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Development of a strategy for the screening of α-glucosidase-producing microorganisms

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α-Glucosidase is a crucial enzyme for the production of isomaltooligosaccharide. In this study, a novel method comprising eosin Y (EY) and α-D-methylglucoside (AMG) in glass plates was tested for the primary screening of α-glucosidase-producing strains. First, α-glucosidase-producing *Aspergillus niger* strains were selected on plates containing EY and AMG based on transparent zone formation resulting from the solubilization of EY by the hydrolyzed product. Conventional methods that use trypan blue (TB) and *p*-nitrophenyl-α-D-glucopyranoside (pPNP) as indicators were then compared with the new strategy. The results showed that EY-containing plates provide the advantages of low price and higher specificity for the screening of α-glucosidase-producing strains. We then evaluated the correlation between the hydrolytic activity of α-glucosidase and diffusion distance, and found that good linearity could be established within a 6–75 U/ml enzyme concentration range. Finally, the hydrolytic and transglycosylation activities of α-glucosidase obtained from the target isolates were determined by EY plate assay and 3,5-dinitrosalicylic acid-*Saccharomyces cerevisiae* assay, respectively. The results showed that the diameter of the transparent zone varied among isolates was positively correlated with α-glucosidase hydrolytic activity, while good linearity could also be established between α-glucosidase transglycosylation activity and non-fermentable reducing sugars content. With this strategy, 7 *Aspergillus niger* mutants with high yield of α-glucosidase from 200 obvious single colonies on the primary screen plate were obtained.

Keywords: α-D-methylglucoside, α-glucosidase, eosin y, hydrolysis, transglycosylation, *Aspergillus niger*

Introduction

α-Glucosidases (EC 3.2.1.20) are glycoside hydrolases belonging to the glycoside hydrolase (GH) family, of enzymes that connect activated glycosyl donors with acceptors. These enzymes catalyze the formation of α ataly linkages (Shimba *et al*., 2009), producing isomaltosyl oligosaccharides, are mainly from family GH13, GH31 and less extent in family GH4, GH63, GH97, and GH122 according to Carbohydrate-Active Enzymes database (CAZy) (Bissaro *et al*., 2015; Gutiérrez-Alonso *et al*., 2016; Hleap and Blouin, 2018). The industrial value of α-glucosidase has been demonstrated by its ability to hydrolyze various substrates, such as maltose and starch, to produce isomaltooligosaccharide (IMO), a prebiotic with biological characteristics (Fernández Arrojo *et al*., 2007).

α-Glucosidase exhibits hydrolytic activity, hydrolyzing the terminal, non-reducing, (1→4)-linked α-glucose residues of its substrates (Song *et al*., 2013; Tagami *et al*., 2015). Numerous α-glucosidase-producing strains have been isolated and screened based on their hydrolytic properties, including *Bacillus subtilis* HTG (Krohn and Lindsay, 1991), *Xanthophyllomyces dendrorhous* ATCC MYA-131 (Marín *et al*., 2006), and *Cellvibrio japonicus* Agd31B (Larsbrink *et al*., 2012). Conventional methods to screen for α-glucosidase-producing microorganisms are based on agar-containing plates, combined with the addition of iodine or *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) (Suzuki *et al*., 1976; Zhou *et al*., 2009; Chen *et al*., 2011). Although some excellent strains have been isolated using these methods, they still have some limitations. For example, plates containing soluble starch or *p*NPG require the addition of an iodine solution or alkaline solutions (sodium carbonate or sodium hydroxide) to provide an alkaline environment, resulting in damage to the target strain. Even though trypan blue (TB) was introduced to replace iodine (Chen *et al*., 2011), the reaction is still susceptible to interference by amylase activity. Moreover, *p*NPG, commonly used as an indicator of α-glucosidase hydrolysis, is expensive and rapidly self-decomposes, which limits the scope of its applications. In addition, the clear zone displayed in blurred yellow fluorescence produced through *p*NPG hydrolysis is difficult to detect in medium that exhibits background color. Therefore, a simple and specific method need be established to overcome these limitations.

α-Glucosidase from different organisms and GH families exhibit a wide variety of catalytic properties (Gutiérrez-Alonso *et al*., 2016; Hleap and Blouin, 2018), which have been exploited for the production of glucose, IMO (Basu *et al*., 2016), and alkyl glucose (Tanaka *et al*., 2002) based on the specific hydrolytic and transglucosylation activities of

