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ORIGINAL PAPER

Breeding and identification of novel koji molds with high activity of acid protease by genome recombination between *Aspergillus oryzae* and *Aspergillus niger*

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Abstract Acid protease is essential for degradation of proteins during soy sauce fermentation. To breed more suitable koji molds with high activity of acid protease, interspecific genome recombination between *A. oryzae* and *A. niger* was performed. Through stabilization with *d*-camphor and haploidization with benomyl, several stable fusants with higher activity of acid protease were obtained, showing different degrees of improvement in acid protease activity compared with the parental strain *A. oryzae*. In addition, analyses of mycelial morphology, expression profiles of extracellular proteins, esterase isoenzyme profiles, and random amplified polymorphic DNA (RAPD) were applied to identify the fusants through their phenotypic and genetic relationships. Morphology analysis of the mycelial shape of fusants indicated a phenotype intermediate between *A. oryzae* and *A. niger*. The profiles of extracellular proteins and esterase isoenzyme electrophoresis showed the occurrence of genome recombination during or after protoplast fusion. The dendrogram constructed from RAPD data revealed great heterogeneity, and genetic dissimilarity indices showed there were considerable differences between the fusants and their parental strains. This investigation suggests that genome recombination is a powerful tool for improvement of food-grade industrial strains. Furthermore, the presented strain improvement procedure will be applicable for widespread use for other industrial strains.

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Introduction

Koji molds are essential as producers of various hydrolyzing enzymes for soy sauce fermentation. *A. oryzae*, a widely used filamentous fungus in traditional fermentation of foods, can produce amounts of amylases and proteases in the koji-making stage [21, 29]. As far as the proteases are concerned, the amount of acid protease (aspartic protease) is relatively deficient compared with neutral and alkaline proteases [13]. The most important protease in sake brewing, however, is acid protease, because of the acid condition of the fermentation mash. Acid protease is considered to contribute to efficient solubilization of steamed soybean in the acid mash [18]. Therefore, it is desirable to breed strains with high production of acid protease for degradation of raw proteins in soy sauce fermentation.

To breed new and more suitable koji molds, it would be useful to introduce foreign genes from organisms of diverse taxa. Gene manipulation techniques are undoubtedly a preferred method, and previous reports have focused mainly on genetic manipulation [14, 19, 32]. However, issues involving use of genetically modified foods have rendered the use of recombinant techniques for improving food-grade industrial stains controversial [15]. Therefore, we attempted to introduce the technique of genome recombination to breed novel strains of *A. oryzae*, which can efficiently improve degradation of soybean proteins in acid mash. Genome recombination is an efficient method for evolution of industrial strains [16]. During the process of genome recombination, genes of the selected strains

with desirable properties are recombined by homologous recombination. This procedure allows rapid evolution of strains with multiple beneficial characteristics, and it has been widely used to combine genes from different taxa for creating strains with desired industrial properties [16, 17, 25]. So far, many trials of intra- or interspecific genome recombination through protoplast fusion among *Aspergillus* have been reported [24, 33, 35]. Interspecific genome recombination between *A. oryzae* and *A. niger*, however, has rarely been reported.

Therefore, our investigation focused on breeding of novel food-grade industrial koji molds with high activity of acid protease by genome recombination between *A. oryzae* and *A. niger*. Furthermore, recognition of the desired fusants was accomplished by analysis of mycelial morphology, esterase isoenzyme profiles, and use of the RAPD technique.

Materials and methods

Microorganism and preparation of conidial suspensions

Parental strains *A. oryzae* HN3.042 and *A. niger* CICC2377 were purchased from Shanghai Difa Brewing Biological Co., Ltd. and Microbial Culture Collection Center of Guangdong Province, respectively. Activation of strains and preparation of conidial suspensions were performed according to the method of Aalbak et al. [1] and Sandhya et al. [30].

Preparation and asymmetric inactivation of protoplasts

Protoplast preparation was carried out according to the methods of de Bekker et al. [6], Ogawa et al. [24], and Ushijima et al. [34] with modifications. About 1.0×10^7 conidia were added to 100 ml MPY lipid medium (1.0% maltose, 1.0% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$) in a 250-ml Erlenmeyer flask, and then were incubated at 30°C and 100 rpm for 20 h. After incubation, the mycelia were collected on filter paper and washed with distilled water under aseptic conditions. Then, the mycelia were added to 20 ml enzyme cocktail consisting of 10 mg/ml lywallzyme, 10 mg/ml cellulose R-10, 10 mg/ml snailase, and 5 mg/ml lysozyme in stabilization buffer (SB; 0.02 M pH 6.0 sodium phosphate buffer, 0.7 M NaCl, 0.4 M sorbitol), followed by incubation at 30°C with slow shaking at 80 rpm for 2 h. The mixture was filtered through two layers of nylon gauze (about 100 μm), and the filtrate was centrifuged at 3,000 rpm and 4°C for 10 min. The sedimented protoplasts were washed and resuspended in 1.0 ml SB. When purification of protoplasts was not satisfactory, they were purified again by centrifugation.

Asymmetric inactivation of protoplasts was done according to the method of Purohit et al. [26] with modifications. The purified protoplasts of *A. oryzae* were placed under an ultraviolet (UV) lamp (15 W) at a distance of 12 cm and irradiated for 15 min. The protoplasts of *A. niger* were incubated at 65°C for 10 min. After this asymmetric inactivation, the survival frequency of protoplasts on regeneration plates was nearly zero. Equal numbers of each type of inactivated protoplasts ($\sim 1 \times 10^6$/ml of each) were mixed and appropriately diluted with SB for subsequent fusion experiments.

