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Changes in the physiological properties and kinetics of citric acid accumulation via carbon ion irradiation mutagenesis of *Aspergillus niger*[*]

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Abstract: The objective of this work was to produce citric acid from corn starch using a newly isolated mutant of *Aspergillus niger*, and to analyze the relationship between changes in the physiological properties of *A. niger* induced by carbon ion irradiation and citric acid accumulation. Our results showed that the physiological characteristics of conidia in *A. niger* were closely related to citric acid accumulation and that lower growth rate and viability of conidia may be beneficial to citric acid accumulation. Using corn starch as a raw material, a high-yielding citric acid mutant, named HW2, was obtained. In a 10-L bioreactor, HW2 can accumulate 118.9 g/L citric acid with a residual total sugar concentration of only 14.4 g/L. This represented an 18% increase in citric acid accumulation and a 12.5% decrease in sugar utilization compared with the original strain.

Key words: Carbon ion irradiation, Physiological properties, Mutation, Citric acid accumulation
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1 Introduction

Citric acid, as an important microbial fermentation product, is widely used in various industrial applications due to its physiological advantages (Mostafa and Alamri, 2012; Angumeenal and Venkappayya, 2013). Several microorganisms (Betiku and Adesina, 2013) can produce citric acid through fermentation, including fungi (*Aspergillus niger*, *Penicillium janthinelum*, and *A. awamori*), yeast (*Yarrowia lipolytica*, *Candida oleophila*, and *Candida tropicalis*), and bacteria (*Bacillus licheniformis*, *Corynebacterium* sp., and *Arthrobacter paraffinens*). However, *A. niger*, as an important microbial cell factory, remains the best choice for the production of citric acid due to its high yield of citric acid and its ability to ferment various cheap raw materials (Grewal and Kalra, 1995; Schuster *et al.*, 2002; Pel *et al.*, 2007; Wang *et al.*, 2015). Different cheap raw materials have been employed to produce citric acid, including starch materials such as corn starch (Hu *et al.*, 2014b), Yam bean starch (Sarangbin and Watanapokasin, 1999), and liquefied corn (Hu *et al.*, 2014a), and cheap agricultural products such as orange peel (Torrado *et al.*, 2011), apple pomace (Dhillon *et al.*, 2011b), whey, and sweet potatoes (Betiku and Adesina, 2013).

Worldwide citric acid production by an industrial-scale process of fermentation is 1.7×10^6 t/a (Dhillon *et al.*, 2011a), and the demand for citric acid is continuously increasing. China accounts for 60%–70% of the citric acid market share and the raw material used in China is mainly corn starch. Both the continuous growing demand for citric acid and the economics of

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fermentation encourage the exploration of different technical approaches to obtain improved varieties of *A. niger* using cheap raw materials for citric acid production (Parekh *et al.*, 2000; Haq *et al.*, 2003). Although physical or chemical mutagenesis agents are not novel and seem to be uneconomic, some remarkable microbial mutants have been obtained through mutagenesis and high-throughput screening (Heerd *et al.*, 2014). Traditional irradiation technologies, such as ultraviolet irradiation, γ-rays, and chemical mutagenesis have been widely and frequently applied to improve the citric acid yield of *A. niger* (Lotfy *et al.*, 2007; Javed *et al.*, 2010). Also, some new and powerful mutagenesis methods have been applied to the breeding of high-yielding industrial strains. These new methods have technological advantages (higher mutation rates and more abundant phenotypic mutations) (Hu *et al.*, 2013; Zhang *et al.*, 2015), and include atmospheric and room temperature plasma mutagenesis technology in Beijing (Li *et al.*, 2008; Wang *et al.*, 2010; Lu *et al.*, 2011; Zhang *et al.*, 2014), and medium or high-energy heavy ion irradiation technology in Lanzhou, China. Specifically, heavy-ion beams, such as $^{12}C^{6+}$, He^{2+}, Ar^{3+}, Zn^{2+}, C^{4+}, and C^{5+} (Yang *et al.*, 2013), as a type of high linear energy transfer (LET) irradiation, have a higher relative biological effect (RBE) compared with X- and γ-rays (Yang *et al.*, 2007; Li S.W. *et al.*, 2011; Ota *et al.*, 2013; Zhou *et al.*, 2013), and are expected to increase mutation frequency and have a wide mutation spectrum. They have been used effectively as a breeding method in plants and microbes (Zhou *et al.*, 2006; Wang *et al.*, 2009; Kazama *et al.*, 2011; Liu Q.F. *et al.*, 2013).

In China, the Heavy Ion Research Facility in Lanzhou (HIRFL) has been founded as a national laboratory and was opened for world-wide use in 1992. It now is an important institute contributing to global microbe breeding. Great progress has been made in radiation breeding of microbes of *Desmodesmus* sp. (Hu *et al.*, 2013), *Nannochloropsis* (Ma *et al.*, 2013), *A. terreus* (Li S.W. *et al.*, 2011), *Dietzia* strains (Zhou *et al.*, 2013), and oleaginous yeast (Wang *et al.*, 2009) via $^{12}C^{6+}$ ion beam irradiation at the Department of Biophysics, Institute of Modern Physics, Chinese Academy of Sciences (IMP, CAS), China. In 2014, a promising *A. niger* mutant, named H4002 (Hu *et al.*, 2014a), was obtained after $^{12}C^{6+}$ ion beam irradiation by IMP, CAS. Based on this mutant, citric acid accumulation can reach up to (187.5±0.7) g/L with extremely high productivity of 3.13 g/(L·h). The mutant has been used widely in industrial scale citric acid production. Citric acid biotechnology of *A. niger* has been significantly advanced by $^{12}C^{6+}$ ion beam irradiation, but there have been few studies of the relationship between changes induced in physiological properties of *A. niger* via $^{12}C^{6+}$ ion beam irradiation and citric acid accumulation.

