Appl Microbiol Biotechnol (1990) 33:671-676

Applied and Microbiology Biotechnology

Intergeneric hybridization between *Monascus anka* and *Aspergillus oryzae* by protoplast fusion

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Received 24 January 1990/Accepted 23 April 1990

Summary. To breed industrially useful strains of a slow-growing, red-pigment-producing strain of *Monascus anka*, protoplasts of *M. anka* MAK1 (*arg*) and *Aspergillus oryzae* AOK1 (*met, thr*) were fused. A mixture of protoplasts prepared from mycelia of *M. anka* MAK1 treated with 2% Usukizyme and of *A. oryzae* AOK1 treated with 2% Usukizyme and 0.2% NovoZym 234 was incubated with 30% (w/v) polyethylene glycol no. 6000. Heterokaryon fusants complementing the auxotrophies of both mutants were isolated on minimal medium, but segregated into red (MAK1) and white (AOK1) sectors after being cultured on a complete medium. After irradiation with UV light, the fusants gave stable heterozygous diploids that formed long white hyphae. These diploids, which had twice as much DNA in the nucleus as their parents, grew more rapidly than the parent strain YZT1, and produced ethanol earlier than the parents. Production of amylase, protease, and kojic acid by the fusants was intermediate in amount between that of the two parents.

Introduction

Species of *Monascus* that have traditionally been used in oriental countries in the production of food colourants and as a fermentation starter for red rice wine produce valuable substances including hydrolytic enzymes such as glucoamylase (Iizuka and Mineki 1977), protease (Tsai et al. 1978), α-galactosidase (Wong et al. 1986), and polypectase (Bridge and Hawksworth 1985). *Monascus pilosus* produces monacolin K, which inhibits the biosynthesis of cholesterols (Endo 1979). Some *Monascus* species abundantly produce red, yellow, and purple pigments such as monascorubrin, rubropunctatin, monascin, anka-flavine, rubropunctamin, and monascorubmin (Nishikawa 1932; Nakanishi et al. 1959; Manchand et al. 1973; Ishiwata et al. 1974).

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The pigment mixtures produced by *M. anka* are commercially produced and widely used to colour flesh foods in the main. The strains that hyperproduce the pigments and also produce ethanol have the practical disadvantage that they grow more slowly and poorly than other industrial moulds. Various strains of *Aspergillus oryzae* that are familiar as *koji* moulds in Japan have been used for a long time as a fermentation starter for the saccharifying process of rice in the production of sake (Japanese rice wine), and can grow rapidly, utilizing various natural materials but not fermenting sugars.

Protoplast fusion, originally developed for plant cells (Kao and Michayluk 1974), has been applied to the breeding and gene analysis of microorganisms. Mutation and genetic crossing have been used in the phenotypic improvement of industrial moulds: intra- or interspecific protoplast fusion of organisms including *Aspergillus* (Kevei and Peberdy 1977; Furuya et al. 1983; Ushijima and Nakadai 1987; Ogawa et al. 1988), *Mucor* (Genthner and Borgia 1978; Ohnuki et al. 1982), *Penicillium* (Anné and Peberdy 1976), and *Trichoderma* (Ogawa et al. 1987) have also been used. Reports on intergeneric fusion are few. We thought that it might be possible to obtain some fast-growing fermenting strains from *M. anka* YZT1, which hyperproduces pigments, by protoplast fusion with *A. oryzae*. Here we describe the construction of phenotypically stable heterozygous diploids and some of their properties.

Materials and methods

Strains of moulds used. Monascus anka YZT1, which hyperproduces red pigments and is used for the production of natural food couloring, is maintained at the Research Center of Yaegaki Zymotecnics Co., Himeji, Japan. *A. oryzae* WS1, which forms conidia poorly and grows white colonies composed of long hyphae, has been used for the production of *amasake* (a sweet drink produced from saccharified rice). *M. anka* MAK1, an arginine-requiring (*arg*) mutant of strain YZT1, and *A. oryzae* AOK1, a mutant requiring methionine and threonine (*met, thr*) of strain WS1, were constructed in this study.

