Feasibility of Citric Acid Production by Gamma Ray Induced Mutant Strains of *Aspergillus Niger* Using Molasses and Maize as Substrates

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Abstract

The worldwide demand for citric acid in industrial microbiology is increasing faster than its production. Therefore, more economical processes for citric acid production are required. In the present study, the feasibility of citric acid production using cane molasses and maize as substrate with an optimum fermentation condition was investigated. Gamma-ray induced second step mutant strains of Aspergillus niger (A. niger) designated as 14/20 and 79/20 were used for citric acid production. The reactions were carried out in the presence and absence of Prescott salt. The result showed an increased rate of citric acid production with the increase of fermentation time. In the absence of Prescott salt, the highest citric acid production was recorded as 13.60 mg/ml and 12.24 mg/ml for A. niger 14/20 and 79/20 strains respectively on the 13th day in mixed fermentation medium prepared with molasses and maize. Aiming to further increase of the production, the previously isolated gamma-ray induced second step mutant 14/20 and 79/20 of A. niger was reradiated at 10, 20, 30 and 40 Krad by ^{60}Co Gamma beam-650 source at a dose rate of 0.674 kGy/hr. In absence of Prescott salt highest titratable acidity (TTA) value (8.11) for production of citric acid was found by further mutated A. niger 14/20 with gamma radiation at 20 Krad in mixed fermentation medium and the lowest TTA value (2.70) was found in maize media by further mutated A. niger 79/20 with gamma radiation at 30 Krad on 13th day. The present study revealed that the possibility of profitable utilization of cane molasses and maize for citric acid production could be explored by using mutationally improved strains of A. niger.

Key words: *Citric acid, Maize, Molasses, Fermentation, Aspergillus niger*

INTRODUCTION

Commercial production of citric acid using the fermentation technique has achieved an important milestone in the field of industrial microbiology in various countries (Munshi et al., 2012). *Aspergillus niger (A. niger)* is a fungus, has been reported to produce different metabolites for commercial interests including citric acid (Munshi et al., 2012; Steinböck et al., 1991; Usami, 1978; Usami & Fukutomi, 1977). *A. niger* has well developed enzymatic system and that is the fact that, this organism is capable of utilizing varieties of substrates.

For the last two decades a number of studies have already been carried out to optimize the citric acid production along with to test the feasibility of using cheap and available raw materials such as molasses and jackfruit (Munshi et al., 2012; Munshi et al., 2013), molasses and pumpkin (Majumder et al., 2010), sugarcane bagasse (Kumar, Jain, Shanker, & Srivastava, 2003; Tuquerres et al., 2017), apple pomace (Shojaosadati & Babaeipour, 2002), palmyra jaggary (Ambati & Ayyanna, 2001), carob pod (Roukas, 1999), corncobs (Hang & Woodams, 1998), okara (Khare, Jha, & Gandhi, 1995) and pineapple waste (Tran & Mitchell, 1995). Not only the raw materials, media is also important for optimization of citric acid production. Dunn and Prescott (Tran & Mitchell, 1995) found a medium (Sucrose 140g/l, NH4NO3, 2.23g/l; K2HPO4, 1.00g/l and MgSO4.7H2O, 0.23g/l) to be most satisfactory for *A. niger* capable of providing high production of citric acid which was known as Prescott salt. If more than 0.25% of ammonium nitrite, 0.15% of potassium monohydrozen phosphate and 0.3% of magnesium sulfate were used oxalic acid formation increased and the yield of citric acid decreased (Prescott & Dunn, 1949).

