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Full Length Article

Factors Analysis in Protoplast Isolation and Regeneration from a Chalkbrood Fungus, *Ascosphaera apis*

Abebe J. Wubie[1,2], Y. Hu[1], W. Li[1], J. Huang[1], Z. Guo[1], S. Xu[1*] and T. Zhou[1*]
[1]*Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture; Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, 100093, Beijing, China*
[2]*Sekota Dry land Agricultural Research Center, Institute of Amhara Agricultural Research, Amhara Region, Sekota, 62, Ethiopia*
*For correspondence: xushufa@caas.cn; ztapis@263.net

Abstract

Current need for genetic engineering for *Ascosphaera apis* and absence of reports have initiated us to target development of an efficient and reproducible protocol to make this fungus amenable to genetic studies and transformation. The fungus was isolated from chalkbrood mummies and checked for its identity. Different enzymolysis and osmotic pressure stabilizing agents along with different growth mediums, incubation periods, pH and temperature have been utilized for isolation and regeneration of protoplasts. The fungus demonstrated varying responses in terms of yield and regeneration rates to different factors tested. Liquid growth medium and shorter incubation periods has yielded the highest isolated protoplast number (34.00×10^5 mL^{-1}) while use of 50mg mL^{-1} driselase was the best enzymolysis agent, yielded 98.36×10^5 mL^{-1} of protoplasts at 5.8 pH and 28°C. Exponentially growing mycelial culture provided the highest viability (90%). Citric acid-monohydrate with NaCl (0.8 mole L^{-1}) as osmotic stabilizer and 240 min of enzymolysis time have supported 53.06% protoplast regeneration, which is the first and highest to be reported for a fungus. With this first time reported protocol, viable protoplasts were obtained and regenerated successfully from *A. apis.* Thus, we believe, an important foundation has been set for efficient genetic manipulation of this fungus.

Keywords: Protoplast; *Ascosphaera apis;* Mycelium; Enzymolysis; Stabilizer; Regeneration

Introduction

Honeybees are accomplishing about 80% of all crop insect pollinations. Furthermore, inadequate pollination is a major constraint to the potential yields of commercial crops (Musallam *et al.*, 2004). However, this economically and ecologically important insect is suffering from various diseases caused by various pathogens. Naturally, honeybees have developed some mechanisms to defend invaders (Balhareth *et al.*, 2012). Chalkbrood is an important invasive mycosis in honeybees produced by *Ascosphaera apis*, Maassen ex Claussen; Olive and Spiltoir, that exclusively affects honeybee brood (Spiltoir, 1955). The disease has been reported to cause about 5-37% reduction in honey production and 80% brood death (Aizen *et al.*, 2009; Aronstein and Murray, 2010; Simlesa, 2010).

Despite the broad range of experimental works conducted towards control of chalkbrood worldwide so far, there is no widely accepted strategy among beekeepers. Up to date, reports for controlling the disease were focusing on the physiological understanding of the pathogen. Scientists are still investigating and studying on molecular aspects and various conditions during the growth of the pathogen.

Different factors are known to affect the growth of this fungus. Among them temperature, humidity and pH, generally, are playing an important role in the viability and germination of fungal spores (Bamford and Heath, 1989). Even though, the optimum temperature for growth of this fungus is 28-30°C, spore germination is facilitated at an incubation temperature of 32-35°C. Even if, spore germination is an important step in its infectivity, knowledge of the control of its infectivity is still limited.

Protoplasts, generally, are referred to as the first organized body of a species that had its cell wall completely or partially removed using either by mechanical or enzymatic means (Peberdy and Ferenczy, 1985; Homolka, 1988). They contain all the intracellular organelles of a cell and form a vital link in transfer of micro-molecules between cyto-organelles (Peberdy and Ferenczy, 1985). The isolated protoplasts may be excellent materials for plasmid DNA transformation or mutagenic treatments and in a variety of genetic manipulation techniques targeting specific genetic information transfer into the fungal species for the development of modified strains.

The presence of rigid cell walls, which are structurally important to fungal hyphae, makes observation of the internal components and manipulations very difficult (Cove, 1979; Peberdy and Ferenczy, 1985; Homolka, 1988; Zhou

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et al., 2008; Gao *et al.*, 2011). In the isolation of protoplasts, previous studies employed methods in which protoplasts were plasmolysed and released either spontaneously or by agitation following mechanical disruption of cell wall (Cove, 1979). However, these methods have yielded very low numbers and less viable protoplasts.

Conversely, longer lytic digestion or liquid culture medium shakings of fungal spores is known to be useful in the production of homogeneous protoplasts (Peberdy, 1979; Chen and Jeffrey, 1993). The technical breakthrough, then after, which led to the isolation of protoplasts in large numbers is the employment of enzymes to degrade the cell walls. In addition, protoplasts shall be osmotically buffered with a variety of solutes including inorganic salts such as NaCl, or sugars such as sucrose to survive the removal of the cell wall (Cove, 1979). This further explains that choice of a solute is also a very critical step for both optimization of the isolation procedure and viability of isolated protoplasts (Zhao *et al.*, 2004).