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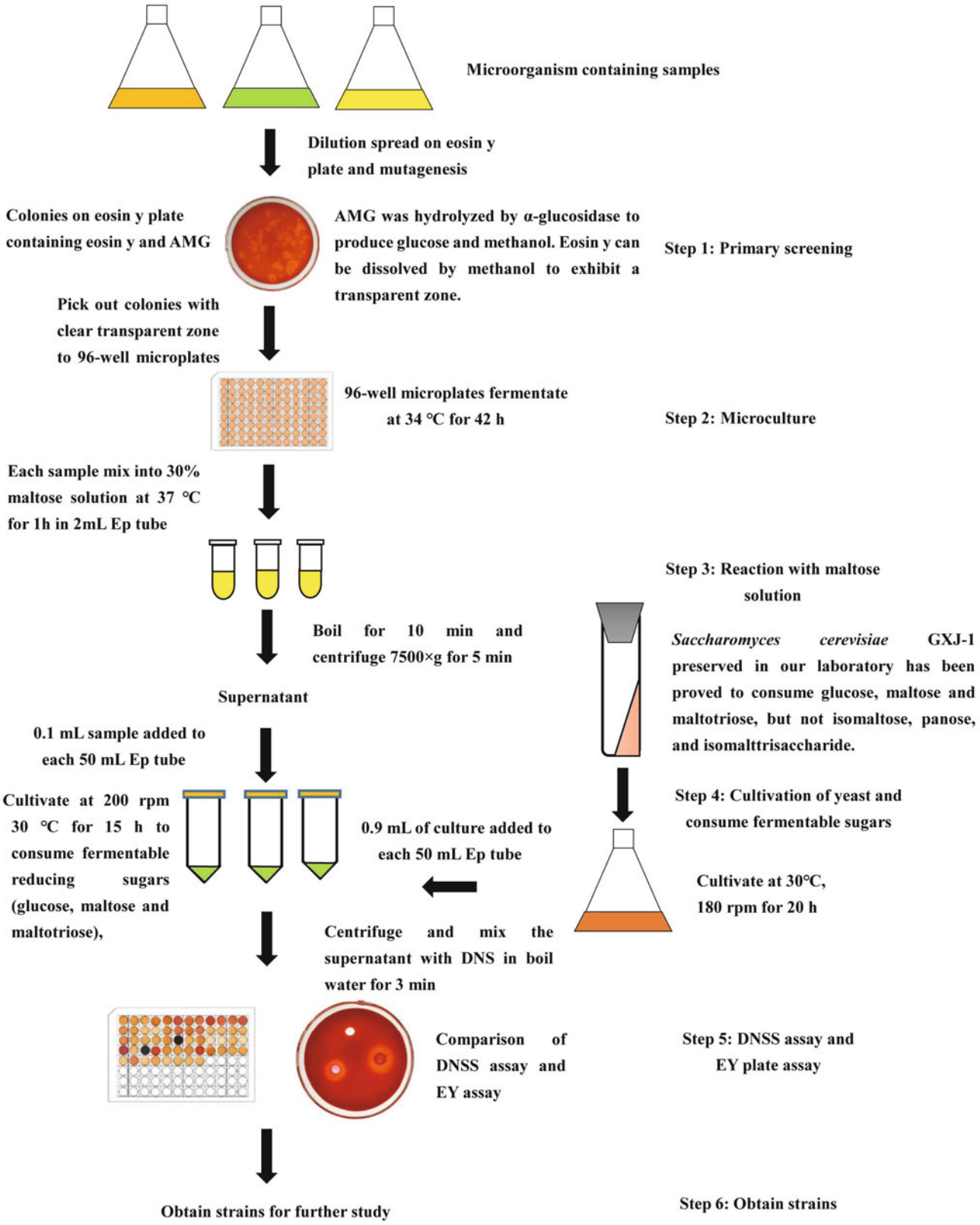

Fig. 1. The integrated screening process of α-glucosidase-producing strains. Step 1: primary screening; Step 2: microculture; Step 3: reaction with maltose solution; Step 4: cultivation of yeast and consume fermentable sugar; Step 5: DNSS assay and EY assay; Step 6: obtain strains.

α-glucosidase. Conventional methods used to detect transglycosylation are based on thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Pan and Lee, 2005; Zhang *et al*., 2010; Basu *et al*., 2016). Although these methods are intuitive and accurate, their application to large-scale fusant screening is hindered by a high cost and low efficiency. Moreover, no studies have used random mutagenesis methods to improve α-glucosidase productivity and properties, possibly owing to the lack of an economical, high-throughput screening strategy. Therefore, it is necessary to develop an efficient and reliable screening strategy by taking both transglycosylation activity and hydrolysis activity into account.

Here, we have proposed an integrated screening strategy to overcome the above-mentioned limitations. First, a primary screen for α-glucosidase-producing microorganisms was performed using eosin Y (EY) dye as an indicator in combination with the α-glucosidase-specific substrate, α-D-methylglucoside (AMG). The strains isolated in the primary screen were then cultured in 96-well microplates, following which transglycosylation and hydrolysis levels were measured by 3,5-dinitrosalicylic acid-*S. cerevisiae* (DNSS) assay and EY plate assay, respectively. Finally, this method was applied to screen for mutants exhibiting increased α-glucosidase transglycosylation and hydrolytic activity. The detailed procedure of this integrated strategy is illustrated in Fig. 1.

Materials and Methods

Microorganisms and reagents

The *Aspergillus niger* F86 strain, isolated from *A. niger* M-1 mutants that can produce intracellular α-glucosidase with high transglycosylation activity (CCTCC M 2014421), was preserved in our laboratory (Zhang *et al*., 2011). The *S. cerevisiae* GXJ-1 strain, was a domesticated strain preserved in our laboratory that can consume fermentable sugars, including glucose and maltose, while barely utilizing IMO (Chen *et al*., 2011). Transglucosidase L was obtained from Amano, α-amylase (EC 3.2.1.1) and *p*NPG were obtained from Sigma-Aldrich. Eosin Y and trypan blue were purchased from Sangon Biotech. All other chemicals used in this study were reagent grade.