Media and culture. Czapek Dox medium (Anné and Peberdy 1976), pH 6.0, was used as the basal minimal medium. When necessary, 50 μg/ml amino acids, 0.2% casamino acid, or 0.2% yeast extract was added. Hennerberg medium used as the complete medium was composed of 10% glucose, 1% peptone, 0.2% KNO_3, 0.2% $NH_4H_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.01% $CaCl_2$. When solid media were prepared, 2.0% agar was added and allowed to solidify. CY medium for the production of pigments was composed of 5% corn starch and 1% yeast extract, pH 6.0. Cultures were carried out at 30° C. Mycelial growth is expressed as grams of wet mycelial mass/100 ml.

Mutagenesis. Conidia of *M. anka* YZT1 or *A. oryzae* WS1 spread onto Hennerberg's agar plates were irradiated with UV light to give a survival rate of 0.1%. An arginine-requiring mutant (AMK1) and a methionine- and threonine-requiring mutant (AOK1) were obtained from strains YZT1 and WS1, respectively. These mutants reverted at frequencies of 10^{-6} to 10^{-7} to the prototypes on agar plates containing minimal medium.

Preparation of protoplast cells from mycelia of MAK1 and AOK1 strains. A loopful of MAK1 or AOK1 conidia was used to inoculate 100 ml Hennerberg medium in a 500-ml shaking flask and the culture was shaken at 100 rpm for 36 h on a reciprocal shaker. The mycelia cultured were harvested by passage through filter paper (no. 2; Toyo Roshi Co., Tokyo, Japan) and washed with 0.6 *M* NH_4Cl, pH 5.6. The wet mycelia (about 150 mg) were suspended in 1 ml of a hypertonic solution containing various concentrations of cell-wall-lytic enzymes, Usukizyme (Wako Pure Chemical Industries, Osaka, Japan) and NovoZym 234 (Novo Industri, Bagsvaerd, Denmark), derived from *Trichoderma* spp., and was shaken at 70 rpm at 35° C for 2–3 h. The protoplasts were counted under a microscope with use of a haemacytometer. After the suspension was passed through glass filters G2 and G3, the protoplasts were harvested by centrifugation (700 *g*, 5 min).

Protoplast fusion. Protoplast fusion was done by a modification of procedures described before (Anné and Peberdy 1976; Furuya et al. 1983). After protoplasts prepared from MAK1 were mixed with those from AOK1 and centrifuged at 700 *g* for 5 min, the mixture of protoplasts was suspended in 1 ml of 0.05 *M* glycine-NaOH buffer, pH 7.5, containing 0.05 *M* $CaCl_2$ and 30% (w/v) polyethylene glycol no. 6000 (PEG) and incubated at 30° C for 15 min. Then the protoplasts were harvested by centrifugation at 700 *g* for 5 min, suspended in 1 ml of hypertonic Czapek Dox medium, and incubated at 30° C for 24 h. The culture was spread onto agar plates of hypertonic minimal or complete medium, covered with the same containing 0.5% agar, and incubated at 30° C for 4–7 days. The colonies that grew on the minimal-medium plates were considered to be those of fused strains lacking any requirement for amino acids. The rates of protoplast fusion were expressed as the numbers of colonies that grew on minimal medium as a percentage of the number that grew on complete medium.

Diploidization of heterokaryons. Conidia of heterokaryon fusants were spread onto minimal and complete agar medium plates and were irradiated with UV to give a survival rate of 50%. After incubation at 30° C for 4–7 days, colonies that did not segregate into the MAK1 and AOK1 sectors were selected as diploid fusants.

Measurement of DNA of conidia. The amount of DNA of conidia was assayed by colorimetric methods described elsewhere (Schmidt and Thannhauser 1945; Schneider 1946; Ceriotti 1954). The A_{490} of 1.0 corresponded to a DNA concentration of 150 μg/ml. Nuclei in the conidia were stained with Giemsa's dye (Toyama and Toyama 1988) and counted under a microscope. The amount of DNA in the conidia is expressed as the amount of DNA per nucleus.

Assay for enzymes. First, 100 g rice was washed with tap water and soaked overnight in water. The water was poured off, and the rice was transferred to a 500-ml flask and steamed for 30 min. One millilitre of the culture fluid shaken at 30° C for 36 h in complete medium was used to inoculate the flask, which was incubated at 30° C for 2–5 days with mixing every 12 h. After culture, 10 g of mouldy rice (known as *koji* seed in Japan) was extracted for 3 h with 100 ml of 0.5% NaCl and filtered through filter paper (Toyo no. 2). The filtrate was assayed for α-amylase (Murao et al. 1977) and protease (Kurimoto et al. 1979) activities.

