TRANSFER OF CELLULOLYTIC CHARACTER OF *ASPERGILLUS NIGER* INTO *ASPERGILLUS KAWACHII* BY THEIR PROTOPLAST FUSION

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The feasibility of obtaining useful interspecific hybrids with high cellulolytic activities by breeding was investigated by a protoplast fusion technique using the citric acid hyper-producer, *Aspergillus kawachii*, and the cellulase hyper-producer, *Aspergillus niger*. Interspecific hybridization between two auxotrophic mutants of *Aspergillus kawachii* (*his*) and *A. niger* (*met*) was achieved by protoplast fusion in the presence of 35% polyethylene glycol as fusogen. Stable diploid strains were obtained from a heterokaryon strain by treatment with 0.1% *d*-camphor. The selected strains were specified by their conidial size and DNA content as well as the conidial germination. One of them showed high cellulase productivity besides the same level of amylase production and sporulation as that of *A. kawachii*, a higher level of citric acid and the lowering of mash viscosity than that of *A. kawachii*. From these results it is suggested that this diploid strain would be very useful for the degradation of cellulosic substances in the mash from shochu fermentation, consequently for its ready disposal thereafter.

Distillery waste from shochu fermentation mash has recently become a social problem as a source of pollution. One of the useful tools for resolution of this problem seems to be the transference of a character to produce cellulase and its related enzymes into the non-cellulolytic fungus, *Aspergillus kawachii* Kitahara, which is the major microbe proliferating in the mash. The potential candidate may be a highly cellulase-producing fungus, *Aspergillus niger*. The cellulosic substances

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therein will be degraded through the treatment to result in lowering the viscosity of the whole mash and they may become easily disposable thereafter. Thus, for example the solid-liquid separation of the fermentation mash, concentration of supernatant, and foraging and composting of the solid-residue may be readily realized. Only a few studies have been made so far for this kind of study by a protoplast fusion procedure. In this work, the feasibility of breeding to obtain useful interspecific hybrids having a high cellulase activity was pursued by the method of protoplast fusion using the citric acid hyper-producer, *A. kawachii*, and the cellulase hyper-producer, *A. niger*.

MATERIALS AND METHODS

Organisms. The citric acid hyper-producer, *Aspergillus kawachii* Kitahara KGS (Kawachi Genichiro Shoten, Kagoshima, Japan) used for shochu fermentation, and a cellulase hyper-producer, *Aspergillus niger* UC (Ueda Chemical Industry Co., Ltd., Osaka, Japan), were employed as the original strains. Conidia of these strains were treated with UV rays in a routine manner. The auxotrophic single mutants obtained were checked for their stability over several vegetative generations, and for their characteristic amylase, cellulase and citric acid productions. Strain KK1 (*his*) is a mutant of *A. kawachii* Kitahara KGS and strain UC (*met*) is a mutant of *A. niger* UC. These parental strains also produced single nuclear conidia on a complete medium.

Mycolytic enzyme sources. Commercial enzyme preparation, Usukizyme (Kyowa Kasei Co., Ltd., Osaka, Japan), *Trichoderma viride* B1A preparation, chitinase (Sigma Chemical Co., St. Louis, U.S.A.) and hemicellulase (Ueda Chemical Ind. Co., Ltd.) were used in this work.

Cultivation of microorganisms. Czapek-Dox medium containing 2% glucose was used as a minimal medium (MM). The minimal medium supplemented with 0.5% yeast extract (Difco Lab., Detroit, U.S.A.) and Casamino acid (Difco Lab.) was used as the complete medium (CM).

Preparation of protoplasts. Protoplast was prepared from mycelia by the method previously reported (8). About 200 mg (wet weight) of mycelia was added to 5 ml of a 3% Usukizyme solution [50 mM malate buffer, pH 5.6, being added for KK1 (*his*)] or to a mixture of 8% *T. viride* B1A preparation, 0.15% chitinase and 0.8% hemicellulase [50 mM malate buffer, pH 5.6 being added for UC1 (*met*)]. The individual mixtures were made to 0.6 M KCl or NaCl [for KK1 (*his*)] or NaCl for UC1 (*met*) and they were incubated under shaking on Monod apparatus at 35°C for 1 h. The reaction mixture was centrifuged at $700 \times g$ for 5 min, and the protoplasts precipitated were suspended in a 1 ml solution (0.6 M NaCl) which was osmotically balanced. The regeneration frequency was defined as the ratio of the number of colonies regenerating from the protoplasts and the total protoplasts used in the suspension. The real colonies regenerating from protoplasts were calculated by means of the water-lysis control test.

