Mutant breeding of Aspergillus niger irradiated by 12C6+ for hyper citric acid production (Postprint)

Authors: HU Wei, Ji-Hong Chen, LI Wen-Jian, Jing Liu, WANG Shu-Yang, WANG Ju-Fang, LU Dong

Date: 2023-06-18T00:00:00+00:00

Abstract

In this study, strains of Aspergillus niger 4# for hyper-production of citric acid were irradiated with different doses of 80 MeV/u 12C6+ ion beams. Seven mutant strains exhibited marked citric acid overproduction and higher productivity than the parental Aspergillus niger 4# strain in shake flask fermentation. The maximum product yield was 132.8 g L^{-1} (the H4002 strain), representing an 8.8% increase over the parental strain. The scale-up experiment was conducted in a 100 L bioreactor. The mutant H4002 could accumulate a citric acid yield of 187 g L^{-1} from starch liquefying supernatant. The citric acid productivity was 2.75 g L^{-1} h^{-1}. Thus, the mutant H4002 possesses rapid sugar catabolism for citric acid production. Meanwhile, the pellet morphology remained compact and spherical throughout the submerged fermentation, which is suitable for citric acid production. The results indicate that mutant H4002 has the potential for rapid citric acid production.

Full Text

Preamble

Mutant Breeding of Aspergillus niger Irradiated by 12C6+ for Hyper Citric Acid Production

HU Wei,[1,2] CHEN Ji-Hong,[1,†] LI Wen-Jian,[1,2,‡] LIU Jing,[1] WANG Shu-Yang,[1] WANG Ju-Fang,[1] and LU Dong[1]

[1]Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Road, Lanzhou 730000, China
[2]University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China

(Received May 6, 2013; accepted in revised form December 30, 2013; published online April 20, 2014)

In this study, strains of Aspergillus niger 4# for hyper citric acid production were irradiated with 80 MeV/u 12C6+ ion beams at different doses. Seven mutant strains showed marked citric acid over-production and faster productivity than the initial Aspergillus niger 4# strain in shake-flask fermentation. The maximum product yield was 132.8 g L^{-1} (achieved by the H4002 strain), representing an 8.8% increase over the initial strain. The scale-up experiment was carried out in a 100 L bioreactor. The mutant H4002 could accumulate a product yield of 187 g L^{-1} citric acid from starch liquefying supernatant, with a productivity of 2.75 g L^{-1} h^{-1}. Thus, the mutant H4002 possesses rapid sugar katabolism for citric acid production. Meanwhile, the pellet morphology remained compact and round throughout the submerged fermentation, which is suitable for citric acid production. These results indicate that mutant H4002 has the potential to produce citric acid rapidly.

Keywords: 12C6+ ion, rapid fermentation, productivity, pellet morphology
DOI: 10.13538/j.1001-8042/nst.25.020302

Introduction

Citric acid fermentation represents one of the largest biotechnological industries, and *Aspergillus niger* has demonstrated good potential for citric acid production. Annual citric acid production through fermentation is estimated at 7.0×10^5 tons, with industrial demand steadily increasing. In 2008, the highest citric acid production level achieved by Chinese producers was 146.9 g L^{-1}, with the best fermentation index reaching 2.7 g L^{-1} h^{-1}, both substantially lower than advanced international levels. Therefore, enhancing the citric acid production capacity of *Aspergillus niger* is vital. To obtain high product yields at production scale, one effective strategy is to improve the microbial production strain.

Strains with superior characteristics, such as enhanced citric acid production and high fermentation index, have been selected after exposing genetic material to physical or chemical mutagenic agents. Heavy ion-induced mutation is a unique physical mutagenesis method. With high linear energy transfer (LET) and relative biological effectiveness (RBE), heavy ions can induce single- or double-strand DNA breaks with low reparability in bombarded tissue and demonstrate powerful capability to improve mutation rates and broaden the mutation spectrum. Progress has been made at the Institute of Modern Physics, Chinese Academy of Sciences (IMP, CAS) in heavy ion mutation breeding of gentamicin-producing microorganisms, alcohol-resistant yeast, and *Streptomyces avermitilis.*

In this paper, we report the selection of *A. niger* mutants for hyper-production of citric acid irradiated by 12C6+ beams from the Heavy Ion Research Facility of Lanzhou (HIRFL). A bioreactor was applied to reveal the fermentation characteristics of the best mutant strain for citric acid production, and the pellet morphology across different fermentation cycles was also analyzed.