Measurement of products. Kojic acid was produced by a culture grown at 30° C for 7 days in the medium described by Ishiie (1966). The culture fluid was filtered through filter paper and then through a membrane filter (diameter 0.45 μm), and the acid in it was measured by HPLC (Hitachi 655A; column, Unisil Q C18, 4.0 × 250 mm; mobile phase, H_3PO_4, pH 3.0; pressure, 15 kg/cm^2; flow rate, 0.8 ml/min; detector, UV 210 nm).

To test for the production of ethanol, mycelia that had been cultured at 30° C for 3 days in Czapek Dox medium supplemented with 0.2% casamino acid and 0.2% yeast extract were harvested by filtration. They were transferred into a screw-capped test tube containing the same medium, and incubated without shaking at 30° C. Portions of the suspension were withdrawn at times and their supernatants were tested for ethanol production by gas-liquid chromatography (Shimadzu, Kyoto, Japan, GC-7AG; column, Chromosorb 101, diameter 3 mm, 1.5 m long; carrier gas, N_2 at 40 ml/min; oven temperature, 180° C; detection by flame ionizer). To determine production of the pigments monascorubrin and rubropunctatin, mycelia that had been cultured for 7 days in 100 ml CY medium in a 500-ml shaking flask and filtered

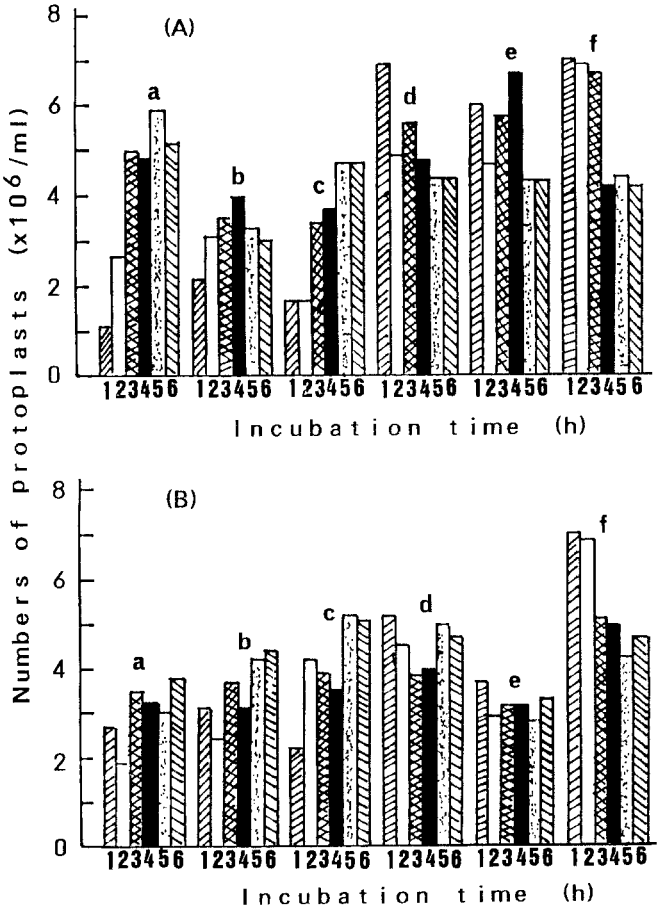

Fig. 1. Effects of 6 *M* D-sorbitol (*a*), mannitol (*b*), sucrose (*c*), NaCl (*d*), KCl (*e*), and $(NH_4)_2SO_4$ (*f*) during protoplast formation from *Monascus anka* MAK1 (**A**) and *Aspergillus oryzae* AOK1 (**B**) using 1% Usukizyme

