Citric acid Production from *Aspergillus niger* using Mango (*Mangifera indica* L.) and Sweet orange (Citrus sinensis) Peels as Substrate

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Abstract— The demand, production and trade of citric acid have increased as it has become a commercially valuable acid due to its application in cosmetics, pharmaceutical and food industries. In the present study, mango peels as novel substrate and sweet orange were utilized for citric acid production by Aspergillus niger and the fermentation parameters were optimized. Maximum yield of citric acid was obtained using mango peel 11%, sucrose concentration 5%, inoculum 2%, potassium dihydrogen phosphate and ammonium nitrate at pH 5 and 32° C after 8 days. In sweet orange peel fermentation, maximum yield was obtained using 11% sweet orange peel, disodium hydrogen phosphate, inoculum 2%, sucrose concentration 25% at 32° C and pH 4 after 6 days.

Index Terms— Citric acid production; Mango peel; Orange peel, Fermentation; Aaspergillus niger.

1 INTRODUCTION

CITRIC acid (2-hydroxy-propane-1, 2, 3-tricarboxylic acid) is a weak organic acid, available in two crystalline forms: anhydrous and monohydrate with 192 and 210 g/mol molecular weight respectively. Its traces are found in virtually all plants and animals [1]. Citric acid can be produced mechanically, chemically and through fermentation. Fermentation technology has established its importance in processing wastes for the production of various vital compounds e.g. enzymes, biomass, organic acids etc [2]. Food processing and agro industrial wastes can be utilized for producing citric acid through fermentation [3].

Food industry uses about 70% of citric acid's total production as a pH adjuster, flavor enhancer, preservative in processed food and as an acidulant in drinks. Pharmaceutical industry consumes 12% of citric acid to enhance flavors of medicines, as an acidulant and anti coagulator. Cosmetic industry uses citric acid in the composition of different cosmetic products. Citric acid has also found other application such as metal cleaning, electroplating, fabric dyeing, detergent etc.

Citric acid has been produced at a commercial scale using microorganisms. Screening of microbes, selection of suitable substrate and optimization of operating variables is crucial for citric acid production. *Aspergillus niger* is highly recommended for industrial production of citric acid due to its property of citric acid accumulation because it produces certain enzymes that help accumulate large quantities of citric acid [4]. It has been found to produce high yields of citric acid by utilizing a variety of substrates. Previously researchers used waste of many fruits such as pineapple waste, orange pulp, orange peel, sweet lime pulp, sweet lime peel, banana peel etc. but there is no report of using mango peel as substrate for citric acid production

Pakistan is an agriculture country and sixth large producer of mango. The food and agricultural industry generate a large amount of agro industrial waste and there is a need to utilize these wastes to produce valuable products. The present study focuses on the utilization of mango and sweet orange peel for the production of citric acid and optimization of fermentation parameters.

2 MATERIAL AND METHODS

The research work was conducted at the Food and Biotechnology Research Centre of PCSIR laboratories Complex, Lahore, Pakistan. Previously isolated and identified *Aspergillus niger* strain was obtained from Molecular biology laboratory at FBRC in PCSIR and revived on potato dextrose agar plates at 30°C for 24 for 5-7 days.

2.1. Preparation of Inoculum

Spores of *Aspergillus niger* from PDA plates, were aseptically inoculated to the sucrose salt media in shake flask and incubated in an orbital shaker at 30°C and 250 rpm for 48 hr. At the end of this period the small pellets which had formed in the flasks were used as inoculum for optimization experiments, using an inoculum level of 2%.

2.2. Pretreatment of Substrates

The substrates, mango (*Mangifera indica* L.) and sweet orange (*Citrus sinensis*) peels were washed, air dried and then dried in a hot air oven at 70 °C for about 2-3 hours. Peels were then ground to about 1-2 mm size. The ground peels were collected by passing them through a sieve of 2 mm size. The peels were then stored at 4 °C in an air tight jar.

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2.3. Preparation of substrate Media

Fermentation media of sweet orange and mango peel were prepared by adding 2.5 g of substrate to 50 ml of distilled water. Media was autoclaved at 121°C for 25 minutes and then brought to room temperature. Control media with same concentrations were also prepared.

