International Journal of Advancement in Life Sciences Research

Online ISSN: 2581-4877

journal homepage http://ijalsr.org

Original Article

Development of *Termitomyces* Protoclones and Characterizations Through PCR Based ISSR And RFLP of rRNA-ITS Gene

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Abstract

A total of five protoclones were successfully cultured on PDA medium out of regenerated twenty two colonies of *Termitomyces* protoplast and further studied. Liquid MYG grown mycelial tissue is used for protoplast isolation by enzymatic digestion in a mixture containing Lysing enzyme 2% and Cellulase R10 2% in 0.6 M mannitol. The incubation conditions like temperature, shaking and time were standardized at 24ºC with shaking 60 rpm for 10 hours, respectively for healthy protoplasts liberation. The purified protoplasts showed 31.60±9.31% regeneration on specific medium and 77.12±2.72% viability by FDA test. Four ISSR primers were used in this study with a total of 27 reproducible bands with mean value 6.75 and they showed similar banding pattern in all with zero percent polymorphism ranged from 280 bp - 2700 bp. The amplified rRNA-ITS gene showed ~ 600 bp size in gel and found a single restriction site for enzyme Hae*III* in all the protoclones and parent with similar fragment size in all.

Keywords: *DNA markers, Edible mushroom, Genome stability, Protoplast regeneration.*

Introduction

Edible mushrooms are rich in protein content and thus used as food supplement worldwide. They can be commercially cultivated but still the wild collection is very popular to the poor tribal family in Asian and African countries. *Termitomyces sp.* is such popular wild edible mushroom grows in termite gut and found in Birbhum, Purulia and Midnapore districts of West Bengal, India. Apart from protein, *Termitomyces* is an affluent source of sugar, fibre, lipid, vitamin, mineral in addition to medicinal value which is used in lower blood pressure, rheumatism, kwashiorkor, obesity, diarrhea and purgative (Apetorgbor *et al*, 2005; Rajoriya *et al*, 2014). So, it is now an important issue to improve the *Termitomyces* strains in terms of substrate specificity, yield and nutritional attributes. The mushroom crop improvement includes the two major aspects i.e., genome shuffling via protoplast technology and development of transgenic via genetic engineering. Due to the lack of available genetic resources and many other disadvantages, researchers prefer the protoplast fusion technology for mushroom breeding where even mating can be possible between the sexually incompatible strains (Mallick and Sikdar 2014, 2015a). However, it has been proved that the protoplasts are good source material for mushroom crop

improvement (Chakraborty and Sikdar, 2010; Mallick and Sikdar, 2014, 2015a) where researchers had successfully developed somatic hybrids between the different genera followed by fruit body production. Development of *Termitomyces* protoplast and study about their genome stability is a crucial factor for its breeding with other strains. Sometimes, the fruit body derived mushroom strains show genome instability with their parent (Mallick and Sikdar, 2015b) analyzed by molecular DNA markers.

In the past, Nazrul and Yin-Bing (2010) developed ten protoclones of *Agaricus bisporus* and characterized those using morphological and molecular parameters. They showed the genome instability in the protoclones analyzed by ISSR markers. ISSR is a reproducible molecular DNA marker for genetic analysis (Bornet and Branchard 2001) and hence have been used to study the genetic diversity of mushrooms (Guan *et al.*, 2008; Nazrul and Yin-Bing, 2010; Malekzadeh *et al.*, 2011; Mallick and Sikdar, 2014, 2015a).

However, in this study we wanted to study about the genome stability of *Termitomyces heimii* protoclone developed by mycelial protoplast and characterization of those by PCR based molecular DNA markers.

Materials and Methods

Protoplast isolation, purification and culture:

The *Termitomyces heimii* fruit body was collected from Bankura district of West Bengal, India and routinely maintained the vegetative culture on PDA medium (Potato Dextrose Agar, pH 6.2) at 28°C. The 7 days old MYG (10 g/l malt extract, 4 g/l yeast extract and 10 g/l glucose, pH 6.2) grown mycelial tissue was used for enzymatic digestion. Protoplast isolation and purification was done according to the protocol of Mallick & Sikdar, 2014. Protoplast yield was measured using a hemocytometer and viability was calculated by FDA staining (Widholm, 1972). Culture media containing MYG supplemented with 0.7M NaCl as an osmotic stabilizer and 1.5% agar with purified protoplast was kept at 28°C for regeneration.

