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Enhancing fructooligosaccharides production by genetic improvement of the industrial fungus *Aspergillus niger* ATCC 20611

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Highlights

- An efficient PEG-mediated protoplast transformation system with *ptrA* as the selectable marker was developed in *Aspergillus niger* ATCC 20611.
- The EGFP reporter gene (*egfp*) was co-transformed with *ptrA* and the efficiency reached approx. 82%.
- The activity-improved variant of β-fructofuranosidase, FopA(A178P), was successfully overexpressed in *A. niger* ATCC 20611.
- The genetically engineered strain, CM6, exhibited a 58% increase in β-fructofuranosidase activity and achieved a 40% reduction in the time required for completion of FOS synthesis.

Abstract *Aspergillus niger* ATCC20611 is one of the most potent filamentous fungi used commercially for production of fructooligosaccharides (FOS), which are prospective components of functional food by stimulating probiotic bacteria in the human gut. However, current strategies for improving FOS yield still rely on production process development. The genetic engineering approach hasn't been applied in industrial strains to increase FOS production level. Here, an optimized polyethylene glycol (PEG)-mediated protoplast transformation system was established in *A. niger* ATCC 20611 and used for further strain improvement. The pyrithiamine resistance gene (*ptrA*) was selected as a dominant marker and protoplasts were prepared with high concentration (up to 10^8 g^{-1} wet weight mycelium) by using mixed cell wall-lysing enzymes. The transformation frequency with *ptrA* can reach 30~50 transformants per μg of DNA. In addition, the efficiency of co-transformation with the EGFP reporter gene (*egfp*) was high (approx. 82%). Furthermore, an activity-improved variant of β-fructofuranosidase, FopA(A178P), was successfully overexpressed in *A. niger* ATCC 20611 by using the transformation system. The transformant, CM6, exhibited a 58% increase in specific β-

fructofuranosidase activity (up to 507 U/g), compared to the parental strain (320 U/g), and effectively reduced the time needed for completion of FOS synthesis. These results illustrate the feasibility of strain improvement through genetic engineering for further enhancement of FOS production level.

Keywords Fructooligosaccharide; *Aspergillus niger*; Transformation system; Genetic engineering

1. Introduction

Prebiotics are nondigestible food ingredients that selectively stimulate the growth and/or activity of beneficial bacterial species already resident in the host intestine (Sangeetha et al., 2005; Simmering and Blaut, 2001). Among the commonly used prebiotics, fructooligosaccharides (FOS) have been regarded as important food ingredients due to their excellent nutrition- and health-relevant properties, such as possessing low calorie, reduction of total serum cholesterol level, as well as enhancement of calcium absorption (Maiorano et al., 2008; Dominguez et al., 2014). FOS generally refer to a series of homologous oligosaccharides that are mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1^F-β-fructofuranosyl nystose (GF_4), in which two, three, and four fructosyl units are bound at the β-2,1 position of glucose, respectively (Mutanda et al., 2014). Naturally, FOS are found in plants such as onion, banana, jerusalem artichoke and some grasses (Maiorano et al., 2008). However, the FOS production yield derived from these natural plant hosts is low and also quite limited by seasonal conditions (Yun, 1996). Commercially, the production of FOS is primarily carried out from sucrose by the transfructosylation activity of β-fructofuranosidases (Ffase, EC 3.2.1.26) (Hirayama et al., 1989; Ghazi et al., 2007; Kurakake et al., 2010).

β-fructofuranosidases belong to the family 32 glycoside hydrolase (GH32) enzymes that act on sucrose and related β-D-fructo-furanosides (Cantarel et al., 2009; Trollope et al., 2015). They intrinsically have both hydrolysis (Uh) and fructosyl transfer (Ut) activities, but the ratio (Ut/Uh) of each enzyme varies depending on the

specific source organism (Yanai et al., 2001). The enzymes that are potential for industrial production generally come from several fungi including *Aspergillus niger*, *A. japonicus*, and *Aureobasidium pullulans* (Hidaka et al., 1988; Kurakake et al., 2010; Ghazi et al., 2007). In particular, *A. niger* ATCC 20611 showed high productivity of β-fructofuranosidase (namely FopA), and FopA demonstrated much higher transfructosylation activity than hydrolysis activity (Hidaka et al., 1988). Therefore, *A. niger* ATCC 20611 is considered as one of the most suitable strains for FOS production (Yanai et al., 2001; Trollope et al., 2015).

The FOS synthesis catalysed by the FopA enzyme from *A. niger* ATCC 20611 has been scaled up in industry for more than 20 years (Hidaka et al., 1988; Nishizawa et al., 2001). So far, optimization of nutritional and culture parameters of fermentation processes is still the main approach to increase enzyme yield and productivity (Sangeetha et al., 2005; Mutanda et al., 2014). Dorta et al. (2006) found that the inexpensive cane molasses and yeast powder were as good as sucrose and peptone in the enzyme and FOS production, thus reducing the cost of carbon and nitrogen sources. Moreover, response surface methodology (RSM) was applied to optimization of medium composition and a high productivity of β-fructofuranosidase was obtained by using a slowly metabolized nitrogen source and limiting the carbon content conditions (Dinarvand et al., 2013). These traditional approaches typically rely on optimization of the fermentation conditions and there is an upper limit to the degree of enzyme production that can be increased. Recently, the *A. japonicus* FopA was semirationally engineered using a combined crystal structure- and evolutionary-

guided approach and 15 single-amino-acid-substitution variants displayed improved catalytic activities, with the FopA(A178P) identified as the most improved variant (Trollope et al., 2015). However, these improved FopA variants were all expressed in yeast system and not tested in the industrial *Aspergillus* strains. It is known that improvement of the production strain could offer the greatest opportunity for cost reduction without significant capital outlay (Chiang, 2004). Genetic engineering is a powerful approach for filamentous fungi in order to increase productivity, which can help to transfer desired phenotypic trait more quickly and accurately than conventional breeding (Meyer, 2008). However, before genetic manipulation can become available, the establishment of a suitable transformation system is an essential prerequisite. Although genetic transformation of *Aspergillus niger* strains was developed over the years, most of the strains used in the literature are non-industrial ones (Kelly and Hynes, 1985; Sureka et al. 2014). To date, no available transformation descriptions have been reported for the industrial strain *A. niger* ATCC 20611, which limits strain improvement in the FOS industry.

In the present study, an efficient PEG-mediated protoplast transformation system with *ptrA* as the selectable marker was developed in *A. niger* ATCC 20611. High efficiency of co-transformation with the EGFP reporter gene (*egfp*) was demonstrated. Then construction of genetically engineered strains, for the first time, with increased FOS production by overexpressing activity-improved β-fructofuranosidase was achieved.

