RESEARCH ARTICLE

Characterization of yeast in hapludands soil with biotechnological potential

Paola E. Díaz[1*], Camila Aranda[1], Oscar Martínez[1], Roberto Godoy[2], Aldo Gonzales[3] Eduardo Valenzuela[1]

[1]*Institute of Biochemistry and Microbiology, Faculty of Sciences, University Austral of Chile. Casilla 167, Valdivia 5090000, Chile, Phone: 0056 632221296.* [2]*Institute of Environmental and Evolutionary Sciences, Faculty of Science University Austral of Chile. Casilla 567, Valdivia 5090000, Chile.* [3]*Severo Ochoa Centre for Molecular Biology (CBMSO), University Autónoma of Madrid, C/Nicolás Cabrera, 1. 28049 Madrid, España.* *Corresponding author: paola.diaz@postgrado.uach.cl*

Abstract

Yeasts play an important role in nature, and soil is one of the largest reservoirs of yeasts. Seventy-seven yeast strains were isolated from southern Chilean volcanic ash soil. These 77 strains were composed of 30 strains derived from soils used as permanent grassland, 30 strains from rotating grassland, and 17 strains from native forest (considered as the control). These strains were identified using restriction fragment length polymorphisms (PCR-RFLP) in conjunction with ITS-5.8S rDNA sequencing. Additionally, a physiological characterization of the assimilation of carbon and nitrogen and the fermentation of sugars was performed. Various features of the strains were evaluated including their growth in vinasse medium, nitrogen and phosphorus content, and lipid production. Lastly, the use of strains as biofertilizers was analyzed via the cultivation of bell pepper seedlings in soil. From the RFLP profiles, the 77 strains clustered into 10 groups. Of these, only three groups could be identified to the species level, and another was classified to the level of genus (*Devaryomyces hansenii, Pichia fermentan, Kazachstania exigua, Candida* sp.). All strains grew in vinasse medium. Six strains (PP1, PP4, PR4, PR10, PR27 and PR29) showed a high capacity to accumulate lipids, and three strains (PP1, PP21 and PR28) have the potential to be used as biofertilizers.

Keywords: Yeast, PCR-RFLP, ITS-5.8S, lipids, biofertilizers

1. Introduction

The conditions found in soil facilitate the development of certain species of yeast; thus, a high diversity of yeast contributes to the soil microbial community (Wuczkowski and Prillinger, 2004). The diversity of yeasts in soil has been examined globally from Antarctica to tropical regions (Slavikova and Vadkertiova, 2000), and soil type, climatic features, and anthropic disturbance have been found to influence the presence of yeasts in soil. Recently, several studies have demonstrated how human activity influences the diversity and community structure of yeasts in soil. Slavikova and Vadkertiova (2003) compared yeasts in soils where different crops (maize, beet and potatoes) were grown versus non-tilled soils and determined that there is a decrease in yeast population in cultivated soils likely due to the use of pesticides and fungicides. Yurkov *et al.* (2012) analyzed yeast diversity in soils whose use had changed from forest to pasture, and they found that pasture soils preferentially harbor Ascomycete yeasts as opposed to forest soils where the community structure is more heterogeneous.

The economic potential of yeast to agriculture, medical, or environmental applications warrants their molecular identification. Currently, molecular barcoding employing rDNA genes or the internal transcribed spacer (ITS) is the most common technique used to characterize yeast (Pham *et al*., 2011). Molecular barcoding is fast, reliable, accurate and therefore, suitable for rapid screening of isolated strains. It has been shown that ITS regions (non-coding and variable) and the 5.8 rRNA gene (coding and conserved) may be useful for the measurement of phylogenetic relationships since these regions present high intraspecific differences (Fernández-González *et al*., 2001). Currently, there are various applications for the use of yeast in industry including in the production of biofertilizers and lipids. Biofertilizers are composites of beneficial microorganisms, which, after application, can increase nutrient availability and thus help improve soil health (Kupper *et al.*, 2006). Given that many agricultural systems are characterized by low efficiency in the use of nutrients, agricultural practices that minimize the excessive addition of N and P are urgently needed in order to ensure sustainable food cultivation Yurkov *et al.* (2012). El-Tarabily and Sivasithamparam (2006) note that yeast taxa have been registered as plant endophytes, as promoters of plant growth, as producers of enzymes (e.g., β-1,3-glucanasa), and as agents of biological control. Additionally, Ignatova *et al.* (2015) have shown that yeast directly or indirectly provide beneficial services to plants. Some of these services include nitrogen fixation, the production of plant hormones such as auxins, gibberellins, and cytokines, the synthesis of vitamins, the production of antifungal and antibiotic compounds, the ability to solubilize minerals such as phosphorous, and the capacity to degrade numerous chemically toxic substances.

Additionally, yeasts have been reported (Chang *et al*., 2013) as the most promising species for the production of lipids. Specifically, the high intercellular lipid accumulation (up to 80% of cell dry weight) of some species, namely *Rhodosporidium* sp., *Rhodotorula* sp., *Lipomyces* sp., and *Yarrowia lipolytica*, make them very useful for the production of fats (Rakicka *et al.*, 2015). Many of these yeasts accumulate lipids in the form of trialkylglycerols (TAGs), mainly in the cytoplasmic membrane, and the production of TAGs increases during nutrient limitation (Arous *et al*., 2015). Currently, research on the accumulation of yeast lipids and lipids generated by other oil-producing microorganisms is mainly focused on: (i) optimizing pilot-scale lipid production including residual raw materials as a source of energy (Polburee *et al*., 2015),

(ii) the search for strategies in the culture to optimize the lipid production process and (iii) the determination of metabolic and molecular aspects involved in lipid accumulation (Ledesma-Amaro *et al.*, 2016). The objective of the present study was to identify and characterize yeasts that are present in volcanic ash soils in the Los Rios Region of Chile. Furthermore, we evaluated the biotechnological potential of these yeasts by growing them in an industrial waste liquid known as vinasse.

2. Materials and Methods

2.1. Collecting soil samples

Samples were collected from various sites with volcanic ash soil (Hapludands) in the town of Marquina, Valdivia, Chile (673.75° E, 5625.5° N UTM, Quadrant H18). Specifically, 400 g samples were collected from the first 10 cm of the soil. The sample sites included permanent grassland (PP), rotating grassland (PR), and native forest (B) that was considered as the control. Two samples were randomly collect from each site. The soil samples were deposited independently in sterile plastic bags and transferred to the Mycology Laboratory of the Institute of Biochemistry and Microbiology of the Austral University of Chile.

2.2. Isolation of yeasts

Isolation of yeasts was done by serial dilution whereby 10 g of the respective soil samples were weighed and placed in a flask. Then, 90 mL of sterile distilled water was added, and the obtained mixture was shaken manually until total dissolution of the soil was achieved. Following this, 1 mL of the mixture was extracted using a sterile pipette and placed in a tube containing 9 mL of sterile distilled water (10^{-2} dilution). Hence, dilutions were made up to 10^{-6}. Subsequently,

1 mL of the respective dilution was deposited in an empty Petri dish to which 0.5 mL of antibiotic mixture (penicillin and streptomycin 1: 1) and 15 mL of Sabouraud agar were added. Once the agar had solidified, the plates were incubated at 23 ± 2 °C for seven days. After the incubation, 30 yeast colonies were selected for each grassland site, and 17 colonies were selected for the control site. Colonies were selected using morphological criteria, including the color and the size of the colony. Overall, 77 yeast strains were selected for downstream analysis.

