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Effect of Gamma-Ray Induced Mutant Strains of *Aspergillus niger* on Citric Acid Fermentation Using Molasses and Jackfruit Based Medium

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Abstract: *Aspergillus niger* is most commonly known among the various fungal strains screened for citric acid production, when cultivated in carbohydrate-rich medium of fermentation. In the present study, a number of indigenous raw materials such as molasses and jackfruits have been used for citric acid production. The previously isolated gamma-ray induced second step mutants 14/20 and 79/20 of *A. niger* were reradiated by ^{60}Co Gamma beam-650 source at a dose rate of 0.674 kGy/hr aiming to further increase the production of citric acid. In absence of Prescott salt and in mixed fermentation medium the highest production of citric acid was found by further mutated. *A. niger* 14/20 with gamma radiation at 20 Krad which was about 16.16 mg/ml and lowest citric acid production was found in molasses media by further mutated *A. niger* 14/20 with gamma radiation at 10Krad which was found 9.22 mg/ml on the day 13 but it was higher than that produced by the un irradiated strain. In absence of Prescott salt highest production of citric acid was found by further mutated *A. niger* 79/20 with gamma radiation at 20 Krad in mixed fermentation medium which was about 15.94 mg/ml. Lowest citric acid production was found in molasses media by further mutated *A. niger* 79/20 with gamma radiation at 10 Krad which was found 9.08 mg/ml on the day 13 but it was higher than that produced by the un irradiated strains.

Key words: Gamma Radiation · Citric Acid · *Aspergillus niger* · Molasses · Jackfruit

INTRODUCTION

Citric acid, widely used in food, pharmaceutical and chemical industries, is abundantly produced in the world and commercially one of the most important organic acids [1-3]. *Aspergillus niger (A. niger)* is a fungus utilized for the production of different metabolites of commercial interest especially citric Acid.

According to the estimates, globally citric acid produced through fermentation is 7.0×10^5 ton/annum [4, 5]. The techniques of ultraviolet irradiations, gamma rays or N-methyl, N-nitro-N-nitroso-guanidine (MNNG) induced mutagenesis are useful to improve the yield of various secondary metabolites by *A. niger* [6]. The basic substrates for citric acid fermentation using submerged technique of fermentation are beet or cane-molasses [7]. Owing to the steadily increasing demand of citric acid for industrial purposes, its manufacture from cane or beet molasses has proved to be of great importance to the sugar industry [7]. The filamentous fungus *Aspergillus niger* is the most commonly used microorganism for citric acid production [8]. Until about 1920, all commercial CA was produced from lemon and lime juices [9]. Rohr (1983) reported that CA can be produced by fermentation process using species of microorganisms namely *Aspergillus niger*, a fungus which was used commercially for the first time in 1923 [10].

The production of citric acid by fermentation on a commercial basis has been a highly important achievement in the field of industrial microbiology in different countries. Global production of citric acid has now reached 1.4 to 1.5 million tones and there is annual growth of 3.5-4.0 % in demand/consumption. Bangladesh at present has to import cent?? percent citric acid from foreign countries [11].

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High production of citric acid depends to a great extent on the strain used and its response to the composition of the medium can show a great deal of variability [12]. According to Bangladesh Sugar and Food Industry, there are 19 sugar mills in Bangladesh[**]. The climate in Bangladesh is perfect for the cultivation of jackfruit trees and in addition to its nutritional value; the Jackfruit is an inexpensive fruit and can be bought or grown in the poorest communities. With so many uses, low cost and high nutritional value, it is no surprise that the jackfruit is the national fruit of Bangladesh[***].

Keeping in view the future requirements and also the availability of cheap raw material, efforts were made to develop the process for citric acid fermentation, based on our local resources such as molasses from sugar mills and jackfruits from farmers. Similar type of research are also carried out by using molasses, pumpkin, baggage and other starchy substrates [4, 11]. So the purpose of present study was to describe the feasibility of using raw and cheap materials such as molasses and jackfruit 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 and 79/20 of *A. niger*.

[**]http://www.bsfic.gov.bd/centerCont.php?pid5=18
[***]http://www.bangladesh.com/blog/jackfruit-national-fruit-of-bangladesh

MATERIALS AND METHODS

Microorganisms Used: Citric acid producing strains of *A. niger* designated as 14/20 and 79/20 [13] were used in this study. 14/20 and 79/20 are second step mutants derived from the strain HB3 which is the first step mutant from the wild type strain CA16 [13, 14]. The culture was maintained on agar slants containing 1% malt extract, 1% yeast extract, 1.5% dextrose and 2.5% bacto agar.