Alignment and fusion of protoplasts

Alignment and fusion of the inactivated protoplasts were done according to the method of Ushijima et al. [35] with modifications. An Electro Cell Fusion Generator LF201 (Japan, 1.0 MHz) and fusion chamber (3 mm gap) were used as the fusion apparatuses. A mixture (1.0 ml) of equivalent numbers of each type of protoplast was added to the fusion chamber under aseptic conditions, and a sinusoidal voltage range of 200–750 V/cm was screened for inducing alignment of protoplasts. Protoplast electrofusion was performed according to the method of Ushijima et al. [35] with slight modifications. After electrofusion, diploidization of heterokaryons was carried out with *d*-camphor according to the method of Ogawa et al. [24]. After successive subcultures for 10 generations on camphor medium, stabilization and haploidization of the fused diploids were done with benomyl according to the method of Ushijima et al. [34].

Screening of fusants

Preliminary screening of fusants

Preliminary screening for fusants with high proteolytic activity was done according to the method of Aalbak et al. [1], Adinarayana et al. [2], Lazim et al. [20], and Rajamani and Hilda [28] with modifications. Fusants with fast growth rates were harvested from potato dextrose agar (PDA) medium after 4 days culturing, and the conidia were washed from cultures with distilled water, adjusting to final concentration of $\sim 10^3$ conidia/ml. The suspensions were used to inoculate acid casein agar plates (pH 4.0), and triplicate cultures were incubated at 25°C for 96 h, after which the diameters of the colonies and the clear zones were measured using a Vernier caliper. Proteolytic activity was evaluated by the formula: Hc (activity) = H (clear zone diameter)/l (colony diameter). Fusants with higher hydrolytic activity were selected for further screening.

Further screening of fusants

Fusants exhibiting higher proteolytic activities on acid casein agar plates were selected and used to make koji for further quantitative analysis. The acid protease activities of selected strains in koji cultures were assayed as described by García-Gómez et al. [12] and Guo and Ma [14] with modifications. Proteolytic activity was determined using 2% casein in 0.1 M citric acid-sodium citrate buffer (pH 3.5) as substrate. Substrate (0.1 ml) was incubated with 0.1 ml appropriately diluted enzyme solution at 40°C for 10 min. The reaction was terminated by addition of 0.2 ml 0.5 M trichloroacetic acid (TCA), and the mixture was incubated for 20 min to allow unhydrolyzed protein to settle. The unhydrolyzed protein was removed by centrifugation at 12,000 rpm for 10 min. Folin-Ciocalteu reagent (0.1 ml, 1.0 M) and sodium carbonate solution (0.5 ml, 0.40 M) were mixed with 0.1 ml of the supernatant. For color development, the mixture was incubated at 40°C for 20 min and the absorbance of 0.2 ml supernatant was measured spectrophotometrically at 660 nm (infinite 200; Tecan, USA). One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg tyrosine per minute under the above conditions.

Confirmation of acid protease activity

Confirmation of acid protease activity of screened fusants was carried out by milk clotting assay. Milk clotting activity of the acid protease in the culture supernatant of screened fusants was checked by the milk agarose plate assay. The composition of the milk agarose was as follows: 1.0% agarose, 1.0% casein, 100 mM sodium acetate, pH 4.0. After supplementation with 10 mM $CaCl_2$, melted agarose solution was poured into sterile Petri plates to uniform thickness of 0.5 cm. Stainless-steel cylinders (inside diameter × outside diameter × height = 6 × 8 × 10 mm^3) were placed on the surface of the milk agarose plates, and then 100 μl crude enzyme solution was added to the cylinders. Then, plates were incubated at 37°C overnight. Milk clotting activity was observed by formation of clear zones around the test wells.

The protein content of the crude extracts was determined by the method of Bradford [5] with slight modifications. On a 96-well plate, 10 μl crude extract was mixed with 200 μl Bradford reagent. After 5 min for color development, the absorbance of the plate was read spectrophotometrically at 595 nm. To determine the protein concentration, a standard curve of protein concentrations over the range 0–10 mg/ml was used.

Identification of fusants

Mycelial morphologies of fusants and parental strains

Mycelial morphology of fusants and their parental strains was observed using a phase-contrast microscope (BX51; Olympus, Japan). A specific amount of minimal medium (about 1 cm^2 in area and 1 mm in thickness) was placed on glass slides, and the conidia of fusants and parental strains were inoculated on the rim of the minimal medium, followed by incubation at 30°C and 90% relative humidity for 48 h. Mycelial morphology was observed at a certain magnification, and representative zones were photographed. The average diameter of mycelia was measured using a micrometer attached to the microscope.

Expression profiles of extracellular proteins

Extracellular proteins were produced when the fusants were cultured on a 4:1:5 mixture of wheat bran, flour, and distilled water. About 1×10^5 conidia were inoculated onto the bran and wheat flour medium and were cultured at 30°C for 72 h. The extracellular proteins in the koji were extracted with water containing 0.1 M sodium chloride (bran:solvent = 1:4) at 40°C and 150 rpm for 1 h. The extract was centrifuged at 10,000 rpm and 4°C for 10 min. Then the supernatant was passed through a 0.45-μm ultrafiltration membrane (Minisart NML; Sartorius AG, Goettingen, Germany), and the filtrate was collected as crude protein solution. The protein in each solution was adjusted to the same content with distilled water. An amount of 500 μl of the resulting solution was used as the sample for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), performed according to the method of Laemmli [19].

Esterase isoenzyme profiles

Mycelia were produced in 250-ml Erlenmeyer flasks containing 50 ml MPY. Flasks were inoculated with 1 ml conidial suspension ($\sim 10^5$/ml). They were incubated at 30°C and 100 rpm for 20 h. Each sample was filtered through gauze and was ground into powder in liquid nitrogen using a pestle. Extraction buffer (3 ml; Tris–HCl 50 mM, pH 8.0, sucrose 10%) was added to the mycelial powder. The mixture was agitated with a supermixer for 2 min and then centrifuged at 4°C and 12,000 rpm for 20 min. The supernatant containing soluble proteins was collected using a syringe, taking care to avoid the lipid layer that floated on the surface after centrifugation, and then the soluble proteins were dispensed into Eppendorf tubes for storage at −20°C until analysis.