2.4. Fermentation

Surface culture fermentation methodology was adopted for the study. The fermentation media of sweet orange and mango peels were inoculated by aseptically transferring 2% of starter culture to the fermentation media. The media was stirred and then incubated at 30 °C for 7 days in the incubator. Citric acid was then estimated titrimetrically.

2.5. Assay Procedure for Citric acid

The fermentation media was taken out of incubator, diluted with autoclaved water and shaken for 60 minutes to mix all the contents. The contents were then passed through a mesh of about 2 mm size to collect all the substrate and biomass particles. The resultant solution was passed through a filter paper. The filtrate was added to an Erlenmeyer flask and 2- 3 drops of 0.1 % phenolphthalein indicator were added. 0.1 M NaOH was then added drop wise to the filtrate until the color changed which indicated the end point of titration. The volume of NaOH used was recorded and citric acid was calculated. Percentage citric acid was then converted to g/l. [5, 6]

2.6. Optimization of Fermentation Parameters

The parameters of mango and sweet orange peel fermentation using *Aspergillus niger* were optimized. The effect of phosphate sources, nitrogen sources, substrate concentration, incubation temperature, incubation period, Prescott salt and inoculum level, were observed. Except for the tested parameter, all others were set constant.

2.6.1. Effect of phosphate sources

All phosphate sources were added at a concentration of 0.1 % and fermentation of sweet orange and mango peels was carried out at 30° C, keeping all other parameters constant.

2.6.2. Effect of nitrogen sources

Ammonium nitrate and ammonium sulphate were tested as nitrogen sources at a concentration of 0.25 %. For both mango and sweet orange, this experiment was carried out at 30° C.

2.6.3. Effect of Prescott salt

Prescott salt is a mixture of salts that affects citric acid production. K2HPO4 0.1%, NH4NO3 0.25% and MgSO4 0.025% were added to the media to examine the effect of this salt mixture on citric acid production at 30° C. This fermentation experiment was done to identify the most suitable fermentation media, salt solution or distilled water.

2.6.4. Effect of inoculum level

The amount of vegetative inoculum used, affects citric acid production in relation to the carbon source available. Inoculum levels of 1-3% were tested for maximum citric acid production at 30°C for mango peel and sweet orange peel at 30°C.

2.6.5. Effect of substrate concentrations

Substrate concentration 5%, 7%, 9%, 11% and 13 % were checked for highest citric acid production. For mango, this parameter was tested at 30° C. For sweet orange peels, substrate concentrations of 5%, 7%, 9% and 11% were checked for citric acid production.

2.6.6. Effect of incubation Time

The effect of incubation time was observed by carrying out fermentation of two fruit peels at 30°C. All other parameters were kept constant.

2.6.7. Effect of incubation temperature

Temperatures of 25 °C, 28 °C, 30 °C, 32 °C, 34 °C and 36 °C were tested to identify the optimum temperature for citric acid production using sweet orange and mango peel. For sweet orange peel, the fermentation media was prepared with a composition of substrate 11 %, sucrose 5 % and KH2PO4 0.1 %. The pH of media was set at 4 and was inoculated with 2 % inoculum. For mango peel, the fermentation media had the composition Na2HPO4 0.1 %, NH4NO3 0.25 %, substrate 11 % and sucrose 15 %. The pH of media was set at 5 and was inoculated with 2 % inoculum.

2.7. Partial Purification of Citric acid

Fermentation of mango and orange peels was carried out with almost all parameters optimized and the fermented media was then filtered. For partial recovery of citric acid, lime (200 g/l) was added gradually to continuously agitated filtrate at 70° C (placed on hot plate magnetic stirrer). After the pH of medium had reached 7.0, the tricalcium precipitates were filtered using a Whattman no. 1 filter paper. These precipitates were then dissolved in 70 % sulphuric acid while being heated at 60° C for 15 minutes. The CaSO4 precipitates formed were then filtered to obtain citric acid solution [1, 6].

