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Region based Diversity Studies of *Saccharomyces cerevisiae* and *Lentinula edodes*

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ABSTRACT

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Microbial diversity is the main key point during the studies of microbial ecology. The diversity of edible fungal species in an area affects the nutritional behavior of that community. Hence, the present study has been designed for assessing the diversity of two very important fungal genera involved in eating habits of the Indians i.e. *Saccharomyces cerevisiae* and *Lentinus edodes* using different molecular as well as protein markers. The results revealed the divergent nature of these two genera on the basis of their geographical origins. PCR-RFLP analysis of *S. cerevisiae* strains from Northern India clustered them into two groups one with four strains from different locations and other with fourteen strains, most of which from the same geographical location i.e. Lahaul and Spiti. Two indigenous strains of *L. edodes* from Tamil Nadu Agricultural University, Coimbatore (LeC and LeI) showed different characteristics as compared to other strains as seen by protein profiling using SDS-PAGE and isozymes. This study gives an indication of these techniques to be a powerful tool to discriminate these fungal species at intraspecies level for their diversity analysis.

Introduction

India is a rich repository of microbial genetic diversity. There are number of fungal species that are associated with the eating habits and health benefits of the Indians. The most famous yeast comes under ascomycetes family of fungi, *Saccharomyces cerevisiae*, which is associated with most of the fermented foods of the Indian origin (Walia *et al.*, 2014). The fermented foods are more common among

people belonging to the rural areas, especially in hilly and tribal people. The quality of a fermented product is consequence of the starter and progressive presence of microbial flora during the process of fermentation. To obtain the best strain, the knowledge of natural yeast diversity available in the given area or yeast(s) associated with a particular product, is of prime importance (Kanwar and Keshani 2014). Eighteen *S. cerevisiae* strains isolated from various fermented foods of Western

Himalayas had already been available with the Department of Microbiology, Himachal Pradesh Agricultural University, Palampur were used in the present study. These strains were discriminated at genetic level using internal transcribed spacer (ITS) region and were found to possess potential fermentation traits both in baking as well as brewing aspects (Keshani *et al.*, 2015). However, to study their diversity on the basis of different geographical locations and to discriminate them at intraspecies level other molecular technique is needed to be used.

The other most important fungal species is *Lentinula edodes*, of basidiomycetes family, commonly known as Xianggu or shiitake is one of the white-rot fungi growing on the dead tree or sawdust and ranks second in the global mushroom market after *Agaricus bisporus* (Bisen *et al.*, 2010). This mushroom grows throughout East and Southeast Asia and possesses abundant amount of proteins, bio-active polysaccharides such as β -D-glucan, heteroglucan, xylomannan, lentinan and eritadenine; free sugars including arabinose, arabitol, mannose, mannitol, trehalose and glycerol; vitamins (B2, B12, D2) and dietary fibre (Hobbs 2000). These nutritional components render shiitake overwhelmingly popular with consumers in Asian countries. The presence of different bioactive components in *L. edodes* aids in its pharmacological and therapeutical potential against hypertension, hyperlipidemia and cardiovascular complications, depressed immunity, hepatic disorders and cancer. Mostly, morphological parameters such as the size, shape, form, gills, basidium, and basidiospores are used for basidiomycetes identification, but differentiations of closely related cultures at molecular level require extensive molecular techniques. These molecular approaches are considered as one of the efficient biotechnological tool for assessment of genetic diversity (Mehmood *et*

al., 2008) and have been proven to be effective in the classification of fungal taxonomic groups including mushrooms (Le *et al.*, 2008).

Therefore, the present study have been planned with the objective of exploring intraspecies variability among eighteen *S. cerevisiae* strains at molecular level using PCR-RFLP technique and seven *Lentinula edodes* strains at protein level using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isozyme analysis.

Materials and Methods

***S. cerevisiae* strains**

Eighteen strains of *S. cerevisiae* used for PCR-RFLP had been available with the Department of Microbiology, Himachal Pradesh Agricultural University, Palampur. These strains were isolated from various fermented foods of Western Himalayas from different tribal geographical locations.