Native polyacrylamide gel electrophoresis was performed on vertical slab gels (Bio-Rad Protean 10 Cell). The separating gel composition was 12% (w/v) T and 2.6% C, in Tris–HCl buffer (50 mM, pH 8.9), and the stacking gel was 5% (w/v) T and 2.6% C, in Tris–HCl buffer (50 mM, pH 6.9). The running buffer was composed of Tris–HCl buffer (25 mM, pH 8.9). The gels were run at 80 V for 5 h. All sample volumes were set as 20 µl per lane. After electrophoresis, the gels were incubated for 1 h in 100 ml phosphate buffer at pH 6.5 containing 100 mg naphthyl acetate (added to 10 ml 50% aqueous acetone). Gels were then stained for nonspecific esterase by adding 100 mg Fast Blue RR salt to 100 ml buffer.

RAPD analysis of genomes

DNA extraction was done according to the method of Murray and Thompson [23], and genomic DNA quality was tested on 0.8% (w/v) agarose gels (USB; MB Grade, USA) and then observed and photographed under UV light using a Molecular Imager ChemiDoc XRS system (Bio-RAD, USA). The primers 5′-GGCAGATAAGATAGCGGAA G-3′ (P1) and 5′-CCGACAGACTGTAGCCACC-3′ (P2) were selected for RAPD after 10 random primers had been screened. Polymerase chain reactions (PCR) were performed in a 25 µl reaction system, containing 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Takara, Japan), 2.5 µl PCR buffer, 1 U DNA polymerase (Takara, Japan), 0.6 µM single 10-base-pair primer (Invitrogen, USA), 50 ng template DNA, 2 mM $MgCl_2$, and ultrapure water to give the final volume. Amplifications were carried out in a Mastercycler Gradient PCR system (Eppendor, Germany) programmed for 1 cycle at 94°C for 5 min, 45 cycles of 94°C for 1 min, 33°C for 1 min, and 72°C for 2 min, followed by a final elongation stage of 72°C for 10 min, and then PCR products were directly analyzed or stored at −80°C until use. Amplified products (5 µl) were analyzed by electrophoresis using 1.5% agarose gels with ethidium bromide in 1× TBE buffer at 100 V for 1 h. Gels were scanned with UV in the Molecular Imager ChemiDoc XRS system (Bio-RAD, USA). The contrast and gray balance of the entire image were adjusted to reduce background to achieve high-resolution images.

The numerical analysis of RAPD bands was done according to the method of Feng et al. [11] with slight modifications. Only strong and reproducible RAPD bands were scored, with the help of Quantity One 4.5.2 software (Bio-Rad, USA). The observed profiles were scored as discrete variables, using 1 and 0 to indicate presence and absence, respectively. The genetic distance (D) between two samples was calculated using the following formula: $D = 1 - F$, where F is an estimation of similarity calculated as $2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of shared amplified DNA fragments in both samples x and y, N_x is the total number of fragments scored in sample x, and N_y is the total number of fragments found in sample y.

Statistical analysis

All determinations were performed in triplicate, and data are reported as mean ± standard deviation (SD) values. Significant differences were evaluated using generalized linear model (with $P < 0.05$). Hierarchical clustering analysis was done by SPSS 13.0 (SPSS Inc., Chicago, USA) for Windows. The average linkage between groups was calculated and Euclidean distance was determined according to the software.

Results

Alignment and fusion of protoplasts

Alignment of protoplasts, a prerequisite for fusion, was induced by exposure to a certain electric field strength for a set period of time. Distinct pearl chain formation was observed by microscopy when the voltage strength was increased from 400 V/cm, and sufficient pearl chains of protoplasts were formed at 500 V/cm, but the rotation of protoplasts was also observed when the voltage strength increased above 650 V/cm. The voltage dependence of pearl chain formation is shown in Fig. 1a. The voltage strength needed to align protoplasts was slightly lower than that described by Ushijima et al. [33], which might be explained by differences in apparatus and materials for fusion. Influences of field strength and number of direct-current pulses on incidence of protoplast fusion were examined. The results showed that maximum count of fusant colonies was reached at strength of direct-current pulse voltage of 3.0 kV/cm, and the optimum number of pulses for fusion was two (Fig. 1b).

Fusants were obtained by regeneration of the fused protoplasts on regeneration medium, and the results are shown in Fig. 1c, d. Figure 1c illustrates the regeneration efficiency of controls. The negative control (first line), without asymmetric inactivation treatment, showed high regeneration efficiency, whereas the positive control (second line) exhibited nearly zero regeneration efficiency on regeneration medium, which indicated that the inactivation treatment was suitable for subsequent screening. The colonies that appeared on the regeneration medium (Fig. 1d) were regarded as fusants because of the occurrence of complementary repairing.