2. Materials and methods

2.1 Strains, plasmids and growth conditions

A. niger ATCC 20611, a commercial strain for FOS production, was used as transformation host and routinely maintained on potato-dextrose agar (PDA, containing 20.0 g/L D-glucose and 20.0 g/L agar). Minimal medium (MM, as described by Penttilä et al. (1987)), supplemented with 2 μg/ml pyrithiamine, was applied for fungal growth during genetic transformation. For β-fructofuranosidase production, *A. niger* ATCC 20611 and transformants were cultured in fermentation medium (FM) at 30℃, 200 rpm. The FM medium contained sucrose 20 g/L, yeast extract 15 g/L, KCl 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, K_2HPO_4 5 g/L, $NaNO_3$ 2 g/L. The initial pH of FM was adjusted to 5.5 using 0.1 M HCl. After incubation for 48 h, the cultures were centrifuged and cells were separated for enzyme assays.

Plasmid pME2892 (Krappmann et al., 2005), which contains the *ptrA* selectable marker gene, was adopted for genetic transformation. Plasmid pIG1783 (Pöggeler et al., 2003), which carries the enhanced green fluorescent protein-encoding gene (*egfp*) as a reporter, was used for detection and analysis of heterologous gene expression in *A. niger*. The pMD19-T cloning vector (Takara, Otsu, Japan) was employed for TA cloning. *Escherichia coli* DH5α was the host for cloning of constructs and cultured in LB broth supplemented with appropriate antibiotics at 37℃.

2.2 Protoplast preparation and transformation of A. niger ATCC 20611

Protoplast preparation was carried out as previously described with modification (Penttilä et al., 1987; Liu and Friesen, 2012). *A. niger* spores were inoculated in liquid

modified MM (with substitution of glucose for sucrose as the carbon source) and cultured for 30 h at 28℃, 200 rpm. The mycelia were harvested by centrifugation at 13,000 rpm for 10 min and about 1 g (wet weight) of mycelia was added into 20 ml of Solution I (1.2 M sorbitol-0.1 M KH_2PO_4, pH 5.6), in which cell wall-digesting enzymes, including lysing enzymes from *Trichoderma harzianum* (Sigma Corp., St Louis, MO), snailase (Dingguo Corp., Beijing, China) and lysozyme (Genview, Houston, TX), were dissolved at appropriate concentrations and sorbitol was used as the osmotic stabilizer. Incubation was performed at 28℃ with gentle shaking for 2 h. Then the mixtures were filtered aseptically through 4 layers of glass wool to remove hyphae and other debris, and the protoplasts were centrifuged at 2500 rpm and finally resuspended in 200-1000 μl of Solution II (1 M Sorbitol-10 mM $CaCl_2$-10 mM Tris−HCl, pH 7.5). The formation of protoplasts was monitored by microscopic observation.

Protoplasts were transformed with pME2892 or cotransformed with pME2892 and pIG1783. The transformation procedure was a modification of that of Yelton et al. (1984). Specifically, 200 μl of protoplasts suspension, 2~5 μg of plasmid DNA and 50 μl of PEG/$CaCl_2$ solution (25% PEG 6000, 50 mM $CaCl_2$) were mixed and incubated on ice for 20 min, followed by addition of 500 μl of the same PEG/$CaCl_2$ solution and incubation for 5 min at room temperature. The protoplasts were then plated on selective regeneration medium (MM medium containing 1 M sorbitol, with 2 μg/ml of pyrithiamin) and incubated at 30℃ for 3-4 days. Finally, well-growing

transformants were selected and purified to uninuclear clones by plating single spores on selective medium.

2.3 PCR and Southern blot analysis

Chromosomal DNA was isolated from fungal transformants and analyzed by PCR using primer pair ptrA-F/ptrA-R for amplification of a 853-bp *ptrA* fragment, and egfp-F/egfp-R for amplification of a 720-bp *egfp* fragment, respectively. Southern hybridization and detection was carried out with the DIG Easy Hyb kit (Roche Diagnostics, Germany) according to the manufacture's instruction. The *A. niger* chromosomal DNA was digested with *Hind* III, size-fractioned by gel-electrophoresis and transferred to a Hybond N^+ nylon membrane (Amersham Biosciences, Piscataway, NJ). The probe was prepared by PCR amplification of a 853-bp *ptrA* fragment from plasmid pME2892 using primer pair ptrA-F/ ptrA-R.

2.4 Enzyme determination and TLC detection

To determine the β-fructofuranosidase activity, the reaction mixture consisted of 25% (W/V) sucrose (2 ml) as the substrate, 0.1 M citrate buffer (pH 5.0, 3 ml) and adequate amount of the collected cells. The enzymatic reaction was carried out at 50 ℃ for 2 h with moderate shaking and terminated by heating the mixture in boiling water for 10 min. The β-fructofuranosidase activity was determined by measuring the amount of glucose released from sucrose using the 3, 5-dinitrosalicylic acid (DNS) method as described previously (Ghazi et al., 2007). One unit of activity (U) was defined as the amount of enzyme required to produce 1 μmol of glucose per min.

Qualitative identification of FOS production and composition was conducted using the thin layer chromatography (TLC). TLC was done by using the silica gel 60 F_{254} plates (Millipore). Aliquots (1-2 μl) of the reaction solutions were spotted 10 mm from the bottom of the plates with 2.5 μl micro syringe pipettes. Then plates were run using a mixture of solvents (butanol, isopropanol, acetic acid, and water in volume (ml) at proportions of 7:5:2:4, respectively). After development was complete, the plates were dried and the sugars were visualized using a spray of diphenylamine–aniline–phosphoric acid (DPA) reagent (Buchan and Savage, 1952).

2.5 Fluorescence and light microscopy

For visualization of EGFP expression, spores of the fungal strains, transformed with pIG1783, were cultured in liquid MM medium and grown for 10 h at 30℃. Mycelia were collected and used directly for microscopic observation by using fluorescence and light microscopy (Nikon Eclipse 80i fluorescence microscope).

2.6 Construction of FopA(A178P)-overexpression A. niger strains

The FopA(A178P)-overexpression cassette, which carries the *mfopA* gene encoding the activity-improved β-fructofuranosidase variant FopA(A178P) (Trollope et al., 2015), was constructed as follows. Firstly, the 5'- and 3'-regions of the *mfopA* gene from the *A. niger* ATCC 20611 genome were amplified using primer pairs fopA-F/mfopA-542R and mfopA-525F/fopA-R, respectively and fused together to form the full-length *mfopA* gene using the primer pair fopA-F/fopA-R. At the same time, the fragments of *gpdA* promoter and *trpC* terminator were amplified from plasmid pAN7-

1 (Punt and Hondel, 1992) with prime pairs gpdA-F/gpdA-R and trpC-F/trpC-R, respectively. Then, the full-length *mfopA* gene, the *gpdA* promoter and the *trpC* terminator were mixed and ligated together to amplify the FopA(A178P)-overexpression cassette with primer pair gpdA-F/trpC-R by using Double-joint PCR method (Yu et al., 2004). Finally, the overexpression cassette was cloned into pMD19-T plasmid (Takara, Japan) to generate the final donor vector, named pGF-mfopA. Primers used in this study are listed in Table 1. All of the PCR experiments were carried out using standard methodology and the PCR fragments were purified with Gel Extraction Kit (Omega, USA).

