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Polyploid Formation Between *Aspergillus niger* and *Trichoderma viride* For Enhanced Citric Acid Production From Cellulose

Ramida Watanapokasin · Nitisak Sawasjirakij · Shoji Usami · Kohtaro Kirimura

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Abstract The first-stage heterokaryons, obtaining from intergeneric protoplast fusion between *Aspergillus niger* (Y-b) and *Trichoderma viride* (M5S51), showed slow growth and mixed morphologies on minimal medium. The fusants were classified into heterokaryon and prototrophic haploid, showing the morphology as that of *A. niger*. The heterokaryon strains formed conidia with the same nutritional requirements as those of the original auxotrophic mutant strains. After several subcultivations on minimal medium containing *d*-camphor, some heterokaryon strains formed larger two to seven nuclei/conidium as compared to one nucleus/conidium of the auxotrophic mutant and prototrophic strains, indicating that the new hybrids were generated. Interestingly, three fusant strains AT 11-2-3, AT 11-2-10, and AT 11-2-14 produce 19.2, 6.1, and 10.5 g/l citric acid, respectively, in semisolid culture containing cellulose, whereas *A. niger* Yang no. 2 could not use carboxymethyl cellulose as the sole carbon source for citric acid production. In addition, the average maximum β-glucosidase and carboxymethylcellulase productions from AT 11-2-3, AT 11-2-10, and AT 11-2-14 were about 16- and 4-folds higher than those of *A. niger*, respectively.

Keywords *Aspergillus niger* · Intergeneric fusant · Citric acid production · Polyploid · *Trichoderma viride*

R. Watanapokasin (✉)
Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University,
Bangkok 10110, Thailand
e-mail: ramidawa@yahoo.com

N. Sawasjirakij
Faculty of Information Technology, North Bangkok College, Bangkok 10220, Thailand

S. Usami · K. Kirimura
Department of Applied Chemistry, School of Science and Engineering, Waseda University,
Tokyo 169-8555, Japan

Introduction

Citric acid is one of the heavily utilized commodities, which mainly produced from molasses or starch hydrolysates by a fermentation process with the filamentous fungus, *Aspergillus niger* [1, 2]. Although, there is a high demand of citric acid used each year, it is provided at a low price as a commodity chemical. Therefore, it is necessary to establish a method for citric acid production from inexpensive and readily available raw materials in industrial processes [3–8] to achieve technical and economical benefits.

Cellulose is the most abundant organic polymer [9] on the planet and is an important energy renewable source along with sugars and starches. Cellulose represents the major constituent of plant cell wall polysaccharides and consists of a linear polymer of β-1, 4-linked D-glucose residues [10]. Complete degradation of cellulose requires a large number of extracellular enzymes working synergistically to allow hydrolysis to smaller oligosaccharides and finally to the corresponding monomers. Four classes of enzymes, cellulase system, are involved in the biodegradation of cellulose. Endoglucanases (EC 3.2.1.4) hydrolyze cellulose to glucooligosaccharides. Cellobiohydrolases (EC 3.2.1.91) release cellobiose from crystalline cellulose. β-Glucosidases (EC 3.2.1.21) degrade the oligosaccharides to glucose. Exoglucanases release glucose from cellulose and glucooligosaccharides. The difference between exoglucanases and cellobiohydrolases is not always clear because of differences in the methods used to study these enzymes [11–17]. Enzymatic hydrolysis of cellulose is of major importance in both natural and engineered systems in which utilization of cellulosic biomass occurs. In particular, biologically mediated cellulose hydrolysis could be widely used to sustainably produce fuels and chemicals.

Thus, this work aims to study citric acid production from cellulose, which is mainly scrapped without use as agricultural waste, especially in Southeast Asian countries. At present, direct production of citric acid from cellulose has not yet succeeded, because *A. niger* usually cannot degrade nor assimilate crystalline cellulose as a sole carbon source. *Trichoderma* species in particular, are well known as efficient producers of plant cell wall-degrading systems, cellulolytic and xylannolytic activities [18–20].

