Improving Citric acid Production of an Industrial Aspergillus Niger CGMCC 10142: Identification and Overexpression of a High-affinity Glucose Transporter with Different Promoters

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Research

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Abstract

Background: The transporter of glucose plays an important role in the fermentation of citric acid. In this research, a high-affinity glucose transporter (HGT1) was identified and overexpressed in industrial strain *A. niger* CGMCC 10142. The overexpressed-HGT1 strains were obtained to verify the glucose transporter functions with different promoters P*gla*A and P*aox1*.

Result: The sugar contents and citric acid production during fermentation were tracked. As speculated, the HGT1-overexpressed strains showed higher citric acid production and lower residual sugar contents. The best-performing strain on the fermentation is *A. niger* 20-15 transformant of which the total sugar content and residual reducing sugar both were reduced by 31% and 44.9% comparing to *A. niger* CGMCC 10142, and the final citric acid production was correspondingly increased to 174.1 g/L comparing to *A. niger* CGMCC 10142 with 162.3 g/L. Besides HGT1, the transcription levels of related genes as *citrate synthase* and *glucokinase* in transformants were also significantly higher than *A. niger* CGMCC 10142.

Conclusion: The results indicate that HGT1 understandably alleviates the metabolic restriction caused by insufficient sugar transporters and finally improves the utilization of sugar. This study confirms the important role of glucose transporter HGT1 in the citric acid fermentation process of *Aspergillus niger*.

Background

Citric acid (CA), a natural product that is widely used in food, chemistry, medicine is among the most commonly used organic acid in the world [1, 2]. And the CA demand of microbial fermentation is increasing with a rate of 3.7% in recent years. Nowadays, CA is often produced by *Aspergillus niger* (*A. niger*) as microbial cell factory fermentation on the industrial scale. As a saprophytic fungus, *A. niger* could secrete sufficient hydro-enzymes which are beneficial to CA producing [3, 4].

A. niger is a kind of important industry strains with a great amount of value. With the development of sequencing and genomics technology, direct molecular evolution and modification instead of random mutagenesis are selected to increase CA fermentation efficiency on *A. niger* [5]. To date, the principal metabolic engineering areas to the CA industry focus on the improvement to the main flux and respiratory chain related to energy generation in *A. niger* [6–8], such as knockout and overexpression of key genes which involving in EMP, TCA, and electron transport [9–11]. During recent years, researches about energy metabolic and electron transporter were further and further [12, 13]. As the metabolic process of *A. niger* involving complex metabolism and replenishment pathways, overexpression of some crucial genes presents little effect on citric acid fermentation. For example, overexpression of *citrate synthase* could not improve citric acid production [14, 15]. Maybe the gene expression has already reached saturation condition in the fermentation process.

Fungi that for CA fermentation cannot use polysaccharides directly as carbon sources on account of that no polysaccharide can go into the cell. Generally, polysaccharides are firstly hydrolyzed to monosaccharides, then which can be used in the metabolic process [16, 17], and this means CA

production began even before the polysaccharide go into the cell. Therefore, Torres calculated the key points of citric acid production are the intake and phosphorylation of hexose [18]. The CA production peak period of the fermentation process will be advanced, but the final yield will not increase when maintaining high glucose concentration during citric acid fermentation [19]. In general terms, the type of carbon source and its concentration both are the critical roles in citric acid fermentation. Xu et al confirmed that the sugars as maltose, sucrose, mannose and fructose each with 10% w/v concentration led to the highest yields of citric acid while glucose with 7.5% w/v concentration gave the best results. Furthermore, citric acid production was ceased in the media including less than 2.5% sugars [20]. Meanwhile Mischak et al obviously showed that the uptake of glucose was inhibited because of small quantities of extracellular citric acid over 0.5 mM concentration [21]. That is, citric acid production and its rate are strongly related to glucose concentration and its uptake rate. Furthermore, the absorption rate of glucose inhibits a simple linear relationship to the glucose concentration, and glucose as a kind of polar molecules cannot pass through the cell membrane with the phospholipid bilayer structure in a freely diffused form. So facilitated diffusion and active transporter becomes the main way for cells to absorb glucose [22–23].

There are two kinds of glucose transporters in *A. niger*, they are low-affinity glucose transporter with K_m of 3.67 mmol/L and high-affinity glucose transporter with K_m of 260 μmol/L, respectively. And Torres confirmed that high glucose concentration (> 50 g/L) was a prerequisite for the function of low-affinity glucose transporter which was capable of providing the high flux of glucose required for citrate production [18]. The analysis of transcriptome of *A. niger* H915-1 in fermentation process revealed that a low-affinity glucose transporter was maintained a high level but other 5 high-affinity glucose transporters were not [22, 23]. Based on low-affinity glucose transporter with high K_m and high-affinity glucose transporters with extremely low-expressed levels, so where may affect citrate yields and maintain more residual glucose in the fermentation [24, 25]. Controlling and coordinating these proteins can change the nutrient uptake ability of the strains in a targeted manner, to be more suitable for industrial fermentation. Therefore, glucose transport efficiency of *A. niger* is a key step to speed up the CA production and may be one of the limiting factors for a higher CA yields [24, 25].

In fungi, many putative sugar transporters have been identified. For instance, several newly discovered xylose transporters in *A. nidians*, *A. niger*, and *T. reesei* can transport glucose [26, 27]. Besides, a low affinity glucose transporter HxtB involving in glucose signaling were characterized [28]. So far, there are a few reports on the *A. niger* specific glucose transporters. Based on a conserved protein domain search, 86 putative sugar transporter genes in *A. niger* were identified and annotated [29]. Two new putative high-affinity glucose transporters, denoted MstG and MstH, were identified and biochemically characterized in *A. niger* by membrane-associated proteome analysis [30]. It has been determined that MstA is a high-affinity glucose transporter in *A. niger*, and its disruption resulted in a 2- to 5-fold reduction of cellular glucose affinity [31]. This protein exists in the enzyme-producing strain *A. niger* CBS 513.88 and the original acid-producing strain *A. niger* ATCC 1015 [32], but it does not exist on the *A. niger* CGMCC 10142. Additionally, two glucose transporters were identified in *A. niger* H915-1, they are a low-affinity glucose

transporter with high expression at high glucose concentration and a high-affinity glucose transporter with extremely low-expression level, respectively [22, 23] L Liu accelerated the glucose transport rate and shorten the fermentation cycle by overexpression of a low-affinity glucose transporter [33]. Meanwhile, a glucose transporter HGT1 (high-affinity glucose transporter 1) is reported in *Kluyveromyces lactis*, which also is identified in the *A. niger* CBS 513.88 and *A. niger* CGMCC 10142 [34].

The primary nutrition of the *A. niger* CA industry are liquefied corn medium with a high amount of total sugar including glucose, disaccharide, and polysaccharide. *A. niger* could release plenty of glucoamylases that can hydrolyze polysaccharide into easily intaked glucose. Thus, the main sugar at the beginning of the fermentation is glucose, and a more adequate supply of carbon sources can greatly increase the CA production. Beyond all doubts, the transport rate of glucose understandably becomes a limiting factor for the increase in CA yields. Therefore, it is considered to accelerate the transport efficiency of glucose during the metabolism of *A. niger* for improving the citric acid production performance at the same time.

In this work, the CA industrial production strain *A. niger* CGMCC 10142 was used as a parent strain on genetic modifications. A high-affinity glucose transporter (HGT1) coding gene was identified and amplified from the parent strain. After sequence alignments, promoter P*aox1* and P*gla*A were chosen to overexpress HGT1. Subsequently, overexpressed-HGT1 strains were successfully constructed and characterized. The transcription levels of key enzyme genes involved in CA production was revealed during fermentation by real-time qPCR. Furthermore, the pellet and sugar were tracked and analyzed between transformants and the parent strain.

Results And Discussion

Sequence analysis of the native HGT1 from parent strain

There are two kinds of glucose transporters in *A. niger*, one is high-affinity and the other is low-affinity. *HGT1* gene, as a high-affinity glucose transporter, was amplified from *A. niger* CGMCC 10142 genome. Then *HGT1* sequence was compared with *A. niger* CBS 513.88 *HGT1* sequences (GenBank: AM269996.1). The coding region totals 1644 bp and the coherence is 100%. The similarity of the amino acid sequences between *A. niger* HGT1 and MstA, LGT1, and *A. nidulans* MstE were 27.83%, 25.71%, 25.91%, respectively (shown in Figure 2B). Between HGT1 from *A. niger* and its homolog from *Kluyveromyces lactis*, the identity of amino acid sequence reached 40.87%, which was higher than with other glucose transporters. In this study, the structure of HGT1 from *A. niger* was predicted through homology modeling using Swiss-Prot (www.swissmodel.expasy.org/). The glucose transporter and D-xylose transporter were among the most similar related proteins (data not shown).

The phylogenetic tree of sugar (glucose) transporters from different fungi is shown in Figure 2A. At least several glucose transporters have been identified for both *A. niger* and *A. nidulans*. The HGT1 of *A. niger* CGMCC 10142 can be identified as the *A. niger* CBS 513.88 HGT1 (XP_001399197.1), which is located at

the lower side of the phylogenetic tree. Several other glucose transporters that are close to HGT1 were XP_001394117.2 and XP_001390064.1. What's more, The MstA in *A. niger* and most of the sugar transports identified in *A. nidulans* were located on the upper side of the phylogenetic tree.