***L. edodes* strains**

Seven strains of *L. edodes* used for SDS-PAGE and isozyme analysis included five strains, namely, LeS, OE-38, OE-142, OE-329, and OE-388 procured from the germplasm collection of edible mushrooms, Directorate of Mushroom Research, Solan, India and two indigenous strains (LeC and LeI) obtained from Tamil Nadu Agricultural University, Coimbatore, India.

PCR-RFLP of ITS region of *Saccharomyces cerevisiae* strains

DNA extraction, amplification and visualization

Thermo Scientific Phire Plant Direct PCR Kit,

F-130 was used to perform polymerase chain reaction (PCR) directly from the yeast cells. Based on optimization trials, the standardized PCR protocol for 50µl reaction mixture; included, 25 µl of 2X phire plant PCR buffer, 0.6 µl of 10µM of primer set containing forward and reverse primers, 1 µl of phire hot start II DNA polymerase, 0.5µl of yeast cells grown in potato dextrose broth (PDB) and sterilized milli-Q water to make up the reaction volume. The PCR products were resolved in 2.0 % agarose gel in 1x tris acetate EDTA buffer containing ethidium bromide (0.5 µg/ mL) at 75 V for 90 minutes.

Digestion of PCR products

The PCR products were digested without further purification with four restriction endonucleases i.e. *AluI*, *HaeIII*, *HinfI* and *TaqI* by the following protocol:

PCR product:	10 µl
Buffer:	2 µl
Restriction enzyme:	0.5 µl
Distilled water:	7.5 µl

The mixture was incubated at 37 °C for two hours except for *TaqI*, where incubation temperature was 60 °C, the restriction fragments were separated on 1.2 % agarose gel in 1X TAE buffer. The gels were stained with ethidium bromide, visualized under UV light and photographed by UVITEC, Cambridge, GeNei™. Sizes of different bands were estimated by comparison with a DNA length standard. The PCR-RFLP analyses were carried out using Treeconw software program.

SDS-PAGE and isozyme analysis of *Lentinula edodes* strains

Pure mycelial cultures of the seven *L. edodes* strains were aseptically inoculated in 100 mL potato dextrose broth (PDB) medium and incubated at 25±2°C for 4 weeks. The

mycelial mat was harvested by filtering the broth through Whatman no. 1 filter paper and harvested mycelium was used for SDS-PAGE and isozyme extraction.

Extract preparation

About 10g of fresh mycelium of each strain was taken in 50 mL of 0.5M Tris-HCl buffer (pH 6.8), homogenised in a tissue homogeniser and transferred to the centrifugation tubes. The homogenized mycelium were then centrifuged (10,000 rpm, 10min). The supernatant was then saturated to 80 % by ammonium sulphate, centrifuged (10,000 rpm, 20 min). Supernatant was discarded and the residue was dissolved in a minimum amount of 0.5M Tris-HCl buffer (pH 6.8), dialysed to remove the impurities. After dialysis the solutions were again centrifuged at 4°C at 6000 rpm for 5 min, the supernatant was discarded and the residue was dissolved in minimum amount of buffer.

Protein estimation

The protein content of the samples was estimated by Lowry's method (Lowry et al.1951) with bovine serum albumin (BSA) as the internal standard.

Gel electrophoresis

SDS-PAGE was carried out according to the modified method of Laemmli (1970) under reducing conditions (2-mercaptoethanol) with 12 % resolving gel and 5% stacking gel. Protein bands were visualized by staining the gel with 45% (V/V) methanol, 10% (V/V) acetic acid for 30 min and stained with 0.25 (m/V) Coomassie Brilliant Blue R-250 in fixing solution and later destained by incubating the gel in 40% (V/V) methanol, 7.5% (V/V) acetic acid. The molecular masses of protein bands were calculated using the broad range SDS-PAGE molecular mass standards.