The efficiencies of protoplast formation and viability vary widely among different species, probably because of starting material physiological characters and enzymatic factors (Cundliffe, 1968). Scientists are still looking for wide range of options for appropriate protoplast isolation methods from a variety of species (Cocking, 1960; 1979). Because of the fact that considerable care is paramount important during protoplast preparation, it is also necessary to study the critical factors affecting the protoplast formation and regeneration of protoplasts used in biotechnology (Sankara and Prakash, 1995; Zhao *et al.*, 2004).

Protoplast formation is affected by many factors and the cell wall composition varies among species. So it is safe to assume that various solutes at different concentrations should be optimal for different species. More specifically, type and concentration of enzymes, osmotic stabilizers and pH should be emphasized and prioritized to cause substantial effects on protoplast preparation in various experiments. Even if, osmotic stabilizers are important to protect protoplasts from being broken during enzymatic actions and further improve enzyme activities, up to now, there are no recommendations about kinds of suitable osmotic pressure stabilizers and their appropriate concentration to be used in certain fungus species.

Although different protoplast isolation procedures have previously been developed to isolate from mycetes (fungi and mushrooms) and other species (Chen and Jeffrey, 1993; Sikandar and Christos, 2010), development of an appropriate method is thus, paramount important for better understanding of genetic properties through protoplast manipulation (Eguchi *et al.*, 1990; 1998; Tamai *et al.*, 1990).

However, as to our knowledge, we did not find protocols and descriptions available for generation of protoplasts from *A. apis*. This led us to conduct experiment targeting the development of a simple and convenient protocol for viable protoplast isolation and regeneration to make its genetic engineering through mutagenesis and transformation very convenient. The objectives of this study were to examine the effect of different factors important for appropriate protoplast isolation and develop an efficient and reproducible protocol for protoplast production from *A. apis* with the ultimate purpose of making this fungal species amenable to genetic studies and transformation.

Materials and Methods

Research Material

The fungal material, *A. apis*, was isolated and obtained from diseased larvae mummies of *Apis mellifera*, collected by apicultural research institute of Chinese academy of agricultural sciences, Beijing, China. And this fungus was used throughout the study as an experimental material.

Media and Reagents Preparation

During the experiment, potato dextrose agar (PDA), Liquid Regeneration Medium (0.2 – 1.0 mole L^{-1} of Glucose, 1% tryptone, 1% yeast extract, 1.2% maltose, 0.5% potassium phosphate dibasic anhydrous), solid regeneration medium (0.2 - 1.0 mole L^{-1} of Glucose, 0.5% yeast extract, 1% agar, 0.6% maltose), Sodium buffer (1 mole L^{-1} disodium hydrogen phosphate and 1 mole L^{-1} of sodium di-hydrogen phosphate), DF (0.2 – 1.0 mole L^{-1} of KCl), Liquid II (0.2 mole L^{-1} disodium hydrogen phosphate dodecahydrate, 0.2 – 1.0 mole L^{-1} of KCl), and finally different Enzymes (Lytic enzyme, cellulase, snailase and driselase at 50 mg mL^{-1} concentration) were prepared. All stabilizers (0.2 - 1.0 mole L^{-1} of glucose, KCl, sucrose, citric acid monohydrate plus with NaCl and NaCl) were used independently in each of the mediums and reagents used. All these growth media and reagents were adjusted to a pH value of 5.5 – 6.0 and autoclaved at 121^{o}C for 15 min. Chemicals were obtained from Sigma Chemical Co. Ltd., Beijing, China.

Fungal Spores Preparation

The collected fungal material, the chalkbrood mummy, was grown on to different growing mediums and incubation temperatures. After incubation at a temperature of 28-31^{o}C, spores were washed and collected for mycelia growth at a concentration of 1 x 10^{7} spores g m^{-1} in a liquid medium. The spores were then incubated in a reciprocal shaker at 180 rpm and 28-31^{o}C temperature for different hours and protoplast production efficiency of each were checked and better mycelial density incubation period was selected.

The incubated mycelium mat was then kept at -20°C for further use. Stock cultures were also maintained at -80°C using mineral oil and 40% (w/v) glycerol for further preservation and were checked for viability every 15 days.

Effect of Mycelium Age on Protoplasts Formation

Mycelium was subsequently picked up from liquid medium shakings at different incubation periods (24, 36, 48, 72 and

96 h) and was subjected to enzymatic digestions for different time gradients (2-6 h of enzymolysis) and temperature setups. The obtained protoplast was then tested for viability and other parameters accordingly.

