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An Efficient Protoplasting/Regeneration System for *Agaricus bisporus* and *Agaricus bitorquis*

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Abstract. Conditions for efficient protoplasting and regeneration of *Agaricus bisporus* and *A. bitorquis* are described. Especially for *A. bisporus* protoplasts, high regeneration frequencies were obtained (up to 30%). The protoplasting/regeneration system can be used for routine isolation of homokaryons of *A. bisporus*. Such homokaryons, derived from protoplasts containing one type of nucleus only, can easily be identified by analyzing isoenzyme banding patterns.

The white button mushroom *Agaricus bisporus* is commercially the most important edible mushroom, accounting for a world production of 1250 million kg in 1985 [18]. Despite the long cultivation tradition, little genetic improvement has been achieved in strains of this species compared with other commercially important crops. This is partly owing to the unique life cycle of *A. bisporus*, which is a secondary homothallic [16]. Upon germination, most spores from a multinucleated heterokaryotic mycelium which is capable of producing fruit bodies. In addition, it is known that the available strains are genetically uniform to a high degree [3, 9, 11]. For breeding purposes it is, therefore, necessary to introduce new genetic traits from *A. bisporus* collected in the wild or from other *Agaricus* species. Because of interspecies sterility, the latter is not possible through conventional crosses [13, 15]. An efficient protoplasting/regeneration system could provide a way to achieve the introduction of foreign genes through protoplasty fusion or DNA-mediated transformation.

Until now, only a few reports have appeared on the development of a protoplasting/regeneration system for *Agaricus* species [3, 6, 20]. The methods reported all share low regeneration percentages, 0.1% or less. This low regeneration capacity can be a major drawback in recovering the desired products after transformation or fusion.

We therefore tried to improve the yield of protoplasts and the regeneration of protoplasts of *A. bisporus* and *A. bitorquis*. This has led to a more efficient protoplasting/regeneration system. Especially with *A. bisporus* protoplasts, high regeneration percentages were obtained (up to 30%). This allows for a routine isolation of mycelia derived from regenerated single protoplasts (protoclones) and, therefore, for the isolation of homokaryotic mycelia. The homokaryons can be distinguished from heterokaryons by comparing isoenzyme banding patterns.

Materials and Methods

Strains. The following strains were used from the collection of the Mushroom Experimental Station: *Agaricus bisporus* homokaryons 39 and 97 and the derived heterokaryon Horst U1; *Agaricus bitorquis* homokaryons 2/69 and 4/53 and the derived heterokaryon K26. All were maintained on wheat agar slope cultures.

Culture condition, protoplast production, and isolation. Medium used to obtain mycelium for the production of protoplasts was modified after Dijkstra [5] and had the following composition (DT80 medium): (per liter) 30 g glucose, 3.23 g L-aspargine, 0.62 g L-phenylanaline, 0.13 g L-histidine, 0.44 g L-valine, 0.15 g DL-methionine, 1.36 g K_2HPO_4, 0.64 g $NaH_2PO_4 \cdot H_2O$, 0.40 g $MgSO_4 \cdot 7H_2O$, 20.00 mg $CaCl_2 \cdot 2H_2O$, 10.00 mg $FeCl_3 \cdot 6H_2O$, 7.20 mg $MnCl_2 \cdot 4H_2O$, 8.82 mg $ZnSO_4 \cdot 7H_2O$, 1.25 mg $CuSO_4 \cdot 5H_2O$, 0.40 mg thiamine-HCl, and 0.50 g Tween 80, pH 7.0. Ten to 15 petri dishes with DT80 agar covered with uncoated cellophane were inoculated, each with 10 plugs of mycelium, and incubated at 24°C in a CO_2 incubator (1% CO_2). The colonies were removed from the cellophane after 10 days and fragmented in liquid DT80 with a Waring blender (20 s, low speed). The suspension was used to inoculate six 2-liter Fernbach flasks (150 ml DT80 per flask) and incubated for 4 days at 24°C (stationary culture). Mycelium was filtered over nylon cloth (300 μm mesh) and rinsed with water. After suspension of the mycelium in approximately 100 ml water, one volume of 2 × concentrated protoplasting medium was added (4 mg/ml *Trichoderma harzianum* cell wall-lytic enzymes [17], 0.1 mg/ml chitinase (*Streptomyces*