As genetic manipulation for strain improvement in *A. niger* has been hampered by the lack of a sexual cycle, therefore, genetic recombination via the parasexual cycle was used for genetic studies. Protoplast fusion in filamentous fungi has proved to be a highly efficient procedure for obtaining hybrids such as heterokaryons and diploids [21, 22]. Series of studies on the intraspecific protoplast fusion of *A. niger*, aiming to develop a new breeding system, have successfully obtained new strains with enhanced citric acid production or with some advantageous characters, different from those of the parental strains [23–25].

With the aim of breeding new fungi that produce citric acid from cellulolytic materials, 2-deoxyglucose-resistant mutant strains of *A. niger* were induced, showing 49.6 g/l citric acid from 100 g/l cellobiose in semisolid culture [26]. In other trials, the intergeneric protoplast fusion between auxotrophic mutant strains of *A. niger* and *T. viride* known as cellulase producer were also carried out [23, 24]. Two types of intergeneric fusants were successfully obtained. The first type of the fusants was haploid and formed prototrophic conidia containing one nucleus with colonies of an *A. niger*-type morphology. The second type of the fusants was heterokaryon, forming conidia with one nucleus but showing the same nutritional requirements as those of the original auxotrophic mutants. In addition, the fusant strains retained potential abilities for both cellulase production of *T. viride* and citric acid production of *A. niger*. In this report, these fusant strains were named as the first-stage heterokaryons.

Besides industrial application, the fusant strains between *A. niger* and *T. viride* might serve as interesting models for improving the citric acid production from cellulolytic

material. Therefore, the new hybrids derived from the first-stage heterokaryons were further induced and characterized. Although the "new" hybrids were confirmed to be heterokaryons as determined by conidial sizes and DNA contents, they generally formed uniform colonies on the complete, supplemented, and minimal medium (MM) and mostly formed prototrophic conidia containing two nuclei. These properties are distinct from those of the first-stage heterokaryons. The direct production of citric acid from cellulose by the new hybrids in semisolid culture as well as extracellular β-glucosidase and carboxymethylcellulase (CMCase) activities were also obtained.

Materials and Methods

Microorganisms

A. niger Yang no.2, a citric acid hyper-producer in semisolid culture, and *T. viride* WU-36B, the cellulase producer, were used as the prototrophic parental strains. *A. niger* Yang no.2 accumulates yellow pigment in the mycelium, and its conidiophores are sparse especially on complete medium (CM) such as *koji*-extract agar medium (Ballg. 10^0, pH 6.0). *T. viride* WU-36B does not accumulate yellow pigment in the mycelium (white mycelia) and produces abundant green conidia. Some properties of the strains mainly used in this study are shown in Table 1.

Media and Fermentation Conditions

Czapek's medium containing 30 g/l glucose as the sole carbon source was used as the MM or a basal medium. MM containing 2 g/l casamino acid (Difco, USA) and 2 g/l yeast extract (Difco) was used as the supplemented medium (SM) that allows conidiation, detected by conidial size, DNA content, number of nuclei, and stability of colonies. For solidifying, 20 g/l agar was added to each medium. *Koji*-extract agar medium (Ballg. 10^0, pH 6.0) was used as a CM for strain maintenance. A synthetic (SS) medium used for citric acid

Table 1 Properties of *Aspergillus niger*, *Trichoderma viride*, and hybrids used in this study.

Strain[a]	Phenotype	Conidial colour	Sucrose utilization[b]
Parents			
A. niger Yang no.2	Prototrophic	Black	+
T. viride WU-36B	Prototrophic	Green	−
Auxotrophic mutants			
A. niger Y-(b)	$N.a^-$	Brown	+
T. viride M5S51	Leu^-	Green	−
Hybrids			
AT 11-2-3	(Prototrophic)	Brown	+
AT 11-2-10	(Prototrophic)	Brown	+
AT 11-2-14	(Prototrophic)	Brown	+

$N.a^-$ Requirement for nicotinic acid; Leu^- requirement for leucine

[a] *A. niger* Y-(b) and *T. viride* M5S51 were induced from Yang no.2 and WU-36B, respectively, by UV-irradiation [6]. Reversion frequencies of them are very low [6]. The hybrid strains were induced from fusant strains obtained through protoplast fusion between *A. niger* Y-(b) and *T. viride* M5S51.