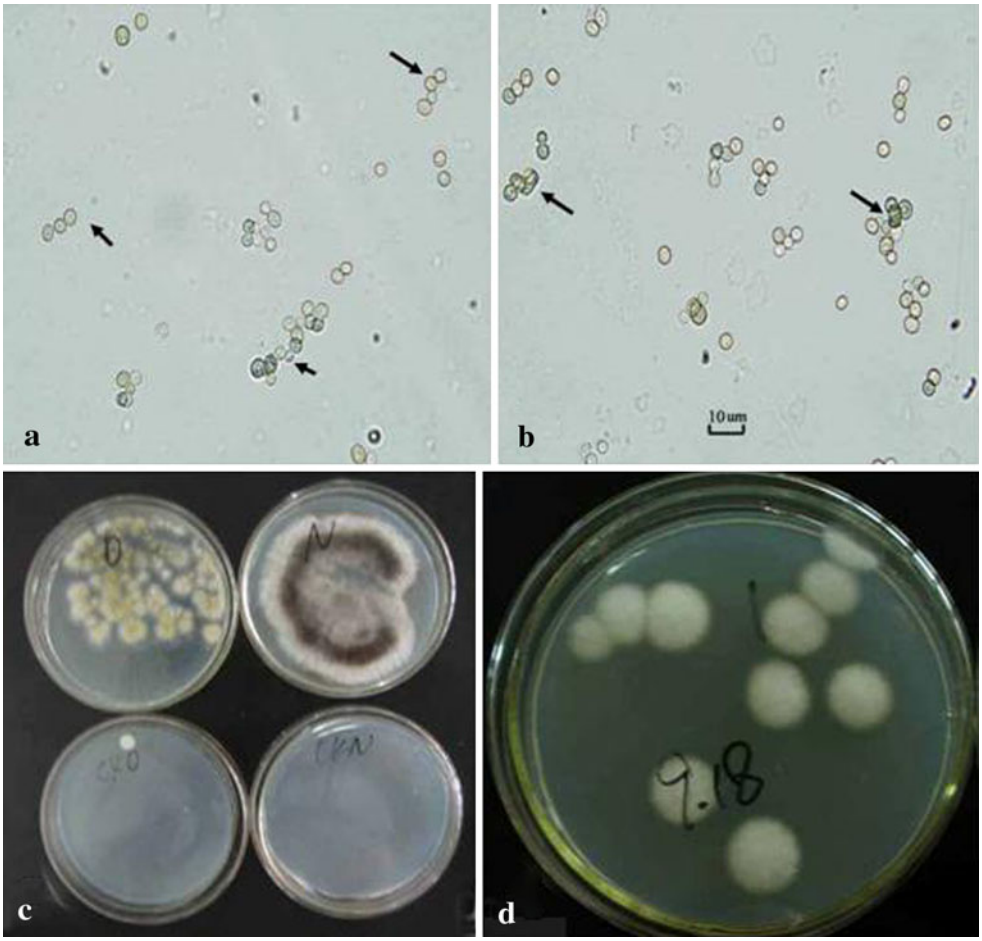

Fig. 1 Electrofusion of protoplasts between *A. oryzae* and *A. niger* (**a**, **b**) and the colonies of partial fusants and their parental strains on agar plates (**c**, **d**). **a** Pre fusion (pearl chain formation); **b** post fusion, fused protoplasts indicated by *arrow*; **c** negative control (*first line*, neither asymmetric inactivation nor fusion between parental strains) and positive control (*second line*, with asymmetric inactivation but without fusion); **d** fusants regenerated on the regeneration medium (with asymmetric inactivation and fusion); the parameters of asymmetric activation and fusion are presented in the "Materials and methods" section

Screening for fusants with high activity of acid protease

Isolation of heterozygous diploids

After electrofusion and regeneration, the colonies that appeared on regeneration medium were regarded as fusants, and the fusants showing a faster growth rate than the parental strain *A. oryzae*, were isolated from the regeneration medium. After successive subcultures on minimal medium (MM) containing *d*-camphor at concentration of 500 mg/l for five generations, the diploids were induced and stabilized. Haploidization of these diploids was subsequently induced by benomyl at concentration of 1 mg/l and stabilized by subculturing on MM for five generations. Finally, 654 fusants with faster growth rates than the parental strain *A. oryzae* were picked and preliminarily screened to identify fusants with high proteolytic activity on acid casein agar plates.

Preliminary screening for fusants with high proteolytic activity

By plate screening, 50 out of 654 strains were isolated; some representative strains are displayed in Fig. 2a, and data on their proteolytic activities are listed in Table 1. The clear zones surrounding representative strains exceeded the area occupied by the colony, which indicated that enzyme production or substrate degradation in these representative strains was higher than that of the parental strain *A. oryzae*. Interestingly, the value of Hc of the parental strain *A. niger* was lower than that of the parental strain *A. oryzae* and of the fusants. The reasons for the low Hc value of *A. niger* might be its faster growth rate in mycelial form than other strains, and thus although it displayed equivalent zones, its Hc value was low. Furthermore, this observation was consistent with the report by Bizukojc and Ledakowicz [4]. Therefore, in spite of its low Hc value, *A. niger* is commonly known as a

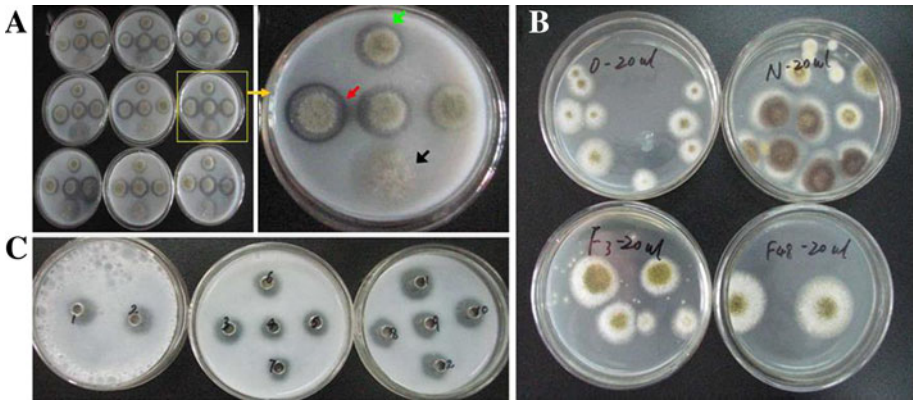

Fig. 2 Screening of fusants. **a** Differentiation of the value of Hc between partial fusants and their parental strains; **b** comparison of the growth speed of partial fusants and their parental strains on CD medium; **c** comparison of the area of clear zones on acid casein agar plates of the koji extract of partial fusants and their parental strains; *O A. oryzae*, *N A. niger*, *F* fusant, *1* F2, *2* F16, *3* F42, *4* F59, *5* F87, *6* F48, *7* F37, *8* F76, *9* F98, *10* F110, *11 A. niger*, *12 A. oryzae*

