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ARTICLE

Phylogenetic analysis of isolated biofuel yeasts based on 5.8S-ITS rDNA and D1/D2 26S rDNA sequences

Abd El-Latif Hesham [a,*], Virginia Wambui [b,e], Henry Ogola J.O. [c], Julius M. Maina [d]

[a] *Genetics Department, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt*
[b] *Kenya Industrial Research and Development Institute, P.O. Box 30650-00100, Nairobi, Kenya*
[c] *School of Agriculture, Food Security and Biodiversity, Bondo University, P.O. Box 210-40601, Bondo, Kenya*
[d] *Department of Food Science, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00100, Nairobi, Kenya*
[e] *Institute of Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00100, Nairobi, Kenya*

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Abstract The utilization of agro-industrial wastes such as whey as raw materials for the production of bio-ethanol is gaining importance as a result of the attractiveness of renewable fuel alternatives due to exhaustion of fossil fuel sources coupled with the positive impact to the environment. Here, we report the isolation of two *Kluyveromyces* spp. designated as BM4 and P41, able to produce ethanol as main fermentation product from fermenting whey. Three different molecular biological approaches including, the RFLP analysis of the 5.8S-ITS rDNA, the sequence of the 5.8S-ITS rDNA region and the sequence of the D1/D2 domain of the 26S rRNA gene were applied for accurate identification. While RFLP analysis of 5.8S-ITS region failed to accurate the differentiation between the two species, sequencing of this region and D1/D2 region of the 26S rRNA gene verified the identification. PCR amplification and sequence analysis of 5.8S-ITS rRNA and D1/D2 domain of the 26S rRNA genes revealed that the isolates BM4 and P41 were highly related to *Kluyveromyces marxianus* and *Kluyveromyces lactis* with homology of 99% for both. In addition, phylogenetic analysis indicated that both BM4 and P41 shared a cluster with *K. marxianus* and *K. lactis*, respectively. The fermentative performance of both strains on cheese whey to produce

* Corresponding author. Tel.: +20 1062069551; fax: +20 88 2331384.
E-mail address: hesham_egypt5@yahoo.com (A.E.-L. Hesham).

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ethanol was evaluated at different parameters such as incubation temperature, initial pH, whey sugar concentrations, and yeast concentrations. Results show that the maximum ethanol productions achieved at pH 4.5 and 35 °C were 5.52% and 5.05% for *K. marxianus* and *K. lactis*, respectively. Our results demonstrated that *K. marxianus* and *K. Lactis* could be recommended for cheese whey bioremediation in the environment and produce renewable biofuel.

1. Introduction

Currently, bio-ethanol is being vigorously promoted globally as a renewable and environmentally friendly alternative energy resource to fossil-based fuel. Ethanol (ethyl alcohol and bio-ethanol) is the most employed liquid biofuel in terms of volume and market value, either as a fuel or as a gasoline enhancer [28,40]. The massive utilization of fuel ethanol in the world requires its production technology to be cost-effective and friendly to environment. However, the current first generation process of bio-ethanol production is based on fermentation of starch and sugar from maize and sugar cane, which only constitutes about 1–2% of the total plant biomass. The merits of resource-intensive crops to bio-ethanol process in terms of net emissions, cost, and energy balance also remain under debate [14,28,40]. In addition, the "fuel vs. food" debate suggests that growing food crops for biofuel negatively impact the world's poorest populations has also risen [9,46]. This has led to renewed momentum in the search for new and cost-effective alternative non-food sources or second-generation processes feedstock for bio-ethanol production.