The purpose of this study was to obtain different phenotypic *A. niger* mutants via $^{12}C^{6+}$ ion beam irradiation. To our knowledge, it was the first time to study the relationships between alterations in the physiological properties of *A. niger* induced by $^{12}C^{6+}$ ion beams and its secondary metabolite accumulation. This study indicated that the physiological characteristics of conidia in *A. niger* were closely related to citric acid accumulation, which can provide a new alternative way for screening high-yield citric acid *A. niger* mutants in the future.

2 Materials and methods

2.1 Strains

The original strain, named H4002 (Hu *et al.*, 2014a; 2014b), is preserved in IMP, CAS. Mutants HW2 and H4 were obtained after $^{12}C^{6+}$ ion beam irradiation.

2.2 Conidial diameter

Conidia of mutants and the original strain cultivated on potato dextrose agar (PDA)-containing slopes for 6 d were harvested in sterile distilled water, and then filtered through sterile filter paper with an aperture size of 20–25 μm. The filtered liquids were injected into a flow cytometer (MACSQuant™, Germany or FlowSight, USA) for measurement of conidial diameters.

2.3 Conidial viability

Conidia of mutants and the original strain cultivated on PDA-containing slopes for 6 d were harvested in sterile PDA-containing lipid medium (without agar). The conidial concentrations of the mutants and the original strain were all 4.65×10^6 conidia/ml. A volume of 400 μl of conidial suspensions of mutants and original strain were added to wells in 24-well plates, and were cultivated for 2 h in a

shake flask at a speed of 200 r/min. Then, 100 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) liquid (5 mg/ml) was added to each well for 2 h at 50 °C. After standing, 500 μl hydrochloric acid (1 mol/L) was added to each well in the shake flask which was then shaken at a speed of 200 r/min for 10 min, followed by centrifugation (12 000 r/min, 5 min) and supernatant removal. A volume of 400 μl of isopropanol was added to the Eppendorf (EP) tubes for 10 min. After standing, the extraction supernatants of the mutants and original strain were collected followed by centrifugation (12 000 r/min, 5 min). Finally, 200 μl of each extraction supernatant was added to each well in a 96-well plate, and analyzed using an Infinite M200 PRO (Switzerland) microplate reader. The absorbance of each extraction supernatant was read at 560 nm.

2.4 Scanning electron microscopy

Conidia of the mutants and the original strain cultivated on PDA-containing slopes for 6 d were harvested using a sterile bamboo stick, and were shifted to Eppendorf tubes with 200 μl sterile water, followed by centrifugation (12 000 r/min, 5 min). The supernatants were removed after two washing steps with absolute ethyl alcohol, and then viewed using a JSM-5600LV scanning electron microscope (Japan).

2.5 Colony growth rates

Conidia of the mutants and the original strain cultivated on PDA-containing slopes for 6 d were harvested in sterile distilled water. The conidial concentrations were all 3.7×10^5 conidia/ml. Details of procedures were reported by Liu *et al.* (2014). The solid plate medium had the following composition: 4.5 g/L glucose or 4.5 g/L maltose or 10 g/L starch as different carbon sources, 3 g/L sodium nitrate, 1 g/L dipotassium phosphate, 0.5 g/L magnesium sulfate, 0.5 g/L potassium chloride, 0.01 g/L ferric sulfate, 20 g/L agar, and 0.2 g/L bromocresol green. The cultivation conditions were 37 °C for 76 h.

2.6 Citric acid accumulation

The fermentation medium contained 120 g/L corn starch and 10.6 g/L nitrogen source material, which were hydrolyzed by α-amylase at 95–98 °C for 30 min in a 10-L bioreactor. Then, the whole medium was autoclaved at 115–118 °C for 30 min. Finally, equiponderant bran seeds of mutant HW2 and the original strain were injected into the 10-L bioreactor. The fermentation temperature was 37 °C, and the rotation speed (450±30) r/min.

2.7 Analytical methods

The concentrations of citric acid, total sugar, and biomass accumulation were measured using Fehling reagent (Hu *et al.*, 2014a). The growth rates of the mutants and original strain were measure by colony diameters. The ratio of acid spot diameter to lawn diameter is defined to RALD. Each experiment was carried out three times.

3 Results

3.1 Conidial diameters and viability of mutants and the original strain

Mesquita *et al.* (2013) reported that flow cytometry can be an effective tool to assess the size and complexity of *A. niger* conidia after γ radiation, and that forward-scattered light (FSC) can provide information on spore size. Stentelaire *et al.* (2001) reported that MTT assay can be used to measure fungal conidial viability. In this study, we used flow cytometry and MTT assay to assess the average diameters and viabilities of conidia of the mutants and the original strain. The HW2 and H4 mutants had slightly larger conidia than the original strain (Table 1).

Using MTT assay, we found that conidial vitality of the H4 strain was higher than that of the original strain. This implies that the metabolic activity of the H4 strain was higher than that of the original strain. The conidial vitality of the HW2 strain was not significantly different from that of the original strain (Fig. 1).