Effect of Osmotic Stabilizers and Other Factors

Among the several compounds proposed as osmotic stabilizers for fungal protoplast isolation and regeneration, KCl, NaCl, citric acid monohydrate with NaCl, sucrose and glucose were tested at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 M L^{-1} in order to determine their most suitable levels. Additionally, factors such as lytic buffers, pH (5.5 - 6.0), and temperatures (28-31°C) were tested throughout the experiment and yields at different levels were determined.

Effects of Enzymes on Protoplast Isolation

Different concentrations of lysing enzyme, cellulase, snailase and driselase were tested for their degrading efficiency for the release of viable and regenerable protoplasts from *A. apis.* The efficiencies of these enzymes were checked for different pH levels for cell wall degradation times (2-6 h) (Zhou *et al.*, 2006). The chemicals were obtained from Biodee Biotechnology Co. Ltd., Beijing, China.

Protoplast Isolation

The incubated fungal mycelium was collected from the liquid medium by centrifugation at 6400 xg and 4°C for 45 sec. The collected mycelium mat was retained and washed with sterilized water three times. 2 mg of collected mycelia mat was taken and added into 4 Erlenmeyer flasks, each containing 50 mL of a wash buffer solution and 0.005% of DTT, and incubated in a reciprocal shaker at 90 rpm and 28-31°C for 30 min. Similarly, 8ml of each of the enzymes (at a concentration of 50 mg mL^{-1}) with a stabilizer were added separately into each of the 4 flasks and incubated again in a reciprocal shaker at 100 rpm and 28-31°C temperature for different time periods (2- 6 h).

Mycelia samples from each of the enzymolysis time periods (2, 3, 4, 5 and 6 h) were taken and filtered three times into new eppendorf tubes and centrifuged at 2200xg and 4°C for about 15 mins. The retained precipitate was then received 1mL of Liquid II and was centrifuged again at 2200xg and 4°C for 10 mins. Finally, 8-10 mL of 0.2-1.0 mole L^{-1} of different stabilizers was added in to each of the retained precipitates (protoplasts). Protoplasts counts were determined per ml per gram of fresh weight of mycelia using haemocytometer (Beyotime Institute of Biotechnology, Beyotime®, Beijing, China). Microscopic pictures of protoplasts were also taken and documented. The obtained protoplasts were then stored at -20°C for further use.

Protoplast Viability and Regeneration Assessment

Based on the experimental setup for protoplast preparation, viability and regeneration tests, various mycelia incubation times were used. The obtained protoplast from each of the incubation times were diluted to 10^5 mL^{-1} with osmotic pressure stabilizers and tested for their viability using Fluorescein Diacetate (FDA) at 5 mg mL^{-1} concentration and observation through fluorescence microscope with a final concentration of 0.01~0.1% with protoplast according to the recommendations made by (Zhou *et al.*, 2006). Regeneration test was conducted by distributing 100 µL of protoplast suspension onto a single and double layer solid regeneration medium and incubated at 28-31°C for 72 or more h until hyphal development is visible as a result of regeneration. Regeneration rate (%) = (A-B)/plated protoplast number × 100 was then used as a formula to calculate regeneration rate where A is colony number regenerated from protoplasts diluted with 0.7 mole L^{-1} NaCl and B is colony number regenerated from protoplasts diluted with sterile water. Growth performance was also observed starting from 24 h of incubation.

Protoplast Release and Growth Morphology

The protoplast releasing process was examined by isolating protoplasts from samples enzymolysed for 2, 3, 4, 5 and 6 h and observing through a microscope (Chongguang Co. LMT, Chongqing, China) with appropriate dilution. Observed protoplasts and their releasing stages were pictured and documented accordingly. Subsequently, protoplasts were dispensed on to a solid medium and their growth paten and morphology was observed starting from 24 h or incubation at 28-31°C.

Data Analysis

Data were analyzed with one-way analysis of variance (ANOVA), using SPSS statistical software and the significance between treatments in each experiment was evaluated by Duncan's multiple range test at $P<0.01$. The values are expressed as means S.D. $P<0.01$ was defined as statistically significant.

Results

Protoplast Isolation

Protoplast formation is affected by many factors. Protoplast preparation technique, mycelium age, type of culture medium, type and concentration of osmotic stabilizers and enzymes, pH and temperature of the lytic mixture are the frontline causes. More specifically, since cell membranes had a direct contact with the media and are therefore, more likely to be affected by subtle environmental changes, growth mediums, type and concentration of enzymes, osmotic stabilizers and pH have been prioritized to cause substantial effects on protoplast preparation. Thus, appropriate analysis of each of the factors has been carried out in this experiment.