[b] Utilization of sucrose instead of glucose as the sole carbon source is shown. The symbols + and − show positive and negative, respectively.

production composed of (per liter of distilled water): NH_4NO_3, 2 g; KH_2PO_4, 10 g; $MgSO_4 \cdot 7H_2O$, 250 mg; $FeCl_3 \cdot 6H_2O$, 20 mg; $MnSO_4$, 14 mg. For semisolid culture, 140 g/l glucose or cellobiose (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added as the sole carbon source. Unless otherwise indicated, for semisolid culture, 100 g/l carboxymethyl cellulose (Wako Pure Chemical Industries Ltd.) was included. The pH was initially adjusted to 4.25. For β-glucosidase and CMCase production in shake culture, a medium containing (per liter of distilled water): KH_2PO_4, 2 g; $(NH_4)_2\ SO_4$, 1.4 g; urea 300 mg; $MgSO_4 \cdot 7H_2O$, 300 mg; $CaCl_2 \cdot 2H_2O$, 400 mg; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4$, 1.4 mg; $ZnSO_4 \cdot 7H_2O$, 2.5 mg; $CoCl_2$, 2 mg and Bacto peptone (Difco), 1 g, was used. All cultivations were carried out at 30 °C.

Protoplast Formation

Conidia of *A. niger* Yang no. 2 were suspended at a concentration of 1×10^6/ml in 50 ml of SS medium containing 80 g/l of glucose and cultivated with orbital shaking (120 rpm) in 500-ml Sakaguchi flasks at 30 °C for 24 h. For the auxotrophic mutant Y-(b), 5 g/l of yeast extract (Difco) was added to the SS medium. Protoplast formation from mycelia of *A. niger* was performed in a lytic enzyme solution containing 1.0 g/l of Zymolyase-20T (Kirin Brewery Co., Tokyo, Japan), 1.5 g/l of Chitinase GODO (GODO Shusei Co., Tokyo, Japan), and 8.0 g/l Hemicellulase (Miles Laboratory, Inc., Elkhart, USA).

In *T. viride* WU-36B, conidia were suspended in 100 ml of liquid SM at a concentration of 1×10^6/ml and then incubated aerobically with orbital shaking at 120 rpm in 500-ml Sakaguchi flask at 30 °C for 24 h. The formation of protoplasts from mycelia of *T. viride* was carried out in a lytic enzyme solution containing 1.25 g/l of Zymolyase-20T (Kirin Brewery Co.) and 0.625 g/l of Chitinase GODO (GODO Shusei Co.).

Protoplast was incubated for 120 min at 30 °C in 50-ml Erlenmeyer flask with gentle shaking at 60 rpm. The incubation mixture was filtered through 3G3-sintered glass filters (Shibata Scientific Technology, Ltd., Tokyo, Japan) to remove mycelial debris. Protoplasts in the filtered lysate were counted in a Thoma hemacytometer under microscope.

Protoplast Fusion

Protoplasts prepared from the auxotrophic mutant strains were mixed in a 30% (*w*/*v*) polyethylene glycol solution containing 0.01 M $CaCl_2$ and 0.5 M KCl in a 0.05 M glycine–NaOH buffer, pH 7.5 (total volume, 5 ml). After incubation at 30 °C for 10 min, the mixture was slowly diluted with stabilized buffer (0.05 M potassium phosphate buffer in 0.7 M KCl, pH 6.0), then centrifuged at 800 g for 15 min.

Protoplast Regeneration

Aliquots of protoplast suspension (0.2 ml) were plated on 20 ml of MM containing 0.7 M KCl (SM containing 0.7 M KCl) and 20 g/l of agar and then overlaid with 10 ml of 10 ml of MM containing 0.7 M KCl (SM containing 0.7 M KCl) and 5 g/l of agar, preincubated at 40 °C. Protoplast were incubated at 30 °C for 4 days.

Benomyl Treatment

Mycelial mats (5×5 mm) of each strain were plated onto SM containing 1.25 mg/l of benomyl as a haploidizing agent. After 14 days cultivation at 30 °C, the segregants were

checked for morphological stability on fresh SM containing benomyl. Cultures were then purified and characterized by phenotype.

Analysis of Conidia

The nuclei were stained with 20 μg/ml of Hoechst 33258 (Wako Pure Chem. Ind.). Conidial DNA contents were then determined [27], using the diphenylamine agent with calf thymus DNA (Sigma, St. Louis, USA) as a standard.

