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MEDIA OPTIMIZATION STUDIES ON MEDIA COMPONENTS AFFECTING CITRIC ACID PRODUCTION BY SOIL FUNGI *A.NIGER*

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Abstract: Citric acid has got several other applications in various fields. Currently, the global production of citric acid is estimated to be around 7,36,000 tones/year and the entire production is carried out by fermentation. In Brazil, almost the entire demand of citric acid is met through imports. There is constant increase (3.5-4%) each year in its consumption, showing the need of finding new alternatives for its manufacture. Citric acid is produced by mycological fermentation on an industrial scale using crude sugar solutions such as molasses and strains of Aspergillus niger. In the present study Plackett burman design was used to evaluate the components of the media affecting the production of citri acid using Aspergillus niger. Two major components $NaNo_3$ and KCL was found to be most effective giving maximum yield and the citric acid was crystalized in the laboratory.

Index Terms – Citric acid, plackett Burman design, sodium nitrate, potassium chloride

I. INTRODUCTION

Citric Acid is naturally occurring weak organic acid which contains 3 carboxyl groups. The chemical name of Citric acid is 2-Hydroxy Propane- 1, 2, 3- tricarboxylic acid. The molecular formula of citric acid is $C_6H_8O_7$ and molecular weight of anhydrate citric acid is 192.12 g/mol and monohydrate citric acid is 210.14 g/mol. The world production of this citric acid (2- Hydroxy Propane- 1, 2, 3 – tricarboxylic acid) by fermentation is rapidly increasing. Today over 99 % of the Worlds output of citric acid is produced by microbes by various fermentation processes, substrates and microorganisms. (1)

A.niger is primarily found within soil. It is a haploid filamentous fungus found most commonly in mesophillic environment such as decaying vegetation, plants or soil. *A.niger* is a thermo tolerant and Xorephillic fungi. *A.niger* has a metabolic system which is composed of the cytoplasm, mitochondria & peroxisomes.

Currie in 1917 (2) discovered that some strains of *A.niger* grew abundantly in a nutrient medium that had a high concentration of sugar & mineral salts and initial pH of 2.5-3.5. *A.niger* was a known producer of Oxalic Acid but low pH of Currie's work suppressed both the formation of Oxalic acid & Gluconic Acid.

Citric acid is produced by mycological fermentation on an industrial scale using crude sugar solutions such as molasses and strains of *Aspergillus niger*. The natural source of citric acid is citrus fruit e.g. lemon, which contain about 7 to 9% citric acid.

II. MATERIALS AND METHODS

2.1 . Sample Collection

The natural source of Aspergillus niger is soil. For the isolation of high citric acid yielding strains of *Aspergillus niger*, so soil are collected from different location i.e. 1) Dr. Babasaheb Ambedkar University campus Aurangabad.2) Aurangabad Caves hill bottom3) Sillod farm soil, Aurangabad. The soil samples were collected in presterilised plastic bags and brought in the laboratory and were used on the same day.

2.2. Isolation and Identification

Each sample was diluted in sterile saline so as to get 1/100, 1/1000 and 1/10000 dilution of each sample. These dilutions (0.1ml) of each sample were spread separately on sterile czepeck's Dox agar medium containing gms/L quantity of $NaNO_3$- 3.0, MgSO4.7H2O, 1.0, KCL-0.5, FeSO4.7H2O- 0.5, Sucrose- 0.01, Agar- 30.00, Distilled water- 1000ml, pH- 7.3.A dilute concentration of streptomycin was added to the media to avoid growth of bacteria. Plates were incubated at 28°C for 48 hours. Isolated culture were observed and they were purified by sub culturing on czepeck's Dox agar slant. The morphological characteristization was performed using camera lucida and standard monograph.

2.3. Screening for Organic acid Producers

All the isolated cultures were subjected for screening of organic acid productions, the fungal strain producing organic acid from carbon substrates spot inoculated on Czepeck's Dox agar medium incorporated with pH indicators dye Bromocresol green A color change of medium from blue to yellow in vicinity of colony indicated the organic acid producer.

2.4. Fermentation Media

Sterile czepeck's Dox agar medium containing gms/L quantity of $NaNO_3$- 3.0, $MgSO_4$.7H2O, 1.0, KCL-0.5, $FeSO_4.7H_2O$- 0.5, Sucrose- 0.01, Distilled water- 1000ml, pH- 7.3 was inoculated with the identified strains of *A.niger*. The flask containing inoculated media was kept at 28^{o}C for 48 hours and growth of the fungi was observed. The flasks were incubated for 11 days and titrable acidity was calculated every day.

2.5. Plackett Burman Design

The Plackett - Burman Design was used for screening of the factor (media components) that significantly influenced citric acid production. The design considers the main effect of these variables but not their interaction effects. It can be represent by first order polynomial equation

$Y = \beta 0 + \Sigma \beta i\, Xi \qquad (i = 1, \ldots. k)$ (Equation 1)

Where, Y is the estimated target function Where, Y is the estimated target function, $\beta 0$ is a constant, $\beta 1$ *is* the regression coefficient, X is independent variable and k is number of variables. Each variable was represented in two levels, i.e. high(+) and low(-).