Culture conditions

Spores of *A. niger* F86 strain were grown on solid medium 3% (v/v) wheat bran extract, 30 g/L maltose, and 20 g/L agar, pH 5.0. EY plates contained 40 g/L AMG, 15 g/L peptone, 2 g/L yeast extract, 2 g/L sodium nitrate, 0.5 g/L dipotassium phosphate, 0.25 g/L anhydrous calcium chloride, and 20 g/L agar, with an initial pH of 5.0 ± 0.1. Typically, 0.3% (w/v) alcohol-soluble EY was uniformly distributed into the medium. The carbon source for the TB and *p*NPG plates was 1% (w/v) soluble starch. The fermentation medium contained 80 g/L corn starch, 20 g/L corn steep liquor, and 0.1 g/L potassium chloride, pH 5.0 ± 0.1. *S. cerevisiae* GXJ-1 strain GXJ-1 was cultured on yeast extract peptone dextrose medium (YPD): 20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, and 20 g/L agar, pH 5.0 ± 0.1.

Construction of mutation library

Spores harvested from solid medium were suspended in 0.85% (w/v) sterile saline and diluted to 10^8 spores/ml. The suspension containing the ungerminated spores was irradiated at 800 W microwave output power (5 sec of irradiation followed by 10 sec in an ice bath, 480 sec of irradiation in total). The spores were then germinated at 34°C for 3 h and exposed to ultraviolet radiation at 253.7 nm for 200 sec. The mutation library was stored at 4°C for further screening.

Primary screening for α-glucosidase-producing mutants

Lithium chloride (0.8% [w/v]) was added to plates for mutant screening. After 40 h of incubation at 37°C, all the surviving colonies containing a specific transparent zone resulting from the interaction with the acid dye were considered as high-yielding, α-glucosidase-producing strains and selected for further analysis. The clear zones were compared among the EY, TB, and *p*NPG plates.

Microbial culture

Rapidly growing single colonies surrounded by clear, transparent zones were picked from the plates, inoculated into 96-well microplates containing 200 μl of fermentation medium per well, and cultured at 34°C for 42 h.

The *S. cerevisiae* GXJ-1 strain was inoculated into a 3,000 ml flask containing 600 ml of YPD without agar, cultured at 30°C with shaking at 180 rpm for 20 h, and then used to consume the fermentable sugars.

Analytical methods

Starch hydrolysis was measured using 3,5-dinitrosalicylic acid (DNS) and the hydrolytic activity of α-glucosidase was measured by *p*NPG assay (Coleri *et al*., 2009; Nwokoro and Anthonia, 2015). An aliquot (10 μl) of the fermented sample was added to an equal volume of 5 mM *p*NPG precooled at 4°C for 30 min into a 96-well microplate. Then, 90 μl of citrate phosphate buffer (20 mM, pH 4.5) preheated at 37°C for 30 min was added to each well, and the mixture was incubated for 15 min at 37°C in a spectrophotometric microplate reader. Then, 100 μl of 1 M pre-cooled sodium carbonate was immediately added to the 96-well plate. The absorbance of *p*-nitrophenol (PNP) was measured at 405 nm using a spectrophotometric microplate reader. One unit of α-glucosidase activity was defined as 1 μmol of released PNP per min. A standard curve was generated by plotting the average blank-corrected absorbance of PNP versus its concentration in micromole per liter (0–100 μmol/L).

The hydrolytic activity of α-glucosidase was also determined by measuring the diffusion distance of the transparent zone on EY plates. In brief, 15 ml of 0.3% EY and a 2% agar-deionized water solution were used to prepare one 9-cm-diameter agar-containing petri dish, and 4-mm diameter holes were made in the solidified agar using a puncher. Samples (50 μl) from selected mutants were pipetted into the holes. After a 1 h incubation at 37°C, the diffusion distance of the transparent zones was measured using a vernier caliper. A correlation between hydrolytic activity and diffusion distance was developed by comparing the *p*NPG and EY plate assays.

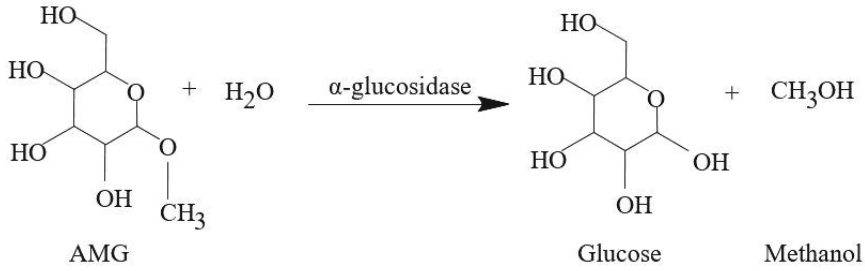

Fig. 2. The degradation of AMG by α-glucosidase.

The yield of non-fermentable reducing sugars, including isomaltose, panose, and isomaltotriose, was measured by 3,5-dinitrosalicylic acid-*S. cerevisiae* (DNSS) assay to determine the transglycosylation activity of α-glucosidase (Chen *et al.*, 2011). In brief, 30% maltose was mixed into each sample and incubated for 1 h at 37°C. The reaction was terminated by placing the sample in a boiling water bath for 10 min, and then a 9-fold volume of yeast culture was added to consume the fermentable sugars. The yield of non-fermentable reducing sugars was measured by DNS assay after incubation at 30°C for 15 h with shaking (200 rpm). A standard curve was generated by plotting the average blank-corrected absorbance of glucose at 540 nm versus its concentration in micromole per L (0.25–2.5 μmol/L). One unit of transglycosylation activity was defined as the synthesis of 1 micromole non-fermentable reducing sugars per min.

Non-fermentable reducing sugar yield was also analyzed by ion-exchange chromatography using an HPLC system equipped with an Ecosil NH_2 column (5 μm, 4.6 mm × 250 mm) and a Waters Sugar-Pak 1 column (10 μm, 6.5 mm × 300 mm) as previously reported (Chen *et al.*, 2011). Glucose, maltose, isomaltose, maltotriose, panose, and isomaltotriose were used as the standards.