Isozyme analysis was carried out for three different enzymes namely alcohol dehydrogenase, malate dehydrogenase and peroxidase as native PAGE using 2.5 % stacking gel and 12 % resolving gel. After electrophoresis, gel was stained using specific staining systems in the presence of the specific substrates for different enzymes. Different staining systems were used for different isozymes viz., alcohol dehydrogenases (Tanksley 1979), malate dehydrogenases (Brown *et al.*, 1978) and peroxidases (Guikema and Sherman 1980)

After destaining, the protein bands appeared in the gel was visualized for the polymorphism among the different strains. The relative mobility (R_m) was calculated by measuring the distance moved by the dye and different protein bands as follows:

$R_m =$

Distance travelled by the protein band (cm)

Distance travelled by the tracking dye (cm)

Results and Discussion

PCR-RFLP of ITS region of *S. cerevisiae* strains

The ITS regions of the indigenous yeast isolates were amplified using primers ITS1 and ITS4. The ITS region of all the strains amplified about 880 bp sequences. The PCR products were digested with four restriction endonucleases: *Alu I*, *Hae III*, *Hinf I* and *Taq I*. The RFLP analysis grouped these strains in two groups; one with 90% similarity and the other with 85% similarity (Fig. 1). All eighteen *S. cerevisiae* strains shared almost same restriction patterns with endonucleases *Alu I*, *Hae III*, *Hinf I* and *Taq I* (Table 1), suggesting a relationship among them in the restriction pattern of internal transcribed spacer region, however, with a divergent

nature as the grouping of strains was not location based (Fig. 2). The second cluster consisted of strains from Sangla, Lahaul & Spiti and Bharmour region whereas, all the other strains were grouped in first cluster.

S. cerevisiae has been studied extensively due to its importance in the production of various fermented foods and beverages (Kanwar and Keshani, 2016). However, this species is also able to cause spoilage (Martorell *et al.*, 2005); therefore, food industry is interested in defining methods that can discriminate between isolates of *S. cerevisiae*. A typing technique, in which restriction fragment length polymorphism in the rDNA repeat of various isolates of *Candida albicans* was generated, showed that this technique enabled the discrimination at intraspecies level (Magee *et al.*, 1987). Chromosomal length polymorphism has also been shown in baker's (Casey *et al.*, 1990) and brewer's yeast (Pedersen 1986) strains. Walther *et al.*, (2014) also stated that by using PCR-RFLP two types of lager yeasts could be distinguished, showing it to be a powerful technique for discrimination at intra-species level. Thus, the use of restriction enzymes to generate restriction patterns from the amplified rDNA regions seems to be a beneficial approach to discriminate *S. cerevisiae* at intra-species level. Molina *et al.*, (1993) have amplified part of the 3' external transcribed spacer and the intergenic spacer, which after digestion with *MspI* allowed discrimination of strains between and within the species *S. cerevisiae*, *S. pastorianus* and *S. carlsbergensis*. It has been shown that the ITS regions have a significant level of length and sequence polymorphism across fungal species (Feibelman *et al.*, 1994).

SDS-PAGE and isozyme analysis of *L. edodes*

The protein profile of all the seven strains was evaluated for relatedness amongst the strains.

The samples used for molecular characterization by SDS-PAGE were estimated for the protein content before being used in generating the protein profiles (Table 2). The maximum protein content was obtained in OE-388 ($176.6 \mu\text{g ml}^{-1}$) followed by LeS ($152.9 \mu\text{g ml}^{-1}$) and OE-38 ($151.2 \mu\text{g ml}^{-1}$).

Slight variation was observed in the banding pattern among the strains (Table 3). Five strains (LeS, OE-38, OE-142, OE-329 and OE-388) showed one band having similar relative mobility value of 0.78. Maximum number of bands (8 bands) were observed in OE-329 while minimum (5 bands) were in LeI and OE-142. Three bands having similar relative mobility were obtained in three strains (OE-38, OE-142 and OE-388). The protein profiles were obtained for all the strains. The banding pattern obtained after electrophoresis was used to evaluate the similarities and dissimilarities among the strains (Plate 1).

Results of SDS-PAGE indicated similarity among LeS, OE-38, OE-142, OE-329 and OE-388. The one band formed at relative mobility of 0.78 had molecular weight of 18.7 kDa, respectively. However, the maximum similarity was observed in three stains of *L. edodes* (OE-38, OE-142 and OE-388). The three bands formed at relative mobility of 0.02, 0.36 and 0.78 had molecular weight of > 97 kDa, 44 kDa and 18.7 kDa, respectively.