Effects of Growth Medium and Culture Method on Protoplast Preparation Rate

PDA and different liquid mediums were used as a growth medium in the whole process of protoplast preparation. Isolated protoplast yield evaluated at different growth mediums with different incubation times has confirmed that the maximum protoplast yield (15.00×10^5 mL^{-1}) was obtained from liquid culture medium at 24 h of incubation time (Fig. 1). The lowest yield was obtained from PDA cultured samples. It is, maybe, because PDA is suitable for fungus sporulation while liquid growth mediums are more luxurious for mycelium growth and hyphal development. The other possible reason is that the importance of incubation time as younger mycelium age is very important for maximum protoplast isolation than older samples.

Effects of Enzymes on Protoplast Yield

Different kinds of commercial enzymes Cellulose, Lysing enzyme, Snailase and Driselase with a concentration of 50 mg mL^{-1} have been tested for their ability to yield high quantity protoplast yield and we have found that Driselase was the best treatment (yielded 98.36 $\times10^5$ mL^{-1}) with 4 h incubation time at a temperature of 28°C (Fig. 2). Even if, driselase was confirmed to maintain highest protoplast release at 28°C, temperature increase from 28 to 31°C during tissue incubation, in fact, has aided tissue digestion further but has decreased protoplasts yield. Results showed that Lysing efficiency of enzymes to degrade the cell wall, as a grant for the release of protoplasts, depends on fungal growing stage. The younger the mycelium age, the higher will be the protoplast yield within the given factors.

Enzyme activity time in protoplast release has been confirmed to bring about significant differences on the type and amount of protoplast produced (Fig. 3a-g). Shorter time enzymolysis time was observed to yield very small amounts, which are not separated from each other (Fig. 3a). Further enzymolysis time of 4 h yielded most appropriate number of individual protoplasts which was observed to simplify further protoplast manipulations (Fig. 3b-c). It was also possible to yield individual protoplasts at enzymolysis time greater than 4 h up to 6 h (Fig. 3d). However, it has been confirmed that even though we can have individual protoplasts, the quantity and its further viability was much lower than that of 4 h enzymatic digestion.

Effects of Stabilizers on Protoplast Yield

Osmotic stabilizers are playing an important role for the isolation of viable protoplasts. A variety of osmotic pressure stabilizers have been used in this experiment. Among them, 0.8 Mol L^{-1} citric acid monohydrate with NaCl was found to assist the isolation of maximum protoplast yield 34.00×10^5 mL^{-1} followed by 0.6 Mol L^{-1} of citric acid monohydrate with NaCl yielded 11.10×10^5 mL^{-1} (Table 1).

The lowest protoplast yield (only 0.001×10^5 mL^{-1}) was obtained from 0.2 Mol L^{-1} of sucrose. The very interesting result from this test we found was use of citric acid monohydrate with NaCl as an osmotic stabilizer have surprisingly enable the isolation of individual protoplasts (with no aggregation), which in turn simplified protoplast manipulation other than all the osmotic stabilizers used in this experiment, while others have given protoplasts with aggregations being cultures having sole NaCl and KCl as an osmoticum with the highest aggregation (Table 1, Fig. 4).

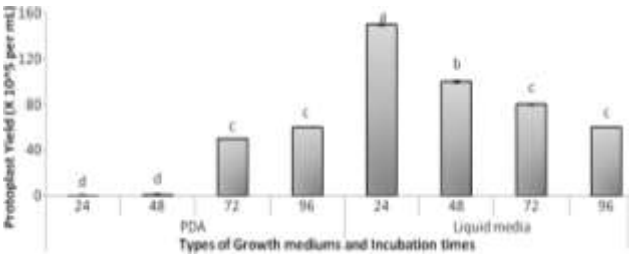

Fig. 1: Effects of growth medium and culture method was a result from an experiment conducted to examine the type of culture medium (PDA and Liquid medium) and incubation time (24, 48, 72 and 96 h) for optimum protoplast yield. a, b, c and d stand for significant differences ($p<0.01$)

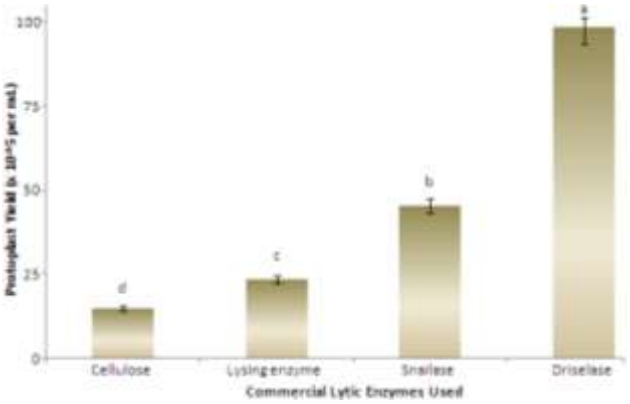

Fig. 2: Effects of enzymes on protoplast yield. The tested commercial lytic enzymes were used at a concentration of 50 mg mL^{-1} with degradation time of 240 minutes at 28°C. a, b c and d stand for significant differences ($p<0.01$)

