Chapter 8
Appendix
Production and molecular characterization of somatic hybrids between *Pleurotus florida* and *Lentinula edodes*

Pijush Mallick • Samir Ranjan Sikdar

Received: 29 April 2013 / Accepted: 11 April 2014
© Springer Science+Business Media Dordrecht 2014

Abstract Nine inter-generic somatic hybrids named as *pfl* were produced through PEG-mediated protoplast fusion between *Pleurotus florida* and *Lentinula edodes* using double selection method. Hybridity of the newly developed strains was established on the basis of colony morphology, mycelial growth, hyphal traits, fruit-body productivity and inter single sequence repeat (ISSR) marker profiling. Hybrid population was assessed with different phenotypic variables by one-way analysis of variance. Principal component matrices were analyzed for the six phenotypic variables in scatter plot showing maximum positive correlation between each variable for all strains examined. Six ISSR primers generated 66 reproducible fragments with 98.48 % polymorphism. The dendrogram thus created based on unweighted pair-group method with mathematic averages method of clustering and Euclidean distance which exhibited three major groups between the parents and *pfl* hybrids. Though *P. florida* parent remained in one group but it showed different degrees of genetic distance with all the hybrid lines belonging to the other two groups while *L. edodes* was most distant related to all the hybrid lines. *L. edodes* specific sequence-rich ISSR amplicon was recorded in all the hybrid lines and in *L. edodes* but not in *P. florida*. All the fruit body generating *pfl* hybrid lines could produce basidiocarp on paddy straw in sub-tropical climate and showed phenotypic resemblance to the *P. florida* parent.

Keywords Basidiocarp • ISSR • *Lentinula edodes* • *Pleurotus florida* • Polymorphism • Principal component analysis • Somatic hybrid

Abbreviations
- ANOVA Analysis of variance
- FDA Fluorescein diacetate
- IOD Iodacetamide
- ISSR Inter single sequence repeat
- MYG Malt-yeast extract-glucose
- MYGNa MYG supplemented with 0.7 M NaCl
- PDA Potato-dextrose-agar
- PCA Prinicipal component analysis
- PIC Polymorphic information content
- SCAR Sequence characterized amplified region
- UPGMA Unweighted pair-group method with mathematic averages

Introduction

Production of new edible mushroom strains through conventional sexual mating in inter-generic or inter-order is restricted due to incompatibility barrier but the same through para-sexual mating became possible. Protoplast fusion proved as a feasible method for inter-specific and inter-generic hybridization for strain improvement among edible mushrooms (Peberdy and Fox 1999). Production of somatic hybrid strains in edible mushrooms through protoplast fusion had been attempted in the past in intra-
Appendix

World J Microbiol Biotechnol

specific, inter-specific, inter-generic (Ogawa 1999) and even inter-order combinations (Yoo et al. 2002; Yoo 2004), but successful basidiocarp formation in those strains are meagre. However, Chakraborty and Sikdar (2008, 2010) successfully produced basidiocarp in somatic hybrid strains between Pleurotus floridus + Volvariella volvacea and P. floridus + Calocybe indica. Previous report on somatic hybrids with compatible and incompatible species in genera Pleurotus and Ganoderma showed a different colony morphology and basidiospore development pattern (Yoo et al. 2000).

Somatic hybrids and their progenies in fungi have been characterized by various methods, e.g., colony morphology and genetic character segregation (Kevei and Peberdy 1984; Yonagui et al. 1988; Pan et al. 1991; Sonnenberg et al. 1991; Pan et al. 1992; Sumagawa and Miura 1992; Wang et al. 1992), fruit body morphology and productivity (Li and Chang 1989; Ohnma 1986), isozyme polymorphism (Anne and Peberdy 1991; Kim et al. 1997, 2000), and DNA polymorphism (Witte et al. 1989; Chakraborty and Sikdar 2008, 2010).

Pleurotus floridus is a common oyster mushroom in India, having good nutritive values and can be commercially cultivated in the sub-tropical climate (temperature range 22–26 °C). Lentinula edodes, the shiitake mushroom requires low temperature (10–18 °C) and specific substrate (Oak log) for fruiting. But, in the sub-tropical climate of West Bengal L. edodes do not grow. The aim of this study was to develop hybrid strains between L. edodes and P. floridus and to see whether the hybrid could grow easily in sub-tropical climate of West Bengal and having some medicinal properties of L. edodes so that it could serve dual purpose as food and as nutraceuticals.

Materials and methods

Strains and culture condition

The mother culture of two parental strains viz., P. floridus and L. edodes were obtained from National Research Centre for Mushroom, Solan, Himachal Pradesh, India. Routine maintenance of both the strains was done in potato dextrose agar (PDA, pH 6.2) medium. For protoplast preparation mycelial tissue was grown in liquid Malt-yeast extract-glucose (MYG) (10 g/l malt extract, 4 g/l yeast extract and 10 g/l glucose, pH 6.2) medium for 4–5 days and 10–12 days for P. floridus and L. edodes respectively at 24 ± 1 °C.

Protoplast preparation

From liquid MYG culture, mycelial mat of both the strains was collected separately, washed thrice with sterile double distilled water and twice with osmotic stabilizer solution (0.6 M mannitol with 0.2 % CaCl₂, 2H₂O), chopped into pieces and subjected to enzymatic digestion for protoplast liberation. Different combinations of Lysing enzyme (Sigma-Aldrich) and Cellulase Onozuka R-10 (Yakult) prepared in 0.6 M mannitol with 0.2 % CaCl₂, 2H₂O (pH-5.5) were tested. To obtain healthy and good protoplasts, mycelial tissues of both the strains were digested in enzyme mixture at 24 °C, for 12 h for P. floridus and 18 h for L. edodes with shaking at 70 rpm. For purification, liberated protoplasts were sieved through cotton wool pad and centrifuged at 3,000 rpm for 5 min to collect the pellet. The pelleted protoplasts were washed thrice with osmotic stabilizer solution by suspending and pelleting through centrifugation. Finally, purified protoplast pellet was suspended in 200 μl osmotic stabilizer solution for further use.

Total yield was calculated using a hemocytometer. Viability (%) was tested using FDA staining (Widholm 1972).

Strategy for hybrid selection

Pleurotus floridus is known to be a salt tolerant strain (Chakraborty and Sikdar 2010). To test the salt tolerance level of L. edodes, protoplasts of L. edodes were grown in different concentrations of NaCl (0.1–1.0 M) containing MYG medium with a view to use this character for hybrid selection. It was found that L. edodes protoplasts could not grow beyond 0.2 M NaCl concentration. So the high salt tolerance (0.7 M NaCl) property of P. floridus and low salt tolerance property of L. edodes (0.2 M NaCl) were used for hybrid selection between P. floridus and L. edodes. Parental protoplasts of P. floridus were inactivated (cytoplasmic gene function) using 10 mM iodoacetamide (IOA) in the dark condition for 5 min before protoplast fusion with normal L. edodes protoplasts. The fused protoplasts were plated in a medium containing 0.7 M NaCl. In high NaCl containing medium neither the L. edodes protoplasts (because of its salt sensitivity) nor P. floridus protoplasts (because of inactivation of its cytoplasmic gene function) will grow. However, hybrid protoplasts could grow due to complementation of parental genome or nucleo-cytoplasmic interaction.

