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Gamma-Ray Induced Mutants 14/20 and 79/20 of *Aspergillus niger* Increases Citric Acid Production from Molasses and Pumpkin

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Abstract: Among the various fungal strains screened for citric acid production, *Aspergillus niger* is known to produce considerable amounts of citric acid and other organic acids when cultivated in carbohydrate-rich medium of fermentation. A number of indigenous raw materials such as cane molasses and pumpkin have been carried out in present study for citric acid production by using gamma ray induced mutant strains 14/20 and 79/20 of *Aspergillus niger* under surface culture condition. Aiming to further increase the production of citric acid, 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 production of citric acid was found by further mutated *A. niger* 14/20 with gamma radiation at 20 Krad in mixed fermentation medium which was about 19.32 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 9.65 mg/ml on the day 13 but it was higher fermentation of citric acid than by the unirradiated strain. In absence of Prescott salt higher production of citric acid was found by further mutated *A. niger* 79/20 with gamma radiation at 20 Krad in mixed fermentation medium than *A. niger* 79/20 which was about 17.58 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 8.92 mg/ml on the day 13 but it was higher fermentation of citric acid than by the unirradiated strains.

Key words: Gamma radiation · Citric acid · *Aspergillus niger* · Molasses · Pumpkin

INTRODUCTION

Citric acid is abundantly produced in the world. Commercially, citric acid is one of the most important organic acids and it is widely used in food, pharmaceutical and chemical industries [1-3].

According to the estimates, 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]. 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 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 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. High production 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 [8]. Industrial production of this chemical by fermentation using cheap raw materials is helpful in economic development of our country. 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

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pumpkin. So the purpose of present study describes the feasibility of using raw and cheap materials such as molasses and pumpkin 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 *Aspergillus niger*.

MATERIALS AND METHODS

Microorganism Used: Citric acid producing strain of *Aspergillus niger* designated as 14/20 and 79/20 [9] were used in this present 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 [9, 10]. 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 (b) Pumpkin
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.

Preparation of Pumpkin Medium: Pumpkin was washed with tap water. Then pumpkin was sliced thinly and dried in dryer at 50°C. The substrates were powdered in a grinding machine. Dried powder of pumpkin 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.

Preparation of Mixed Substrate Medium: Equal amount of pumpkin 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).

Irradiation of the Organism and Further Induction of High Yielding Mutants: With the help of gamma ray, several high citric acid yielding mutants of *Aspergillus 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 [11, 12]. Recently, the induction of further mutation for higher yield of citric acid has been attempted using mutant strains 14/20 and 79/20 as the parent strain. Conidia were irradiated with gamma radiation respectively 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 medium, pumpkin medium and cane molasses-pumpkin 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.

RESULTS AND DISCUSSION

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

It can be observed from the figures that the citric acid concentration in the molasses, pumpkin and mixed substrate media for *A. niger* 14/20 increase with incubation period up to 13 days reaching a value of 4.88, 2.87 and 4.76 mg/ml respectively with Prescott salt and 7.72, 10.35 and 14.86 mg/ml respectively in cane molasses, pumpkin and mixed substrate media without Prescott salt. And the citric acid concentration in the molasses, pumpkin and mixed substrate media for *A. niger* 79/20

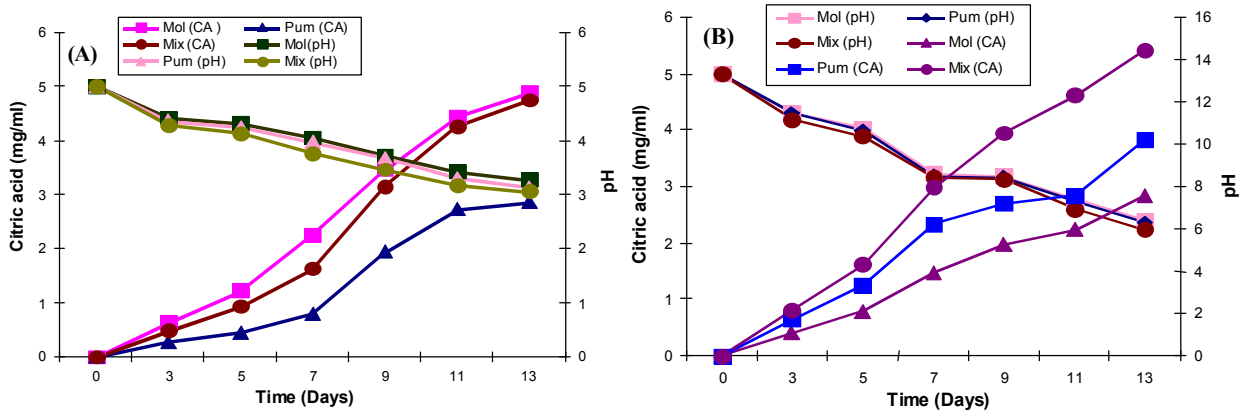

Fig. 1: Time course of citric acid production and the pH evolution obtained with *A.niger* 14/20 in various fermentation media. (A) With Prescott salt and (B) without Prescott salt. Mol, Pum, mix indicates molasses, pumpkin and mixed substrates respectively