Cheese whey, as the main dairy by-product, is increasingly becoming an attractive source of many bioactive valuable compounds [22]. It is characterized by abundant amounts of lactose (ca. 5% w/v) and other milk nutrients, which represents a significant environmental problem as a result of its high biological demand. Consequently, due to the large lactose surplus generated, its conversion to bio-ethanol has long been considered as a possible solution for whey bioremediation. The fermentation of whey lactose using yeasts has been frequently reported in the literature [1,10,18,42] and the ability to metabolize lactose to ethanol has been demonstrated in *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Candida pseudotropicalis* [5,16]. Presently, there are a few established industrial processes to produce ethanol from whey utilizing these yeast strains, which has been done in some countries [41,44]. But there occur several challenges and limitations in the process of utilization of whey lactose to ethanol. Yeast strains exhibiting lactose fermenting ability are still rare in nature [16], and there is an urgent research need to isolate, identify and develop novel microbial strains that can ferment lactose to ethanol with very high efficiency to increase the industrial attractiveness of whey-to-ethanol bio-processes.

Yeasts are important components of the microflora of many food products due to their ability to grow on a substrate rich on proteins, lipids, sugars and organic acids [34]. Furthermore, yeasts have great advantage due to their robustness with a wide range of physiochemical tolerance. For yeast identification and characterization, a combination of classical culture-dependent, biochemical and genotyping techniques is used. In particular, molecular fingerprinting techniques have gained importance due to their improved speed and accuracy in identification due to their established and comprehensive databases for comparisons of yeast strains [13,47].

Research studies by Cai et al. [6] and James et al. [30], have demonstrated that the complex ITS (internal transcribed spacer) regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved), are useful in measuring close fungus genealogical relationships. As ribosomal regions have evolved in a concerted fashion, they generally show a low intraspecific polymorphism and a high interspecific variability [36]. Therefore, the use of two universal and two species-specific primers derived from the D1/D2 region of the 26S rDNA and subsequent sequencing of this domain has facilitated rapid and accurate species identification [23–25,29,45], including *Saccharomyces* species [27], *Kluyveromyces* species [3] and a small collection of wine yeast species [21].

Accordingly, the present study aimed to isolate and characterize whey lactose fermenting yeasts using API® 20C AUX Kit, and sequencing of the domains D1/D2 of the 26S rRNA gene and the 5.8S-ITS rDNA region for the discrimination and identification of yeast isolates. In addition to, the ability of two selected isolates (BM4 and P41) for ethanol production as renewable biofuel from whey was examined.

2. Materials and methods

2.1. Sampling, isolation and characterization of yeast strains

The raw whey, wastewater and swab samples for the study were obtained from Browns Cheese Industry Ltd. in Nairobi, Kenya and transported under cold storage at 4 °C in autoclaved sterile Borosil bottles for laboratory analysis. Collected samples were used for isolating yeast on YEPL (yeast extract peptone lactose) agar medium containing yeast extract 1.0%, peptone 2.0%, dextrose 2.0%, pH 5.5 supplemented with 50 mg chloramphenicol. Plates were incubated at 30 °C for 48 h, then different colonies were picked up on the basis of colony shape and color and purified by streaking 3 times on YEPL media. The isolates were characterized based on their physiological and morphological properties according to Barnett et al. [2]. The tests included assimilation of carbon compounds, growth at 25, 30, 37, 42 and 47 °C and growth at pH 3, 4, 4.5, 5.5 and 6.

2.2. DNA isolation and PCR amplification of the 5.8S-ITS rDNA region

The yeast isolates were sub cultured on YEPL media for 28 h at 30 °C and then DNA extraction was carried out using a GentraPuregene® Yeast/Bacteria kit (Qiagen, USA) according to manufacturer's instructions. ITS1-5.8S-ITS2 rDNA region was amplified using the following primer pair: forward ITS-1

(5′-TCC GTA GGT GAA CCT GCG G-3′), and reverse ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) [48]. Amplification was carried out in 50 μl reaction mixture containing 25 μl Taq Master mix (Roche, USA), 18 μl PCR water, 1 μl of each primer (0.5 μM), and 1 μl (200 ng) DNA template. The PCR condition was: 40 cycles including an initial denaturation at 95 °C for 4 min, subsequent denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 2 min followed by final extension at 72 °C for 7 min and holding at 4 °C. 10 μl of PCR products was then analyzed using 1.5% 0.59 TBE agarose gel electrophoresis. The gel was stained with ethidium bromide, visualized under UV light, and photographed. Approximate sizes of amplicons were determined using a standard molecular weight marker 100-bp DNA ladder (Qiagen, USA).