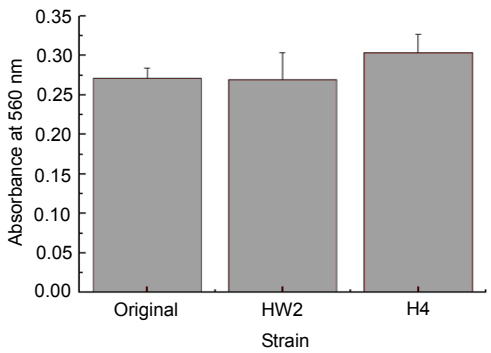

Fig. 1 Comparison of the vitality of conidia among the original strain, HW2, and H4
Error bars indicate the standard deviation of the mean (n=3)

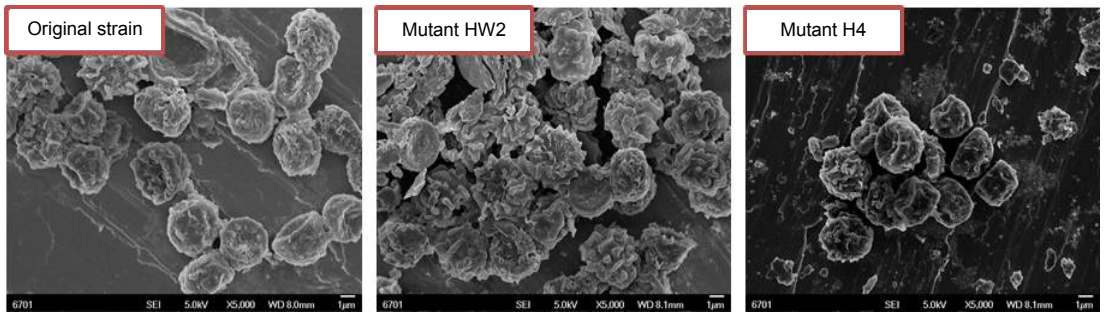

Fig. 2 Conidial surface morphologies of the original strain, HW2, and H4
All photos were taken at the same magnification (×5000)

Table 1 Comparison of conidial diameters among the original strain, mutant HW2, and mutant H4

Strain	FSC
Original strain	95.16±2.79
H4	103.76±8.22
HW2	96.72±10.90

FSC: forward-scattered light

3.2 Differences in conidial morphologies and colony growth rates between mutants and the original strain

Scanning electron microscopy showed that the proportion of conidia with a wrinkled surface in the HW2 strain was higher than that of the original strain. The proportion in the H4 strain was not significantly different from that of the original strain (Fig. 2).

Growth rate experiments showed that when glucose, starch, or maltose was the carbon source, the lawn diameter of HW2 became smaller than that of the original strain, whereas the lawn diameter of the H4 strain was larger when starch or maltose was supplied as the carbon source (Figs. 3 and 4).

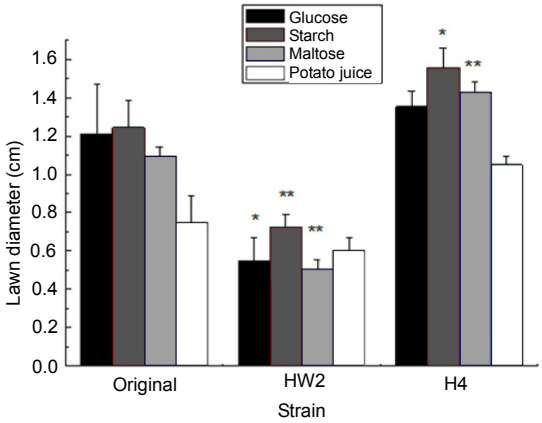

Fig. 3 Effect of carbon source on the lawn diameters of the original strain, HW2, and H4
Error bars indicate the standard deviation of the mean (n=3). $^{*}P<0.05$, $^{**}P<0.01$, compared with the original strain

The diameters of transparent halos are often used to screen mutants with a high yield of organic acids (Bai *et al.*, 2004; Li S.C. *et al.*, 2011). We analyzed differences in RALD between mutants and the original strain. When glucose, starch, or maltose was supplied as the carbon source, the RALD of HW2 was significantly larger than that of the original strain, whereas the RALD of the H4 strain was not significantly different from that of the original strain (Fig. 5). We conclude that mutant HW2 may exhibit enhanced citric acid accumulation.

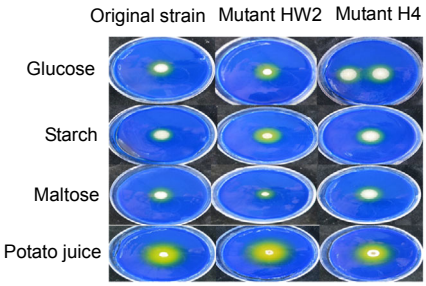

Fig. 4 Variation in lawn diameter among the original strain, HW2 strain, and H4 strain in response to different carbon sources

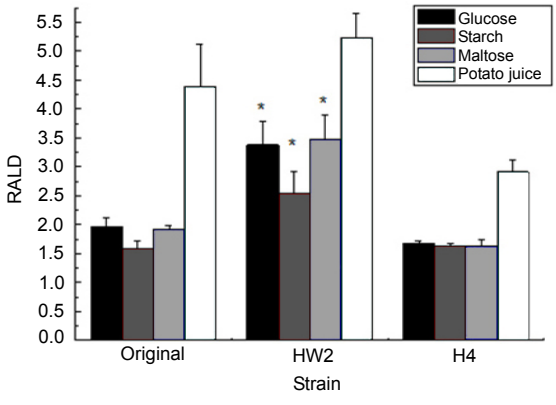

Fig. 5 Effects of different carbon sources on the RALD of the original strain, HW2, and H4
Error bars indicate the standard deviation of the mean (n=3). $^{*}P<0.05$, compared with the original strain

3.3 Differences in citric acid accumulation between mutant HW2 and the original strain in the shake flask and bioreactor

Using corn starch as a carbon source, HW2 accumulated citric acid and subdued biomass accumulation under different fermentation time in the shake flask (Fig. 6). The genetic stability of the mutant HW2 was also investigated. Through four consecutive generations of citric acid production in the shake flask (48 h), mutant HW2 produced 37–38 g/L citric acid (Fig. 7). This suggests that mutant HW2 had a stable ability to produce citric acid.