It was found that in fermentations at relative lower glucose concentration (<50 g/L), the low-affinity glucose transporter is the limiting factor for glucose import into the cell [23]. The sugar concentration of the liquefied corn medium for CA fermentation is quite high at about 180 g/L and low-affinity glucose transporters can transport sugar fast at first. After 32 h fermentation, when the sugar concentration is declined quickly to less than 50 g/L, the low-affinity glucose transporters could not function, while the high-affinity glucose transporters could function even at the end of fermentation when the sugar concentration is extremely low. It's weird that the high-affinity glucose transporters show extremely low-transcription level even no transcription. To improve the glucose utilization rate, the high-affinity glucose transporter HGT1 in *A. niger* CMCC 10142 was chosen to overexpress.

Construction of *HGT1* overexpression cassettes and Screening of high CA production strains

The P*gla*A, P*aox1*, and *HGT1* segments were obtained from the parent strain genome. Then the P*gla*A, *HGT1*, and P*aox1*, *HGT1* was recombined into p80-HSVtk linear plasmid by MultiS kit (Vazyme Biotech Co., Ltd) to construct the new overexpression plasmids p20 and p21, respectively (shown in Figure 1B and Figure 1C). Both the plasmid p20 and p21 was successfully constructed by PCR and sequencing as showed in Figures S1a and S1b.

The ratios of the transparent halo zones due to acid production and colony diameter were calculated to select transformants with high CA production (Table S1). Then, genomic DNA was extracted from the transformants that showed a high CA production rate to confirm the correct insertion of the overexpression cassettes (promoter to *HGT1*) as shown in Figure S1c and S1d. Finally, eight transformants (*A. niger* 20-15, 20-16, 20-25, 20-27, 20-29, *A. niger* 21-8, 21-28 and 21-32) were selected for the ratios of the transparent halo zones (Figure 3B) and shake flask fermentation to screen higher transformants (Figure 3A).

Verification of genetic stability of the transformants by inserted expression cassettes on PCR

The inserted expression cassettes in the positive transformants were further verified. Firstly, The *HGT1-hyg* cassette of the transformants was verified as shown in Figure S1e. Then. the integration location was tested using primers of the *ku70* total length. As shown in Figure S1f, except for p20-16 in the 8th lane, the *ku70* original length (1800 bp) was amplified from all genomic DNA samples, and not the target fragment (5710 bp). These results indicated that the *ku70* gene was partly destroyed in the strains 20-16.

However, other transformants retained the intact *ku70* gene, and the whole P*gla*A-*HGT1-hyg* segment was inserted. The overexpression frame from the upstream to the downstream homologous arm of *ku70* was very long, totaling nearly 6000 bp. What's more, it is common for filamentous fungi to lose heterologous genes, especially during long cultivation and passage. Therefore, the 8 strains were passaged for 15 generations to verify the transformants' genetic stability. Then, conidia were prepared from the 8 identified stable transformants to verify their CA production ability in shake-flask fermentations.

Screening of higher CA production strains in shake-flask fermentations

The transformants (*A. niger* 20-15, 20-16, 20-25, 20-27, 20-29, *A. niger* 21-8, 21-28 and 21-32) were selected for the ratios of the transparent halo zones (Figure 3B) and shake flask fermentation to screen higher transformants (Figure 3A). As shown in Figure 3A and 3B, the CA production and the ratios of the transparent halo zones of all the eight transformants were higher than that of the *A. niger* CGMCC 10142. Two strains with the higher improvement of CA production were *A. niger* 20-15 and *A. niger* 21-8, with 15.8% and 12.8% comparing to the strain 10142. And the ratios of the transparent halo zones of *A. niger* 20-15 and *A. niger* 21-8 were 3.73 and 3.55 higher than strain 10142 with 2.85. The results indicated that the CA production ability of p20 transformants was higher than that of p21 transformants in shake flask fermentation. The higher production indicated that the P*gla*A promoter presents a higher transcription than the P*aox1* promoter, leading to higher-level of HGT1 protein synthesis. Therefore, the two transformants *A. niger* 20-15 and 21-8 were chosen for further analysis by 30 L bioreactor-scale fermentation and real-time quantitative PCR.

CA fermentation and statistical analysis

As shown in Figure 4A, the morphology of the parental *A. niger* CGMCC 10142 strain as well as the transformants *A. niger* 20-15 and 21-8 for each sampling time are normal. The diameter of the mycelial pellets increased quickly from 8 h to 24 h, showing that the strains had entered the rapid growth phase. After 24 h the pellets of fungi showed a little increase and the colony began to sprout small mycelium, indicating that has begun to enter the period of CA production. Papagianni et al found reduction of mycelial clumps for the first 48 h of fermentation [35]. But in our research, the colony morphology didn't change significantly from the period of CA production to the end of fermentation. Yet the mycelial pellets of *A. niger* 21-8 still showed some growth from 24 h to 32 h and maintained morphological stability to the end, too.

The CA production in the bioreactor-scale fermentations was also measured every 8 hours, as shown in Figure 4B. The CA production increased rapidly from 8 h, and the transformants showed a gradually improved CA fermentation ability. The CA production of *A. niger* 20-15 and 21-18 increased to 174.1 g/L and 169.4 g/L comparing to 10142 strain with 162.3 g/L which each improved by 7.3% and 4.4%,

respectively, at the end of the fermentation. The productivity rates of each time stage of the fermentation process were calculated, as shown in Figure 4C. The highest productivity rates of *A. niger* 20-15 was reaching to 4.4 g/L/h which was higher than *A. niger* 21-8 and 10142 both with 4.1 g/L/h at 24 h of fermentation. The highest productivity rates of *A. niger* 21-8 was reaching to 4.2 g/L/h which was approximately equal to *A. niger* 20-15 but higher than *A. niger* CGMCC 10142 with 4.0 g/L/h at 32 h, and maintained higher level at 40 h. This indicated that the 24-h CA metabolism level of *A. niger* 20-15 was higher than that of *A. niger* 21-8 and *A. niger* CGMCC 10142, probably due to the fact that 21-8 did not have such a strong HGT1 overexpression burden. The biggest differences in productivity rate with the starting strains were among 56 h-64 h at the end of fermentation. Among 56 h-64 h, the CA productivity rates of *A. niger* 20-15 and *A. niger* 21-8 were 1.6-0.6 g/L/h and 1.0-0.7 g/L/h, respectively, which were 2.1-1.5 and 1.3-1.8 times higher than the *A. niger* CGMCC 10142 with 0.7-0.4 g/L/h. The above results further confirmed that the overexpression of HGT1 promoted CA production at the later stage of fermentation when the nutrition were running out.

During the fermentation process, the total sugar in the fermentation broth was measured every 8 hours. The results of residual total sugar at 48 h, 56 h and 64 h were 36.6 g/L, 21.7 g/L, 10.6 g/L of *A. niger* 20-15 which were higher than *A. niger* 21-8 with 33.8 g/L, 18.9 g/L and 8.8 g/L but lower than *A. niger* CGMCC 10142 with 37.4 g/L, 22.9 g/L and 12.7 g/L, respectively (shown in Figure 5A). This indicated that the overexpression of HGT1 had a positive influence in the transformant strains at 48 h, 56 h and 64 h of the fermentation. This result was consistent with an earlier study [36]. What's more, HGT1 did not function effectively because of the high sugar concentration (data not shown). Earlier studies found that low-affinity glucose transporter is formed when the glucose concentration is 15% and is highly expressed when it drops to more than 8% [23, 24]. Our results also showed that the most significant changes were observed when the fermentation 32 h sugar concentration dropped below 8% HGT1 began to play a more important role. At the end of fermentation (64 h), the total residual sugar of *A. niger* 20-15 and *A. niger* 21-8 decreased 16.5% and 30.7% compared with *A. niger* CGMCC 10142. This result indicated that the overexpression of HGT1 significantly improved the sugar consumption rate.

A proportion of the initial sugar is lumping which was unutilized by microorganisms during CA fermentation leading to waste of raw materials. Therefore, the total reducing sugar concentrations in the fermentation broth were measured at 48 h, 56 h, and 64 h as shown in Figure 5B. The total reducing sugar concentrations at 48 h, 56 h, and 64 h of *A. niger* 20-15 were 16.0 g/L, 5.9 g/L, and 2.1 g/L which were less than *A. niger* 21-8 with 18.1 g/L, 7.4 g/L, and 2.7 g/L. Both of them were less than *A. niger* CGMCC 10142 with 19.1 g/L, 8.3 g/L and 3.8 g/L, and the utilization rates of the total reducing sugar of *A. niger* 20-15 and 21-8 were improved 44.7% and 26.3% comparing to *A. niger* CGMCC 10142 at the end of fermentation. What's more, we found that the reducing sugar concentrations were increased rapidly during the first 16 h, and especially during the first 8 h. It then decreased in the subsequent fermentation period with increasing CA production, during which a large amount of glucose was used to synthesize CA. Consequently, the reducing sugar concentration decreased rapidly after 24 h (data not shown). Therefore, the overexpressed-HGT1 transformants showed the higher glucose utilization ability and also improved CA production.