Table 1 Preliminary screening for fusants with high activity of acid protease on acid casein agar plates

Strains	Clear zone diameter, *H* (cm)	Colony diameter, *l* (cm)	Hc
A. oryzae	2.1 ± 0.06[a]	1.9 ± 0.04	1.11
A. niger	3.0 ± 0.02[b]	3.0 ± 0.06	1
F42	2.8 ± 0.02[b]	2.3 ± 0.01	1.22
F48	2.4 ± 0.09[b]	1.8 ± 0.07	1.33
F16	2.3 ± 0.03[b]	2.0 ± 0.09	1.15
F98	2.6 ± 0.01[b]	2.1 ± 0.02	1.24
F2	2.7 ± 0.02[b]	2.4 ± 0.04	1.13
F87	2.7 ± 0.03[b]	2.3 ± 0.02	1.17
F76	2.5 ± 0.02[b]	2.1 ± 0.05	1.19
F110	2.8 ± 0.04[b]	2.4 ± 0.04	1.17

Data are mean ± standard deviation values of three determinations. Data in a column followed by the same superscript letter are not significantly different ($P \leq 0.05$, by Tukey test)

Table 2 Acid protease activity of fusants in koji cultures and their comparison with parental strain *A. oryzae*

Strains	Enzymatic activity of acid protease (U/g dry weight koji culture)	Degree of enhancement of acid protease activity of fusant over *A. oryzae* (%)
A. niger	1,503 ± 376[d]	98.28
F110	1,386 ± 149[bc]	82.85
F42	1,266 ± 317[b]	67.02
F48	1,407 ± 119[c]	85.62
F76	1,381 ± 237[bc]	82.19
F98	1,274 ± 157[b]	68.07
F87	1,194 ± 259[b]	57.52
F16	1,142 ± 290[b]	50.66
F2	1,218 ± 307[b]	60.69
A. oryzae	758 ± 231[a]	0

Data are mean ± standard deviation values of three determinations. Data in a column followed by the same superscript letter are not significantly different ($P \leq 0.05$, by Tukey test)

strain with high activity of acid protease and thus could be used as the reference strain. Subsequently, the isolates selected on the basis of plate screening were used to make koji for further quantitative screening.

Further screening of the picked fusants

The results for acid protease activities of fusants in koji cultures and their comparison with parental strains of *A. oryzae* are shown in Table 2. The data listed in Table 2 clearly indicate that fusants F48, F110, and F76 exhibited higher acid protease activity than the parental strain *A. oryzae* ($P < 0.05$), amounting to 85.62%, 82.85%, and 82.19% increases, respectively. To evaluate the extracellular acid protease activity and stability in the koji-making process, five fed-batch cultivations were done in wheat bran, and the data are listed in Table 3. The results clearly showed that the fusants expressed significantly higher acid protease activity than the parental strain *A. oryzae* ($P < 0.05$) and that the difference in acid protease activity among the five generations was not significant ($P > 0.05$), suggesting that these fusants were genetically stable and suitable for practical use.

Moreover, to further evaluate other properties of the screened fusants, the growth rate of the koji mold and other hydrolytic enzyme activities (neutral and alkaline proteases, α- and β-amylase) produced by the screened fusants were also detected and compared with those of *A. oryzae*. The results are presented in Fig. 2b and Table 4. Figure 2b illustrates that the fusants had higher growth rate than *A. oryzae* on Czapek-Dox (CD) medium, and the data for other enzyme activities showed that the screened fusants exhibited similar hydrolytic ability towards proteins and

Table 3 Stability test for the acid proteases produced by partial fusants and their parental strains

Strains	Generations				
	First	Second	Third	Fourth	Fifth
A. oryzae	867 ± 231^{aA}	699 ± 131^{aA}	763 ± 261^{aA}	876 ± 104^{aA}	738 ± 158^{aA}
A. niger	1,613 ± 376^{aB}	1,542 ± 284^{aB}	1,438 ± 166^{aB}	1,644 ± 411^{aB}	1,537 ± 275^{aB}
F2	1,266 ± 317^{aB}	1,176 ± 254^{aB}	1,042 ± 309^{aB}	1,318 ± 225^{aB}	1,287 ± 198^{aB}
F16	1,362 ± 149^{aB}	1,269 ± 224^{aB}	1,194 ± 178^{aB}	1,304 ± 206^{aB}	1,297 ± 351^{aB}
F98	1,386 ± 237^{aB}	1,217 ± 168^{aB}	1,174 ± 294^{aB}	1,428 ± 312^{aB}	1,309 ± 178^{aB}
F42	1,574 ± 257^{aB}	1,613 ± 184^{aB}	1,421 ± 169^{aB}	1,396 ± 228^{aB}	1,278 ± 309^{aB}
F48	1,381 ± 259^{aB}	1,244 ± 177^{aB}	1,178 ± 328^{aB}	1,043 ± 255^{aB}	956 ± 197^{aB}
F76	1,605 ± 290^{aB}	1,524 ± 246^{aB}	1,476 ± 335^{aB}	1,569 ± 178^{aB}	1,437 ± 252^{aB}
F87	1,367 ± 307^{aB}	1,288 ± 414^{aB}	1,052 ± 358^{aB}	1,175 ± 291^{aB}	1,094 ± 371^{aB}
F110	1,549 ± 219^{aB}	1,621 ± 206^{aB}	1,428 ± 277^{aB}	1,519 ± 184^{aB}	1,377 ± 292^{aB}