Protoplast fusion. The methods of Ann and Peberdy (2) and the method reported previously (9,10) were used under a slight modification. The fusion frequency was defined as the percentage ratio of the number of colonies developing on MM and CM agar after the fusion treatment.

Diploidization of heterokaryon. The same method as reported previously was used (8).

Determination of DNA. *Conidia* (10^9 /ml) cultured at 25C for 3 days were treated with 5 ml of 0.25 N HClO₄ at 0C for 30 min to eliminate low molecular weight substances in conidia. After elimination of the low molecular weight substances, nucleic acid was extracted with 4 ml of 0.5 N HClO₄ at 70C for 25 min. The extraction was repeated 3 times. Determination of DNA was carried out in the same way as the method of Burton (3) after extraction by the method of Schneider (15). The DNA content per conidium was calculated as follows: $100 \times (\text{the amount of DNA in the conidia}) / (\text{the number of conidia tested}) (10^{-6} \mu\text{g})$.

Enzyme assays. Amylase activity was measured by the method reported previously (9,10). The reducing sugar content was measured by the method using 3,5-dinitrosalicylic acid (16). Avicel saccharifying, CM-cellulose saccharifying, and β -1,3-glucanase activities were measured by the methods reported previously (11,12). One unit of these enzyme activities was defined as an amount of enzyme which produced 1 μ mol of reducing sugar as glucose per min. Filter paper degrading activity was also assayed by the method reported previously (17).

Culture for enzyme preparation. Solid culture for amylase production was performed by the method previously reported (8). Citric acid was measured by the method of Kusunose (7). Solid and submerged cultures for cellulase production were performed by the following methods. In a 300 ml Erlenmeyer flask, 10 g of wheat bran was mixed with tap-water at ratio of 1 : 1 for the solid culture. The flask was then autoclaved at 120C for 20 min, followed by an inoculation with small pieces of a solid seed culture. The flask was incubated at 30C for 6 days. With the submerged culture of *T. viride*, 0.25 g of Avicel PH-101 and 0.25 g of wheat germ were added to the medium in a 100 ml Erlenmeyer flask (1). These flasks were autoclaved at 120C for 20 min, followed by an inoculation with small pieces of fungal mat grown on the slope. The flask was incubated with shaking on a rotary shaker (Iwashiyama K, Sawada Co., Ltd., Tokyo, Japan) at 160 rpm 25C for 5 days.

Small-scale shochu mash. The small-scale shochu mashes consisting of 50 g of rice grain *koji* and 80 ml of distilled water (for 1st mash), and 250 g of steamed sweet potato and 120 ml of distilled water (for 2nd mash) were fermented at 25C and pH 3.6 for 5 days (for 1st mash), and pH 4.0 for 8 days (for 2nd mash).

Viscosity measurement of sweet potato shochu mash. Viscosity of a sweet potato shochu mash was measured using an electric rotary viscometer, Vismetron (Tokyo Keisoku Co., Ltd., Tokyo, Japan).

RESULTS

Protoplast fusion

Protoplasts ($3.4 \times 10^6/\text{ml}$) of KK1 (*his*) were formed from mycelia by incubation with *Trichoderma* lytic enzyme preparation for 1 h in a hypertonic stabilizing solution with 0.6 M KCl or NaCl. However, protoplasts from UC1 (*met*) could not be formed from mycelia with only a single lytic enzyme system in using *Trichoderma* lytic enzyme preparation, but required a multilytic enzyme system for the protoplast ($3.0 \times 10^6/\text{ml}$) formation. Regeneration frequency of the KK1 (*his*) and UC1 (*met*) strains were 15.6 and 11.5%, respectively. Protoplasts from KK1 (*his*) and UC1 (*met*) were fused using 35% polyethylene glycol (m.w. 6,000) as reported previously (8,9). After 5 days of incubation, fusants could be selected with nutritional complement as colonies appearing on MM agar after fusant treatment. The fusion frequency of the protoplasts was estimated to be 0.51%, i.e., more than 10^4 reversion rates of the fused auxotrophs (Table 1). The fusants that appeared on MM agar were transferred onto new MM slant. These fusants showed fine growth on MM agar and their conidia segregated into KK1 (*his*) and UC1 (*met*) of the parental auxotrophs showing segregation rates of 16:84 (Table 2). These results obviously verified that the fusants are heterokaryon.