Results

Optimization of primary screening conditions

α-Glucosidase specifically catalyzes the hydrolysis of α-glucosidic bond from the non-reducing end of its substrates (Charron *et al.*, 1986). Eosin Y, a macromolecular complex, is well known for its ability to dye proteins and compounds through electrostatic interactions (Jones *et al.*, 1984; Lin *et al.*, 1991; Jin *et al.*, 2018). In this report, AMG, a α-glucosidase-specific substrate that can be degraded to glucose and methanol by this enzyme, was used to detect target strains. The α-glucosidase-catalyzed hydrolysis of AMG is illustrated

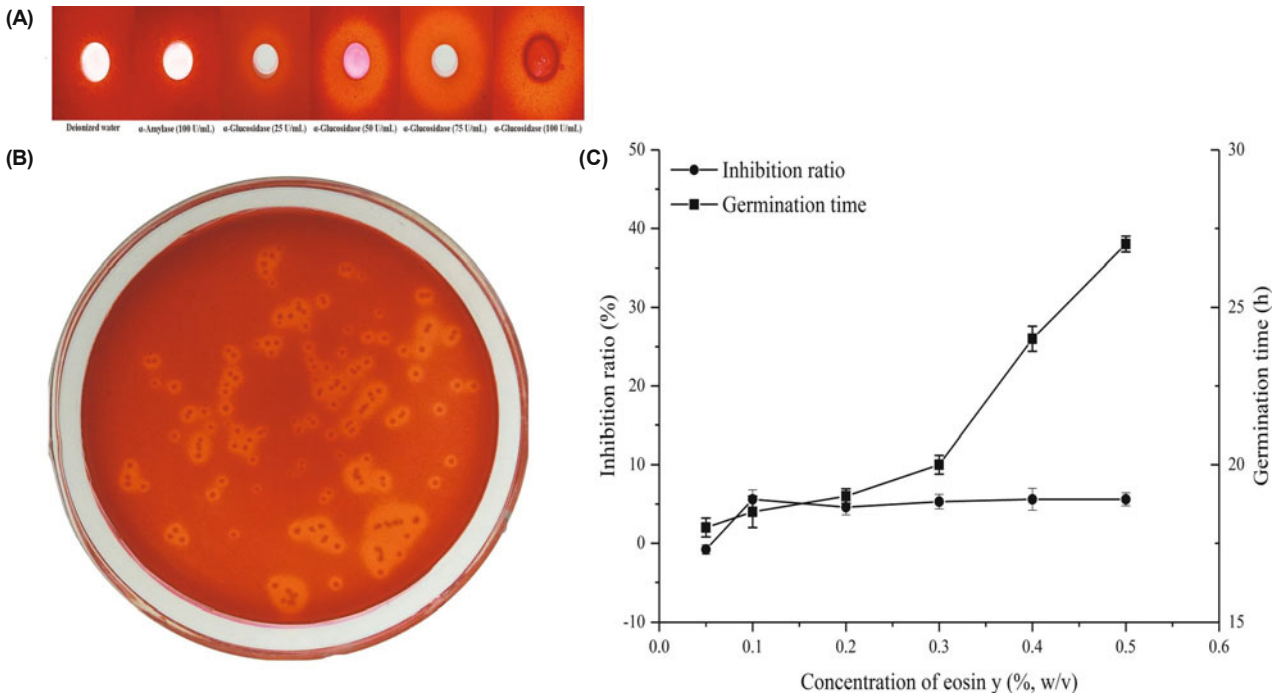

Fig. 3. (A) A comparison of the transparent zone on the primary screen plate. From left to right are deionized water, α-amylase (100 U/ml), α-glucosidase (25 U/ml, 50 U/ml, 75 U/ml, and 100 U/ml). The control was deionized water placed 50 μl into the hole (4 mm in diameter). (B) α-Glucosidase-producing colonies in isolation plates containing acidic dye EY (0.3%, w/v). (C) The correlation between inhibition rate of strains, germination time of spores, and concentration of EY. The data represent mean values of triplicate and independent experiments.

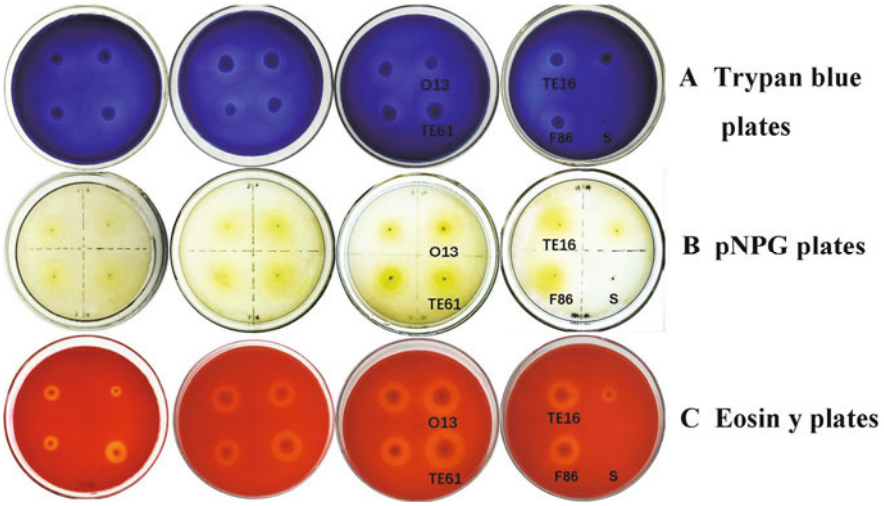

Fig. 4. The detection of hydrolysis activity of α-glucosidase from fourteen random mutants on agar plates. *A. niger* F86 (F86) and *S. cerevisiae* GXJ-1 (S) were used as controls, *A. niger* O13 (O13), *A. niger* TE61 (TE61) and *A. niger* TE16 (TE16) were positive mutants and the unmarked strains were false or negative mutants, all strains were incubated at 37°C for 40 h. (A) Plates contained 0.02% typany blue. (B) Plates contained 0.05% *p*NPG, incubated plates were flooded with 1 M sodium carbonate for 3 min and washed three times by deionized water. (C) Plates contained 0.3% eosin Y.