2.3. RFLPs of 5.8S-ITS rDNA

PCR products of the 5.8S-ITS region were digested without further purification with the restriction endonucleases *Hae*III and *Hinf*I (Roche, USA) according to manufacturer's instructions. Restriction fragments were separated on 3% agarose gels, in 0.5% TBE buffer for 2 h at 80 V. Band sizes were estimated by comparison against 50-bp DNA ladder (Qiagen, USA).

2.4. PCR amplification of D1/D2 domain of 26S rDNA region

The D1/D2 domain of 26S rDNA region was amplified using the primers NL1 (5′-GCATATCAATAAGCGGAGGAA AAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) [25,33]. PCR was performed in a final volume of 50 μl as mentioned above. The amplification was carried out by PCR under the following conditions: initial denaturation at 95 °C for 5 min, followed by 36 cycles at 94 °C for 2 min, 52 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 7 min, holding at 4 °C. 5 μl of PCR products was then analyzed using 1.5% 0.59 TBE agarose gel electrophoresis. The gel was stained with ethidium bromide, visualized under UV light, and photographed.

2.5. Sequencing of 5.8S-ITS rDNA and D1/D2 26S rDNA regions

Amplified PCR of 5.8S-ITS and partial D1/D2 26S rDNA products were purified and directly sequenced using the Big-Dye Terminator v3.1 Cycle Sequencing kit in an ABI 3730 automated sequencer. PCR primers NL-1 and NL-4 of the D1/D2 26S rRNA gene and ITS1 and ITS4 of 5.8S-ITS region were used in the sequencing reactions to read both DNA strands.

2.6. Alignment and phylogenetic analysis

The sequences obtained were aligned with known 26S rDNA and 5.8S-ITS sequences in the Genbank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), and percent homology scores were generated to identify yeast isolates. Phylogenetic trees were constructed with MEGA version 4.0 using a neighbor-joining algorithm, plus the Jukes–Cantor distance estimation method with bootstrap analyses for 1000 replicates was performed [27].

2.7. Whey fermentation, ethanol production and HPLC analysis

The raw material whey was obtained from Browns cheese industry in Nairobi-Kenya, and characterized in terms of its total carbohydrates, proteins, minerals and pH. Batch fermentation [1,19] was carried out using the collected industrial whey adjusted to a pH of 4.5 and the two yeast isolates BM4 and P41 previously sub-cultured on YEPL media at 30 °C for bio-ethanol production.

The effect of various parameters such as incubation temperature (30, 35 and 40 °C), initial pH (4.5 and 6), whey sugar concentrations (10%, 12% and 15% brix), and yeast concentrations (10% and 20% w/v) on ethanol production was examined [19]. The amount of ethanol in the fermented whey samples was determined in the 10 AT High Performance liquid Chromatography (HPLC, Shimadzu Corp., Kyoto, Japan) equipped with 10 A refractive index detector (Shimadzu Corp., Kyoto, Japan) using discovery® HSC18 (Supelco, USA) reverse phase column at a temperature of 30 °C, and 0.005 M H_2SO_4 as a mobile phase at a flow rate of 0.7 ml/min with a refractive index detector and control temperature of 40 °C. Samples were filtered with a 0.45 mm membrane filter prior to injection into the machine with 1%, 2%, 4%, 6% and 10% ethanol (Scharlab S.L., Spain) as the control.

2.8. Nucleotide sequence accession number

The sequences of the 5.8S-ITS rDNA region and the D1/D2 domains of the 26S rRNA gene of strains MB4 and P41 reported in this study have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession Nos. KC987956 and KC987957 for 5.8S-ITS rDNA and KC512906 and KC512908 D1/D2 domains of the 26S rRNA gene, respectively.

