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Comparison of locally available carbon rich substrates for augmented microbial production of citric acid with mutant *Aspergillus niger* S-6 strain

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Abstract
The aim of the present study was to compare three carbon sources for Citric acid (CA) production by using mutant strain of *Aspergillus niger* S-6. UV rays at a wavelength of 365 nm were used as the mutagen on a locally isolated *Aspergillus niger* strain, arbitrarily named as *A. niger* S-6. The fungus was exposed to 30 minutes of UV irradiation. Cane molasses, pumpkin and sweet potato were taken as the carbon sources for preparation of fermentation media. CA yield was quantitatively estimated by calculating total titrable acidity (TTA) of fermentation media. Mutagenesis with UV radiation led to more than twofold higher CA production in all fermentation media. Sweet potato medium showed higher CA production of 11.84 $\pm$ 0.13 g% in comparison to cane molasses (11.37 $\pm$ 0.11 g %) and pumpkin (10.15 $\pm$ 0.06 g %) Sweet potato.

Keywords: Citric acid, fermentation, UV rays, *Aspergillus niger*, cane molasses, sweet potato, pumpkin, total titrable acidity

Introduction
Citric acid (CA) is an organic acid which is produced as a primary metabolic product during the tricarboxylic acid cycle of aerobic respiration. CA has multifarious uses in a wide range of industries. It is used in food, beverages, pharmaceuticals, flavour enhancement, cosmetics, chemicals and other industries. It is also used as a preservative, plasticizer and synergistic sequestering agent [1, 4]. It is also used in bioremediation and agricultural industries, electroplating, bioleaching, toiletry and detergent industries [5]. The wide applicability of CA in industries is attributed to its low or non-toxicity, high solubility, biodegradability and palatability [6].
As compared to the growing demand of CA for different industries, the amount of the acid that can be produced from natural sources is quite less. The production of CA by fermentation on a commercial basis has been a highly important achievement in the field of industrial microbiology. A large number of micro-organisms have been used for CA production but only a few of them can produce the acid on an industrial scale [4]. Bacteria such as *Arthrobacter* spp., *Bacillus* spp., *Corynebacterium* spp., etc., fungi such as *Aspergillus* spp., *Penicillium* spp., etc. and yeasts such as *Candida*, *Saccharomyces* spp., *Torula* spp., *Torulopsis* spp., etc. have been used by various workers for CA production [7]. The ever-increasing demand of CA can only be satisfied by biotechnological processes [8, 9]. Physical mutagens like x rays, UV rays, Gamma rays have been used by various workers to induce mutation in fungi and many of these mutagenic treatments have been found to produce high yield of CA when grown in different sugar-rich substrates [10].
Keeping in view the requirements and the availability of cheap raw materials, efforts need to be made to develop the process of CA fermentation, based on local resources, by using mutagens. Therefore, this study was conducted with the aim of hyperproduction of CA from locally isolated *Aspergillus* spp. using cheap and easily available carbon sources by mutagenesis with UV radiation.

Materials and method
The study was conducted in the Microbiology Laboratory of the Department of Botany, Cotton University, Guwahati, Assam, India.

Isolation and identification of *Aspergillus* spp.
Soil samples were collected aseptically from the garden and serial dilution method was Used for the isolation of *Aspergillus* spp. on Potato Dextrose Agar (PDA) medium [11]. The pH of the Medium was adjusted at ~6.0 by adding 1M HCl and a temperature of 30±1 °C was

maintained in the incubator. The *Aspergillus* spp. were isolated on the basis of microscopic observation of morphological characteristics, with the help of a manual [12]. Ten isolates of *Aspergillus* spp. were identified, of which an *Aspergillus niger* isolate, arbitrarily named as *Aspergillus niger* S-6 strain was cultured for the study.

Induction of mutation with UV rays

4 mL of the inoculum containing *Aspergillus niger* S-6 spores were taken in sterilized petri-plates. The petri-plates were kept inside the UV chamber and exposed to UV rays of 365 nm wavelength for 30 minutes. Once the stipulated time of exposure was over, the UV exposed petri-plates were kept in dark inside the UV chamber overnight to avoid photo-reactivation. The irradiated spores were then streaked on petri-plates containing sterile Czapek's Dox Agar medium and incubated for four days. After four days, the plates were checked for colonies that showed survival rate of less than 1%, based on their morphological characteristics [13]. The surviving mutant spores were then sub-cultured and maintained on agar slants containing 1% malt extract, 1% yeast extract, 1.5% dextrose and 2% agar at 4 °C [14, 15].