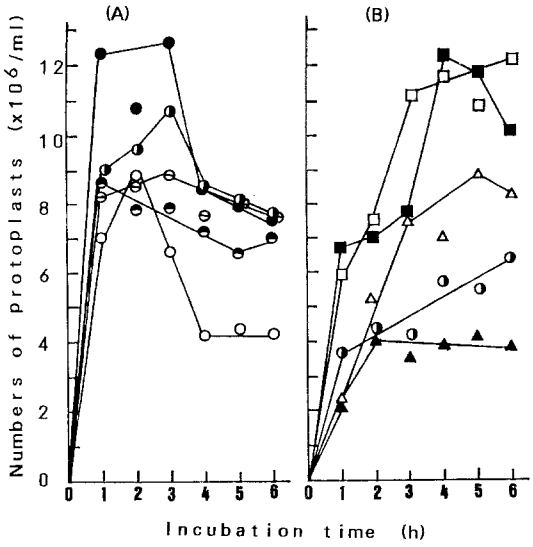

Fig. 2. Effects of lytic enzymes on protoplast formation. *M. anka* MAK1 (**A**) was treated with Usukizyme at concentrations of 1% (○), 2% (›), 3% (◑), 5% (⊖), or 8% (◓), and *A. oryzae* AOK1 (**B**) was treatment with 2% (◑) or 4% (▲) Usukizyme, or else with 0.2% (□), 0.4% (■), or 0.6% (△) NovoZym 234 in addition to 2% Usukizyme

were extracted with 50 ml of 95% ethanol. The ethanol extract, either diluted or concentrated, was subjected to HPLC (Shimazu LC-8A; column, Lichro CART manu-fix 50-4 100RP-18, diameter 4.0 × 50 mm; mobile phase, 60% CH_3CN and 0.05% trifluoroacetic acid; flow rate, 2 ml/min; detector, SPD-6A, absorbance at 470 nm).

Results and discussion

Conditions for formation of protoplasts from mycelia of M. anka MAK1 (arg⁻) and A. oryzae AOK1 (met⁻, thr⁻)

Many cell-wall-lytic enzymes from various sources are commercially available for the preparation of protoplasts from mould mycelia. We found in preliminary tests that Usukizyme and NovoZym 234 could be used for protoplast formation from *M. anka* MAK1 and *A. oryzae* AOK1. D-Sorbitol, mannitol, sucrose, NaCl, KCl, and $(NH_4)_2SO_4$ were first tested as hypertonic solutions for protoplast formation using 1% Usukizyme (Fig. 1). With 6 *M* $(NH_4)_2SO_4$, protoplasts were formed from mycelia of both strains at a frequency of about 7×10^6/ml. Next, the lytic enzymes used were tested for optimum concentration. Protoplasts of MAK1 were formed at a frequency of 1.3×10^7/ml with 3% Usukizyme alone, and protoplasts of AOK1 were formed at a frequency of 1.2×10^7/ml with a combination of 2% Usukizyme and 0.4% NovoZym 234 (Fig. 2). Protoplast formation was most satisfactory at pH 6.0 and 35° C.

As reported by others (Nohmi and Ichihara 1982; Furuya et al. 1983), protoplasts formed from these species of moulds were yeast-like spheres or ovoids in the presence of 6 *M* $(NH_4)_2SO_4$, and burst when the suspension was diluted with water. When protoplasts of MAK1 and AOK1 were incubated in Czapek Dox medium containing 6 *M* $(NH_4)_2SO_4$ (regeneration medium), mycelia regenerated at a frequency of 36.5% and 21.7%, respectively. On the other hand, the auxotrophies of both mutants reverted at frequencies of the order of only 10^{-6} during regeneration (Table 1).

Protoplast fusion

When a mixture of 8.20×10^5 MAK1 protoplasts and 5.36×10^5 AOK1 protoplasts was treated to cause cell fusion and incubated on regeneration medium without auxotrophic requirements for 7 days, 1.97×10^3 red colonies grew; the frequency was 0.14% (Table 1). No colonies grew on a non-hypertonic medium without ammonium sulphate. Thus, the colonies that grew were probably fusants between *M. anka* and *A. oryzae*. All fusants had morphological properties of both MAK1 and AOK1; the mycelia had long (*A. oryzae*-like) red (*M. anka*-like) hyphae. When the fusants were grown on complete medium for a long time, the colony segregated into red and white portions (Fig. 3A). These results showed that the fusants were heterokaryons.