∗ Supported by the Project of Science and Technology for Gansu of Chinese Academy of Sciences (KJZG-2011-002) and West Light Foundation of The Chinese Academy of Sciences (2012)
† chjh@impcas.ac.cn
‡ wjli@impcas.ac.cn

II. Materials and Methods

A. Microorganism

The initial *Aspergillus niger* strain was provided by the biophysics laboratory of IMP, CAS. It was maintained on potato dextrose agar slants and stored at 4 °C. Spores of *Aspergillus niger* were inoculated into bran medium (20% potato, 2% saccharose, 2% agar) for 6 days.

B. Irradiation

The conidial suspension from 6-day-old slant cultures of *Aspergillus niger* 4# in saline water was transferred to irradiation dishes. The colony-forming units per milliliter (CFU/mL) were maintained at 1×10^6 cells/mL. Seven groups of initial *Aspergillus niger* strains were prepared and irradiated at doses of 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy, 50 Gy, and 60 Gy. The 12C6+ ion beams of 80 MeV/u had an LET of 40 keV μm^{-1}.

C. Screening of Mutant Strains for Hyper Citric Acid Production

Colony suspensions irradiated at different doses were diluted to a 10^{-5} gradient. Then, 0.1 mL of diluted colony suspension was daubed onto solid plate medium. Strains with a high ratio between acid spot diameter and lawn diameter were selected for shake-flask fermentation. All fermentation media were composed of 16% corn starch liquefying supernatant and 0.43% mixed soybean cake powder. Initial pH was adjusted to 6.1-6.4. The solid plate medium composition was 20% potato juice, 2% sucrose, 2% agar, and 0.01% bromcresol green. Liquid media were sterilized at 115 °C for 30 min.

D. Bioreactor Fermentation

Fermentation was carried out in a 100 L stirred tank reactor. The fermentation media were prepared as follows: water to corn starch ratio of 3:1, with 25 mL of thermostable α-amylase added to the 100 L bioreactor at 95 °C for 30 min. Liquefying supernatant was obtained and diluted with tap water to the desired sugar concentration, after which the nitrogen source was added. Liquid media were sterilized at 115 °C for 30 min.

E. Analysis

The concentration of citric acid in culture filtrate was measured by titration with 0.1429 N sodium hydroxide using 0.5% phenolphthalein as an indicator, and the purity of citric acid in the fermentation broth was determined by high-performance liquid chromatography (HPLC). Total and residual sugars were hydrolyzed into glucose and fructose using 6 N hydrochloric acid at boiling temperature for 10 min and analyzed by the Fehling reagent method.

III. Results and Discussion

A. Mutagenesis and Selection for Citric Acid Hyper-Production Mutants

In this study, the initial *Aspergillus niger* 4# strain and seven mutants were examined for citric acid production from 160 g L^{-1} (w/v) carbohydrates using corn starch liquefying supernatant in 250 mL Erlenmeyer flasks. The 60 Gy group exhibited the highest fatality rate and positive mutation rate. The H3002, H4001, H4002, H5001, H6003, H6005, and H6007 strains showed marked citric acid over-production and faster productivity (Table 1), with the 40 Gy H4002 strain performing the best.

B. Analysis of Fermentation Characteristics and Pellet Morphology of H4002 Strain in the Bioreactor

Citric acid overproduction requires a unique combination of high sugar concentration, H^+, and dissolved oxygen—several unusual nutrient conditions. In 2008, the best citric acid production level achieved by Chinese companies was 146.9 g L^{-1}, with the best fermentation index being 2.7 g L^{-1} h^{-1}. However, the H4002 strain obtained in our laboratory can convert high starch liquefying sugar to citric acid more efficiently. The results (Fig. 1) show that the citric acid concentration achieved by mutant H4002 (187 g L^{-1}) is much higher than that of the initial strain (136 g L^{-1}), representing an increase of 37.5%. Productivity by mutant H4002 (2.75 g L^{-1} h^{-1}) is 1.20 times higher than the initial strain (2.3 g L^{-1} h^{-1}) and exceeds the result in Ref. [13]. The mutant H4002 strain can convert sugar to citric acid with an average productivity of 2.12 g L^{-1} h^{-1} in the later phase of submerged fermentation (44–69 h). Furthermore, the pH in the broth is very low (pH $\leq$ 1.32). Therefore, it can be deduced that the H4002 strain is far more resistant to high sugar and citric acid concentrations than current *Aspergillus niger* strains.