However, the worldwide demand for citric acid is increasing faster than its production leading to the necessity of the economical production processes (Alvarez-Vasquez, González-Alcón, & Torres, 2000). However, high production of citric acid is highly dependent on the strain used and its response to the composition of the medium used which can show a great deal of variability (Oshoma & Ikenebomeh, 2005). Bangladesh is a lower middle income country which is transforming its economy from agro-based to the industry-based for the last two decades. Therefore, the industries in Bangladesh require a large quantity of citric acid every year. Currently, there is no production facility of citric acid in Bangladesh. Hence, the country is importing 100 percent of citric acid from different countries to meet its own demand (Majumder et al., 2010). For the last two decades, Bangladesh had enormous development in the field of food and pharmaceutical industry. Therefore, any increase in citric acid production would be of potential interest and hence there is an obvious need to consider all possible ways in which this might be achieved. Considering the future requirements and the availability of cheap and raw material, efforts were made to develop the process for production of citric acid based on local resources like molasses and maize. So the purpose of study refer to the feasibility of raw and cheap materials such as molasses and maize for citric acid fermentation and to use gamma-ray induced mutants for further genetic improvement of the high citric acid yielding mutant strains 14/20 & 79/20 of *A. niger*.

MATERIALS AND METHODS

2.1 Microorganism used

The strains of *A. niger* designated as 14/20 and 70/20 were used for citric acid production (Hannan, Sarwar, Baten, & Chaudhury, 1976). Strains 14/20 and 79/20 are the second step mutants derived from the strain HB3 which is the first step mutant from the wild type strain CA16 (Faruk et al., 2014; Hannan et al., 1976). The strains were sub cultured on potato dextrose–agar slants containing 1% malt extract, 1% yeast extract, 1.5% dextrose and 2% bacto agar.

2.2 Substrates used

Cane molasses and Maize powder were used as substrate.

2.3 Preparation of molasses medium

Molasses was clarified by appropriate dilution with tap water and boiling the solution for half an hour. The boiling molasses was then preserved overnight for sedimentation of suspended particles. In order to remove the abrasive particle from the solution it was filtered through absorbent cotton and dregs was cast-off.

2.4 Preparation of maize medium

After separation of maize then washed thoroughly with tap water and there after dried in dryer at 50 ºC. The maize was powdered in a grinding machine. Dried powder of maize was hydrolyzed in 300 ml solution of 0.05 N HCl and autoclaved at 121 º C temperatures less than 15 lbs pressure for 20 min. The hydrolyzed maize powders were then filtered through thin cloth.

2.5 Preparation of mixed substrate medium

Equivalent amount of maize powder and molasses were hydrolyzed in 300 ml solution of 0.05 N HCl and autoclaved at 121 ºC temperatures less than 15 lbs pressure for 20 min. The hydrolyzed solution was then sieved through thin cloth. The media was then conserved overnight for sedimentation of suspended particles be existent in molasses.

The following parameters were selected to find out which one was better for citric acid fermentation: Sugar with Prescott salt and Sugar without Prescott salt.

2.6 Irradiation of the organism and further induction of high yielding mutants

Some citric acid yielding mutants of *A. niger* have been isolated and the induction of further mutation for higher production of citric acid has been attempted with the help of gamma irradiation. Conidia were irradiated with gamma radiation at 10, 20, 30 and 40 krad/ hour respectively, using by ^{6o}Co Gamma beam-650 source. The strains were sub cultured on dextrose agar slants containing 1% malt extract, 1% yeast extract, 1.5% dextrose and 2% bacto agar. Conidia were harvested in sterile distilled water after 7 - 9 days of growth at 30 ºC and inoculated to the fermentation media. One ml spore suspension was taken from the test tube by micropipette and dropped into fermentation media. After exposure of gamma ray and the breaking of DNA, the cell can repair the damaged genetic material and genetic improvement may occur.

2.7 Inoculum

Conidiophores suspension of the concentration $2X10^7$ spores/ml prepared from 7 to 10-day old culture of *A. niger* were used as inoculums in the experiment. The conidia of the strains, 14/20 and 79/20 was inoculated in cane molasses, maize and mixed media modified to 14% sugar concentration, pH 5 and at 30°C under stationary condition. Initial and residual sugar concentrations, total titratable acidity (TTA), citric acid and pH level in the fermentation broth were determined 48 h up to 13 days of growth.

2.8 Citric acid production process

Citric acid were produced according to the flow chart adopted from Munshi *et al,* (2012) (Munshi et al., 2012). After inoculation of conidiophores of the strains 14/20 and 79/20 in cane molasses, maize and mixed media, citric acid and pH level in the fermentation broth were determined at every 2 days up to 13 days of growth.