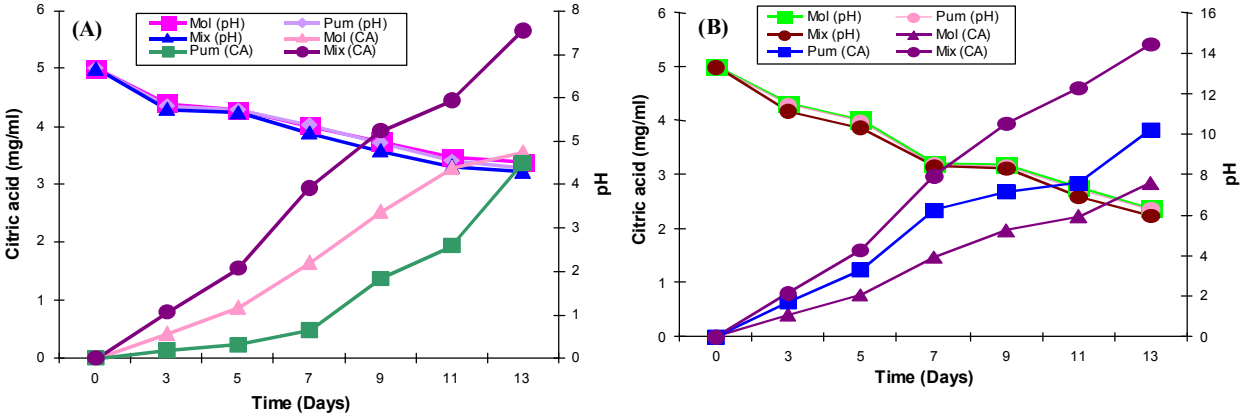

Fig. 2: Time course of citric acid production and the pH evolution obtained with *A.niger* 79/20 in various fermentation media. (A) With Prescott salt and (B) without Prescott salt. Mol, Pum, mix indicates molasses, pumpkin and mixed substrates respectively.

increase with incubation period up to 13 days reaching a value of 4.75, 2.57 and 4.48 mg/ml respectively in cane molasses, pumpkin and mixed substrate medium with Prescott salt and 7.57, 10.21 and 14.44 mg/ml respectively in cane molasses, pumpkin and mixed substrate medium without Prescott salt. In the presence of Prescott salt citric acid production was found lower than the absence of Prescott salt in both cases.

For the strain, *A. niger* 14/20 the pH of the medium declined from initial value of pH 5 to 3.28, 3.14 and 3.05 respectively in cane molasses, pumpkin and mixed substrate medium with Prescott salt and 2.26, 2.12 and 2.06 respectively in cane molasses, pumpkin and mixed substrate media without Prescott salt at the end of fermentation period of 13 days. At the same time for the strain *A. niger* 79/20 the pH of the medium declined from initial value of pH 5 to 3.38, 3.28 and 3.24 respectively in cane molasses, pumpkin and mixed substrate medium with Prescott salt 2.39, 2.36 and 2.24 respectively in cane molasses, pumpkin and mixed substrate medium without Prescott salt at the end of fermentation period of 13 days.

Irradiation of 14/20 and 79/20 with Gamma-ray and Further Improvement in the Yield of Citric Acid: The *Aspergillus niger* mutants, 14/20 and 79/20 have been confirmed in the present study to give high yield of citric acid in molasses, pumpkin 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]. Further mutagenic improvement of strains 14/20 and 79/20 are essential to find new strains which would be industrially more

Table 1: Total titratable acidity, citric acid, residual sugar concentration and mycelial dry weight in different medium (in absence of Prescott salt) by gamma-ray (0.1, 0.2, 0.3 and 0.4kGy) induced mutants of *Aspergillus niger* strain 14/20 on 13th day at pH 5 and temperature 30ºC under stationary condition