Table 1. Fusion frequency between protoplasts of auxotrophic mutants.

Protoplast pair	Reversion rate (less than)	Protoplast regenerated on hypertonic CM		Fusant formed on hypertonic MM	Fusion frequency (%)
		Before fusion treatment	After fusion treatment		
KK1 (<i>his</i>) ^a	2.9×10^{-7}	8.0×10^5	7.5×10^4	3.8×10^2	0.51
UC1 (<i>met</i>) ^b	2.2×10^{-6}	6.5×10^5			

Czapek-Dox medium containing 2% glucose was used for the minimal medium (MM). MM supplemented with 0.5% yeast extract and Casamino acid was used as complete medium (CM). Fusion was done by suspending the protoplasts in 35% PEG (m.w. 6,000), 0.05 M CaCl₂, 0.05 M glycine-NaOH buffer, pH 7.5 (25°C for 15 min). After being washed with 0.6 M NaCl, a series of appropriately diluted protoplast suspensions were plated onto hypertonic MM and CM agar.

^a KK1 (*his*): An auxotrophic mutant from *A. kawachii* Kitahara KGS.

^b UC1 (*met*): An auxotrophic mutant from *A. niger* UC.

Table 2. Segregation of heterokaryons after fusion.

Protoplast pair	Segregation (%)
KK1 (<i>his</i>) ^a	16
UC1 (<i>met</i>) ^b	84

Appearance frequency of diploids was represented by the ratio of the numbers of conidia regenerating on CM and MM agars.

^a KK1 (*his*): An auxotrophic mutant from *A. kawachii* Kitahara KGS.

^b UC1 (*met*): An auxotrophic mutant from *A. niger* UC.

Fig. 1. Conformation of heterokaryon by growth on minimal medium.

Czapek-Dox medium agar was used for as a minimal medium. The photograph was taken after 6 days of incubation at 25°C. AK (*his*): KK1 (*his*); AN (*met*): UC1 (*met*).

Fig. 2. Germination of diploid conidia on minimal medium.

The photograph was taken after 20h of incubation at 25°C. The bar marker represents 45 μ m.

Preparation of heterozygous diploid

A loopful of mycelia mixed with conidia from a heterokaryon strain, H-KK1-UC1, was inoculated onto a Petri dish of MM agar containing 0.1% *d*-camphor (14), followed by incubation at 30°C for 5 days. The mycelia showed nice growth and sporulation, some sectors evidently being produced. The conidia heads in the sectors were either black or brown (Fig. 1). Heterozygous diploids could be selected on the basis of germinated conidia on MM agar, as shown in Fig. 2. The

Table 3. DNA content of conidia of haploids and diploids.

Strain	DNA	
	10^{-8} μ g per conidium	10^{-8} μ g per nucleus
<i>A. kawachii</i> Kitahara	13.41	13.41
<i>A. niger</i> UC	12.76	12.76
KK1 (<i>his</i>) ^a	12.13	12.13
UC1 (<i>met</i>) ^b	12.58	12.58
Heterokaryon (H-KK1-UC1)	12.00	12.00
Diploid (D-KK1-UC1)	20.86	20.86

DNA was measured by the method of Burton after extraction by the method of Schneider.

^a KK1 (*his*): An auxotrophic mutant from *A. kawachii* Kitahara KGS.

^b UC1 (*met*): An auxotrophic mutant from *A. niger* UC.

Table 4. Size of conidia of haploids and diploids.

Strain	Diameter ^c	(Ave. μ m)
KK1 (<i>his</i>) ^a	2.4-4.8	(4.6)
UC1 (<i>met</i>) ^b	2.3-4.5	(4.3)
Heterokaryon (H-KK1-UC1)	2.5-4.8	(4.5)
Diploid (D-KK1-UC1)	4.5-7.1	(5.3)

^a KK1 (*his*): An auxotrophic mutant from *A. kawachii* Kitahara KGS.