in Fig. 2. Following AMG degradation by α-glucosidase, eosin Y was dissolved by methanol, a product of AMG hydrolysis, and a transparent zone formed around α-glucosidase-producing colonies in the plate. In addition, transglucosidase L (100 U/ml) was also pipetted into EY plates (Fig. 3A), and reflected the hydrolytic activity of α-glucosidase. In contrast, deionized water and α-amylase (100 U/ml) did not produce a transparent zone. Clear zones, representing solubilization of the acidic dye, appeared around the colonies when *A. niger* F86 was grown on primary screening plates (Fig. 3B), indicating that the α-glucosidase hydrolytic activity of the samples can be determined by the size of the transparent zone.

Because eosin Y is an acidic dye and can inhibit the growth of microorganisms, the initial concentration of dye required optimization. The dye inhibition rate was less than 6% at EY concentrations ranging from 0.01% to 0.5% (Fig. 3C). However, the time of germination and growth of *Aspergillus niger* strain F86 was delayed in an EY-concentration-dependent manner, and the spore production time increased with increasing concentration of the dye. Compared with the control (without dye), the germination time increased from 18–27 h at an EY concentration of 0.5%, and the time required for spore production increased from 40–72 h. At dye concentrations below 0.25%, the medium in the plates was too transparent to observe the formation of transparent zones. In contrast, with a dye concentration of 0.4%, the medium was too dark to visualize the transparent zone in the early stages of growth. Based on these results, 0.3% eosin Y was selected as the optimal screening concentration. By doing so, it is possible to simultaneously detect more than 100 colonies on each plate. Therefore, this plate method is suitable for screening a large number of samples.

Table 1. The hydrolytic activity and transglycosylation activity of mutants from isolated α-glucosidase-producing *Aspergillus niger* strains

Strain	Hydrolytic activity[a] (U/ml)	Transglycosylation activity[b] (U/ml)
H9-30	12.52 ± 0.88	318.18 ± 4.71
TE61	12.42 ± 0.72	312.37 ± 5.12
O13	11.73 ± 0.28	305.16 ± 6.83
TE7	11.13 ± 0.21	300.83 ± 2.74
TW16	11.36 ± 0.66	299.53 ± 6.88
N32	11.09 ± 0.87	288.28 ± 2.13
TE16	11.07 ± 0.42	286.47 ± 4.42
F86[c]	8.43 ± 0.33	220.66 ± 5.77

[a] Determined by *p*NPG assay
[b] Determined by DNSS assay
[c] Original strain

Comparison of TB, *p*NPG, and EY plates

Fourteen mutants were randomly screened for α-glucosidase hydrolytic activity on TB, *p*NPG, and EY plates to test the efficacy of the developed plate assay. As shown in Fig. 4A, all 14 isolates produced clear zones when grown on TB plates, indicating that the soluble starch had been hydrolyzed. The diameters of the clear zones on the TB plates were similar among the different strains. However, the results for the α-glucosidase hydrolytic activity of these fourteen strains on the TB plates showed that there were three positive mutants (O13, TE61, and TE16; Table 1) distributed in the plates of the third and fourth columns, and the rest were either false or negative mutants. Moreover, the α-glucosidase produced by the *A. niger* F86 strain has been proved cannot hydrolyze soluble starch (Zhang *et al.*, 2011). The amylase difference of all strains was less than 0.32 U/ml, and the activity of F86 amylase was 3.58 U/ml (date not shown). These results indicated that the use of TB plates to screen for α-glucosidase-producing strains was not so reliable.

These experiments were repeated on *p*NPG plates (Fig. 4B) and EY plates (Fig. 4C). All the strains showed specific hydrolysis zones on the plates, and the clear zones matched the results of hydrolytic activity. However, the blurred ranges generated by PNP were difficult to distinguish under natural light. A maltose analog (*p*NPG) was added to the plate as a substrate and the production of PNP, which generates a yellow color, can be detected by spectroscopy in an alkaline environment containing sodium carbonate or sodium hydroxide (Krolicka *et al.*, 2018). Therefore, it is only commonly used for detection of enzyme properties and rarely added to plate culture medium for preliminary screening of α-glucosidase-producing microorganisms owing to spontaneous

decomposition, high price, and limited conditions (Li *et al.*, 2018). These disadvantages can be overcome by using AMG instead of *p*NPG as a specific substrate of α-glucosidase. In addition, compared with the 50 USD/g market price (Sigma-Aldrich) for *p*NPG, the cost of AMG is 0.2 USD/g, which reduces the screening cost in a large extent.