2.3. Metabolic determination of yeast strains

Each strain was characterized using the traditional methodology proposed by Barnett *et al*. (2000). Using an auxanogram, we determined how the yeasts used different carbon sources (succinic acid, citric acid, starch, cellobiose, Arabinose, D-glucitol, D-mannitol, D-xylose, D-ribose, DL-lactic acid, Galactitol, Galactose, Glycerol, Inositol, Lactose, L-aravinose, L-Rhamnose, L-sorbose, Maltose, Melibiosa, Melizotosa, Rafinosa, Ribitol, Sucrose, Trehalose). Specifically, 0.5 mL of yeast suspension (McFarland 0.1 turbidity = 10^2 CFU yeast / mL) was mixed with 10 mL of Yeast Nitrogen Base (YNB) agar, and the obtained mixture was deposited in a sterile and empty Petri dish. Once solidified, the selected carbon sources were deposited at four points that were equidistant from the plate edge. Glucose was used as a control. Following additions, the plates were incubated at 23 °C and examined every two days for one week. The qualitative growth of the yeast strain was given as positive (+), weakly positive (+/-), or negative (-). Nitrate, nitrite, and urea nitrogen assimilation was also tested by changing the base medium to Yeast Carbon Base (YCB) agar. Additionally, fermentation was evaluated. Specifically, glucose was used as a positive control, and the following were added at a 2% final

concentration: Starch, D-Galactose, D-xylose, Fructose, Lactose, Maltose, Sucrose, Trehalose. As with the broth culture medium, 0.1% of yeast extract broth was used. To this, the sugar to be evaluated was added at a 2% final concentration. Overall, 3 mL of culture medium and 1.5 mL of the sugar to be tested were added to individual tubes. Then, 0.1 mL of the respective yeast suspension (at McFarland 0.1) was added to each tube, and then these were incubated at 23 °C for one month. Each day, visual checks for turbidity and gas in the Durham tubes were performed to determine whether fermentation was occurring.

2.4. DNA extraction and amplification of the ITS rDNA region

The same yeast isolates used in the metabolic analyses were also cultured in Yeast Peptone Dextrose (YPD) for 30 h at 25 °C. Following this, the DNA of the strains was extracted using an E.Z.N.A.® Yeast DNA Kit following the supplier's instructions. The 5.8S-ITS region of the nuclear ribosomal DNA (rDNA) was amplified using the ITS1 primer (5' TCCGTAGGT-GAACCTGCG G-3´) and ITS4 (5´ TCCTTCCGCT-TATTGATATGC G-3´). Additionally, the 5.8S-ITS region and partial 26S gene of the nuclear ribosomal DNA (rDNA) was amplified using the ITS1 primer (5' TCC GTA GGT GAA CCT GCG G 3´) and NL4 primer (5´ GGT CCG TGT TTC AAG ACG G 3´). The PCR reaction was carried out in 25 μl which was composed of GoTaq® Green Master Mix (Taq DNA polymerase, dNTPs, MgCl2) (Promega) and 0.5 μl of the forward and reverse primers. Amplification conditions for the first primers, ITS1-ITS4, were: initial denaturation (2 min at 95 °C), final denaturation (1 min at 95 °C), annealing (2 min at 56 °C), extension (2 min at 72 °C), and final extension (10 min at 72 °C). For the first ITS1-NL4, the conditions were: initial denaturation (2 min at 95 °C), final denaturation

(94 °C for 1 min), annealing (40 s at 52 °C), extension (1 min at 72 °C), final extension (10 min at 72 °C), each for 30 cycles. 4 μl of the PCR product were analyzed on 1.5% TAE 1X agarose gels and visualized on a FOTO/UV21 Transilluminator. A 100-bp DNA molecular weight marker (Thermo Scientific) was used. The DNA concentration was measured on a Nanodrop, Infinite M200. The amplified DNA was purified and sent for sequencing to Macrogen, Korea.

2.5. PCR-RFLP technique

The 5.8S-ITS PCR products (5 μl) were digested with the restriction enzymes *Cfo*I, *Hae*III (Promega), and *Hinf*I (New England Biolabs) according to the supplier conditions; these were incubated at 37 °C for 2.5 hr. The fragments were separated on a 3.5% agarose gel in 1x TAE for 2 h at 80 V. Band sizes were estimated by comparison with a 100-bp DNA marker (Thermo Scientific). For the identification of the obtained sequences, the Genbank database was used with the BLAST tool, http://www.ncbi.nlm.nih.gov/BLAST. For in silico characterization, RFLP restriction patterns were predicted by the Restriction Mapper program at: http://www.restrictionmapper.org/.

2.6. Determination of lipids, proteins, and total phosphorus in yeast strains

Independently, flasks containing 50 mL of vinasse broth (medium) were inoculated with the yeast strains (10^2 CFU / mL); 77 strains were tested independently. After inoculation, the flasks were incubated at 23 ± 2 °C for 5 days and were maintained on a shaker at 150 rpm. After this time, total lipids (TL) were evaluated by the colorimetric Sulfo-Phospho-Vanillin (SPV) method; absorbance (A) at 520 nm was measured. Total Phosphorus (TF) was determined by acid digestion and absorbance (A) at 400

nm. Total protein (TP) content was determined by Biuret colorimetry; absorbance (A) at 595 nm was measured. All determinations were undertaken in triplicate. The determination of the lipid profile was undertaken in a gas chromatograph (Varian 2200 GC/MS, Varian CP-3800 GC & FID Detector) using a VF-23 ms, FS 60 m, 0.25 mm ID 0.25 μ column (VARIAN). The fatty acids were derived as methyl esters, and 35 methyl ester analytical standards (Sigma) were used for the identification.

The obtained data were submitted to statistical analysis using R. Data were analyzed using a one-way analysis of variance (ANOVA) and student's-t tests. The differences observed at $p \leq 0.05$ were considered significant.

2.7. Determination of yeast strain potential for use in biofertilizers

Based on the results of the metabolic analyses, the yeast strains PP1, PP21 and PR28 were selected to be tested as biofertilizers. Specifically, these strains had high nitrogen content (in protein) and high total phosphorus compared to the other strains (PP1: 1.4 ± 0.03 mg total phosphorus/g dry yeast and 130.5 ± 15 mg protein/g dry yeast. PP21: 0.9 ± 0.00 mg total phosphorus/g dry yeast and 251.3 ± 7.0 mg dry yeast protein/g. PR 28: 4.83 ± 0.01 mg of total phosphorus/g dry yeast and 124.9 ± 6 mg protein/g dry yeast). To test their utility as biofertilizers, each strain (10^2 CFU/mL) was cultivated in vinasse medium for 72 h, then the culture was centrifuged at 20,000 rpm for 20 min, and the obtained pellet was dried at 70 ºC for 72 h. This pellet was then ground to ≤ 0.1 cm, and the resulting powder constituted the respective biofertilizers (B1; B21; B28). To determine if the biofertilizers positively affected plant growth, they were added to bell pepper seedlings that were grown until the transplanting stage. Each

plant was grown in 2 kg of volcanic ash soil with guano (49.4 g/tray) and NH_4NO_3 (42.1 g/tray) additions. B1, B21, B28 (24.6 g/tray), or no fertilizer (control) were added separately to each tray. Bell pepper seeds were added to each tray, which were kept in a greenhouse for 40 days with a 16 h light/8 h dark photoperiod, temperatures between 12 and 30 °C, and a water regimen of distilled water and monthly irrigation with Hoagland solution without nitrogen and phosphorus. Fifteen seedlings per control and treatment were measured in cm: the total length; long stem, long root and the number of true leaves were counted. The numerical results underwent analysis of variance and the Tukey test ($p < 0.05$) using the R program. The differences observed at $p \leq 0.05$ were considered significant.

