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Regeneration of protoplasts from hyphal strands of *Volvariella volvacea*

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Abstract A series of experiments on the preparation and regeneration of protoplasts from hyphal strands of *Volvariella volvacea* (Bull. ex. Fr.) Singer were conducted with the aim of optimizing the conditions for its efficient regeneration. One commercial (Vvc1) and two wild (EAAC-0001 and EAAC-0002) strains of *V. volvacea* from the Philippines were used and subjected to varying conditions to determine the most efficient means for regeneration of their protoplasts. The effects of age and type of strain, pH, type and concentration of osmotic stabilizer, enzymatic composition, treatment time, temperature, reciprocal frequency during enzymatic lysis of the cell wall, and centrifugation conditions were investigated. Results showed that the three strains of *V. volvacea* had varying responses in terms of yield, size, and ability of their protoplasts to regenerate into the protoplast regeneration medium. Among the three strains, EAAC-0002 had the highest rate of regeneration. The 5-day-old culture of *V. volvacea*, when subjected to a combination of 2% Novozyme 234 and 0.2% chitinase in 0.6M mannitol (pH 6.0) for 3h at 30°C, 90 strokes/min and centrifuged at 1100g for 10min, produced an efficient yield of protoplasts with a relatively high regeneration rate.

Key words Fungal protoplast · Mushroom · Protoplast technology · *Volvariella volvacea*

Introduction

Volvariella volvacea, which ranks fifth among the most important edible mushrooms in terms of world production,[1] is cultivated in tropical and subtropical regions. Though regarded as one of the easiest mushrooms to cultivate owing to its short cropping period of 14 days, its yield of fruiting bodies is low and unstable. The development of an improved strain is therefore important. This mushroom, as a primary homothallic species, is not known to produce a clamp connection. Primary homothallism makes conventional breeding activity difficult. Thus, a nonconventional breeding strategy such as protoplast technology offers the possibility of improving the strains of this mushroom. For fungi, which have no convenient natural mechanism for sexual or parasexual genetic exchange, protoplast fusion is suggested as the most efficient way of carrying out genetic crosses in the laboratory.[2,3]

The success of protoplast technology depends on optimum conditions for the efficient regeneration of protoplasts. A large number of research studies have been reported on the optimum conditions for the preparation and regeneration of protoplast in Basidiomycetes.[4-14] Among edible mushrooms, extensive studies on the preparation of protoplast in the genera *Agrocybe*,[15] *Pleurotus*,[16-27] *Schizophyllum*,[28] and *Lentinus*[29] have been reported, but, literature on the regeneration of protoplast from *V. volvacea* is limited.[30-34]

Our initial studies[35] on the isolation of *V. volvacea* showed lower yield but large protoplasts with early regeneration. This finding led us to conduct a thorough experiment on the optimization of physical conditions for the regeneration of protoplast.

Materials and methods

The general procedure for the preparation and regeneration of mushroom protoplast based on the works of Higaki and Eguchi[27] was adapted and modified. To determine optimum conditions for the efficient release and cell wall regeneration of protoplasts from the hyphal strands of *V. volvacea*, a series of experiments were planned with the following factors manipulated.

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Strain and age of culture

Pure cultures of two wild strains of *V. volvacea* designated EAAC-0001 and EAAC-0002 were obtained from the Culture Collection of the Center for Tropical Mushroom Research and Development at the Central Luzon State University (CLSU-CTMRD-Philippines). One commercial strain (Vvc1) was donated by Mr. Celso Apigo, the owner of Apigo's Mushroom Farm in Munoz, Nueva Ecija, Philippines. Three-, five-, and seven-day-old SMY (containing 10 g saccharose, 10 g malt extract, and 4 g yeast extract in 1000 ml distilled water) broth cultures of these strains were collected separately using previously sterilized nylon mesh.

pH of buffer solution and osmotic stabilizer

The hyphal strands of the three strains were washed separately with 0.05 M maleic acid plus 1 M NaOH as buffer solution (pH 4.0–9.0) containing 0.6 M mannitol as osmotic stabilizer. In the succeeding experiments, pH 6.0 was used.

