African Scientist Vol. 23, No. 2 June 30, 2022
Printed in Nigeria

1595-6881/2021 $10.00 + 0.00

http://www.niseb.org/afs

AFS2022012/23204

Mutational Effect and Genetic Regulation in the Production of Citric Acid from *Aspergillus niger*

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(Received May 3, 2022; Accepted in revised form May19, 2022)

ABSTRACT: *Aspergillus niger* popularly known as industrial workhorse due to its fitness for industrial fermentation, has the potentials to secret citric acid. In view of surges in demand and growing markets for citric acid, the major attempt in this work was to determine how the product titres can be maximized. *A. niger* strains were isolated from various food substances. These strains were exposed to UV radiation at 356nm followed by subsequent subjection of the mutant strains to a medium containing chlorate as mutagen. The mutant strains from the chlorate medium were further subjected to another round of mutation using the medium containing chlorate for the second time. Thus, mutation proceeded from UV-PDC1-PDC2. These mutants strains (UV-mutants, PDC1-mutant and PDC2-mutants) with the wild strains as control, were then employed in submerged fermentation for the production of citric acid. The product titres of all the citric acids produced were analyzed. UV mutation proved to be very significant in preparing the fungus for a higher production of citric acid. The effect of repeated mutations showed that the citric acid production can be genetically regulated. Thus the ability to engineer the genes encoding the morphological variation or the constitutive expression of the genes encoding the citric acid may increase the level of citric acid production to the maximum.

Keywords: *Aspergillus niger*, Mutant, Chlorate, Citric acid, Mutagenesis, Gene

Introduction

Aspergillus niger regarded as one of the most industrially useful organism (Gautam *et al* ., 2011) is widely distributed all over the world. It is well known as industrial workhorse due to its unique integral physiological characters and better fitness for industrial fermentation (Legisa and Mattey, 2007; Papagianni, 2007; Show *et al*., 2015). As a filamentous dull or dark black looking fungus, it has ability to produce extracellular organic acids including citric acid. *A. niger* also has powerful polymer degrading enzymes system that hydrolyze many polymeric substrates, enabling it to rapidly grow and ferment on a variety of low-cost raw materials such as corn meal and molasses (Majumdor *et al*., 2010). Some other microorganisms have been used for the production of citric acid, however *Aspergillus niger* remains the choice organism following its enlistment as a source of citric acid production by the FOA (Food and Drug Administration).

Improvement on the strains of *A. niger* for higher product titres of citric acid has been a major interest in the industry. There is increasingly fierce competition demanding for high yield, titre and productivity that strain improvement appears to be crucial. In favour of the above view, it is reported recently that, strain development efforts commonly occur through random mutagenesis and screening processes that supplied several mutants with great industrial performance, which constitutes a bottleneck for further improvement (Meyer *et al*., 2016).

The first step toward the genetic regulation of the production of citric acid is creation of mutants of which, the morphology of the mycelium is very informative. According to Sun *et al.,* (2018), the morphological change in the

mycelium causes a decrease in viscosity and subsequent improvement in oxygen and mass transfer efficiency which may be conducive for citric acid accumulation. In this research, we employed repeated mutagenic approach on the same sets of *A. niger* strains, as to check possibly, how the production of citric acid is regulated. Our objective was to underpin the point at which the production of citric acid titre is at its peak and then suggests the way or direction the production of citric acid can be improved

The reason *A. niger* secretes citric acid into the external media is yet unknown (Upton *et al*., 2017). Recently, genetic engineering of the genes encoding TCA enzymes and substrate transporters has been reported to have varied success in citric acid biotechnology (Cairns *et al*, 2021). This gave us the impetus to try repeated mutation (thrice) on *A. niger* strains isolated from various food substances.

Materials and methods

Strains preparation: *A. niger* strains used in this work were isolated from various food substances in the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria. Following standard laboratory procedure, strains were regularly cultivated on Potato dextrose agar medium. After obtaining pure culture of each strain, the conidia were harvested and then stored in McCartney bottles in 4^{o}C refrigerator until use.