The plasmid pGF-mfopA was co-transformed with pME2892, which consists of the *ptrA* selectable marker gene, into *A. niger* ATCC 20611 protoplasts to get the FopA(A178P)-overexpression strains. Transformants were selected on MM medium with 2 μg/ml pyrithiamine.

2.7 Fructooligosaccharides synthesis and HPLC analysis

A mixture containing 50% (W/V) sucrose and cells (6 units of enzyme per g sucrose) of *A. niger* strains in 50 mM citrate phosphate buffer (pH 5.5) was stirred at 50℃. After 5 h, the reaction was stopped by treatment in boiling water for 5 min. Then the mixture was centrifuged at 1,600 *g* for 10 min and the supernatant was collected. Quantitative analysis of the FOS product was performed using HPLC (LC-6A, Shimadzu, Japan) with a Agilent Zorbax NH_2 column (5 μm, 4.6 mm×250 mm) (Agilent Technologies, Santa Clara, CA) coupled with a refractive index detector

(2414, Waters, USA). The mobile phase was acetonitrile: water (70:30, v/v) at a flow rate of 1.0 ml/min. The identification and quantification of each FOS was carried out by a calibration curve built with authentic standards of 1-kestose, nystose and 1^F-β-fructofuranosyl nystose (Sigma Corp., St Louis, MO).

3. Results

3.1 Sensitivity of A. niger ATCC 20611 to antibiotics

As previously reported, filamentous fungi are relatively resistant to many antibiotics (Penttilä et al., 1987; Meyer, 2008). In this study, three generally used antibiotics for fungal species, including hygromycin B, pyrithiamine and sulfonylurea, were selected to test the sensitivity of the *A. niger* ATCC 20611. The diluted spore suspensions were plated on MM solid medium containing 0-300 μg/ml hygromycin B or 0-6 μg/ml of pyrithiamine and sulfonylurea, and then cultivated at 30℃ for 3 d. The sensitivity of this fungus to the antibiotics is shown in Table 2. *A. niger* ATCC 20611 displayed resistance to high concentrations of sulfonylurea and hygromycin B, and still grew in the presence of 300 μg/ml hygromycin B or 6 μg/ml sulfonylurea. However, it was highly sensitive to pyrithiamine at concentrations as low as 2 μg/ml, indicating that the pyrithiamine resistance gene (*ptrA*) could be used as a dominant selectable marker for genetic transformation of *A. niger* ATCC 20611.

3.2 Protoplast preparation from mycelium of A. niger ATCC 20611

PEG-mediated protoplast transformation system is the most commonly used method in filamentous fungi and preparation of high-yield and high-activity

protoplasts is the key to successful application of this technology (Liu and Friesen, 2012). Protoplast preparation is often affected by many factors, such as the types and concentrations of cell-wall-digesting enzymes, the age and morphology of mycelium, and the molarity of osmotic stabilizer (Collings et al., 1988). To our knowledge, there are no protocols and descriptions available for protoplast generation from *A. niger* ATCC 20611. Here, three types of cell wall-digesting enzymes, including lysing enzymes from *T. harzianum*, snailase and lysozyme, were adopted individually or combinely for preparing protoplasts. It was found that any individual enzyme in different concentrations could not crack mycelium easily and quickly and only few protoplasts were released (data not shown). When these enzymes were combined together, protoplasts could be prepared much more efficiently. And the optimal enzyme mixture, consisting of 60% lysing enzymes from *T. harzianum*, 30% snailase and 10% lysozyme, was confirmed to produce viable protoplasts from *A. niger* ATCC 20611 mycelium (Fig. 1A, B). The concentration of protoplasts prepared in this situation could reach 9×10^6 /g wet weight mycelium and the regeneration frequency was 57% (Fig. 1C, D).

Just as mentioned above, the morphology of fungal mycelium is another important factor for preparation protoplasts. In the present study, different carbon sources were also used for cultivation of *A. niger* ATCC 20611. It was found that the mycelium was stubby and formed compact pellets when sucrose or glucose was used as carbon source, while the mycelium grew rapidly and formed loose mycelium pellets when cane molasses was used as carbon source (Fig. S1). These mycelia were

then applied for protoplast preparation, and cane molasses-cultivated mycelium could produce high yield of viable protoplasts, with the concentration of up to 1×10^8 /g wet weight mycelium and the regeneration frequency of 59% (Fig. 1C, D), demonstrating that the looser mycelium could be more beneficial to protoplast preparation. Therefore, a high concentration of *A. niger* ATCC 20611 protoplasts was successfully prepared and could be used for further genetic transformation.

3.3 PEG-mediated protoplast transformation of A. niger ATCC 20611 with the ptrA gene as a dominant selectable marker

To confirm if the *ptrA* gene is available as a selectable marker for the FOS-producing industrial strain *A. niger* ATCC 20611, the frequency of PEG-mediated protoplast transformation was examined using a plasmid pME2892, which contained the *ptrA* gene expression cassette. In the presence of 2 μg/ml pyrithiamine, approximately 100 resistant transformants were obtained per μg DNA, while no colonies were regenerated in the control experiments with no plasmid (Fig. 2A). The putative transformants were purified on MM medium containing pyrithiamine to assess the mitotic stability. Six transformants, which had been proved to retain their mitotic stability, were randomly selected for further analysis. PCR was carried out to confirm the integration of *ptrA* into the genomes of transformants. Reactions with *ptrA*-specific primers yielded the expected amplification products in each of the six transformants respectively, but not in the parental strain (Fig. 2B). In order to determine the copy number of *ptrA* in the fungal genomes, Southern blot analysis was performed for the transformants. As shown in Fig. 2C, all the transformants appeared

to have two to three copies of *ptrA* integration into the genomes, indicating that the *ptrA* gene is a suitable selectable marker for genetic transformation of *A. niger* ATCC 20611.

To investigate whether the *ptrA* integration affects the *fopA* expression, the transformants were cultured and determined for the β-fructofuranosidase activity. There were no significant difference in the enzyme activity between the transformants and parental strain (Fig. 2D). The typical TCL profiles of FOS products prepared using β-fructofuranosidases from the fungal strains were all roughly of equal size (Fig. 2E). These results suggested that the *ptrA* integration randomly located in the genomes and did not influence the ability of the fungal strains to synthesize FOS.