Similar results were obtained using a 10-L bioreactor (Fig. 8). Under optimized culture conditions in the 10-L bioreactor, when the initial total sugar concentration was 120 g/L and fermentation time was 53 h, mutant HW2 could produce 118.9 g/L citric acid and the residual total sugar was only 14.4 g/L, whereas, the original strain produced 100.8 g/L citric acid and residual total sugar of 16.2 g/L. Mutant HW2 showed an 18.0% increase in citric acid accumulation and a 12.5% decrease in sugar utilization compared with the original strain. In other words, mutant HW2 own higher sugar-acid conversion rate than the original strain. Hence, the HW2 strain is a more promising strain than the original strain for industrial production of citric acid. Furthermore, during the whole metabolism process, HW2 showed more round and compact pellets than the original strain (Fig. 9).

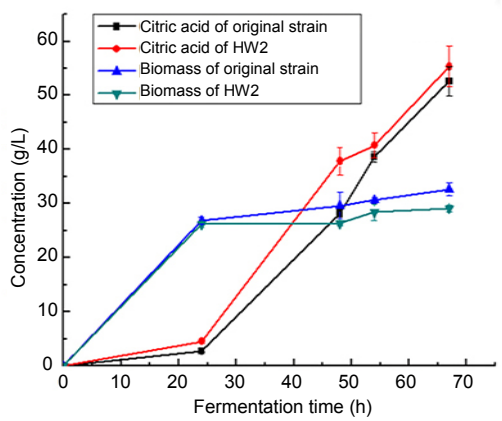

Fig. 6 Citric acid accumulation of the original strain and HW2 with starch as raw material under different fermentation time in a shaking flask

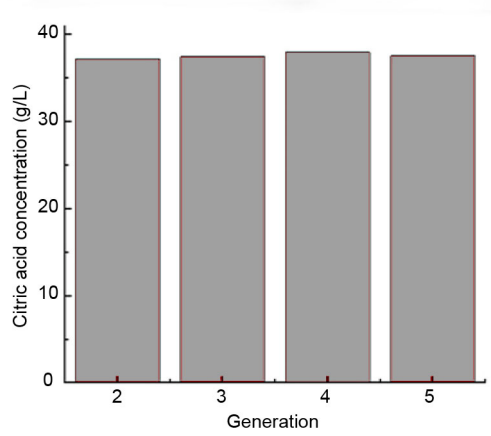

Fig. 7 Genetic stability of the mutant HW2 (48 h)

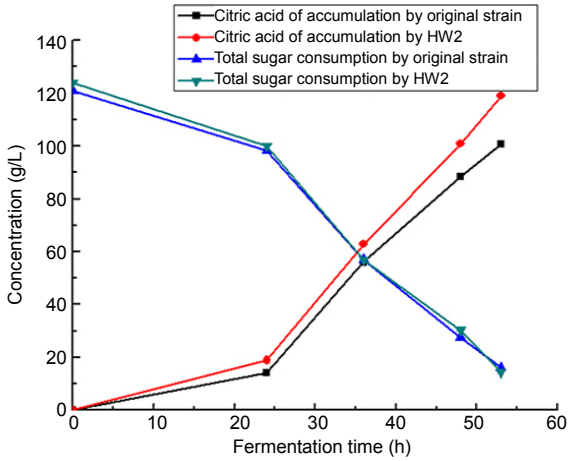

Fig. 8 Accumulation of citric acid by the original strain and HW2 in a 10-L bioreactor

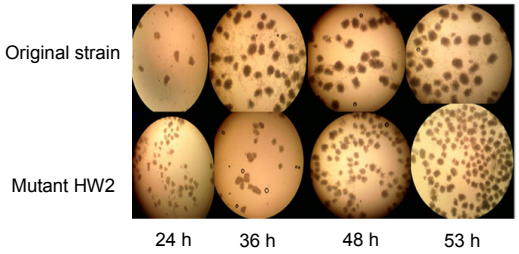

Fig. 9 Pellet morphologies of the original strain and HW2 after different fermentation periods in a 10-L bioreactor
All photos were taken at the same magnification (×10)

4 Discussion

Conidia are the main physiological structures in *Aspergillus* (van Leeuwen *et al.*, 2013), and have important physiological functions. The physiological

properties of conidia have drawn the attention of biologists and breeding experts. Butler and Day (1998) reported that conidia in *A. niger* showed strong tolerance to UV-rays due to the presence of melanin in conidia. Similar results were found by Liu *et al.* (2014). de Nicolas-Santiago *et al.* (2006) reported that differential mannanase production in original *A. niger* and mutants was associated with different conidial morphologies and diameters. These results proved that the physiological properties of fungal spores were related to their physiological metabolic functions. Changing in the physiological characteristics of microbes via mutagenesis indicated differential secondary metabolite accumulation (Zhang *et al.*, 2014).