Meanwhile, we calculated the glucose-CA conversation rate based on the measured values of total sugar consumption and CA production, as shown in Figure 5C. As expected, the overexpressed-HGT1 strains showed higher conversation rates than the original strain. The final conversation rates of *A. niger* 20-15 and 21-8 were 102.4% and 100.4%, respectively. The CA fermentation performance was in the order *A. niger* 20-15>*A. niger* 21-8>*A. niger* CGMCC 10142. It is worth noting that the overexpression strain with the P*glaA* promoter showed the best performance.

Real-time quantitative PCR

The relative transcription levels of the *HGT1* gene (XM_001399160.2), *citrate synthase* (CS) gene (XM_001393946), and *glucokinase* gene (XM_001395875.2) during the fermentation process were measured to explore whether the overexpression of HGT1 influences the metabolism and accumulation of CA in *A. niger*. Mycelial pellet simples of *A. niger* 20-15 and 21-8 were taken at 12 h and 48 h, separated from the culture broth through filtration, and the total RNA was extracted for real-time quantitative PCR.

The relative expression of *HGT1* is shown in Figure 6A. The expression level of *HGT1* in the overexpression strains was much higher than in the control at 12 h and 48 h. The overexpression of *HGT1* in *A. niger* 20-15 was about 219 times and 208 times higher than the original strain at 12 h and 48 h, respectively, which indicated that the glucose utilization efficiency of the HGT1 overexpression strain was also higher than that of the *A. niger* CGMCC 10142. What' s more, the HGT1 overexpression strain with the P*gla*A promoter showed 2.5 times higher expression than the strain with P*aox1* at 12 h, as well as 5 times higher at 48 h. P*gla*A is a widely used strong fungal promoter that can be induced by starch or dextrose [37]. P*aox1* is the promoter of alternative oxidase in *A. niger* [8]. The overexpression of the low-affinity glucose transporter increased the substrate uptake at high glucose concentrations, partly releasing the limit of glucose consumption in the strains. The HGT1 transporter accelerated sugar transport at the end of CA fermentation when the remaining glucose concentration was low. This could reduce the final total residual sugar and improve the economic value of the fermentation.

Notably, the relative expression levels of glucokinase in the HGT1 overexpression strains were higher than in the parent strain, especially at 48 h (Figure 6B). The glucokinase expression of *A. niger* 20-15 was 8.4 times higher than in the parent strain and that of *A. niger* 20-8 was 1.4 times higher at 48 h, which indicated that the overexpression strains had a higher glucose utilization rate and improved CA producing ability. The results indicated that the overexpression of HGT1 increased the glucose uptake efficiency, which provided adequate carbon flux for increased CA production in the transformants. Glucokinase plays a key role in the metabolic activation of glucose to 6-p-glucose, and its activity is controlled via feedback inhibition by 6-p-glucose and ADP. The increased glucokinase activity could therefore directly improve the CA production at the end of fermentation process.

As shown in Figure 6C, the expression of *citrate synthetase* (*CS*) presented a similar trend to glucokinase. The CS expression of the engineered *A. niger* 20-15 was 6.4 times higher than that of the parent strain at

48 h. CS is necessary for the synthesis of CA as indicated by its name, and therefore plays a key role in CA metabolism. When the CS gene in *A. niger* is deleted, CA production falls to almost zero. High expression of HGT1 therefore increased the expression of *CS* and contributed to abundant CA accumulation. The changes in the relative expression of *CS* indicated that high carbon flux increased the CA yields of the transformants. It is therefore possible that substrate limitation is one of the reasons for low enzyme activity in the parent strain.

In this study, we obtained some strains which overexpress the glucose transporter HGT1 using two promoters P*gla*A and P*aox1*. The results of real-time quantitative PCR indicated that P*gla*A is a stronger promoter than P*aox1*. P*gla*A is the native promoter of glucoamylase in *A. niger*, and it is widely used as a strong promoter. By contrast P*aox1* is the native promoter of alternative oxidase in the alternative respiratory chain. Our past research found that the overexpression of *aox1* also could effectively increase CA production [8]. Therefore, these two promoters were used to induce HGT1 overexpression in the present study. It is reasonable that the P*gla*A-induced strain *A. niger* 20-15 had higher HGT1 transciption level than the strain using P*aox1* as shown in Figure 6A.

Notably, although P*gla*A is a stronger promoter than P*aox*1, there were 1.5 times higher transcription levels of *glucokinase* and *CS* induced by P*aox*1 than P*gla*A at 12 h where the strains were still in the active growth phase (shown in Figure 6B and 6C). When mycelium grows vigorously at 12 hours, dissolved oxygen and nutrition may become limiting factors. In this case, P*aox1* activates the expression of the key enzyme Aox1 in the secondary respiratory chain that does not require high amounts of oxygen, which may increase the metabolic flow, thereby increasing the expression of *glucokinase* and *citrate synthase* [6]. Interestingly, at 48 h of the fermentation time in which the carbon source becomes limited, P*aox1* cannot influence the secondary respiratory chain. Therefore, the P*gla*A-containing transformant *A. niger* 20-15 showed higher transcription levels of *glucokinase* and *CS* as shown in Figure 6B and 6C.

L. Wang et al. found that when a native promoter of the original strain is used in an engineered strain, could not only drive the expression of the target genes, but it may also increase the expression of the original gene of the promoter itself [38]. So we speculated that P*aox1* may not only drive the expression of the HGT1 gene but also activate its own *aox1* gene to some extent and thereby indirectly increase the fermentation performance observed in *A. niger* 21-8. In view of this, it may be useful to verify the relative expression of *aox1* and related enzymes in further studies. What's more, insertion locus or copy numbers of the overexpression cassettes may also influence the expression levels. The genes that are important for CA synthesis, such as *CS*, and *glucokinase* were expressed at higher levels in the HGT1 overexpression strains than in the *A. niger* CGMCC 10142. It is possible that sufficient glucose increased the expression of these genes and thereby improved the CA production performance of *A. niger* [23]. Besides, the strain used in this study is already an industrial CA production strain, and its metabolite levels are higher than in undomesticated wild-type strains.

Glucose transport is the first step from sugar to CA, but it does not influence the CA fermentation directly. In this study, we found that the overexpression of *HGT1* improved the CA fermentation performance in

terms of reducing sugar consumption and total residual sugar in the fermentation process. Furthermore, the final glucose-CA conversation rates of *A. niger* 20-15 and 21-8 both increased to 102.4% and 100.4%, which nearly to theoretical glucose-CA conversion rate of just 106.7% when all the resources are used to produce CA and none for growth [25]. Finally, the CA production of the engineered strains also increased by 7.3% for *A. niger* 20-15 and 4.4% for *A. niger* 21-8. Therefore, sugar intake may still be a bottleneck in the fermentation process.

Conclusions

In this study, we investigated the effects of overexpressing the glucose transporter *HGT1* gene on industrial CA fermentation and found that it is beneficial for CA production in both shake flasks and a 30 L laboratory-scale bioreactor. The increase of CA production can be explained by an increased sugar utilization rate and indirect positive effects on the expression of key metabolic genes. Overexpression of glucose transporter genes can be used to reduce the cost of industrial-scale production and increase the yield of citric acid.

Material And Methods

Strain, reagent and culture condition

All the strains and plasmids used in this work were obtained from the Tianjin Key Laboratory of Industrial Fermentation Microbiology, Tianjin, China, or constructed in this work, as shown in Table 1. The parent strain *A. niger* CGMCC 10142 was cultivated on potato dextrose agar (PDA) at 35 ℃. The *E. coli* DH5α was cultured on LB medium at 37℃ for enriching plasmid. All the regents were analytically pure. The *Hygromycin* was purchased from solarbio. Co., Ltd. The reagents about plasmid construction and real-time qPCR were bought from Takara Co., Ltd.

The spores were obtained from cultures on PDA plates which were grown at 35 °C for 4 days, and cultured in liquefied corn medium (RZBC Co., Ltd., Rizhao, Shandong, China) with spore concentration up to 1×10^5 spores/mL. The organism was cultured at 35℃ for 72 h under constant shaking at 330 rpm in 50/500 mL shake flasks. The CA fermentation in 30 L fermenters was the same as described previously [38].

Table 1
Strains and plasmids used in this work

Name	Genetic characteristics	From
Aspergillus niger CGMCC 10142	CA producer, parent strain	The laboratory
Aspergillus niger 20-15	P*gla*A induced *HGT1* overexpression strain, *hyg*^{r}	This work
Aspergillus niger 21-8	P*aox1* induced *HGT1* overexpression strain, *hyg*^{r}	This work
E. coli DH5α		The laboratory
pGM-*HGT1*	T-vector with *A. niger HGT1* DNA, *Amp*^{r}	This work
p80-HSVtk	*ku70* gene knockout plasmid, *hyg*^{r} *Kan*^{r}	The laboratory
p20	*HGT1* overexpression plasmid with promoter P*gla*A, *hyg*^{r} *Kan*^{r}	This work
p21	HGT1 overexpression plasmid with promoter P*aox1*, *hyg*^{r} *Kan*^{r}	This work

Construction of *HGT1* gene overexpression plasmids and strains

All the primers about plasmid construction were listed in Table 2. the *HGT1* gene, promoter *gla*A (P*gla*A), and promoter *aox1* (P*aox1*) was cloned from *A. niger* CGMCC 10142 genome. The genome was extracted from young hyphae growth on PDA medium. Using *A. niger* CBS 513.88 genome date as reference to design primers. After sequencing at BGI Genomics Co., Ltd, we conducted sequence alignment based on NCBI to see the coherence about the HGT1. The plasmid p80-HSVtk that contains HSVtk, *ku70* upstream sequence, HYG resistant marker, and *ku70* downstream sequence was used as the parent plasmid. The *ku70* deleting displays improved efficiency of transformation and the HSVtk inserting show increased rate of homologous recombination [39]. The p80-HSVtk was restricted by *Kpn* I and then promoter and *HGT1* gene segments were ligated by Multis (Vazyme Biotech Co., Ltd) to form the plasmid p20 or p21 (Figure 1B, 1C). The *HGT1* gene was promoted by P*gla*A in the p20, and the *HGT1* gene was promoted by P*aox1* in the p21.