3.4 Co-expression of enhanced green fluorescent protein (EGFP) with the ptrA gene

EGFP was chosen as the reporter to verify the practicability of the established genetic transformation system in *A. niger* ATCC 20611 with the *ptrA* selectable marker. The plasmid pIG1783, carrying the EGFP expression cassette, was co-transformed with pME2892 into the protoplasts of *A. niger* ATCC 20611. 45 pyrithiamine-resistant transformants were obtained. Among these, the 720-bp fragment of the *egfp* gene could be amplified from 42 transformants, indicating that the co-transformation rate of *ptrA* and *egfp* reached up to 82%. Fig. 3A showed the PCR result of seven randomly selected transformants, in which five transformants

contained both *ptrA* and *egfp*. The EGFP expression was further analyzed by fluorescence microscopy (Fig. 3B). The mycelium showed intense fluorescence emission, demonstrating successful expression of the *egfp* gene co-integrated into the genome. This result exhibited the validity and efficiency of the transformation system for genetic manipulation in the industrial stain *A. niger* ATCC 20611.

3.5 Overexpression of an activity-improved FopA variant for improving FOS production

The β-fructofuranosidase is the key enzyme catalyzing the biotransformation of sucrose to FOS (Yanai et al., 2001). Because of its key role in the FOS biosynthesis, the FopA from *A. niger* ATCC 20611 has recently been engineered to generate activity-increased variants, including the most improved single-amino-acid-substitution variant FopA(A178P), using a semirational directed evolution strategy (Trollope et al., 2015). However, these FopA variants were only expressed in yeast host and have not been applied in an industrial *Aspergillus* strain to increase the ability of FOS biosynthesis. Here, the β-fructofuranosidase variant FopA(A178P) was selected for strain improvement by genetic engineering. The FopA(A178P) expression cassette containing the *gpdA* promoter and the *trpC* terminator was constructed and cloned into pMD19-T to generate the expression vector, pGF-mfopA (Fig. 4A). The resultant vector was then co-transformed with pME2892 into the protoplasts of *A. niger* ATCC 20611. Purified transformants were grown in FM medium and screened for production of β-fructofuranosidase. The best three transformants, CM2, CM6 and CM9 were selected and each of them produced over 40% higher β-fructofuranosidase

activity than the parental strain (Fig. 4B). Especially, CM6 could produce specific β-fructofuranosidase with activity of up to 507 U/g, which was 1.58 times of the parental strain (320 U/g).

Furthermore, production of FOS by *A. niger* ATCC 20611 and the FopA(A178P)-overexpression strains was investigated. Equal amounts of mycelium cells were used to produce FOS under conditions according to the method described in 2.7. Results of FOS formation detected by HPLC were summarized in Table 3. During the initial reaction up to 1 h, markedly higher GF_2 levels for the overexpression transformants (31.1% for CM2, 32.9% for CM6 and 31.3% for CM9) than the parental strain (25.5%) highlighted the enhancements in catalytic activities, as GF_2 is the initial FOS species produced from sucrose. As the reaction progressed, larger amounts of GF_3 and GF_4 also accumulated for the transformants, further confirming the effectiveness of overexpression of FopA(A178P) in the industrial strain *A. niger* ATCC 20611. Generally, the endpoint for a typical FOS synthesis reaction was regarded as the time when the content of total FOS was approx. 60% (Hidaka et al., 1988; Nishizawa et al., 2001). All the transformants reached over 59% FOS in 3 h while it took the parent 5 h to produce similar amount of FOS, indicating that overexpression of FopA(A178P) achieved a 40% reduction in the time required to complete the reaction. These results displayed an enhanced effectiveness for FOS production by strain improvement via genetic engineering, suggesting the industrial relevance of the engineered strains to improve process economics.

4. Discussion

For more than decades, genetic engineering has been used as a fruitful strategy for strain improvement in filamentous fungi, as it substantially circumvents the shortage of conventional breeding (Yelton et al., 1984; Chiang, 2004). Before designing a genetic engineering strategy, development of an efficient transformation system is essential to a specific filamentous fungus. Many fungal species have been transformed and a variety of protocols now exist, including the widely used PEG-mediated protoplast transformation (Meyer, 2008). However, no common rule could be applicable to predicting the availability of a particular protocol for the fungus of interest. Individual species, especially the industrial strains, have to be considered independently and the most appropriate method for each fungus need to be identified and optimized. In this study, a PEG-mediated protoplast transformation system was established in *A. niger* ATCC 20611 by determination of the dominant selectable marker and optimization of protoplast preparing conditions, and furthermore applied to strain improvement for FOS production.

Filamentous fungi generally display a considerable metabolic diversity, which confer them relatively resistant to many antibiotics. Selection of an appropriate selectable marker is an important prerequisite for stable genetic transformation of certain fungus species (Punt and Hondel, 1992; Zhong et al., 2007). There have been several commonly used markers conferring resistance to antibiotics for selection of fungal transformants, such as the hygromycin B resistance gene (*hph*), the sulfonylurea resistance gene (*sur*) and the pyrithiamine resistance gene (*ptrA*). The *hph* gene encodes a phosphotransferase that could phosphorylate and inactivate

hygromycin B, an aminoglycoside antibiotic that interrupts the translocation step of protein synthesis (Punt and Hondel, 1992). Although *hph* has been widely used as an effective marker for transformation selection in many fungi, some species display high resistance to this robust antibiotic, including the filamentous ascomycetes *Colletotrichum acutatum* and *Beauveria bassiana* (Sureka et al., 2014). The *sur* gene from *Magnaporthe grisea* encodes acetolactate synthase (ALS) required for valine/isoleucine biosynthesis, and overexpression of *sur* in a fungus could eliminate the inhibition of ALS by sulfonylurea, thus resulting in sulfonylurea resistance (Chung et al., 2002). The two mentioned-above fungi *C. acutatum* and *B. bassiana* were reported to be successfully transformed by using *sur* as the dominant selectable marker (Chung et al., 2002; Zhang et al., 2010). Pyrithiamine, a potent antagonist of thiamine, could inhibit the fungal growth and the *ptrA* gene would confer high resistance to pyrithiamine (Kubodera et al., 2000). More recently, *ptrA* has been proven to be particularly useful for transformation of different *Aspergillus* sp., including *A. oryzae* and *A. aculeatus* that are insensitive to various drugs (Kubodera et al., 2002; Kanamasa et al., 2003). In this study, the sensitivity of *A. niger* ATCC 20611 to hygromycin B, sulfonylurea and pyrithiamine were examined and found that pyrithiamine inhibited its growth effectively, even in the concentration as low as 2 μg/mL (Table 2). Consequently, the *ptrA* gene was selected as the dominant selectable marker for genetic transformation of *A. niger* ATCC 20611. Furthermore, protoplast preparation is another important element to consider with the choice of the resistance to antibiotic for fungal transformation (Liu and Friesen, 2012; Zhang et al., 2016).

Two crucial parameters, i.e., cell wall-lysing enzymes and fungal cultures, necessary to be considered for proper generation of protoplasts were examined. Our results confirmed that lysing enzymes from *T. harzianum*, snailase and lysozyme were more effective when used as mixtures rather than individually. And high yield of protoplasts were obtained using the molasses-cultured fungal mycelia with concentrations of 1×10^8/g wet weight mycelium (Fig. 1C). Finally, the protoplasts of *A. niger* ATCC 20611 were successfully transformed using *ptrA* as the dominant selectable marker. This is the first report on the genetic transformation of the FOS-producing industrial strain and the efficiency of transformation reaches 30~50 transformants per μg of DNA.