3. Results

3.1. Physiological characterization of the yeast strains

From the sequencing of the 77 yeast strains, 10 unique taxa were found. Thus, the strains identified as a given taxon were grouped, yet the physiological characteristics of all 77 strains were determined to verify consistency within taxa. Regarding the assimilation of carbon (Table 1), the strain identified as P. fermentans (B27) had a carbon assimilation profile that was similar to that similar to that described by Kreger-van Rij (1994) for the same yeast specie. Interestingly, none of the strains from the present study were able to assimilate Inositol, and all were able to assimilate Glycerol. The strains grouped into the genus *Kazachstania* assimilated all of the tested carbon sources with the exception of Inositol, L-Rhamnose, and Salicin. Strains grouped in the *Trichosporon* genus differed in their carbon assimilation profiles. Specifically, PP2, PR11, and PR12 were identified as

T. lignícola, yet the strain PR11 did not assimilate D-Arabinose, D-Ribose, Eritritol, or L-Rhamnose while PR12 and PP2 did assimilate these sugars. Regarding *T. dulcitum*, the strain PR27 did not assimilate D-Arabinose, D-Ribose, L-Arabinose, L-Rhamnose or compared to the other strains identified as *T. dulcitum* which did assimilate these sugars (PR1, PR3, PR4,

PR5, PR8, PR10, PR23, PR24, PR25, PR26). There were differences in the assimilation of some carbon sources by strains identified as *T. porosum* (PP4, B2, B3, B5, B6, B16, B21). Specifically, the strain PP4 was capable of assimilating D-Arabinose, D-Ribose, L-Rhamnose, Eritritol and Starch while the other strains weakly assimilated these sugars.

Table 1. Example of assimilation of carbon sources for some yeast strains studied in this work

Species	Cn*	Cs*	Dh*	Ke*	Pf*	Uf*	Td*	Tl*	Tm*	Tp*
Strain	**PR28**	**PR16**	**PP1**	**PP10**	**B27**	**PR9**	**PR10**	**PR11**	**PR29**	**B6**
N°of strains in this work	1	1	9	38	1	5	11	3	1	7
Cellobiose	+	+	+	+	-	+	w	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+
D-Arabinose	w	w	-	+	w	w	(-/+)	(-/+)	+	(-/+)
D-Glucitol	+	+	+	+	-	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+
DL-Lactate	w	w	+	+	+	-	+	+	w	-
D-Ribose	-	-	w	+	-	-	(-/+)	(-/+)	-	(-/+)
D-Xylose	+	+	w	+	+	+	+	+	+	+
Erythritol	-	-	+	+	-	-	+	(-/+)	+	(-/+)
Glycerol	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	w	w	+	w	+	(-/+)	+	+	+
L-Rhamnose	-	-	w	-	-	-	(-/+)	(-/+)	-	(-/+)
L-Sorbose	+	+	w	+	-	+	w	+	+	+
Melezitose	+	+	+	+	-	+	+	+	+	+
Melibiose	+	+	-	+	-	+	(-/+)	+	w	+
Raffinose	+	+	w	+	-	+	+	+	+	+
Ribitol	+	+	+	+	w	+	+	+	+	+
Salicin	+	+	+	-	w	+	+	+	+	w
Starch	+	w	+	+	w	w	+	w	+	(-/+)
Succinate	+	+	+	+	+	+	+	w	+	w

*****Abbreviations name yeast:** *Candida* sp. (Cn), strain PR28; *Candida saitoana* (Cs), strain PR16; *Devaryomyces hansenii* (Dh), strains PP1, B1, B4, B7, B9, B18, B20, B26, B28; *Kazachstania exigua* (Ke), strains PP5, PP6, PP7, PP8, PP9, PP10, PP11, PP12, PP13, PP14, PP15, PP16, PP17, PP18, PP19, PP20, PP21, PP22, PP23, PP24, PP25, PP26, PP27, PP28, PP29, PP30, PR5, PR13, PR14, PR15, PR17, PR18, PR19, PR20, PR21, PR22, PR30, B29; *Pichia fermentans* (Pf), strain B27; *Uncultured fungus* (Uf), strains PR2, PR7, PR9, B10, B11; *Trichosporon ducitum* (Td), strains PR1, PR3, PR4, PR5, PR8, PR10, PR23, PR24, PR25, PR26, PR27; *Trichoporon lignícola* (Tl), strains PP2, PR11, PR12; *Trichosporon miniiliforme* (Tm), strain

The analysis of fermentation (Table 2) indicated that with the exception of *D. hansenni, S. starkeyiihenricii*, and strains grouped in the genus *Trichosporon* (*T. ducitum; T. lignicola; T. miniiliforme* and *T. porosum*), the other strains fermented Glucose. Further to this, species of the genus *Trichosporon* were unable to ferment other carbon sources. The strains PR16 and PR28 showed a weak positive assimilation of urea. Additionally, the strain PR16 positively assimilated nitrate while PR28 was not able to assimilate nitrate. Additionally, the carbon assimilation profile of the strains PR16 and PR28 were similar; both positively assimilated glucose. However these strains differed in the assimilation of nitrogenous compounds.

Table 2. Example of fermentation of carbon sources and assimilation of nitrogen sources for some yeast strains studied in this work.

Species	Cn*	Cs*	Dh*	Ke*	Pf*	Uf*	Td*	Tl*	Tm*	Tp*
Strain	PR28	PR16	PP1	PP10	B27	PR9	PR10	PR11	PR29	B6
N°of strains in this work	1	1	9	38	1	5	11	3	1	7
Fermentation										
D-Galactose	-	-	-	-	-	-	-	-	-	-
D-Glucose	+	+	-	+	+	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-	-	-	-
Fructose	+	-	-	+	w	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-
Starch	+	+	+	+	w	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-
α,α-Trehalose	-	-	-	-	-	-	-	-	-	-
Nitrogen sources assimilation										
Nitrate	-	+	-	-	-	-	+	-	+	-
Nitrite	w	-	+	w	w	w	+	w	-	+
Urea hydrolysis	w	w	+	w	-	+	w	+	-	+

*Abbreviations name yeast: *Candida* sp. (Cn), strain PR28; *Candida saitoana* (Cs), strain PR16; *Devaryomyces hansenii* (Dh), strains PP1, B1, B4, B7, B9, B18, B20, B26, B28; *Kazachstania exigua* (Ke), strains PP5, PP6, PP7, PP8, PP9, PP10, PP11, PP12, PP13, PP14, PP15, PP16, PP17, PP18, PP19, PP20, PP21, PP22, PP23, PP24, PP25, PP26, PP27, PP28, PP29, PP30, PR5, PR13, PR14, PR15, PR17, PR18, PR19, PR20, PR21, PR22, PR30, B29; *Pichia fermentans* (Pf), strain B27; *Uncultured fungus* (Uf), strains PR2, PR7, PR9, B10, B11; *Trichosporon ducitum* (Td), strains PR1, PR3, PR4, PR5, PR8, PR10, PR23, PR24, PR25, PR26, PR27; T*richoporon lignícola* (Tl), strains PP2, PR11, PR12; *Trichosporon miniiliforme* (Tm), strain PR29; *Trichosporon porosum* (Tp) strains PP4, B2, B3, B5, B6, B16, B21. Scored for response to tests: negative = -, positive = +, weakly positive = w, positive or negative for one or more strains within the group = (-/+).