*Mutagenesis of A. niger strains***:** The following *A. niger* strains: AspnB, AspnG, AspnM, AspnO, AspnPe, AspnPo and AspnY previously isolated in our laboratory (Eboigbe and Omoregbe, 2020) were exposed to UV radiation at 356 nm for 30 mins. These isolates were subculture twice for stability. The mutants obtained were then further subjected to another round of mutation (twice) using potato dextrose agar medium amended with chlorate(PDC1 and PDC2). Chlorate is well known as mutagen (Cove, 1979)

Preparation of molasses Sugar cane was cut into tiny bits, and then grinded. The mixture was separated using a filter cloth, the greenish yellow liquid obtained was then boiled with low heat and stirred for about 180 minutes. The molasses was collected in clean, durable plastic container and stored at room temperature for further use.

Inoculum preparation: Spore suspension was prepared from 14 days old culture of parental and mutant *A. niger* strains respectively. The conidia spores were harvested with sterilized water, and spore density was adjusted to 10^6 spores/ml of the suspension.

Screening of isolates for citric acid production: Seven isolates of *A. niger* (*A. niger*-Br, *A. niger-Ga*, *A. niger*-Mi, *A. niger*-On, *A. niger*-Pe, *A. niger*-Po and *A. niger*-Ya) from the wild, seven UV mutants, seven PDC1 mutant and seven PDC2 mutants were screened for citric acid production using submerged fermentation in shake flask, as follows; Add 100ml of water into 250ml conical flasks respectively, mix with 14 g of molasses then add 0.005 mg $FeSO_4$ .6HO, 0.8 mg KH_2 PO_4 , 0.018 mg $MgSO_4$ .7HO, 0.5 mg $(NH4)_2$ SO_4 , 0.032 mg $CuSO_4$ The mixture was stirred vigorously until it dissolves; 10^6 spores/ml of the specific wild and mutant strains were used to inoculate each flask and incubated for a period of 14 days. Lime [$Ca(OH)_2$] was added to the solution (5 mg) for each of the flasks, mixed properly and allowed to stay for 1 hour; sulfuric acid was then added and incubated overnight. After incubation period, the culture broth from each flask was filtered through *Whatman* filter paper for mycelia separation. The clear culture filtrate was employed for determination of citric acid.

Citric acid yield determination by titration with NaOH: The concentration of citric acid in supernatant was estimated trimetrically (AOAC, 1995) by using 0.1M NaOH and phenolphthalein as indicator. Hence, 20ml of the analyte (citric acid) was placed in a titration flask and the burette filled by a standard solution (0.1M NaOH) until it reached the zero mark using a funnel, two drops of phenolphthalein was placed in the titration flask with the citric acid. The base was slowly added from the burette, drop by drop, until the phenolphthalein suddenly achieves the intermediate color (pink) between that of the acid and the color of the base. The titration ceases when the system was neither acidic nor basic referred to as the endpoint, once the endpoint was attained, the volume of NaOH used (titre) on the burette was read and titration was completed, this process was done thrice for each strain, and the average titre value was used in calculation to determine concentration of citric acid yield.

Statistical analysis: One way ANOVA was used in order to differentiate the mean of the various titre value of citric acid produced between the wild and mutants strains and also between the mutant strains. ANOVA was also used to check if there was any significant difference in the width of the hypha strand and width of sporangia heads between the wild and the mutant strains. Significant differences were determined at $P>0.05$. Correlation analysis was used in order to check the link between the sporangium head and the hypha strand.

Results

The mutation of the fungal strains used in this work for the production of citric acid proceeded from UV radiation followed by chemical mutagenesis using potato dextrose agar medium amended with chlorate (PDC). The results of the mutagenic approach shown below revealed a clear phenotypic variance proceeding from UV-radiation to PDC1 and PDC2 (Plate1). Interestingly, this phenotypic variance affected the mycelia (hypha strands) especially the PCD2 which appears somewhat pellets morphology and mycelia less dense.