Data are mean ± standard deviation values of three determinations. Data in a column followed by the same superscript letter are not significantly different ($P \leq 0.05$, by Tukey test)

Table 4 Distribution and stability of proteases and amylases from partial fusants and their parental strains after five-generation subculture (U/g dry weight koji culture)

Strains	Acid protease	Neutral protease	Alkaline protease	α-Amylase	β-Amylase
A. oryzae	867 ± 231^{a}	3,634 ± 236^{b}	2,899 ± 556^{b}	1,101 ± 365^{c}	13,709 ± 852^{b}
A. niger	1,613 ± 376^{c}	178 ± 35^{a}	186 ± 80^{a}	9,009 ± 760^{e}	7,494 ± 750^{a}
F2	1,466 ± 317^{b}	3,554 ± 180^{b}	2,872 ± 672^{b}	1,622 ± 344^{c}	13,645 ± 835^{b}
F13	1,362 ± 149^{b}	3,437 ± 397^{b}	3,116 ± 493^{b}	1,001 ± 275^{c}	13,392 ± 937^{b}
F38	1,486 ± 237^{b}	3,663 ± 286^{b}	3,266 ± 390^{b}	1,067 ± 232^{c}	13,800 ± 995^{b}
F42	1,574 ± 157^{bc}	3,279 ± 455^{b}	2,713 ± 327^{b}	678 ± 99^{a}	13,967 ± 553^{b}
F48	1,381 ± 259^{b}	3,318 ± 246^{b}	2,729 ± 634^{b}	701 ± 219^{a}	14,310 ± 890^{b}
F76	1,605 ± 290^{c}	3,303 ± 250^{b}	2,676 ± 578^{b}	793 ± 148^{a}	14,400 ± 609^{b}
F79	1,467 ± 307^{b}	3,816 ± 275^{b}	3,970 ± 671^{c}	830 ± 180^{b}	15,507 ± 823^{c}
F110	1,549 ± 119^{b}	3,436 ± 437^{b}	3,357 ± 798^{c}	1,212 ± 340^{c}	13,912 ± 697^{b}

Data are mean ± standard deviation values of three determinations. Data in a column followed by the same superscript letter are not significantly different ($P \leq 0.05$, by Tukey test)

starches under neutral conditions to *A. oryzae* (Table 4). To further evaluate the acid protease activity of the screened fusants, the koji extracts were all adjusted to the same protein concentration, and 100 µl of the extracts was added to cylinders on milk clotting assay plates. The milk clotting activities of the fusants and parental strains were compared by the area of clear zones around the test wells, and the results clearly illustrated that the diameters of clear zones of fusant extracts were larger than those of *A. oryzae*, indicating enhanced levels of acid protease in the fusants (Fig. 2c).

Identification of fusants

Mycelial morphology of representative fusants

Morphological variations were monitored with an Olympus microscope at 40 × magnification, and representative observations are shown in Fig. 3a. Figure 3a illustrates that mycelial morphology varied between the representative fusants and their parental strains in terms of diameter, roughness, and degree of branching. Compared with the parental strain *A. oryzae*, mycelia of F76 and F110 exhibited more sturdy and less branching characteristics, typical of *A. niger* to some extent. Phenotypes are a reflection of the genetic makeup of the strain, and so the variations in phenotypic characteristic must have resulted from mutations, deletions or insertions in the genome [37]. In addition, some research has shown that aerial mycelia are not only extremely important for respiration by filamentous fungi on solid-state medium, but also are intimately associated with the process of biomass accumulation and enzyme secretion [7, 27]. Therefore, the variation in mycelial morphology observed between representative fusants and their parental strains may be a result of extensive rearrangements of the