^b UC1 (*met*): An auxotrophic mutant from *A. niger* UC.

^c The measurement was done on at least 200 conidia of each strain and the ranges of conidial diameters are shown.

Table 5. Properties of heterokaryon and diploid.

Strain	pH	Citric acid (mg/1 g of wet <i>koji</i>)	Amylase (units/ml)
<i>A. kawachii</i> Kitahara	4.78	7.66	157.5
<i>A. niger</i> UC	4.92	7.44	218.3
KK1 (<i>his</i>) ^a	5.35	5.13	59.2
UC1 (<i>met</i>) ^b	4.17	7.92	192.4
Heterokaryon (H-KK1-UC1)	4.62	7.50	168.2
Diploid (D-KK1-UC1)	5.05	8.01	185.0

The *koji* extract was prepared by extraction of the solid culture with one volume (for a fresh culture) of distilled water at room temperature for 1 h.

^a KK1 (*his*): An auxotrophic mutant from *A. kawachii* Kitahara KGS.

^b UC1 (*met*): An auxotrophic mutant from *A. niger* UC.

frequency of diploidization was 5.6×10^3 . A diploid strain, D-KK1-UC1, showed 1.6-fold DNA content per nucleus (Table 3). The conidia diameter of this diploid strain was much greater than those of the original strains (Table 4). Nuclear number of the diploid conidia was verified to be single nucleus by DAPI staining methods (5). Citric acid and amylase productivities of a diploid strain, D-KK1-UC1.

Table 6. Cellulase activity (filter paper degrading activity) of heterokaryon and diploids.

Strain	Solid culture	Submerged culture
	FPD activity ^c (units/mg)	FPD activity ^c (units/mg)
<i>A. kawachii</i> Kitahara	250	200
<i>A. niger</i> UC	1,000	500
KK1 (<i>his</i>) ^a	200	188
UC1 (<i>met</i>) ^b	600	500
Heterokaryon (H-KK1-UC1)	429	333
Diploid (D-KK1-UC1)	1,500	600

^a KK1 (*his*): An auxotrophic mutant from *A. kawachii* Kitahara KGS.^b UC1 (*met*): An auxotrophic mutant from *A. niger* UC.^c Filter paper degrading activity.

Table 7. Cellulase activities of heterokaryon and diploid.

Strain	Avicel saccharifying activity	CM-cellulose saccharifying activity	β -1,3-Glucanase activity
	(units/ml)	(units/ml)	(units/ml)
<i>A. kawachii</i> Kitahara	1.2	0.2	1.4
<i>A. niger</i> UC	6.4	1.5	4.4
KK1 (<i>his</i>) ^a	0.5	0	0.4
UC1 (<i>met</i>) ^b	5.3	1.0	3.5
Heterokaryon (H-KK1-UC1)	4.4	0.3	2.6
Diploid (D-KK1-UC1)	6.5	0.5	4.8

Enzyme solution was obtained by submerged culture.

^a KK1 (*his*): An auxotrophic mutant from *A. kawachii* Kitahara KGS.^b UC1 (*met*): An auxotrophic mutant from *A. niger* UC.

The diploid strain was assayed for the citric acid and amylase productivities. The citric acid and amylase productivities levels in the diploid strain cultured on solid rice grain were enhanced as compared with those of original strain, *A. kawachii* Kitahara KGS (Table 5). This strain produced phenotypically black conidia derived from *A. niger* UC.

Cellulase activities of a diploid strain, D-KK1-UC1

A diploid strain, D-KK1-UC1, showed 6-fold filter paper degradation for activity as compared with that of the original strain, *A. kawachii* Kitahara KGS, on solid and submerged culture (Table 6). Avicel and CM-cellulose saccharifying and β -1,3-glucanase activities of D-KK1-UC1 also showed higher (i.e., 6.3- and 2.5-fold) activities than those of *A. kawachii* Kitahara KGS, respectively (Table 7). These results confirmed that cellulase producing character originated in *A. niger* UC has been transferred into *A. kawachii* Kitahara by the protoplast fusion technique.

Table 8. Lowering the viscosity of shochu mash using a cellulase-inducible fusant.