3. Results and discussion

3.1. Yeast isolation, screening and phenotypic characterization

A total of 28 yeast isolates were isolated from Kenyan dairy products (pH ~5.2) and were screened for their ability to ferment cheese whey. Two isolates exhibiting higher whey lactose-to-ethanol fermentation ability, designated as BM4 and P41, were selected for further analysis. Initially, the yeast strains were cultured for the characterization on the basis of methods summarized according to Barnett et al. [2]. While both strains were able to grow best at pH 4.5–6.0, BM4 was characterized by an optimum growth temperature of 30–37 °C in contrast to strain P41 that had an optimum growth at 25–30 °C. Strain BM4 was able to assimilate all the carbon sugars which included, glucose, glycerol, calcium 2-keto gluconate, L-arabinose, D-xylose adonitol, xylitol, D-galactose, inositol, D-sorbitol, methyl-D-glucopyranoside, *n*-acetyl-glucosamine, D-cellobiose, D-lactose, D-maltose, D-saccharose, D-trehalose, D-melezitose and D-raffinose, while the Strain P41 was able to assimilate the sugars glucose, glycerol, calcium 2-keto gluconate, L-arabinose, D-xylose, D-galactose, D-sorbitol, *n*-acetyl-glucosamine, D-cellobiose, D-lactose, D-maltose, D-saccharose, D-trehalose, D-melezitose and D-raffinose, but was not able to assimilate adonitol, xylitol, inositol and methyl α-D-glucopyranoside.

Morphological traits and physiological abilities, on which identification and characterization of yeast species and strains were based, are not reliable and may give false results [2,21].

3.2. Molecular genetic characterization

3.2.1. PCR-RFLP Analysis of rDNA

PCR products of the5.8S-ITS rDNA regions for the isolates MB4 and P41 were approximately 750 and 740 bp long, respectively. The digestion of the PCR products with the enzymes *Hae*III, and *Hinf*I yielded different fragments which ranged in size from 80 to 650 bp and from 80 to 250 bp for strain BM4 by using *Hae*III and *Hinf*I, respectively, while for the strain P41 the results ranged from 80 to 655 bp for *Hae*III and from 65 to 290 bp for *Hinf*I (Table 1). These results failed to accurate the discrimination between the two isolates, BM4 and P41, which were identified as *K. marxianus* and *K. Lactis* after comparing the molecular mass of the restriction products with those previously described in the literature [12,13] as well as matched the restriction patterns to different yeast species using the http://www.yeast-id.com database.

ITS-PCR has been used for the taxonomic study and rapid identification of yeasts in dairy products, wine and other foods [12,17,35]. Several authors have proven that the RFLPs of 5.8S-ITS rDNA are identical between strains within the same species [11,13,21], although other authors have also found different patterns within the same species due to heterogeneous 5.8S-ITS rDNA regions [4,13]. Therefore, we confirmed the identification of the isolates by sequencing of 5.8S-ITS rDNA and D1/D2 26S rDNA regions.

3.2.2. Ribosomal DNA sequence analysis and phylogenetic tree construction

In order to confirm the correct affiliation of these two strains, 5.8S-ITS rDNA and D1/D2 26S rDNA regions were sequenced and compared with the sequences of 5.8S-ITS rDNA and 26S rDNA available in the GenBank for each by means of BLAST search of the National Center for Biotechnology Information (NCBI) databases. Alignment results of the rDNA sequences of these isolates show that the sequences of strain MB4 were found to have 99% similarity with the 5.8S-ITS rDNA and D1/D2 26S rDNA sequences of strain *K. marxianus*, respectively. While the sequences of strain P41 were found to have 99% similarity with the 5.8S-ITS rDNA and D1/D2 26S rDNA sequences of strain *K. lactis*, respectively.