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griseus, Sigma) in 1.2 *M* sucrose). The suspension was mixed, transferred to a Fernbach flask, and incubated for 4 h at 24°C (stationary). Other lytic enzyme preparations used were Helicase (Industrie Biologique Francaise, France), Zymolase (Sigma, USA), and Novozym 234 (Novo Biolabs, Denmark). The mixture of hyphal fragments and protoplasts was filtered over nylon cloth (500 and 150 μm mesh) and glass wool prerinsed with 0.6 *M* sucrose. Protoplasts were collected by centrifugation (15 min, 500 *g*) and washed twice by centrifugation in 0.6 *M* sucrose. After suspension of the final pellet in 0.6 *M* sucrose, protoplasts were counted with a hemocytometer.

Regeneration. Regeneration studies were performed on the following three types of solid media: MMP (1% malt extract; 0.5% mycological peptone; 10 mM KMOPS (3-[N-morpholino] propanesulfonic acid), pH 7.0), DT80, and CMP (7.5% compost extract; 0.5% mycological peptone, pH 7.0). The compost extract was prepared by boiling peak-heated, dried, and ground compost (7.5 g/l) for 1 hour in water [7]. After cooling and centrifugation of the suspension (20 min, 6000 *g*), the supernatant was used for preparing the CMP medium. All media were prepared with 1.5% agar and osmotically supported by 0.6 *M* sucrose. Protoplasts were diluted in 0.8% low melting point agarose (35–38°C) and 0.6 *M* sucrose to a concentration of 300–500 protoplasts/ml. One ml of the protoplast suspension was plated per Petri dish and incubated in a CO_2 incubator (24°C, 1% CO_2).

Protoclone isolation and identification by isoenzyme banding. Regenerated single protoplasts (protoclones) were isolated after 3–5 days under a stereo microscope (SR, Zeiss) and transferred to MMP agar plates covered with uncoated cellophane. After 10–14 days of incubation at 24°C, the mycelium was removed from the cellophane. The original inoculum was avoided. Approximately 0.5 g (wet wt) of the mycelium was placed in a microcentrifuge tube (Eppendorf) with an equal volume (vol/wt) of distilled water. Samples were quickly frozen in liquid nitrogen and stored at −70°C. Before isoelectric focusing, (IEF) samples were partly thawed at room temperature and homogenized with a rotating teflon rod fitting the microcentrifuge tube. The crude homogenates were centrifuged at 14,000 *g* for 10 min (4°C, Eppendorf centrifuge) and the supernatants used for electrophoresis. The IEF experiments were carried out with gels 0.5 mm thick consisting of a mixture of 6% polyacrylamide, 10% glycerol, 1.6% ampholyte pH 2–4, 1.6% ampholyte pH 4–7 (servalyt, Serva), 0.0195% ammonium persulfate, and 0.0019% silver nitrate. The IEF runs were performed on a flatbed apparatus (LKB 2117 Multiphor) connected to a high-voltage power supply (LKB2197) at constant power of 9 watt. Gels were stained for α-esterase with α-naphthylacetate according to Allendorf et al. [1].

Fruiting trials. Fruiting tests for protoclones of *A. bisporus* heterokaryon Horst U1 were carried out in 3.5-l polypropylene jars with a surface of 170 cm^2 [7]. Protoclones that produced more fruitbodies than the control strain (U1) were tested on a larger scale, i.e., on beds of 1.3 m^2 in a cropping house.