Formation of heterozygous diploids

Heterokaryons can be made diploid by treatment with UV light (Oda and Iguchi 1963; Ushijima and Nakadai 1987). After irradiation with UV light that gave a 50% survival rate, eight colonies that did not segregate even after being subcultured ten times in complete media were isolated. On a complete medium plate, one of these colonies, tentatively designated AOFD-1, showed long white hyphae (Fig. 3B) with perithecia at the tips

Table 1. Frequency of fusion between protoplasts of *Monascus anka* MAK1 (*arg*) and *Aspergillus oryzae* AOK1 (*met, thr*)

Strains used	Reversion[a]	Numbers of protoplasts		Numbers of fusants
		Before[b]	After[c]	
M. anka MAK1	3.8×10^{-6} (*arg*)	8.2×10^5	8.3×10^5	2.0×10^3
A. oryzae AOK1	5.2×10^{-6} (*met*), 2.2×10^{-6} (*thr*)	5.4×10^5	5.4×10^5	

[a] Reversion frequencies of auxotrophies of the mutants
[b] Numbers of mycelial colonies growing on regeneration medium before protoplast fusion treatment
[c] Numbers of mycelial colonies growing on regeneration medium after protoplast fusion treatment

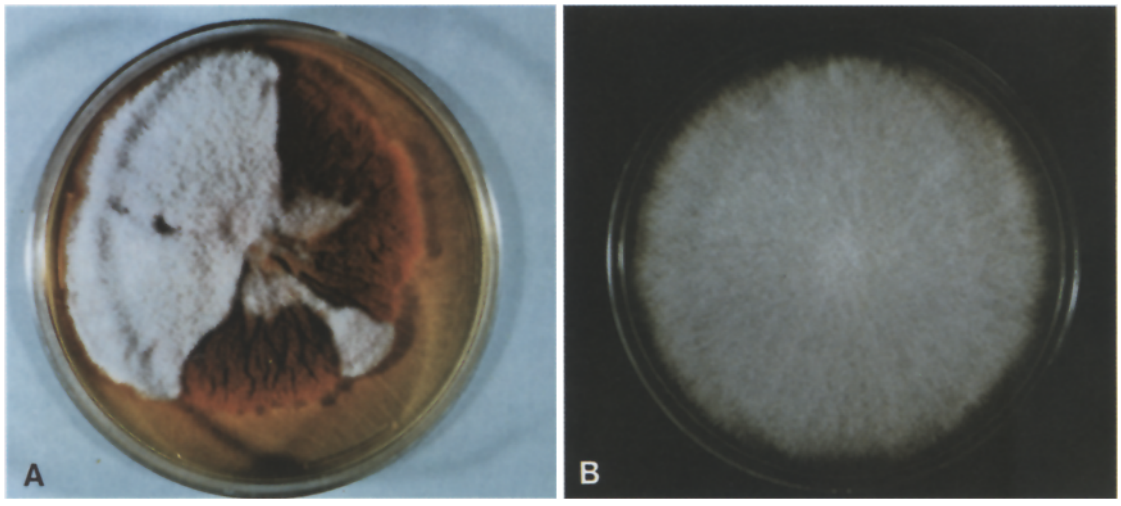

Fig. 3. Growth of heterokaryonic (**A**) and diploid (**B**) fusants on Hennerberg medium plates

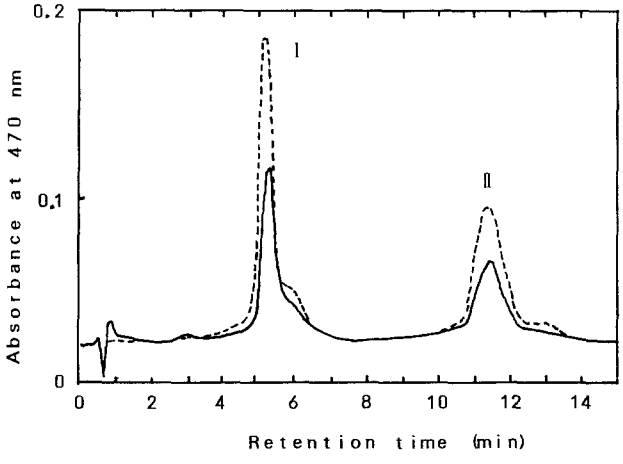

Fig. 4. HPLC of the pigments monascorubrin (*I*) and rubropunctatin (*II*), produced in mycelia of the diploid strain AOFD-1 (*solid curve*) and the parent strain *M. anka* YZT1 (*dotted curve*) cultured for 7 days in CY medium

and conidia (angled spheres) characteristic of *M. anka* (not shown). However, on a minimal medium plate or in CY medium the growth was red. The pigments produced in CY medium by the parent strain MAK1 and the strain AOFD-1 were then assayed by HPLC. As Fig. 4 shows, the diploid produced the red pigments monascorubrin (I; 67%) and rubropunctatin (II; 33%) as did the parent strain, but the former strain produced only 3.4% of the total amount of pigments produced by the latter strain.