Effects of Mycelium Age on Protoplast Preparation

Efficiency of cell wall degrading enzymes and use of different osmotic pressure stabilizers, as a grant for the release of protoplasts, depends foremost on fungal growing stage (mycelium age). From the experiment, we have found that age or the physiological state of the mycelium was one of the important factors affecting the protoplast formation and confirmed that young and exponentially growing culture was the most suitable state. Hence, the highest protoplast yield ($34.00\pm0.32\times10^5$ mL^{-1}) was obtained from younger mycelium which has been incubated only for 24 h at a temperature of 28°C (Table 2). On contrary, the lowest

Table 3: Effect of pH of osmotic stabilizers on protoplasts yield

pH	Tested stabilizers			
	Citric acid monohydrate with NaCl	NaCl	Sucrose	KCl
5.5	0.30±0.43f	0.01±0.04f	0.001±0.44 f	0.01±0.62 e
5.6	5.40 ±0.66d	0.16±0.22d	0.01±0.16 d	0.01±0.18 f
5.7	11.10±0.14b	3.1±0.18 b	0.22±0.38 c	0.28±0.74 c
5.8	34.00±0.32a	3.4±0.30 a	0.35±0.02 b	0.58±0.52 a
5.9	5.5±0.52c	0.25±0.72c	0.62±0.80 a	0.55±0.30 b
6.0	0.34±0.31e	0.11±0.00e	0.002±0.14 e	0.04±0.12 d

Note: a, b, c, d, e and f stand for significant differences ($p<0.01$)

Table 4I: Effects of growth medium on protoplast regeneration

Mediums	Ingredients	Visibility time (h)	Regeneration rate (%)
Solid medium	Basic medium	96	12.00±0.58c
Solid medium	Basic regeneration medium	72	51.06±0.58a
Liquid medium	Liquid medium	72	15.00±0.58c
Liquid medium	Liquid regeneration medium	72	32.88±0.58b

Note: a, b and c stand for significance of difference ($p<0.01$)

Table 5: Effects of osmotic stabilizers on protoplast regeneration

Osmotic stabilizers	Concentration (Mole L^{-1})	Time (h)	Regeneration rate (%)
	0.2	72	0.20±0.08 e
Citric acid monohydrate with NaCl	0.4	72	5.30±0.52 d
	0.6	72	10.82±0.56 c
	0.8	72	51.06±0.58 a
	1.0	72	32.88±0.58 b
	0.2	72	0.10±0.38 e
	0.4	72	3.20±0.14 d
KCl	0.6	72	8.20±0.56 b
	0.8	72	18.42±0.76 a
	1.0	72	6.54±0.32 c
	0.2	72	0.04±0.97 e
	0.4	72	0.48±0.08 d
Sucrose	0.6	72	4.60±0.52 c
	0.8	72	21.48±0.77 a
	1.0	72	5.42±0.95 b
	0.2	72	0.06±0.87 e
	0.4	72	0.62±0.01 d
Glucose	0.6	72	6.40±0.12 c
	0.8	72	19.87±0.32 a
	1.0	72	7.22±0.25 b

Note: a, b, c, d and e stand for significance of difference ($p<0.01$)

number of protoplast (only 0.04±0.56 $\times 10^5$ mL^{-1}) was obtained from samples incubated for only 12 h at the same temperature and other conditions. The results indicated that early mycelium age is not able to support the release of protoplasts and 24 h of incubation is more appropriate for protoplast isolation from *A. apis*. The concentration of the mycelium in the lytic mixture also has been found to affect the protoplast yield, apparently explaining the accessibility of hyphae to the lytic agents. Protoplast numbers have been observed to decrease with increasing age of the culture, perhaps reflecting maturity of large parts of the mycelia mat is not accessible for enzymatic actions.

Table 1: Effects of stabilizers' concentration to protoplast yield

Stabilizers	Concentration (Mol L^{-1})	Protoplast Yield (x 10^5 mL^{-1})	Cell Aggregation
	0.2	0.01±0.04 e	†
	0.4	0.16±0.22 d	††
NaCl	0.6	3.1±0.18 b	†††
	0.8	3.4±0.30 a	†††
	1.0	0.25±0.72 c	†
	0.2	0.30±0.43 e	-
Citric acid with	0.4	5.40 ±0.66 d	-
monohydrate	0.6	11.10±0.14 b	-
NaCl	0.8	34.00±0.32 a	-
	1.0	5.5±0.52 c	-
	0.2	0.01±0.62 d	†
	0.4	0.01±0.18 e	†††
KCl	0.6	0.28±0.74 c	††††
	0.8	0.58±0.52 a	††††
	1.0	0.55±0.30 b	††††
	0.2	0.001±0.44 e	†
	0.4	0.01±0.16 d	†
Sucrose	0.6	0.22±0.38 c	†
	0.8	0.35±0.02 b	†
	1.0	0.62±0.80 a	†