Compared with traditional irradiation methods, LET heavy charged particles show denser ionization along their trajectories and higher RBE (Kiefer, 1992; Goodhead, 1999), and result in complex DNA damage in the body, such as large deletions, rearrangements, or translocations, which can generate abundant mutants (Hu *et al.*, 2013). The establishment of different high-throughput screening methods has also enabled some promising mutants to be obtained quickly. These mutants can be useful in functional gene research (Shikazono *et al.*, 2005; Murai *et al.*, 2013). As a novel and efficient method for generating mutations, $^{12}C^{6+}$ ion beam irradiation has been widely applied in the breeding of industrial microorganisms in China due to its characteristics of high LET, high RBE, and high mutation rate.

In this study, we firstly showed that carbon ion irradiation may induce alterations in the physiological characteristics of conidia in *A. niger*. Our results suggested that changes in the physiological properties of *A. niger*, such as lower growth rate and viability of conidia, may provide a new strategy for screening high-yielding citric acid producing strains. To achieve economic production of citric acid in an industrial-scale process, a supply of cheap raw materials, such as starch materials, and high-yielding strains are highly desirable (Suzuki *et al.*, 1996; Haq *et al.*, 2003). Presently, China produces more than 60% of global citric acid (Hu *et al.*, 2014a), and the raw material for citric acid production in China is mainly corn starch. Therefore, using corn starch as raw material, differences in citric acid accumulation between mutant HW2 and the original strain in shake flask and in a 10-L bioreactor were investigated. HW2 exhibited enhanced citric acid production and sugar-acid conversion rates compared with the original strain. Hu *et al.* (2014b) reported that, when using corn starch as a carbon source, the original strain used in this study could accumulate 187 g/L citric acid within 68 h. Therefore, we conclude that a more promising citric acid-producing strain was obtained via carbon ion irradiation. Morphological differences in pellets between mutant HW2 and the original strain during the fermentation process were also observed. It has been suggested that the morphology of filamentous fungi is strongly related to their productivity (Paul *et al.*, 1999; Grimm *et al.*, 2005). In this study, it was observed that, compared with the original strain, smaller pellets in fermentation broth in *A. niger* mutants may enhance citric acid accumulation (Fig. 8). Thus, heavy ion irradiation can be an effective tool for inducing significant alterations in physiological characteristics in microbes, including conidial growth rate, conidial vitality, and pellet morphology, which are related to secondary metabolite accumulation.

Studies of the relationship between morphology and citric acid production in *A. niger* have been reported (Papagianni *et al.*, 1999; Ikram-Ul-Haq *et al.*, 2003). Paul *et al.* (1999) discussed how morphology in *A. niger* affected citric acid accumulation and several other variables, such as oxygen uptake, glucose uptake, and carbon dioxide production. Chitin synthase genes are important in determining the hyphal morphology and conidial development of *A. nidulans* (Borgia *et al.*, 1996; Fukuda *et al.*, 2009). Liu *et al.* (2013b) reported that the chitin synthase gene can influence the morphology of *P. chrysogenum*, and morphological changes induced by class III chitin synthase gene silencing were related to penicillin production by *P. chrysogenum* (Liu *et al.*, 2013a). In our study, the mutants HW2 and H4 had different colony and pellet morphologies from those of the original strain. Citric acid accumulation by *A. niger* may be associated with mutation in chitin synthase genes induced by carbon ion irradiation. We also found preliminary evidence that the expression levels of key genes involved in the pathway of starch bio-degradation, such as the glucoamylase gene and starch degradation regulation gene, showed obvious differences between the mutant and original strains. These findings suggest possible mechanisms to explain why the mutant strains HW2 and H4 showed

differential citric acid accumulation compared with the original strain. This may be worthy of further study in the future.

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Compliance with ethics guidelines

Wei HU, Ji-hong CHEN, Shu-yang WANG, Jing LIU, Yuan SONG, Qing-feng WU, and Wen-jian LI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Angumeenal, A.R., Venkappayya, D., 2013. An overview of citric acid production. *LWT-Food Sci. Technol*., **50**(2): 367-370. http://dx.doi.org/10.1016/j.lwt.2012.05.016

Bai, D.M., Zhao, X.M., Li, X.G., *et al*., 2004. Strain improvement of *Rhizopus oryzae* for over-production of L(+)-lactic acid and metabolic flux analysis of mutants. *Biochem. Eng. J*., **18**(1):41-48. http://dx.doi.org/10.1016/S1369-703X(03)00126-8

Betiku, E., Adesina, O.A., 2013. Statistical approach to the optimization of citric acid production using filamentous fungus *Aspergillus niger* grown on sweet potato starch hydrolyzate. *Biomass Bioenerg*., **55**:350-354. http://dx.doi.org/10.1016/j.biombioe.2013.02.034

Borgia, P.T., Iartchouk, N., Riggle, P.J., *et al*., 1996. The *chsB* gene of *Aspergillus nidulans* is necessary for normal hyphal growth and development. *Fungal Genet. Biol*., **20**(3):193-203. http://dx.doi.org/10.1006/fgbi.1996.0035

Butler, M.J., Day, A.W., 1998. Fungal melanins: a review. *Can. J. Microbiol*., **44**(12):1115-1136. http://dx.doi.org/10.1139/w98-119

de Nicolas-Santiago, S., Regalado-Gonzalez, C., Garcia-Almendarez, B., *et al*., 2006. Physiological, morphological, and mannanase production studies on *Aspergillus niger* uam-gs1 mutants. *Electron. J. Biotechnol*., **9**(1):50-60. http://dx.doi.org/10.2225/vol9-issue1-fulltext-2