To test whether the fusants were diploids, numbers of nuclei and the DNA in conidia from a colony of fusants were investigated. Single conidia of *M. anka* and *A. oryzae* had a mean of five nuclei, and single conidia of the fusant had three. That is, the mean number of nuclei in the fusant conidia was half that of the parents, so the fusant was diploid. The total DNA in single conidia of the fusant and the parents was almost the same, but the mean amount in single nuclei of the fusant was double that of the parents (Table 2). It was additionally

Table 2. Mean amounts of DNA in a nucleus of diploid fusant AOFD-1 and other strains

Strains	Amount of DNA per conidium (10^{-7} µg)	Numbers of nuclei per conidium[a]	Amount of DNA per nucleus (10^{-7} µg)
Prototrophic strains			
M. anka YZT1	5.24	4–6 (5)[b]	0.87–1.31 (1.1)[b]
A. oryzae WS1	4.81	4–6 (5)	0.80–1.20 (1.0)
Auxotrophic strains			
M. anka MAK1	5.40	4–6 (5)	0.90–1.35 (1.1)
A. oryzae AOK1	4.86	4–6 (5)	0.81–1.22 (1.0)
Diploid fusant			
AOFD-1	5.91	2–3 (3)	1.48–2.96 (2.2)

[a] One hundred colonies were tested
[b] Mean values

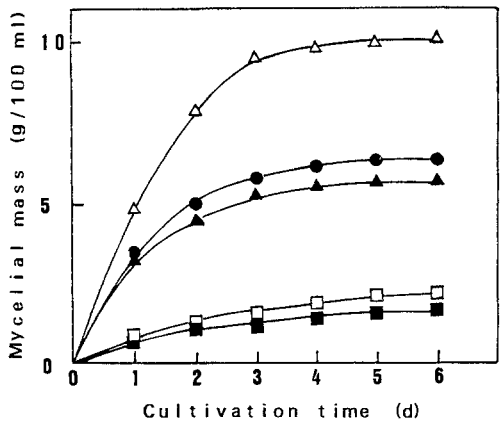

Fig. 5. Growth curves of *M. anka* YZT1 (□), *M. anka* MAK1 (■), *A. oryzae* WS1 (△), *A. oryzae* AOK1 (▲), and a diploid fusant AOFD-1 (●) in Hennerberg medium

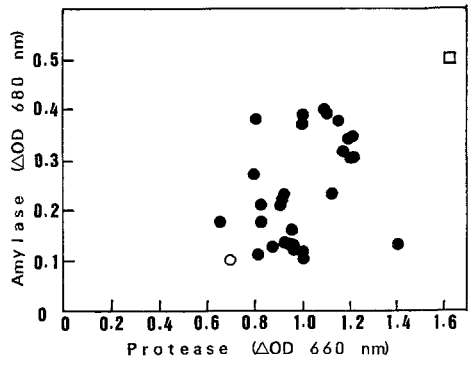

Fig. 6. Production of amylase and protease by *M. anka* YZT1 (○), *A. oryzae* WS1 (□), and 30 diploid fusants (●)

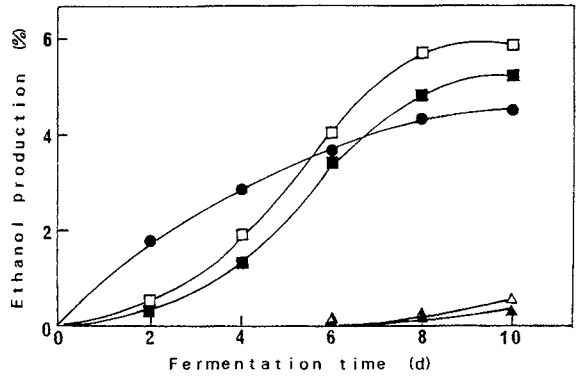

Fig. 7. Production of ethanol by *M. anka* YZT1 (□), *M. anka* MAK1 (■), *A. oryzae* WS1 (△), *A. oryzae* AOK1 (▲), and a diploid fusant AOFD-1 (●)

found that a 3-kb *Eco*RI fragment from MAK1 DNA, which was cloned on pHSG299 in *Escherichia coli* JM105, hybridized to one of the *Eco*RI digests of DNA from strain AOFD-1 (not shown). These results showed that the constructed strain AOFD-1 was a hybrid between *M. anka* and *A. oryzae* in spite of showing an *A. oryzae*-like appearance of the growth on solid medium.