Results

Protoplast formation. The efficiency of protoplast production in filamentous fungi can be affected by several factors: the composition of the culture medium, the age of the culture, the lytic enzymes, and the osmotic stabilizer [12]. In optimizing protoplast production of *Agaricus* spp., we examined all these factors.

Although complex media containing yeast extract, malt extract, peptone, or compost extract provided good growth of the mycelium, they were less suited for protoplast production. In these media *Agaricus bisporus* forms crystals of Ca-oxalate [2, 19], which gave hyphae a "bottle-brush"-like appearance. When mycelium grown on cellophane-covered agar plates was fragmented and used to inoculate liquid media, large numbers of these crystals were set free and were difficult to remove from the medium. The crystals were released during protoplasting of the mycelium and co-isolated with the protoplasts, causing clustering and lysis of part of the protoplasts during pelleting. Contrary to the results with complex media, hardly any Ca-oxalate crystals were formed in a chemically defined medium (DT80).

In preliminary experiments, protoplast production of *A. bisporus* and *A. bitorquis* was examined with varying culture age on DT80 medium. In these experiments, colonies were grown on cellophane-covered agar plates and compared with those grown in liquid cultures inoculated with fragmented mycelium derived from colonies grown on cellophane.

Microscopic examination revealed that protoplasts were released from young hyphae only at the margin of colonies so that protoplast yield per gram mycelium was low. Besides, colonies were hydrophobic, and this made it difficult to submerge the mycelium in the protoplasting medium. Although in liquid cultures protoplasts were also released mainly from young hyphae (Fig. 1), protoplast production was higher because the ratio of the number of young cells to total cell number was higher than with surface-grown colonies. The liquid cultures were prepared by homogenizing colonies mildly (Waring blender, 20 s, low speed), because small fragments (one to five cells long) created by more vigorous homogenization grow very poorly. Inoculation density influenced the lag time of the culture. At densities below 0.01 mg dry wt/ml, the lag time could be as long as 4–7 days, whereas at inoculation densities of 0.1 mg dry wt/ml or higher, growth could be observed within 1 day. We used inoculation densities between 0.4 and 0.8 mg dry wt/ml. All liquid cultures were stationary incubated, because shaken cultures tended to form mycelial pellets, which liberated protoplasts at their surfaces only. The optimal culture age for protoplast production was 4–5 days.

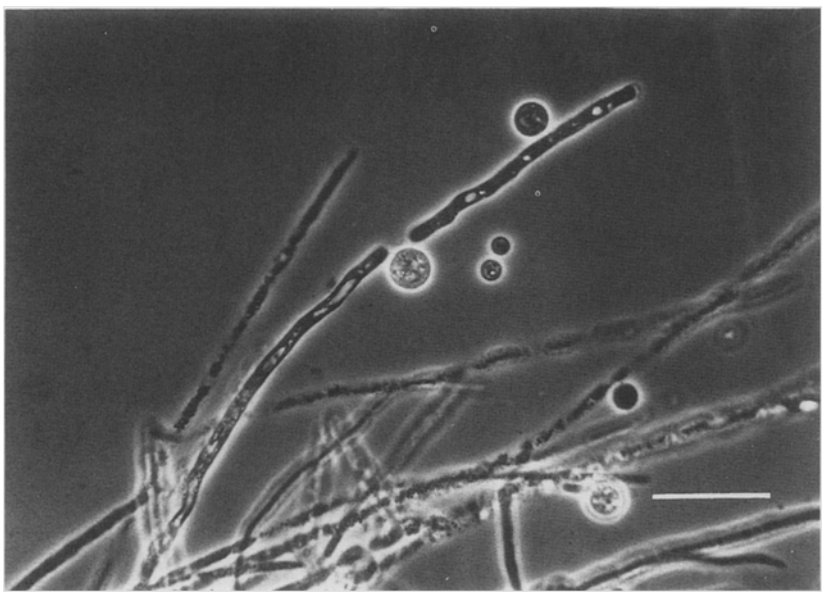

Fig. 1. Formation of protoplasts from *Agaricus bisporus* Ul. The mycelium was treated with TE enzyme mixture as described in *Materials and Methods*. The sample was viewed after 1 h of incubation at 24°C.