A possible explanation is that the H4002 strain has a strong ability to secrete highly active glycolytic enzymes, which enhance polysaccharide catabolism. It is accepted that high activities of glycolytic enzymes can increase the rate of citric acid accumulation. Ambient pH strongly influences the synthesis of secreted enzymes, permeases, and metabolites in a wide range of microorganisms. The low pH (pH $\leq$ 1.32) may induce over-expression of some genes in the H4002 strain, enabling it to adjust to changing environmental conditions. Of course, this explanation requires experimental verification.

The dissolved oxygen (DO) curve shows that the DO value remained higher than 62.5%, which is necessary for normal citric acid fermentation throughout the entire process. Because high DO can induce strain aging while low DO can decrease productivity, the optimized fermentation conditions (aeration rate 18 L min^{-1}, rotation speed 400 r min^{-1}) can meet the production requirements of citric acid by the H4002 strain.

High-performance liquid chromatography (HPLC) was applied to determine the citric acid concentration in fermentation broth. A linear regression between sample concentration (x) and corresponding peak area (y) was performed, yielding $y = 1.93 \times 10^9 x + 46895$ with a correlation coefficient of $R^2 = 0.9998$. The final citric acid concentration of 187 g L^{-1} and the minimal heteroacid peaks in the chromatogram (Fig. 2) of fermentation broth (69 h) are encouraging. Based on the ratio of citric acid monohydrate (indirectly measured by HPLC) to total acid (directly measured by acid-base titration), the purity of citric acid in the fermentation broth is 98.4%.

[Figure 2: see original paper]

Figure 3 shows the pellet morphology of the H4002 strain in liquid medium at 24 h, 48 h, and 60 h. Most pellets formed by the H4002 strain are compact and round with short hyphae around the pellet surface. In contrast, the pellet morphology of the 4# strain is relatively loose with relatively long hyphae around the pellet surface. It is accepted that mycelial morphology of *Aspergillus niger* in citric acid fermentations is a critical parameter for industrial application. Shorter and highly branched hyphae are the only parameter that can be helpful for pellet formation, which benefits citric acid enhancement. However, as citric acid concentration increases and fermentative sugar decreases, the physical properties of the fermentation broth change, seriously influencing pellet morphology, which can no longer remain compact and round, causing the rate of citric acid production to become slower.

[Figure 3: see original paper]

While genetic alteration is important for mutant strains to achieve hyper citric acid production, culture conditions also play a significant role. Through genetic alteration, strains may become relaxed in regulation and capable of devoting their metabolic machinery to producing key biosynthetic enzymes, resulting in over-production of metabolites to levels needed for economical industrial use. In the present study, we focused on screening mutant strains and conducting fermentation experiments. Whether key genes have changed between the H4002 strain and the 4# strain will be investigated in future experiments.

Currently, several mutagenesis methods are employed to improve microbial strains, each with distinct advantages. Based on its unique physical and chemical properties, heavy ion mutagenesis has been used to improve the production capacity of microorganisms and has proven to be an efficient method for microbial breeding.

IV. Conclusion

This study demonstrates that 12C6+ beam irradiation can cause significant changes in the fermentation characteristics and pellet morphology of *Aspergillus niger*. These changes lead to increased citric acid production by the H4002 strain, suggesting that mutant H4002 possesses enhanced ability for sugar katabolism and citric acid production. It is confirmed that heavy ion irradiation is an efficient method for breeding hyper citric acid-producing strains.

Acknowledgements

The authors are grateful to all staff at the Heavy Ion Research Facility of Lanzhou (HIRFL) for providing the carbon beams and dosimetry, and to our colleagues in the Radiobiology Department for their invaluable suggestions and cooperation.

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Note: Figure translations are in progress. See original paper for figures.