Plasmids p20 and p21 were used individually to transform *A. niger* CGMCC 10142 by *Agrobacterium*-mediated transformation for efficient DNA transformation according to the method reported before [40, 41]. The cells were cultured on complete medium (CM) with 200 μg/mL *hygromycin* to screen the correct transformants and the CM medium shown in Michielse's report [42].

Table 2
The primers used in this work

Name	Primer sequence (5′→3′)	Amplification product	Length (bp)
Hyg-F	GTCGACGTTAACTGATATTG	*hygromycin* resistance gene (*hyg*	1389
Hyg-R	TTTGCCCTCGGACGAGTGCT		
Ku70-L-F	GGGGTACCGAGCTCGAGGCCAAACAGGCAG	*ku70*	1800
Ku70-R-R	CCCAAGCTT TCTAGATAACTGTACATCGCCT		
P*gla*A-F	CGATAGATCTGGATCCCTGCTCTCTCTCTGCTCT	*gla*A promoter	1493
P*gla*A-R	CAACAACATGTGAGGAGGTGAACGAA		
HGT1-F	CACCTCCTCACATGTTGTTGATTGGCAACAT	*HGT1*	1819
HGT1-R	CGACTCTAGAGGATCCTTATGCTGTGGCCTCCTGGG		
P*aox1*-F	CGATAGATCTGGATCCGACACCGAGCACATGA	*aox1* promoter	1404
P*aox1*-R	GTTGCCAATCAACATCGGGTATAGAACCACAG		
GKqrt-F	CCGCAATGAGAAGAATGG	qPCR for glucokinase	
GKqrt-R	CATCGGGAATGTTGAAGC		
CSqrt-F	ACGGCAAGACCAAGAACC	qPCR for citrate synthase	
CSqrt-R	CACGGGAAACACCGAAGA		
HGT1qrt-F	CGGTATGCTCGTTGTTGG	qPCR for HGT1	
HGT1qrt-R	GGCGAAAGTTCACTGATGTA		
18Sqrt-F	TCGCTACTACCGATTGAA	qPCR for 18S rRNA	
18Sqrt-R	CACCTACGGAAACCTTGT		
The *EcoR* I, *Kpn* I, *Pst* I and *Hind* III sites are underlined			

Analytical methods for CA and strain generation stable

The fresh hyphae of transformants that on the CM + *Hygromycin* medium were first inoculated on CM + 0.5% $CaCO_3$ plates at 35℃ for 72 h to check the CA yields of strains via comparing the ratios of transparent halo zones to the colony diameters. The larger acid cycle means the larger production acid potential. Then the genome types of transformants that showed large transparent halo zones were checked at first. After verifying the insert segments, the spore suspension of the correct transformants were prepared for the shake flask fermentation.

Selected transformants were subcultured on CM medium for 15 generations, and then the genome was extracted to verify their genetic stability. What's more, CA production in shake flask fermentation were tested again.

Determination of sugar and citric acid yields in the 30 L fermenter

The pellets were checked in a microscope every 8 hours in the 30 L fermentation process. The initial total sugar of the fermentation medium was 16.95%. Total sugar and reducing sugar and CA concentration were tracked every 8 hours too. The contents of residual total sugar and residual reducing sugar were determined by the DNS method [43]. Citric acid was measured according to the manufacturer's instructions (Test Kit Cat. No:10139076035, Roche).

Real-time quantitative PCR

To further investigate how the the glucose transporter affects the metabolism and accumulation of CA in *A. niger*, real-time quantitative PCR was conducted during the fermentation. The relative expression of *HGT1* and the genes encoding key rate-limiting enzymes related to the citric acid cycle, citrate synthase and glucokinase, were measured. The primers used for the qPCR were listed in Table 2. The pellet of 12 h and 48 h in CA fermentation were filtered to extract total RNA. Then, reverse transcription was conducted.

The relative gene transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the values were normalized to the 18S rRNA (CGMCC 10142). A standard curve using a dilution series of the cloned amplicon was used to calculate the gene-specific real-time qPCR efficiency. The correlation coefficient (R^2) of the slope of the standard curve was used to calculate each gene's PCR amplification efficiency as described before [8].

Statistical analysis

All the results were based on three independent experiments, and samples with $P<0.05$ were considered significant.

Abbreviations

HGT1: high-affinity glucose transporter 1;

CS: citrate synthase;

CA: citrate acid.

Declarations

Conflicts of interest

The authors declare no conflicts of interest.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The authors declare that all relevant data supporting the findings of this study are available within the article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FB and XX performed the experiments and draft the manuscript, DW conceived the study and designed the experiments, BL and JL and do the fermentation experiments and draft the figures. JZ and QG embellished the manuscript. All authors revised and approved the final manuscript.

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Not applicable.

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Figures

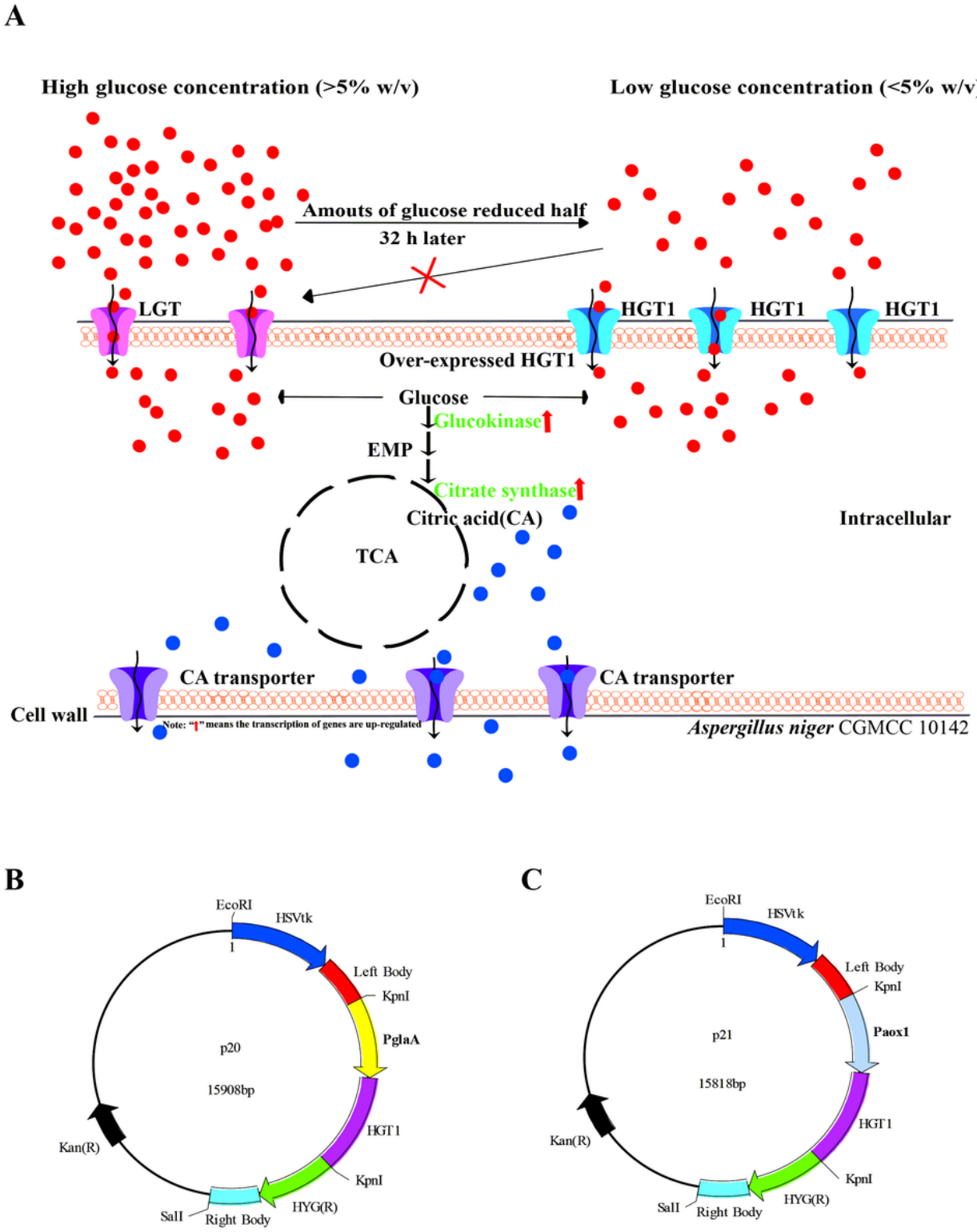
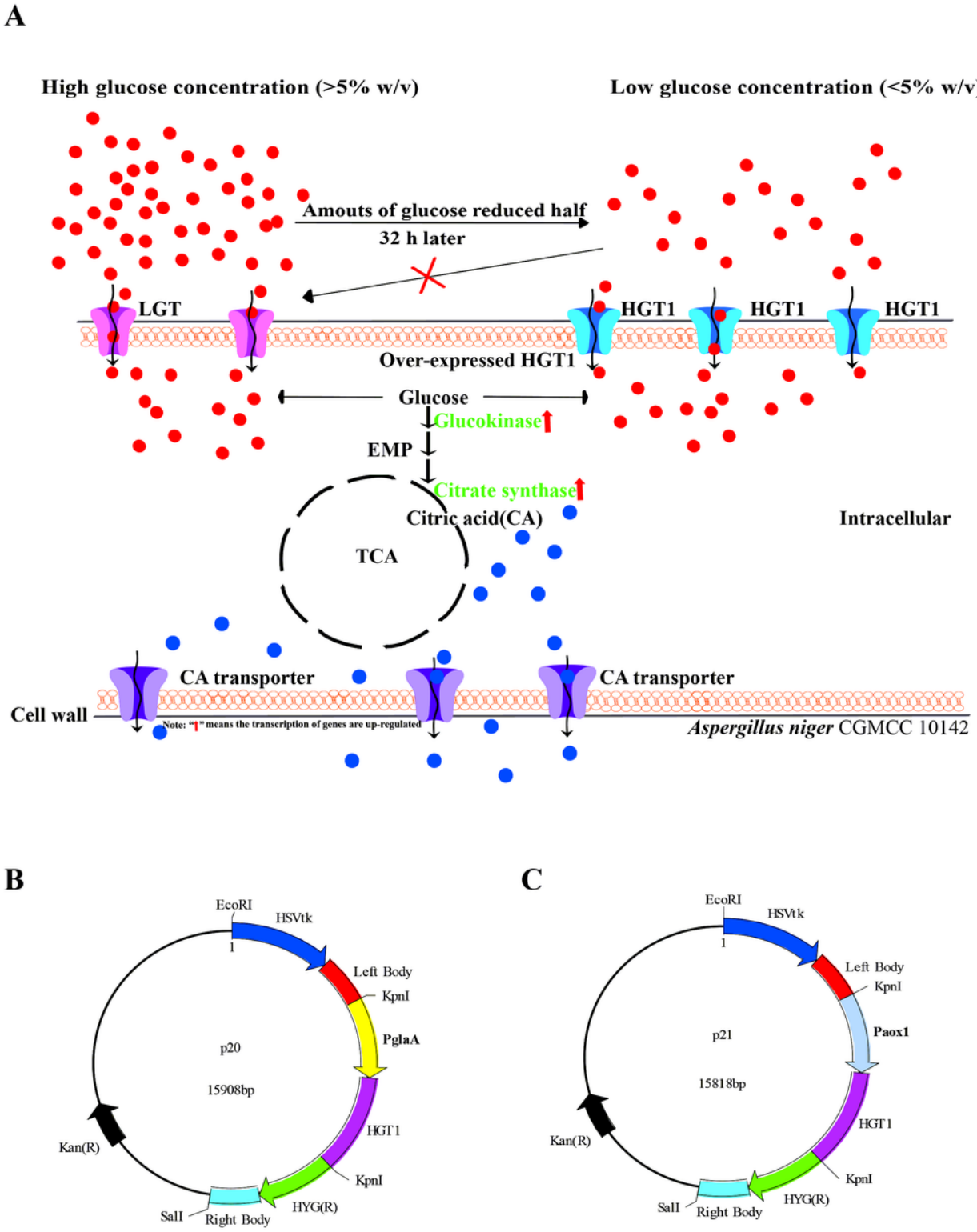