Protoplast fusion and culture

Protoplast fusion was carried out according to Anne and Peberdy (1976) with some modifications. Equal volume (50 μl each) of IOA treated P. floridus and normal L. edodes protoplasts were taken in a centrifuge tube at a density of 10⁶–10⁷ protoplast/ml, mixed the suspension well and kept at 24 °C for 5 min. Equal volume of 30 % PEG solution (30 % PEG MW-3350. 0.05 M glycine-NaOH, 50 mM CaCl₂, 2H₂O, pH-7.5) was added and kept at the same
temperature for 5 min and observed protoplast fusion. The PEG treated protoplasts were then diluted 6 times with osmotically balanced MYGNa liquid culture medium (MYG + 0.7 M NaCl, pH 6.2). Aliquot of 100 μl was plated with 1 ml of the same culture medium containing 1.5 % agar. Normal viable protoplasts of P. floriae (positive control), L. edodes and TOA-treated P. floriae (negative control) protoplasts were also plated on the same medium. Normal viable protoplasts of L. edodes were plated on MYG + 0.6 M mannitol, pH 6.2 as positive control. All the fusion and control plates were maintained at 24 ± 1 °C, till micro-colony formation. The presumptive somatic hybrid colonies were transferred to PDA medium for further growth after macro-colony formation. The hybrid colonies were tested for spawn and fruit body production as per standard protocol used for oyster mushroom cultivation. To test the growth of the putative nine hybrid lines on slide, mycelium of all the hybrid strains along with the parents were placed on a drop of liquid potato-dextrose medium separately on individual slide in aseptic condition. The slides were kept in the moist condition within petri dishes and maintained at 24 ± 1 °C temperature.

Spawn and fruit-body production

The hybrid colonies those could be maintained on PDA medium were used finally for spawn preparation on paddy grains. Spawn packets were prepared using autoclaved grains of 200 g each. The matured spawn was finally used for fruit body production on overnight soaked and then autoclaved paddy straw substrate (100 g spawn/0.65 kg dry straw) taken in a cylindrical polypropylene packet. After spawning, when the mycelia showed complete colonization in the substrate, several pores were made all over the surface of the polypropylene packet (about 2 cm apart). The bags were placed in the mushroom cultivation room at 24 ± 1 °C. After pin head emergence through the pores on the polypropylene packets, high humidity was maintained by misting the room time to time.

Barrage reaction

The selected individual hybrid lines and individual parental lines were inoculated on the same plate on PDA medium and maintained at 24 ± 1 °C for 5 days after which the point of contact zone was observed for barrage reaction.

Extraction of genomic DNA and PCR conditions

Genomic DNA was isolated from young fruit bodies/actively growing mycelia using the modified CTAB method (Dellaporta et al. 1983). PCR reactions for inter single sequence repeat (ISSR) and L. edodes genome specific ISSR were conducted with slight modification of Borret and Branchard (2003) in 25 μl reaction mixture containing 1X Taq buffer with KCl salt (Fermentas), 75 ng template DNA, 2 mM dNTP mixture (four nucleotide triphosphate, Fermentas), 25 mM MgCl2, 20 μM ISSR primer, and 5U/μl Taq DNA polymerase enzyme (Fermentas). The ISSR amplification condition was: 5 min initial denaturation at 94 °C; 40 cycles consisting of 1 min denaturation at 94 °C, 1 min primer annealing ranges from 37 to 61 °C and 3 min extension at 72 °C and a final extension for 10 min at 72 °C.

Gel analysis

Amplified ISSR and L. edodes genome-specific ISSR fragments were separated on 1.8 % agarose gel pre-stained with ethidium bromide solution using 1X TAE buffer. The gels were run at 80 V for 3 h for ISSR and 2 h for L. edodes genome-specific ISSR products. Separated band profiles were visualized under UV Transilluminator. All the fingerprint profiles were recorded with Molecular Analyst Gel Documentation System. The size of the amplified fingerprints were determined by using DNA Ruler Plus (100-3,000 bp) ladder (MBI, Fermentas) as a standard molecular weight marker.

Statistical analysis

Morphological variations among the hybrids were analyzed by one-way analysis of variance (ANOVA) and the Tukey Post hoc test for multiple comparisons of means, standard deviation and level of significance (Zar 1998). A bivariate correlation was made among the hybrids with their parents using IBM SPSS software, version 19. Principal component analysis (PCA) was used for multivariate phenotypic data analysis. Through PCA the component matrix was extracted and data were plotted in Microsoft Excel sheet. Scatter diagram was constructed of component matrix for phenotypic traits. From DNA profiling, ISSR bands were scored as present (1) or absent (0) for each primer genotypic combinations. Data generated from 6 ISSR primers for each hybrid strain were entered into a binomial matrix. The bivariate 0-1 data were analyzed using IBM SPSS software, version 19. Jaccard’s proximity matrix (Jaccard 1901) was generated through SPSS and a dendrogram of hybrids and their parents was constructed employing the unweighted pair group method with mathematic average (UPGMA) algorithm.

Polymorphic information content (PIC) value

Polymorphic information content value is used to refer to the relative value of each marker with respect to the
amount of polymorphism exhibited. It was estimated according to Weir (1996), using the formula: 
PIC = [1 - (Σ Pi2)], where ‘i’ is used as the total number of alleles detected from ISSR marker, and ‘Pi’ is the frequency of the ith plus allele in the set of the pflc genotype.

Results and discussion

Protoplast isolation, protoplast inactivation and generation of hybrid lines

A combination of cell wall degrading enzymes of 2 % Cellulase Onozuka R-10 and 2 % Lysing enzyme in 0.6 M mannitol + 0.2 % CaCl2, 2H2O (pH-5.5) successfully produced protoplasts in both P. florid and L. edodes (1.5 x 102 protoplast/g tissue) and L. edodes (1.6 x 102 protoplast/g tissue) with 14 and 18 h incubation, respectively. Viability of the protoplasts as observed after FDA staining was found to be 95.45 ± 3.25 % in P. florid and 68.15 ± 2.95 % in L. edodes (Fig. 1a). When freshly isolated protoplasts of both the strains were plated in regeneration medium (MYG + 0.6 M mannitol, pH 6.2) regeneration percentage of P. florid protoplast was found to be 33.45 ± 9.02 while in case of L. edodes it was 18.50 ± 11.30. However, the plating efficiency (%) was scored as 29.15 ± 3.40 and 14.12 ± 2.90 % for P. florid and L. edodes, respectively.