3.2. Molecular identification of yeast strains

Differences were found in the sizes of the amplified yeast ITS-5.8S rDNA regions; amplified fragments ranged in size from 421 to 678 bp. Additionally, ten distinct profiles (i.e. ten taxa) were found from the RFLP analysis with *Cfo*I, *Hae*III, and *Hinf*I (see Table 3).

Of these ten taxa, only four groups, *C. saitoana, D.hansenii, K. exigua* and *P. fermentan*, could be identified to species level by comparing the RFLP profiles to those available in the literature, (Esteve Zarzozo *et al.*, 1999). Figure 1 shows the restriction profiles obtained for the yeast group with the greatest number of strains (38).
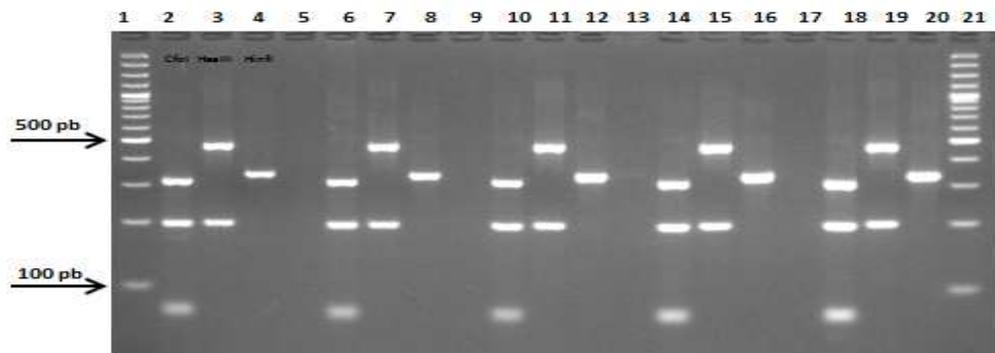

Figure 1. Restriction Enzyme Digestion *Cfo*I, *Hae*III, *Hinf*I for strains PR17 to PR21 (*Kazachtania exigua*). Lane 1; molecular marker; Lanes 2 (*Cfo*I), 3 (*Hae*III), and 4 (*Hinf*I) strain PR17, Lanes 6 (*Cfo*I), 7 (*Hae*III), and 8 (*Hinf*I) strain PR18, Lanes 10 (*Cfo*I), 11 (*Hae*III), and 12 (*Hinf*I) strain PR19, Lanes 14 (*Cfo*I), 15 (*Hae*III), and 16 (*Hinf*I) strain PR20, Lanes 18 (CfoI), 19 (*Hae*III), and 20 (*Hinf*I) strain PR21, Lane 21; molecular marker

In order to identify the unknown strains (those not identified by PCR-RFLP) and to confirm the identity of the others, the PCR products of 77 strains were sequenced and compared to those available in the GenBank database. From this, the six groups unidentified using PCR-RFLP were identified as *C. saitoana, T. ducitum, T. miniiliforme, T. lignícola, T. porosum,* and *S. starkeyiihenricii* with greater than 99% max identity. Additionally, the identity of the strains identified using the RFLP method was confirm with the sequencing. The *in silico* RFLP analysis of the obtained sequences allowed us to identify the fragments that would have been obtained from the endonuclease cutting (Table 3) and to compare

these with the empirically determined fragments (see Figure 1). The in silico restriction profiles for the following species *C. saitoana; D. hansenii; K. exigua; P. fermentan; S. starkeyiihenricii* and *Candida* sp. were clearly unique. This was not the case for species of the genus Trichosporon where, for example, the restriction patterns for *T. dulcitum, T. lignícola* and *T. porosum* were similar. For *T. miniiliforme*, although the *in silico* RFLP profile and profiles generated with the enzymes and cuts with the enzymes *Cfo*I and *Hinf*I were similar to those obtained for the other *Trichosporon* species, there was a clear difference in the restriction pattern obtained with the enzyme *Hae*III where no cut occurred.

Table 3. Identification of yeast strain, size of PCR products and fragments analysis of restriction of the 5.8S-ITS gene.

* Yeast strain	Query cover	%Identity	Identified taxa/Accession GenBank Number	bp	*Cfo*I	*Hae*III	*Hinf*I
Sequencing of the 5.8S-ITS and partial sequence of the 26S rRNA gene				**Analysis RFLP *In* silico the region ITS-5.8S.(rRNA)**			
PR28	100%	97%	*Candida saitoana* **JX188109.1**	616	556+50	385+132+99	303+209+96
PP1	100%	99%	*Devariomyces hansenii* **FR686593.2**	640	295+284+51	421+139+80	316+316
B27	100%	99%	*Pichia fermentans* **GQ458040.1**	447	157+92+88+86+69	334+78+35	250+197
PR9	100%	99%	*Uncultured Fungus* **KF617918.1**	572	304+268	*n.c*	275+173+124
PR10	99%	99%	*Trichosporon ducitum* **FR716597.1**	533	269+264	479+54	229+139+87+61
PR11	99%	97%	*Trichoporon lignícola* **AB180199.1**	533	267+266	479+54	229+149+89+61
PR29	100%	99%	*Trichosporon miniiliforme* **AB180198.1**	530	266+264	*n.c*	287+235
B6	99%	99%	*Trichosporon porosum.* **JX243939.1**	528	265+263	474+54	234+225+89+61
PR16	100%	99%	*Candida sp.* **AJ549823.1**	421	216+205	*n.c*	226+207
PP10	100%	97%	[A]*Kazachstania exigua.* **JQ808008.1**	678	319+201+76+73	476+202	340+337

bp = base pair; *n.c* = not cut site

*Yeast strains isolated in this work.

A: In Barnett *et al.*, (2000) corresponds to *Saccharomyces exiguu*

3.3. Selection of strains with biotechnological potential

After five days of incubation in vinasse, there was cellular growth of all of the strains (77); growth ranged from 0.18 g of yeast /L (strain PR27) to 5.58 g of yeast /L (strain B3). The strain PP12 had the highest amount of total phosphorous (1.55 mg/g of dry yeast), and the greatest amount of total proteins was found in strain PP4 (409 ± 18.5 mg of protein/g of dry yeast). Overall, statistically significant differences (p < 0.05) were found for all three parameters (growth, total phosphorous, and total amount of proteins). The highest values of lipid production were determined for the following strains PP1, PP4, PR4, PR10, PR27 and PR29. As such, these strains were selected as yeast with oleaginous potential. Overall, the statistical analysis indicated that

there were statistically significant differences in production of lipids among strains (p < 0.005). The yeast selected to be cultivated in vinasse had an average of 214 ± 36 mg of lipids / g of dry yeast. The strain with the highest percentage of accumulation of lipids was PP4 (25.8% lipids with respect to its dry weight of 2.46 g/L, see Table 4). According to the molecular identification, he strains selected or growth in vinasse belonged to the genera *Devaryomyces* (PP1) and *Trichosporon* (PP4, PR4, PR10, PR27 and PR29). Two strains, PP1 (*Devaryomyces hansenii*) and PR27 (*Trichosporon dulcitum*) were selected for lipid profile analysis. From this, it was found that 34 % of the fatty acids of PP1 and 33 % of the fatty acids of PR27 (($W_{oleic acid}$ /$W_{fatty acids total}$)*100) were C18:1 oleic acids (cis9).