Extraction of genomic DNA and ISSR:

The genomic DNA was extracted from MYG grown mycelial tissues using modified CTAB method (Dellaporta *et al.*. 1983). Four ISSR primers were used (Table1) in each single reaction according to the protocol of Bornet and Branchard (2001) with slight modifications. The PCR was performed in 25 µl of reaction volume in a DNA thermal cycler (Applied Bio-systems 2027) containing 25ng template DNA, 10X Taq buffer (+KCl), 25mM $MgCl_2$, 2mM dNTPs mix, primer and Taq DNA polymerase (5U/µl). The initial denaturation at 94ºC for 5 min, followed by 40 cycles consisting of denaturation at 94ºC for 1 min, primer annealing (37-61ºC) for 1 min and extension at 72ºC for 3 min and a final extension for 10 min at 72ºC.

Amplification of rRNA-ITS gene and RFLP:

According to the protocol of White *et al.* (1990), the rRNA-ITS gene was amplified by PCR in a 25µl of reaction volume containing 15ng template DNA, 10X Taq buffer (+KCl), 25mM $MgCl_2$, 2mM dNTPs, primer ITS1-F(5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4-R(5′ TCCTCCGCTTATTGATATGC 3′) (Table1), Taq DNA polymerase (5U/µl). The amplification was conducted in a DNA thermal cycler (Applied Bio-systems 2027) by preliminary denaturation at 95°C for 4 min followed by 40 cycles; denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, initial extension at 72°C for 1 min 30 sec, followed by a final extension at 72°C for 8 min.

For restriction analysis, PCR products were purified by sodium acetate precipitation. 10µl of purified PCR product was used for restriction digestion according to the supplier's specification with enzyme *Hae*III (Fermentas).

Gel run:

ISSR fragments, rRNA-ITS products and restriction digested products were separated on 1.8%, 1.5% and also 1.5% agarose gel with pre-stained ethidium bromide solution using 1X TAE buffer, respectively. The gels were run at 80 volts for 3 hrs (ISSR), 2 hrs (rRNA-ITS) and 3 hrs (RFLP) and banding profiles were visualized under UV light using a transilluinator system. Data were recorded in a Molecular Analyst Gel Documentation System. Gene

Ruler 100 bp plus DNA ladder, (MBI, Fermentas) is used as a standard molecular weight marker in each case.

Results

Protoplast culture and regeneration:

The enzyme mixture containing lysing enzyme 2% and cellulase R10 2% in 0.6 M mannitol as osmoticum showed the best result with gentle shaking at 60 rpm for 10 hrs at 24ºC. The average yield of purified protoplast was 1.2×10^7 cells/gm of 7 days old tissues (Fig.1a) as optimum. They showed the maximum 31.60±9.31% regeneration and 77.12±2.72% viability (Fig.1c). The purified protoplasts (Fig.1b) were cultured on MYG medium supplemented with 0.7M NaCl and 1.5% agar, pH 6.2 and kept at 28°C for regeneration.

Table1: Details of primers used in this study.

Sl No.	Primer Name	Sequence (5′ - 3′) with repeat motif	No. of amplified bands	Size range in bp
1.	ISSR-01	GACAGACAGACAGACA [(GACA)4]	5	520-1850
2.	ISSR-02	CAGCAGCAGCAGCAG [(CAG)5]	7	350-2200
3.	ISSR-11	CACCACCACGC [(CAC)3GC]	6	500-2000
4.	ISSR-12	GAGGAGGAGGC [(GAG)3GC]	9	280-2700
5.	ITS1-F ITS4-R	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	1	600