Protoplasts of L. edodes could produce colonies in MYG medium containing a maximum of 0.2 M NaCl concentration and no regeneration was observed in the same medium when NaCl concentration was increased further. While those of P. florid could tolerate a concentration up to 0.7 M NaCl (Chakraborty and Sikdar 2010). Protoplasts regeneration was found in the fusion medium after 10-12 h of PEG treatment (Fig. 1b). So for hybrid selection, the MYG supplemented with 0.7 M NaCl (MYGNa) medium

Fig. 1. Production of hybrid strains from fused protoplasts between Pleurotus florid and Lentinula edodes and barrage reaction between a hybrid strain and L. edodes parent. a FDA stained viable protoplasts of L. edodes. b Fused protoplasts (marked by arrows) after PEG treatment. c The culture of L. edodes along with a hybrid line pflc 1a on the same plate showing thick, brownish barrage at the zone of contact. d pflc 6c; hybrid culture showing yellowish colour at mature stage on PDA medium. e Vegetative cultures of six fruit-body producing strains with L. edodes and P. florid parents. Size bar corresponds to 80 μm in a, 40 μm in b, 40 μm in c, 15 mm in d, and 3 cm in e. (Color figure online)
Appendix

was used where only fused hybrid colonies were developed. Not a single protocline were developed from any of the parental protoplasts in this selection medium. *P. floridens* protoplasts could not regenerate as these protoplasts were inactivated with 10A and *L. edodes* protoplasts also could not regenerate because this strain did not grow in high NaCl (0.7 M) containing medium. Only hybrid protoplasts could regenerate in MYGNA medium due to complementation of parental genome. A total of 58 putative hybrid colonies were regenerated from three fusion experiments involving 3 x 10^7 protoplasts (plating efficiency 0.000193%). Micro-colonies were scored in three spells i.e., after 6, 15 and 23 days from the fusion plates. No regeneration from the negative control plates and normal regeneration in the positive control plates of parental protoplasts strongly support the success of double selection strategy. Micro-colonies of 58 putative hybrid lines were transferred to PDA medium and maintained at 24 ± 1°C for their further growth. Only 9 hybrid lines (pfe 1o, pfe 1p, 1q, 1r, 1s, 1v, 6s, 6w and 6z) could be maintained in PDA medium routinely for further characterization.

Assessment of morphological traits

Based on the mycelial growth and cell width on slide culture (4 days old), and colony morphology and diameter on PDA medium (7 days old) all the variables were tabulated in Table 1. Barrage reaction between parent *L. edodes* and hybrid *pfe 1s* showed thick, brownish barrage at their contact zone on PDA medium (Fig. 1c). Growth of the hybrid lines on slide culture ranged from 7.04 ± 0.12 cm (pfe 1p) to 5.24 ± 0.26 cm (pfe 6w) compared to 5.55 ± 0.08 cm in *P. floridens* and 4.24 ± 0.26 cm in *L. edodes*. Among nine hybrid strains, only six (pfe 1o, 1p, 1q, 1r, 1s, 1v) were very close to *P. floridens*, one (pfe 6s) was intermediate and rest two (pfe 6w, 6z) showed completely different type of mycelial nature on morphological observations. At the initial stage the mycelial colour of *pfe 6w* and *pfe 6z* hybrid lines remained white (12 days old) that turned yellowish at fully mature stage (18 days old) (Fig. 1d) on both PDA and MYG medium. Colony diameter of the hybrid lines (7 days old) ranged between 4.82 ± 0.04 cm (pfe 6w) to 2.21 ± 0.02 cm (pfe 1p) compared to 3.82 ± 0.11 cm in *P. floridens* and 4.24 ± 0.26 cm in *L. edodes* (Table 1). The variation of growth in slide culture and PDA medium may be due to different cultural conditions maintained during culture. Strengthen condition maintained in slide culture would probably responsible for faster growth of mycelium on slide compared to PDA medium. Among the hybrid lines, clamp was found in pfe 1v and pfe 1r only that is the characteristic feature of *P. floridens*. Hyphal cell width also varied among the hybrid lines, it was minimum in *pfe 1q* (14.47 ± 2.19 μm) and maximum in *pfe 6s* (50.52 ± 1.03 μm). Interestingly, phenotypic data showed that *pfe 6c* and *pfe 6w* were very close to each other in many respect from mycelial nature, colour and cell-width (35.50 ± 0.89 and 32.45 ± 0.52 μm, respectively).

Production of spawn and fruit body

Time requirement for spawn maturation on paddy grain varied from hybrid to hybrid. Hybrid *pfe 1o*, *pfe 1q*, and *pfe 1r* required 10 days like that of *P. floridens* parent, *pfe 1v* and *pfe 1s* required 14 days and *pfe 1p* required 18 days. Hybrid *pfe 6w* and *pfe 6z* required 2 months for spawn maturation and the quality of spawn was not good and *pfe 6s* could not produce any spawn at all on paddy grain, however, the other parent *L. edodes* needed 22 days for maturation of spawn. As paddy straw substrate was used for fruit body production, *P. floridens* could produce fruit body in it, whereas *L. edodes* could not. This may be due to substrate specificity and very low temperature requirement for cultivation of *L. edodes* that was not met in this particular experiment. Six hybrid lines *pfe 1o*, *pfe 1v*, *pfe 1q*, *pfe 1r*, *pfe 1s* and *pfe 1v* (Fig. 1c) could also produce basidiocarp on the paddy straw substrate. The fruit bodies of these hybrids are shown in Fig. 2a, b, c and d. Primordial initiation required different time for different hybrid lines. Hybrid *pfe 1p* and *pfe 1o* required 30–32 days (starting from bed preparation) for primordial initiation, where parent *P. floridens* required 19–22 days. Oyster-shaped fruit bodies generated in the first flush of hybrid *pfe 1p* are shown in Fig. 2e. Maximum stipe length (6.87 ± 0.14 cm) was found in *pfe 1v* with white, oyster shaped pelus (3.99 ± 0.02 cm diameter) and minimum stipe length was found in *pfe 1r* (2.24 ± 0.12 cm) with small, lobed, centrally stipitate, oyster shaped pelus (3.29 ± 0.08 cm diameter). All the fruit body producing lines showed *P. floridens* type fruit bodies. Fruit body producing hybrid line *pfe 1s* showed maximum crop yield (698.15 ± 36.55 g with 107.4 % bioefficiency) among all hybrids (Table 2). While yield of *P. floridens* was 715.5 ± 40.31 g with 110.23 % bioefficiency. Minimum crop yield was found in *pfe 1o* hybrid with 44.22 % bioefficiency. In *pfe 6w* and *pfe 6z* after spawn run the beds turned yellow and no fruit body was produced even after 5 months of observation (Fig. 2f). *P. floridens* type fruit body of the six hybrid hybrid lines may be either due to the dominance nature of *P. floridens* genome over *L. edodes* genome in expressing basidiocarp phenotype or due to elimination of the *L. edodes* chromosomes responsible for expression of basidiocarp phenotype in the hybrid environment.
two strains might be due to retention of more L. edodes chromosomes that hinders development of basidiocarp in non-specific substrate like paddy straw. The inability of pfe 1s to grow on rice grain (for production of spawn) might be due to some defect occurred at the gene level for the synthesis of enzymes responsible for digestion of lignocellulosic substrate. The basidiocarp formation in four (pfe 1p, 1q, 1s, 1o) out of six pfe hybrid lines without mating became possible may be due to triggering of an FRTI type gene (Horton and Raper 1991). The control of fruiting competence by genes other than the mating type genes may be of general occurrence in basidiomycetes, but this has not been extensively investigated.