Table 4, Determination of dry weight, total proteins, total phosphorus and total lipids in strains of yeasts isolated from permanent grassland (PP),), rotating grassland PR and native forest (B) grown in vinasse medium.

Strain	*D.W	*P	*T.P	*T.L	Strain	*D.W	*P	*T.P	*T.L
PR1	1926±28,3	87,5±11,9	0,3±0,004	161,0±14,6	PP10	811±225	135,7±8	0,7±0,00	166,3±2,6
PR2	439±52,3	157,7±12	0,3±0,001	256,8±20,5	PP11	844±158	267,0±4	0,8±0,00	150,3±21
PR3	1680±48,1	204,9±13,6	0,3±0,002	199,4±5,8	PP12	990±14	160,7±21	1,5±0,00	128,2±9
PR4	1485±145	107,8±17,8	0,3±0,00	158,2±7,5	PP13	689±72	228,3±15	1,2±0,00	198,1±1,3
PR5	439±128	121,4±21,5	0,2±0,001	114,1±17,7	PP14	1529±197	170,7±6	0,8±0,00	141,8±5,8
PR6	1633±15,6	122,5±7	0,3±0,003	258,9±16,3	PP15	1333±254	168,0±7	0,6±0,00	110,8±6,5
PR7	168±5,7	121,2±9,5	0,2±0,003	261,0±13,9	PP16	1377±24	150,7±20	0,7±0,00	149,3±4,7
PR8	1584±39,6	91,8±2,1	0,2±0,001	128,5±6,1	PP17	920±63	200,0±8	0,6±0,00	185,0±3,5
PR9	346±25	177,6±4,7	0,5±0,002	217,2±4,7	PP18	1260±8	258,3±6	0,6±0,00	95,6±6,7
PR10	1566±76	135,4±14,5	0,2±0,002	240,0±12	PP19	959±83	185,0±4	0,6±0,00	174,2±4,1
PR11	2175±193	106,4±27,5	0,1±0,002	130,7±13,6	PP20	1069±115	198,3±7	0,5±0,00	120,2±14,3
PR12	2425±123	78,0±5,8	0,2±0,002	248,7±17,8	PP21	876±45	251,3±7	0,9±0,00	317,2±12,1
PR13	1966,5±229	176,1±7,5	0,2±0,004	160,6±11,5	PP22	1111±72	212,7±6	0,6±0,00	108,4±6
PR14	841±7,1	174,7±8,1	0,1±0,006	261,0±7	PP23	1124±175	193,7±23,5	0,3±0,00	232,3±12,5
PR15	994±53,7	81,0±2	0,0±0,002	148,7±1,5	PP24	1177±33	259,0±5	0,5±0,00	148,7±13,2
PR16	1311±43,8	110,1±17,7	0,2±0,003	69,3±17,2	PP25	1407±49	176,7±5	0,7±0,00	107,3±5,2
PR17	1844±263	107,1±16,3	0,1±0,003	211,0±16,3	PP26	1729±144	135,7±15	0,7±0,00	86,4±2,6
PR18	2065±12,7	164,1±13,9	0,4±0,003	287,4±13,9	PP27	951±120	234,3±41	0,5±0,00	204,3±3,1
PR19	1945±43,8	142,1±6,1	0,1±0,003	131,7±6,1	PP28	991±122	93,3±14	1,1±0,00	220,4±14,4
PR20	1566±167	151,6±4,7	0,1±0,003	187,5±4,7	PP29	859±105	243,7±5	0,5±0,00	168,3±12,9
PR21	2237±112	232,2±14,9	0,2±0,003	72,7±14,9	PP30	982±120	273,7±34	0,7±0,00	140,6±13,7
PR22	2357±41	166,2±10,9	0,3±0,003	132,9±10,9	B1	2123±174	198,0±10	1,3±0,00	206,6±15,5
PR23	2782±62	229,9±8,5	0,1±0,003	143,1±8,5	B2	1465±35	168,7±5,1	0,4±0,00	215,8±19,7
PR24	1831±202	258,6±2	0,4±0,003	176,2±15,4	B3	6002±286	135,7±3	0,5±0,00	82,5±6,3
PR25	3314±40	133,7±17,7	0,1±0,003	97,3±14,9	B4	3261±369	121,0±9	0,4±0,00	154,1±5,5
PR26	2042±149	207,5±16,3	0,1±0,003	162,6±10,9	B7	3824±458	114,7±10	0,2±0,00	98,6±6
PR27	2067±10	131,8±13,9	0,2±0,003	287,9±8,5	B8	6106±161	178,0±3	1,0±0,00	199,7±3

	D.W	P	T.P	T.L		D.W	P	T.P	T.L
PR28	2261±61	124,9±6	0,0±0,003	128,8±15,4	**B9**	3262±337	96,3±16	1,0±0,00	253,2±3
PR29	2858±59	226,0±5	0,3±0,003	212,6±3,3	**B10**	1898±26	221,7±12	0,5±0,00	211,4±20
PR30	2170±240	174,1±0,5	0,2±0,003	127,0±11,5	**B11**	1620±447	232,3±3	0,4±0,00	221,8±9
PP1	3924±124	130,5±15	1,4±0,003	227,1±2	**B13**	6300±161	194,3±25	0,3±0,00	181,2±8
PP2	5576±57	189,0±9	0,7±0,003	128,9±14,9	**B16**	3351±337	128,3±4	0,3±0,00	224,1±13
PP3	1229±239	272,0±18	0,6±0,003	199,9±13	**B18**	4488±25	97,3±13	0,7±0,00	81,6±14
PP4	2463±115	409,0±18,5	1,3±0,003	258,2±8,3	**B20**	5686±345	116,3±7	0,6±0,01	182,9±14
PP5	684±31	148,3±12	1,1±0,003	199,6±21,5	**B21**	5591±400	136,3±3	0,2±0,01	170,2±16
PP6	798±168	150,3±18,4	0,9±0,003	285,8±10,7	**B26**	5357±237	141,7±13	0,6±0,01	78,1±12
PP7	759±12	181,3±12	0,5±0,003	296,2±6,2	**B27**	5618±234	369,7±2	1,6±0,00	111,2±9
PP8	1245±100	207,4±14	0,7±0,00	152,2±5,5	**B28**	3449±496	189,3±15	0,6±0,00	162,4±8
PP9	1143±30	190,3±16	0,9±0,00	89,9±2,2					

*Values represent the average of three determinations ± standard deviation. D.W= Dry weight (g/L); P =Total protein (mg/g dry yeast); T.P= Total Phosphorus (mg/g dry yeast); T.L = Total lipid (mg/g dry yeast).