Of the lytic enzymes tested, zymolase and helicase were almost inactive. The lytic enzyme preparation from the culture filtrate of *Trichoderma harzianum* (TE) [17] was, however, very active in a concentration of 2–4 mg/ml. Higher concentrations did not appreciably increase the number of protoplasts. Protoplasts appeared within 15 min of incubation after addition of enzymes. Addition of low concentrations of chitinase (0.05–0.1 mg/ml) stimulated protoplast production. It was difficult to determine the exact number of protoplasts produced, because large numbers of protoplasts remained attached to the mycelium. Only the number of protoplasts obtained after filtration could be counted exactly and varied between 1 and 2×10^7 protoplasts/g dry wt mycelium. A commercially available enzyme preparation, Novozym 234, which is also derived from *T. harzianum*, when tested in the same concentration range, produced only 1% of the number of protoplasts liberated by TE. The maximum number of protoplasts produced by Novozym 234 was $1–2 \times 10^6$/g dry wt mycelium at an enzyme concentration of 10–20 mg/ml.

The protoplasting experiments mentioned were all performed with sucrose as an osmotic stabilizer, with an optimum concentration of 0.6 *M*. Other osmotic stabilizers such as $MgSO_4$, mannitol, or sorbitol were less effective. The average diameter of the protoplasts was 7 μm, with a range of 4–11 μm. All protoplasts seemed to be free of wall remnants, because no fluorescence was observed after incubation with the optical brightener Calcofluor White, and no intact protoplasts could be detected microscopically after a 10× dilution of the protoplast suspension in water.

In none of the osmotic stabilizers tested, protoplasts floated, so that protoplasts had to be purified by filtration. The best results were obtained when the mycelium–protoplast suspension was filtered sequentially through 500-μm nylon mesh, 150-μm nylon mesh, and glass wool. Despite the stepwise filtration and rinsing of the filters with 0.6 *M* sucrose, many protoplasts remained attached to the mycelium as revealed by microscopic examination of the suspension left on the filters.

Regeneration. Table 1 describes the effect of the medium composition on the regeneration of *A. bisporus* and *A. bitorquis* protoplasts prepared with TE. All media were osmotically supported by 0.6 *M* sucrose. On complex media, very high regeneration percentages could be obtained for *A. bisporus*. The regeneration percentages of *A. bitorquis* protoplasts were, however, considerably lower and could not be improved by replacing sucrose with other osmotic stabilizers such as $MgSO_4$, mannitol, or sorbitol. In fact, hardly any regeneration of *A. bitorquis* protoplasts was observed in media containing these osmotic stabilizers. The regeneration percentages of *A. bisporus* protoplasts prepared with Novozym 234 were more variable than of protoplasts prepared with TE. With Novozym, regeneration percentages of *A. bisporus* protoplasts were in general 5–10 times lower than with protoplasts prepared with TE. The presence of 1% CO_2 in the atmosphere stimulated growth but did not influence the regener-