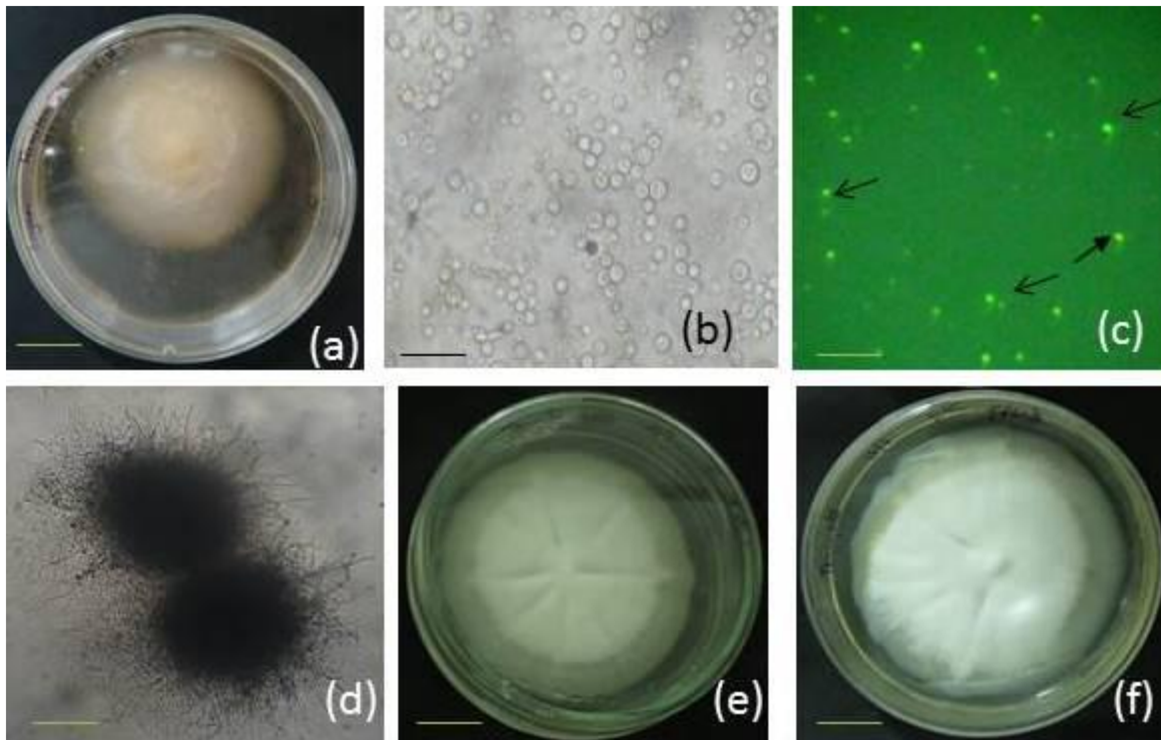

Fig.1: (a) 7days old liquid culture of Termitomyces, (b) Purified protoplasts in 0.6M mannitol isolated from liquid-grown tissue, (c) FDA stained viable protoplasts, (d) Developed 3 days old micro-colonies on regeneration medium, (e) 7days old PDA culture of TPC1, (f) 7days old PDA culture of TPC5. Bar size: 20mm in (a), (e) & (f). 60 µm in (b), 100 µm in (c) and (d)

Protoplast started germ tube formation in 24 hrs followed by micro-colonies (Fig.1d) formation in 72 hrs onwards. A total of twenty two macro-colonies were observed in four replicate regeneration plates after 5, 7 (from two replicate) and 8 days, respectively. Immediately, the colonies were transferred in PDA medium for further growth and kept at 28°C. Among them only five putative protoclones were selected based on their growth performance and named as *TPC1*, *TPC2*, *TPC3*, *TPC4* and *TPC5* (Fig.1e&f).

ISSR profile:

Four reproducible ISSR primers amplified a total of 27 bands in all the TPC lines including parent with mean value of 6.75. ISSR-01 produced the lowest number of band i.e., 5 and ranged from 520 bp -1850 bp where ISSR-12 produced the highest number of band i.e., 9 and ranged from 280 bp – 2700 bp (Table1). All the TPC lines showed similar banding patterns in size and number with the parent using ISSR primers (Fig.2). However, the ISSR profiles resulted that there is no dissimilarities or polymorphism in all the protoclones with respect to the parent.

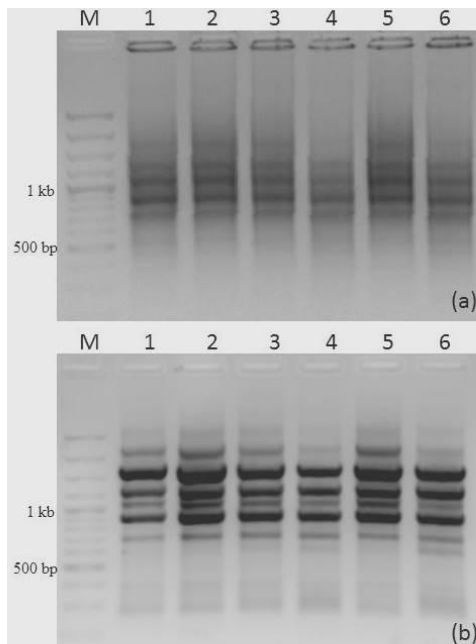

Fig. 2: ISSR profiles of TPC cultures and parent Termitomyces generated by (a) ISSR-11 and (b) ISSR-12. Lanes: M – molecular weight markers, 100 bp DNA ruler plus, lane1 – parent Termitomyces and lane 2 – 6 is TPC 1 – 5, respectively

Amplification of rRNA-ITS region and RFLP analysis:

The primer set ITS1(F) and ITS4(R) amplified the rRNA-ITS gene in all the TPC lines and parent and the size was analyzed in agarose gel. The PCR product showed a single band in all the lines with approx. 600 bp in size (Fig.3a). The primer set ITS1(F) and ITS4(R) can amplify only the ITS1-5.8S-ITS2 region which is basically a conserved sequence. We also found that the TPC lines are genetically similar with the parent by the restriction enzyme digestion. The restriction enzyme *Hae*III showed only single restriction site in the entire ITS1-5.8S-ITS2 region in all the lines including parent followed by producing two bands in the gel (Fig.3b). It was also noted that the sizes of restriction fragments were same in all.