Citric Acid Production

Conidia of each strain were suspended in the SS medium at a concentration of 1×10^6 per ml. The 1-ml suspension was added to the solid medium, containing 3.9-g sugar cane bagasse as a carrier and 15-ml SS medium, in a 9-cm diameter petri dish. Cultivation was carried out at 30 °C for 3 days, unless otherwise indicated. Citric acid was enzymatically measured using a test kit (Wako Pure Chem).

Culture Filtrate Preparation

After cultivation, the samples in the petri dishes were broken into pieces, and 200-ml distilled water at 60 °C was added. Only for the β-glucosidase and CMCase assays were distilled water at 30 °C added to prevent enzyme inactivation. The mixture was vigorously stirred for 15 min and filtered through Whatman GF/A with suction. The filtrate was then used as the semisolid culture extract for measurement of citric acid, β-glucosidase, CMCase, and the like. On the other hand, in shake culture, the culture broth obtained after filtration on Whatman GF/A was used for measurement.

β-Glucosidase and Carboxymethylcellulase Assays

β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as a substrate [28]. One unit (U) of enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the assay conditions. CMCase was assayed on carboxymethyl cellulose sodium salt (Sigma, USA) by measuring the reducing sugar as glucose, using dinitrosalicylic acid (DNS) method as previously described [29]. Enzyme activity is expressed as micromole of glucose released per minute under the assay conditions.

Reducing Sugar, Glucose, and Cellobiose Measurement

Total reducing sugars were measured by the colorimetric method using DNS at 540 nm. Glucose was enzymatically measured using the glucose B-test kit (Wako Pure Chem.). Amount of cellobiose was calculated by subtracting the amount of glucose from that of the total reducing sugars and corrected by using the cellobiose standard [29].

To examine whether or not other saccharides but glucose or cellobiose were produced, the culture filtrate was analyzed with a high-performance liquid chromatography system (Shimadzu, Japan) equipped with an LC-6A pump, a refractive index detector of RID-6A, and an SCR-101H column (7.9 mm diameter × 300 mm). Pure water was used as a mobile phase with flow rate of 0.6 ml/min at 40 °C.

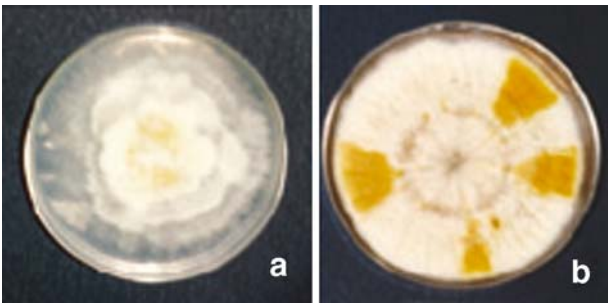

Fig. 1 Colonies of the hybrid strain AT 11-2-3. **a** Colony of the hybrid strain AT 11-2-3 cultivated on MM at 30 °C for 7 days. The mycelia were lighter yellow than those of *A. niger* Yang no.2 and Y-(b). **b** Colony of AT 11-2-3 cultivated on CM at 30 °C for 7 days. Sectors occasionally formed in a colony

Results and Discussion

New Hybrid Strain Formation

Our study aims to increase citric acid production from cellulose through protoplast fusion between *A. niger* Y-(b) and *T. viride* M5S51. Many strains were isolated as the first-stage heterokaryons, showing slow growth and mixed morphologies between those of Y-(b) and M5S51 on MM agar plates. The colonies showed segregated sectors of the component strains or showed a morphology similar to either *A. niger* Y-(b) or *T. viride* M5S51 after transferring their mycelial mat on MM agar plates or CM and SM agar plates. Almost all of these heterokaryon strains maintained their morphologies when any part of mycelial mat was picked up and/or subcultivated for more than ten times on MM. During subcultivations on MM agar plates, 3 among 100 strains showed much faster growth than the first-stage heterokaryons and as fast as the prototrophic parental strain *A. niger* Yang no.2. These three hybrid strains were designated as AT 11-2-3, AT 11-2-10, and AT 11-2-14, which showed fine growth on MM agar plates, accumulated little yellow pigment in mycelia, and formed sparse conidiophores similar to *A. niger* Yang no.2 and Y-(b) (Fig. 1). On CM and SM agar plates, the strains AT 11-2-3, AT 11-2-10, and AT 11-2-14 usually show stable growth with uniform morphologies, but with a frequency of approximately 1% segregated sectors of the component auxotrophic strains as shown in Fig. 1. Notably, the first-stage heterokaryons formed separate conidia of *A. niger* Y-(b) and *T. viride* M5S51 on MM agar plates, whereas AT 11-2-3, AT 11-2-10, and AT 11-2-14 usually formed globular-shape conidia like those of *A. niger* but not ellipsoidal ones of *T. viride* when cultivated on MM, CM, and SM. Less than 0.5% of the ellipsoidal conidia were found in the hybrid strains on MM, CM, and SM agar plates after 7-day cultivation.