**Barrage reaction**

Barrage formation was noticed in all the sexual mating experiments involving P. florid a × pfe hybrids and L. edodes × pfe hybrids. This indicates that all the strains generated through protoplast fusion are genetically different from the parental strains, so they are hybrid strains.

---

**Table 1** Morphological features of mycelia of the pfe hybrids and their parents

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture maintenance temp (°C)</th>
<th>Growth after 4 days in cm³ (F = 9.25)</th>
<th>Colony diameter after 7 days in cm² (F = 31.87)</th>
<th>Cell width in μm² (F = 48.31)</th>
<th>Clamp connection</th>
<th>Colony Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. florid a</td>
<td>24</td>
<td>5.55 ± 0.08</td>
<td>3.82 ± 0.11</td>
<td>25.37 ± 1.05</td>
<td>Present</td>
<td>White fluffy growth radiated from the centre</td>
</tr>
<tr>
<td>pfe 1s</td>
<td>24</td>
<td>5.24 ± 0.26</td>
<td>4.82 ± 0.04</td>
<td>32.45 ± 0.52</td>
<td>Absent</td>
<td>Yellowish-brown, thin hyphal growth</td>
</tr>
<tr>
<td>pfe 1p</td>
<td>24</td>
<td>6.84 ± 0.27</td>
<td>2.21 ± 0.02</td>
<td>20.45 ± 1.14</td>
<td>Absent</td>
<td>White fluffy, radiated from the centre</td>
</tr>
<tr>
<td>pfe 1q</td>
<td>24</td>
<td>6.34 ± 0.10</td>
<td>3.21 ± 0.20</td>
<td>14.47 ± 2.19</td>
<td>Absent</td>
<td>Discrete colonies, white spike-like mycelial growth</td>
</tr>
<tr>
<td>pfe 1v</td>
<td>24</td>
<td>7.04 ± 0.12</td>
<td>2.65 ± 0.07</td>
<td>13.10 ± 0.98</td>
<td>Present</td>
<td>White fast growing mycelia</td>
</tr>
<tr>
<td>pfe 1r</td>
<td>24</td>
<td>5.81 ± 0.29</td>
<td>2.25 ± 0.06</td>
<td>18.72 ± 1.67</td>
<td>Absent</td>
<td>Scattered white mycelial growth</td>
</tr>
<tr>
<td>pfe 6s</td>
<td>24</td>
<td>5.83 ± 0.20</td>
<td>3.04 ± 0.07</td>
<td>50.52 ± 1.05</td>
<td>Absent</td>
<td>White fluffy thread-like mycelia</td>
</tr>
<tr>
<td>pfe 6z</td>
<td>24</td>
<td>6.31 ± 0.09</td>
<td>4.25 ± 0.06</td>
<td>35.50 ± 0.89</td>
<td>Absent</td>
<td>Yellowish-brown, thin hyphal growth radiated from the centre</td>
</tr>
<tr>
<td>pfe 1o</td>
<td>24</td>
<td>5.44 ± 0.10</td>
<td>3.60 ± 0.02</td>
<td>18.35 ± 1.06</td>
<td>Absent</td>
<td>White straight, continuous mycelia with appressed colonies</td>
</tr>
<tr>
<td>pfe 1t</td>
<td>24</td>
<td>6.40 ± 0.45</td>
<td>2.65 ± 0.06</td>
<td>21.12 ± 0.88</td>
<td>Present</td>
<td>White fluffy, dense mycelia radiated from the centre</td>
</tr>
<tr>
<td>L. edodes</td>
<td>24</td>
<td>4.24 ± 0.26</td>
<td>1.86 ± 0.14</td>
<td>28.15 ± 0.47</td>
<td>Absent</td>
<td>Off-white at advance stage, leathery, very slow growing mycelia</td>
</tr>
</tbody>
</table>

The values of hyphal growth, colony diameter and cell width are mean ± SD of five repeated experiments. Results of one-way ANOVA for each parameter are represented as F values, P < 0.05 (considering the F value 3.48 for this experiment, the tabulated are greater). The difference between any two mean values (of any two different strains) of same parameter (i.e. within a column) is to be considered as significant at 5 % level if the said difference between the means exceeds the calculated CD (critical difference) values provided at bottom of the same column. *a* a Mean ± SD, data taken from five slide culture, *b* Mean ± SD, data taken from five PDA slab culture.

d Some mutant strain bears clamp connection.
Appendix

Fig. 2 Morphological characteristics of the fruit bodies generated from different somatic hybrid strains between L. edodes and P. florid. a Fruit bodies from colonized bed of hybrid strain pffe 10 (left), pffe 1r (middle) and pffe 1q (right). b Fruit bodies derived from hybrid strain pffe 1v. c Bunch of oyster shaped and brownish basidiocarp developed from pffe 1p hybrid strain. d White fruit bodies generated from hybrid strain pffe 1s. e Fruit bodies of the first flush of pffe 1p hybrid strain. f Three months old colonized bed of pffe 6c hybrid showing yellowish colour. Size bar corresponds to 40 mm in a, 20 mm in b, c, d and e, 15 mm in f. (Color figure online)

Table 2 Comparison of sporophore characters of the pffe somatic hybrid lines

<table>
<thead>
<tr>
<th>Strain</th>
<th>Spawn production time (d)a</th>
<th>Primordial initiation time (d)a</th>
<th>Stipe length in cmb (F = 67.12)</th>
<th>Pellic diameter in cmb (F = 68.25)</th>
<th>Wt. of 3 flushes in gmc (F = 82.13)</th>
<th>BE (%c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. florid.</td>
<td>10</td>
<td>15–20</td>
<td>6.38 ± 0.12</td>
<td>4.08 ± 0.31</td>
<td>716.5 ± 40.31</td>
<td>110.23</td>
</tr>
<tr>
<td>pffe 1p</td>
<td>18</td>
<td>30-32</td>
<td>2.56 ± 0.25</td>
<td>4.04 ± 0.11</td>
<td>426.25 ± 90.15</td>
<td>65.57</td>
</tr>
<tr>
<td>pffe 1q</td>
<td>10</td>
<td>20-22</td>
<td>3.08 ± 0.11</td>
<td>5.81 ± 0.04</td>
<td>428.05 ± 19.73</td>
<td>65.85</td>
</tr>
<tr>
<td>pffe 1v</td>
<td>14</td>
<td>28-30</td>
<td>6.87 ± 0.14</td>
<td>3.99 ± 0.02</td>
<td>698.0 ± 23.34</td>
<td>107.38</td>
</tr>
<tr>
<td>pffe 1s</td>
<td>14</td>
<td>25-27</td>
<td>2.97 ± 0.99</td>
<td>4.43 ± 0.07</td>
<td>698.15 ± 36.55</td>
<td>107.4</td>
</tr>
<tr>
<td>pffe 1o</td>
<td>10</td>
<td>30-34</td>
<td>3.09 ± 0.07</td>
<td>4.30 ± 0.04</td>
<td>287.45 ± 60.18</td>
<td>44.22</td>
</tr>
<tr>
<td>pffe 1r</td>
<td>10</td>
<td>28-30</td>
<td>2.24 ± 0.12</td>
<td>3.29 ± 0.08</td>
<td>361.75 ± 26.52</td>
<td>55.65</td>
</tr>
<tr>
<td>SE (Mean)</td>
<td>–</td>
<td>–</td>
<td>0.71</td>
<td>0.30</td>
<td>49.95(6.03)</td>
<td>–</td>
</tr>
<tr>
<td>p value</td>
<td>–</td>
<td>–</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>–</td>
</tr>
</tbody>
</table>