Concentration and type of osmotic stabilizer

Mannitol, magnesium sulfate, and saccharose at concentrations ranging from 0.4 to 0.9 M were evaluated. The hyphal strands were subjected to mannitol at 0.6 M concentration in the next test.

Enzymatic treatment conditions

To determine the effects of enzymatic treatment conditions on the release and regeneration of protoplast, experiments on enzymatic treatment time, composition, treatment temperature, and reciprocal frequency were set up as follows. Five-day-old hyphal strands of the three strains were immersed separately in 2% Novozyme 234 (Novo Industries A/S Enzyme Division, Bagsvaard, Denmark), which was previously sterilized in a membrane filter (0.45 μm cellulose acetate; Advantec Toyo, Japan). Enzymatic treatment was undertaken at 1, 2, and 3 h respectively, in a reciprocal shaker at 30°C with 85 strokes/min.

Five-day-old hyphal strands of *V. volvacea* were incubated separately in 1% Novozyme 234, 2% Novozyme 234, 2% Novozyme 234 + 0.1% chitinase, and 2% Novozyme 234 + 0.2% chitinase for 3 h at 30°C with 85 strokes/min. The temperature of the reciprocal shaker was set from 25°–40°C at 5°C intervals during the enzymatic treatment of 5-day-old hyphal strands of *V. volvacea* submerged in a mixture of 0.05 M maleic acid/1 M NaOH, 0.6 M mannitol, and 2% Novozyme 234 + 0.2% chitinase (pH 6.0) for 3 h at 85 strokes/min. To determine the appropriate reciprocal frequency, the reciprocal shaker was set at varying freqencies (80–95 at an interval of 5 strokes/min) during the enzymatic treatment of the hyphal strands of *V. volvacea*. Incubation was undertaken at 30°C for 3 h in a mixture of 2% Novozyme 234 + 0.2% chitinase dissolved in osmotic stabilizer-buffer solution consisting of 0.6 M mannitol and 0.05 M maleic acid/1 M NaOH (pH 6.0).

Centrifugation conditions

After the enzymatic treatment, liberated protoplasts were separated from the hyphal debris using previously sterilized miracloth (Calbiochem; Novabiochem, USA) and washed twice in 0.05 M maleic acid/1 M NaOH, 0.6 M mannitol (pH 6.0) by centrifugation at 400, 700, 1100, and 1600 g for 5–20 min at 5-min intervals.

The protoplasts were plated on osmotically and pH-stabilized SMY containing 2% agar (pH 6.0) and 0.6 M mannitol. The plated protoplast were overlaid with the same medium but containing a reduced percentage of agar (0.7%). The percentage plating efficiency was determined based on the colony count in the protoplast regeneration medium versus the actual count of protoplast per milliliter of sample.

Results and discussion

The success of protoplast preparation and regeneration depends on (1) the nature and status of the mushroom organism being used; (2) the pH of the buffer solution, which prevents a drastic shift in the pH of the medium; (3) the type and level of osmotic stabilizer, which may minimize osmotic shock to the protoplasts and thus ultimately lead to maintenance of the osmotic balance of the cell wall-less protoplasts; and (4) conditions for enzymatic treatment, which facilitates degradation of the cell wall, exposing the naked protoplast; and (5) the centrifugation condition. In this study, the influences of the previously mentioned factors were examined for the efficient release and regeneration of protoplasts from hyphal strands of *V. volvacea*.

Influence of strain and age of culture

The results on the influence of the age of culture and strain on the plating efficiency of protoplasts from hyphal strands of *V. volvacea* are presented in Fig. 1. The 3-, 5-, and 7-day-old cultures of one commercial and two wild strains of *V. volvacea* exhibited varying degrees of response as far as the ability of the protoplasts to regenerate back to their mycelial forms is concerned. Moreover, even though the three strains belonged to the same genus and species, variation existed. This observation strengthens our previous findings[36] on the differences in the mycelial performances of our wild strains. Toyoda et al.[29] noted the same trend during protoplast isolation of their three strains of *Lentinus edodes*. A 5-day-old culture of *V. volvacea* yielded a relatively lower number of protoplasts but higher plating efficiency compared to 3- and 7-day-old cultures, respectively.