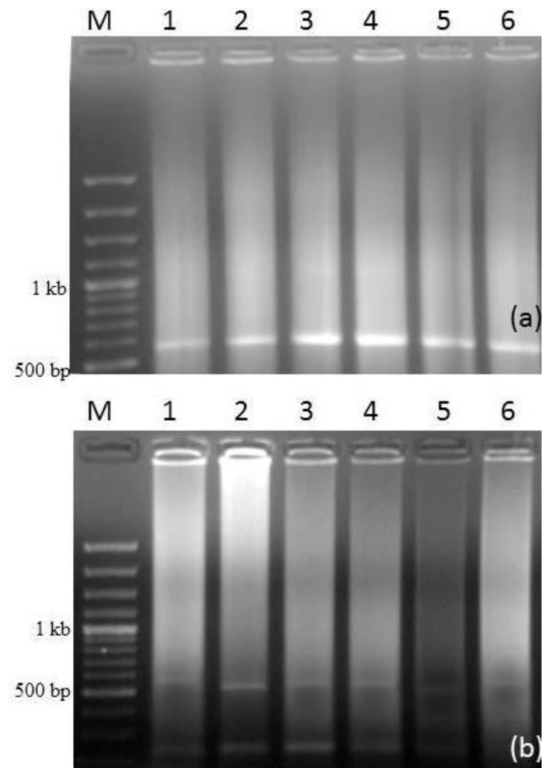

Fig.3: The rRNA-ITS gene and RFLP profile of TPC lines and parent Termitomyces. (a) Amplified rRNA-ITS gene showing approx. 600 bp size in gel, (b) RFLP profile by restriction enzyme HaeIII. Lanes: M – molecular weight markers, 100 bp DNA ruler plus, lane1 – parent Termitomyces and lane 2 – 6 is TPC 1 – 5, respectively

Discussion

Development of protoclone of agricultural crop usually helps in breeding for development of superior quality lines. In plant system several reports have been made where protoclone is developed from the protoplast and interestingly they showed phenotypic diversity where genotypic identity was maintained (Gill *et al.* 1986) and *vice-versa* (Kane *et al.* 1992). Theoretically, protoclones are identical lines of the existing parent whether it may be plant, animal, fungi or other living organisms. For rapid multiplication of crop plant and mammalian cell lines this technique is used in tissue culture industry.

Reports on characterization of mushroom protoclone are limited and it depends on the regeneration percentage of protoplast in the suitable culture medium. Isolation and purification of *Termitomyces* protoplast was carried out following the standardized protocol with slight modifications and the protoplasts were cultured in such suitable medium where the regeneration percentage was highly achieved. However, the resulted genetic structures were identical in all the TPC lines with parent based on ISSR profile and RFLP of rRNA-ITS gene. The ISSR, RFLP of rRNA-ITS gene has already been proved as reproducible and suitable genetic markers for analysis of mushroom somatic hybrids and fruit body derived lines (Mallick and Sikdar 2014, 2015a & 2016). Even the mushroom fruit body derived lines showed genetic diversity which might be occurred due to the gene environmental factor followed by elimination or rearrangement of genome structure during subsequent culture (Mallick and Sikdar, 2015). In this case, all the *TPC* lines are genetically identical with little morphological dissimilarity like heterogeneous colony nature, growth rate on PDA medium (not details in this manuscript). This might be happen due to the presence of inactive compounds in the medium or the protoplast cultures were not exposed to the environment where direct gene environmental factor works.

Conclusion

The morphological variation may be due to the effects of vegetative culture or the growth responsive gene might be in the dominant allele or induced during sub-culturing. Once the genetic makeup remains identical in this case, we could claim that these lines are clone which are derived from the protoplast. The further study regarding their yield, detail morphological attributes, nutritional profiles of the existing cloud give a good conclusion.

Acknowledgement:

Authors are thankful to the Director of Bose Institute, Head, Division of Plant Biology section, Vice Chancellor and Registrar of Brainware University, Kolkata, India for providing all the necessary facilities and support.

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