Almost similar molecular weight proteins have been reported in *Lentinus* spp. by several workers. Kobayashi *et al.*, (2003) isolated two new RNase T2 Ribonucleases, RNase Le37 and Irp3 with a molecular mass of 45kDa from basidiomycetous fungi, *L. edodes* and *Irpexlacteus*. Vetchinkina *et al.*, (2008) isolated a number of lectins varying in polypeptide composition and carbohydrate

specificity from *L. edodes* at different stages of its morphogenesis: non pigmented mycelium, brown mycelium film and fruiting body. SDS-PAGE identified three lectins at the non pigmented mycelium stage, two of them being dimers consisting of 16 and 45 kDa and 16 and 42 kDa subunits; the third was a tetramer of 16, 39, 42 and 45 kDa subunits. The fractions with lectin activity obtained at the brown mycelium film stage contained polypeptides of 24, 30 and 38 kDa. Thetsrimuang *et al.*, (2011) reported that the crude polysaccharides from the fresh and the dried fruiting bodies of *Lentinus* sp. (RJ-2) had protein bands which had molecular weights lower than 20.1 kDa and around 66.0-97.0 kDa.

Isozyme analysis of *L. edodes* strains

Isozyme analysis was carried out for three different enzymes namely alcohol dehydrogenase, malate dehydrogenase and peroxidase as native PAGE.

Alcohol dehydrogenase

Two bands corresponding to two isozymes were obtained for alcohol dehydrogenase (Plate 2 a). The relative mobility (Rm) values of the two bands were 0.13 and 0.24. A single band at Rm value of 0.13 was observed in four strains (LeS, OE-38, OE-142 and OE-329). An additional band at Rm value of 0.24 was observed in OE-142 along with another band at Rm value of 0.13 (Table 4). However, the additional band of Rm value (0.24) was also observed in OE-388 strain. Chakraborty and Sidkar (2008) obtained two ADH isozymes in protoplasts between *P. florida* and *V. volvacea*. Chakraborty and Sidkar (2010) again obtained two isozymes for fusion products of *P. florida* and *Calocybeindica*

Table.1 Restriction analysis of the ITS1-5.8S-ITS2 region from eighteen *S. cerevisiae* strains

Strains	Place of Collection	Restriction Fragments (size)			
		<i>Alu I</i>	<i>Hae III</i>	<i>Hinf I</i>	<i>Taq I</i>
Sc01	Lahaul&Spiti	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc02	Lahaul&Spiti	660+50	310+250+110+60	360+300+70	280+250+130+70
Sc03	Lahaul&Spiti	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc04	Lahaul&Spiti	660+50	310+250+100+70	360+300+70	280+250+140+60
Sc05	Lahaul&Spiti	660+50	310+250+100+70	360+320+50	250+230+150+80
Sc06	Lahaul&Spiti	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc07	Lahaul&Spiti	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc08	Lahaul&Spiti	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc09	Lahaul&Spiti	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc10	Sangla	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc11	Sangla	660+50	310+250+120+50	360+320+50	300+250+130+50
Sc12	Sangla	660+50	310+250+120+50	360+320+50	300+250+130+50
Sc15	Bharmour	660+50	310+250+100+70	360+320+50	280+250+140+60
Sc16	Pangi	660+50	310+250+120+50	360+300+70	280+250+140+60
Sc19	Sangla	660+50	310+250+120+50	360+300+70	300+250+130+50
Sc20	Sangla	660+50	310+250+120+50	360+320+50	280+250+140+60
Sc21	Sangla	660+50	310+250+120+50	360+320+50	280+250+140+60
Sc22	Palampur	660+50	310+250+120+50	360+300+70	280+250+140+60

Table.2 Estimation of protein content in *L. edodes* strains

Strains	Protein content ($\mu\text{g ml}^{-1}$)
LeC	45.53
LeI	68.30
LeS	152.9
OE-38	151.2
OE-142	96.30
OE-329	115.69
OE-388	176.6
CD at 5%	1.72

Table.3 Relative mobility of the proteins for *L. edodes* strains

Strains	No. of bands	Relative Mobility (Rm)
LeC	6	0.11, 0.43, 0.53, 0.66, 0.72, 0.81
LeI	5	0.10, 0.42, 0.64, 0.71, 0.80
LeS	7	0.04, 0.22, 0.32, 0.39, 0.46, 0.67, 0.78
OE-38	6	0.02, 0.20, 0.36, 0.44, 0.66, 0.78
OE-142	5	0.02, 0.36, 0.45, 0.66, 0.78
OE-329	8	0.04, 0.10, 0.27, 0.37, 0.46, 0.61, 0.68, 0.78
OE-388	7	0.02, 0.22, 0.30, 0.36, 0.45, 0.68, 0.78

