Short Communication

PROTOPLAST FROM *MALBRANCHEA PULCHELLA* VAR. *SULFUREA*: ISOLATION AND REGENERATION

S. VIRK, B. N. JOHRI,* AND SATYA P. SINGH

*Department of Microbiology, College of Basic Sciences & Humanities,
G. B. Pant University of Agriculture & Technology,
Pantnagar, India-263145*

(Received October 4, 1991)

Protoplasts of *Malbranchea pulchella* var. *sulfurea* were isolated from 14 h culture grown in YpSs and production media or 36 h culture from minimal medium; protoplast yields were, 1×10^4 , 7.0×10^6 and 7.2×10^6 /ml, respectively. A cell density of 40 mg/ml in phosphate buffer (0.05 M, pH 5.5) containing 0.6 M KCl as stabilizer, was most suitable; Novozym 234 + β -glucuronidase were effective as lytic system. Protoplasts were easily maintained at 50°C for 60 min without loss of viability. A 70–90% regeneration frequency was achieved in five different liquid and solid media.

Fungal protoplasts have been used in a variety of studies including strain improvement and fusion to prepare hybrids for genetic analysis (1,8). However, a majority of these investigations deal almost exclusively with the mesophilic fungal species. Thermophilic fungi have gained considerable interest in biotechnology in view of their strong enzymatic machinery and thermal stability of the proteins (10, 12, 13, 17). However, several important species such as *Humicola lanuginosa*, *Malbranchea pulchella* var. *sulfurea*, *Sporotrichum thermophile* and *Scytalidium thermophilum* are imperfect and genetic improvement is difficult. Recently, protoplasts of *S. thermophile* were prepared and immobilized for lipase production (6, 11). *Malbranchea pulchella* var. *sulfurea* is characterized by its sulfur-colored, somewhat mealy growth, yellow conidia in chains and production of penicillins (3). Since it produces a wide array of hydrolytic enzymes (16), an attempt was made to develop a protoplasting system for strain improvement.

Malbranchea pulchella var. *sulfurea*, a coal mine isolate, was maintained on YpSs agar containing (g/l): yeast extract, 4.0; K₂HPO₄, 1.0; MgSO₄·7H₂O, 0.5; soluble starch, 15.0; agar, 20, pH 7.0. The mold was grown in production medium

* Address reprint requests to: Dr. B. N. Johri, Department of Microbiology, College of Basic Sciences & Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar, India-263145.

which contained (g/l): NaNO_3 , 3.0; KCl , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; KH_2PO_4 , 1.0; glucose, 40.0; yeast extract, 2.0. The minimal medium contained (g/l): NaNO_3 , 6.0; KCl , 0.52; KH_2PO_4 , 1.52; glucose 10.0; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4×10^{-3} ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.2×10^{-3} at pH 6.0. Twenty ml of medium was dispensed in 150 ml Erlenmeyer flask and then inoculated with spore suspension to a final concentration of $1.0 \times 10^7/\text{ml}$. Cultures were incubated at 45°C for 16–18 h in a water bath shaker.

After harvesting ($3,000 \times g$, 15 min), the mycelium was washed with 0.05 M phosphate buffer (pH 5.5) and suspended in same buffer containing 0.6 M KCl . Lytic enzymes were added in various combinations (details under results) and the mixture incubated at 35°C under shaking for up to 3 h. Protoplast formation was monitored under phase contrast microscope and population density calculated using a Thoma Neuberg counting chamber. Protoplast viability was based on dye exclusion method (4); protoplast suspension (0.1 ml) was added to 1 ml of 0.5% trypan blue in phosphate buffer (pH 5.5). Protoplasts exhibiting dye exclusion after 1–2 h at 35°C were taken as viable. Regeneration of protoplasts was carried out in both liquid and solid basic salt media supplemented with various carbon sources (details under results).