Nutritional Requirements of Hybrid Strains

Conidia of hybrid strain (> 94%) formed colonies on MM as shown in Table 2, indicating that almost all of the conidia were prototrophic. Several segregants, obtaining from single-colony isolation of the hybrid strain conidia on MM agar plates, were classified into two types: one is an N.a$^-$ auxotrophic *A. niger* and the other is a Leu$^-$ auxotrophic *T. viride*. The frequency of the auxotrophic segregants was approximately 2–6% of total conidial progeny. Approximately 1% of conidial progeny, showing stable growth and segregated sectors of the component auxotrophic strains, was similar to the parental hybrid strains AT 11-2-3, AT 11-2-10, and AT 11-2-14 on CM and SM agar plates, whereas on MM agar plates, they showed growth with stable morphology as those of the original hybrid strains without

Table 2 Nutritional requirements of hybrid strains between *A. niger* and *T. viride.*

Hybrid strains	Relative numbers[a] of colonies formed on the minimal medium containing			
	No addition	Leu	N.a	Leu + N.a
AT 11-2-3	94	94	98	100
AT 11-2-10	97	98	99	100
AT 11-2-14	96	97	99	100

[a] Conidia of each fusant strain in a petri-dish, 9 cm in diameter, were collected and plated on the MM and MM containing leucine and/or nicotinic acid, and the cultivated at 30 °C for 7 days to allow colony formation. The numbers of colonies formed on each medium are expressed relative to those on MM containing both leucine and nicotinic acid. Values are the means for 50 independent measurements.

Leu Leucine; *N.a* nicotinic acid

sectoring, nor growth cessation. Because the segregation frequency was not affected by the addition of benomyl to CM or SM, we thus presumed that the hybrid strains were heterokaryons or unusual haploids but not polyploids nor aneuploids.

The hybrid strains and their progeny showed similar growth rate to the prototroph *A. niger* Yang no.2 on MM, CM, and SM agar plates. It is noteworthy that the hybrid strains and their progeny showed growth on MM agar plates as well as in MM solution, containing sucrose instead of glucose as the sole carbon source, whereas neither *T. viride* WU-36B nor M5S51 could utilize sucrose as the sole carbon source (Table 1).

DNA Contents of Hybrid Strains

To determine whether or not the hybrid strains were heterokaryons, single colony was isolated from conidia of each strain on MM agar plates three times, then the biochemical properties were tested. As described previously [23, 24], the conidia of *A. niger* Yang no.2 and Y-(b) or *T. viride* WU-36B and M5S51 are uninucleated upon staining with Hoechst 33258 by fluorescent microscopic observation. However, many conidia of the hybrid strains were multinucleated and usually showed larger sizes than those of *A. niger* and *T. viride* (Tables 3 and 4). The numbers of nuclei per conidium of AT 11-2-3 varied from one to seven (Table 3) and those of AT 11-2-10 and AT 11-2-14 from one to five (Table 4). In addition, the hybrid strains formed most frequently conidia containing two nuclei (Fig. 2).

Table 3 Variation of nuclei numbers per conidium and conidial size of new hybrid strains.

No. of nuclei Per conidium	Frequency[a] (%)			Conidial diameter[b] (μm)		
	AT 11-2-3	AT 11-2-10	AT 11-2-14	AT 11-2-3	AT 11-2-10	AT 11-2-14
1	39	37	37	3.9	3.8	3.9
2	51	50	51	4.7	4.5	4.6
3	5	3	2	5.5	5.5	5.3
4	1	4	4	6.0	6.1	5.9
5	2	6	6	6.5	6.6	6.5
6	1	N.D.	N.D.	6.9	N.D.	N.D.
7	1	N.D.	N.D.	7.2	N.D.	N.D.