Table.4 Rm values for different isozymes for *L. edodes* strains

Strains	Alcohol dehydrogenases		Malate dehydrogenases		Peroxidases	
	No. of bands	Rm values	No. of bands	Rm values	No. of bands	Rm values
LeC	0	-	0	-	1	0.66
LeI	0	-	0	-	1	0.66
LeS	1	0.13	2	0.35, 0.54	1	0.69
OE-38	1	0.13	0	-	1	0.62
OE-142	2	0.13, 0.24	1	0.35	1	0.62
OE-329	1	0.13	2	0.35, 0.54	1	0.62
OE-388	1	0.24	0	-	1	0.62

Fig.1 Dendrogram showing relationship between different *S. cerevisiae* strains generated after PCR-RFLP analysis of ITS region using Treeconw software program

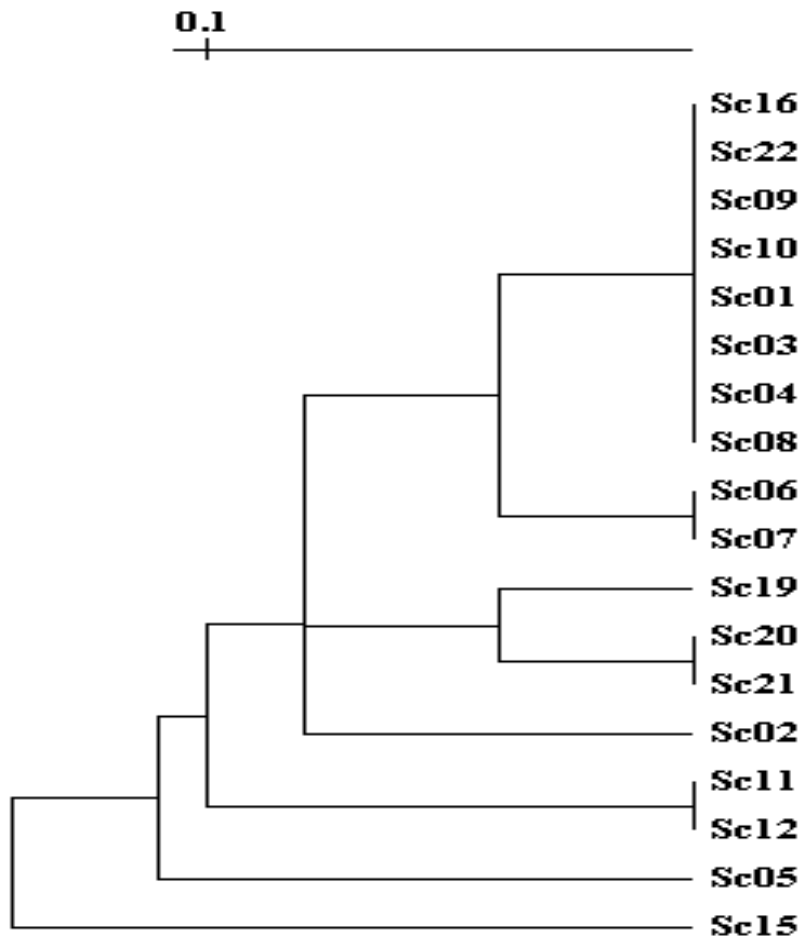


Plate.1 Protein profiling of 7 strains of *L. edodes* using SDS-PAGE

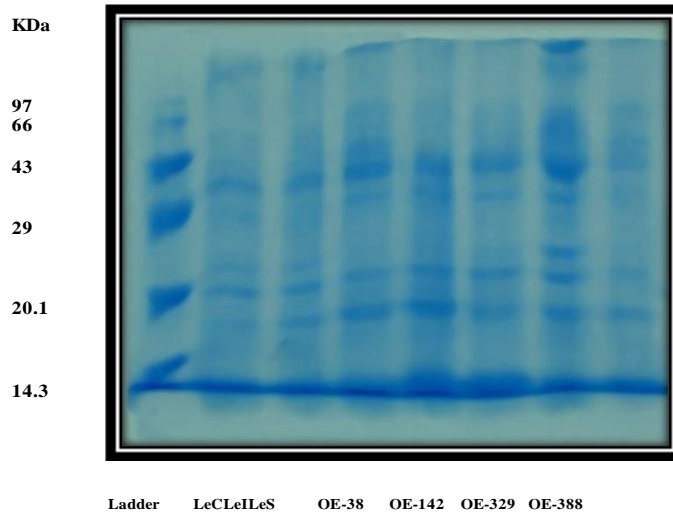


Fig.2 Restriction pattern shown by different *S. cerevisiae* strains after digestion with *Alu I*, *Hae III*, *Hinf I* and *Taq I* endonucleases

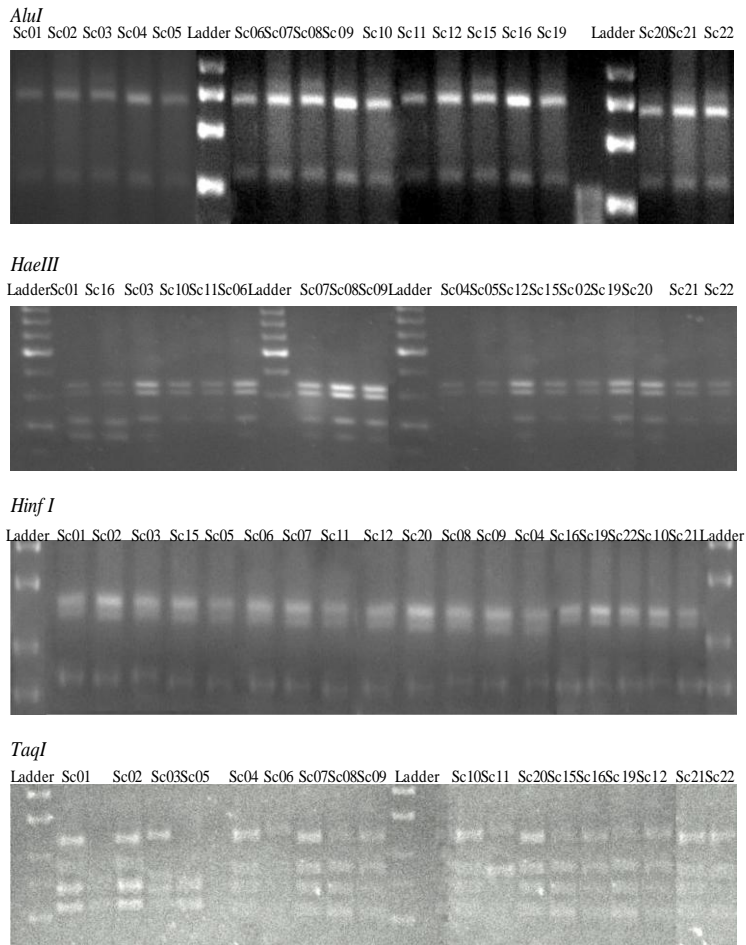
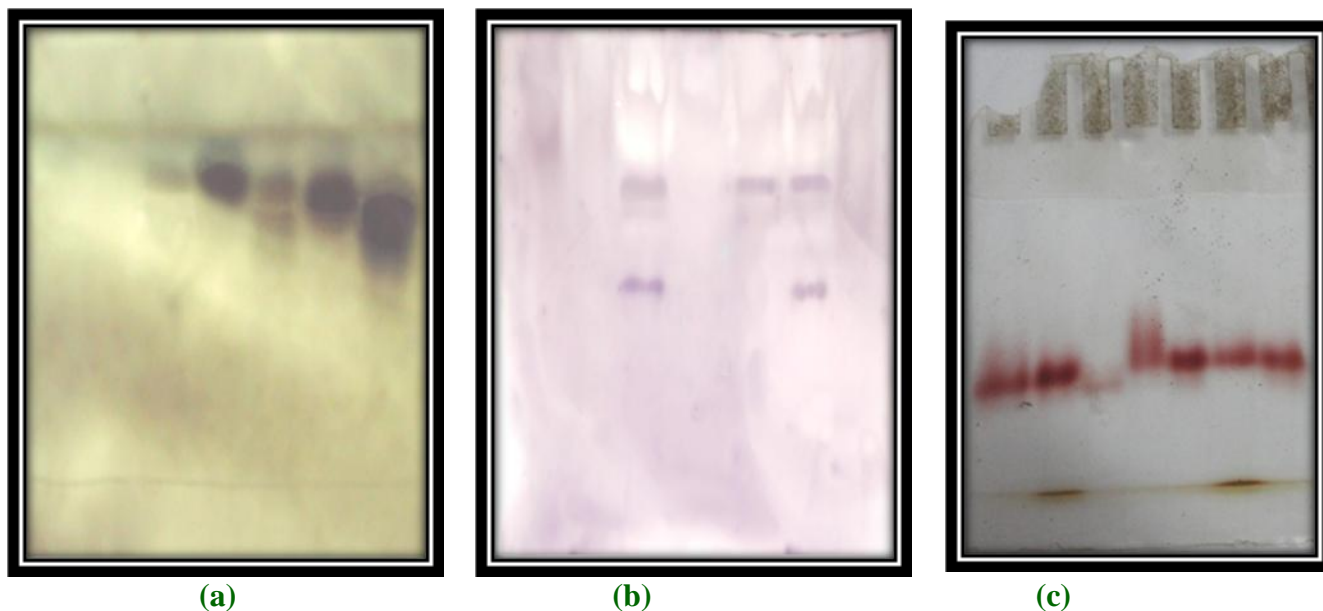