To confirm the position of each strain in phylogeny, a number of sequences were selected from the Genbank database for the construction of a phylogenetic tree using the MEGA4 program. As shown in Figs. 1 and 2, the phylogenetic tree of 5.8S-ITS rDNA and D1/D2 26S rDNA sequences indicated that the strain BM4 and *K. marxianus* shared one clade cluster. Therefore, the strain BM4 was identified as *K. marxianus*. For strain P41, the phylogenetic tree of 5.8S-ITS rDNA and D1/D2 26S rDNA sequences indicated that the strain and *K. lactis* were in the same clade cluster. Therefore, the strain P41 was identified as *K. lactis* (Figs. 3 and 4).

The molecular methods based on the PCR amplification and partial sequencing of the ITS region of the rDNA and the D1/D2 domain of the large sub-unit 26S of the rDNA have been used to identify the yeasts isolated from different sources. It was found that these molecular methods are rapid and precise compared with the physiological method for the yeast identification, and have also been applied to study the phylogeny of different yeast groups [7,8,24–27,33,37,38].

3.3. Ethanol production by K. marxianus strain BM4 and K. lactis strain P41

Ethanol was produced from whey using selected yeast strains at different operating conditions, i.e., pH (4.5 and 6), temperature (30, 35 and 40 °C), yeast concentration (10% and 20%) and sugar levels (10%, 12% and 15%). The ethanol concentrations were recorded as shown in Table 2. The results showed that the optimal pH conditions for the yeast strains were 4.5 with ethanol concentrations of 5.25 and 5.05%, for BM4 and P41, respectively (Table 2, Fig. 5). High ethanol concentration was obtained at 10% brix, 30 °C temperature and 10% yeast concentration. Results in Table 2 revealed that variation in temperature and yeast concentration did not yield high ethanol concentration in comparison to variation in sugar concentration.

A pH of 4.5 was the best parameter for fermentation whey to produce bio-ethanol, whereas ethanol yields were 5.25% and 5.05% for strains, BM4 and P41, respectively. This was also observed by Kadar et al. [31], where the highest ethanol per gram of sugar (0.51 g ethanol/g lactose) was achieved in the experiment using whey type 1 which was fermented at 30 °C and at 4.5 pH, this is because the low pH in the whey forces the yeast to use energy pumping out H ions out of the cell instead of using the energy on biomass formation, consequently giving a higher ethanol yield because more lactose is used for production of energy instead of formation of biomass.

Strains of *Kluyveromyces* spp. have been considered the most appropriate for bio-conversion of lactose in whey [15,43]. However, incomplete or slow fermentations have been observed for many *Kluyveromyces* strains when concentrated whey or lactose-enriched substrates have been employed. These effects have been attributed to the toxicity of the ethanol produced and/or to inhibition by high salt concentrations, resulting in elevated osmotic pressure [20].

Table 1 Sizes of amplified products of the 5.8S ITS regions and restriction fragments (bp) from the isolates with endonuclease *Hae*III *and Hinf*I.

Isolates code	PCR sizes	Restriction fragments (bp)		Identification
		*Hae*III	*Hinf*I	
MB4	750	650 + 80	250 + 190 + 120 + 80	*Kluyveromyces marxianus*
P41	740	655 + 80	290 + 180 + 120 + 80 + 65	*Kluyveromyces Lactis*

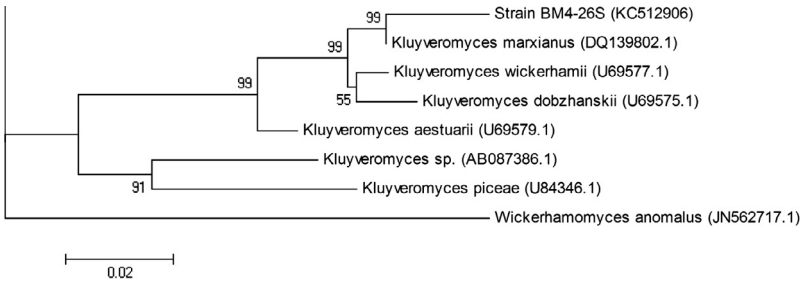

Figure 1 Phylogenetic relationship between *Kluyveromyces marxianus* strain BM4 and other 26S rRNA sequences of published strains. In the phylogenetic tree, BM4 and *Kluyveromyces marxianus* were clustered together as one clade segments corresponding to an evolutionary distance of 0.02 are shown with bars. Numbers above branches are bootstrap values, shown greater than 50%. GenBank accession numbers are given in parentheses. *Wickerhamomyces anomalus* was used as an outgroup.