Appropriate age of culture is an important factor in protoplast formation.[37] Old mycelia become resistant to the action of mycolytic enzymes, and very young mycelia are more sensitive, which may lead to lysis of the whole cell including its protoplast.[38] This probably explains the low percentage regeneration in the 3- and 7-day-old cultures of the three strains. Among the two wild isolates, 5-day-old

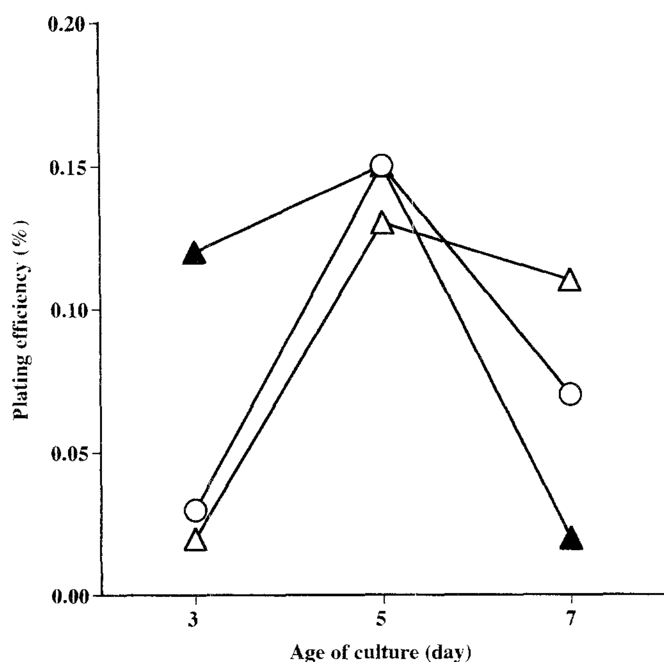

Fig. 1. Influence of age of culture on the regeneration of *Volvariella volvacea* protoplasts. *Open circles*, Vvcl; *open triangles*, EAAC-0001; *filled triangles*, EAAC-0002

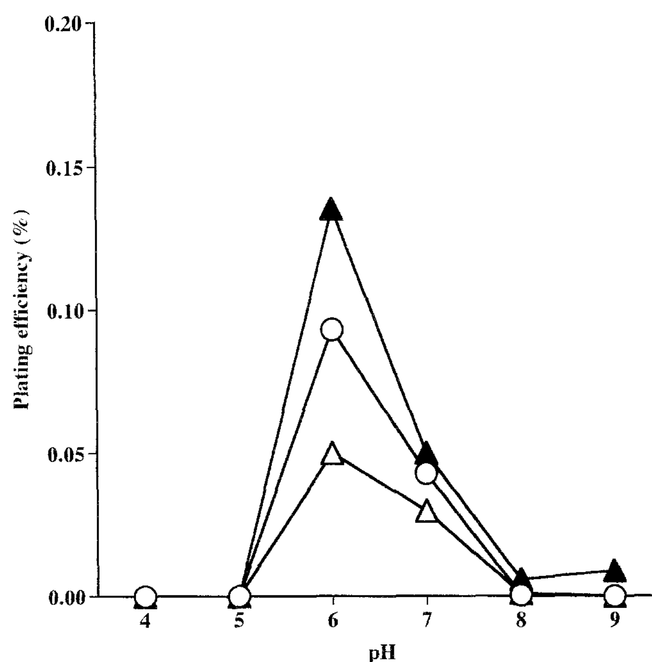

Fig. 2. Influence of varying levels of pH on the regeneration of *Volvariella volvacea* protoplasts. *Open circles*, Vvcl; *open triangles*, EAAC-0001; *filled triangles*, EAAC-0002

EAAC-0002 is comparable with the commercial strain of the same age on the basis of the ability of the protoplasts to revert to their mycelial forms.