2.9 Data analysis

The data were analyzed using “MicrosoftExcel-2003”. The statistical program SPSS 12.0 for windows was used to test the level of significance. Probability (p) value of 0.05 or less ($p<0.05$) was considered as significant.

RESULTS AND DISCUSSION

3.1 Kinetics of citric acid fermentation by the A. niger mutant strains 14/20 and 79/20 in different media

In the present study, reversible results were obtained for the concentration of citric acid and pH value. The concentration of citric acid was reported to be increased with incubation period, at the same time the pH value has been reported to be declined. Out of three substrate media and two strains tested, the highest citric acid concentration was recorded in mixed substrate media for *A. niger* 14/20 strain with incubation period of 13th day. The value of citric acid production was recorded as 11.92 mg/ml with Prescott salt (figure-1, A) and 13.60 mg/ml without Prescott salt (figure-1, B).

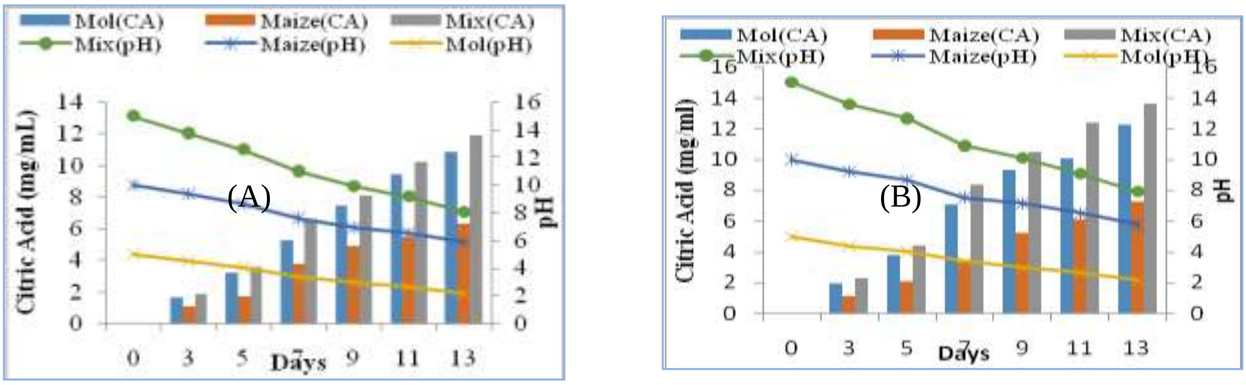

Figure (1): Time course of citric acid production and the pH evolution obtained with A.niger 14/20 in various fermentation media. (A) With Prescott salt and (B) without Prescott salt. Mol, Maize Mix indicates molasses, maize and mixed substrates respectively. Significantly different (P < 0.05).

At the same time, citric acid concentration was recorded in mixed substrate media for *A. niger* 79/20 strain on 13th days as 11.81 mg/ml (figure-2, A) and 12.24 mg/ml with Prescott salt and without Prescott salt (figure-2, B) respectively.

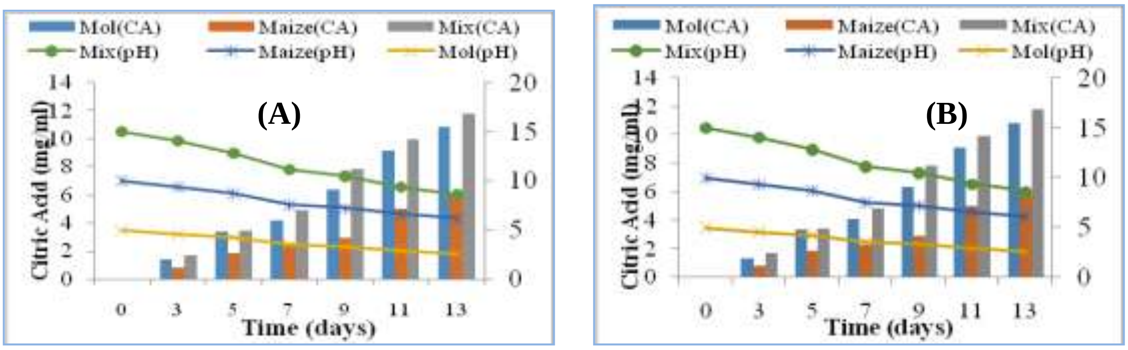

Figure (2): Time course of citric acid production and the pH evolution obtained with *A. niger* 79/20 in various fermentation media. (A) With Prescott salt and (B) without Prescott salt. Mol, Maize, Mix indicate molasses, Maize and mixed substrates respectively. Significantly different (*P* < *0.05*).