FOS is now produced commercially from sucrose using the enzymes with high transfructosylating activity (Maiorano et al., 2008). Such enzymes can be derived from plants, bacteria or fungi, with the latter affording the greatest yields (Dominguez et al., 2014). Different FOS-producing fungi have been identified and characterized for their potential application to FOS production. *A. niger* ATCC 20611 was the first fungus reported to achieve a high yield of FOS production with the maximum FOS conversion from sucrose reaching 55-60% (w/w) of total sugar (Hidaka et al., 1988). An *A. japonicus*, TIT-KJ1, was isolated from air and found to produce a significantly high transfructosylating activity (Duan et al., 1993). Another promising fungus *Aureobasidium pullulans* for FOS production was investigated and gave rise to industrial process as well described by Yun et al. (1992). Other candidate fungi, including *A. oryzae*, *A. aculeatus*, *Penicillium citrinum*, *P. expansum* and *Calviceps*

purpurea, have also received particular attention (Sangeetha et al., 2005; Yun, 1996; Mutanda et al., 2014). So far, most investigations with the aim to obtain higher FOS yields and productivities are mainly based on the development of more efficient production processes (Dominguez et al., 2014). Chen and Liu (1996) firstly examined the effect of various carbon and nitrogen sources in cultivation of *A. japonicus* for FOS production and found that sucrose and yeast extract were the best ones, respectively. The effect of the medium pH in fungal growth has also been evaluated. pH of 5.5 was found to be the best initial value for *A. oryzae*, *A. japonicus*, and *P. purpurogenum* (Dominguez et al., 2014). In addition, several agricultural by-products like cereal bran, corn products and sugarcane bagasse were used as the low-cost substrates for the growth of the FOS-producing strain *A. oryzae* CFR 202 (Sangeetha et al., 2004). Moreover, immobilized enzymes or cells have been employed for the continuous production of FOS. Calcium alginate, synthetic polymer polymethacrylamide as well as gluten have been adopted as the matrix for entrapping fungal mycelia to achieve the high-level production of FOS (Chien et al., 2001; Sangeetha et al., 2005). However, there are few reports concerning strain improvement and genetic manipulation. Genetic engineering can be a powerful strategy for filamentous fungi in order to increase productivity and thereby to maximize the capacity of a particular metabolic process (Meyer, 2008). In the present study, an efficient genetic transformation system was developed in *A. niger* ATCC 20611 and, furthermore, the reporter protein EGFP was successfully expressed based on this system (Fig. 3). These results indicated the potential feasibility of strain

improvement of *A. niger* ATCC 20611 for higher FOS productivity by genetic manipulation.

Principal FOS such as 1-kestose, nystose and 1^F-β-fructofuranosyl nystose are synthesized by the fructosyltransferase activity of β-fructofuranosidase (also designated as β-fructosyltransferase). Fructosyltransferases derived from fungi provide high yields of FOSs and their mass production is not complicated (Madlová et al., 2000). For example, *A. niger* ATCC 20611 produces a unique β-fructofuranosidase, which has a high transfructosyating activity and a high regiospecificity for fructosyl transfer to the 1-OH group of terminal fructofuransides (Hirayama et al., 1989). The fructosyltransferase from *Aureobasidium pullulans* also showed high activity and regiospecificity in the fructosyl transfer reaction (Hayashi et al., 1990). An important property of these enzymes is that they catalyze almost exclusively transfructosylation reaction at rather high concentration of sucrose solution (over 500 g/L) to produce a mixture of FOS and glucose (Nishizawa et al., 2001). And the typical values of recommended conditions for the production of FOS are the pH of about 5-5.5 and temperature of 55-60℃ (Madlová et al., 2000). Therefore, industrial sucrose-to-FOS biotransformation is generally conducted in 50 to 60% (w/v) sucrose solution with the reaction proceeding at 50 to 60℃ for up to 20 h (Madlová et al., 2000; Trollope et al., 2015). However, operating at large volumes of substrate loading and long-term high temperatures, the enzyme activity is prone to be impaired by thermal instability. To address these limitations, Trollope et al. (2015) used a combined crystal structure- and evolutionary-guided approach for the directed

evolution of the *A. japonicus* β-fructofuranosidase and acquired a library of functionally enriched variants displaying improved thermostability and increased specific activities. Here, the most improved single-amino-acid-substitution variant FopA(A178P) was overexpressed in *A. niger* ATCC 20611 through the genetic transformation system. The FopA(A178P)-encoding gene *mfopA* under the control of the strong promoter, P*gpdA*, was successfully transformed into the protoplasts of *A. niger* ATCC 20611 (Fig. 4A). All the transformants showed improved β-fructofuranosidase activity and the transformant, CM6, exhibited the highest activity (507 U/g) (Fig. 4B). When used for FOS synthesis, the transformants displayed an increased catalytic effectiveness by reducing the time to complete the reaction. Thus, when applying the genetic engineered strains for commercial FOS production, the required enzyme loading could be reduced to improve industrial process economics.

In conclusion, this study developed an efficient PEG-mediated protoplast transformation system for the industrial strain *A. niger* ATCC 20611, which is based on the use of *ptrA* as the selectable marker. Further strain improvement for FOS production was achieved by overexpression of FopA(A178P) in *A. niger* ATCC 20611. The high efficiency of genetic manipulation achieved using this transformation system will further facilitate new developments in genetic engineering of *A. niger* ATCC 20611, thus strengthening the role of this strain as an important microorganism in functional food industry.

Acknowledgment

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References

Buchan, J., Savage, R., 1952. Paper chromatography of some starch conversion products. Analyst. 77, 401–406.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V., Henrissat, B., 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res. 37, D233–D238.

Chen, W.C., Liu, C.H., 1996. Production of β-fructofuranosidase by *Aspergillus japonicus*. Enzyme Microb. Technol. 18, 153-160.

Chiang, S.J., 2004. Strain improvement for fermentation and biocatalysis processes by genetic engineering technology. J. Ind. Microbiol. Biotechnol. 31, 99-108.

Chien, C.S., Lee, W.C., Lin, T.J., 2001. Immobilization of *Aspergillus japonicus* by entrapping cells in gluten for production of fructooligosaccharides. Enzyme Microb. Technol. 29, 252-257.

Chung, K.R., Shilts, T., Li, W., Timmer, L.W., 2002. Engineering a genetic transformation system for *Colletotrichum acutatum*, the causal fungus of lime anthracnose and postbloom fruit drop of citrus. FEMS Microbiol. Lett. 213 (1), 33-39.

Collings, A., Davis, B., Mills, J., 1988. Factors affecting protoplast release from some mesophilic, thermophilic and thermotolerant species of filamentous fungi using Novozym 234. Microbios. 53, 197-210.