3 Results

3.1. Effect of phosphate sources

For mango peel, 7.52 g/l, 7.35 g/l and 4.62 g/l of citric acid was produced when potassium dihydrogen phosphate, disodium hydrogen phosphate and dipotassium hydrogen phosphate were used as phosphate sources respectively (Fig. 1). Sweet orange peel fermentation in the presence of potassium dihydrogen phosphate, dipotassium hydrogen phosphate and disodium hydrogen phosphate, gave 8.78 g/l, 6.60 g/l and 11.01 g/l citric acid production.

3.2. Effect of nitrogen sources

Ammonium nitrate and ammonium sulphate as nitrogen sources, gave same effect in mango peel fermentation (Fig. 2). In sweet orange peel fermentation, ammonium salts decreased the citric acid production.

3.3. Effect of Prescott salt

Prescott salt had negative effect in mango and sweet orange peel fermentation in our studies, citric acid production was

declined to 2.36 g/l and 2.30 g/l respectively in the presence of this salt combination (Fig. 3).

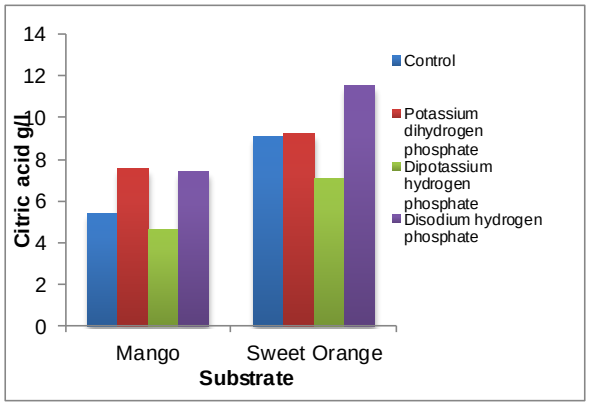

Fig. 1. Effect of phosphate sources on citric acid production in fermentation of mango and sweet orange peel

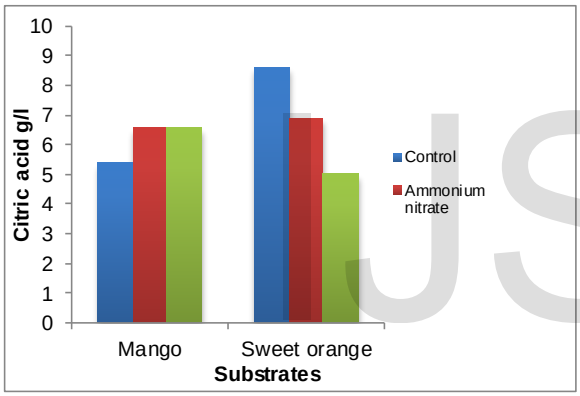

Fig. 2. . Effect of nitrogen sources on citric acid production in mango and sweet orange peel fermentation

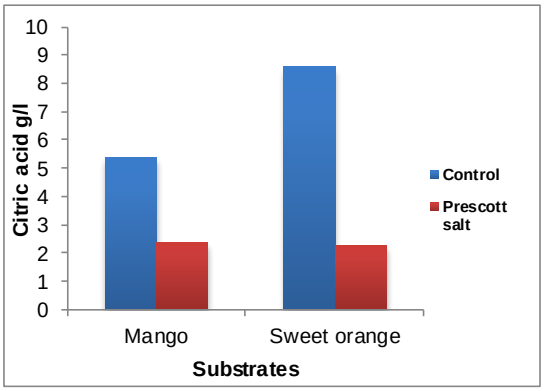

Fig. 3. Effect of Prescott salt on mango and sweet orange peel fermentation.

3.4. Effect of inoculum level

Figure 4 showed effect of vegetative inoculum size of Aspergillus niger on the production of citric acid. In mango peel fermentation 0.438, 5.477 and 0.483 g/l of citric acid was produced using 1%, 2% and 3 % inoculum respectively, while citric acid produced using these inoculum sizes in sweet orange peel fermentation were 3.719, 6.568 and 3.01 g/l respectively.

3.5. Effect of substrate concentrations

After subtracting control values, the citric acid produced by varying substrate concentration to 5, 7, 9, 11 and 13 % in mango and sweet orange peel fermentation were shown in fig. 5. 11% substrate concentration was found optimum.