Correlation between traditional methods and the developed method

Correlation between spectrophotometric and EY plate assays : An equal volume of transglucosidase L (0–200 U/ml) was added to the holes in EY plates to test the correlation between hydrolytic activity and diffusion distance. Analyses of the diffusion distance and hydrolytic activity (Fig. 5A) indicated that the relationship between enzyme activity and diffusion distance was not a positive linearity singly. It was not in proportion with the enzyme activity between 0–4 U/ml, while a good linear relationship could be observed between 6–75 U/ml, with a linear correlation coefficient greater than 0.99. The linear relationship was lost again at 75 U/ml, and a blurred and irregular transparent circle could be seen at 100 U/ml (Fig. 3A). This suggests that the concentration of AMG was relatively low compared to enzyme activity and exceeded the detection range of the assay. In addition, the detection limit of α-glucosidase was 0.07 U/ml which calculated by the quantities' results (S/N=3). These results above suggested that the EY plate assay could be applied to rapidly determine the hydrolytic activity of α-glucosidase during the screening process for α-glucosidase producing strains.

A calibration curve for the *p*NPG assay was generated based on the average blank-corrected absorbance of PNP versus its concentration in micromole per liter (Fig. 5B) which showed a good correlation coefficient of 0.99965. The correlation between the traditional *p*NPG assay and the EY plate assay was explored to determine whether the results of the EY plate assay could represent the hydrolytic activity of the strains. Twenty-eight mutants with clear transparent zones on the EY plates were randomly screened out and the correlation efficiency between the spectrophotometric and EY plate assays was examined. As shown in Fig. 5C, there was a positive correlation (Adj. R-Square = 0.93261) between the diffusion distance and hydrolytic activity. The results suggested that

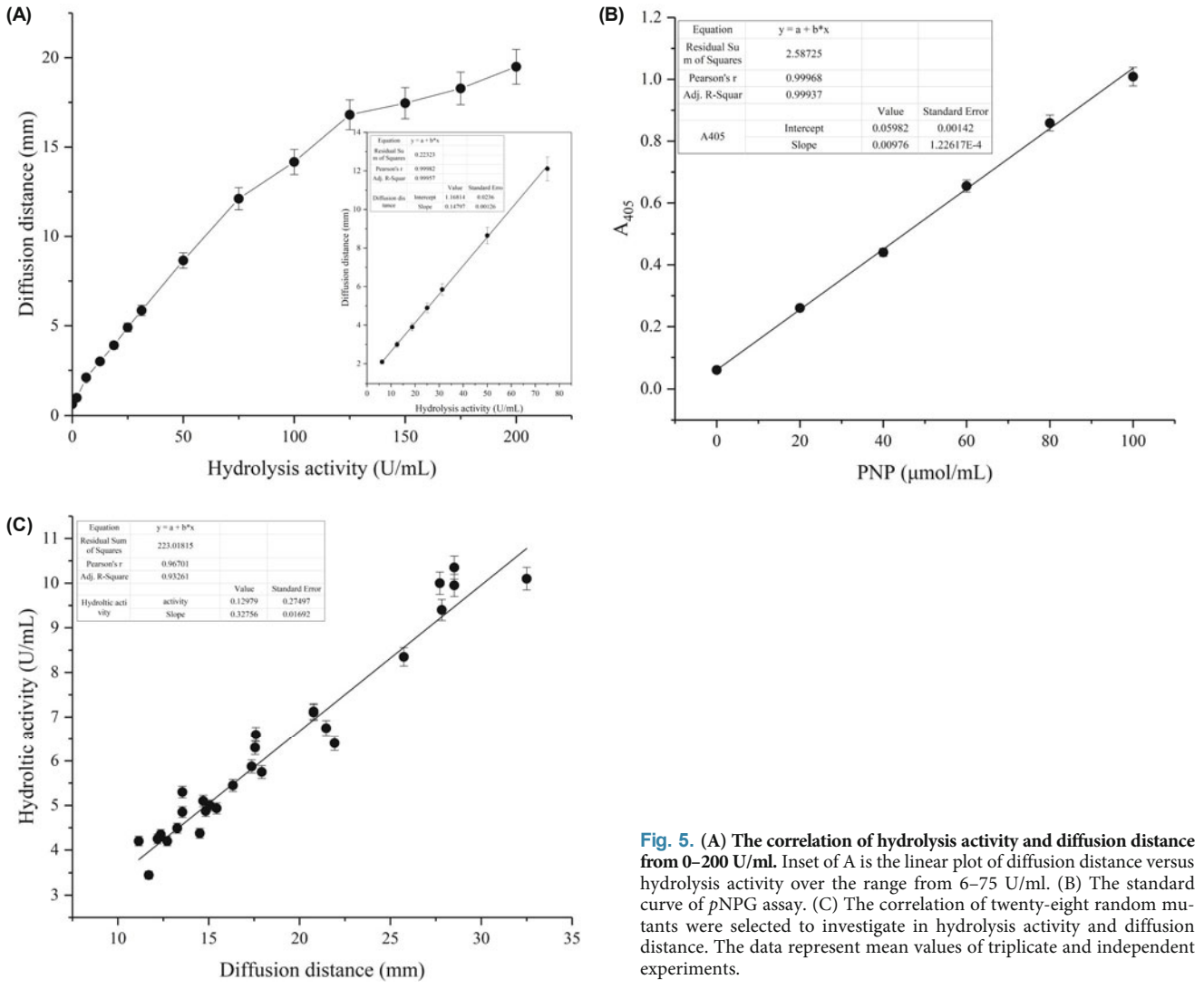

Fig. 5. (A) The correlation of hydrolysis activity and diffusion distance from 0–200 U/ml. Inset of A is the linear plot of diffusion distance versus hydrolysis activity over the range from 6–75 U/ml. (B) The standard curve of *p*NPG assay. (C) The correlation of twenty-eight random mutants were selected to investigate in hydrolysis activity and diffusion distance. The data represent mean values of triplicate and independent experiments.