The values of stipe length, pellic diameter and yield are mean ± SD of five repeated experiments. Results of one-way ANOVA for each variable are shown as F values, P < 0.05 (considering the F value 3.48 for this experiment, the tabulated are greater)

a Data taken at 24 ± 2°C temperature
b Mean ± SD, data taken from ten observations
c Mean ± SD, data taken from five bags
d BE = Biological efficiency
Molecular genetic analysis

ISSR profile

Among the fifteen ISSR primers tested, six were chosen for their clear and reproducible banding patterns. Details including ISSR repeat sequences, number of amplified fragments with sizes and PIC values of these six ISSR primers are shown in Table 3. All the chosen six primers amplified a total of 66 fragments with 98.48% polymorphism across the eleven strains (nine hybrids and two parents) studied, with an average of 11 bands per primer. The amplified fragments were ranged from 250 bp to 3,000 bp in size. Variation of band number occurred in each ISSR primer due to dissimilar genome sequences against primer repeat sequence in individual hybrid and parental strains. ISSR-01 and ISSR-11 primers generated DNA profiles are depicted in Fig. 3. Among all the six primers tested, ISSR-11 [(CAC)_2G] produced highest scorable bands, i.e., 13 (300-3,000 bp) followed by ISSR-12 [(GAG)_2G] 12 bands (300-2,500 bp). Minimum amplification was found in primer ISSR-01 which produced only 9 bands (550-3,000 bp). Based on this ISSR PCR-amplification, the polymorphism information content values were calculated and it showed differences for each ISSR primer. The recorded mean PIC value was 0.485 for six primers when the lowest was found 0.466 in ISSR-02 and the highest was found 0.499 in ISSR-15.

The DNA banding profiles generated by six ISSR primers revealed that the banding profile of neither of the somatic hybrids represent the sum total of the parental banding profile nor they resemble each other among the hybrid strains; this strongly proved that all the hybrid strains were not heterokaryons of the parental nuclei rather after protoplast fusion synkaryon was formed that was followed by chromosome elimination and somatic recombination (Primrose 1987, Chakraborty and Sikdar 2008, 2010).

Jaccard’s proximity matrix was generated based on the amplified DNA fragments of the pfe hybrids and parents. Genetic distances between hybrid to hybrid, hybrid to parent and parent to parent were tabulated in this matrix (Supplementary Table 1). Within this population, the dissimilarity coefficient was varied and it ranged from 1.00 to 6.481. Based on the Jaccard’s proximity matrix data it appeared that parent L. edodes and hybrid pfe 6w were genetically most distantly related (6.481) among the population studied, while the most closely related hybrid lines were pfe 1r and pfe 1o (1.00). The variation of genetic distances explained that parent L. edodes genome is highly dissimilar to hybrid pfe 6w and similar to hybrid pfe 1s within hybrid population. Another parental strain P. floridza showed highest dissimilarity with hybrid pfe 6c and similarity with hybrid pfe 1r. Based on the dendrogram (Supplementary Fig. 1) study three individual clusters were found, in which P. floridza, pfe 1o and pfe 1r formed one group; pfe 1p, pfe 1s and pfe 1q formed second group, and pfe 6w, pfe 6c and pfe 1v formed the third group. L. edodes showed maximum genetic distance with all hybrid lines than the other parental strain P. floridza.

![Fig. 3 ISSR profiles of the parents and their hybrid lines. a) Primer ISSR-11 and b) Primer ISSR-01. Lane M-marker, 100 bp DNA ruler plus, Lane 1 P. floridza, Lane 2 pfe 6w, Lane 3 pfe 1p, Lane 4 pfe 1q, Lane 5 pfe 1v, Lane 6 pfe 1s, Lane 7 pfe 6s, Lane 8 pfe 6c, Lane 9 pfe 1o, Lane 10 pfe 1r and Lane 11 L. edodes; left arrows depict bands from P. floridza and right arrows from L. edodes parent.](image-url)
L. edodes sequence-rich ISSR marker profile

Lentinula edodes specific sequence-rich ISSR ([aagag]$_n$) marker was used to examine the genome contribution of L. edodes to the hybrid lines. This ISSR amplification was used earlier by Tan et al. (2006) to develop strain-specific sequence characterized amplified region (SCAR) markers for Lentinula edodes. This profile generated polymorphic bands in all the hybrid lines (all phenotypically looked like P. floridensis parent) but no amplification was found in P. floridensis (Fig. 4). Minimum and maximum number of bands was generated in the hybrid line pfle 1p and pfle 1v respectively. This indicates that less percentage of L. edodes genome was contributed in pfle 1p and more in pfle 1v. The dendrogram data generated based on other ISSR markers also partly support these observations i.e., pfle 1v is closer to L. edodes compared to pfle 1p. Moreover, the polymorphic banding profile generated by using L. edodes specific sequence-rich ISSR marker in all the P. floridensis like pfle hybrid line strongly support that all are hybrid between P. floridensis and L. edodes.

Principal component analysis of pfle hybrid lines

Variations associated with phenotypic traits of pfle hybrid lines were analyzed. Dimension reduction factors helped to extract the principal component matrices using SPSS software. Six essential phenotypic characters such as, mycelial growth on slide culture, colony diameter, cell width, yield in first three flushes, stipe length and pelvis diameter of pfle hybrid lines were used to check the dimension of each trait between each variable through graphical presentation. Two component matrices were extracted and plotted on Microsoft Excel sheet for two dimensional scatter plot preparation. Data of principal component 1 (PC1) represented on X-axis and principal component 2 (PC2) represented on Y-axis. Variable factors were associated with each dimension in scatter plot (Fig. 5). In pfle hybrids, colony diameter and stipe length scattered points indicate that they are greater influential variables than other and lesser influential variables are growth rate and pelvis diameter. The dimension reduction was higher in colony diameter, cell width and stipe length and lower in growth rate and pelvis diameter. Growth on slide culture of all pfle hybrid lines showed a negative value ($-0.017$) in component matrix one but in component matrix two it showed the reduced dimension for the variables yield, cell width and pelvis diameter. Thus all the six phenotypic characters of pfle hybrids graphically expressed their dimensions between each particular character to others. Correlation of all phenotypic variables among the hybrids and parents is shown in Table 4. Growth on slide culture of all pfle hybrid lines showed a negative correlation with other variables, except stipe length ($-0.285$). Colony diameter of all pfle hybrid lines were also negatively correlated ($-0.108$) with pelvis diameter. Rest data showed significant positive correlation between each variable. As per PCA analysis, the most influential variables were found between colony diameter and stipe length so these two parameters could be considered for selection of successful hybrid lines at the initial stage of growth.