Dinarvand, M., Rezaee, M., Masomian, M., Jazayeri, S.D., Zareian, M., Abbasi, S., Ariff, A.B., 2013. Effect of C/N ratio and media optimization through response surface methodology on simultaneous productions of intra-and extracellular inulinase and invertase from *Aspergillus niger* ATCC 20611. Biomed Res. Int. 2013, 508968.

Dominguez, A.L., Rodrigues, L.R., Lima, N.M., Teixeira, J.A., 2014. An overview of the recent developments on fructooligosaccharide production and applications. Food Bioproc. Tech. 7, 324-337.

Dorta, C., Cruz, R., Oliva-Neto, P.D., Moura, D.J.C., 2006. Sugarcane molasses and yeast powder used in the fructooligosaccharides production by *Aspergillus japonicus* -FCL 119T and *Aspergillus niger* ATCC 20611. J. Ind. Microbiol. Biotechnol. 33, 1003-1009.

Duan, K.J., Sheu, D.C., Chen, J.S., 1993. Purification and characterization of β-fructofuranosidase from *Aspergillus japonicus* TIT-KJ1. Biosci. Biotechnol. Biochem. 57, 1811-1815.

Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Plou, F.J., Ballesteros, A., 2007. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. J. Biotechnol. 128, 204-211.

Hayashi, S., Nonokuchi, M., Imada, K., and Ueno, H., 1990. Production of fructosyl-transferring enzyme by *Aureobasidium* sp. ATCC 20524. Ind. Microbial. 5, 395-400.

Hidaka, H., Hirayama, M., Sumi, N., 1988. A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 52, 1181-1187.

Hirayama, M., Sumi, N., Hidaka, H., 1989. Purification and properties of a fructooligosaccharide-producing β-fructofuranosidase from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 53 (3), 667-673.

Kanamasa, S., Yamaoka, K., Kawaguchi, T., Sumitani, J.I., Arai, M., 2003. Transformation of *Aspergillus aculeatus* using the drug resistance gene of *Aspergillus oryzae* and the *pyrG* gene of *Aspergillus nidulans*. Biosci. Biotechnol. Biochem. 67 (12), 2661-2663.

Kelly, J. M., Hynes, M. J., 1985. Transformation of *Aspergillus niger* by the *amd*S gene of *Aspergillus nidulans*. The EMBO J. 4(2), 475-9.

Krappmann, S., Bayram, O., Braus, G.H., 2005. Deletion and allelic exchange of the *Aspergillus fumigatus* veA locus via a novel recyclable marker module. Eukaryot. Cell 4, 1298–1307.

Kubodera, T., Yamashita, N., Nishimura, A., 2000. Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. Biosci. Biotechnol. Biochem. 64 (7), 1416-1421.

Kubodera, T., Yamashita, N., Nishimura, A., 2002. Transformation of *Aspergillus* sp. and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. 66 (2), 404-406.

Kurakake, M., Masumoto, R., Maguma, K., Kamata, A., Saito, E., 2010. Production of fructooligosaccharides by β-fructofuranosidases from *Aspergillus oryzae* KB. J. Agric. Food Chem. 58 (1), 488-492.

Liu, Z., Friesen, T.L., 2012. Polyethylene glycol (PEG)-mediated transformation in filamentous fungal pathogens. Methods Mol. Biol. 835, 365-375.

Madlová, A., Antosová, M., Baráthová, M., Polakovic, M., Stefuca, V., Báles, V., 2000. Screening of microorganisms for transfructosylating activity and optimization of biotransformation of sucrose to fructooligosaccharides. Chem. Papers 53 (6), 366-369.

Maiorano, A.E., Piccoli, R.M., da Silva, E.S., de Andrade Rodrigues, M.F., 2008. Microbial production of fructosyltransferases for synthesis of pre-biotics. Biotechnol. Lett. 30 (11), 1867-1877.

Meyer, V., 2008. Genetic engineering of filamentous fungi — Progress, obstacles and future trends. Biotechnol. Adv. 26 (2), 177–185.

Mutanda, T., Mokoena, M.P., Olaniran, A.O., Wilhelmi, B.S., Whiteley, C.G., 2014. Microbial enzymatic production and applications of short-chain fructooligosaccharides and inulooligosaccharides: recent advances and current perspectives. J. Ind. Microbiol. Biotechnol. 41 (6), 893-906.

Nishizawa, K., Nakajima, M., Nabetani, H., 2001. Kinetic study on transfructosylation by β-fructofuranosidase from *Aspergillus niger* ATCC 20611 and availability of a membrane reactor for fructooligosaccharides. Food Sci. Technol. Res. 7 (1), 39-44.

Penttilä, M., Nevalainen, H., Rättö, M., Salminen, E., Knowles, J., 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61 (2), 155-164.

Pöggeler, S., Masloff, S., Hoff, B., Mayrhofer, S., Kück, U., 2003. Versatile EGFP reporter plasmids for cellular localization of recombinant gene products in filamentous fungi. Curr. Genet. 43, 54–61.

Punt, P.J., van den Hondel, C.A., 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Methods Enzymol. 216, 447-457.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2004. Production of fructosyl transferase by *Aspergillus oryzae* CFR 202 in solid-state fermentation using agricultural by-products. Appl. Microbiol. Biotechnol. 65 (5), 530-537.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2005. Recent trends in the microbial production, analysis and application of fructooligosaccharides. Trends Food Sci. Technol. 16, 442–457.

Simmering, R., Blaut, M., 2001. Pro- and prebiotics--the tasty guardian angels? Appl Microbiol Biotechnol. 55(1):19-28.

Sureka, S., Chakravorty, A., Holmes, E.C., Spassibojko, O., Bhatt, N., Wu, D., Turgeon, B.G., 2014. Standardization of functional reporter and antibiotic resistance cassettes to facilitate the genetic engineering of filamentous fungi. ACS Syn. Biol. 3 (12), 960-962.

Trollope, K.M., Görgens, J.F., Volschenk, H., 2015. Semirational directed evolution of loop regions in *Aspergillus japonicus* β-fructofuranosidase for improved fructooligosaccharide production. Appl. Environ. Microb. 81 (20), 7319-7329.

Yanai, K., Nakane, A., Kawate, A., Hirayama, M., 2001. Molecular cloning and characterization of the fructooligosaccharide-producing beta-fructofuranosidase gene from *Aspergillus niger* ATCC 20611. Biosci. Biotechnol. Biochem. 65 (4), 766-773.

Yelton, M.M., Hamer, J.E., Timberlake, W.E., 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. U. S. A. 81, 1470–1474.

Yu, J.H., Hamari, Z., Han, K.H., Seo, J.A., Reyes-Domínguez, Y., 2004. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fung. Genet. Biol. 41 (11), 973-981.

Yun, J.W., 1996. Fructooligosaccharides—occurrence, preparation, and application. Enzyme Microb. Technol. 19, 107-117.