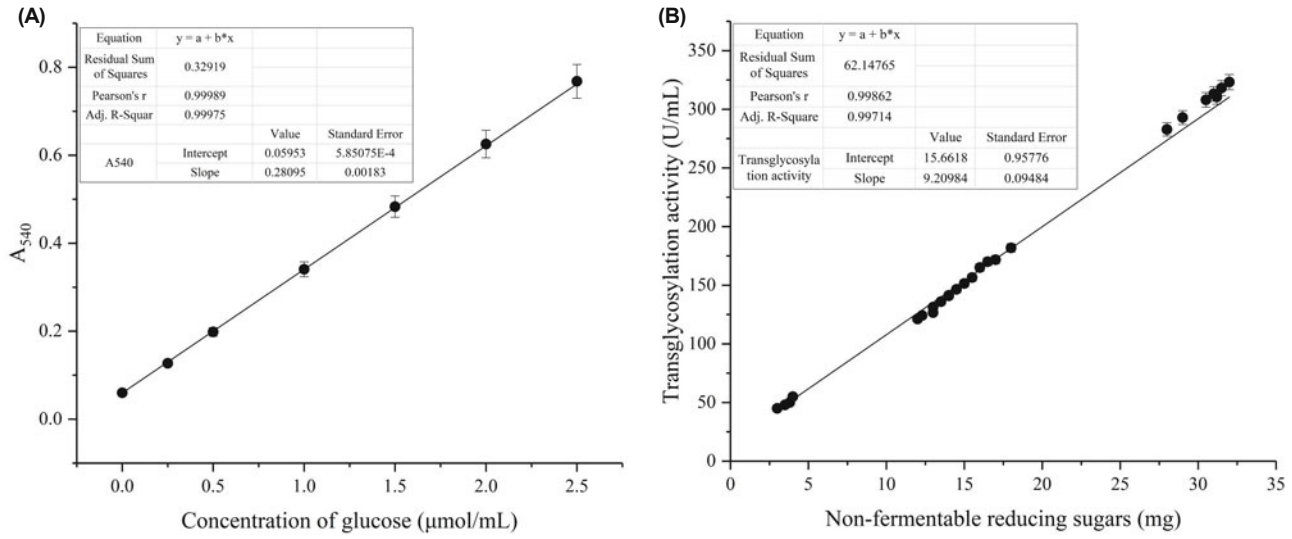

Fig. 6. (A) The standard curve of DNS assay. (B) The correlation of twenty-eight random mutants were selected to investigate in non-fermentable reducing sugars and transglycosylation activity. The data represent mean values of triplicate and independent experiments.

the EY plate assay was effective for the screening of α-glucosidase-producing microorganisms.

Correlation between non-fermentable reducing sugars and transglycosylation activity : To further define the transglycosylation activity of the enzyme, 28 colonies were also selected to measure the transglycosylation activity and the correlation efficiency between the HPLC and DNSS assays was examined. The calibration curve of the DNSS assay is shown in Fig. 6A. As demonstrated in Fig. 6B, the yield of non-fermentable reducing sugars and transglycosylation activity presented a good correlation (Adj. R-Square = 0.99714). This result suggested that the DNSS assay was suitable for the quantification of α-glucosidase transglycosylation activity and could replace the HPLC assay.

Screening for α-glucosidase-producing microorganisms using the developed strategy

Using this screening strategy, approximately 6,000 single *A. niger* F86 strain mutants producing α-glucosidase were identified on the EY plates. According to the size of the transparent circles, 200 strains were selected and cultured in fermentation medium, and 7 colonies with high α-glucosidase activity were shown in Table 1. Of which, *A. niger* H9-30 showed the highest hydrolytic and transglycosylation activities, which were 48.5% and 44.2% higher than those of the original strains, respectively.

Discussion

Compared with conventional screening methods (Suzuki *et al.*, 1976; Nakao *et al.*, 1994; Fernández-Arrojo *et al.*, 2007; Ganzlin and Rinas, 2008; Chen *et al.*, 2011), the screening approach reported in this study presented several important advantages. First, our method could effectively isolate α-glucosidase-producing strains on EY plates, while the single and unique carbon source prevented contamination by non-α-glucosidase-secreting microorganisms. Second, this method can be used to screen a large number of samples more economically than conventional methods. Finally, α-glucosidase hydrolytic and transglycosylation activity can be rapidly detected when combined with a DNSS assay, thereby improving screening efficiency and throughput. In brief, this strategy can be used for the rapid and cost-effective screening of strains producing α-glucosidase of high yield and differing catalytic properties.

Numerous dyes, such as methylene blue (Nishikawa and Ogawa, 2002) and neutral red (Zeng *et al.*, 2013), are commonly used for the screening of microorganisms. To the best of our knowledge, this is the first study to use a combination of EY and AMG to screen for α-glucosidase-producing strains. In the report of Nishikawa and Ogawa (Nishikawa and Ogawa, 2002), the growth of the single colony was inhibited by acidic dye. However, in our study, this inhibition was not observed, even at a 0.5% EY concentration. In the primary screen, a linear relationship was maintained between the diffusion distance and hydrolytic activity ranging from 6 U/ml to 75 U/ml. However, this relationship was lost (Fig. 5A) as the enzymatic activity exceeded 75 U/ml, and an irregular, gelatinized, hydrolytic circle was observed at 100 U/ml (Fig. 3A). This suggests that, at 100 U/ml, the enzymatic activity exceeded the detection range due to a lack of substrate (Lee *et al.*, 2001). Linearity was reestablished when the concentration of AMG in the medium was increased. Both the AMG content and detection range greatly influence its practical application, which should be considered before screening. In this report, 4% was chosen as the optimal AMG concentration and, under this condition, enzymatic activity can be detected within a range of 6–75 U/ml in the EY plate assay.

