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A mutation of *Aspergillus niger* for hyper-production of citric acid from corn meal hydrolysate in a bioreactor*

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Abstract: The properties of the screened mutants for hyper-production of citric acid induced by carbon ($^{12}C^{6+}$) ion beams and X-ray irradiation were investigated in our current study. Among these mutants, mutant H4002 screened from $^{12}C^{6+}$ ion irradiation had a higher yield of citric acid production than the parental strain in a 250-ml shaking flash. These expanded submerged experiments in a bioreactor were also carried out for mutant H4002. The results showed that (177.7–196.0) g/L citric acid was accumulated by H4002 through exploiting corn meal hydrolysate (containing initial 200.0–235.7 g/L sugar) with the productivity of (2.96–3.27) g/(L·h). This was especially true when the initial sugar concentration was 210 g/L, and the best economical citric acid production reached (187.5±0.7) g/L with a productivity of 3.13 g/(L·h). It was observed that mutant H4002 can utilize low-cost corn meal as a feedstock to efficiently produce citric acid. These results imply that the H4002 strain has the industrial production potentiality for citric acid and offers strong competition for the citric acid industry.

Key words: Mutation, Citric acid, Corn meal hydrolysate, *Aspergillus niger*
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1 Introduction

Citric acid fermentation is one of the largest biotechnological industries (Ikram-ul *et al*., 2004), and it has been proven that *Aspergillus niger* is the most effective strain for citric acid production by fermentation of carbohydrates (Lesniak *et al*., 2002) through secreting large amounts of enzymes into the environment for degrading carbon sources into small sugar molecules that are then used to serve as nutrients (de Bekker *et al*., 2011). According to various estimates, citric acids produced through fermentation are 1.7×10^6 t/a (Dhillon *et al*., 2011), but the industrial demands for citric acid are still increasing. Presently, China occupies a large market share in the citric acid industry all over the world, and the yield reached 0.94×10^6 t/a (Yang *et al*., 2013), but conventional citric acid fermentation biotechnology in China is not yet economically competitive. For instance, in 2008, the best citric acid production level of Chinese manufacturers was 146.9 g/L and the best productivity was 2.7 g/(L·h) (Gao and Yang, 2010). A patent with citric acid concentration of 173 g/L and productivity of 2.66 g/(L·h) was also reported by Zhang *et al.* (2013). Both are much lower than the advanced levels in the rest of the world (Hu *et al*., 2014).

High yield depends mainly on the strains used and the industrial production of citric acid by fermentation using cheap raw material is helpful to the

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economic development of a country (Majumder *et al*., 2009). Aiming to obtain high yields at a production scale level, a possible strategy is to improve the properties of the microbial production strain (Hu *et al*., 2014). Presently, as a novel mutagenesis technique, heavy ion irradiation has been referred to as a means of breeding new mutants of different microorganism, such as $^{12}C^{6+}$ ion irradiation (Wang *et al*., 2009; Zhou *et al.*, 2013). When compared with traditional physical or chemical mutagenic agents, such as X-rays or γ-rays, heavy ion beams induce a higher mutation rate and create a wide range of mutation types due to their higher linear energy transfer (LET) and greater relative biological effect (RBE) (Du *et al*., 2014). Progress has also been made in heavy ion mutation breeding of *Clostridium tyrobutyricum*, microalga *Desmodesmus* sp., etc. (Hu *et al*., 2013; Zhou *et al*., 2014). However, few reports concerning breeding of *A. niger* by heavy ion beams uniting corn meal hydrolysate as the feedstock in a bioreactor for hyper citric acid have been published (Hu *et al*., 2014).

In this study, we successfully screened a high yield strain of *A. niger* for citric acid by $^{12}C^{6+}$ ion irradiation. In addition, the kinetics of citric acid production and sugar consumption in a bioreactor by this strain under different sugar concentrations was also studied.

2 Materials and methods

2.1 Organism and culture maintenance

A parental strain, preserved at the biophysics lab in the Institute of Modern Physics, Chinese Academy of Sciences, was maintained on potato-dextrose agar slants and stored at 4 °C in a refrigerator.