The yeast strains that were tested for potential use as biofertilizers were PP1, PP21, and PR28, which were identified as the following genera *Devaryomyces, Kazachstania*, and *Candida,* respectively. The strain PP1 had 1.4 ± 0.03 mg of total phosphorous/ g of dry yeast and 130.46 ± 0.5 mg of protein/ g of dry yeast. The strain PP21 had 0.87 ± 0.06 mg of total phosphorous/ g dry yeast and 251.3 ± 3.6 mg of protein/ g of dry yeast. The strain PR28 was found to have 4.83 ± 0.01 mg of total phosphorous/ g of dry yeast and 124.93 ± 12.73 mg of protein/g of dry yeast. The potential use of these strains (PP1, PP21, and PP28) as biofertilizers was determined by measuring the morphometric parameters of bell pepper seedlings suitable for transplantation. The highest mean total length (9.53 ± 0.85 cm) was found for bell pepper seedlings grown in the control soil (Figure 2A), and there were significant differences ($p < 0.05$) in the mean total length of these seedlings compared to those grown in guano and with B28 and B1 addition. The highest average stem length (5.66 ± 0.99 cm) was found in seedlings treated with B1 (Figure 2B); there were significant differences ($p < 0.05$) in the average stem length of plants fertilized with guano and with additions of B1, B21, and B28 compared to plants grown in the control soil and those fertilized with NH_4NO_3. The highest mean root length (5.6 ± 1.59 cm) was found in seedlings fertilized with NH_4NO_3 (Figure 2C), and these values differed significantly ($p < 0.05$) from the mean root length of plants fertilized with B1; B21 and B28. Lastly, the highest average number of leaves 3.93 ± 0.70 was recorded for bell pepper seedlings treated with B1.
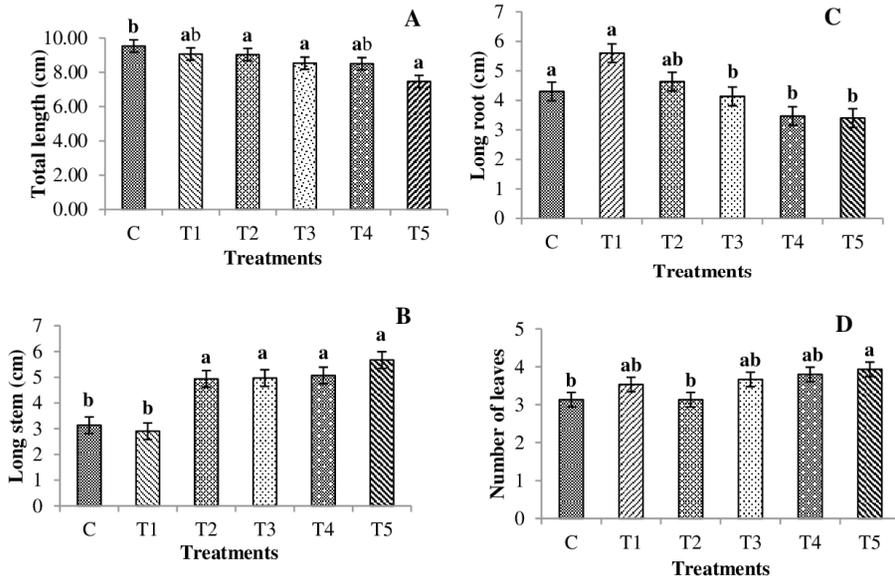
Figure 2. Morphometric parameters determined in bell pepper seedlings. C = control soil. T1 = soil with NH_4NO_3. T2 = soil with guano. T3 = soil with B28. T4 = soil with B21. T5 = soil with B1. Different letters in the columns indicate statistically significant differences (p < 0.05). A=total length in bell pepper seedlings; B= Long stem: The stem is the central part of aboveground portion of a land plant C=Long root: Main root, D= Number of leaves

4. Discussion

This study demonstrates that physiological analysis and molecular characterization using PCR-RFLPs and sequencing are useful tools for the identification of yeast, and this, in turn, can facilitate the development of biotechnological applications for these microorganisms. The assimilation and fermentation profiles obtained from the physiological characterization of these yeasts were similar with those described in the literature for yeast reference strains. For example, the assimilation of carbon by the strain *P. fermentans* (B27) was similar to that found by Caputo *et al*. (2012). Furthermore, all of the strains identified as belonging to the genus *Kazachstania* assimilated the tested carbon sources with the exception of Inositol, L-Rhamnose, and Salicin. These results are similar to those obtained by Liu *et al*. (2008) for *K. exigua*.

The carbon assimilation profiles differed among species from the genus *Trichosporon*. For example, the strain PR27 identified as *T. dulcitum* did not assimilate D-Arabinose, D-Ribose, L-Arabinosa, Ramhnosa, or Melibiosa while the other strains identified as this species (PR1, PR3, PR4, PR5, PR8, PR10, PR23, PR24, PR25, PR26) did assimilate these sugars. Regardless, the carbon assimilation profiles of some *T. dulcitum* reference strains [CBS 8257, CBS 5785, CBS 5786, CBS 7608, CBS 8258 (http://www.cbs.knaw.nl/)] also differ. Differences were also found among the strains identified as *T. porosum* (PP4, B2, B3, B5, B6, B16, B21). Specifically, the strain PP4 positively assimilated D-Arabinosa, D-Ribosa, L-Rhamnose, Erythritol and Starch, while the other strains minimally assimilated these sugars. These results are similar to those presented by Middelhoven *et al*. (2001) for *T. porosum* (reference strains: CBS 2040, 8396, 8397 and 8522) only that in the mentioned study starch is positively assimilated. Colombo *et al*. (2011) suggest that these differences between strains depend on the source from which the strains are isolated.

Regarding the fermentation of carbon sources, the results presented here indicate that all of the studied strains (77) ferment glucose with the exception of *D. hansenni, S. starkeyiihenricii*, and strains identified as belonging to the genus *Trichosporon (T. ducitum, T. lignícola, T. miiniliforme* and *T. porosum)*. With respect to the genus *Candida*, Yan *et al*. (2014) have shown that *Candida mycoderma* obtained from soil from rice cultivation in the Jiangxi province of Chine assimilate nitrogen compounds including KNO_3 and Urea. Similarly, Kurztman *et al*. (2011) indicate that strains from the genus *Candida* can be nitrate positive or negative and usually do not hydrolyze Urea. The strains PR16 and PR28 were weakly positive for Urea. Additionally, the strain PR16 positively assimilated nitrate, while PR28 did not. With respect to the yeast identification, there are many molecular techniques that employ the use of rDNA gene sequences and/ or ITS regions (Honnavar *et al.*, 2016). Recently, the use of mitochondrial DNA (mtDNA) and ribosomal DNA (RFLP) has been widespread (Kopecká *et al*., 2016). Here, both RFLP analysis and ribosomal DNA sequencing were used for the identification of yeast. Differences were found in the sizes of the amplified ITS-5.8S regions of the yeast strains (regions ranged in size from 421 to 678 bp). Taking into account this variability, the 77 strains analyzed here clustered into ten unique groups according to the RFLP profiles. Of these ten groups, four could be identified to species level, and one group could be identified to the genus level (*D. hansenii, P. fermentan, K. exigua, Candida* sp.). Despite this, it has been shown that restriction profiles often do not differ drastically, thus identification to the species level is complicated (Segura *et al*., 2010). This was the case for the restriction profiles for strains identified as belonging to the genus *Trichosporon*.

In order to further resolve these profiles, other restriction enzymes should be used to differentiate the strains. Alternatively, sequencing other DNA regions,

such as intergenic spaces (IGS) located within 26S and 18S, would help resolve species identifications (Romero *et al*., 2005). At the very least, similarity in the restriction patterns of closely related species is to be expected. Middelhoven *et al*. (2001) have shown that the ITS-5.8S is highly homologous in species of the genus *Trichosporon*; thus, this genus is considered phylogenetically monophyletic. Further to this, Segura *et al*. (2010) reports that the RFLP band patterns for the yeast species *Kuyvemyces lactis* and *Kluveromyces marcianus* are very similar, thus correct identification using RFLPs or morphophysiological measurements is complicated. The restriction patterns for the strains identified to the genus *Kazachstania* were similar to those obtained in other studies (for examples see Pham *et al*., 2011 and Esteve-Zarzoso *et al.*, 1999).