Dhillon, G.S., Brar, S.K., Verma, M., *et al*., 2011a. Recent advances in citric acid bio-production and recovery. *Food Bioprocess Technol.*, **4**(4):505-529. http://dx.doi.org/10.1007/s11947-010-0399-0

Dhillon, G.S., Brar, S.K., Verma, M., *et al*., 2011b. Utilization of different agro-industrial wastes for sustainable bioproduction of citric acid by *Aspergillus niger*. *Biochem. Eng. J*., **54**(2):83-92. http://dx.doi.org/10.1016/j.bej.2011.02.002

Fukuda, K., Yamada, K., Deoka, K., *et al*., 2009. Class III chitin synthase chsB of *Aspergillus nidulans* localizes at the sites of polarized cell wall synthesis and is required for conidial development. *Eukaryot. Cell*, **8**(7):945-956. http://dx.doi.org/10.1128/Ec.00326-08

Goodhead, D.T., 1999. Mechanisms for the biological effectiveness of high-LET radiations. *J. Radiat. Res.*, **40**: S1-S13. http://dx.doi.org/10.1269/jrr.40.S1

Grewal, H.S., Kalra, K.L., 1995. Fungal production of citric acid. *Biotechnol. Adv*., **13**(2):209-234. http://dx.doi.org/10.1016/0734-9750(95)00002-8

Grimm, L.H., Kelly, S., Krull, R., *et al*., 2005. Morphology and productivity of filamentous fungi. *Appl. Microbiol. Biotechnol*., **69**(4):375-384. http://dx.doi.org/10.1007/s00253-005-0213-5

Haq, I.U., Ali, S., Iqbal, J., 2003. Direct production of citric acid from raw starch by *Aspergillus niger*. *Process Biochem*., **38**(6):921-924. http://dx.doi.org/10.1016/S0032-9592(02)00201-7

Heerd, D., Tari, C., Fernandez-Lahore, M., 2014. Microbial strain improvement for enhanced polygalacturonase production by *Aspergillus sojae*. *Appl. Microbiol. Biotechnol*., **98**(17):7471-7481. http://dx.doi.org/10.1007/s00253-014-5657-z

Hu, G.R., Fan, Y., Zhang, L., *et al*., 2013. Enhanced lipid productivity and photosynthesis efficiency in a *Desmodesmus* sp. mutant induced by heavy carbon ions. *PLoS ONE*, **8**(4):e60700. http://dx.doi.org/10.1371/journal.pone.0060700

Hu, W., Liu, J., Chen, J.H., *et al*., 2014a. A mutation of *Aspergillus niger* for hyper-production of citric acid from corn meal hydrolysate in a bioreactor. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **15**(11):1006-1010. http://dx.doi.org/10.1631/jzus.B1400132

Hu, W., Chen, J.H., Li, W.J., *et al*., 2014b. Mutant breeding of *Aspergillus niger* irradiated by $^{12}C^{6+}$ for hyper citric acid. *Nucl. Sci. Tech*., **25**(2):020302. http://dx.doi.org/10.13538/j.1001-8042/nst.25.020302

Ikram-Ul-Haq, Ali, S., Qadeer, M.A., *et al*., 2003. Control of *Aspergillus niger* morphology to enhance citric acid production under liquid culture. *Pak. J. Bot*., **35**(4): 533-539.

Javed, S., Asgher, M., Sheikh, M.A., *et al*., 2010. Strain improvement through UV and chemical mutagenesis for enhanced citric acid production in molasses-based solid state fermentation. *Food Biotechnol*., **24**(2):165-179. http://dx.doi.org/10.1080/08905436.2010.482014

Kazama, Y., Hirano, T., Saito, H., *et al*., 2011. Characterization of highly efficient heavy-ion mutagenesis in *Arabidopsis thaliana*. *BMC Plant Biol*., **11**(1):161. http://dx.doi.org/10.1186/1471-2229-11-161

Kiefer, J., 1992. Heavy-ion effects on cells: chromosomal-aberrations, mutations and neoplastic transformations. *Radiat. Environ. Bioph*., **31**(4):279-288. http://dx.doi.org/10.1007/BF01210208

Li, G., Li, H.P., Wang, L.Y., *et al*., 2008. Genetic effects of radio-frequency, atmospheric-pressure glow discharges with helium. *Appl. Phys. Lett.*, **92**(22):221504. http://dx.doi.org/10.1063/1.2938692

Li, S.C., Zhu, Z.Y., Gu, S.B., *et al*., 2011. Mutation-screening in L-(+)-lactic acid producing strains by ion implantation. *Indian J. Microbiol*., **51**(2):138-143. http://dx.doi.org/10.1007/s12088-011-0161-y

Li, S.W., Li, M., Song, H.P., *et al*., 2011. Induction of a high-yield lovastatin mutant of *Aspergillus terreus* by $^{12}C^{6+}$ heavy-ion beam irradiation and the influence of culture conditions on lovastatin production under submerged fermentation. *Appl. Biochem. Biotechnol*., **165**(3-4):913-925. http://dx.doi.org/10.1007/s12010-011-9308-x