2.2 Isolation of mutants

H4002, H1, H3, H4, and H5 were obtained after carbon ion irradiation. XHW2 and XHW3 were obtained after X-ray irradiation. The large diameter of transparent halos and morphology by a single colony on agar plates were employed for preliminary screening mutants. The agar plate contained 200 g/L potato juice, 20 g/L sucrose, 20 g/L agar, and 0.2 g/L bromocresol green. The fermentation medium was prepared with 0.25 g/ml corn meals, which were hydrolyzed by α-amylase at 95–98 °C for 30 min in a 10-L bioreactor. After being filtered, the supernatant hydrolysate was obtained with an initial pH 5.15. Corn meals were obtained from smashing corn grains. The medium used in all the studies of citric acid fermentation in the shake flash (250 ml) contained corn meal hydrolysate with sugar concentration 166.2 g/L and 5 g/L soybean cake. The medium was then autoclaved at 115 °C for 30 min.

2.3 Conversion of corn meal hydrolysate into citric acid by mutant H4002

Fermentation was carried out in a 50-L stirred tank reactor. The fermentation medium was prepared with the following source of carbohydrates. Corn meals (0.25 g/ml) were hydrolyzed by α-amylase at 60–72 °C for 30 min and 95–98 °C for about 10 min in the 50-L bioreactor. After centrifugal filtration, liquefying supernatant was obtained and diluted with tap water to the desired sugar concentration. The medium was then autoclaved at 118 °C for 30 min.

2.4 Analytical methods

The concentration of citric acid in culture filtrate was measured by titration with 0.1429 mol/L sodium hydroxide using 0.5% phenolphthalein as an indicator. Total sugar was hydrolyzed by 6 mol/L vitriol at boiling temperature for 10 min into glucose and fructose, and then analyzed by a Fehling reagent (Jin and Cen, 2004). For the calculation of dry cell mass, the mycelium was thoroughly washed with tap water and dried to a constant weight after centrifugation of 5 ml fermentation broth at 4000 r/min for 5 min. All the experiments were repeated two or three times.

3 Results and discussion

3.1 Isolation of mutants for citric acid production induced by $^{12}C^{6+}$ ion and X-ray irradiations

In the present study, numerous mutants induced by $^{12}C^{6+}$ ion and X-ray irradiations were primarily screened on the selective plate medium. Compared with the parental strain, the citric acid production by the screened strains H4002 and XHW3 was greatly improved in terms of the final citric acid concentration and productivity (Fig. 1). The parental strain and both mutants H4002 and XHW3 could synthesize (70.25±5.30), (76.55±1.48), and (72.80±0.56) g/L

citric acids, respectively, and produced (34.62±0.71), (31.62±0.31), and (32.17±1.49) g/L dry biomass. The increase in citric acid production was substantial for the H4002 and XHW3 strains, especially H4002, which showed the highest capacity in citric acid production with an increase of 8.9%.

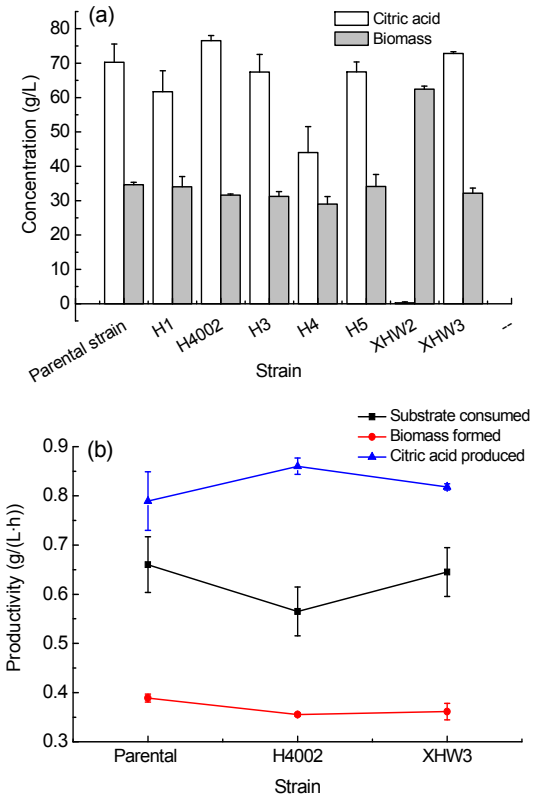

Fig. 1 Citric acid productions of the parental strain and its mutant derivatives induced by $^{12}C^{6+}$ ion and X-ray irradiations when grown in corn meal hydrolysate
(a) Citric acid and biomass concentrations. (b) Kinetic parameters for citric acid production. Culture conditions: temperature (36.8±0.4) °C, initial pH 5.15, fermentation time 89 h. Data are expressed as mean±standard deviation (SD), n=3 or 4