Figure 1

The schemes of the passway on glucose-citric acid conversion and recombinant plasmids. (A) the passway on glucose-citric acid conversion including that how LGT and HGT1 transport glucose; (B) the recombinant plasmid (p20) of overexpreesion HGT1 transcripted by glaA promoter (PglaA); (C) the recombinant plasmid (p21) of overexpreesion HGT1 transcripted by aox1 promoter (Paox1).



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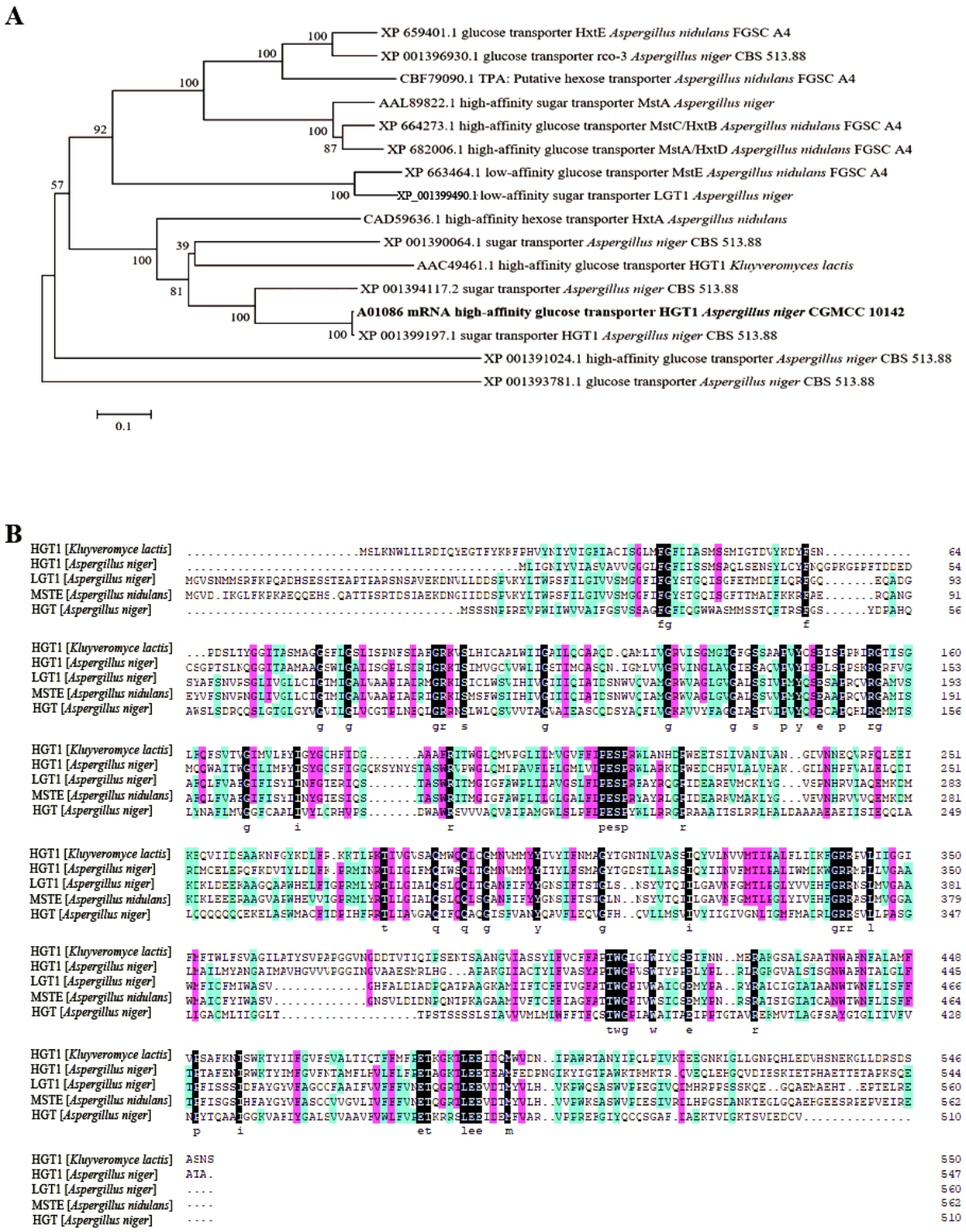

Figure 2

Phylogenetic tree and multiple sequence alignments of HGT1 from Aspergillus niger CGMCC 10142 (A01086) with other homologs. (A) Phylogenetic tree of HGT1 and other homologs from Aspergillus nidulans FGSC A4 (XP 659401.1), Aspergillus niger CBS 513.88 (XP 001396930.1), Aspergillus nidulans FGSC A4 (CBF79090.1), Aspergillus nidulans FGSC A4 (CBF79090.1), Aspergillus niger (AAL89822.1), Aspergillus nidulans FGSC A4 (XP 664273.1), Aspergillus nidulans FGSC A4 (XP 682006.1), Aspergillus