Present study reported a case of inter-generic somatic hybridization between two heterothallic genera, P. floridensis and L. edodes. In the past two somatic hybridization reports in edible mushroom from this laboratory showed that phenotypic character of only one parent is expressed in the hybrid strains i.e., Pleurotus phenotype was expressed in the somatic hybrids between P. floridensis + V. volvacea (Chakraborty and Sikdar 2008) and Cloocybe phenotype.

---

**Fig. 4** Lentinula edodes genome-rich ISSR DNA profile of pfle hybrid lines and the parents. Lane 1 (extreme left) Marker (100 bp DNA ruler plus), Lane 2 Control, Lane 3 Pleurotus floridensis showing no amplification, Lane 12 (extreme right) Lentinula edodes showing amplification. Lane 4 to Lane 12 pfle hybrid lines showing variable amplification with this specific primer.

**Fig. 5** Scatter plot of principal component matrices for six phenotypic variables of pfle hybrid lines. The geometric steps for finding the main directional component of principal component analysis indicating by the combination of each variable PC1 data on X-axis (horizontal) and PC2 data on Y-axis (vertical). Out of six, two variables are in first quadrant, one in second and rest three are in fourth quadrant, indicating different influential concept between each other.
Table 4 Correlations using principle component matrices between six variable phenotypes of *pfe* hybrids (SPSS version.19, bivariate correlation)

<table>
<thead>
<tr>
<th></th>
<th>Growth Colony</th>
<th>Cell width</th>
<th>Spite</th>
<th>Peliss diameter</th>
<th>Yield of three flushes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlations</td>
<td>Growth</td>
<td>Colony diameter</td>
<td>Cell width</td>
<td>Spite</td>
<td>Peliss diameter</td>
</tr>
<tr>
<td>Growth</td>
<td>1</td>
<td>0.073</td>
<td>-0.307</td>
<td>0.310</td>
<td>0.285</td>
</tr>
</tbody>
</table>

was expressed in the somatic hybrids between *P. florida* + *C. indica* (Chakraborty and Sikdar 2010). In the present report also only *Pleurotus* character was expressed in the *pfe* hybrid strains. Though the *pfe* hybrids looked like *P. florida* but the barricade reaction, recombinant banding pattern of ISSR strongly proved their hybrid nature. Structural investigation of polysaccharides of *pfe* 1r (Maji et al. 2012, 2013) and *pfe* 1q (Maiti et al. 2013) showed that they were different from the polysaccharides isolated from fruit bodies of parental strains *P. florida* and *L. edodes*; these also proved their hybridity. So through the present endeavour hybrid edible mushrooms could be produced between *P. florida* and *L. edodes* those could grow on paddy straw in sub-tropical climate.

Acknowledgements We acknowledge fellowship support to the first author from Bose Institute, Kolkata. We also thank to Dr. Sonnath Bhattacharya, Reader, Bidhan Chandra Krishi Viswa Vidyalya, Mohanpur, Nadia, West Bengal for his help in statistical analysis.

References

Anne J, Peberdy JF (1976) Induced fusion of fungal protoplasts following treatment with polyethylene glycol. J Gen Microbiol 92:413-417


Chakraborty U, Sikdar SR (2010) Intergeneric protoplast fusion between *Calosybe indica* (milkly mushroom) and *Pleurotus floridus* aids in the qualitative and quantitative improvement of sporophore of the milky mushroom. World J Microbiol Biotechnol 26:213-225


Appendix

Genetics and breeding of Agaricus. Pudoc, Wageningen, pp 57–61


A heteropolysaccharide from an edible hybrid mushroom *pfla 1p*: structural and immunostimulating studies


Department of Chemistry & Technology, Udaipur University, Malda 732102, West Bengal, India
Department of Biotechnology, Indian Institute of Technology (Indian School of Mines), Ranchi 835215, Jharkhand, India
Division of Plant Biology, Bose Institute, Kolkata 700004, West Bengal, India

ARTICLE INFO

Article history:
Received 21 February 2013
Received in revised form 3 April 2013
Accepted 7 April 2013
Available online 13 April 2013

Keywords:
Hybrid mushroom
Heteropolysaccharide
NMR experiments
Immunostimulating activity

ABSTRACT

A water-soluble heteropolysaccharide (PS-I) having molecular weight $2.1 \times 10^2$ Da was isolated from hot aqueous extract of the fruit bodies of hybrid mushroom *pfla 1p*. The hybrid mushroom *pfla 1p* was obtained through intergenic protoplast fusion between *Pleurotus florida* and *Lentinula edodes*. The heteropolysaccharide contained $\beta$-glucose, $\alpha$-galactose, and $\alpha$-mannose in a molar ratio of nearly 4:2:1. The structural investigation of PS-I has been carried out using sugar and methylation analyses as well as 1D/2D NMR experiments ($^1$H, $^13$C, DEPT-135, DQF-COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC). Based on the results of these experiments, the repeating unit of the PS-I was established as:

\[
\begin{align*}
\beta\text{-d-glucopyranosyl(1\rightarrow2)}\beta\text{-d-glucopyranosyl(1\rightarrow2)}\alpha\text{-d-galactopyranosyl(1\rightarrow2)}\alpha\text{-d-galactopyranosyl(1\rightarrow3)}\alpha\text{-d-mannopyranosyl(1\rightarrow4)}\beta\text{-d-glucopyranosyl(1\rightarrow2)}
\end{align*}
\]

PS-I showed in vitro macrophage activation by NO production and also stimulated splenocytes and thymocytes.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Mushrooms are enriched with various bioactive molecules. Among them, $\beta$-glucans have been extensively studied and considered as the most potent antitumor and immunostimulating materials.

Lentinan, a $\alpha$-(1\rightarrow3)-[1\rightarrow6]-$\beta$-glucan isolated from *Lentinula edodes* is well known for its high antitumor activity. Different types of glucans from *Pleurotus florida* also exhibit significant immunostimulating properties. In addition to the antitumor $\beta$-glucans, researchers have focused on heteroglycan which also has significant biological activities.

The structure of different heteroglycans may contain backbones of $\alpha$-(1\rightarrow6)- and $\alpha$-(1\rightarrow3)- or both $\alpha$-(1\rightarrow6)-[1\rightarrow3]-linked $\alpha$-galactose, $\alpha$-glucose, or $\beta$-glucose with mainly fucose, mannnose or glucose as nonreducing residues. Recently, Dey et al. and Patra et al. have isolated different immunostimulating heteroglycans from edible mushroom and hybrid mushroom. Protoplast fusion between the strains of *Pleurotus florida* and *Lentinula edodes* produced nine new hybrid strains adopting the procedure as applied earlier out of which six strains, that is, *pfla 1e*, *pfla 1p*, *pfla 1q*, *pfla 1r*, *pfla 1s*, and *pfla 1v* produced fruit bodies. Recently, a $(1\rightarrow3)-, (1\rightarrow6)-\beta$-glucan, and a heteroglycan have been isolated from one such hybrid strain *pfla 1r*. Two water soluble polysaccharides (PS-I and PS-II) were isolated from the fruit bodies of *pfla 1p*. PS-I was found to consist of $\alpha$-glucose, $\alpha$-galactose, and $\alpha$-mannose in a molar ratio of nearly...
Appendix