Specific productivities (g/(L·h)), such as citric acid produced, biomass formed, and substrate consumed, are shown in Fig. 1b. Mutant H4002 exhibited the maximum production improvement (increased by 8.97%), low substrate consumption (decreased by 13.9%), and low biomass production (decreased by 8.66%) over the parental strain. In another word, H4002 utilized lower sugar consumption to produce more citric acids, which was helpful for material utilization. Consequently, it was proven that the mutant H4002 showed a high citric acid production and productivity through the shaking flash culture.

3.2 Conversion of corn meal hydrolysate into citric acid by mutant H4002

To produce citric acid on a large scale for industrial applications, a 50-L bioreactor was implemented to produce citric acid by mutant H4002 due to its good physical parameters, such as dissolved oxygen. Meanwhile, the type and concentration of the carbon source are probably the most crucial parameters for successful citric acid production (Karaffa and Kubicek, 2003).

Fig. 2 shows the kinetic parameters for citric acid production at different concentrations of corn meal hydrolysates as a carbon source by mutant H4002 in a 50-L agitator bioreactor. Under the optimized culture condition (data not shown), when the initial sugar concentration increased from (200±0.0) to (235.7±2.4) g/L, the produced citric acid also increased from (177.7±3) to (196±0.0) g/L after 60 h fermentation (Fig. 2b), and the citric acid productivity was 2.96 and 3.27 g/(L·h) (Fig. 2c), the final sugar concentration decreased to (26.7±0.7) and (36.9±0.8) g/L (Fig. 2a), and the conversion rate was 88.85% and 83.1% (Fig. 2c), respectively. Especially, when the initial sugar concentration reached 210 g/L, the mutant H4002 exhibited the best citric acid accumulation of (187.5±0.7) g/L, with a high productivity of 3.13 g/(L·h), a high conversion rate of 89.25%, and a low residual sugar concentration of (26.0±0.4) g/L. Furthermore, during the whole fermentation process, round and compact pellets were being observed all the time, which improved the properties of the process and enhanced the citric acid production (Pera and Callieri, 1997; Ikram-ul *et al*., 2001).

An important issue for citric acid production is whether the process is economical. A low cost of feedstock is a very important factor in establishing a cost-effective technology (Mojovic *et al*., 2006). Recently, in order to increase the efficiency of citric acid production, different mutants and feedstock have been studied, but the productivity achieved was not high enough for the competitive economical environment in an industrial operation. Compared with the best production levels of 146.9 g/L reported by Gao and Yang (2010) and a patent with citric acid concentration of 173 g/L and productivity of 2.66 g/(L·h)

reported by Zhang *et al.* (2013), there are significant increases in both citric acid concentration and productivity by mutant H4002. Consequently, these findings suggest that the mutant H4002 strain possesses enhanced the efficiency for citric acid production from corn meal hydrolysate metabolism.