[a] Analysis was done on 100 conidia of each strain, and number of strains counted is shown as frequency.

[b] Mean conidial diameters are shown. The mean conidia diameters of *A. niger* Yang no. 2 and *T. viride* WU-36 B are 4.1 and 3.6 μm, respectively.

Table 4 DNA contents per conidium and per nucleus of parental and hybrid strains.

Strain[a]	DNA content per conidium (10^{-8} μg)	No. of nuclei per conidium^{-}	DNA content per nucleus (10^{-8} μg)
Auxotrophic mutants			
A. niger Y-(b)	3.64	1.00	3.64
T. viride M5S51	6.31	1.00	6.31
Hybrid strains			
AT 11-2-3	8.87	1.83 (1–7)	4.85
AT 11-2-10	8.92	1.92 (1–5)	4.65
AT 11-2-14	8.88	1.91 (1–5)	4.65

[a] The contents of 100 conidia were counted as shown in Table 3, and mean values are shown. In parentheses, distributions are shown.

The mean DNA content per nucleus of the strains used is shown in Table 4. Although *A. niger* Y-(b) and *T. viride* M5S51 were haploids, their DNA contents per nucleus were different. The DNA content per nucleus of AT 11-2-3, AT 11-2-10, and AT 11-2-14 were in between those of *A. niger* Y-(b) and *T. viride* M5S51 and lower than double contents of *A. niger* Y-(b). The results, therefore, indicated that the hybrid strains were heterokaryons containing haploid nuclei from the component strains. The hybrid strains showed various conidial nuclei numbers and the DNA content per nucleus were still maintained, after more than ten subcultivations.

Attempts to improve the productivity of enzymes and metabolites have been made by manipulating the ploidy of filamentous fungi. As to citric acid production by *A. niger*, most research has been directed toward the formation of diploids (or polyploids) by the anastomosis method using auxotrophs derived from the same parental strain. Although Ikeda

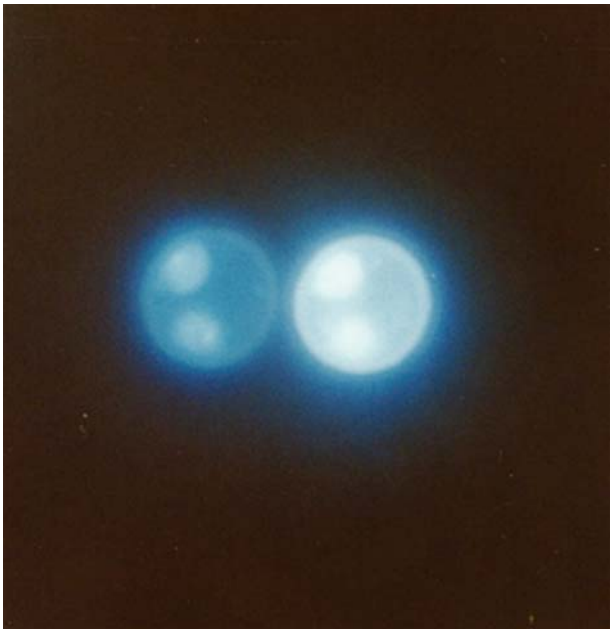

Fig. 2 Fluorescent micrograph of conidia from the hybrid strain AT 11-2-3 stained with Hoechst 33258. *Bar* 10 μm

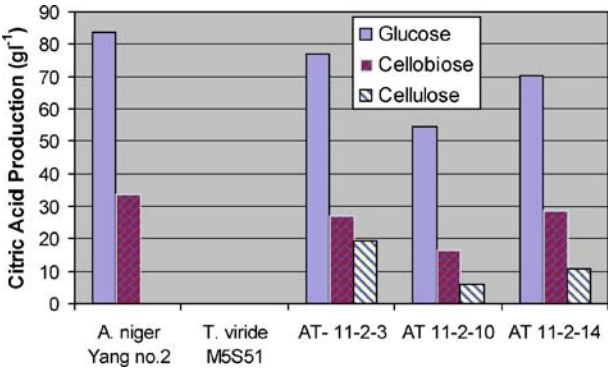

Fig. 3 Citric acid production by parental strains, *A. niger* Yang no.2, and *T. viride* M5S51, as compared to hybrid trains, AT 11-2-3, AT 11-2-4, and AT 11-2-10, using various carbon sources in semisolid culture

[30] reported successful results, other researchers showed that the citric acid productivities of the diploids formed did not surpass those of the respective parental strains [31, 32].