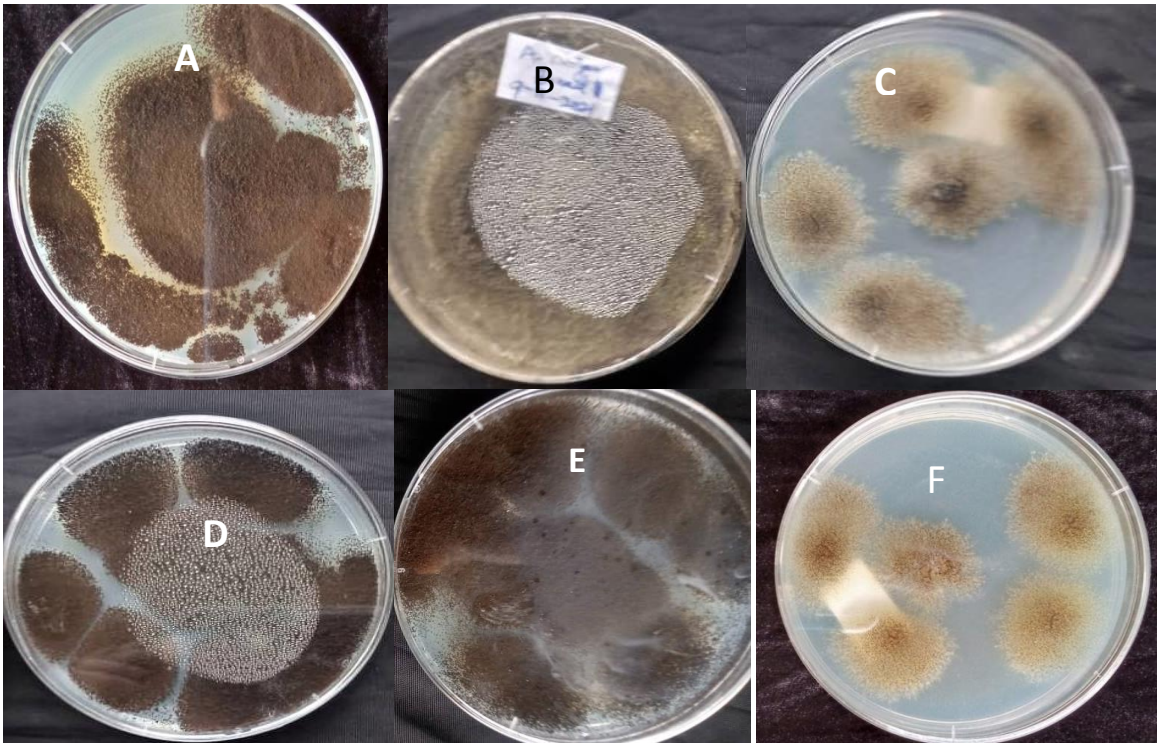

Plate 1: A and D are UV- mutants from AspnB and AspnPo respectively, B and E are PDC1 mutants From AspnB and AspnPo respectively, C and F are mutants from PDC2 respectively. From clear observation the mutation seem progressive from UV-irradiation to PDC2 which shows some deviation from filamentous to pellet morphology and less dense mycelia.

The measurement of the width of hypha strands and sporangia heads showed some level of variation (Fig. 1 & 2). Here we used the width of the hypha strands as a measure of compactness of the mycelia. This trait according to Sun *et al* (2018) is expected to influence the production rate of the citric acid. The variation in the width of hypha strand showed a significant difference. This variation was more pronounced between the wild and various mutants. With the analysis of variance (ANOVA), the variation between the various mutants also showed significant difference. This result is a true reflection of the mutational effect between the wild and the various mutants. The same trend was observed in the measurement of the width of the sporangia heads. As a result, we decided to check if there is correlation between the width of the sporangia heads and hypha strands. There is a positive correlation between the width of the sporangium head and hypha strand (R^2=0.66). There is positive interaction within the fungus' cell structure.