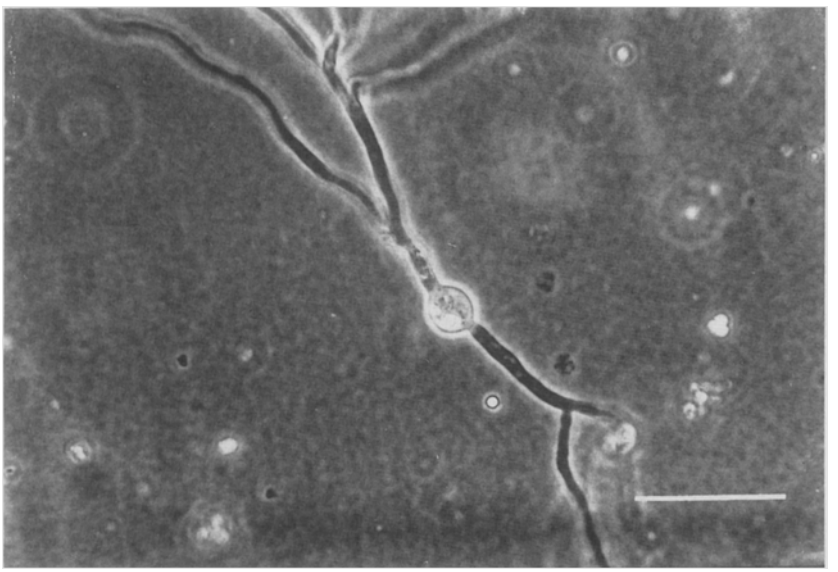

Fig. 2. Regeneration of an *Agaricus bisporus* protoplast, viewed after 3 days of incubation on CMP medium as described in *Materials and Methods*.

Table 1. Regeneration percentages of protoplasts of *Agaricus bisporus* and *A. bitorquis* on different solid media

Medium	Regeneration %	
	A. bisporus	*A. bitorquis*
CMP	25–30	1–2
MMP	15–20	<0.1
DT80	7–10	<0.1

ation frequency. In ambient air, colonies were visible after approx. 10 days, whereas in the presence of 1% CO_2, colonies could be detected within 1 week. The number of colonies formed on media lacking sucrose was less than 0.1% of the number of colonies formed on media with sucrose; this indicates that almost all colonies were derived from regenerated protoplasts.

Microscopic examination revealed that regeneration started within 2–3 days with the formation of one to three hyphae per protoplast (Fig. 2). Regeneration of *A. bisporus* protoplasts also started within 2 days in liquid CMP medium. Regeneration percentages, however, could not be determined because of clustering of the regenerated protoplasts. Regeneration percentages of homokaryons of *A. bisporus* and *A. bitorquis* were similar to those of the derived heterokaryons, i.e., high for *A. bisporus* and low for *A. bitorquis*.

Protoclones. Small colonies derived from single regenerated protoplasts (protoclones) of *A. bisporus* heterokaryon U1 were isolated after 4–5 days and transferred to MMP agar plates. The isolation was done under a stereomicroscope to ascertain that the isolated mycelia were derived from single protoplasts. The colonies exhibited a variety of growth rate and morphology. Many of the slow-growing colonies showed the morphology of the homokaryons used to produce the heterokaryon U1 (colony color, appressed mycelium). To verify the formation of homokaryons, 90 protoclones were screened for α-esterase isoenzymes on IEF-gels (Fig. 3). The analysis of the isoenzyme banding patterns showed that many of the mycelia grown from regenerated protoplasts were homokaryotic (Table 2) and confirmed the preliminary identification of homo- and heterokaryons by colony morphology and growth rate. We also observed that, with IEF, homo- and heterokaryons of other *Agaricus* spp. could be identified (*A. bisporus*, *A. bitoquis*, and *A. arvenis*; results not shown).

Sixty protoclones were tested for fruiting in 3.5-l polypropylene jars, and the number of fruitbodies was determined after 5 picking weeks. Table 2 shows that the protoclones identified as heterokaryons by isoenzyme analysis produced more fruitbodies than the homokaryons. Seven protoclones, identified as heterokaryotic, yielded more fruitbodies than the control strain U1. However, fruiting trials on a small scale are not very reliable. We decided, therefore, to test three protoclones with the highest yield on a larger scale (1.3 m^2). These protoclones now showed a considerably lower yield when compared with the original strain U1, i.e., a