1. Introduction

Mushrooms which are used as foodstuff for their taste and nutritional value from ancient times, also exhibit important medicinal properties. During the last few decades polysaccharides isolated from different mushrooms have become an interesting topic for the researcher in the field of biochemistry and pharmacology for wide application of their anti-tumor, immunostimulating, and antioxidant properties. A series of polysaccharides like (1→6)-α-D-glucan, (1→3)-, (1→6)-α-1,6-glucan, heteroglycan consisting of glucose, mannose and galactose and (1→3),(1→6)-β-D-glucan have been isolated from water soluble, insoluble and NaCl soluble fractions of an edible mushroom Pleurotus florida and reported earlier. Immunostimulating heteroglycans consisting of galactose, glucose and fucose and (1→3), (1→6)-β-D-glucan were also reported from the aqueous and alkaline extracts of another edible mushroom Lentinus squarrosulus (Mongt.) Singer, respectively. Twelve new hybrid strains were developed through intergeneric protoplast fusion between Pleurotus florida and Lentinus squarrosulus (Mongt.) Singer by adopting the method reported earlier, out of which six strains that are, pfs1h, pfs 1j, pfs 1k, pfs 1m, pfs 1n, and pfs 1p were found to produce fruit bodies. Aqueous extract of fruit bodies of the hybrid mushroom strain, pfs 1h yielded two polysaccharides, glucan (PS-I) and heteroglycan (PS-II). Detailed structural characterization and immunostimulating activities of the (1→3)-, (1→6)-β-D-glucan (PS-I) obtained from the hybrid mushroom, pfs1h were reported recently. In the present investigation structural characterization and immunoenhancing activities of the heteroglycan (PS-II) were carried out and reported herein.

2. Results and discussion

2.1. Purification and chemical analysis of PS-II

The fresh fruit bodies (500 g) of the hybrid mushroom pfs1h were washed with distilled water followed by extraction with hot water, precipitation in alcohol, dialysis, centrifugation and freeze drying to yield crude polysaccharide (1.9 g). Two fractions of purified polysaccharides were obtained after fractionating water soluble crude polysaccharide (30 mg) through a Sepharose 6B column. Two fractions, fraction I and fraction II were collected and freeze dried, yielding purified polysaccharide of 9 mg PS-I and 11 mg PS-II, respectively. PS-II showed a specific rotation of $[\alpha]_D^{25} +45.8 (< 0.1$ water). The molecular
Structural study of an immunoenhancing polysaccharide isolated from an edible hybrid mushroom of Pleurotus florida and Lentinula edodes

Saikat Maity\textsuperscript{a}, Eshita Kar Mandal\textsuperscript{a}, Kousik Maity\textsuperscript{a}, Sanjoy K. Bhunia\textsuperscript{a}, Birendra Behera\textsuperscript{b}, Tapas K. Maiti\textsuperscript{b}, Fijush Mallick\textsuperscript{c}, Samir R. Sikdar\textsuperscript{d}, Syed S. Islam\textsuperscript{b,\ast}

\textsuperscript{a}Department of Chemistry & Chemical Technology, Visva-Bharati University, Santiniketan, West Bengal 731235, India
\textsuperscript{b}Department of Biotechnology, Indian Institute of Technology (IIT) Kharagpur, Kharagpur, West Bengal 721302, India
\textsuperscript{c}Division of Plant Biology, Bose Institute, P-1/12, C.I.T. Scheme VII M, Kolkata, 700054 West Bengal, India

ABSTRACT

The water-soluble polysaccharide isolated from hot aqueous extract of the fruit bodies of somatic hybrid mushroom (pfle 1q) obtained through intergenic protoplast fusion between Pleurotus florida and Lentinula edodes consists of \(\alpha\)-galactose and \(\alpha\)-mannose in a molar ratio of nearly 2:1. The polysaccharide showed immunoenhancing activity by stimulating the macrophage, splenocyte, and thymocyte. Based on the results of acid hydrolysis, methylation analysis, periodate oxidation, and NMR experiments (\(^{1}H, ^{13}C\), DEPT-135, DOF-COSY, TOCSY, NOESY, HMQC, and HMBC) the repeating unit of the purified polysaccharide was established as:

\[
\beta-D-Manp \rightarrow 6) \rightarrow 6) \rightarrow \alpha-D-Galp \rightarrow (1 \rightarrow 2) \rightarrow \alpha-D-Galp \rightarrow (1 \rightarrow 1)
\]

1. Introduction

Mushrooms have been consumed globally as tasty food and nutritional supplements. Mushroom polysaccharides have been recognized as the most potent antitumor and immunostimulating materials (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Namba, Hamaguchi, & Kuroda, 1987; Mizuno et al., 1996). Various mushroom polysaccharides such as Lentinan derived from Lentinula edodes, Krestin (PSK) derived from Coriolus versicolor have been recommended for use as anticancer drugs (Sasaki & Takasuka, 1976; Sakagami, Aoki, Simpson, & Tanuma, 1991). A variety of immunoenhancing polysaccharides from edible mushrooms (Bhunia et al., 2010) and edible hybrid mushrooms (Patra et al., 2011) were reported by our group. Four different immunostimulating polysaccharides isolated from Pleurotus florida were also reported by our group (Rout, Mandal, Chakraborty, Pramanik, & Islam, 2004; Rout, Mandal, Chakraborty, & Islam, 2006, 2008). Water insoluble (Sasaki & Takasuka, 1976) and soluble \(\beta\)-glucans (Yu et al., 2010) extracted from L. edodes were reported. Protoplast fusion between the strains of \(P.\) florida and \(L.\) edodes produced nine new hybrid strains adopting the procedures as applied earlier (Chakraborty & Sikdar, 2010), and among them six strains, i.e., pfle 1q, pfle 1p, pfle 1q, pfle 1r, pfle 1s, and pfle 1v produced fruit bodies. Recently a \((1 \rightarrow 6)\rightarrow 4\)-\(\beta\)-glucan was isolated from fruit bodies of one of the hybrid strains pfle 1r (Maji et al., 2012). Aqueous extract of the fruit bodies of another hybrid strain, pfle 1q, yielded a
Glucan of a somatic hybrid mushroom, *pfis1h*: structural characterization and study of immunological activities

Ipsita K. Sen, Praloy K. Maji, Birendra Behera, Tapas K. Maiti, Pijush Mallick, Samir R. Sikdar, Syed S. Islam

*Department of Chemistry and Chemical Technology, Vidyasagar University, Midnapore 721102, West Bengal, India*

*Department of Biotechnology, Indian Institute of Technology (IIT) Kharagpur, Kharagpur 721302, West Bengal, India*

*Division of Plant Biology, Bose Institute, Central Building, P-112, C.L.T. Scheme VII M, Kolkata 700054, West Bengal, India*

**ABSTRACT**

A water-soluble glucan (PS-I) was isolated from the aqueous extract of the fruit bodies of a hybrid mushroom, *pfis1h* of *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer. Structural characterization of PS-I was carried out using total hydrolysis, methylation analysis, periodate oxidation, and NMR experiments. A DEPT-135, DQF-COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC. Methylation analysis revealed that PS-I was composed of (1→3), (1→6), (1→6)-linked and terminal β-D-glucopyranosyl residues in a relative proportion of approximately 1:1:1. The repeating unit of the glucan consists of a backbone chain of two (1→6)-β-D-glucopyranosyl residues, one of which is branched at 2-0 position with (1→3)-β-D-glucopyranosyl and terminated with a β-D-glucopyranosyl residue. Study of immunological activity revealed that PS-I stimulates the spleenocytes, thymocytes and macrophages.