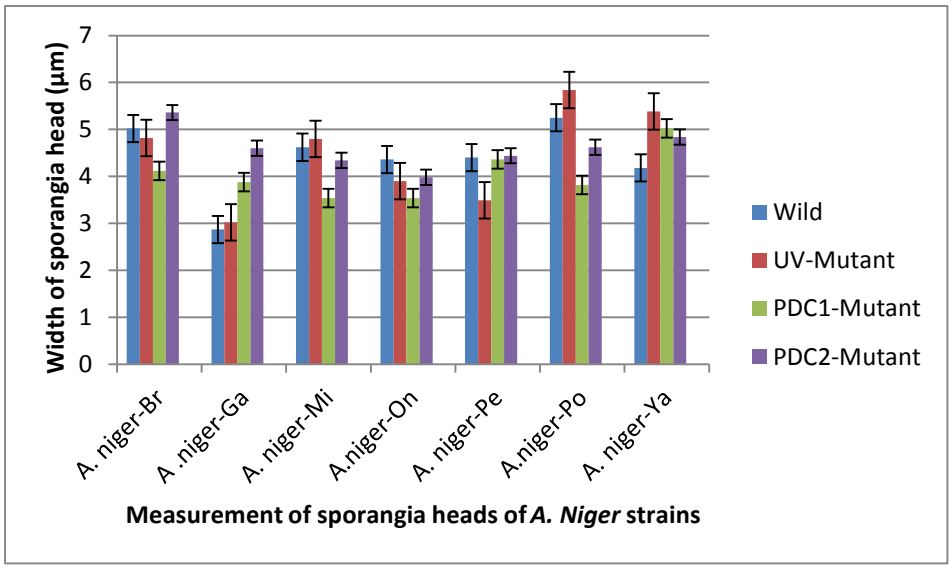

Fig. 2: Measurement of the width of sporangial heads in the wild and mutant strains. There is significant difference between the wild and the mutant strains and also within the mutants strains. Thus the effect of the repeated mutation may have some contribution to these variations

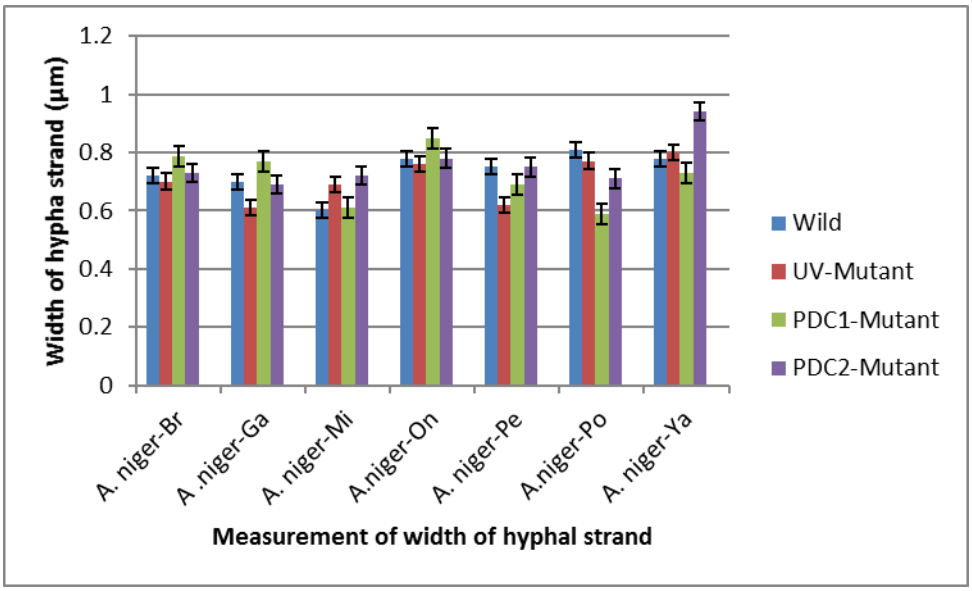

Fig 2: Measurement of the width of hypha strands of the wild and mutant strains of *A. niger*. There is significant difference between the wild and the mutant strains and also within the mutants strains.

Based on the above repeated mutational effect on the whole parts of the fungi, we proceeded with the submerge fermentation of molasses for the production of citric acid using the wild and mutant strains. The citric acid product titres of the UV-mutants strains were higher than that of the wild (Fig. 3). The repeated mutation with PDC1 and PDC2 did not show significant difference in the product titres of the citric acid from the UV-mutants. However the PDC1 and PDC2 mutants' product titres were also higher than that of the wild. Thus, the repeated mutation showed a clear difference in the product titres of citric acid between the wild and mutants (Fig. 3). The differences in the product titres were observed to be significantly difference between the wild and the UV mutants. However, between the UV mutants and others mutants (PDC1 and PDC2), there was no significant difference.