Note: symbols (†) stands for cell aggregation was seen, (††††) stands for cell aggregation was serious, while (-) stands for cell aggregation was not seen. a, b, c, d and e stand for significant differences ($p<0.01$)

Table 2: Effects of mycelium age to protoplast preparation

Mycelium age	Temperature (°C)	Protoplast yield ($\times 10^5$ mL^{-1})
Incubated for 12 h	28	0.04±0.56 d
Incubated for 24 h	28	34.00±0.32 a
Incubated for 48 h	28	26.00±0.58 b
Incubated for 72 h	28	10.50±0.34 c

Note: a, b, c and d stand for significant differences ($p<0.01$)

Effects of pH Value and Incubation Temperature on Protoplast Isolation

Like other very important factors, protoplast formation was also influenced by pH of incubation stabilizers. Based on the protoplast count, results showed that the highest protoplast number was obtained from citric acid monohydrate with NaCl at 5.8 pH value (34.00 × 10^5 mL^{-1}) and declined with either side of the range at 28°C (Table 3), while 5.5 pH value was found to be the lowest in assisting protoplast release (only 0.001 × 10^5 mL^{-1}) (Table 3). Furthermore, higher pH values did not favor the release of optimum protoplasts. The results also confirmed that increased incubation temperature from 28°–31°C significantly reduced the time needed for rapid release of protoplasts. In this protocol, optimal incubation temperature was 28 – 29°C and pH of 5.7 – 5.9 for better protoplast yield (Table 3).

Protoplast Regeneration

Protoplast regeneration provides a relative measure of the effects of enzyme treatment on cell visibility. Protoplasts that lack the ability to regenerate presumably either lack nuclei or were damaged at some point during or after the enzyme treatment. The types of regeneration and growth mediums

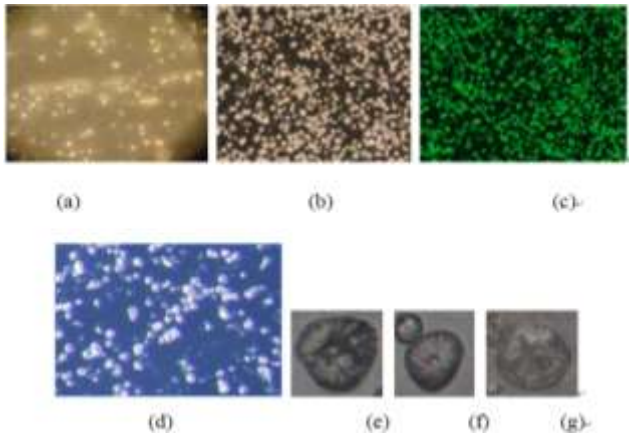

Fig. 3: Isolated protoplasts from *Ascosphaera apis*. (a) is indicating an incomplete process of protoplast formation obtained from 2-3 h of enzymatic digestion (enzymolysis time) at 28℃. (b) and (c) are figures indicating individual optimum number of individual protoplasts obtained as a result of 4 h enzymolysis time. (d) is showing the protoplasts obtained from 6 h of enzymatic digestion. (e), (f) and (g) are 400×magnifications of individual protoplasts released from 4 h of enzymatic digestion

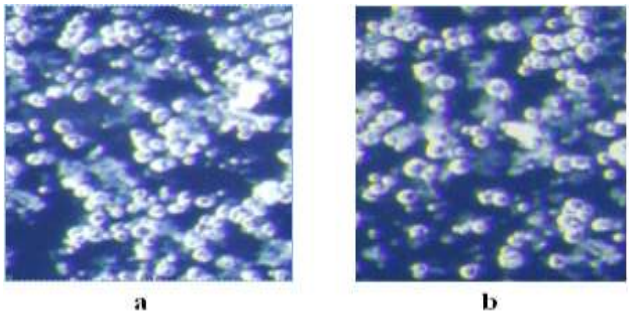

Fig. 4: Protoplasts aggregation isolated from cultures having sole KCl (a) and NaCl (b) as an osmoticum

and different osmotic stabilizers used in the experiment have been believed to have significant effects on the regeneration of the protoplasts. Furthermore, as days of protoplast storage increased, regeneration was observed also to decrease. Based on the microscopic observation, viability of isolated protoplasts after Fluorescein Diacetate (FDA) treatment was surprisingly higher with nearly 90% viability (Fig. 5).