This result indicated that *A. niger* 14/20 strain was superior than that of *A. niger* 79/20 in mixed substrate medium for citric acid production. In the absence of Prescott salt citric acid production was found most effective than the absence of Prescott salt in all cases. Similar findings were reported by Laboni *et al.*, (Majumder et al., 2010) and Munshi *et al.*, (Munshi et al., 2012). For the strain *A. niger* 14/20 the lowest pH was recorded (2.19) for mixed substrates followed by cane molasses (2.20) with Prescott salt. However, in absence of Prescott salt the pH value was recorded as 2.13 for mixed substrates followed by 2.16 in cane molasses on 13thdays (figure-1, A and figure-1, B). At the same time for *A. niger* 79/20 the pH of the medium declined from initial value of 5 to 2.57, 3.62 and 2.47 with Prescott salt and 2.35, 3.58 and 2.24 without Prescott salt respectively in cane molasses, maize and mixed substrate medium on 13thday (figure-2, A and figure-2, B). It is reported that the maintenance of a favorable pH is very essential for the successful fermentation of citric acid. Decrease in pH caused increase of citric acid production. A higher initial pH leads to the accumulation of oxalic acid (Shadafza, Ogata, & Fazeli, 1976). Another study carried out by Hossain *et al,* (Hossain, Brooks, & Maddox, 1985) demonstrated that citric acid production is depended by various factors such as quality of strain, sugar type concentration, temperature and pH etc.

3.2 Further mutagenic improvement of *A. niger* strains with gamma-ray and effect on citric acid yield

Attempts were made to achieve further improvement of 14/20 and 79/20 in the yield of citric acid by using starchy substrates through stepwise mutational process applying gamma ray as mutagen. In the present investigation, un-irradiated 14/20 and 79/20 were inoculated as control and compared the variation in citric acid production to irradiated cultures.

3.2.1 On the molasses fermentation medium

The highest of total titratable acidity (TTA) value was recorded as 7.0 for citric acid production by further mutated *A. niger* 14/20 strain with gamma radiation dose at 20 Krad on the 13th day in the absence of Prescott salt on the molasses fermentation medium (Figure 3A). Furthermore, highest amount of TTA value (6.42) was recorded for citric acid production by further mutated *A. niger* 79/20 with gamma radiation at 20 Krad on the 13th day on the molasses fermentation medium (Figure 4A).

3.2.2 On the maize fermentation medium

The TTA value for citric acid production in maize media on day 7, 9, 11 and 13 by *A. niger* 14/20 without Prescott salt was found as 2.01, 2.55, 3.02 and 3.51 respectively. On the other hand, TTA value for citric acid production by further mutated *A. niger* 14/20 with gamma radiation at various radiation doses on day 7, 9, 11 and 13 respectively has been shown in figure-3B. Significantly higher amount of TTA value (3.53) for citric acid production was recorded by further mutated *A. niger* 14/20 with gamma radiation at 20 Krad on the day 13 for maize fermentation medium. The TTA value for citric acid was found 1.30, 1.75, 2.32 and 3.02 respectively on day 7, 9, 11 and 13 by *A. niger* 79/20 without Prescott salt. Higher amount of TTA value (3.39) for citric acid production was found by further mutated *A. niger* 79/20 with gamma radiation at 20 Krad on day 13 on the maize fermentation medium (Figure 4B).