Medium	Strain no.	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	10	140	4.425	9.65	27.97	55.69	84.31	60.22	6.89
	20	140	4.94	10.97	31.16	57.30	82.70	59.07	7.83
	30	140	4.535	10.05	28.75	56.00	84.00	60.00	7.17
	40	140	4.585	10.15	29.20	56.62	83.38	59.56	7.25
Pumpkin	10	140	6.07	12.92	27.24	56.15	83.85	59.89	9.23
	20	140	7.25	15.45	30.55	58.32	81.68	58.34	11.04
	30	140	6.08	12.83	29.78	57.89	82.11	58.65	9.16
	40	140	6.56	13.63	30.18	58.18	81.82	58.44	9.74
Mixed Substrate	10	140	6.85	13.74	28.63	57.44	82.56	58.97	9.81
	20	140	8.85	19.32	32.98	54.26	85.74	61.24	13.8
	30	140	7.15	15.06	28.83	57.37	82.63	59.02	10.76
	40	140	7.525	14.85	30.05	56.00	84	60	10.6

Table 2: Total titratable acidity, citric acid, residual sugar concentration and mycelial dry weight in different medium (in absence of Prescott salt) by gamma-ray (0.1, 0.2, 0.3 and 0.4kGy) induced mutants of *Aspergillus niger* strain 79/20 on 13th day at pH 5 and temperature 30ºC under stationary condition.

Medium	Strain no.	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	10	140	4.41	9.53	27.11	58.21	81.79	58.42	6.8
	20	140	4.72	10.50	30.54	59.08	80.2	57.29	7.5
	30	140	4.48	9.86	28.00	58.66	81.34	58.10	7.04
	40	140	4.53	10.11	29.12	58.00	82	58.57	7.22
Pumpkin	10	140	5.73	12.47	27.52	60.25	79.75	56.96	8.9
	20	140	6.76	14.36	30.58	59.86	80.14	57.24	10.28
	30	140	5.50	12.38	29.23	59.97	80.03	57.16	8.84
	40	140	6.11	13.49	29.75	59.58	80.42	57.44	9.63
Mixed Substrate	10	140	6.15	13.58	28.54	58.91	81.09	57.92	9.7
	20	140	8.22	18.22	32.28	55.97	84.03	60.02	13
	30	140	6.65	14.62	28.65	58.12	81.88	58.49	10.44
	40	140	7.05	14.18	30.00	57.84	82.51	58.93	10.57

feasible. So in the present investigation attempts were made to obtain further improvement in the yield of citric acid in cane molasses, pumpkin and mixed media by these strains through stepwise mutational process using gamma ray as mutagen.

An unirradiated sample serving as control was also plated. Unirradiated 14/20 and 79/20 were inoculated as control and tested for comparison of the variation in citric acid production by irradiated and unirradiated cultures.

On the Molasses Fermentation Medium: The TTA value for citric acid production by *Aspergillus niger* 14/20 was 2.215, 2.81, 3.31 and 4 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 2.17, 2.81, 3.67 and 4.43; for 20Krad 2.545, 3.17, 4.235 and 4.94; for 30Krad 2.32, 2.955, 3.835 and 4.535 and finally for 40Krad it was found 2.46, 3.06, 3.815 and 4.585 on day 7, 9, 11 and 13 respectively. Significantly 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 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 1.85, 2.38, 2.83 and 3.55 respectively. Significantly higher amount of TTA value 4.81 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 on the molasses fermentation medium.

On the Pumpkin 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 3.05, 4.01, 4.51 and 5.795 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 3.425, 4.44, 5.145 and 6.07; at 20Krad 4.875, 6.05, 6.645 and 7.25; at 30Krad 3.85, 4.825, 5.66 and 6.08 and at 40Krad it was found 4.42, 5.175, 6.05 and 6.56 on day 7, 9, 11 and 13 respectively. Significantly 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 on the pumpkin fermentation medium.

The TTA value for citric acid was found 2.93, 3.38, 3.6 and 4.9 respectively on day 7, 9, 11 and 13 by *A. niger* 79/20 without the presence of Prescott salt. Significantly higher amount of TTA value 6.675 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 on the pumpkin fermentation medium.

On the Mixed Fermentation Medium: Without Prescott salt TTA value for citric acid production by *Aspergillus niger* 14/20 was found 3.81, 4.905, 5.84 and 6.94 on day 7, 9, 11 and 13 respectively. Significantly highest amount of TTA value 8.85 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 3.35, 4.5, 5.25 and 6.15 on day 7, 9, 11 and 13 respectively. Significantly highest amount of TTA value 8.245 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 mixed 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* 79/20 produces significantly less amount of citric acid than *A. niger* 14/20.

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