Plate.2 Native-PAGE of a. Alcohol Dehydrogenase b. Malate Dehydrogenase c. Peroxidase



Malate dehydrogenase

Bands were obtained at Rm values corresponding to two isozymes (Plate 2 b). Two bands were obtained in two strains of *Lentinus* (LeS and OE-329) at Rm values of 0.35 and 0.54, a single band was found at Rm value of 0.35 in case of OE-142 while no bands were found in LeC, LeI, OE-38 and OE-388 (Table 4). Ohamasa and Furukawa (1986) classified forty different strains of *Lentinus edodes* into five groups according to the typical bands of malate dehydrogenase (MDH) and esterase. To analyze the genetic variability in the natural population of the fungus, electrophoretic patterns of nine enzymes from the mycelia of 93 strains of *L. edodes* from different countries were studied by Fukuda and Tokimoto (1991). They observed that the strains showed a high variation in esterase and malate dehydrogenase systems and suggested that geographical distance reduces the genetic similarity in the natural population of *L. edodes*. Tokimoto *et al.*, (1998) observed six types of malate dehydrogenase enzymes in *L. edodes*.

Peroxidase

Three isozymes corresponding to Rm values of 0.62, 0.66 and 0.69 could be observed (Plate 2c). One band at Rm value of 0.66 was observed in LeC and LeI while in LeS, the band corresponding to Rm value of 0.69 was present (Table 4). However, one band at Rm value of 0.62 was present in OE-38, OE-142, OE-329 and OE-388. Baocheng *et al.*, (1995) studied esterase and peroxidase isozyme pattern and showed that fusant strains of *P. sapidus* and *P. ostreatus* had bands from both the parents. Park *et al.*, (1997) assessed the genetic similarity of sixty three isolates of *L. edodes* by isozyme polymorphisms and by combining the isozyme patterns of esterase, peroxidase and acid phosphatase, each isolate showed its own distinct electrophoretic phenotypes. The results of RAPD-PCR and ITS sequencing also revealed the divergent nature of LeC and LeI strains (Sharma *et al.*, 2014).

In conclusion, the results of the present study revealed divergent nature of *Saccharomyces cerevisiae* as well as *Lentinus edodes* on the

basis of their geographical origins. Four *S. cerevisiae* strains clustered separately from other strains corresponding to different locations during PCR-RFLP analysis. Two indigenous strains of *L. edodes* from Tamil Nadu Agricultural University, Coimbatore (LeC and LeI) showed different characteristics as compared to other strains as seen by protein profiling using SDS-PAGE and isozymes. This study shows these techniques to be a powerful tool to discriminate these fungal species at intraspecies level for their diversity analysis.

Conflict of Interest: Authors declare no conflict of interest.

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