3.2.3 On the mixed fermentation medium

Without Prescott salt TTA value for citric acid production by *A.niger* 14/20 was found 3.90, 4.78, 5.64 and 6.53 on days 7, 9, 11 and 13 respectively. Significantly highest amount of TTA value 8.11 for citric acid production was found by further mutated *A. niger* 14/20 with gamma radiation at 20 Krad on the day 13 in mixed fermentation medium (Figure 3C). Without Prescott salt TTA value for citric acid production by *A. niger* 79/20 was found 3.53, 4.67, 5.41 and 6.10 on day 7, 9, 11 and 13 respectively. Significantly highest amount of TTA value 7.95 for citric acid production was found by further mutated *A. niger* 79/20 with gamma radiation at 20 Krad on the day 13 on the mixed fermentation medium (Figure 4C).

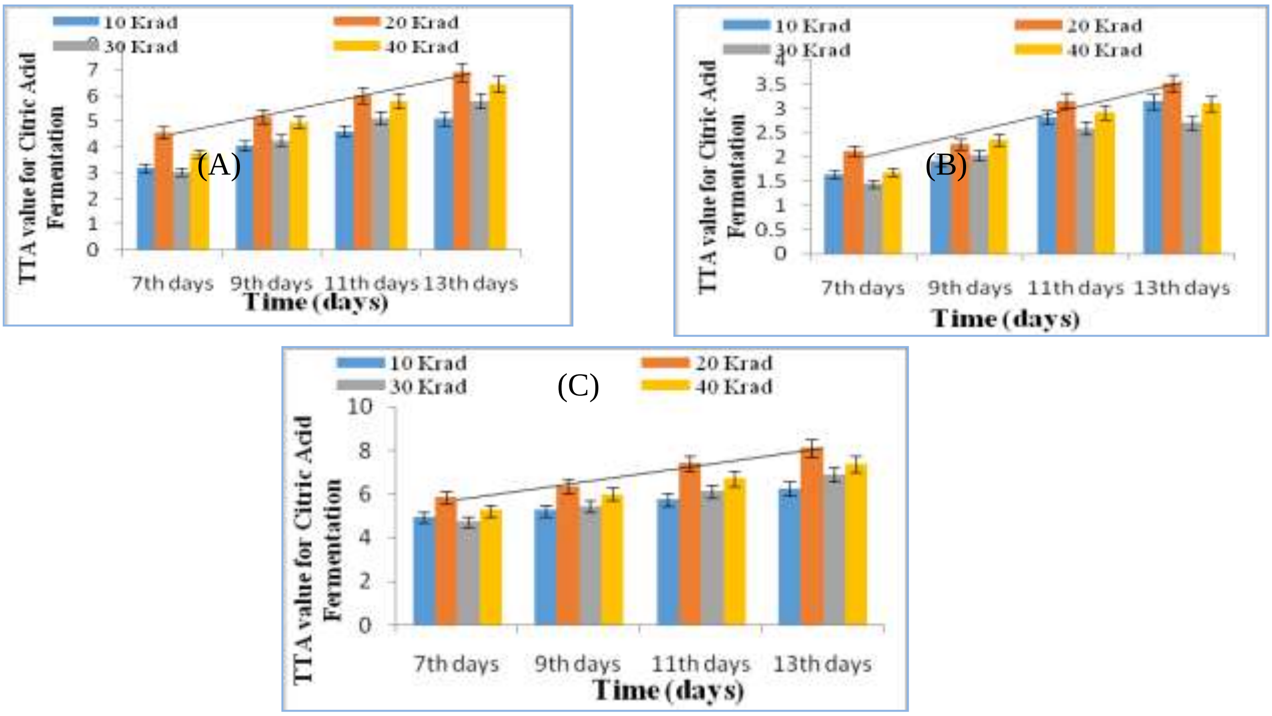

Figure (3): Total titratable acidity (TTA) for citric acid production at different days of fermentation in various media by further mutated *A. niger* 14/20. Here, (A), (B) and (C) indicate molasses, maize and mixed substrates. Significantly different (*P < 0.05*).