Substrates Used: (a) Cane molasses and (b) Jackfruits.
Fermentation medium:

Preparation of Molasses Medium: Molasses was clarified by appropriate dilution with tap water and boiling the solution for half an hour. The clarified molasses was then kept overnight for sedimentation of suspended particles. In order to remove the coarse particle in the solution it was filtered through absorbent cotton and sediment was discarded [11].

Preparation of Jackfruits Medium: Jackfruits was washed with tap water. Then jackfruits was sliced thinly and dried in dryer at 50°C. The substrates were powdered in a grinding machine. Dried powder of jackfruits was hydrolyzed separately in 300 ml solution of 0.05 N HCl and autoclaved at 121°C temperature, 15 Ibs pressure for 20 minutes. The hydrolyzed materials were then filtered through thin cloth [11].

Preparation of mixed substrate medium: Equal amounts of jackfruits and molasses were hydrolyzed in 300 ml solution of 0.05 N HCl and autoclaved at 121°C temperature, 15 Ibs pressure for 20 minutes. The hydrolyzed materials were then filtered through thin cloth. The media was then kept overnight for sedimentation of suspended particles resides 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 (NH_4NO_3 2.23 g/l; K_2HPO_4, 1.00 g/l and $MgSO_4$. $7H_2O$, 0.23 g/l) [11].

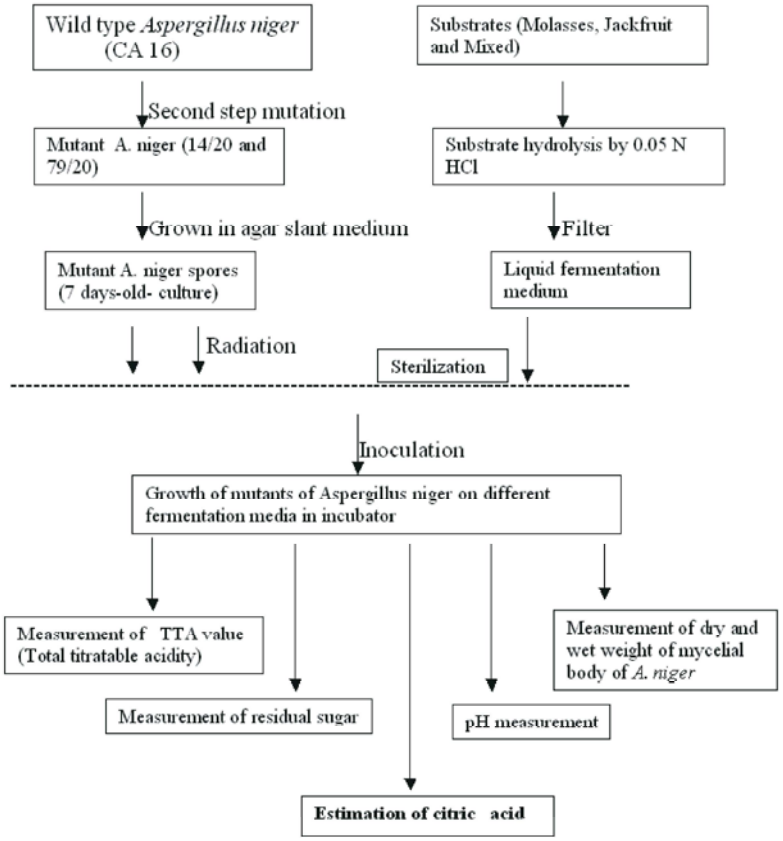
Irradiation of the Organism and Further Induction of High Yielding Mutants: With the help of gamma rays, several high citric acid yielding mutants of *A. niger* (HB3, 14/20 and 79/20 etc.) have been isolated and their preliminary characterizations, semi-pilot scale studies in cane molasses medium were made [13-16]. Recently, the induction of further mutation for higher yield of citric acid has been attempted using mutant strains 14/20 and 79/20. Conidia were irradiated with gamma radiation at 10, 20, 30 and 40 Krad / hour by ^{60}Co Gamma beam-650 source.

The strain was 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°C and inoculated to the fermentation media. 1 ml spore suspension was taken from the tube by micropipette and dropped into fermentation media. Cane molasses, jackfruit and cane molasses-jackfruit mixed media were used for fermentation.

A gamma ray is a packet of electromagnetic energy (photon) emitted by the nucleus of some radionuclides following radioactive decay. After gamma-irradiation and the breaking of the DNA double-strands, the cell can repair the damaged genetic material in the limit of its capability and genetic improvement may occur.