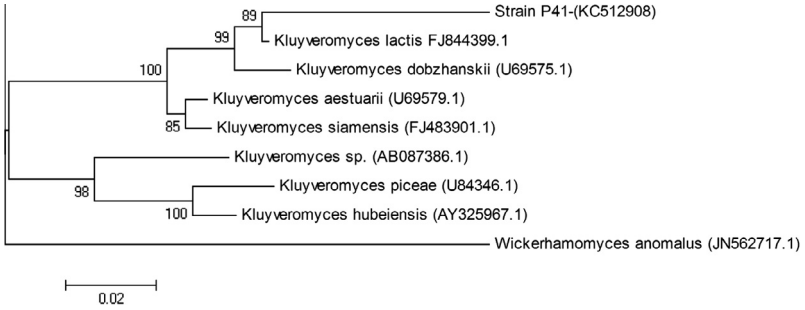

Figure 2 Phylogenetic relationship between *Kluyveromyces lactis* strain P41 and other 26S rRNA sequences of published strains. In the phylogenetic tree, P41 and *Kluyveromyces lactis* were clustered together as one clade segments corresponding to an evolutionary distance of 0.02 are shown with bars. Numbers above branches are bootstrap values, shown greater than 50%. GenBank accession numbers are given in parentheses. *Wickerhamomyces anomalus* was used as an outgroup.

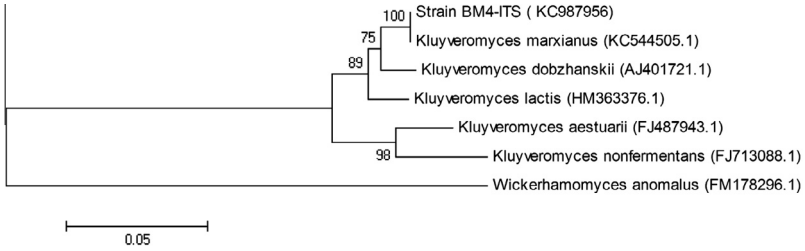

Figure 3 Phylogenetic relationship between *Kluyveromyces marxianus* strain BM4 and other ITS1–5.8S-ITS2 rDNA sequences of published strains. In the phylogenetic tree, BM4 and *Kluyveromyces marxianus* were clustered together as one clade segments corresponding to an evolutionary distance of 0.05 are shown with bars. Numbers above branches are bootstrap values, shown greater than 50%. GenBank accession numbers are given in parentheses. *Wickerhamomyces anomalus* was used as an outgroup.

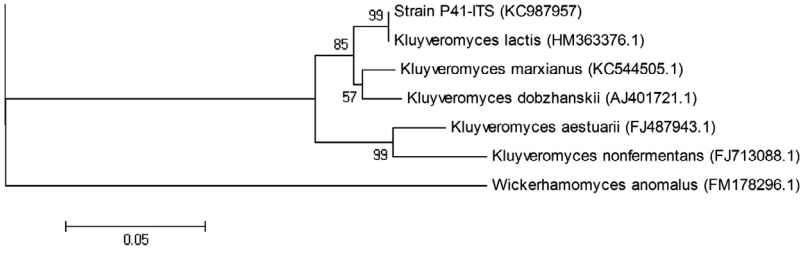

Figure 4 Phylogenetic relationship between *Kluyveromyces lactis* strain P41 and other ITS1–5.8S-ITS2 rDNA sequences of published strains. In the phylogenetic tree, P41 and *Kluyveromyces lactis* were clustered together as one clade segments corresponding to an evolutionary distance of 0.05 are shown with bars. Numbers above branches are bootstrap values, shown greater than 50%. GenBank accession numbers are given in parentheses. *Wickerhamomyces anomalus* was used as an outgroup.