Total six macronutrient Czepeck's Dox medium from were screened include $NaNO_3$, K_2HPO_4, $MgSO_4.7H_2O$, Kcl, $FeSO_4$ Sucrose,. Each independent variable was investigated at a high (+1) and a low (-1) level which represents two different nutrient concentrations as shown in Table 1. The levels of micronutrient in all experiments were kept constant. (3).

Table- 2.1 The Plackett Burman design for macronutrients from the fermentation media.

Trials	$NaNO_3$	K_2HPO_4	$MgSO_4.7H_2O$	KCl	$FeSO_4$	Sucrose
T_1	4*	2	0.6	0.4	0.00	40
T_2	4	0	0.6	0.6	0.02	20
T_3	4	2	0.4	0.4	0.02	20
T_4	2	2	0.4	0.6	0.02	40
T_5	4	0	0.4	0.6	0.00	40
T_6	2	2	0.6	0.6	0.00	20
T_7	2	0	0.4	0.4	0.00	20
T_8	2	0	0.6	0.4	0.02	40

Table- 1 The Plackett Burman design for macronutrients from the fermentation media.
*Values in the parenthesis are expressed in gms/L quantity

2.6 Estimation of Citric acid using titrable acidity

Citric acid was estimated using standard method of estimation using 0.1 N NaOH which was standardized using 1 N Succinic acid than control medium and then titrate fermentated broth against 0.1 N NaOH using phenolphthalein as an indicator and end point colorless to faint pink color was obtained. (4).

2.7 Estimation of residual sugar by Folin-Wu method.

Standard protocol was used to estimate residual sugar using Folin-Wu method, using alkaline copper sulphate reagent and phosphomolybdic acid solution. Blue color thus obtained, which is read in a colorimeter with standard calibration curve for glucose. (5)

2.8 Recovery of Citric acid

Mycelia mats from the surface process are easily separated from the fermentation fluid. Further filtration steps are usually combined with the precipitation of oxalate.

This is done at pH value below 3.0 by the addition of lime in slight excess. Calcium oxalate thus formed is filtered off.

In order to attain the formation of large crystals of higher purity, lime is added at an empirically determined slow rate at temperature of about 90°c and at ph value close to 7. Thus losses are reduced and due to the increased solubility of impurities relatively pure crystals are obtained which may be effectively washed.

The moist precipitation is treated with (60 to70%) sulphuric acid maintaining a slight excess (1 to 2 gm/1) in order to secure complete reaction. The temperature is kept about 50°c. Then this citric acid was subsequently evaporated at 40°c. (6)

IIIIICitric acid monohydrate was formed at temperature below the transition temperature of 36.5°c. Drying of citric acid monohydrate is usually performed temperature below 36.5°c. (Bauer, U.; Marr, R.; Rueckl, W.; Siebenhofeer, M.: Extraction

of citric acid from aqueous solutions. Chem. Biochem. Eng. 2

(1988) 230±232

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III. RESULT

3.1 Isolation and Screening of Fungal cultures-

Total 10 isolates were obtained from the soil samples collected from different sites and were screened for production of organic acid. Based on the ability of the isolates to degrade bromo-cresol green dye one isolate form Sillod farm soil proved to be potential producer of citric acid. The same isolate was taken for further study.