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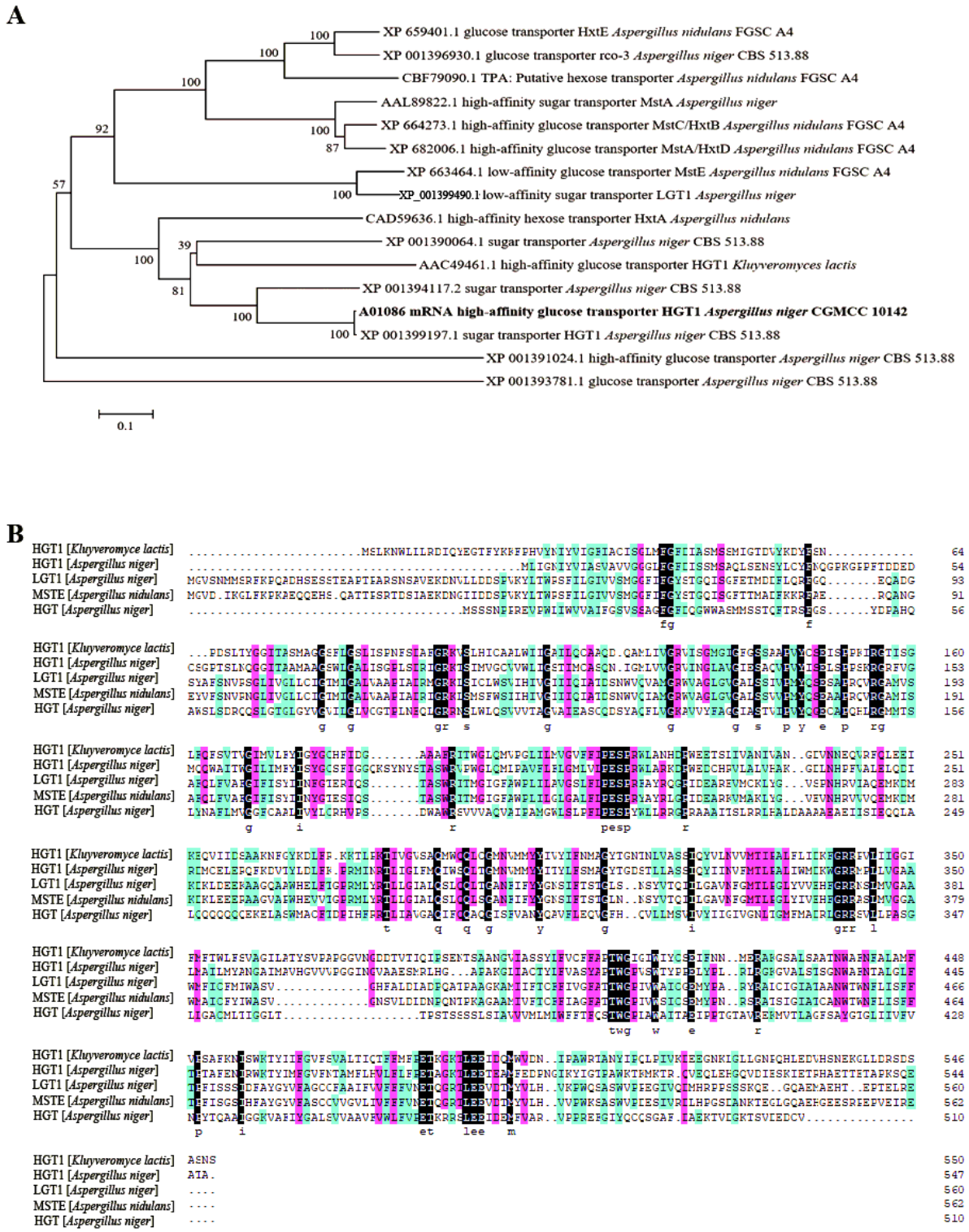

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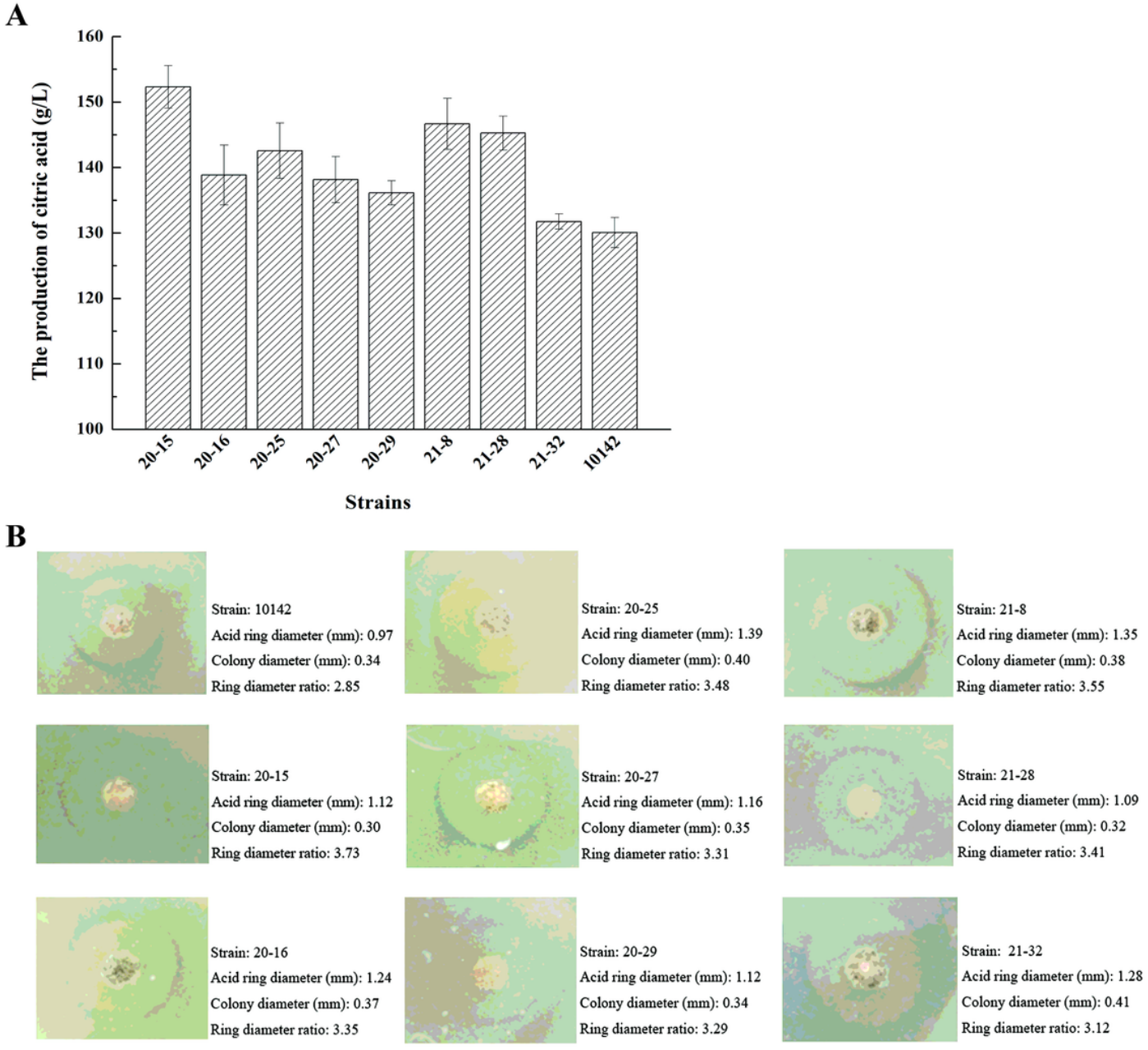
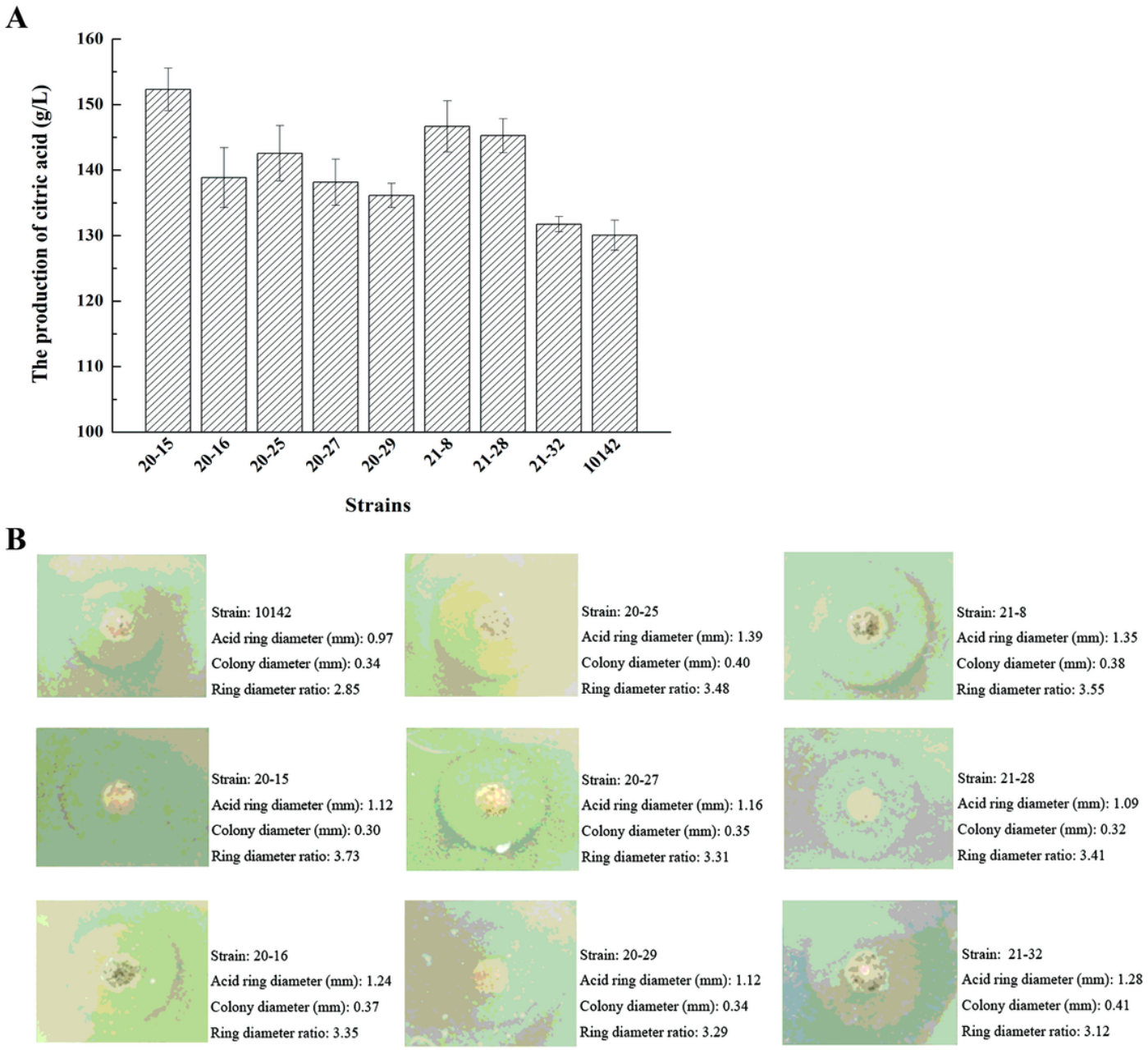