Citric Acid Production Process: In this production technique, which is still the major industrial route to citric acid used today, cultures of *A. niger* were fed on sucrose to produce citric acid. The diagrammatic presentation of citric acid production process is given as below [11]:

Diagrammatic presentation of citric acid production process:



Data Analysis: The data were analyzed using "Microsoft Excel-2003.

RESULTS AND DISCUSSION

Kinetics of Citric Acid Fermentation by the *A. niger* Mutants, 14/20 and 79/20 in Different Media: The changes in citric acid concentration and pH levels in the medium, kinetics of citric acid fermentation was followed in more detail with time over a long period of fermentation. Under stationary condition the conidia of the strains, 14/20 and 79/20 were inoculated in cane molasses, jackfruit and mixed media adjusted to 14% sugar concentration, pH 5 and at 30°C. Citric acid and pH level in the fermentation broth were determined 48 hours up to 13 days of growth and the results were shown in following figures (Fig. 1 and 2).

From figure-1 (A), it can be observed that the citric acid concentration in the molasses, jackfruit and mixed substrate media for *A. niger* 14/20 increased with incubation period up to 13 days reaching a value of 6.50, 5.0 and 6.40 mg/ml respectively with Prescott salt and from the figure-1 (B), 9.0, 15.50 and 16.50 mg/ml respectively in cane molasses, jackfruit and mixed substrate media without Prescott salt. From the figure-2 (A), it is also observed that, for *A. niger* 79/20, the citric acid concentration in the molasses, jackfruit and mixed substrate media with Prescott salt increased with incubation period up to 13 days reaching a value of 6.22, 5.0 and 5.90 mg/ml respectively and from Figure-2 (B), 9.08, 15.45 and 15.94 mg/ml respectively in cane molasses, jackfruit and mixed substrate media without Prescott salt. In the absence of Prescott salt citric acid production was found higher than the presence of Prescott salt in both cases and it was also found that, citric acid production for *A. niger* 14/20 is higher than that of *A. niger* 79/20.

At the end of fermentation period of 13days, in cane molasses, jackfruit and mixed substrate media, for the strain, *A. niger* 14/20 the pH of the medium declined from initial value of pH5 to 3.25, 3.09 and 3.15 respectively with Prescott salt and 2.24, 2.19 and 2.07 respectively without Prescott salt in same substrate medium (Fig. 1 and 2).

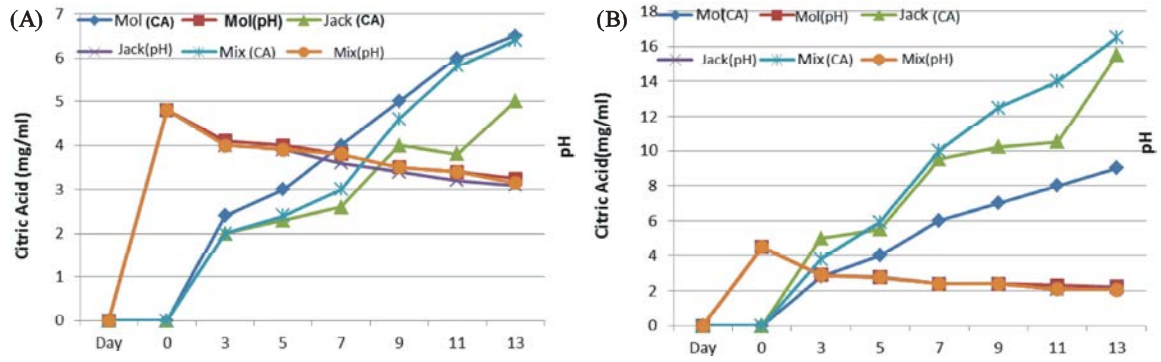


Fig. 1: Time course of citric acid production and the pH evolution obtained with *A. niger* 14/20 in various fermentation media. (A) With Prescott salt and (B) without Prescott salt. Mol, Jack and Mix indicate molasses, Jackfruit and mixed substrates respectively. CA denotes citric acid.