The diffusion speed and distance can be influenced by temperature (Zeng *et al.*, 2013). In the experiment, there was a better linear relationship between diffusion distance and enzyme activity at 37°C. Additionally, this linear relationship

could be affected by the concentration of agar based on the degree of crosslinking. In this study, 37°C and 2% agar were determined as the optimal conditions for the EY plate assay.

Trypan blue is an acidic dye that is widely used to detect cell membrane integrity and cellular survival (Tennant, 1964; Jauregui *et al*., 1981; Strober, 2001). In addition, a blue complex can be formed by combining polysaccharides, and a transparent circle appears following the hydrolysis of polysaccharide substrates by the corresponding enzymes (Ma *et al*., 2007). Trypan blue is used as an indicator in the screening of polysaccharide hydrolase-producing strains, such as those that producing cellulase, amylase, and α-glucosidase (Ma *et al*., 2000; Margesin *et al*., 2003; Chen *et al*., 2011). Interestingly, starch is also used as a substrate to screen for α-glucosidase and amylase, and also through the visualization of transparent areas (Ma *et al*., 2000; Chen *et al*., 2011). However, most reported α-glucosidases show a preference for hydrolyzing oligosaccharides with a degree of 2-6 polymerization and have a low or no affinity for polysaccharide substrates (Nakao *et al*., 1994; Hostinová *et al*., 2005). In this work, amylase interference was observed in the TB plates and false-positive strains were screened out. This suggested that using TB plates to screen for α-glucosidase-producing strains is likely to be unreliable. In contrast, the EY plates showed strong specificity and reliability, and α-amylase can also be excluded with the EY assay. Moreover, we also observed a good relationship between transparent zones and hydrolytic activity. This indicates that the hydrolytic activity of target strains on EY plates can be determined based on the size of the transparent zones produced by the activity of specific enzymes, and that EY plates are better than TB plates for the screening of α-glucosidase-producing microorganisms.

p-Nitrophenyl-α-d-glucopyranoside in solution is a widely used assay (*p*NPG assay) to determine the hydrolytic activity of α-glucosidase, for which it has a high affinity (K_m is low) (Saha and Zeikus, 1991; Coleri *et al*., 2009; Hu *et al*., 2011). Furthermore, *p*NPG can be degraded by α-glucosidase to produce PNP, which fluoresces yellow in alkaline conditions, and its concentration shows a good linear relationship with the fluorescence intensity at 405 nm, as shown in Fig. 5B (Dej-Adisai and Pitakbut, 2015). A low detection limit was estimated at between 6.6×10^{-4} U/ml to 7.6×10^{-4} U/ml based on the reports of Li and Tang (Tang *et al*., 2017; Li *et al*., 2019). According to the calibration curve (Fig. 5B), a quantitative range from 0–150 U/ml could theoretically be calculated for the *p*NPG assay, which is higher than that for the EY assay (6–75 U/ml), and lower when compared with the detection limit of EY plate assay (0.07 U/ml). However, the *p*NPG assay is not suitable for high-throughput and rapid screening because *p*NPG can be easily degraded and is expensive. The *p*NPG plate method is a modification of the *p*NPG assay, which simplified the operation to some extent. In our study, all the *A. niger* strains showed specific hydrolytic zones with a *p*NPG concentration of 0.05% (w/v). However, the use of *p*NPG plates for the screening of random mutant strains has not been reported, possibly owing to the following reasons: (1) *p*NPG is expensive, costing 250 times more than eosin Y in unit mass; (2) *p*NPG is easily degradable, and reduced the culture time of the strains to less than 60 h; moreover, the detection time was reduced to less than 3 h (data not shown), and the chromogenic reaction could not be detected because of the spontaneous degradation of *p*NPG; (3) both *p*NPG and the associated alkaline environment are harmful to microorganisms, which is not conducive to the selection of target strains for further study. In contrast, the novel EY plate method proposed in this study could overcome these shortcomings, is simple to operate, and is more suitable for high-throughput screening.

In this study, two rapid characterization methods were used to distinguish and screen for α-glucosidase exhibiting different catalytic properties. Hydrolytic activity can be examined by the EY plate assay proposed in this report. DNS is an important method to measure reducing sugar levels (Tanaka *et al*., 2002). Non-fermentable reducing sugars and transglycosylation activity presented a good correlation, as determined by HPLC and DNSS assays. This indicates that the DNSS assay can represent transglycosylation activity through the levels of non-fermentable reducing sugars. Importantly, this step can significantly increase the throughput and efficiency of sample analysis relative to HPLC and TLC assays.

In summary, we developed a specific, simple, and convenient plate method for the screening of α-glucosidase-producing microorganisms. In this study, in a primary screen, 200 colonies surrounded by clear, transparent zones were selected, from which seven strains with high enzymatic activity were screened out. Our results indicated that this strategy can be widely used to screen for α-glucosidase-producing microorganisms.

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Conflict of Interest

The authors declare that have not any conflict of interest in this work.

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