Studies on The Production of Citric Acid By "Mucor Sp" Using Different Solid Substrates

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Abstract- *Citric acid is the most important organic acid. It plays a vital role in food, pharmaceutical and chemical industries. It is produced mainly by submerged fermentation. Four different fungi were isolated from different soil samples and rotten food for the production of citric acid. The different fungal isolates were differentiated on the basis of morphological characteristics. After pure culture the fungi were named as USTM17, USTM18, USTM19, USTM20. The isolate USTM19 identified as Mucor sp. which were found to be best among all the 4 fungi. Optimum growth of Mucor sp was found in 30ᴼ±1ᴼC and pH 6.5. Environmental factors have shown effect on growth of fungi and production of citric acid. Among different carbon sources sucrose (0.1g%) shows maximum citric acid production at optimum temperature 30ᴼ±1ᴼC. And phosphate source, K2HPO4 (0.4g%) and nitrogen supplement ammonium nitrate at concentration (0.5g%) gave higher yield, where as molasses 25g% was found optimum for obtaining maximum yield of citric acid. medium volume 50 ml and fermentation period 72 hours. Under such conditions, citric acid yield is 2 w/v whereas in cane molasses citric acid yield is 3.12 w/v was recorded for Czapex Dox medium and cane molasses respectively. In all cases of the treatment conditions, the amount of citric acid produced were measured by*

Acid base titration methods and the results obtained were expressed in terms of weight by volume (w/v) and all results showed that chemically pretreated pumpkin peels (SSF) relatively produced more citric acid than those Czapek Dox broth media.

The Mucor species produced higher concentration (12.85w/v) of citric acid at the pH 6by 100% pretreated pumpkin peels. At 30°C in solid state fermentation Mucor produced maximum amount of citric acid (30.94 w/vl) after 4 days. . A cost reduction in citric acid production can be achieved by using less expensive substrates. The use of agro-industrial residues as support in solid-state fermentation is economically important and minimizes environmental problems.

Keywords- citric acid, Mucor sp., Pharmaceutical, submerged fermentation.

I. INTRODUCTION

Citric acid (2-hydroxy-1, 2, 3-propanetricarboxylic acid) is the most important organic acid found virtually in all plants and animals formed by Krebs cycle process (Nadeem et al., 2010). Citric acid is extensively used in the food and beverage industry as it combines a pleasant taste with low toxicity and sweetness. It serves several functions in the food formulation, like flavor fixation and enhancement, and standardization of acid levels (Jainlong, 2010; Knuf, 2014). The use of citric acid in food industry is because of its pleasant acid taste and high solubility in water, preserving and buffering properties in the food and beverages particularly in soft drinks (Kumar and Jain, 2010). The annual production of citric acid has been estimated at about 1.4 to 1.5 million tons per year and its demand is estimated to be growing at a rate of about 3.5 to 4.0% annually. India ranks 6th in the production of citrus fruits in the world. About 70% of citric acid is utilized by the food industry; about 12% is utilized by pharmaceutical industries as flavouring, anticoagulant and preservative while the remaining 18% is utilized by other industries such as cosmetics, detergent, textile, oil recovery and paper etc (Soccol et al., 2006; Lazar et al., 2011). There are three principal methods which are available for the microbial production of citric acid i.e., the surface culture, submerged and solid state fermentation. The submerged technique is widely used for the production of citric acid. It is estimated that about 80% of world production is obtained by submerged fermentation. It also presents several advantages such as higher productivity and yields lower labour costs, lower contamination risks and labour consumption.

Among microbial fermentation of citric acid production, a lot of interest has been shown in producing citric acid via SSF. Through this process, a substantial cost reduction in citric acid production can be achieved by using cheap agricultural wastes such as apple and grape pomace, sugar cane baggage, pineapple waste, orange peel, kiwi fruit peel, cotton waste, soya- residue, vegetable peels and cane molasses as growth substrates (Baei et al., 2008; Imandi et al., 2008). In the history of citric acid fermentation, in the last hundred years, various strains of genera fungi, yeast and bacteria were reported such as: Penicillium luterum, Penicillium purpurogenum, Penicillium restrictum, Penicillium janthinellum, Penicillium citrinum, Paecilomyces divaricatum, Mucor piriformis, Trichoderma viride, Sacharomycopsis lipolitica, Arthrobacter paraffineus, Corynebacterium sp. et al.(Dawson MW, Maddox IS, Boag IF, Brooks JD (1988)). However, only mutants of Aspergillus and yeasts genus Candida have almost exclusively been utilized.