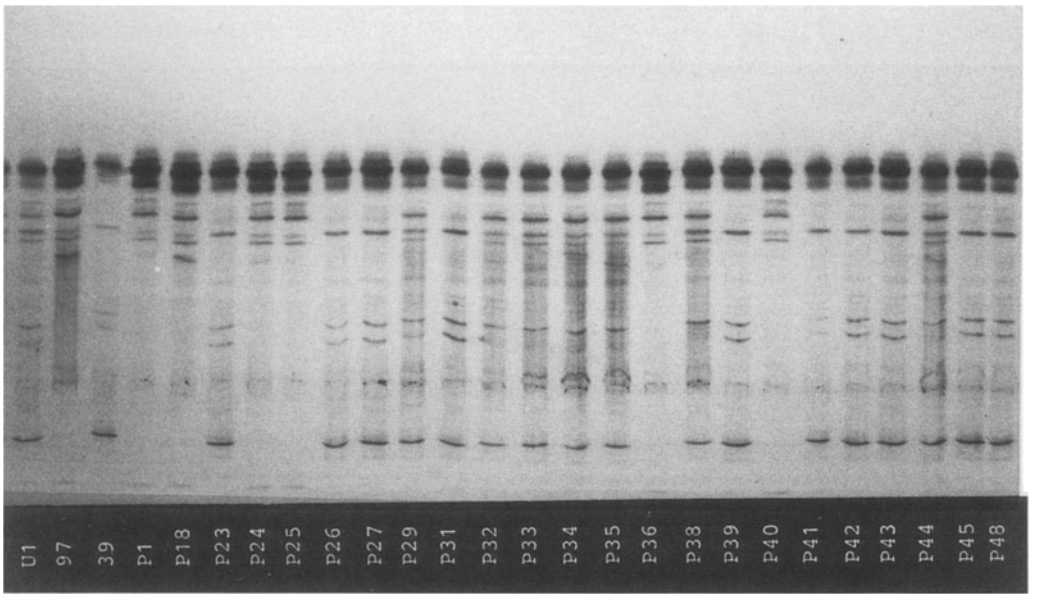

Fig. 3. Isoenzyme banding patterns of α-esterase after isoelectric focusing of mycelial extracts of the homokaryons and the derived heterokaryon of *Agaricus bisporus* and protoclones derived from regenerated single protoplasts of *A. bisporus* heterokaryon. Ul, *A. bisporus* heterokaryon; 97 and 39, homokaryons of *A. bisporus* Ul; P, protoclones of the heterokaryon *A. bisporus* Ul. The zymogram identifies P1, P18, P24, P25, P36, and P40 as carrying the nucleus of 97, P23, P26, P27, P31, P39, P41, P43, P45, and P48 as carrying the nucleus of 39. The other protoclones are apparently heterokaryons carrying both types of nuclei.

reduction of 26%, 30%, and 43% in total weight of the fruitbodies after 4 picking weeks. The mushrooms were growing in clusters, leaving large open areas on the beds. Many mushrooms had no veil, and gills were absent or hardly developed, an aberrant morphology known as 'hard gill'. The possibility was tested that this poor quality was just a transitional stage due to the protoplasting method itself. One of the protoclones was transferred several times to fresh plates and re-used for fruiting trials. This gave no qualitative improvement. When, however, a tissue culture was prepared from one of the fruitbodies with a normal morphology produced by a heterokaryotic protoclone, the original yield and fruitbody morphology were restored.

Table 2. Identification of protoclones of *Agaricus bisporus* Ul by analysis of isoenzyme banding patterns of α-esterase and number of fruitbodies produced

Isoenzyme banding pattern	Number of protoclones[a]	Number of fruitbodies[b]	SD[c]
Type Ul (heterok.)	49 (30)	28.5	16.8
Type 97 (homok.)	14 (9)	5.0	6.9
Type 39 (homok.)	27 (21)	6.7	4.3
Control Ul (heterok.)	(5)	45.6	2.7
Totals:	90 (60)		

[a] Number of protoclones tested for fruiting trials in brackets.
[b] Number of fruitbodies given per 170 cm² when grown in 3.5-l polypropylene jars with compost.
[c] Standard deviation from the mean.