**ARTICLE INFO**

Article history:

Received 12 June 2012

Received in revised form 5 November 2012

Accepted 13 November 2012

Available online xxx

**Keywords:**

Poly saccharide NMR Spleenocytes and thymocytes Macrophages

**1. Introduction**

In recent years, mushroom polysaccharides have drawn the attention of chemists and immunologists for their wide application as antinecancer, immunostimulating, and antioxidant materials [1–4]. (1→3), (1→6)-α-D-Glucan [5], (1→3), (1→6)-α, β-D-glucan [6], a heteroglycan consisting of glucose, mannose and galactose [7] and (1→3), (1→6)-β-D-glucan [8] from *Pleurotus florida* were reported by our group. A heteroglycan consisting of galactose, glucose and fucose [9] from the aqueous extract and a (1→3), (1→6)-β-D-glucan [10] from the alkaline extract of *Lentinus squarrosulus* (Mont.) Singer were also reported by our group. Intergeneric protoplast fusion between *P. florida* and *L. squarrosulus* (Mont.) Singer produced twelve new hybrid strains using the methods adopted earlier [11] out of which six strains i.e., *pfis1h, pfis1, pfis3, pfis1m,* *pfis1tm* and *pfis1p* were found to produce fruit bodies. Aqueous extract of fruit bodies of one of such hybrid mushroom strain, *pfis1h* yielded two polysaccharides, glucan (PS-I) and heteroglycan (PS-II) consisting of mannose, galactose and glucose. The main objective of the present work is to investigate whether PS-I is structurally different from the polysaccharides isolated either from aqueous or alkaline extract of the parent mushrooms *P. florida* and *L. squarrosulus* (Mont.) Singer. Hence, the detailed structural characterization of the glucan (PS-I) was carried out along with immunostimulating studies and reported herein.

**2. Materials and methods**

2.1. Preparation of hybrid strains from edible mushrooms *P. florida* and *L. squarrosulus* (Mont.): Singer through protoplast fusion

The hybrid mushroom strain *pfis1h* was produced through polyethylene glycol (30%) mediated protoplast fusion between *P. florida* and *L. squarrosulus*. Hybrid strains were selected based on double selection method and afterwards maintained in potato-dextrose-agar medium ( manuscript under preparation). Spawn of the hybrid strain was produced on paddy straw and mushroom was produced on paddy straw substrate.

2.2. Isolation, purification and determination of molecular weight of the polysaccharide

The fresh fruit bodies of the hybrid mushroom (pfis1h) of *P. florida* and *L. squarrosulus* (Mont.) Singer (500 g) were collected from Falta Experimental Farm, Bose Institute, Kolkata and were washed with distilled water, followed by boiling with hot water for 4 h. The aqueous extract was kept overnight at 4°C and then filtered through linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratus centrifuge) for 45 min at 4°C.
Appendix

Structural characterization and study of immunoenhancing properties of a glucan isolated from a hybrid mushroom of *Pleurotus florida* and *Lentinula edodes*

Pratoy K. Maji, Ipsita K. Sen, Birendra Behera, Tapas K. Maiti, Pijush Mallick, Samir R. Sikdar, Syed S. Islam

*Department of Chemistry and Chemical Technology, Vidyasagar University, Midnapore 721 102, West Bengal, India*

*Department of Biotechnology, Indian Institute of Technology (IIT) Kharagpur, Kharagpur 721 102, West Bengal, India*

*Division of Plant Biology, Bose Institute, Centraline Building, P-1/12, CIT, Scheme V/II, Kolkata 700094, West Bengal, India*

**ABSTRACT**

A water soluble glucan isolated from hot aqueous extract of fruit bodies of an edible hybrid mushroom *P/E* of *Pleurotus florida* and *Lentinula edodes* showed macrophages, splenocytes, and thymocytes activation. The glucan consists of terminal, (1→3,6)-linked, and (1→6)-linked β-D-glucopyranosyl moieties in a molar ratio of nearly 1:1:3. On the basis of acid hydrolysis, methylation, periodate oxidation study, and NMR studies (*H*, *C*, DEPT-135, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC), the structure of the repeating unit of the glucan was established as:

\[
\beta-D-GlcP_3
\]

© 2012 Elsevier Ltd. All rights reserved.

Mushrooms are important for its medicinal value.1 Mushroom polysaccharides have gained importance because of their immunomodulatory,2 free radical scavenging,3,4 and antitumor5-6 activity. Various immunoenhancing polysaccharides from edible mushrooms7-12 and hybrid mushrooms13-17 were reported by our group. Four different polysaccharides isolated from *Pleurotus florida*13-15 were also reported by our group. Lentinula, a biologically active water insoluble polysaccharide from *Lentinula edodes* containing (1→3), (1→6)-β-D-glucan17 has been reported and widely used for cancer therapy. Water soluble polysaccharides18-20 from *L. edodes* are also reported. Protoplast fusion between the strains of *Pleurotus florida* and *Lentinula edodes* produced nine new hybrid strains adopting the procedures as applied earlier21 out of which six strains *PflE*1, *PflI*, *PflIq*, *Pfl1r*, *Pfl1s*, and *Pfl1v* produced fruit bodies. Aqueous extract of the fruit bodies of one of the hybrid mushroom strains, *PflI* yielded two polysaccharides, glucan (PS-I) and a heteroglycan (PS-II) consisting of glucose, mannose, and galactose. Structural investigation of PS-I showed that it is different from the polysaccharides isolated from either of the aqueous or alcohol extract of parent mushrooms *Pleurotus florida* and *Lentinula edodes*. The structural characterization and immunoenhancing studies of PS-I isolated from the aqueous extract of fruit bodies of a hybrid mushroom strain *PflI* have been carried out and reporting herein.

This pure polysaccharide (PS-I) had a specific rotation [α]D29 × 12 (c 0.8, water). Molecular weight19 of PS-I was estimated as 1.80 × 10^5 Da from a calibration curve prepared with standard dextran. PS-I was hydrolyzed with 2 M triflouroacetic acid and then aldol acetate22 was prepared for GLC analysis. GLC analysis of aldol acetate of hydrolyzed product of PS-I confirmed the presence of glucose only. The absolute configuration of the glucose residue was determined as α by the method of Gerwig et al.24 This PS-I was methylated according to Ciosca and Kerek25 method followed by hydrolysis and then aldol acetate was prepared to know the linkages of sugar moieties. The GLC-MS analysis of the partially methylated aldol acetate of PS-I revealed the presence of 1,3,5,6-tetra-0-acetyl-2,4,6-di-0-methyl-gluclot, 1,5,6-tri-0-acetyl-2,3,4,tri-O-methyl-gluclot, and 1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl-gluclot in a molar ratio of nearly 1:3:1. These results