3.6. Effect of incubation time

Citric acid production was recorded at different incubation time for mango and sweet orange peel. Citric acid recorded at 5,6,7,8, 9 and 10 incubation days showed decline after day 8 and day 6 in mango peel and sweet orange peel fermentation respectively (Fig. 6).

3.7. Effect of incubation temperature

Citric acid production with above optimum parameters was recorded for sweet orange and mango peel fermentation at 25° C, 28° C, 30° C, 32° C and 34 °C as shown in fig. 7. Aspergillus niger exhibited no significant difference in citric acid production using sweet orange peel as substrate at various temperatures. For mango peel at 32 °C citric acid production was enhanced to 30 g/l.

3.8. Partial Recovery of Citric Acid

From fermented mango, sweet orange peel media, 0.751 g/ 300 ml, 3.119g/ 300ml of tricalcium citrate was obtained respectively using recovery methods.

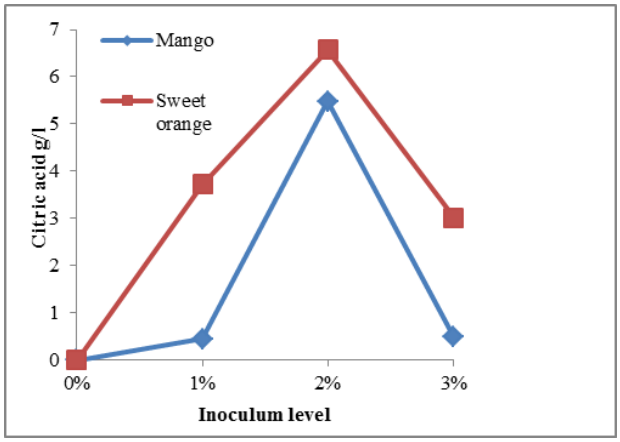

Fig. 4. Effect of inoculum level on citric acid production in fermentation of mango and sweet orange peel.

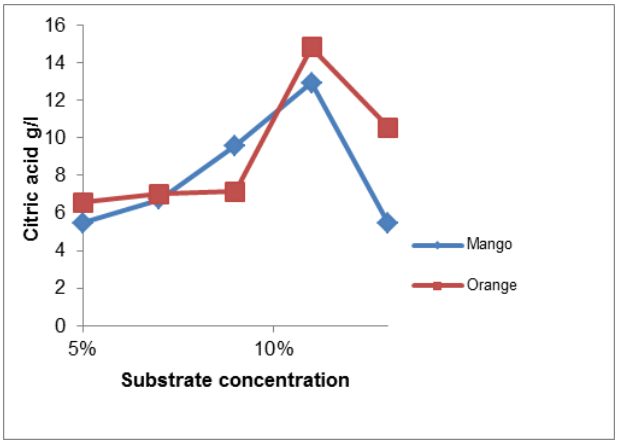

Fig. 5. Effect of substrate concentrations on citric acid production in fermentation of mango and sweet orange peel.

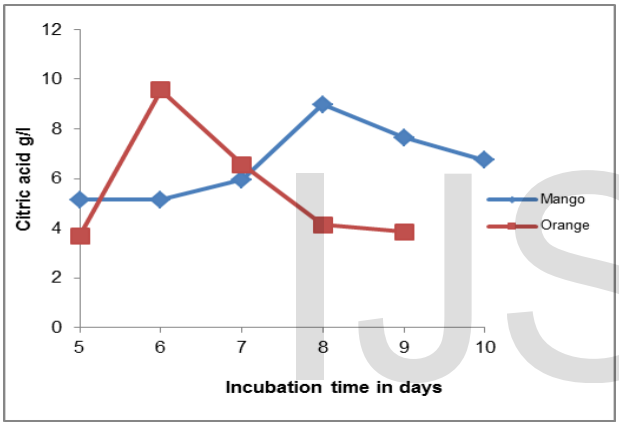

Fig. 6. Effect of incubation days on citric acid production in mango and sweet orange peel fermentation.