Discussion

Agaricus bisporus can be typified by the absence of those characteristics that a fungus should have to be the ideal candidate for breeding [14]: a heterothallic sexuality, quick germination of spores at a high incidence, a short life cycle, clearly distinguishable sexual interactions between compatible infertile strains, and a high genetic variability of strains. The difficulties in isolating and identifying homokaryons and the low genetic variability are particularly responsible for the relatively small genetic improvement that *A. bisporus* strains have undergone. Some of these difficulties can be overcome by the use of protoplasts. As shown, homokaryons can be isolated from protoplasts derived from heterokaryotic strains. Protoplasts also offer the prospect of genetic transformation and somatic hybridization with related *Agaricus* spp. with a higher variety in genetic traits such as *A. bitorquis* [3, 9]. Especially

for *A. bisporus* protoplasts, high regeneration percentages can be obtained. This makes it feasible to isolate desired products after transformation and fusion, possibly even in the absence of strong selection markers.

The main reason for the high efficiency of the regeneration for *Agaricus* protoplasts reported in this study is probably the superiority of the enzyme system used. In former reports [3, 6], Novozym 234 was used to produce protoplasts from *A. bisporus* and *A. bitorquis*. Although Novozym 234 is isolated from the same organism as the enzyme system used in this study, i.e., *Trichoderma harzianum*, the laboratory-prepared enzyme mixture [17] is more effective. It can be used at a much lower concentration (2–4 mg/ml) than Novozym 234 (10–20 mg/ml) to produce protoplasts, and the regeneration frequency of the protoplasts is considerably higher. It is known that in Novozym 234 enzymes with undesirable activities, such as proteases, are present [8]. Such proteases are not present in the TE enzyme mixture used in this study [4]. Because Novozym has to be used at a much higher concentration than TE, such undesirable activities can severely decrease the viability of the protoplasts. Another disadvantage of Novozym 234 is the variation of activities among different batches. No differences were found in protoplast production and regeneration with different batches of the laboratory-prepared enzyme system.

An efficient protoplasting/regeneration system is particularly helpful in routine isolation of homokaryons of *A. bisporus*. In this study we have used isozyme banding patterns to identify homokaryons and heterokaryons among the protoclones; this method probably provides an easier way of identifying clones than restriction fragment length polymorphism (RFLP) of DNA [3]. Homokaryotic and heterokaryotic protoclones of *A. bisporus* Ul showed considerable variations in colony morphology, growth rate, and yield of fruitbodies. These differences could arise from the accumulation of different mutations in individual nuclei during vegetative growth. Possibly this is one cause for the degeneration of strains observed when mycelium is vegetatively propagated over a long period of time. Protoplasts might be used to purify a strain and recover it from degeneration by isolating protoclones with the original genetic traits. Even strains with superior qualities may be isolated by this procedure. Magae et al. [10] reported that some of the mycelia derived from single regenerated protoplasts of *Pleurotus ostreatus* produced more fruitbodies than the original strain from which the protoplasts were derived. Although fruiting trials on a small scale revealed that some heterokaryotic protoclones of *A. bisporus* did produce more fruitbodies than the control strain, this was not confirmed by fruiting tests on a larger scale. Because only a limited number of heterokaryotic protoclones were used for fruiting trials (29 out of 60 protoclones tested were heterokaryotic, and only 3 were tested on a large scale), conclusions cannot be drawn.

In a previous study [17] we have shown that the electrofusion technique developed by Zimmermann and Scheurich [21] is an efficient method to produce heterokaryons in the basidiomycete *Schizophyllum commune*. By electrofusion of protoplasts of two auxotrophic mutants, nearly 7% of the regenerated protoplasts could be recovered as prototrophic heterokaryotic mycelia. If this method is also effective with *Agaricus* protoplasts, the high regeneration capacity of protoplasts reported in this study, together with the ease of identifying fusion products by isozyme patterns, would open the possibility of obtaining interspecific hybrids without the need for selectable markers such as auxotrophic mutations.

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