Table 2 Effect of different cultural conditions on ethanol production from whey by *K. maxianus* strain BM4 and *K. lactis* strain P41 after 120 h*.

Culture condition	Ethanol production (%)	
	BM4	P41
Initial pH		
4.5	5.25 ± 0.83	5.05 ± 0.14
6.0	2.63 ± 0.04	3.33 ± 0.58
Lactose concentration (% Brix)		
10	4.02 ± 0.01	1.40 ± 0.31
12	1.58 ± 0.02	2.21 ± 0.20
15	3.06 ± 0.01	1.1 ± 0.12
Temperature (°C)		
30	0.84 ± 0.07	1.91 ± 0.07
35	0.68 ± 0.08	0.48 ± 0.01
40	0.58 ± 0.08	0.56 ± 0.15
Yeast concentration (w/v)		
10	1.83 ± 0.33	0.71 ± 0.01
20	1.46 ± 0.01	0.52 ± 0.06

* The results are the means of three separate experiments consisting of three replicates each.

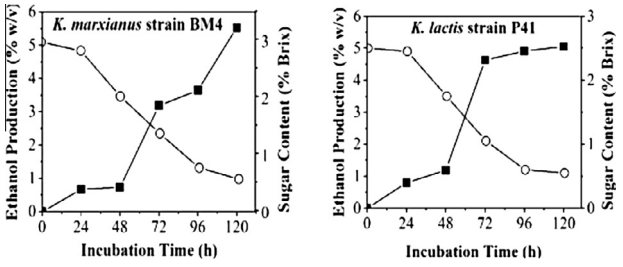

Figure 5 Ethanol production (■) versus sugar depletion (○) in whey by selected yeast isolates BM4 and P41 over varied incubator times. The cultural condition included 10% lactose supplementation, 10% yeast inoculation with whey pH adjusted to 4.5 and incubation performed at 30 °C.

K. lactis is one of the most studied yeast species and making it a model system for comparative studies with *Saccharomyces cerevisiae* [5]. Although not commonly used for ethanol production, *K. lactis* has been exploited for other biotechnological applications such as the production of heterologous proteins using cheese whey as culture media [39]. *K. marxianus* has also received attention due to its biotechnological potential and advantages over *K. lactis*, *K. marxianus* isolates originate from an enormous variety of habitats, accounting for the species broad metabolic diversity and consequent wide range of biotechnological applications [15]. A significant advantage of some *K. marxianus* strains is their ability to grow and ferment at elevated temperatures (>40 °C).

Grba et al. [19] investigated the suitability of five different strains of yeast *K. marxianus* for alcoholic fermentation of deproteinized whey. The selection of yeast strains was performed at different cultivation conditions: temperature ranged between 30 and 37 °C, lactose concentration was between 5% and 15% and pH varied between 4.5 and 5.0. They reported that the optimal temperature was 34 °C for ethanol production with *K. marxianus* which was in agreement with our finding. High temperature alcoholic fermentation of whey also was carried out by Kourkoutas et al. [32] using *K. marxianus* MB3 yeast. Zoppellari and Bardi [49] found that the best performances for ethanol production by *K. marxianus* were reached at low temperatures (28 °C); and high temperatures are also compatible with good ethanol yields in whey fermentations.

4. Conclusion

Two isolated *Kluyveromyces* spp. BM4 and P41, able to produce ethanol as main fermentation product from fermenting whey, were identified. The RFLP patterns of the 5.8S-ITS rDNA failed to differentiate and give the accurate identification for the two isolates. It is clear that *Kluyveromyces* species could be distinguished as *K. marxianus* and *K. Lactis* through the sequence of the 5.8S-ITS rRNA region or the sequence of the D1/D2 domain of the 26S rDNA gene. Maximum ethanol production was achieved from cheese whey by the two species at pH 4.5 and at 35 °C. Our results demonstrated that the yeast strains *K. marxianus* and *K. Lactis* could be recommended for cheese whey bioremediation in the environment.

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