Industrial applicability of the diploid fusants

To explore possible industrial applications of the diploid fusants, their properties were tested. *M. anka* grows slowly, so one of aims of this study was to develop a strain that grew fast by protoplast fusion of *M. anka* and *A. oryzae*. We tested the diploid fusants for speed of growth in Hennerberg's medium. The diploid strain AOFD-1 grew more rapidly than one parent strain, YZT1, but not as fast as the parent strain, *A. oryzae* WS1 (Fig. 5). Next, 30 diploid fusants were tested for production of amylase and protease. The parent strain *M. anka* YZT1 produced less of both enzymes than the other parent, *A. oryzae* WS1; all fusants produced more than the YZT1 strain (Fig. 6). The production of larger amounts of these enzymes would probably allow them to grow more rapidly.

M. anka has an advantage for use in industry in that it ferments glucose to produce ethanol without the participation of yeast, but it also produces pigments during fermentation. This is a disadvantage since coloured sake is unpopular; if coloured sake is required, colourants can be added to colourless products. Although we had expected to construct hybrids that can produce more pigments and grow faster than strain YZT1 before the start of this study, all diploid fusants we obtained produced less pigments, but grew faster. However, these might be more useful for the production of sake.

We compared the production of ethanol by one such fusant with that by the parental strains (Fig. 7). The fusant produced more alcohol at an early stage, but production at 10 days was 4.2% (v/v) less than the 6.0% production by one parent, *M. anka* YZT1. Earlier fermentation might be of industrial use. Next, *koji* was prepared on a laboratory scale with the diploid fusant AOFD-1 using steamed rice in a 1-l flask and on an industrial scale in an automatic *koji* producer. *Koji* prepared in the flask under air-deficient conditions was white, but that produced in the automated apparatus under forced-air conditions was red.

Sake was produced using *koji* of the fusant, and a fruit-flavoured, lightly alcoholic drink was produced. However, the sake had a slightly unpleasant smell, so the *koji* used was assayed. More of the hydrolytic enzymes α-amylase and neutral protease and of aroma substances *n*-propanol, isobutanol, isoamyl acetate, and isoamyl alcohol were produced in the white *koji* than in the red one (Table 3). The greater acidity of the red *koji* may have reflected more oxidation of metabolites such as alcohols due to the forced aeration. Kojic acid, which is a characteristic product of *A. oryzae*, was produced by the two fusants tested, but not as much as by *A. oryzae* (Table 4).

There have been previous no reports on hybridization between *M. anka* and *A. oryzae* by protoplast fusion. Here, we obtained phenotypically stable heterozygous diploids by intergeneric protoplast fusion. Further

Table 3. Components in *koji* prepared with a diploid fusant AOFD-1

Materials	AMase (units)	PRase (units)	PrOH (ppm)	BuOH (ppm)	AmOH (ppm)	AmAC (ppm)	Moisture (%)	Acidity (%)
Red *koji*[a]	79.0	0.030	trace	19.1	38.2	3.4	33.7	0.41
White *koji*[b]	85.0	0.031	74.7	215.2	153.9	16.4	49.7	0.15

[a] *Koji* was a solid culture of the AOFD-1 strain grown on steamed rice, prepared by an automatic *koji* producer under conditions of forced air

[b] As [a] but grown in a 500-ml flask

AMase, α-Amylase; PRase, protease; PrOH, *n*-propanol; BuOH, isobutanol, AmOH, isoamyl alcohol; AmAC, isoamyl acetate

Table 4. Production of kojic acid by parent and derivative strains

Strains	Kojic acid produced (ppm)
Prototrophic strains	
M. anka YZT1	Trace
A. oryzae WS1	44.79
Auxotrophic strains	
M. anka MAK1 (*arg*)	Trace
A. oryzae AOK1 (*met, thr*)	42.18
Heterokaryonic fusants	
AOFY-1	Trace
AOFY-8	3.56
AOFY-43	6.63
Diploid fusants	
AOFD-1	16.10
AOFD-2	12.19

investigations are in progress to make the diploids haploid for breeding more useful industrial moulds.

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