Liu, H., Zheng, Z.M., Wang, P., *et al*., 2013a. Morphological changes induced by class III chitin synthase gene silencing could enhance penicillin production of *Penicillium chrysogenum*. *Appl. Microbiol. Biotechnol*., **97**(8):3363-3372. http://dx.doi.org/10.1007/s00253-012-4581-3

Liu, H., Wang, P., Gong, G.H., *et al*., 2013b. Morphology engineering of *Penicillium chrysogenum* by RNA silencing of chitin synthase gene. *Biotechnol. Lett*., **35**(3): 423-429. http://dx.doi.org/10.1007/s10529-012-1099-9

Liu, J., Zhou, L., Chen, J.H., *et al*., 2014. Role of ozone in UV-C disinfection, demonstrated by comparison between wild-type and mutant conidia of *Aspergillus niger*. *Photochem. Photobiol*., **90**(3):615-621. http://dx.doi.org/10.1111/php.12217

Liu, Q.F., Wang, Z.Z., Zhou, L.B., *et al*., 2013. Relationship between plant growth and cytological effect in root apical meristem after exposure of wheat dry seeds to carbon ion beams. *Nucl. Instrum. Meth. Phys. Res. B*, **305**:9-15. http://dx.doi.org/10.1016/j.nimb.2013.04.046

Lotfy, W.A., Ghanem, K.M., El-Helow, E.R., 2007. Citric acid production by a novel *Aspergillus niger* isolate: I. mutagenesis and cost reduction studies. *Bioresource Technol*., **98**(18):3464-3469. http://dx.doi.org/10.1016/j.biortech.2006.11.007

Lu, Y., Wang, L.Y., Ma, K., *et al*., 2011. Characteristics of hydrogen production of an *Enterobacter aerogenes* mutant generated by a new atmospheric and room temperature plasma (ARTP). *Biochem. Eng. J*., **55**(1):17-22. http://dx.doi.org/10.1016/j.bej.2011.02.020

Ma, Y.B., Wang, Z.Y., Zhu, M., *et al*., 2013. Increased lipid productivity and TAG content in *Nannochloropsis* by heavy-ion irradiation mutagenesis. *Bioresource Technol*., **136**:360-367. http://dx.doi.org/10.1016/j.biortech.2013.03.020

Mesquita, N., Portugal, A., Pinar, G., *et al*., 2013. Flow cytometry as a tool to assess the effects of gamma radiation on the viability, growth and metabolic activity of fungal spores. *Int. Biodeter. Biodegr*., **84**:250-257. http://dx.doi.org/10.1016/j.ibiod.2012.05.008

Mostafa, Y.S., Alamri, S.A., 2012. Optimization of date syrup for enhancement of the production of citric acid using immobilized cells of *Aspergillus niger*. *Saudi J. Biol. Sci*., **19**(2):241-246. http://dx.doi.org/10.1016/j.sjbs.2012.01.004

Murai, K., Nishiura, A., Kazama, Y., *et al*., 2013. A large-scale mutant panel in wheat developed using heavy-ion beam mutagenesis and its application to genetic research. *Nucl. Instrum. Meth. Phys. Res. B*, **314**:59-62. http://dx.doi.org/10.1016/j.nimb.2013.05.026

Ota, S., Matsuda, T., Takeshita, T., *et al*., 2013. Phenotypic spectrum of *Parachlorella kessleri* (Chlorophyta) mutants produced by heavy-ion irradiation. *Bioresource Technol*., **149**:432-438. http://dx.doi.org/10.1016/j.biortech.2013.09.079

Papagianni, M., Mattey, M., Berovic, M., *et al*., 1999. *Aspergillus niger* morphology and citric acid production in submerged batch fermentation: effects of culture pH, phosphate and manganese levels. *Food Technol. Biotechnol*., **37**(3):165-171.

Parekh, S., Vinci, V.A., Strobel, R.J., 2000. Improvement of microbial strains and fermentation processes. *Appl. Microbiol. Biotechnol*., **54**(3):287-301. http://dx.doi.org/10.1007/s002530000403

Paul, G.C., Priede, M.A., Thomas, C.R., 1999. Relationship between morphology and citric acid production in submerged *Aspergillus niger* fermentations. *Biochem. Eng. J*., **3**(2):121-129. http://dx.doi.org/10.1016/S1369-703x(99)00012-1

Pel, H.J., de Winde, J.H., Archer, D.B., *et al*., 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol*., **25**(2): 221-231. http://dx.doi.org/10.1038/nbt1282

Sarangbin, S., Watanapokasin, Y., 1999. Yam bean starch: a novel substrate for citric acid production by the protease-negative mutant strain of *Aspergillus niger*. *Carbohyd. Polym*., **38**(3):219-224. http://dx.doi.org/10.1016/S0144-8617(98)00094-0

Schuster, E., Dunn-Coleman, N., Frisvad, J.C., *et al*., 2002. On the safety of *Aspergillus niger*—a review. *Appl. Microbiol. Biot*., **59**(4-5):426-435. http://dx.doi.org/10.1007/s00253-002-1032-6

Shikazono, N., Suzuki, C., Kitamura, S., *et al*., 2005. Analysis of mutations induced by carbon ions in *Arabidopsis thaliana*. *J. Exp. Bot*., **56**(412):587-596. http://dx.doi.org/10.1093/jxb/eri047

Stentelaire, C., Antoine, N., Cabrol, C., *et al*., 2001. Development of a rapid and highly sensitive biochemical method for the measurement of fungal spore viability. An alternative to the CFU method. *Enzyme Microb. Technol*., **29**(8-9):560-566. http://dx.doi.org/10.1016/S0141-0229(01)00432-X