Figure 3

Verification of citric acid production of transformants by the shake flask fermentation and Ring diameter ratios. (A) Verification of citric acid production of transformants (20-15, 20-16, 20-25, 20-27, 20-29, 21-8, 21-28 and 21-32) by the 50 mL shake flask fermentation; (B) Ring diameter ratios determination of transformants (20-15, 20-16, 20-25, 20-27, 20-29, 21-8, 21-28 and 21-32) by transparent halo zone on CM+CaCO3 medium.



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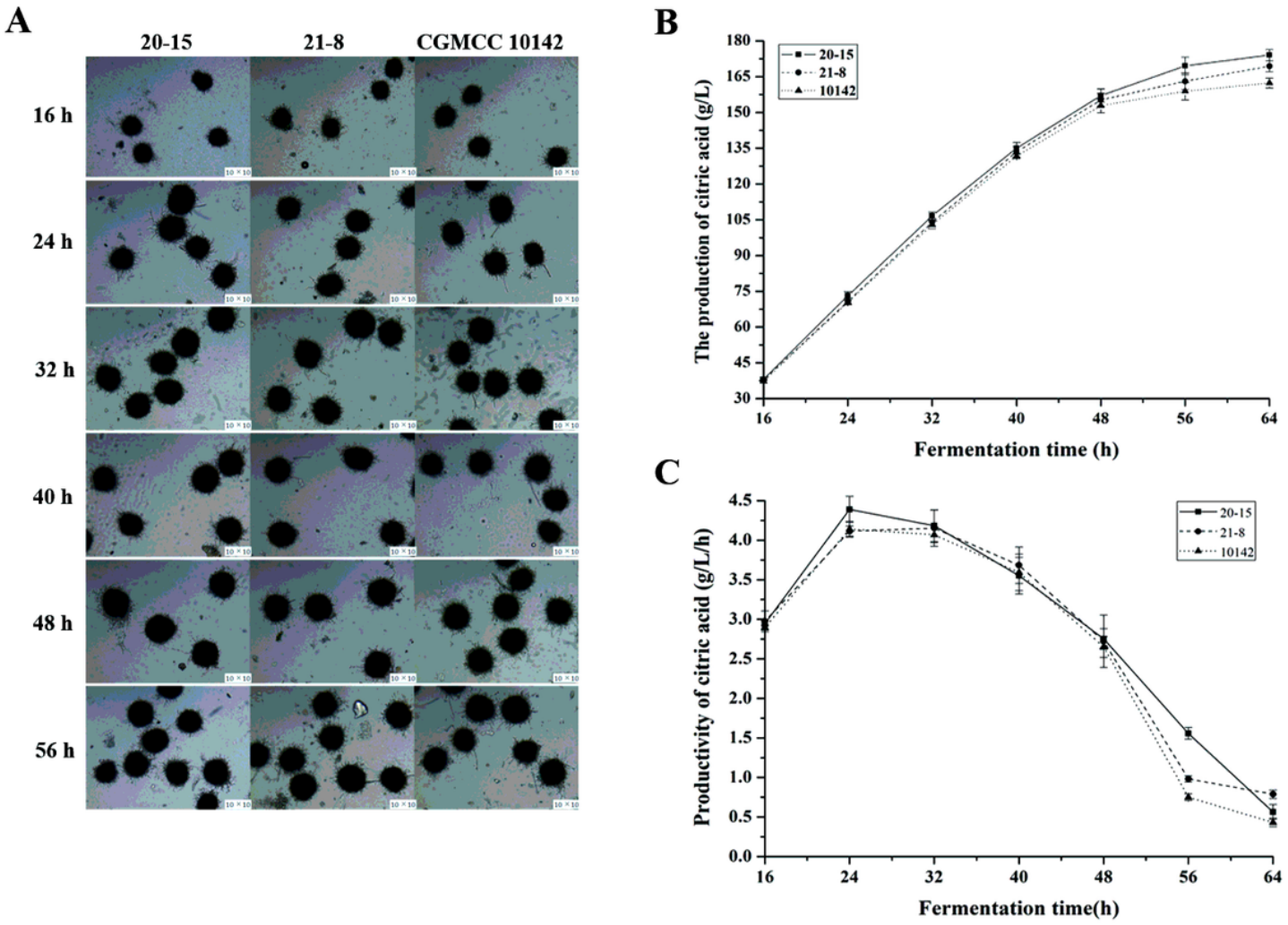
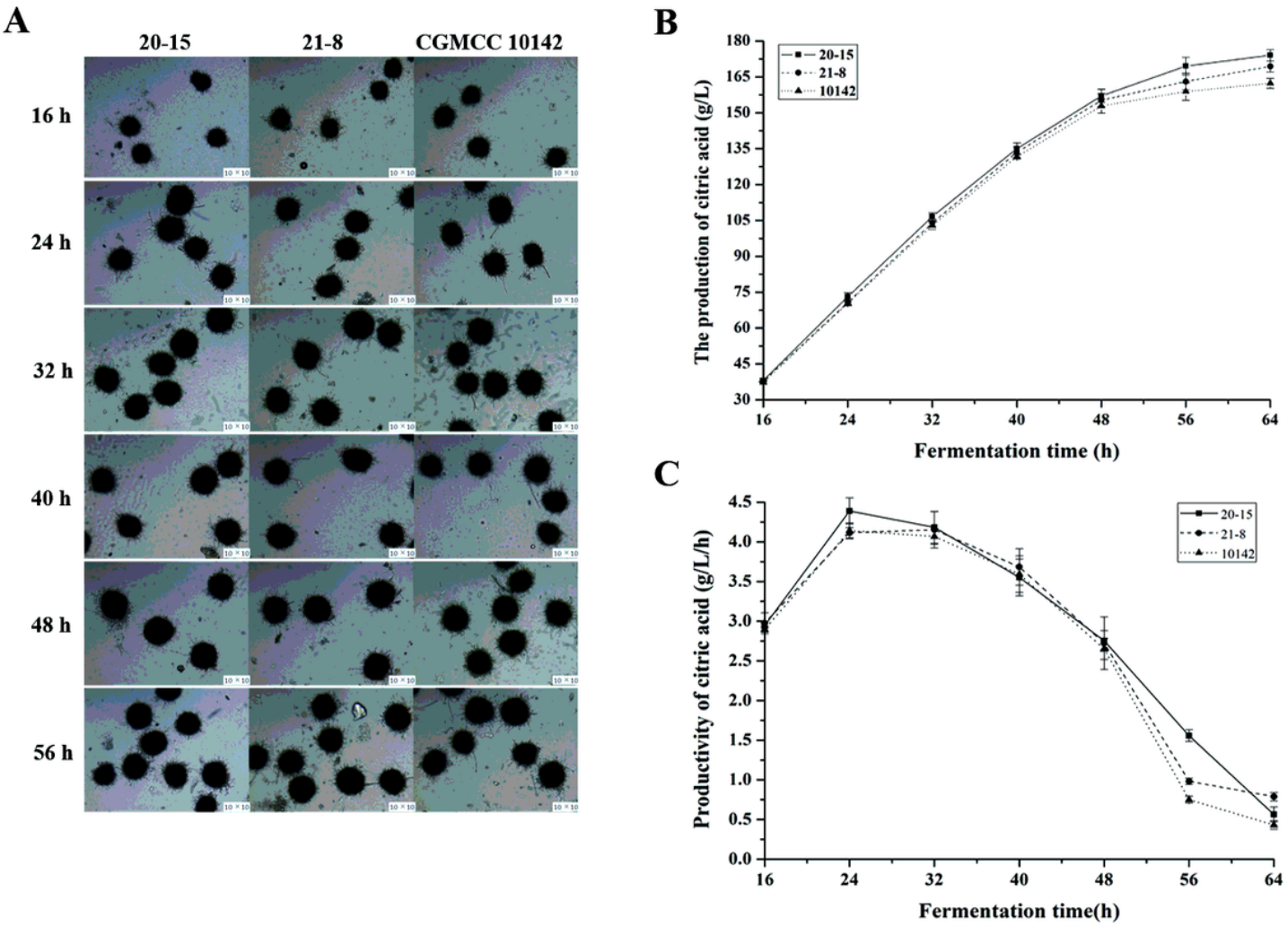


Figure 4

Observation of mycelial morphology and verification of production and productivity of citric acid in the 30 L bioreactor fermentation. (A) Observation of mycelial morphology of A. niger 20-15 (20-15), A. niger 21-8 (21-8) and A. niger CGMCC 10142 (10142) strain at different time points of fermentation; (B) Verification of citric acid production of 20-15, 21-8 and 10142 strain at different time points in the 30 L bioreactor fermentation; (C) Verification of citric acid productivity of 20-15, 21-8 and 10142 strain at different time points in the 30 L bioreactor fermentation.