Yun, J.W., Jung, K.H., Jeon, Y.J., Lee, J.H., 1992. Continuous production of fructo-oligosaccharides by immobilized cells of *Aureobasidium pullulans*. J. Microb. Biotech. 2 (2), 98-101.

Zhang, S., Fan, Y., Xia, Y.X., Keyhani, N.O., 2010. Sulfonylurea resistance as a new selectable marker for the entomopathogenic fungus *Beauveria bassiana*. Appl. Microbiol. Biotechnol. 87 (3), 1151-1156.

Zhang, Y.J, Xie, M., Zhang, X.L., Peng, D.L., Yu, W.B., 2016. Establishment of polyethylene-glycolmediated protoplast transformation for *Lecanicillium lecanii* and development of virulence-enhanced strains against *Aphis gossypii*. Pest Manag. Sci. 72 (10), 1951-1958.

Zhong, Y.H., Wang, X.L., Wang, T.H., Jiang, Q., 2007. Agrobacterium-mediated transformation (AMT) of *Trichoderma reesei* as an efficient tool for random insertional mutagenesis. Appl. Microbiol. Biotechnol. 73(6):1348-54.

Tables

Table 1

Primers used in this study.

Primers	Sequences (5'-3')	Targeted gene
ptrA-F	AGGGTAAGGTCGTGGTTT	*ptrA*
ptrA-R	CGATTATCAAGCCCTTAGTA	*ptrA*
egfp-F	ATGGTGAGCAAGGGCGAG	*egfp*
egfp-R	TTACTTGTACAGCTCGTCCATG	*egfp*
gpdA-F	AGACCTAATACAGCCCCTAC	Promoter of *gpdA*

gpdA-R	GGGTGGTAGTGGTGAGCTTCATTGTCTGCTCAAGC GGGGTAG	Promoter of *gpdA*
fopA-F	ATGAAGCTCACCACTACCACCC	*fopA*
fopA-R	TTTGATGATTTCAGTAACGTTAAGTTCAATTTCTCT CCGGCCAG	*fopA*
mfopA-542R	GGGTGGTCGGGGATGACG	*mfopA*
mfopA-525F	CGTCATCCCCGACCACCC	*mfopA*
trpC-F	ACTTAACGTTACTGAAATCATCAAA	Terminator of *trpC*
trpC-R	GAGTGGAGATGTGGAGTGGG	Terminator of *trpC*

Table 2

Antibiotic resistance of *A. niger* ATCC 20611[a]

	Hygromycin B (μg/ml)				Sulfonylurea (μg/ml)				Pyrithiamine (μg/ml)			
	0	100	200	300	0	2.0	4.0	6.0	0	2.0	4.0	6.0
ATCC 20611	+++	+++	++-	+--	+++	+++	++-	+--	+++	---	---	---

[a] 100 μl diluted spores suspensions (10^5/ml, 10^6/ml and 10^7/ml) are spread on solidified minimal medium plates containing various concentrations of the antibiotic: +++, significant growth; ++ -, growth; + - -, sparse growth; ---, no growth.

Table 3

HPLC analysis of FOS yield and composition at different reaction time by *A. niger* ATCC 20611 (P) and FopA(A178P)-overexpression strains (CM2, CM6 and CM9)[a].

Reaction Time(h)	Strain	Glucose (%)	Fructose (%)	Sucrose (%)	GF_2[c] (%)	GF_3[c] (%)	GF_4[c] (%)	FOS (%)
1h	P	12.8 (±0.64)[b]	0.2 (±0.01)	55.3 (±0.55)	25.5 (±0.21)	5.6 (±0.22)	0.7 (±0.01)	31.8
	CM2	15.8 (±1.31)	0.3 (±0.01)	45.0 (±1.12)	31.1 (±1.72)	7.4 (±0.37)	0.4 (±0.02)	38.9
	CM6	18.6 (±0.18)	0.4 (±0.02)	37.2 (±0.31)	32.9 (±1.31)	9.7 (±0.19)	1.2 (±0.10)	43.8
	CM9	17.3 (±0.43)	0.3 (±0.01)	40.7 (±2.26)	31.3 (±1.56)	9.5 (±0.63)	1.0 (±0.01)	41.8
3h	P	25.5 (±0.21)	0.6 (±0.02)	17.2 (±0.34)	35.7 (±2.97)	18.5 (±0.92)	2.4 (±0.06)	56.6
	CM2	27.0 (±1.50)	0.8 (±0.04)	12.8 (±0.42)	34.6 (±1.73)	22.3 (±1.48)	2.6 (±0.02)	59.5
	CM6	28.8 (±1.15)	1.0 (±0.08)	11.0 (±0.55)	31.2 (±0.78)	23.5 (±1.38)	4.6 (±0.09)	59.3
	CM9	27.7 (±1.47)	0.8 (±0.05)	12.6 (±0.12)	32.7 (±0.27)	22.6 (±0.90)	3.7 (±0.10)	59.0
5h	P	29.5 (±0.73)	1.0 (±0.05)	9.9 (±0.49)	28.8 (±0.96)	25.9 (±0.51)	4.9 (±0.19)	59.6
	CM2	30.2 (±0.60)	1.1 (±0.09)	9.3 (±0.46)	25.9 (±0.64)	28.4 (±1.57)	5.3 (±0.26)	59.6
	CM6	31.9 (±1.06)	1.3 (±0.06)	8.5 (±0.56)	23.5 (±0.22)	27.4 (±0.28)	8.5 (±0.34)	59.4
	CM9	30.6 (±0.61)	1.1 (±0.13)	9.2 (±0.47)	25.5 (±0.62)	27.3 (±0.82)	6.4 (±0.53)	59.2

[a] All reactions were carried out in a 50% (W/V) sucrose solution at 50℃, with shaking at 120 rpm, the enzyme dosage was 6 U per g sucrose for the parent strain (P) and equal amounts of transformants cells.

[b] Values in parentheses are standard errors.

[c] GF_2 : 1-kestose; GF_3 : nystose; GF_4 : 1^F-β-fructofuranosyl nystose.

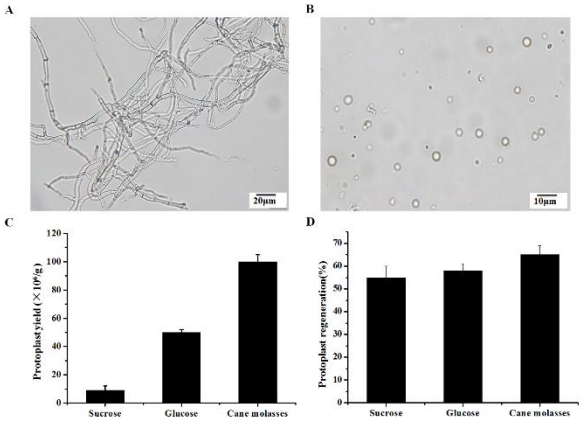
Figure captions

Fig. 1. Microscopic observations of protoplast preparation and the yields of protoplasts from *A. niger* ATCC 20611. (A) The mycelium phenotype of *A. niger* ATCC 20611 that was grown for 30 h in MM liquid media. (B) The micrograph of protoplasts released from *A. niger* ATCC 20611 mycelia. (C) Comparison of the production yields of protoplasts released from mycelia grown in media containing different carbon sources: glucose, sucrose and cane molasses. (D) Comparison of the protoplast regeneration frequencies.