Effects of Protoplast Growth Mediums on Regeneration Rate

Growth performance study of isolated protoplasts was employed on different regeneration mediums at different incubation periods and 28°C until growing hyphae (mycelium mat) became visible. Accordingly, protoplast regeneration was found to be the highest (51.06% mycelia colonies growth) from a single layer basic regeneration medium which has increased regeneration frequencies of *A. apis* while more than 90% of fungal regeneration completes after 72 h of incubation (Table 4; Fig. 6). This result is, thus, classified as the highest protoplast regeneration rate ever reported for a fungus. However, further deeper studies implementing this protocol would have an immense importance for strengthening of this result.

In addition, results showed that Liquid regeneration medium was also effective in favoring regeneration of protoplasts into a mycelium mat within 72 h of incubation at 28°C and 180 rpm shaking assisting for 32.88% of protoplast regeneration (Table 4).

Unlike many other fungal species, protoplasts obtained from the lowest incubation time and pH value of stabilizers, regeneration stops at some stage and the structure decays and disintegrates after a time without giving rise to hyphae, which didn't secure efficient reversion.

Effects of Osmotic Stabilizers on Protoplast Regeneration

Since the cell wall composition varies among species, it is safe to assume that various osmoticum at different concentrations should be optimal for different species. As protoplasts have lost their cell wall because of enzymatic actions, use of osmotic stabilizers is important to protect protoplasts from being broken and further benefit in improving enzyme activities. Of course, up to now, for a certain fungus species, there are no reasonable explanations about which kind of chemical composition are more suitable osmotic pressure stabilizers than others regarding favouring protoplast regeneration.

In this experiment, we have compared Citric acid monohydrate with NaCl, KCl, glucose and sucrose at different concentrations for detection of protoplast regeneration. Results showed that use of citric acid monohydrate with NaCl at 0.8 Mol L^{-1} concentrations as an osmoticum in a culture medium have provided luxurious protoplast regeneration rate (51%) at 72 h of incubation time (Table 5). There was a significant difference among the tested osmotic pressure stabilizers at all concentration levels ($p<0.01$). However, osmotic stabilizers' concentration level 0.8 Mol L^{-1} was better than the other concentration levels in protoplast regeneration rate at the given temperature and incubation periods in this experiment (Table 5).

KC1 is known for its inhibitory effect on protoplast reversion. However, in this test, use of NaCl and KCl as an osmoticum, regeneration rate looks great, but through time colony formation was observed to be slower remarkably. For this reason, glucose and sucrose were used in growing mediums as osmotic henceforth because of the fact that the later are known to be important in both respiration and in synthesis of the cell wall material necessary for protoplast regeneration (Fig. 7). Though, increasing sucrose and glucose concentration in growing mediums generally has improved regeneration efficiency, the effect has been slow and, in some cases, efficiency declined at higher levels. In this experiment, we found that regeneration was minimal when no sugar was present. Therefore, the results suggested

that different fungi species have different rates of sucrose and glucose uptake and utilization.

Effects of Enzymolysis Time on Protoplast Regeneration

Enzymolysis time is one of the important factors affecting protoplast regeneration. Among the different enzymolysis time combined with Citric acid monohydrate with NaCl, KCl, NaCl, Sucrose and Glucose, 240 min of enzyme action upon the mycelia mat, Citric acid monohydrate with NaCl have provided the highest (53%) protoplast regeneration while NaCl at an incubation time of 300 min gave the lowest (only 4.8%) regeneration rate (Fig. 8).

It has been also observed that shorter Lysing times enabled the protoplasts to regenerate better than those subjected to longer Lysing times. It could be due to the fact that protoplast membranes are liable to damage upon longer exposure to lytic enzymes exceeding 6 h.

Discussion

Using the present protocol, the protoplasts isolated from *A. apis* with different levels of enzymes, incubation time, incubation temperature and pH levels used were found to be viable and excellent for further manipulations.

As suggested for other fungal species, this experiment has taken an advantage in using young, exponentially growing culture, which owns a mycelium with active physiological state (Eguchi *et al.*, 1990; 1998; Chen and Jeffrey, 1993). This also confirmed inline result in isolating higher amounts of protoplasts from *A. apis*. Even if, it has been suggested for other kinds of fungal species, on the other hand Chen and Jeffrey (1993) suggested protoplast release and regeneration improved with use of increased culture ages. In this regard, our result has confirmed that there was poor performance in protoplast isolation from older fungal culture based *A. apis* spores and have obtained very low protoplasts, which were also low in regeneration rates.

Furthermore, an important factor affecting quantity and quality protoplast yield was concentration of the mycelium in the lytic mixture which furnished the accessibility of hyphae to the lytic agents for better release of protoplasts. Protoplast regeneration was also decreased with increasing protoplast storage days potentially due to extra maturity.

Compared with the general assumption that inorganic salts are more effective with filamentous fungi for protoplast release, and sugar and sugar alcohols with yeasts and higher plants suggested by Lalithakumari (1996), we have proved that use of inorganic salts and sugars as osmotic pressure stabilizers have important roles in protoplast isolation and protection. In this study, the inorganic salts were found to be optimal osmotic pressure stabilizers protoplasts, which confirmed the contrary viewpoint previously as our fungus is not a filamentous fungus.