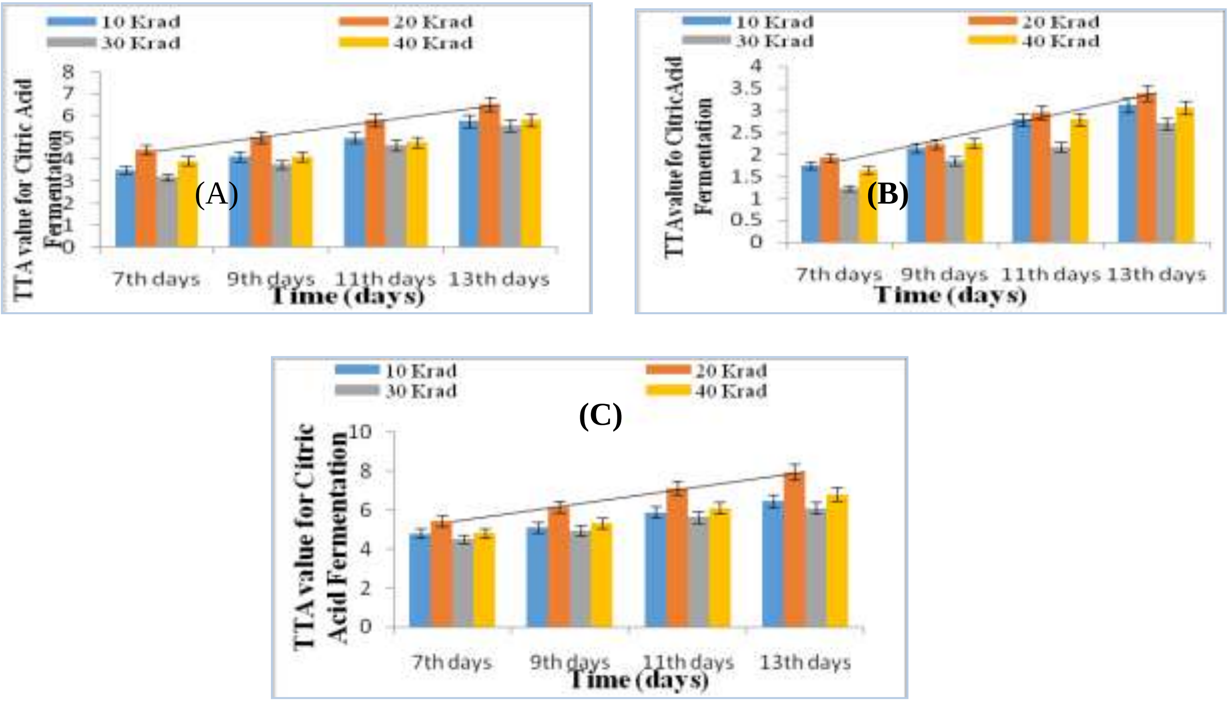

Figure (4): Total titratable acidity (TTA) for citric acid production at different days of fermentation in different media by further mutated *A. niger* 79/20. Here, (A), (B) and (C) indicate molasses, maize and mixed substrates respectively. Significantly different (*P < 0.05*).

According to the present study, highest TTA value was found by further mutated *A. niger* 14/20 with gamma radiation at 20 Krad in mixed fermentation medium throughout the fermentation period and lowest TTA value was found in maize medium by further mutated *A. niger* 79/20 with gamma radiation at 30 Krad. It is notable here that on the same fermentation medium *A. niger* 79/20 produced significantly less amount of citric acid than *A. niger* 14/20. It was reported that highest TTA value was found by further mutated *A. niger* 14/20 with gamma radiation at 20 Krad in mixed fermentation medium in case of pumpkin and molasses (Majumder et al., 2010), jackfruit and molasses (Munshi et al., 2012) throughout the fermentation period. And on the same fermentation medium *A. niger* 79/20 produced significantly less amount of citric acid than *A. niger* 14/20 (Majumder et al., 2010; Munshi et al., 2012; Tuquerres et al., 2017).

CONCLUSION

The present study showed that higher yield of citric acid as desired from industrial point of view could be obtained from molasses and maize (mixed) of Bangladesh and hence the possibility of profitable utilization of cane molasses and maize for citric acid production could be explored by using mutationally improved strains of *A. niger*.

ACKNOWLEDGEMENT

The authors acknowledge with gratitude the financial and all other research facilities rendered by the authority of Institute of Food and Radiation Biology, AERE for carrying out this research work.

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