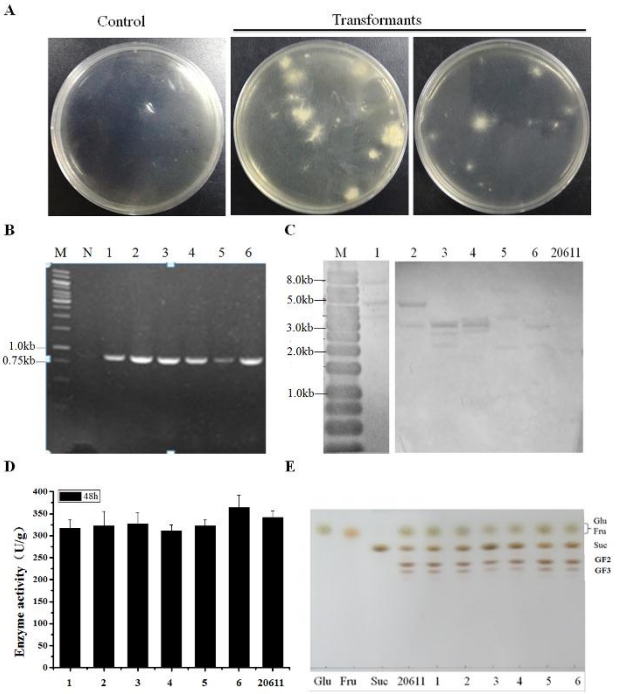
Fig. 2. Transformation of *A. niger* ATCC 20611 using *ptrA* as the selectable marker. (A) The growth plates of *A. niger* ATCC 20611 transformants. The protoplasts were transformed with plasmid pME2892 and grown in regeneration medium containing 2 μg/ml of pyrithiamine for 3 days at 30℃. The control is the plate where only protoplasts were grown. (B) PCR analysis of the *ptrA* transformants. Chromosomal DNA was isolated from six randomly selected transformants (1-6) and analyzed by amplification of 853 bp of *ptrA*. The *A. niger* ATCC 20611 was used as a negative control (N). (C) Southern analysis of the *ptrA* transformants. The chromosomal DNA of the transformants (1-6) and the parental strain (N) was digested with *Hind* III, which is a non-cutter restriction enzyme for the *ptrA* gene, and probed with 853 bp of the *ptrA* fragment. (D) The activities of β-fructofuranosidase produced from the *ptrA* transformants (1-6). (E) TLC analysis of the FOS products synthesized by the transformants (1-6) and *A. niger* ATCC 20611 under standard enzyme assay conditions. Glu, glucose; Suc, sucose; Fru, fructose; GF_2, 1-kestose; GF_3, nystose.

Fig. 3. Co-expression of the enhanced green fluorescent protein-encoding gene (*egfp*) with *ptrA***.** (A) PCR analysis of *A. niger* ATCC 20611(N) and seven randomly selected *ptrA-egfp* co-transformed strains (1-7). Chromosomal DNA of *A. niger* ATCC 20611 and transformants was analyzed by amplification of 853 bp of *ptrA* and 720 bp of *egfp*. (B) Detection of EGFP expression by fluorescent microscopy. Pictures show the typical *A. niger* mycelium from the *ptrA-egfp* transformant after 10 h of incubation investigated by light and fluorescent microscopy.

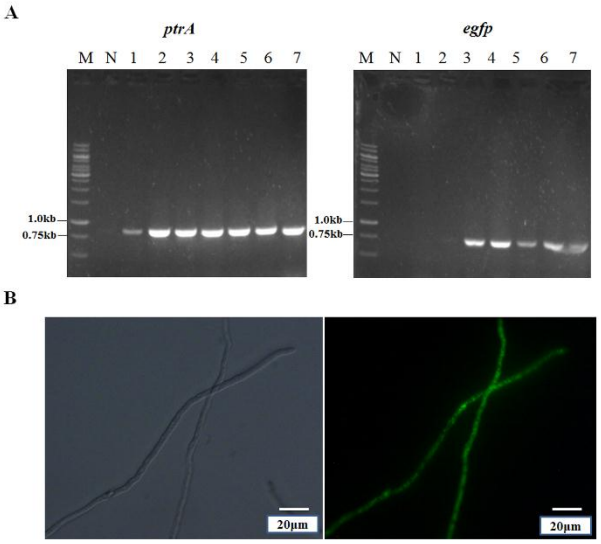
Fig. 4. Overexpression of FopA(A178P) in *A. niger* ATCC 20611**.** (A) Schematic representation of FopA(A178P)-overexpression cassette, which contained the promoter of *gpdA* (P*gpdA*), the FopA(A178P)-encoding gene (*mfopA*) and the terminator of *trpC* (T*trpC*). The fragments of P*gpdA* and T*trpC* were amplified from plasmid pAN7-1, respectively, and the *mfopA* gene, in which the nucleotide G at 532 site was mutated to C, was amplified from the genome of *A. niger* ATCC 20611 as described in Methods. (B) The β-fructofuranosidase activities produced from three *mfopA*-expression transformants (CM2, CM6 and CM9) and *A. niger* ATCC 20611. The activities were measured after 48h incubation at fermentation medium. Data are represented as the mean of three independent experiments. Error bars denote the standard deviations.



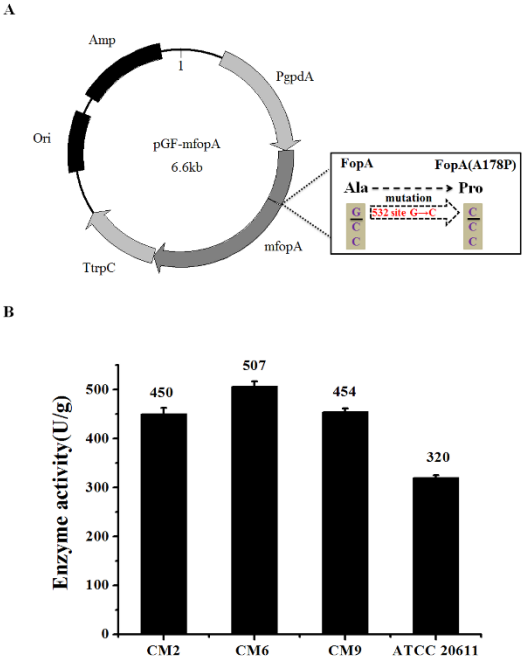
Figr-1



Figr-2



Figr-3



Figr-4