II. MATERIALS AND METHODS

Sources of organisms

The soil sample contaminated with fruit/vegetable waste was collected from Mangaldai (Darrang), Noonmati (Kamrup) and Jalukbari (kamrup) Assam, India.

Isolation, identification and screening of citric acid producing fungi.

The fungal culture was enumerated by soil serial dilution technique and the isolates were maintained on Potato Dextrose Agar media for identification. Four fungal isolates were differentiated and they were named as USTM17, USTM18, USTM19 and USTM20. The potent citric acid producing fungal culture was identified based on its macroscopic and microscopic characteristics by Barnett H.L. Mannual of fungi 1967 and Nagamani et al. Handbook of soil fungi. The isolate USTM19 identified as Mucor sp. which were found to be best among all the 4 fungi. According to Kareem et al, the fungal cultures were screened for citric acid production on Czapek Dox Agar media.

Substrates

Substrate used for production of citric acid were (a) cane molasses (b) fruits/vegetable peels such as Pears peel, Pineapple peels, Sugarcane baggages and Pumpkin peels was obtained from local market of six mile(Guwahati).Before doing fermentation with peels pretreatment is done in three different ways- simple treatment, boil treatment and acid treatment.

Solid state fermentation

Solid-state fermentation, also known as Koji process, was first developed in Japan where abundant raw materials such as fruit wastes and rice bran are available. It is the simplest method for citric acid production and is an alternative method for using agro-industrial residues (Vandenbergheetal.,2000). Solid-state culture is characterized by the development of microorganisms in a low-water activity on an insoluble material that acts both as physical support and source of nutrients (Gonzàlez etal., 1997). The yield of citric acid was dependant on the amount of methanol present in the pomace, the fermentation time and incubation temperature.

Optimization of citric acid productivity

In order to optimize the citric acid production, different chemical and physical parameters such as sugar concentration, nitrogen source, concentration of phosphate and incubation temperature were screened in case of control production medium as well as from an alternate substrate medium.

Fermentation technique

250ml conical flask was employed for solid state fermentation of citric acid fermentation.Vegetative inoculum was transferred to the production medium at a level of 5% (v/v). The incubation temperature was kept at 30 ± 1ºC throughout the fermentation period of 144 hours.

Estimation method

Mycelial wet weight was determined using filtration and centrifugation method and mycelial mat was kept in oven for overnight at 70°C to determine the dry weight also.

Citric acid Estimation

Estimation of citric acid was done by titration method by using 0.1N NaOH for cane molasses and czapek dox and 1N NaOH for Orange peel media with phenolphthalein as indicator.

% citric acid was calculated by using following formula:
% citric acid = N×V1×EqWt / V2 × 10 where; N = Normality of NaOH solution V1 = Volume of 0.1N NaOH for cane molasses and czapek dox / Volume of 1N NaOH for orange peel media.

EqWt. = Equivalent weight of citric acid. V2 = Volume of sample (ml)

Statistical Analysis

All the works were done in triplicates. The results obtained in the present investigation were subject to statistical analysis like Mean (χ) and Standard deviation (SD) by Zar, (1984) Statistical analysis were carried out using Microsoft office excel package

III. RESULT AND DISCUSSION

Effect of Carbon Sources

About 2.28±0.14w/v of citric acid was produced by using sucrose(0.1%conc.) as carbon source (Fig.1.1). Sucrose is the most effective carbon source followed by starch and glucose. It was proved that the increase in sucrose concentration had a positive effect on citric acid production. It was suggested that the mycelial growth and the strain have an extracellular citric acid linked to mycelium and this breaks the sucrose producing energy exactly at the level in which the increase in citric acid production was observed.sucrose is the most favourable car- bon source followed by glucose, fructose and galactose (L.P.S. Vandenberghe, . F. Yokoya,J. Dasgupta).

Effect of Nitrogen Sources

Nitrogen constituent has a profound effect on citric acid production because nitrogen is not only important for metabolic rates in the cells but it is also the basic part of cell proteins. (13.68±0.02w/v) of citric acid was produced by using ammonium nitrate(0.5conc.)as nitrogen source followed by ammonium chloride (13.03 ± 0. 1w/v), result shown in Table 1.2. Minimum citric acid production was observed in ammmonium sulphate. Ammonium compounds are preferred because their consumption leads to pH decreases,which is essential for citric fermentation. However it is necessary to maintain pH values in the first days of fermentation prior to a certain quality biomass production. High nitrogen concentration increases fungal growth and sugar consumption but decrease the amount of citric acid production.(Kiel H, Gurin R, Henis Y.)