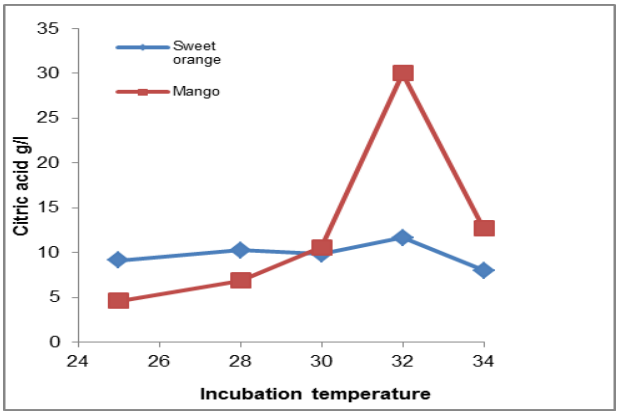

Fig. 7. Effect of incubation temperature on citric acid production in fermentation of mango and sweet orange peel.

4 Discussion

Citric acid has become a commercially important organic acid by virtue of its applications in industries. Many researchers now focus on citric acid production through a cheap substrate that would make the production cost effective. Utilizing fruit peels as a substrate can help in removing waste and in production of a valuable organic acid by fermentation. Aspergillus niger has been found to be a good citric acid producer and is mostly used to produce citric acid commercially. The production of citric acid depends on many factors such as the microorganism used, the type of substrate and other physico-chemical properties such as pH, moisture content, incubation temperature etc. [6,7].

Citric acid produced after fermentation of mango (Mangifera indica L.) and sweet orange (Citrus sinensis) was different for each peel and depended on the chemical properties of the carbon source used. Mango peel used in this study was a novel substrate and no previous studies have reported the use of this peel. Citric acid production decreased by the addition of dipotassium hydrogen phosphate, while the highest amount of citric acid was produced when potassium dihydrogen phosphate was used as a phosphate source for A. niger in mango peel fermentation. This result coincides with that reported earlier [8] when different phosphate sources were used. However, disodium hydrogen phosphate proved to be a good phosphate source in sweet orange peel fermentation.

Nitrogen is a limiting factor and the type and concentration of this source affects citric acid production. Ammonium nitrate has more available nitrogen than ammonium sulphate required for the growth of fungi and hence proved to be a good nitrogen source as in earlier studies [7,8], but no significant difference of citric acid produced using the two nitrogen sources was observed in mango peel fermentation. Prescott salt was found to decrease citric acid production in the present study which coincides with results found by Majumder et al. [9].

Citric acid was found to decrease after 6 days in sweet orange peel fermentation, and after 8 days in mango peel fermentation. 6 days and 8 days of fermentation period were found to be optimum in earlier studies as well [10]. The decrease in citric acid production can be accounted to the decrease in sugar content and the growth phase of fungi [11].

In both mango and sweet orange peel fermentation, an inoculum size of 2% was found to be good for citric acid production. The production decreased on increasing inoculum size to 3%. The reason for this decrease can be the unavailability of sugar content to the fungi [12]. Substrate concentration of 11% was found to be best for both mango and sweet orange peel fermentation. Citric acid production decreased on increasing substrate concentration from 11%. There is a possibility that increased sugar concentration decreased the activity of enzyme involved in citric acid cycle.

Incubation temperature also has an effect on citric acid

production Citric acid produced through mango peel fermentation at the optimized temperature was much greater than that produced by sweet orange peel fermentation. For both mango and sweet orange peel fermentation, a temperature of 32 ° C was found to be best. This finding however was in contrast with the finding of Ali et al. [11]. At a higher temperature, citric acid production was found to decrease. This decrease can be attributed to the inactivity of enzyme citrate synthase at higher temperatures. The yield of citric acid obtained by sweet orange peel fermentation was greater than that reported by Khadir [13]. This difference in production can be attributed to the different variety of fruit peel used and the pre treatment methods.

CONCLUSION

Various fruit wastes are previously reported as substrates for citric acid production but mango peel we used during study was novel. The results revealed that the highest amount of citric acid produced using 11 % mango peel, 5 % sucrose concentration, inoculum 2%, disodium hydrogen phosphate and ammonium nitrate at pH5 and 32° C for 8 days was 30g/l. For sweet orange peel fermentation, optimum parameters were inoculum 2%, sucrose 25%, incubation period 6 days, incubation temperature 32° C, pH 4 and substrate 11 %.

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