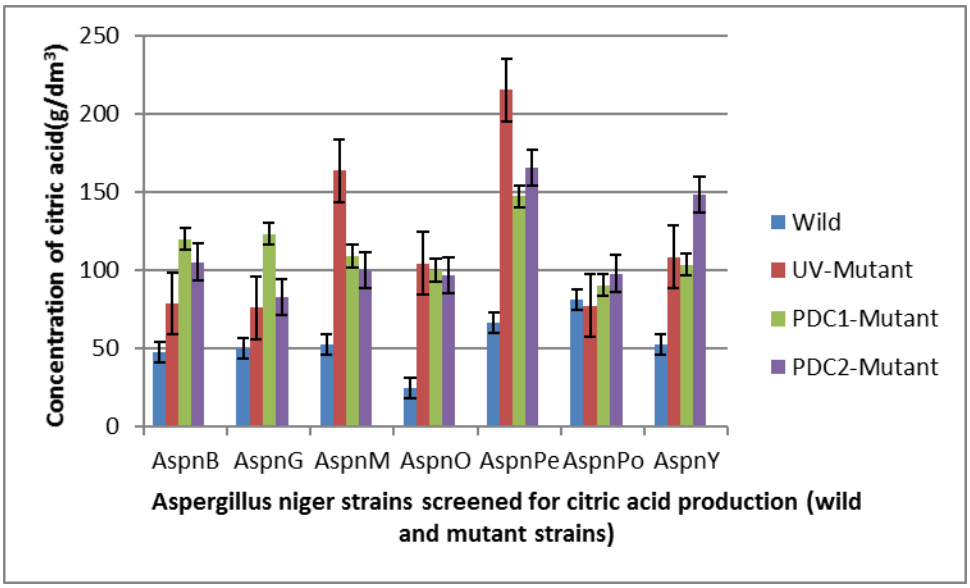

Fig. 3: Concentration of citric acid (g/dm^3) isolated from wild and mutant strains (UV-mutant strains, PDC1-mutants strains and PDC2-mutant strains) of *A. niger*

Discussion

In an attempt to check how morphology contributes to product titres, we decided to engage thrice mutated *A. niger* in citric acid production. According to Cairns *et al*. (2022), macromorphologies are known to have a critical impact on product titres. Our observation during the mutation process of *A. niger* showed that, the organism responded slowly to mutagenic agents especially the UV-irradiation; here there was no visible effect on the colony (Plates 1A and 1D). It was more or less like the wild strains in appearance. It was at the second and the third mutation with PDC that we observed color change in the fungal colony. The effect of the mutation was more pronounced at the second application of the chlorate of which a pelleted form of mycelium growth was seen on the plates (Plates 1C and 1E). Mutation of this nature is usually genetic-based. However, the choice of when to stop the mutation became clear when after the third round of the mutation, the growth of the fungus was hampered.

Since there was no remarkable change in the morphology of the UV-mutant strain when compared with the wild strain, it is expected that there should be little or no difference between the wild and UV-mutant strain in the product titres. Interestingly, the product titres from UV-mutants was higher compare to the wild strain as against our previous findings (Eboigbe and Uhumagho, 2017). We quickly admit that different wavelength was used. Although there was a significant difference between the wild and UV- mutant strains in their product titres, there was no significant difference between the UV-mutants and the other PDC mutants. In fact, there was no consistency in the product titres of citric acid among the mutants. This suggests that the first mutational event in any organism is far more shocking to the organism than the subsequent once.

Citric acid is an important organic acid, with a wide array of uses. Our major interest was to target strains with higher product titres through mutation. Furthermore, the inconsistency in the increase product titres of citric acid among the mutant strains suggests that, genetic engineering of strains is necessary for maximum production of citric acid to be attained. The closest fact in support of this finding is the genetic engineering of the TCA cycle enzymes and substrate transporter (Cairns *et. al*., 2021). From our work, repeated mutation was like a "guesswork" which sometimes leads to increase in the product titres of citric acid as reflected in AspnPO and AspnY in PDC2 mutants (Fig. 3). This outcome is also suggesting the possibility of genetic regulation in citric acid production.

Since by repeated mutations the product titres of citric acid could be increased, it therefore follows that all other nutritional conditions involved could act in synergy in affecting the product titres of citric acid (Kristiansen and Sinclair, 1979; Show *et al*., 2015). Such nutritional conditions as; the level of phosphorus, manganese, nitrogen,

dissolved oxygen concentration, substrate (carbon) concentration, micronutrient concentrations, pH, and ionic strength that can influence or change the morphology of the mycelia from filamentous to pelleted morphology could be very strategic in increasing the production of citric acid (Clark, 1962; Dai *et al*., 2004; Cairns *et al*., 2022). However, this role with respect to the nutritional conditions differs from one fungi to another. The genes that encode these changes should be the target for increasing the product titre. In our approach, as a result of the repeated mutation as shown in the PDC2 mutants, a pellet-like morphology appeared on plates.

In conclusion, this work has highlighted the significance of UV- irradiation (356 nm) as a way to increasing the production of citric acid in *A. niger*. Also, repetitive mutations may lead to improvement in strain for production fitness, if selection is maintained. With this, it is clear that ability to engineer the genes encoding the morphological variation or the constitutive expression of the genes encoding the citric acid may increase the level of citric acid production to the maximum.

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