Effect of phosphate source

At different concentrations two different phosphate sources were chosen for citric acid production. The sources were k2HPO4, KH2PO4.Out of these two source, K2HPO4(0.4%conc.) is suitable for Mucor sp for maximum production of citric acid. Low levels of phosphate have positive effect on citric acid production. This effect acts at the level of enzyme activity and not at the level of gene expression. On the other hand, the presence of excess of phosphate leads to a decrease in the fixation of CO2, which in turn increases the formation of certain sugar acids, and the stimulation of growth.(H.S. Grewal, K.L. Kalra, C.P. Kubicek, M. Röhr, L.P.S. Vandenberghe)

Effect of sucrose

Mucor sp was grown on solid state medium with different concentrations of sucrose (3%,5% and 10%), results are shown in Table 1.4. Maximum citric acid (10.74±0.31w/v) was obtained with initial sucrose concentration of 3%. At higher sucrose concentrations, citric acid production decreased sharply. Hossain et al. (1984) reported that high concentrations of carbon source generally lead to the suppression of α–keto glutarate dehydrogenase. In addition, the mycelium weight is reduced, and its shape is negatively affected (Papagiani etal.,1999).

Effect of temperature

In the present study temperature 30^{0}C of was found to be the optimum for citric acid production. The mold produced only a small amount of citric acid at 25^{0}C in ten days. Sporulation however, was more marked at 35^{0}C than at lower temperatures. At low temperature, the low citric acid production was attributed to low enzyme activity. Further increase in the temperature (above 30^{0}C) results in decreased biosynthesis of citric acid. It may be due to high temperature that can cause denaturation of enzyme citrate synthase and accumulation of other byproduct acids such as oxalic acid and enzyme catabolite repression and it also inhibits the culture development [Panda T, Kundu S, Majumdar SK].

Effect of Ph

Different pH (4 – 6.5) were checked on solid substrates for the citric acid production maximum yield (13.85±0.22w/v), anhydrous citric acid was obtained when pH of the fermentation medium was kept at 6.0 (Figure2.2). Decrease in pH caused reduction in citric acid production.

Incubation periods

The effect of different kinds of incubation time was tested on citric acid production. Maximum amount of citric acid production by Mucor sp was observed in 96 hours (4days) incubation time (30.94±0.35w/v). Minimum amount of citric acid production was obtained in 120 hours of incubation time (25.11±0.49w/v)(Fig.2.3). Further increase in the incubation period resulted in a noticeable decrease in citric acid concentration. This result is comparable with the results of Rehman et al. (2003) who reported four days to be optimum for citric acid production using sucrose salt medium and molasses as substrates

Citric acid production in solid state fermentation + cane molasses

Citric acid production by Mucor using simple treated pumpkin peel with molasses in 250ml conical flask was assessed. Addition of 2% methanol was added. It is clear that treated 2%molasses is better than 3% molasses and 10% molasses.

Maximum citric acid (30.948 w/v) was obtained after 4 days, result shown in table1.5.

IV. CONCLUSION

This study indicates the use of Pumpkin peel waste for fungal production of citric acid represent an efficient method of minimizing waste disposal problem an concomitantly producing organic acid of valuable important for food and pharmaceutical industries etc. Solis state fermentation gives highest production of citric acid as compared to submerged fermentation.The highest production CA (citric acid) in solid state fermentation (30.94±0.35w/v) was obtained at 120hrs of incubation period at pH 6 and temperature 30^{0}C. The study managed to show that Pumpkin peel can be considered as potential substrate for CA production. Due to low cost and abundance, molasses are recommended as an economically attractive substrate for citric acid production especially for a major cane sugar producing country like India.

Table:1.1 Effect of carbon source

Carbon source	Citric acid production in 0.1conc.(%w/v)	Citric acid production 0.3conc.(%w/v)	Citric acid production 0.5conc.(%w/v)
sucrose	2.2827±0.145443	1.14±0.142653	0.7102±0.007328
D-glucose	1.0654±0.023028	1.18±0.49642	1.936±0.2154
Starch	1.057±0.047964	1.936±0.017925	1.194±0.030496