Suzuki, A., Sarangbin, S., Kirimura, K., *et al*., 1996. Direct production of citric acid from starch by a 2-deoxyglucose-resistant mutant strain of *Aspergillus niger*. *J. Ferment. Bioeng*., **81**(4):320-323. http://dx.doi.org/10.1016/0922-338x(96)80584-8

Torrado, A.M., Cortes, S., Salgado, J.M., *et al*., 2011. Citric acid production from orange peel wastes by solid-state fermentation. *Braz. J. Microbiol*., **42**(1):394-409.

http://dx.doi.org/10.1590/s1517-83822011000100049

van Leeuwen, M.R., Krijgsheld, P., Bleichrodt, R., *et al*., 2013. Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. *Stud. Mycol*., **74**(1):59-70. http://dx.doi.org/10.3114/Sim0009

Wang, J.F., Li, R.M., Lu, D., *et al*., 2009. A quick isolation method for mutants with high lipid yield in oleaginous yeast. *World J. Microb. Biot*., **25**(5):921-925. http://dx.doi.org/10.1007/s11274-009-9960-2

Wang, L., Zhang, J.H., Cao, Z.L., *et al*., 2015. Inhibition of oxidative phosphorylation for enhancing citric acid production by *Aspergillus niger*. *Microb. Cell Fact*., **14**(1):7. http://dx.doi.org/10.1186/s12934-015-0190-z

Wang, L.Y., Huang, Z.L., Li, G., *et al*., 2010. Novel mutation breeding method for *Streptomyces avermitilis* using an atmospheric pressure glow discharge plasma. *J. Appl. Microbiol*., **108**(3):851-858. http://dx.doi.org/10.1111/j.1365-2672.2009.04483.x

Yang, Y.N., Ren, N., Xue, J.M., *et al*., 2007. Mutation effect of MeV protons on bioflocculant bacteria *Bacillus cereus*. *Nucl. Instrum. Meth. Phys. Res. B*, **262**(2):220-224. http://dx.doi.org/10.1016/j.nimb.2007.05.016

Yang, Y.N., Liu, C.L., Wang, Y.K., *et al*., 2013. Mutation effects of C^{2+} ion irradiation on the greasy *Nitzschia* sp. *Mut. Res. Fund. Mol. Mech. Mutagen.*, **751-752**:24-28. http://dx.doi.org/10.1016/j.mrfmmm.2013.09.003

Zhang, X., Zhang, X.F., Li, H.P., *et al*., 2014. Atmospheric and room temperature plasma (ARTP) as a new powerful mutagenesis tool. *Appl. Microbiol. Biotechnol*., **98**(12):5387-5396. http://dx.doi.org/10.1007/s00253-014-5755-y

Zhang, X., Zhang, C., Zhou, Q.Q., *et al*., 2015. Quantitative evaluation of DNA damage and mutation rate by atmospheric and room-temperature plasma (ARTP) and conventional mutagenesis. *Appl. Microbiol. Biotechnol*., **99**(13):5639-5646. http://dx.doi.org/10.1007/s00253-015-6678-y

Zhou, L.B., Li, W.J., Yu, L.X., *et al*., 2006. Linear energy transfer dependence of the effects of carbon ion beams on adventitious shoot regeneration from in vitro leaf explants of *Saintpaulia ionahta*. *Int. J. Radiat. Biol*., **82**(7):473-481.

Zhou, X., Xin, Z.J., Lu, X.H., *et al*., 2013. High efficiency degradation crude oil by a novel mutant irradiated from *Dietzia* strain by $^{12}C^{6+}$ heavy ion using response surface methodology. *Bioresource Technol*., **137**:386-393. http://dx.doi.org/10.1016/j.biortech.2013.03.097

中文概要

题　目：碳离子束诱导黑曲霉生理特性的改变与其柠檬酸积累关系的研究

目　的：探讨黑曲霉生理特性的改变对其柠檬酸积累的影响。

创新点：首次报道了碳离子束对黑曲霉生理特性的影响，提出黑曲霉生理特性的改变对其柠檬酸的积累有影响。

方　法：（1）孢子直径和孢子活力的测定：利用流式细胞仪对两株黑曲霉突变菌株以及原始菌株进行了孢子直径测定。通过该实验说明碳离子束可以诱导黑曲霉孢子直径发生改变（表 1）。利用 MTT 法，测定了突变菌株和原始菌株孢子活力。同时，通过该实验表明碳离子束可以诱导黑曲霉孢子活力发生改变（图 1）。（2）生长速率的测定：利用不同糖分的平板生长法测定了突变体和原始菌株生长速度的差异。通过该实验说明碳离子束可以诱导黑曲霉生长速率发生改变（图 3 和图 4）。（3）柠檬酸积累实验：利用摇瓶发酵实验和 10 L 发酵罐扩培实验测定了突变体和原始菌株柠檬酸积累的差异。通过该实验说明生理特性改变的突变体与原始菌株在柠檬酸积累上存在显著差异（图 6~8）。

结　论：通过两株黑曲霉突变体与原始菌株之间生理特性的差异研究，我们发现黑曲霉生理特性的改变对其柠檬酸积累有一定的影响，低的黑曲霉孢子活力以及生长速率对柠檬酸的积累是有益的。最终，获得了一株高产菌株 HW2，其 10 L 发酵罐柠檬酸的积累能力比原始菌株提高了 18%。

关键词：碳离子束辐照；生理特性；突变；柠檬酸积累