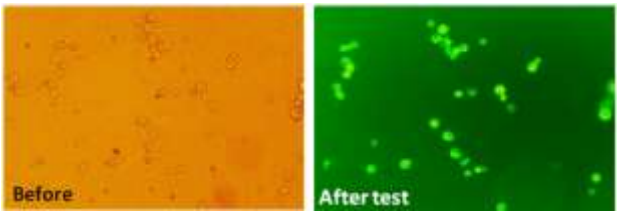

Fig. 5: Microscopic (400×) observation of protoplast viability test in the presence of fluorescein diacetate (FDA) before and after UV light application

Fig. 6: Protoplast regeneration from single layer plates

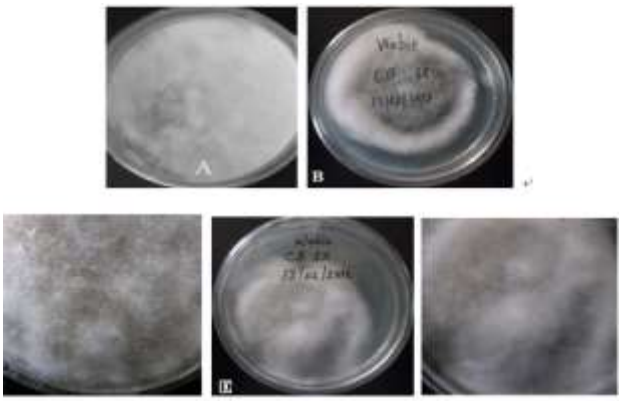

Fig. 7: Regeneration morphology of the *A. apis*. Regeneration mediums containing 0.8 mole L^{-1} of sucrose (A), sodium chloride (B), Citric acid monohydrate with NaCl (C), KCl (D) and glucose (E) were used

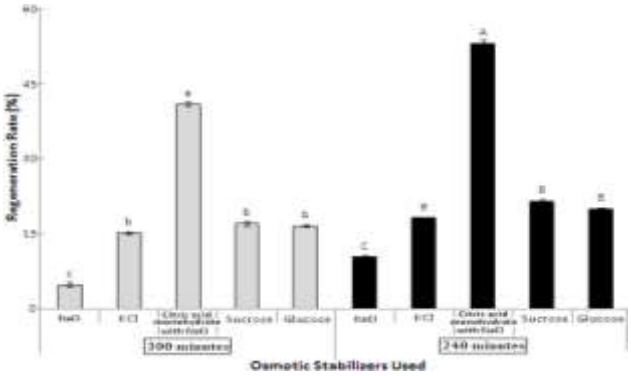

Fig. 8: Effects of enzymolysis time on protoplast regeneration rates of *A. apis*

Unlike results from some other fungal species tested by Zhou *et al.* (2008) and Talkhan *et al.* (2008), protoplasts obtained from *A. apis* in shorter exposure times (2-3 h) to

lytic enzymes have lower capacity to regenerate than those which have been in contact with enzymes for longer periods (4–6 h). This is because longer period lytic exposure enables better protoplast release from swollen hyphae. In addition, even if, it has been blamed by Zhou *et al.* (2008) for other fungal species, use of driselase as a lytic enzyme was found to be best performing enzyme in isolating protoplasts.

In contrast to the generalization made by Peberdy (1991) on protoplast regeneration frequency from fungal species lies between 0.1 to 50%, with our protocol, we were able to obtain better regeneration frequency (>51%). As far as our knowledge is concerned, we didn't find a report with a result which has exceeded our value thus potentially lead us to name our result as the highest protoplast regeneration rate ever reported for a fungus.

The results of this experiment potentially indicated that viable protoplasts can be obtained and regenerated successfully from *A. apis*. Due to the fact that protoplasts are the main starting points for extension of our knowledge to fungal genetic engineering, pathogenecity study and production of various cell wall degrading enzymes and different toxicants against fungal physiology, additional studies would be important for protoplast characterization from the fungus.

In this original study, we developed a method to isolate protoplasts from *A. apis* successfully with impressive results which potentially lead us to investigate and come up with the highest protoplast regeneration rate for a fungus. Our findings are explaining that use of 24 h old liquid medium based cultured mycelium at 28^{o}C and use of 0.8 mole L^{-1} Citric acid monohydrate with NaCl as an osmotic pressure stabilizer in the presence of 50 mg mL^{-1} driselase as a degrading enzyme for 240 min with a pH value of 5.8 is the optimal procedure for protoplast preparations. We finally recommend that use of this technique for successful isolation of protoplasts from this fungus to start with convenient genetic manipulation will be worth enough.

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