Citric Acid Production from Hybrid Strains in Semisolid Culture

Citric acid productivities of the hybrid strains, AT 11-2-3, AT 11-2-10, and AT 11-2-14, in semisolid culture containing 140 g/l glucose, 140 g/l cellobiose, or 100 g/l carboxymethyl cellulose as the sole carbon source are shown in Fig. 3. The maximum amount of citric acid produced, after 5 days in medium containing glucose, by AT 11-2-3, AT 11-2-10, AT 11-2-14, and Yang no.2 were 76.8, 54.4, 70.3, and 83.5 g/l, respectively. Furthermore, when cellobiose was used, the maximum citric acid produced by Yang no.2 was 20–50% higher than those of AT 11-2-3, AT 11-2-10, and AT 11-2-14. Interestingly, when using cellulose, the maximum citric acid production by AT 11-2-3, AT 11-2-10, and AT 11-2-14 were 19.2, 6.1, and 10.5 g/l, respectively, whereas *A. niger* Yang no. 2 could not use carboxymethyl cellulose as the sole carbon source for citric acid production.

2-Deoxy-D-Glucose-Resistant Properties

In this work, we found that AT 11-2-3, AT 11-2-10, and AT 11-2-14 could not grow on minimum medium containing either glucose or cellobiose as the sole carbon source, in the

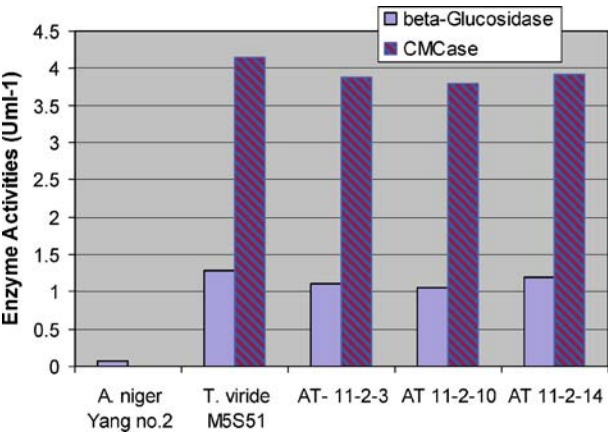

Fig. 4 β-Glucosidase and CMCase production by parental strains, *A. niger* Yang no.2 and *T. viride* M5S51, as compared to hybrid trains, AT 11-2-3, AT 11-2-4, and AT 11-2-10, in shake culture

presence of 1.0 g/l 2-deoxy-D-glucose (DG; data not shown). This result correlated with the previous studies indicating that DG-resistant mutants showed increased citric acid production using glucose and cellobiose as carbon sources [33–35].

β-Glucosidase and Carboxymethyl Cellulase Production in Shake Culture

In semisolid culture, it remains unclear whether or not all of the β-glucosidase and CMCase were extracted in the culture filtrate because there are some possibilities that the bagasse, used as a carrier, containing cellulose or mycelial debris might retain β-glucosidase and CMCase. Therefore, parental and hybrid strains were cultivated in shake culture and examined for the extracellular β-glucosidase and CMCase production as shown in Fig. 4. The results indicated that AT 11-2-3, AT 11-2-10, and AT 11-2-14 have almost the same β-glucosidase and CMCase activities as *T. viride* WU-36B, whereas the maximum β-glucosidase and CMCase productions were 16- and 4-fold s higher than those of *A. niger*, respectively. These results are in accordance with the other studies in enhanced CMCase and glucosidase activities in fusants [36–38].

In conclusion, three fusant strains AT 11-2-3, AT 11-2-10, and AT 11-2-14, derived from intergeneric protoplast fusion between of *A. niger* and *T. viride*, possessed citric acid productivity and β-glucosidase and CMCase activities. This will be beneficial for citric acid fermentation using cellulolytic materials as sole carbon source.

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