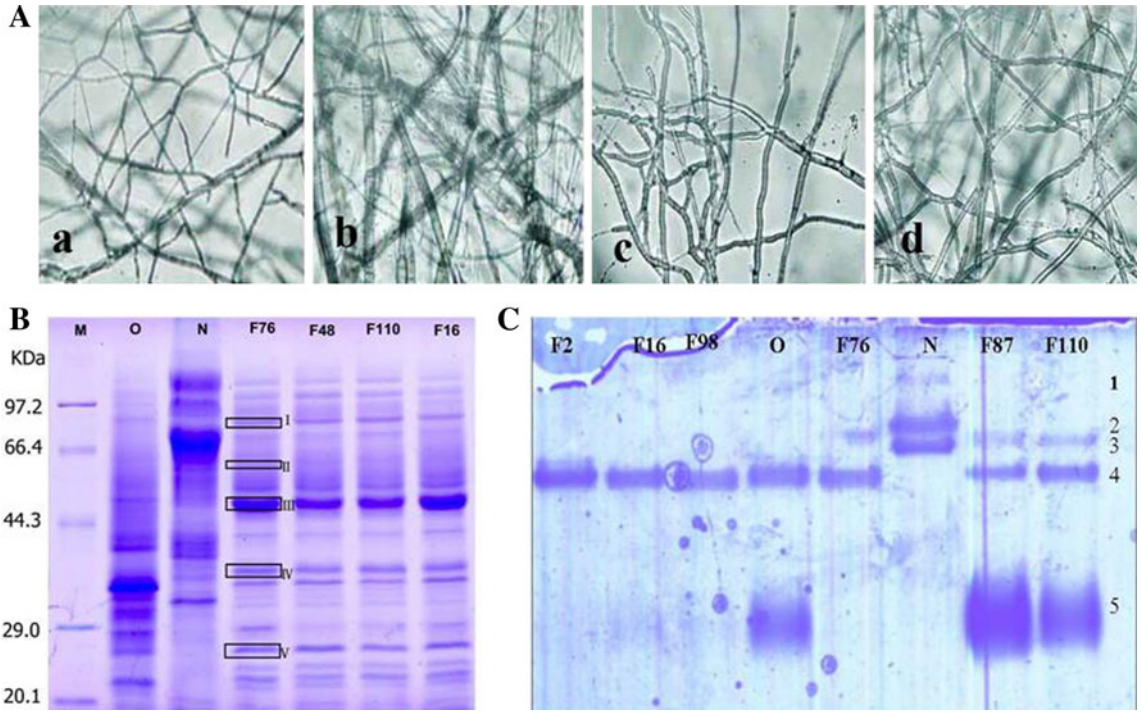

Fig. 3 Identification of the partial fusants. **a** Differences in mycelia morphology: *a A. oryzae*, *b A. niger*, *c* F76, *d* F110. **b** Comparison of SDS–PAGE profiles between fusants and their parental strains: *O A. oryzae*, *N A. niger*, *M* marker. **c** Comparison of esterase isoenzyme profiles between the representative fusants and their parental strains: *O A. oryzae*, *N A. niger*

genomes between the two parental strains, and to some degree, this same rearrangement may be responsible for the differences in hydrolytic enzyme activities between parental strains and fusants.

Whole-cell protein profiles

Whole-cell protein profile analysis was conducted by SDS–PAGE, and the results showed there were considerable differences in protein patterns between *A. oryzae*, *A. niger*, and some fusants (Fig. 3b). While a number of corresponding bands were observed in parental stains and fusants, the protein patterns in fusants were basically different from those of *A. oryzae* and *A. niger*. Among the fusants, however, the protein profiles were similar. Thus, the differentiation of protein profiles between the parental strains and fusants could be demonstrated by comparison of patterns between a representative fusant (F76) and its parents. Unlike those of *A. oryzae* and *A. niger*, the SDS–PAGE profiles of F76 exhibited strong, well-defined, intense bands in the gel (bands III, IV, and V; Fig. 3b). SDS–PAGE profile analysis of whole-cell proteins offers the advantages of a good level of taxonomic resolution at species or subspecies level [8]. It has also been used successfully to solve problems in the identification of closely related species in the genera *Lactococcus* [10]. Thus, it can be safely concluded that the fusants are genomically heterogeneous species, different from the parental strains.

Esterase patterns

Esterase banding patterns of *A. oryzae* and *A. niger* were considerably different, and variation within fusants was also observed (Fig. 3c). When the parental strains were compared, *A. oryzae* was characterized by bands 4 and 5, while *A. niger* was associated with bands 1, 2, and 3. As for the fusants, they exhibited considerable variations. Fusants F2, F16, F98, and F76 showed patterns typical of *A. oryzae* (strong coloration of band 4), whereas fusants F87 and F110 showed banding patterns intermediate between the two parental strains (bands 3, 4, and 5). Furthermore, the strong coloration of band 5 was absent in fusants F2, F16, F98, and F76.

Genome polymorphism

The results of RAPD fingerprint analysis confirmed the hybrid nature that the fusants displayed in comparison with the two parental strains, as seen by the combination of specific parental bands (Fig. 4a). In addition, there were also unique bands in the fusants, revealing the recombination of parental genomes in the fusants. To verify the