Protoplasts first emerged from the hyphal tips but with longer incubation (2–3 h) they were released from all points. In 4–5 h, mycelium was transformed completely into spherical protoplasts. This pattern of protoplast release is evident in most filamentous fungi (7, 9). Protoplast yields were higher when the mold was grown in production ($7.0 \times 10^6/\text{ml}$) or minimal medium ($7.2 \times 10^6/\text{ml}$) than those obtained from YpSs-grown mycelium ($1 \times 10^4/\text{ml}$). Mycelial age significantly affected protoplast formation (Fig. 1). With two combinations of enzymes, viz., β -glucuronidase + cellulase (0.05 + 2 mg/ml, w/v) and Novozym 234 + cellulase (2 + 2 mg/ml, w/v), yields were 8.2×10^6 and 7.2×10^6 respectively with 14-h-old mycelium. Cellulase (EC 3.2.1.4; type V from *Trichoderma viride*) was from Sigma and Novozym 234 from Novo Industries, Copenhagen. Yield, however, decreased with increasing mycelial age (Fig. 1). Attempts to prepare protoplasts from still younger cultures were not successful primarily due to the fragile nature and inadequate density of mycelia leading to inconsistent results. This dependency on culture age, however, implies varying susceptibility of the cell wall towards lytic enzymes (2, 3).

A mycelial biomass of 40 mg/ml supported best yield ($7.2 \times 10^6/\text{ml}$) which was reduced by 28–50% at 80–100 mg/ml density, presumably due to enzyme limitation. A number of osmotic stabilizers were used (0.4–0.8 M). However, KCl (0.6 M) supported stable protoplasts; sucrose and MgSO_4 were not as effective.

Protoplasts were optimally produced at pH 5.5 in phosphate buffer (0.05 M) corresponding to pH optima for protoplasting in *Aspergillus fumigatus* and *A. niger* (15). Optimum temperature for protoplasting was 35°C , where 95% population was viable; at 50°C , protoplast yield was reduced by 70%. Protoplasts prepared at 35°C could be maintained up to 1 h at 30 – 50°C without loss of viability (Table 1). Mycelial cultures of *M. pulchella* var. *sulfurea* are viable up to 55°C . Low

Fig. 1. Effect of mycelial age on protoplast yield from *Malbranchea*. Counts were taken after 3 h incubation at 35°C.

Table 1. Effect of temperature on the stability of protoplast.

Temperature (°C)	Viable protoplasts ^a (× 10 ⁶ /ml) after incubation for (min)			
	15	30	45	60
35	1.0	1.0	0.98	0.98
45	1.0	0.99	0.96	0.96
50	1.0	0.98	0.98	0.97
60	0.7	0.10	0.0	0.0
70	0.5	0.0	0.0	0.0

^a Initial protoplast count in each case was 1.0×10^6 /ml.

protoplast yield at higher temperatures, thus, was due to lytic enzyme inactivation or decreased activity rather than impaired metabolic activities. Drastic decrease in protoplast stability and viability at temperatures beyond 50°C was probably due to agglutination of certain organelles.

In order to use protoplasts as a model to study fungal biochemistry and genetics, the extent of disruption and/or metabolic change, if any, during protoplast preparation must be assessed. Towards this end, we investigated regeneration of protoplasts. The production medium supported best regeneration i.e., up to 90%. This frequency is quite encouraging because low regeneration has been a major drawback (14). In solid medium, hyphal branching led to formation of typically small yellow-colored colonies only after 72 h. A longer incubation, however, did not enhance the number of colonies. Regeneration in liquid medium as monitored by phase contrast microscopy, exhibited two distinct patterns between 2–5 h of incubation. In the first category, protoplasts initially changed to a yeast-like structure and then converted to hyphal segment. In the second pattern, protoplasts proliferated to hyphal structure through a germ tube. N-acetyl glucosamine and yeast extract in minimal medium stimulated regeneration during early hours. This

effect, however, was less pronounced at 72 and 24 h in solid and liquid media, respectively. Metabolic activities of protoplasts were reconfirmed by active uptake and subsequent incorporation of ^{14}C -leucine, ^{14}C -uracil and ^3H -thymidine (results not included) (5). The results thus indicate that viable protoplasts from *Malbranchea* can be isolated, maintained and regenerated for further studies.

We thank Dean, CBS & H and Director Research for facilities. This research was supported by a CSIR grant to Dr. B. N. Johri.

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