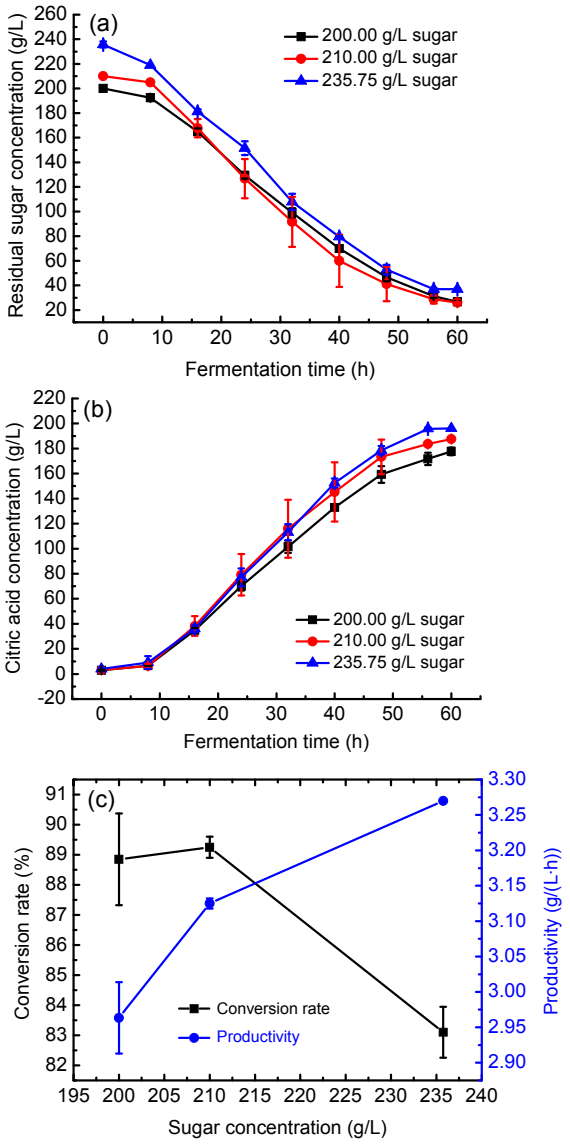

Fig. 2 Kinetics of citric acid production converted from different concentrations of corn meal hydrolysate by mutant H4002 in a 50-L bioreactor

(a) Sugar consumed during fermentation; (b) Citric acid produced during fermentation; (c) Conversion rates and productivity. Data are expressed as mean±standard deviation (SD), n=3

Although the molecular mechanisms of biological action of ion irradiation are not yet understood (Yang *et al.*, 2007), as a novel irradiation technique, it deserves further attention due to its high LET and RBE, which can result in a wider mutation spectrum and higher ratio of mutation. Further work will be carried out to determine any additional benefits from changing the key genes in mutant H4002, compared with the parental strain. The improvement of the substrate consumption rate in the H4002 strain will also be studied.

4 Conclusions

To obtain mutants for hyper citric acid production, we used the accelerated carbon ions and X-rays as a tool to induce mutagenesis of *A. niger*. After the isolation of the irradiated *A. niger*, we successfully obtained a mutant, named H4002, with enhanced citric acid production, which was screened from carbon ion irradiation. Under the optimized culture condition in a bioreactor, H4002 exhibited (187.5±0.7) g/L citric acid accumulation and it implied that the H4002 strain has the industrial production potentiality for citric acid and offers a strong competition for the citric acid industry.

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Compliance with ethics guidelines

Wei HU, Jing LIU, Ji-hong CHEN, Shu-yang WANG, Dong LU, Qing-hua 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.

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中文概要：

本文题目： **高产柠檬酸突变菌株以玉米粉为原料的生物反应器扩大生产研究**

A mutation of *Aspergillus niger* for hyper-production of citric acid from corn meal hydrolysate in a bioreactor

研究目的： 研究高产黑曲霉突变菌株以玉米粉为原料的生物反应器扩大发酵，以期获得适合于工业化生产柠檬酸的发酵工艺。

创新要点： 以玉米粉为原料，系统地研究了筛选得到的高产菌株在 50 L 生物反应器中不同糖浓度发酵生产柠檬酸的特性，最终优化出适合于工业化生产柠檬酸的发酵工艺。

研究方法： （1）利用淀粉酶对粉碎后的玉米进行液化，然后过滤，最终得上清液；（2）以 50 L 生物反应器作为发酵设备，对筛选得到的高产柠檬酸菌株进行扩大培养；（3）通过测定不同培养时期中积累的柠檬酸含量和剩余的残总糖，最终优化出高效率生产柠檬酸的发酵工艺。

重要结论： 以不同糖浓度的液化玉米粉上清液作为碳源，突变菌株 H4002 能积累 177.7~196.0 g/L 的柠檬酸，效率能达到 2.96~3.27 g/(L·h)，尤其当糖浓度为 210 g/L，H4002 菌株表现出最佳的柠檬酸生产水平，如柠檬酸积累 187.5 g/L，生产效率达 3.13 g/(L·h)。上述结果说明了突变菌株 H4002 拥有快速生产柠檬酸的能力。

关键词组： 突变；柠檬酸生产；玉米粉液化液；黑曲霉