Influence of buffer solution pH and osmotic stabilizer

As shown in Fig. 2, slightly acidic pH (pH 6.0) was favorable for the regeneration of protoplast from 5-day-old cultures of *V. volvacea*. The buffer solution and osmotic stabilizer adjusted to an acidic level (i.e., pH 4 and 5) did not positively influence the release and regeneration of protoplasts of the three strains of *V. volvacea*. The same result was also noted at neutral to alkaline levels (i.e., pH 7, 8, and 9). The alkalinity or acidity (pH) of the solution is one of the most important parameters to affect the reactions of the lytic enzyme and the efficacy of the osmotic stabilizer to maintain the osmotic balance of the cell wall-less protoplast.[39]

Influence of type and concentration of osmotic stabilizer

Variations of the response of *V. volvacea* have been observed when 5-day-old hyphal strands of the three strains were subjected to three osmotic stabilizers, including an inorganic salt (magnesium sulfate), sugar (saccharose), and sugar alcohol (mannitol). These osmotic stabilizers influenced the size, regeneration time, and ability of the liberated protoplasts to regenerate back into their mycelial forms 7 days after plating. Hyphal strands of the three strains subjected to mannitol had the highest number with the relatively larger size of the protoplasts (12–15 μm). Saccharose, on the other hand, yielded the smallest protoplasts. The type of osmotic stabilizers also affected the regeneration time of the regenerated protoplasts (Fig. 3). Early regeneration with relatively high plating efficiency was recorded in SMYA plates containing mannitol as osmotic stabilizer. Although magnesium sulfate was singled out by De Vries and Wessels,[40] Anne et al.,[41] and Peberdy et al.[42] as the best osmotic stabilizer (compared to sugar and sugar alcohol), this inorganic salt was remarkable only in terms of early regeneration of protoplasts in this experiment.

Because mannitol was proven the most suitable osmotic stabilizer in this experiment on the bases of regeneration time and plating efficiency, this osmotic stabilizer was examined further. Varying levels of mannitol were evaluated for the regeneration of protoplasts. The highest number of isolated protoplasts was recorded at 0.6–0.7 M. Protoplasts of *V. volvacea* incubated in SMYA containing 0.6 M mannitol had a higher percentage plating efficiency compared to protoplasts in 0.7 M mannitol (Fig. 4). This observation agrees with the previous findings that 0.6 M concentration in protoplast regeneration medium yielded the highest percentage regeneration.[30] Moreover, low percentage plating efficiency was observed with the mannitol concentration that yielded the largest (0.4 M mannitol) and smallest (0.7 M mannitol) protoplasts. It might be that the 0.4 and 0.7 M concentrations of mannitol are hypotonic and hypertonic, resulting in the swelling and shrinking of protoplasts, respectively.