Preparation of fermentation medium

Three types of substrates were used for this study, which included cane molasses, sweet potato and pumpkin. The fermentation media using these substrates were prepared as follows:

The cane molasses were mixed in distilled water and the solution was left undisturbed for 5-6 hours. The sedimentation was removed by decantation. 1 gm of ammonium-sulphate and 1 gm of copper-sulphate were added to the solution and it was left undisturbed overnight. The solution was again filtered to remove the precipitate formed [16]. 10 mL of 1N HCl was added to the solution and the pH was adjusted at 6.0.

The preparation of sweet potato and pumpkin solutions was similar. The sweet potatoes and pumpkin sourced locally were washed thoroughly, peeled, cut into pieces and boiled in water. The pieces were then mashed thoroughly and dissolved in distilled water. The volume of the solution was made up to 1000 mL for both the media. 1 gm of ammonium-sulphate and 1 gm of copper-sulphate were added to the solution and pH was adjusted at 6.0.

Each substrate medium was distributed into 250 mL Erlenmeyer flasks and autoclaved at 15 lbs pressure for 15 minutes.

Fermentation

Submerged fermentation was used for the present study, wherein the liquid fermentation media were inoculated with the mutagen treated *Aspergillus niger* S-6 samples. The spores of the mutagen treated samples were scraped off from the culture slants with the help of a sterile platinum wire-loop and sterile distilled water and then diluted with sterile saline containing 0.1% Tween 80 in a sterilized test-tube [13]. Each flask containing the sterilized fermentation medium was inoculated with 1mL of the inoculum. For each type of inoculum-substrate combination, a set of three Erlenmeyer flasks were inoculated and fermentation was allowed to take place at a temperature of 30±1 °C for a period of 144 hours. Controls were also prepared for each such set of inoculum-substrate combination.

Estimation of CA production

The fermentation broth was filtered using Whatman's filter paper to remove any residual active spores from the solution and also to get a clear filtrate to estimate the level of organic acids present in it. The filtrate obtained, was checked for the pH level using a digital pH meter. The qualitative estimation of organic acids present in the filtrate was done with the help of a few simple biochemical tests. The level of CA in the fermentation medium was calculated titrimetrically [17].

The following process was used with modification:

10 mL of 1N NaOH solution was taken in a 100 mL conical flask and the filtrate obtained from the fermented sample was filled into the burette. The initial volume of the sample was noted down. To the 1N NaOH solution, 1-2 drops of phenolphthalein indicator was added which turned the colour of the solution to pink. Titration was done with this base-indicator mix and the acidic sample in the burette, swirling the flask continuously until the pink colour of the base-indicator mix turned colourless. The difference between the initial and final burette readings gave us the volume of acid required to neutralize the base. The formula applied in this case was:

$S_1V_1 = S_2V_2$, where S_1 = Strength of the base (NaOH solution) = 1N; V_1 = Volume of the base (NaOH solution) = 10 mL; S_2 = Strength of the acid and V_2 = Volume of the acid required for neutralization (final burette reading – initial burette reading).

Sustainability of the effects of the mutagens on the parent strain

Spores from mutagen treated samples were collected and cultured on agar slants containing 1% malt extract, 1% yeast extract, 1.5% dextrose and 2% agar at 4 °C. Inocula were again prepared from these culture slants and used for fermentation in the next generation. This entire procedure was repeated for a total of seven generations and the results obtained, were studied to understand the nature and consistency of the effect of the mutagens on the selected strain of *Aspergillus niger* S-6 with relation to CA production.

Statistical analysis

Data were expressed as mean ± SD. The differences in mean were tested using one way ANOVA and Student-Newman-Keuls test was done for all *post hoc* pairwise comparisons of the substrates. Statistical analysis was done using MedCalc® v12.5.0. The graphical representation of data was done using Microsoft PowerPoint 2010.

Result

The study was conducted over a period of three years from July, 2013 to August, 2016 and a total of 126 samples were studied for CA production. As shown in Figure 1, there has been a consistent increase in CA production across seven generations studied. In cane molasses fermentation medium, there was significant increase in CA production with mutagen treated strains in all generations compared to controls (Table 1). Similar findings were seen in other fermentation media.

There was significant difference in mean CA productions of seventh generation strains using different substrates ($p<0.001$). Using *post hoc* analysis, mean CA production of mutant strains were significantly different using all three substrates with sweet potato giving the highest yield, followed by cane molasses and pumpkin in that order ($p<0.05$ for all comparisons) (Table 2, Figure 2).