Following the identification and characterization of the yeast strains, we found that *K. exigua* was the most abundant species in our samples; this species represented 38% of all of the strains isolated from the Hapludand soils, and it was most prevalent in PP soils. As described by Yurkov *et al*. (2012), yeasts of the genus *Kazaschtania* have been reported in many types of soil including those originating from forests and prairies subjected to different types of management. Likewise, Liu *et al*. (2008) present a new species of the genus *Kazaschtania* from soil from Taiwan. The yeast species that mainly dominated the B soils were *D. hansenii, T. porosum, T. dulcitum* and *S. starkeyihenricii*. In their study conducted in Andian Patagonian forests in Argentina, Mestre *et al*. (2011) have shown that the species *T. dulcitum and T. porosum* actively participate in the decomposition, degradation, and mineralization of plant material. The results obtained here also support the finding that *T. dulcitum*, *T. porosum*, and *Cryptococcus Podzolicus* are involved in the decomposition of complex polysaccharides and some aromatic compounds (Yurkov *et al*., 2012).

Additionally, it has been described that yeast belonging to the following genera *Candida, Cryptococcus, Rhodotorula, Sporobolomyces, Trichosporon, Williopsis* and *Yarrowi*a are capable of directly increasing plant growth (Botha, 2011). Strains identified as belonging to the genus *Kazachstania* were most abundant in the PP soil (87% of the strains isolated from the PP samples). This is consistent with results found by Yurkov *et al*. (2012) that show that with increased intensity of soil management there is a greater proportion of Ascomycete yeast. Overall, two possible biotechnological applications, the production of lipids and use as biofertilizers, have been identified for the yeast isolated in the present study (see Table 4). Specifically, lipid-producing yeast could present an alternative for the production of biodiesel. In the present study, the strains PP1, PP4, PR4, PR10, PR27 Y PR29 had the highest values of lipid production.

According to the molecular identification, these strains are classified as *D. hansenn*i (PP1) and belonging to the genus *Trichosporon* (PP4, PR4, PR10, PR27 and PR29). Interestingly, Breuer and Harms (2006) have indicated that *D. hansenni* is metabolically versatile yeast that is osmotolerant, non-pathogenic, and has the capacity to accumulate lipids. Thus, these authors indicate that this microorganism is attractive from a biotechnological viewpoint. Upon cultivating a new strain of *Devaryomyces etchellsii* in limited nitrogen conditions, Arous *et al*. (2015) found an increase in lipids from the vegetative stage (11.9%) to ascoporogenesis (22.4%). Here it was found that the strain PP1 identified as *D. hansenni* was able to accumulate 22.2% of lipids when grown in vinasse. Some studies have also noted the ability to accumulate lipids by species of the genus *Trichosporon*. For example, Gao *et al*. (2014) found a high accumulation of lipids 12.3g of lipids/ L of hydrolyzed lignocellulose with respect to dry cellular biomass (38.4 g) in *Trichosporon cutaneum* ACCC 20271 when grown in a corncob media

(lignocellulose residue). These values are quite high considering that the cultivation media was a lignocellulose residue, which are known to contain inhibitors (see Kumar *et al*., 2017).

In the present study, after six days of incubation in vinasse, 11.3 g of yeast /L of vinasse were obtained, and the yeast was composed of 30% lipids. Another study evaluating the yeast *Trichosporon oleaginosus* grown in sewage treatment sludge (thermally and chemically pretreated) found a lipid content of 39% (Zhang *et al*., 2014). Here the highest lipid content obtained for strains of the genus *Trichosporon* was found for the strains PP4 and PR27 (25.8 and 21.6%, respectively). Finally, two strains were selected for lipid profile analysis, these included PP1 identified as *D. hansenni* and PR27 identified as *T. dulcitum*. From this, it was found that strain PP1 had 34% (($W_{oleic acid}$ /$W_{total fatty acids}$)*100) oleic acid C18:1 (cis9) while strain PR27 had 33% oleic acid. The composition of these oils indicates that they are suitable as raw material for the production of biodiesel (Soccol *et al*., 2017). The strains PP1 (*D. hansenii*), PP21 (*K. exigua*), and PR28 (*Candida* sp.) were tested for potential use as biofertilizers, and interestingly it has reported that yeast of these genera are promoters of plant growth due to their production of different biomolecules. For example, Fu *et al*. (2016) have isolated 15 species of growth promoting yeast from the phylosphere and rhizosphere of the plant *Drosera spatulata* Lab., and these 15 species include *Kazachstania jiainicus* (FJ196732) and *Candida akabanensis* (EU100744). Yet other studies demonstrate the capacity of isolated yeast, including *Kazachstania siamensis* LM002 and *Candida sirachaensis* LM068, to biosynthesize, Indole-3-Acetic Acid (IAA) (43.3 mg/L IAA and 31.9 mg/L IAA, respectively). Furthermore, Narsian *et al*. (2010) isolated 25 yeast strains and six of these belonging to the genera *Saccharomyces; Hansenula; Klockera; Rhodotorula* and *Debaryomyces* had the highest solubilization indices (ranging from 1.33 to 1.50) when the yeasts were examined *in vitro* with tricalcium phosphate and rock phosphate.

Other studies evaluating the effect of the solubilization of phosphates on the growth of plants have found that inoculation with phosphate solubilizing microorganisms (bacterium) produces increase biomass compared to that found for plants that were not inoculated (Sharon *et al*., 2016). Here it was found that adding yeast to bell pepper seedlings mainly promoted an increase in the number of leaves and an increase in root size produced by the plants (Figure 2). Interestingly, others have found similar effects on root size and number of lateral roots for bean seedlings (Gupta *et al*., 2016).

5. Conclusion

This study has provided initial information on yeast associated with soil with different agricultural management in the region of Los Ríos, Chile. The PCR-RFLP methodology was found to be an appropriate technique (fast and reliable) for the identification of yeast. Despite this, not all RFLP profiles were resolvable, thus not all identifications could be performed. Regardless, when complemented with the sequencing of yeast DNA, the identification of the strains was achieved, and sequencing also confirmed the identifications using the PCR-RFLP methodology. In addition, the physiological characteristics of native yeasts of southern Chile are presented here. Thus, a preliminary evaluation of their biotechnological potential as biofertilizers or for lipid production is given; however, future more detailed studies are warranted to better determine the application of these yeasts to industry.

References

Arous, F., Triantaphyllidou, I., Mechichi, T., Azabou, S., Nasri, M., Aggelis, G. 2015. Lipid accumulation in the new oleaginous yeast *Debaryomyces etchellsii* correlates with ascosporogenesis. Biomass and Bioenergy. 80, 307-315.

Barnett, A.R., Payne, W., Yarrow, D. 2000. Yeast: Characteristics and Identification, Cambridge. Cambridge University Press: 49-77

Breuer, U., Harms, H. 2006. *Debaryomyces hansenii* — an extremophilic yeast with biotechnological potential. Yeast. 23, 415-437.