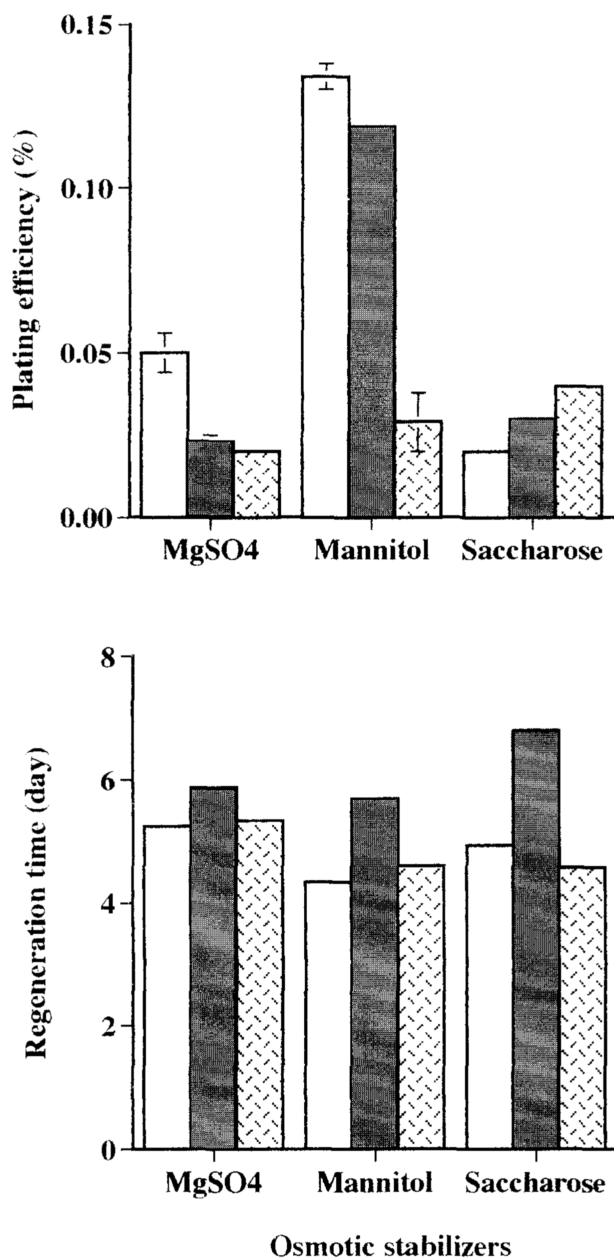

Fig. 3. Influence of various osmotic stabilizers on the regeneration of *Volvariella volvacea* protoplasts. *Open bars*, Vvcl; *shaded bars*, EAAC-0001; *dotted bars*, EAAC-0002. Vertical lines through the bars represent the standard deviation from the mean

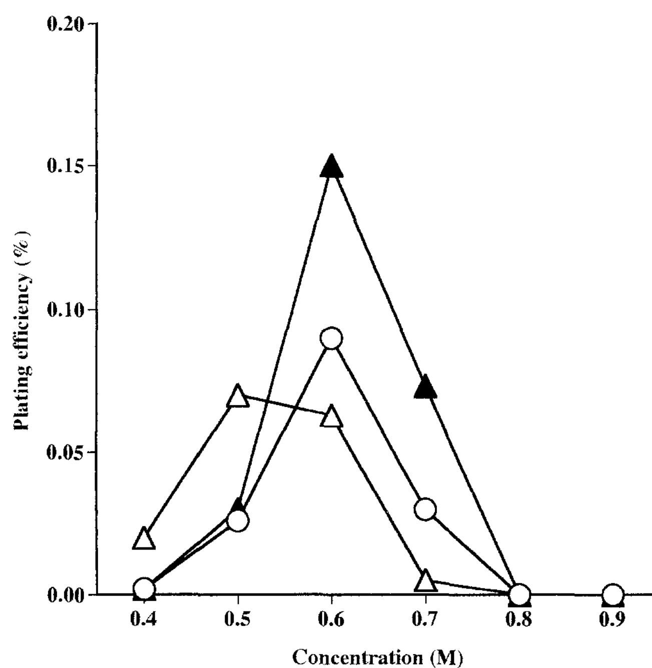

Fig. 4. Influence of varying concentrations of mannitol as osmotic stabilizer on the regeneration of *Volvariella volvacea* protoplasts. *Open circles*, Vvcl; *open triangles*, EAAC-0001, *filled triangles*, EAAC-0002