Table 1: Mean CA production + SD (gram %) in cane molasses fermentation media (All comparisons were statistically significant with $p<0.001$)

Generations	1st	2nd	3rd	4th	5th		
No. of samples							
Control	2.97+	101+	3.08-	3.15+	119-	3.23+	3.28 + 0.11
	0.12	0.05	0.03	0.03	0.04	0.02	
Mutant	11.01:	11.18 +	11.23:	1127+	113+	1135 +	1137+0.11
	0.05	0.04	0.09	0.09	0.05	0.11	

Table 2: CA production with 7ill generation mutant *A. nizer* S-6 strains (glamr9). (Data expressed as Mean ± SD)

Substrates	CA production (gram %)	
	Control (N=3)	Mutant (N=3)
Cane molasses	328 + 0.11	1137 + 0.11
Pumpkin	4.13 + 0.03	10.15 + 0.06
Sweet potato	4.18 + 0.04	11.84 +0.13

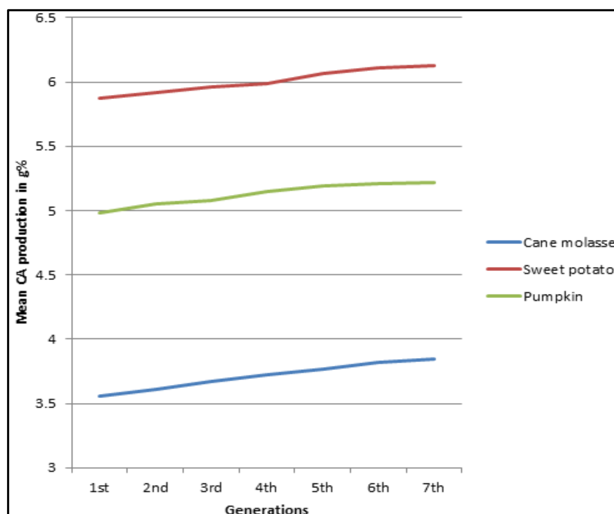

Fig 1: Mean CA production across the 7 generations of mutant *A. niger* S-6 strains in cane molasses, sweet potato and pumpkin media

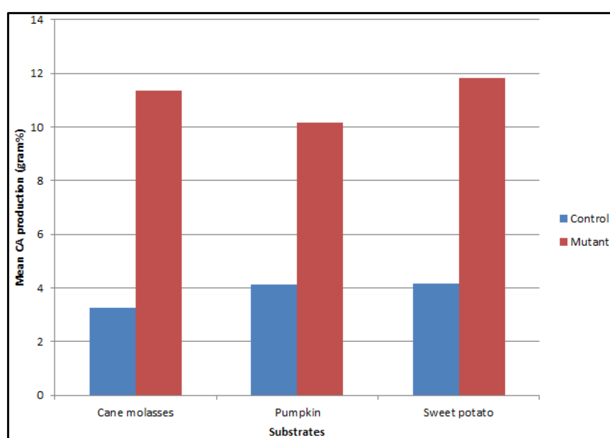

Fig 2: Mean CA production with 7th generation mutant *A. niger* S-6 strains (gram %)

Discussion

High yields of CA depend mainly on the type of microbial strain used and the availability of cheap and local raw materials [18]. Apart from studying the optimization of the basic fermentation parameters such as substrate concentration, nutrient composition, pH of the fermentation medium, incubation period, duration of fermentation, effects of the presence of oxygen, nitrogen, etc, sufficient efforts also need to be made in order to develop sustainable strains of *A. niger* which can produce CA efficiently from a variety of fermentation substrates. This can be achieved by genetic manipulation with the help of classical mutagenesis experiments and rDNA technology. The present study focussed on hyperproduction of CA by focusing on two important parameters of fermentation. These two parameters are (i) the strain of fungus used and (ii) fermentation substrates used. Three alternative carbon sources or sugar rich substrates were used for preparing suitable fermentation media and for comparing the viability of these carbon sources for enhanced CA production. All experiments were done in laboratory scale and the studies were repeated for seven consecutive generations to check the reproducibility of the results. In cane molasses medium, the UV 365 nm treated samples with 30 minutes of irradiation showed 3.4 times more yield of CA than the control. In case of sweet potato medium, the UV 365 nm treated samples produced 2.8 times more CA as compared to the control. In pumpkin medium, the UV 365 nm irradiated samples showed 2.4 times more yield of CA compared to the control. Thus, it was clear from the results obtained, that UV irradiation resulted in more than twice the yield of CA in all the three fermentation substrates, as compared to the parent strain used as control. The changes brought about in the metabolism of the mutagen treated fungal strain remained consistent for the seven generations of study. The results of this study showed similar results obtained in another study of mutagenesis of *Aspergillus niger* using UV rays [19]. Whether the effects of the UV rays in terms of CA production could be further enhanced by elongating the exposure of the parent strain for longer durations of time is a topic that can be further studied.

Conclusion

In conclusion, this study shows the feasibility of augmented production of CA with mutagenesis using locally available carbon rich sources. Use of mutagenesis leads to more than twofold increase in CA production. Sweet potato has been shown as a promising carbon rich source that can be studied further as a substrate for industrial production of citric acid.

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