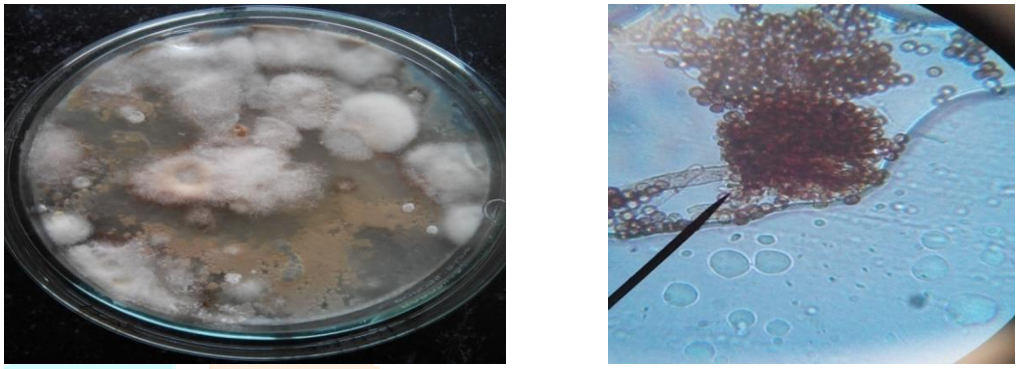

Fig 1 – Primary isolation of soil Fungi

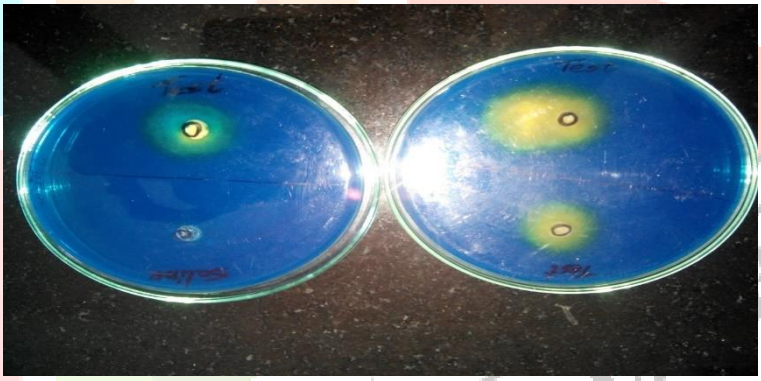

Fig 2 Screening of the fungal isolates for their ability to produce organic acid

3.2 Optimization studies: The 8 run placket Burman Design was conducted and Optical density at 420nm was measure and the amount of citric acid produced was calculated in mg/ml

Trial	$NaNO_3$	K_2HPO_4	$MgSO_4$	KCl	$FeSO_4$	Sucrose	OD at 420 nm	CA produced (mg)
T_1	4*	2	0.6	0.4	0.00	40	0.04	2.1
T_2	4	0	0.6	0.6	0.02	20	0.05	13.3
T_3	4	2	0.4	0.4	0.02	20	0.07	4.2
T_4	2	2	0.4	0.6	0.02	40	0.08	1.4
T_5	4	0	0.4	0.6	0.00	40	0.09	13.3
T_6	2	2	0.6	0.6	0.00	20	0.1	9.8
T_7	2	0	0.4	0.4	0.00	20	0.11	1.4
T_8	2	0	0.6	0.4	0.02	40	0.13	1.4

Table 2- Results of the experimental Design of 8 trials using Plackett Burman Design
The analysis of the Plackett burman design was done and it shows the following result.

Term Constant	Effect	Coefficient 6.8650	Std. Error Coefficient 0.1150	T value 59.70	P value 0.011	
$NaNO_3$	3.0200	1.5100	0.1150	13.13	**0.048**	*** $NaNO_3$**
K_2HPO_4	-0.5800	-0.2900	0.1150	-2.52	0.240	
$MgSO_4$	-1.1200	-0.5600	0.1150	-4.87	0.129	
KCl	9.8700	4.9350	0.1150	42.91	**0.015**	*** KCl**
$FeSO_4$	0.1300	0.0650	0.1150	0.57	0.672	
Sucrose	-0.6200	-0.3100	0.1150	-2.70	0.226	

* Significant

3.3 Crystallization of citric acid-

Citric acid cyatallyzation was done using the method by bauber and the result were as follows

Fig 3- **Pure crystalline form of citric acid**

After the process of crystallization the calculation was done and found to be = 8.5gm/100ml

IV. DISCUSSION

Use of citric acid increases day by day in the food materials, pharmaceutical industries, preservative agent, beverages etc. Lemon fruits are not only sufficient for fulfill the required growing demand of citric acid. Hence find out alternative way to production of citric acid by using micro-organisms. (8) Many microorganisms having capability to produce the citric acid, but in them (Fungus) *Aspergillus niger* is suitable for yielding CA. Hence isolation of *Aspergillus Niger* from soil samples collected from different locations. High yielding strain brought into pure culture form and further used.

The primary objective of any isolation program is the establishment of a clonal culture from a single cell/spore so that one has a genetically uniform strain. The method often found useful is plating and streaking of the sample onto agar plates, allowing colonies to develop from individual cells/spores assuming they can survive and grow on agar plates.In primary isolation we obtained two types of fungus viz. Green and Black colored spores. From these we had selected only black colored spore fungus for our CA production purpose. (9)

The PB design used for the optimization of media for the CA production. The preparation of 8 different trials having variant concentration of nutrients. The ANOVA algorithm is used to PB design analysis to find out highly significant variable in medium and also calculated the T test and P test value. The reading of trial T_2 and T_5 predict that two components of the media. $NaNO_3$ and KCL were reported to be the most significant for the production of citric acids.T_5 trial shows maximum sugar consumption reading than T_2 trial. Srivastava and kamal (7) has reported the significant effect of $NaNo_3$ on the production of citric acid using Aspergillus niger. Nitrogen plays a major role in the metabolism of citric acid. The cell needs nitrogen in form of ammonium to build up cell substances. On the other side too much nitrogen inhibits the production of citric acid due it will enhance the cell to grow and produce biomass.(10). An appropriate level of K enhanced CA concentrations primarily by modulating CA synthesis, as evidenced by the increased activities and gene expressions of citrate synthase (CS) and phosphoenolpyruvate carboxylase (PEPC) was reported by Kongjie Wu (2025).Similar results were obtained during this study of media optimization using Aspergillus niger.

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