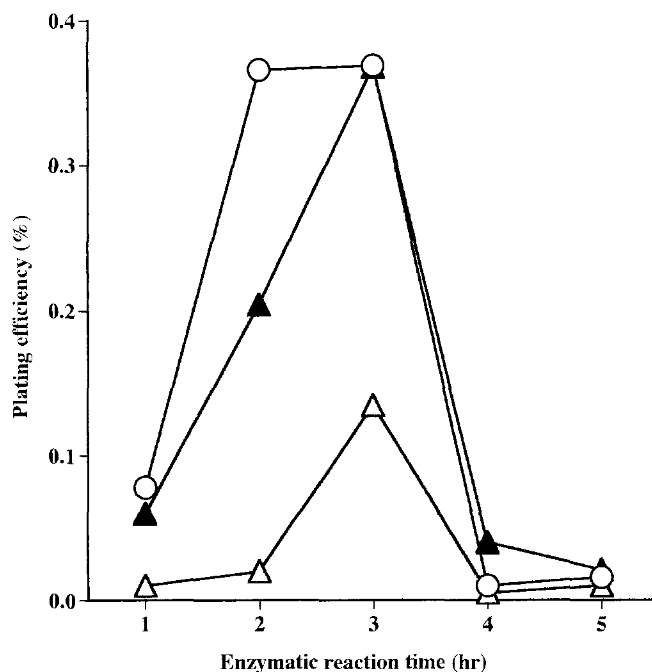

Fig. 5. Influence of enzymatic reaction time on the regeneration of *Volvariella volvacea* protoplasts. *Open circles*, Vvcl; *open triangles*; EAAC-0001; *filled triangles*, EAAC-0002

Influence of enzymatic treatment conditions

The efficient liberation of protoplasts from hyphal strands is basically dependent on the hydrolysis of polysaccharides, which account for 60%–80% of the fungal cell wall.[43] These cell wall components must be lysed to release the protoplasts fully. However, lysis of the cell wall relies on suitable conditions during incubation of hyphal strands in a mixture of enzymes and buffer – osmotic stabilizer solution. We therefore studied the influence of enzymatic reaction time, enzyme composition, and treatment temperature on protoplast regeneration. A 3-h incubation of a mixture of 5-day-old hyphal strands of *V. volvacea*, 2% Novozyme 234, and 0.05 M maleic acid/1 M NaOH with 0.6 M mannitol (pH 6.0) yielded the highest plating efficiency (Fig. 5). The lowest was noted when incubation was undertaken for 4–5 h.

In terms of enzyme composition, the highest plating efficiency was observed when hyphal strands were exposed to a combined action of 2% Novozyme 234 and 0.2% chitinase

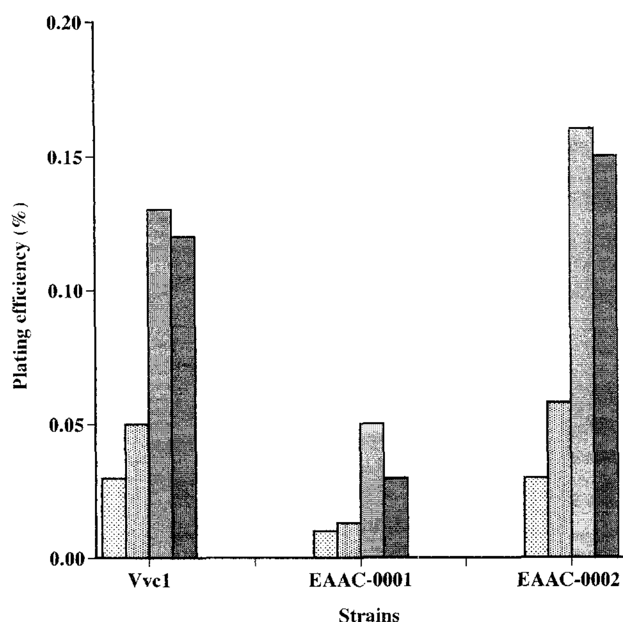

Fig. 6. Influence of enzymatic composition on the regeneration of *Volvariella volvacea* protoplasts. The three groups of four bars, from left to right in each group: 1% Novozyme 234, 2% Novozyme 234, 2% Novozyme 234 + 0.2% chitinase; 2% Novozyme 234 + 0.1% chitinase