Table:1.2 Effect of nitrogen source

Nitrogen source	Production of citric acid in 0.5conc. (%w/v)	Production of citric acid in 0.25conc. (%w/v)	Production of citric acid in 0.75conc(%w/v)	Production of citric acid in 1conc. (%w/v)
NH_4SO_4	10.021±0.92	8.5±0.522	8.1±0.32	6.74±0.58
NH_4NO_3	13.687±0.02032	8.992±0.027372	8.598±0.22419	7.38±0.172253
NH_4Cl	7.01±0.065192	9.65±0.251992	12.652±0.362588	13.038±0.101833

Table:1.3 Effect of different concentration of phosphate source

Phosphate source	Production of citric acid in 0.1conc.(%w/v)	Production of citric acid in 0.2conc.(%w/v)	Production of citric acid in 0.4conc.(%w/v)	Production of citric acid in 0.8conc.(%w/v)
KH_2PO_4	1.384±0.1171	1.004±0.6804	0.842±0.102323	3.008±0.110091
K_2HPO_4	1.03±0.07385	1.654±0.054129	2.1594±0.99784	4.806±0.188892

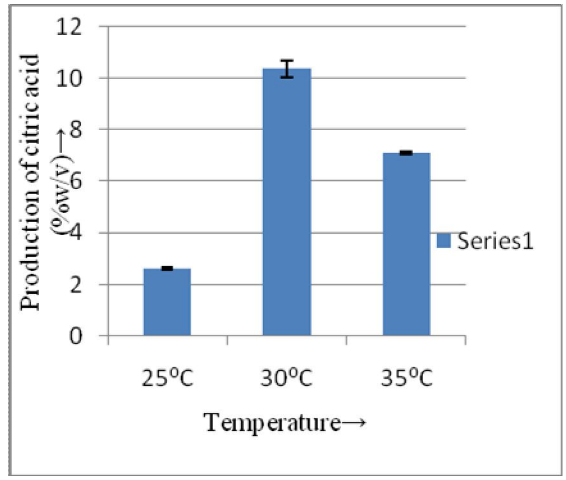

Fig :2.1 showing effect of temperature

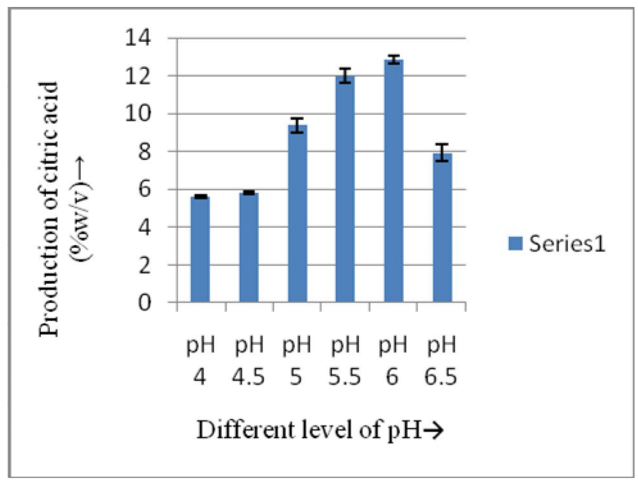

Fig:2.2 Production of CA on different pH level

Table:1.4 Effect of different concentration of sucrose

Concentration of sucrose (%)	Citric acid production (%w/v)
3% sucrose	10.74±0.31
5% Sucrose	9.31±0.28
10% sucrose	8.89±0.28
control	5.79±0.79

Table:1.5 Effect of molasses

Different conc. Of Molasses	Production of citric acid (%w/v)
2% Molasses	30.948±0.35729
3% Molasses	16.02± 0.090554
5% Molasses	15.1152±0.12061
control	6.4816±0.195121

Table:1.6 CA production different incubation period

Fermentation period	Production of citric acid(%w/v)
4^{th} days	30.948±0.3572
5^{th} days	25.1134±0.4937
6^{th} days	13.884±0.811449
7^{th} days	9.64±0.391685

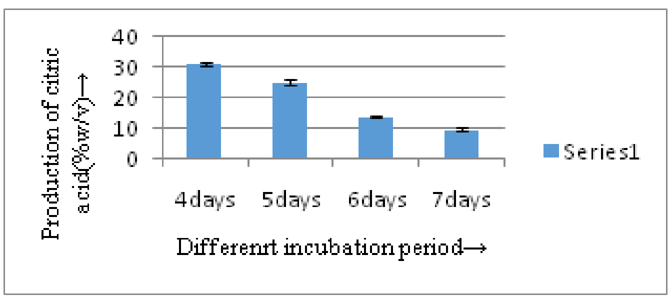

Fig:2.3 Graphical representation of different incubation period

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