Strain	Mash	Ethanol concentration in 2nd mash (%) ^a	Final mash viscosity (CPS) ^b
<i>A. kawachii</i> Kitahara	1st mash		500
	<i>Koji</i> 50 g		
	Water 80 ml		
	2nd mash	14.4	
	Sweet potato 250 g		
	Water 120 ml		
Diploid (D-KK1-UC1)	1st mash		
	<i>Koji</i> 50 g		
	Water 80 ml		
	2nd mash	14.9	
	Sweet potato 250 g		
	Water 120 ml		

Two small-scale shochu mashes consisting of 50 g of rice grain *koji* and 80 ml of distilled water (for 1st mash), and 250 g of steamed sweet potato and 120 ml of distilled water (for 2nd mash), respectively were fermented at 25°C and pH 3.6 for 5 days (for 1st mash), and pH 4.0 for 8 days (2nd mash).

^a Ethanol concentration was assayed by the method of steam distillation.

^b CPS: CGS unit of viscosity rate.

Change of viscosity in sweet potato shochu mash

Change of the viscosity in sweet potato shochu mash fermented with a fusant, D-KK1-UC1, was compared with that of *A. kawachii* Kitahara KGS. The viscosity of the shochu mash was lowered markedly (Table 8). Same result has been shown on shochu mash supplemented cellulase preparation (13).

Furthermore, the D-KK1-UC1 fusant grew well after sporulation under healthy condition on culture, and its productivities of citric acid and amylase, cellulase and related enzymes were found to be very stable even after 6 times successive transfers of culture.

DISCUSSION

A. kawachii Kitahara produces largely citric acid and acidic amylase, although the fungus is unable to produce cellulases and related enzymes such as hemicellulase. Therefore, transferring of the character cellulase and producing cellulase and related enzymes into this strain from some cellulolytic fungi is very useful for degradation of cellulosic substances in mash produced by fermentation industry. Since *A. kawachii* Kitahara is one of the fungi inhabiting the mash from shochu fermentation, we used *A. niger* UC as the donor of such character.

Preparation of mycelial protoplasts from *A. kawachii* Kitahara was first carried out using only lytic enzyme system of *T. viride*, but cooperation of multienzyme system including α - and β -glucanase, chitinase and hemicellulase beside *T. viride*

lytic enzymes was necessary for this purpose in the case of *A. niger* UC. It was suggested that cell wall of this strain contained not only nigeran (4,6) composed of α -1,3- and 1,4-glucans but β -1,3-glucan and chitin.

The protoplast fusants obtained in this way were able to grow on MM agar, and conidia from fusants completely segregated into two types of parental auxotrophs. The result indicates that the protoplast fusants are heterokaryons. After heterokaryon H-KK1-UC1 was incubated on MM agar containing 0.1% *d*-camphor, sectors developed and then germ tubes were developed from a part of the conidia in the sectors.

In this study, we selected a diploid strain D-KK1-UC1 from above sectors. The diploidy of this strain was verified by germination and growth on MM agar of the conidia, and by the diameter and DNA content of the conidia. The conidium from strain D-KK1-UC1, contained an about 1.6-fold amount of DNA and an about 1.3-fold conidial diameter compared with those of original strains. It seemed, therefore, reasonable to assume that strain D-KK1-UC1 is a heterozygous diploid. The diploid strain D-KK1-UC1 was regarded as a useful strain because of its high citric acid and amylase productivities in addition to cellulolytic activity. In the fermented mash of sweet potato including a large amount of parenchymatous cells and crude fibers, the lowering of mash viscosity was not shown by traditional process or by using amylase preparation only (13). When we used a fusant, D-KK1-UC1 for making *koji*, the viscosity in the shochu mash lowered more markedly than that of *A. kawachii* Kitahara KGS. It can therefore be presumed that lowering of the viscosity in the shochu mash is caused by degradation of sweet potato cell walls. By lowering of the viscosity in the shochu mash, the solid-liquid separation of the fermentation mash, elevation of fermentation efficiency (13), and foraging and composting of the solid-residue may be realized. From these results it seems apparent that protoplast fusion technique will practically be a useful tool for transferring the character for the producibility of cellulase and related enzymes from a highly cellulase-producing fungus into a non-cellulolytic one.

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