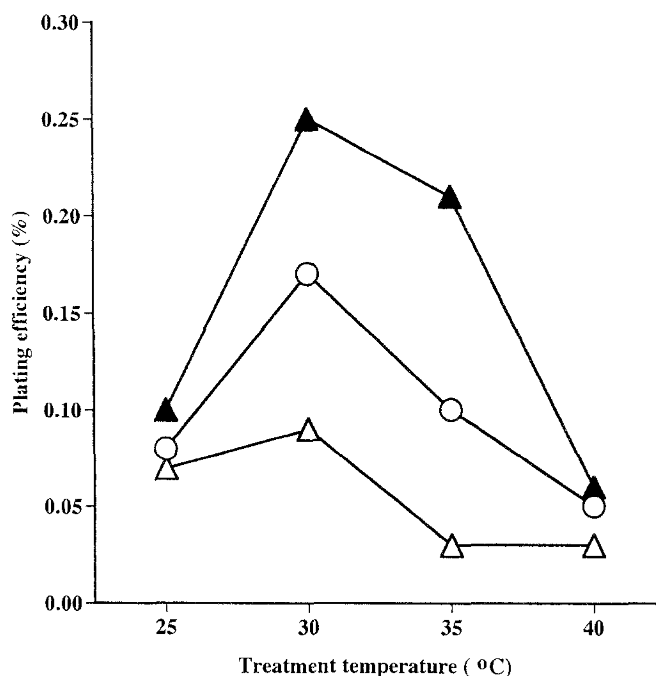

Fig. 7. Influence of enzymatic treatment temperature on the regeneration of *Volvariella volvacea* protoplasts. *Open circles*, Vvcl; *open triangles*, EAAC-0001; *filled triangles*, EAAC-0002

(Fig. 6). Mixtures of these two enzymes are more effective than when they are used singly. This result agrees with the previous findings of Hocart and Peberdy.[44] Our trial on the use of chitinase as the sole enzyme did not produce any protoplast, which may be due to the limited action of this enzyme when used as the sole source of enzyme. Chitinase that was commercially derived from *Streptomyces griseus* (Sigma) only contains 1.1–4.2μg chitinase. Novozyme 234, on the other hand, contains a variety of enzymes, including α- and β1,3-glucanase, laminarinase, xylanase, protease, and a trace of chitinase.

Temperature also influences the activity of the mycolytic enzymes to digest the cell wall, thus exposing the protoplasts. The combined action of 2% Novozyme 234 and 0.2% chitinase was efficient at 30°C. The efficiency of enzyme activity started to decelerate beyond this temperature (Fig. 7).

To facilitate the liberation of protoplast from the hyphal walls, the effects of varying reciprocal frequencies of the reciprocal shaker on protoplast regeneration were examined. Highest plating efficiency was observed when hyphal strands were subjected to 90 strokes/min (Fig. 8). Distorted protoplasts that were not capable of regeneration were observed at 95 strokes/min.

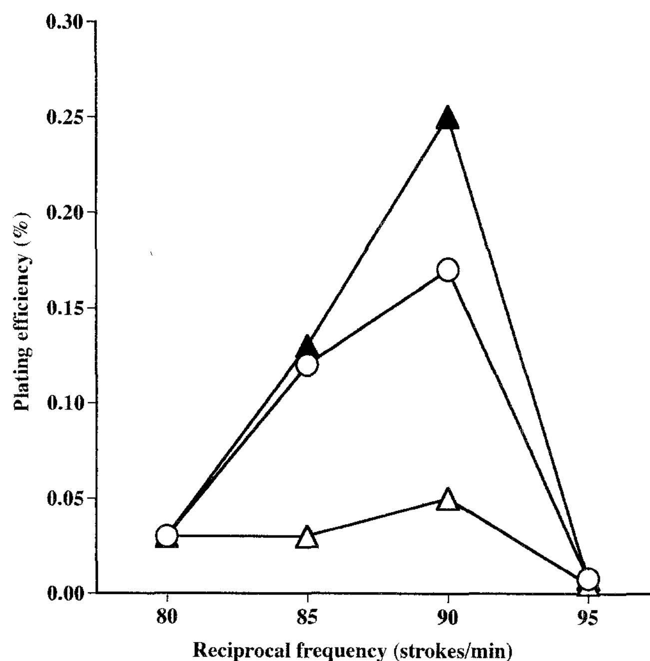

Fig. 8. Influence of reciprocal frequency on the regeneration of *Volvariella volvacea* protoplasts. *Open circles*, Vvcl; *open triangles*, EAAC-0001; *filled triangles*, EAAC-0002

