methanol and gingilly oil (melhanol-gingilly oil basal medium) was used in further studies.

The UV - mutant of *Aspergillus niger* UV_1 was *Aspergillus niger* UV_2. To improve the production of citric acid by *Aspergillus niger* UV_2 the effect of initial glucose concentration in the methanol-gingilly oil basal medium was tried. When 150gl^{-1} glucose was used, the organism utilized 137.7gl^{-1} glucose and hence, the initial glucose concentration in the methanol-gingilly oil basal medium was

changed from 50 to 140gl^{-1}. This medium was named as production medium. With decreased fermentation time (by 3d), 15.8% increase in citric acid production was obtained when 65h old mycelium instead of spore inoculum. *Aspergillus niger* UV_2 was grown in solid state medium (paddy husk impregnated with production medium) at 50% water content. Even though citric acid productivity is higher in solid state fermentation (0.198gd^{-1}) than that in liquid surface fermentation (0.129gd^{-1}), product yield and efficiency were lower (8.9%, 9.01%) in solid state fermentation than in liquid surface fermentation (26.25%, 26.4%). When the water content of the medium was maintained, 75% increase in citric acid production was observed with no change in fermentation time (2d). Since the citric acid production was 4 times higher in liquid surface fermentation than in solid state fermentation it was decided to continue with liquid surface fermentation.

To investigate an extraction method for citric acid, citric acid impregnated to raw paddy husk was manually (hand pressing) and mechanically (screw press) extracted. Total citric acid recovery by mechanical extraction was higher (97.03%) than that by manual extraction (82.6%). Therefore citric acid in moldy husk was extracted mechanically and the recovery was 98%.

The effect of cheap nitrogen sources were studied. Citric acid production was reduced in peptone free production medium. Supplementation of the production medium or peptone free production medium with either soy bean flour or soy meat powder decreased citric acid production while increasing the growth. Increase in NH_4NO_3 concentration in the production medium from 0.5gl^{-1} to 0.75gl^{-1} increased citric acid production from 48.6 to 53.5 gl^{-1} with no change in growth. When the concentration of peptone in the production medium containing 0.75gl^{-1} NH_4NO_3, was doubled (from 7.0gl^{-1} to 14.0gl^{-1} optimized production medium) citric acid production increased to 58.0gl^{-1} from 52.0gl^{-1} with reduced production time (8d).

To find a suitable sugar source for citric acid production from *Aspergillus niger* CM_1 glucose ($140gl^{-1}$) in optimized production medium was substituted with sugars ($80gl^{-1}$) from different sources such as rice flour hydrolysate filtrate (RFHF) and different palmyrah fruit pulp extract (PFP extracts). Citric acid production in media containing PFP extracts were very low (less than $6.5gl^{-1}$) while 38.7% increase in citric acid production ($32.7gl^{-1}$) was observed in the medium containing RFHF when compared with the control. Hence RFHF could be used as a carbon source.

Citric acid from the spent medium was separated as calcium citrate from spent medium using $CaCO_3$. Of the total acid produced (7.28×10^{-2} mole H^+) by *Aspergillus niger* CM_1 80.48% and 7.94% were citric acid and oxalic acid respectively. Palmyrah fruit pulp contains sugar (14-16%). Pulp extracted was 29% (w/w) of the fruit weight and the pulp in the extract was $0.57kgl^{-1}$ with 1.72 folds dilution. To extract the sugar in the pulp, pectin in the palmyrah fruit pulp extract I (PEP extract I) was removed as gel with Ca^{2+} ($0.15gkg^{-1}$) at pH 9.0. The recovery of sugar was 83.3%. By the procedure of acid solubilising the pectin and precipitating with propan-2-ol (1:1v/v), acid soluble pectin was extracted (6.1g kg^{-1} PFP extract I)