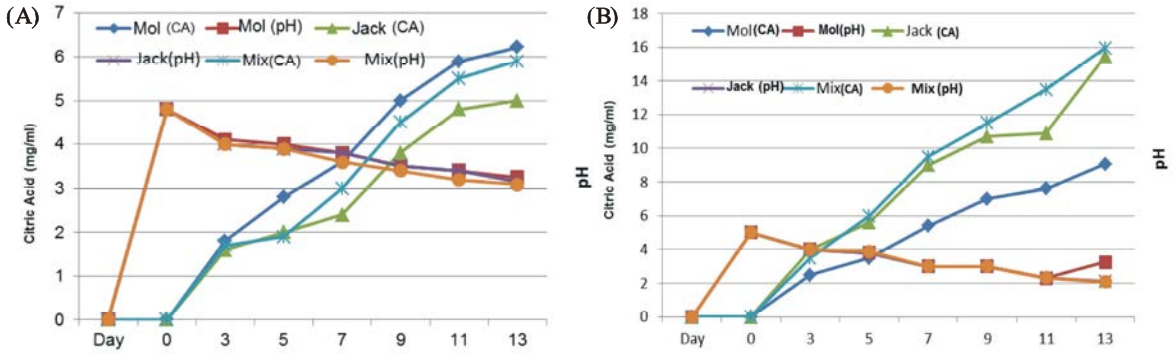


Fig. 2: Time course of citric acid production and the pH evolution obtained with *A. niger* 79/20 in various fermentation media. (A) With Prescott salt and (B) without Prescott salt. Mol, Jack and Mix indicate molasses, Jackfruit and mixed substrates respectively. CA denotes citric acid.

At the same time course, for the strain *A. niger* 79/20 the pH of the medium declined from initial value of pH 5 to 3.25, 3.15 and 3.09 respectively in cane molasses, jackfruit and mixed substrate media with Prescott salt and 3.26, 2.10, 2.09 respectively in same substrates media without Prescott salt. The maintenance of a favorable pH is very essential for the successful fermentation of citric acid. Decrease in pH caused reduction in citric acid production. A higher initial pH leads to the accumulation of oxalic acid as reported by Shadafza. [17].

Irradiation of 14/20 and 79/20 with Gamma-ray and Further Improvement in the Yield of Citric Acid: The *A. niger* mutants, 14/20 and 79/20 have been confirmed in the present study to give high yield of citric acid in molasses, jackfruit and mixed media. It has been mentioned earlier that the strains 14/20 and 79/20 were obtained through stepwise mutagenic improvement of the original parent strain CA16 [13, 18]. Further mutagenic improvement of strains 14/20 and 79/20 are essential to find new strains which would be industrially more feasible. So in the present investigation attempts were made to obtain further improvement in the yield of citric acid in cane molasses, jackfruit and mixed media by these strains through stepwise mutational process using gamma ray as mutagen.

Un irradiated 14/20 and 79/20 strains were inoculated as control and tested for comparison of the variation in citric acid production by irradiated and un irradiated cultures.

On the Molasses Fermentation Medium: The total titratable acidity (TTA) value for citric acid production by *A. niger* 14/20 was 2.4, 3, 3.6 and 4.95 respectively on day 7, 9, 11 and 13 without the presence of Prescott salt. Without the presence of Prescott salt TTA value for citric acid for 10Krad was found 2.67, 3.41, 4.27 and 5.03, for 20Krad 3.15, 3.67, 4.84 and 4.95, for 30Krad 2.92, 3.56, 4.44

Table 1: Total titratable acidity, citric acid, residual sugar concentration and mycelial dry weight of *Aspergillus niger* strain 14/20 on day 13 in different media containing sucrose at pH 5 and temperature 30°C under stationary conditions

Medium	Prescott salt	Sugar supplied (mg/ml)	TTA (ml 0.1N NaOH/ml medium)	Citric acid produced (mg/ml)	Mycelial dry weight (mg/ml)	Residual sugar (mg/ml)	Sugar utilized (mg/ml)	Sugar utilized (%)	Citric acid in relation to sugar supplied (% w/w)
Molasses	With Prescott salt	146.00	2.90	6.38	14.69	94.10	52.50	35.81	4.37
Jackfruit		146.00	2.10	4.40	15.75	98.28	47.12	32.40	3.01
Mixed Substrate		146.00	2.80	6.27	15.65	99.99	47.19	32.02	4.29
Molasses	Without Prescott salt	146.00	4.95	9.22	30.05	62.15	84.65	57.66	6.3
Jackfruit		146.00	7.35	11.85	31.95	62.00	82.50	57.09	8.12
Mixed Substrate		146.00	7.45	16.16	32.70	60.89	85.41	58.38	11.07

Table 2: Total titratable acidity, citric acid, residual sugar concentration and mycelial dry weight of *Aspergillus niger* strain 79/20 on day 13 in different media containing sucrose at pH 5 and temperature 30°C under stationary conditions