Influence of centrifugation condition

After the liberation of protoplasts from hyphal walls, it is important that protoplast be excluded from mycelial debris to ensure its high potential for regeneration. In this experiment, it was done by filtration followed by centrifugation. Protoplast suspension was subjected to varying degrees of centrifugal force at varying durations of centrifugation. The significant results are shown in Fig. 9. Relatively larger protoplasts with high plating efficiency were recorded at 1100G for 10min. Distorted protoplasts, which accounted for low

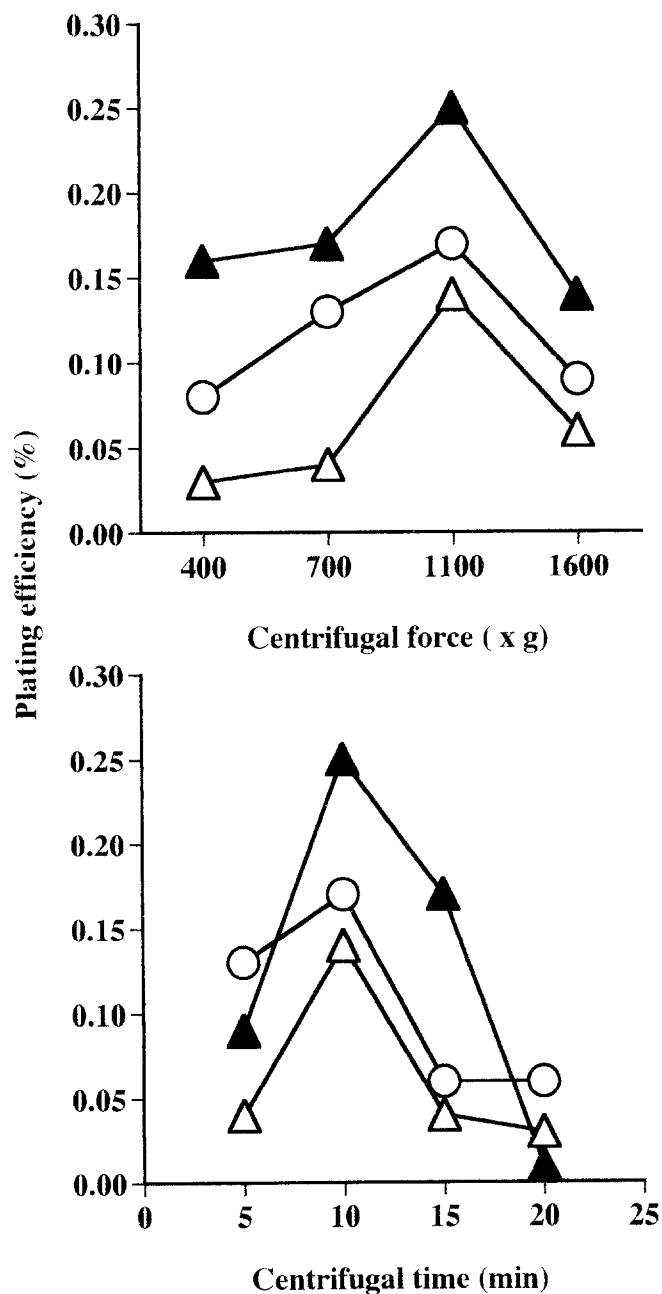

Fig. 9. Influence of centrifugal force and time on the regeneration of *Volvariella volvacea* protoplasts. *Open circles*, Vvc1; *open triangles*; EAAC-0001; *filled triangles*, EAAC-0002

plating efficiency, were noted when the protoplast suspension was subjected to 400 and 1600 G, respectively.

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