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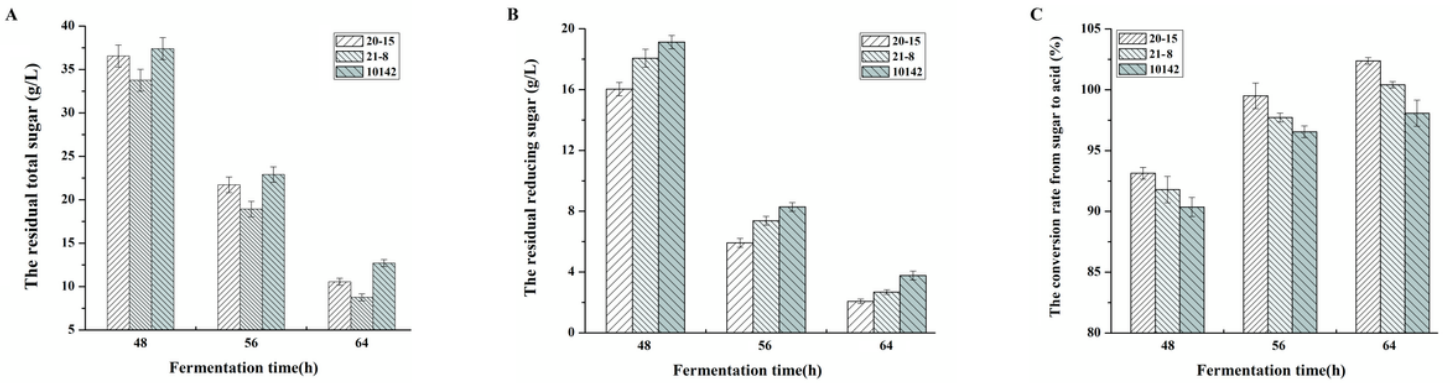
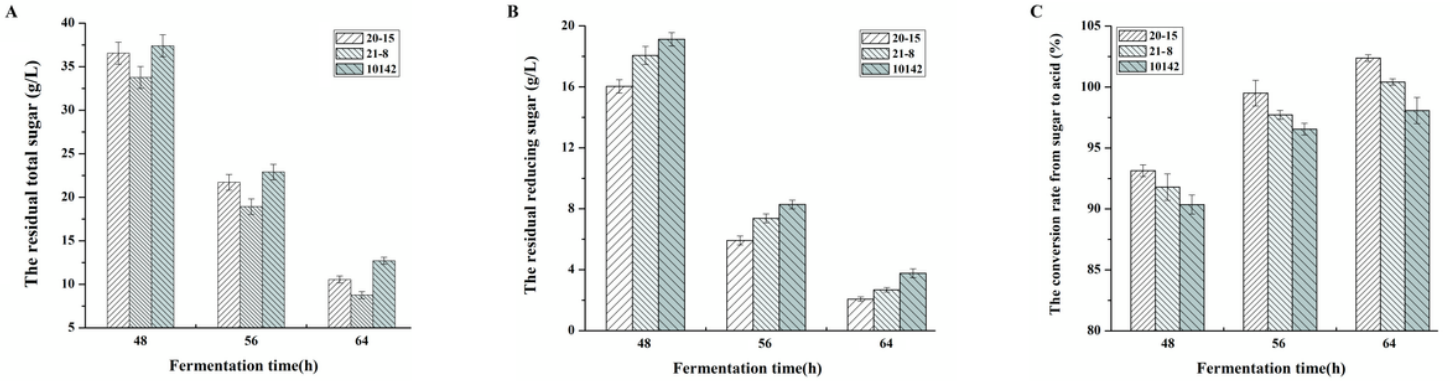


Figure 5

The CA fermentation performance in the 30 L bioreactor. (A) The residual total sugar changes at 48 h, 56 h, and 64 h in the fermentation broth; (B) The residual reducing sugar changes 48 h, 56 h, and 64 h in the fermentation broth; (C) The conversation rate from sugar to CA 48 h, 56 h, and 64 h in the fermentation broth.



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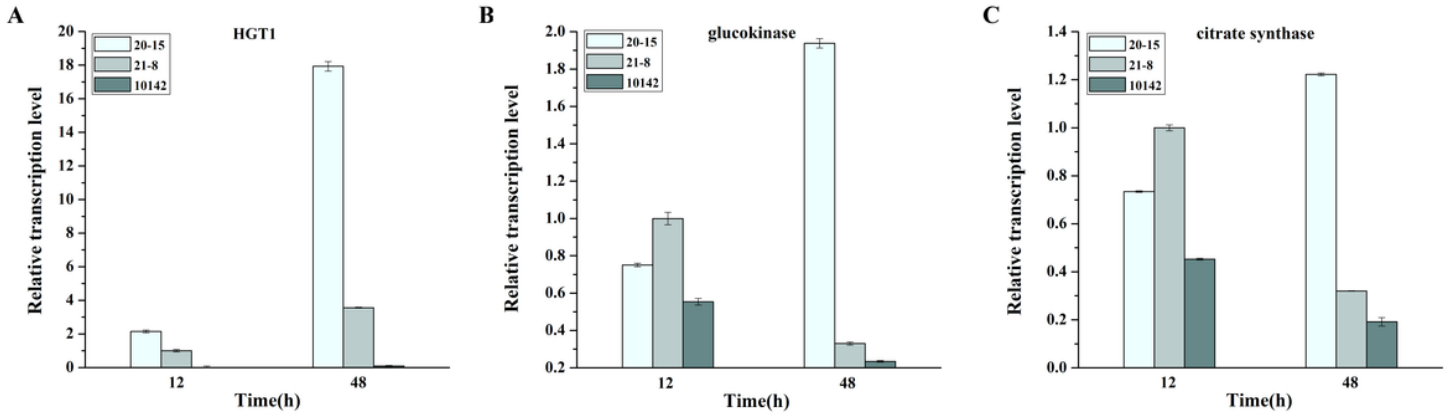
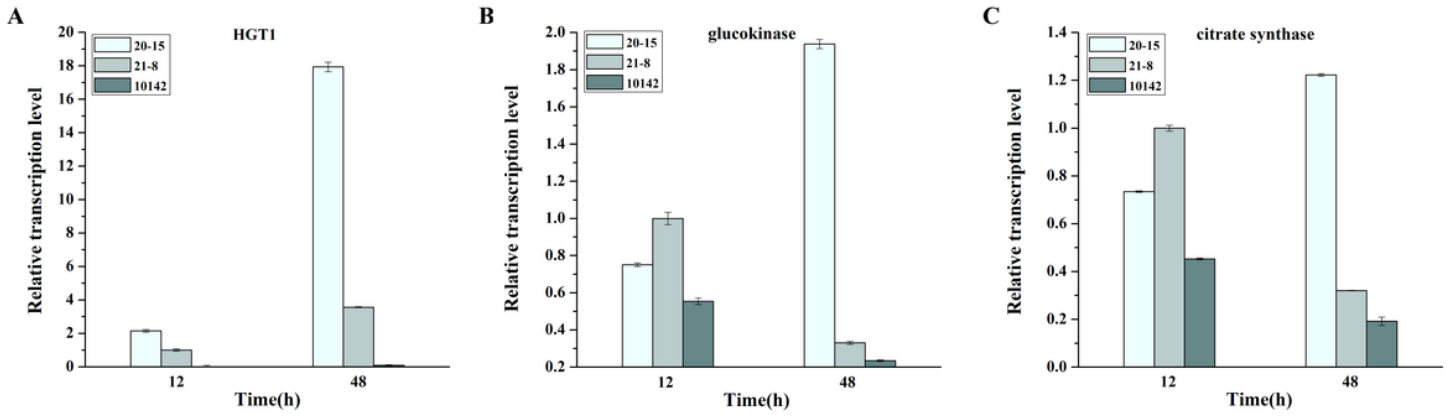


Figure 6

qRT-PCR analysis of the expression profile of HGT1, glucokinase, and citrate synthase at 12 h and 48 h in A. niger 20-15, 21-8 and 10142 strain. The 18S rRNA was used as an internal control. Vertical bars represented the mean ± S.D (n = 3). (A) The relative transcription level of HGT1 at 12 h and 48 h in A. niger 20-15, 21-8 and 10142 strain; (B) The relative transcription level of glucokinase at 12 h and 48 h in A. niger 20-15, 21-8 and 10142 strain; (C) The relative transcription level of citrate synthase at 12 h and 48 h in A. niger 20-15, 21-8 and 10142 strain.



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