Medium	Prescott salt	Sugar supplied (mg/ml)	TTA (ml 0.1N NaOH/ml medium)	Citric acid produced (mg/ml)	Mycelial dry weight (mg/ml)	Residual sugar (mg/ml)	Sugar utilized (mg/ml)	Sugar utilized (%)	Citric acid in relation to sugar supplied (% w/w)
Molasses	With Prescott salt	146.00	2.75	6.22	13.13	98.05	47.95	32.84	4.26
Jackfruit		146.00	1.98	4.20	13.99	100.10	44.99	30.82	2.87
Mixed Substrate		146.00	2.45	5.90	13.05	102.60	44.40	30.41	4.04
Molasses	Without Prescott salt	146.00	3.75	9.08	28.55	61.10	85.10	58.29	6.21
Jackfruit		146.00	5.60	11.75	29.20	65.57	79.43	54.40	8.04
Mixed Substrate		146.00	6.75	15.94	31.05	66.79	80.21	54.94	10.92

and 5.14 and finally for 40Krad it was found 3.06, 3.66, 4.42 and 5.19 on day 7, 9, 11 and 13 respectively. Higher amount of TTA value for citric acid was found by further mutated *A. niger* 14/20 with gamma radiation at 20Krad in the absence of Prescott salt on the day 13 (Table 1) on the molasses fermentation medium.

The TTA value for citric acid production by *A. niger* 79/20 on day 7, 9, 11 and 13 without the presence of Prescott salt was 2.1, 2.78, 3.25 and 3.75 respectively. Higher amount of TTA value for citric acid production was found by further mutated *A. niger* 79/20 with gamma radiation at 20Krad in the absence of Prescott salt on the day 13 (Table 2) on the molasses fermentation medium.

On the Jackfruit Fermentation Medium: The TTA value for citric acid on day 7, 9, 11 and 13 by *A. niger* 14/20 without the presence of Prescott salt was found 3.55, 4.45, 4.95 and 7.35 respectively. On the other hand, without the presence of Prescott salt TTA value for citric acid by further mutated *A. niger* 14/20 with gamma radiation at 10Krad was found 4.03, 5.04, 5.66 and 6.67, at 20Krad 5.48, 6.65, 7.25 and 7.35, at 30Krad 4.45, 5.43, 6.26 and 6.68 and at 40Krad it was found 5.02, 5.68, 6.65 and 7.16 on day 7, 9, 11 and 13 respectively. Higher amount of TTA value for citric acid was found in the absence of Prescott salt by further mutated *A. niger* 14/20 with gamma radiation at 20Krad on the day 13 (Table 1) on the jackfruit fermentation medium.

The TTA value for citric acid was found 3.3, 3.95, 4.3 and 5.6 respectively on day 7, 9, 11 and 13 by *A. niger* 79/20 without the presence of Prescott salt. Higher amount of TTA value for citric acid production was found in the absence of Prescott salt by further mutated *A. niger* 79/20 with gamma radiation at 20Krad on the day 13 (Table 2) on the jackfruit fermentation medium.

On the Mixed Fermentation Medium: Without Prescott salt TTA value for citric acid production by *Aspergillus niger* 14/20 was found 4.3, 5.25, 6.3 and 7.45 on day 7, 9, 11 and 13 respectively. Highest amount of TTA value 7.45 for citric acid production was found by further mutated *A. niger* 14/20 with gamma radiation at 20Krad in the absence of Prescott salt on the day 13 (Table 1) on the mixed fermentation medium.

Without Prescott salt TTA value for citric acid production by *A. niger* 79/20 was found 3.65, 4.95, 5.65 and 6.75 on day 7, 9, 11 and 13 respectively. Highest amount of TTA value 6.75 for citric acid production was found by further mutated *A. niger* 79/20 with gamma radiation at 20Krad in the absence of Prescott salt on the day 13 (Table 2) on the molasses fermentation medium.

Through this result we observed that without the presence of Prescott salt highest TTA value was found by further mutated *A. niger* 14/20 with gamma radiation at 20Krad in mixed fermentation media throughout the fermentation period and lowest TTA value was found in molasses media by further mutated *A. niger* 14/20 with gamma radiation at 10Krad. And on the same fermentation medium *A. niger* 14/20 produces higher amount of citric acid than *A. niger* 79/20.

It was reported that, without Prescott salt citric acid production on day 13 by *A. niger* 14/20 is found 7.72, 10.35 and 14.86 for molasses, pumpkin and mixed substrates respectively and for *A. niger* 79/20 is found 7.57, 10.21 and 14.44 for molasses, pumpkin and mixed substrates respectively [11]. From the present study it was found that, Jackfruits citric acid production rate is higher than that of pumpkin. So, Jackfruits have higher efficacy on citric acid production than pumpkin.

CONCLUSIONS

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

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