Caputo, L., Quintieri, L., Baruzzi, F., Borcakli, M., Morea, M. 2012. Molecular and phenotypic characterization of *Pichia fermentans* strains found among Boza yeasts. Food Research International. 48, 755-762

Chang, Y., Chang, K.S., Hsu, C.L., Chuang, L.T., Chen, C.Y., Huang, F.Y., Jang, H.D. 2013. A comparative study on batch and fed-batch cultures of oleaginous yeast *Cryptococcus* sp. in glucose-based media and corncob hydrolysate for microbial oil production. Fuel. 105, 711-717.

Colombo, A., Padovan, A., Chaves, G. 2011. Current Knowledge of *Trichosporon* spp. and Trichosporonosis. Clinical Microbiology Reviews. 24, 682–700.

El-Tarabily, K., Sivasithamparam, K. 2006. Potential of yeasts as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. Mycoscience. 47, 25-35.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337.

Fernández-González, M., Espinosa, J.C., Úbeda, J.F., Briones, A.I. 2001. Yeasts present during wine fermentation: comparative analysis of conventional plating and PCR TTGE. Syst. Appl. Microbiol. 24, 634–638.

Fu, S.F., Sun, F.P., Lu, H.Y., Wei, J.Y., Xiao, H.S., Fang, W.T., Cheng, B.Y., Chou, J.Y. 2016. Plant growth-promoting traits of yeasts isolated from the phyllosphere and rhizosphere of *Drosera spatulata* Lab. Fungal Biology. 120, 433-448.

Gao, Q., Cui, Z., Zhang, J., Bao, J. 2014. Lipid fermentation of corncob residues hydrolysate by oleaginous Yeast *Trichosporon cutaneum*. Bioresource Technology. 152, 552-556.

Gupta, H., Saini, R.V., Pagadala, V., Kumar, N., Sharma, D.K., Saini, A.K. 2016. Analysis of plant growth promoting potential of endophytes isolated from *Echinacea purpurea* and *Lonicera japonica*. Journal of Soil Science and Plant Nutrition. 16, 558-577.

Honnavar, P., Prasad, G.S., Ghosh, A., Dogra, S., Handa, S., Rudramurthy, S.M. 2016. *Malassezia arunalokei* sp. nov., a Novel Yeast Species Isolated from Seborrheic Dermatitis Patients and Healthy Individuals from India. Journal of Clinical Microbiology. 54, 1826–1834.

Ignatova, L., Yelena, V., Brazhnikova, V., Berzhanova, R., Mukasheva, T. 2015. Plant Growth-Promoting and Antifungal Activity of Yeasts from Dark Chestnut Soil. Microbiol. Res. 175, 78–83.

Kopecká, J., Němec, M., Matoulková, D. 2016. Comparison of DNA-based techniques for differentiation of production strains of ale and lager brewing yeast. J. Appl. Microbiol. 120, 1561-1573.

Kumar, D., Bhaskar, S., Korstad, J. 2017. Utilization of Lignocellulosic Biomass by Oleaginous Yeast and Bacteria for Production of Biodiesel and Renewable Diesel. Renew. Sus. Energ. Rev. 73, 654–71.

Kupper, K.C., Bettiol, W., de Goes, A., de Souzac, P.S., Bellotte, J.A.M. 2006. Biofertilizer for control of *Guignardia citricarpa*, the causal agent of citrus black spot. Crop Protection. 25, 569–573.

Kurztman, C.P., Fell, J.W., Boekhout, T. 2011. The yeasts a taxonomic study. The Fifth Edition. Elsevier.

Ledesma-Amaro, R., Nicaud, J.M. 2016. *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. Progress in Lipid Research. 61, 40-50.

Liu, Y.R., Liu, C.H., Young, S.S., Lee, C.F. 2008. New Records of *Kazachstania* Species in Taiwan. Taiwania. 53, 293-300.

Mestre, M., Rosa, C., Safar, S., Libkind, D., Fontenla, S. 2011.Yeast communities associated with the bulk-soil, rhizosphere and ectomycorrhizosphere of a *Nothofagus pumilio* forest in northwestern Patagonia, Argentina. Microbiology Ecology. 78, 531–541.

Middelhoven, W.J., Scorzetti, G., Fell, J.W. 2001. *Trichosporon porosum* comb. nov., an anamorphic basidiomycetous yeast inhabiting soil, related theloubieri/laibachii group of species that assimilate hemicelluloses and phenolic compounds. FEMS Yeast Research. 1, 15-22

Narsian, V., Samaha, S.M.A.A., Patel, H.H. 2010. Rock phosphate dissolution by specific yeast. Indian J. Microbiol. 1, 50-57.

Pham, T., Wimalasena, T., Box, W.G., Koivuranta, K., Storgårds, E., Smart, K.A., Gibson, B.R. 2011. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery 'wild' yeast contaminants. J. Inst. Brew. 117, 556–568.

Polburee, P., Yongmanitchai, W., Lertwattanasakul, N., Ohashi, T., Fujiyama, K., Savitree, L., 2015. Characterization of oleaginous y easts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol. Fungal Biology. 119, 1194-1204.

Rakicka, M., Lazar, Z., Dulermo, T., Fickers, P., Nicaud, J.M. 2015. Lipid production by the oleaginous yeast *Yarrowia lipolytica* using industrial by-products under different culture conditions. Biotechnology for Biofuels. 8, 104.

Romero, P., Patiño, B., Quirós, M., González-Jaén, M.T., Valderrama, M.J., de Silóniz, M.I., Peinado, J.M. 2005. Differential detection of *Debaryomyces hansenii* isolated from intermediate-moisture foods by PCR-RFLP of the IGS region of rDNA. FEMS Yeast Research. 5, 455-461.

Segura, L., Kirchmayr, M., Flores, E., Gschaedler, A. 2010. PCR-RFLP of the ITS-5.8S regions as an identification tool for yeasts: advantages and disadvantages. e-Gnosis. 8, 1-12.

Sharon, J.A., Hathwaik, L.T., Glenn, G.M., Imam, S.H., Lee, C.C. 2016. Isolation of efficient phosphate solubilizing bacteria capable of enhancing tomato plant growth. Journal of Soil Science and Plant Nutrition. 16, 525-536.

Slavikova, E., Vadkertiova, R. 2000. The occurrence of yeasts in the forest soils. J. Basic Microbiol. 40, 207–212.

Slavikova, E., Vadkertiova, R. 2003. The diversity of yeasts in the agricultural soil. J. Basic Microbiol. 43, 430–436.

Soccol, C.R. et al. 2017. Pilot Scale Biodiesel Production from Microbial Oil of *Rhodosporidium Toruloides* DEBB 5533 Using Sugarcane Juice: Performance in diesel engine and preliminary economic study. Bioresource Technology. 223, 259-268.

Wuczkowski, M., Prillinger, H. 2004. Molecular identification of yeasts from soils of the alluvial forest national park along the river Danube downstream of Vienna, Austria ("National park

donauauen''). Microbiological Research. 159, 263–275.

Yan, M., Yu, L., Zhang, L., Guo, Y., Dai, K., Chen, K. 2014. Phosphatase activity and culture condition-sof the yeast *Candida mycoderma* sp. and analysis of organic phosphorus hydrolysis ability. Journal of Environmental Sciences. 26: 2315-2321.

Yurkov, A., Kemler, M., Begerow, D. 2012. Assessment of yeast diversity in soils under different management regimes. Fungal Ecology. 5, 24-35.

Zhang, X., Yan, S., Tyagi, R., Surampalli, R.Y., Valéro, J. 2014. Lipid production from *Trichosporon oleaginosus* cultivated with pre-treated secondary wastewater sludge. Fuel. 134, 274–282.