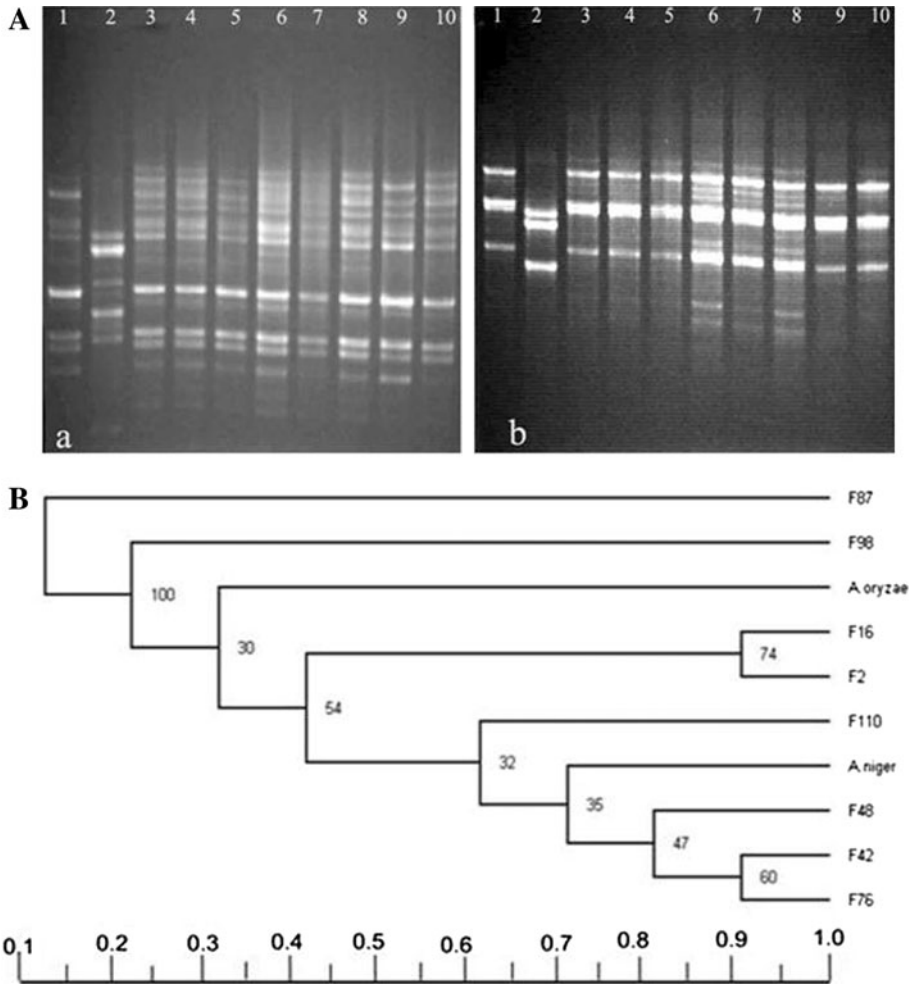

Fig. 4 RAPD fingerprints (**a**) and dendrogram (**b**) of the fusants and their parental strains. *Lane 1 A. oryzae*, *lane 2 A. niger*, *lane 3* F2, *lane 4* F16, *lane 5* F98, *lane 6* F42, *lane 7* F48, *lane 8* F76, *lane 9* F87, *lane 10* F110. *a* Primer 1, *b* primer 2

polymorphism banding data from the agarose gels, dissimilarity indices were calculated for the fusants and their parental strains, and an average linkage dendrogram was constructed based on the RAPD data (Fig. 4b). The average genetic distances between the fusants and their parental strains were also calculated (Table 5), and the results revealed that fusants F42, F48, and F76 were separated by long distances from their parental strain *A. oryzae*, indicating that the process of genome recombination between *A. oryzae* and *A. niger* during protoplast fusion was complex. Genetically, the results could be used to explain why the phenotypes of mycelial morphology and esterase isoenzyme profiles of fusants were intermediate between the two parental strains.

Discussion

Unlike traditional mutation and genetic engineering, genome recombination through protoplast fusion between selected strains with desirable properties is a well-known and widely accepted procedure for industrial strain improvement. In addition, genome recombination allows the creation of strains embodying a range of characteristics from the parents or displaying polygenic traits that would be difficult to construct using more refined methods [16]. Genome recombination has several possible outcomes and can result in genome rearrangements and exchange of genetic material between organisms [36]. In the present study, we applied genome recombination to breed desirable fusants, and the results showed that protoplast fusion successfully induced DNA recombination between *A. oryzae* and *A. niger*. By this efficient technique, we successfully bred several novel fusants with enhanced acid protease production compared with the parental strain *A. oryzae* ($P < 0.05$). More specifically, fusant F76 produced acid protease at a yield of nearly 1,500 U per gram dry seed koji, a value that is higher by 82.19% in activity than the parental strain *A. oryzae* and near to, or even higher than, the parental strain *A. niger*, when grown under the same conditions (Table 2).

In this study, asymmetric inactivation was used as the means to select hybrids from mixtures of heterokaryons. Based on complementary repairing of asymmetric damage,

Table 5 Dissimilarity matrix of genetic distance based on the RAPD bands between partial fusants and their parental strains

	A. oryzae	*A. niger*	F2	F16	F98	F42	F48	F76	F87	F110
A. oryzae	0.000									
A. niger	3.873	0.000								
F2	1.732	4.243	0.000							
F16	1.732	4.243	0.000	0.000						
F98	1.000	4.000	1.414	1.414	0.000					
F42	3.162	4.796	2.646	2.646	3.000	0.000				
F48	2.449	4.359	2.236	2.236	2.236	2.449	0.000			
F76	2.828	4.583	2.646	2.646	2.646	1.414	2.000	0.000		
F87	1.000	4.000	1.414	1.414	0.000	3.000	2.236	2.646	0.000	
F110	1.732	4.000	1.414	1.414	1.414	3.000	1.732	2.646	1.414	0.000

the "double-inactivation" protoplast fusion technique has now been used by a number of groups to produce new strains in different organisms and has revealed universal applicability [22, 31]. This method exploits the "donor-recipient" mechanism in which the nuclei of one fusion partner are inactivated by ionizing irradiation, whilst treatment of the other fusion partner with a toxin or heat inactivates the cytoplasm. Accordingly, we inactivated the protoplasts of *A. oryzae* and *A. niger* by UV irradiation and heat treatment, respectively, and the results showed that this procedure remarkably decreased the occurrence of negative hybrids and enhanced the selection efficiency for preliminary selection. In addition, genetic recombination does not necessarily involve only the targeted enzyme-encoding genes of both strains; it is likely that a wide variety of general housekeeping genes involved in growth are also recombined and thus could produce new interesting genotypes [9]. Therefore, the colonies that produced clearer halos, and produced them faster, in the acid casein agar plate assay were selected for further screening after the genetic segregation of the heterokaryons.

Previously, identification of hybrids was routinely done by phenotypic analyses of properties such as colony morphology, biochemical characteristics, and even product validation, but molecular techniques were rarely introduced. Electrophoretic isoenzyme profiles and RAPD fingerprints are very useful for studying intra- and interspecific genetic diversity. Distinct RAPD profiles may be interpreted as an indication of rearrangements occurring in the primer annealing regions that prevent or allow amplification, as previously observed [3]. In the present study, the resulting fusants, which were morphologically intermediate between the parental strains *A. oryzae* and *A. niger*, had special and unique banding patterns in isoenzyme profiles (Fig. 3c), which meant that some structural genes in the *A. oryzae* genome must have been changed during the rearrangements. In addition, the occurrence of genome recombination during protoplast fusion was evaluated by RAPD fingerprints (Fig. 4); overall, the data (Table 5) confirmed the observations of the mycelial morphology and esterase profiles between fusants and their parental strains, even though they were not exactly the same.

By this combined process of artificial electrofusion and subsequent haploidization, some characteristics of parental strains, especially the high activity of acid protease in *A. niger*, were successfully introduced into the fusants as described above. Moreover, it can be concluded that the procedure of genome recombination associated with an efficient selection process is a powerful tool for genetic improvement of food-grade industrial strains.

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