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APPLICATION OF 26S RRNA GENE SEQUENCING AND RFLP OF ITS1-5.8S-ITS2 ANALYSIS FOR THE IDENTIFICATION OF *KLUYVEROMYCES* STRAIN BM9 PRODUCING BIOFUEL

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ABSTRACT

Cheese whey is a dairy industry waste have high lactose concentration, which case environmental problem. Therefore biofuel production from cheese whey become an interest as renewable energy. In this study 28 yeasts were isolated from Kenyan cheese whey and characterized for lactose fermentation. Out of them one isolate, designated as BM9 had high efficiency for lactose fermentation was selected. For genetically identification PCR amplification of both internal transcribed spacer (ITS) and 5.8 rDNA region (ITS1-5.8S-ITS2) and the variable D1/D2 domain of the 26S rRNA gene were used. RFLP of the PCR-amplified ITS1-5.8S-ITS2 region (750 bp) using two restriction enzymes *Hae*III and *Hin*fI yielded fragments with 650 + 80 bp and 250 + 190 + 120 + 80 bp respectively witch identified the isolate as *Kluyveromyces marxianus*. 26S rRNA of the isolate was sequenced and compared with known 26S rRNA sequences in the GenBank database. Results of 26S rRNA gene confirmed that the isolate was highly related to *K. marxianus* with similarity 99%. Phylogenetic analysis show that BM9 shared a one cluster with *K. marxianus*. The fermentative performance of the strain BM9 on cheese whey to produce bio-ethanol at different parameters such as incubation temperature, initial pH, whey sugar concentrations, and yeast concentrations was evaluated.

Keywords: Whey, 26S rRNA gene, 5.8S-ITS rDNA, phylogenetic analysis, yeast; bio-ethanol

INTRODUCTION

Cheese whey is a major dairy by-product and represents an important environmental problem because of the high volumes produced and its high organic matter content. Therefore convert cheese whey to a biofuel (bio-ethanol) source become an interest. Biofuels are a by-product of degradation of agricultural and industrial wastes by microorganisms (**Solomon, 2010**). Consequently, these microorganisms offer the advantage of both producing fuel and reducing pollution and waste.
The fermentation of whey lactose using yeasts has been frequently reported in the literature (**Barba *et al*., 2001 ; Compagno *et al*., 1993**) and the ability to metabolize lactose to ethanol has been demonstrated in *Kluyveromyces lactis, K. marxianus, and Candida pseudotropicalis* (**Breunig *et al*., 2000 ; Fukuhara, 2006**). Presently, there are a few established industrial processes to produce ethanol from whey utilizing these yeast strains, which has been done in some countries, namely Ireland, New Zealand, United States and Denmark (**Pesta *et al*., 2007; Siso, 1996**) .But there occur several challenges and limitations in the process of utilization of whey lactose to ethanol. Although the yeasts that assimilate lactose aerobically are widespread, those that ferment lactose are rather rare (**Fukuhara, 2006**), and there is an urgent research need to isolate, identify and develop novel microbial strains can ferment lactose to ethanol with very high efficiency to increase the industrial attractiveness of whey-to-ethanol bioprocesses.
The molecular methods based on the PCR-RFLP of the ITS region of the rDNA and the partial sequencing of the D1/D2 domain of the large sub-unit 26S of the rDNA have been used to identify the yeasts isolated from different sources (**Kurtzman and Robnett, 1998; Esteve – Zarzoso *et al*., 1999; Hesham *et al*., 2006a**).
The aims of this study were to use the restriction-fragment length polymorphism (RFLPs) of 5.8S-ITSrDNA region and sequencing of the domains D1 and D2 of the 26SrRNA gene for the identification of yeast isolated from Kenyan dairy products. The ability of selected isolate for ethanol production from whey was examined.

MATERIAL AND METHODS

Sampling, isolation and conventional identification of yeast strains:

The raw material whey was obtained from Browns cheese industry in Nairobi-Kenya, and the yeast strains were isolated from the dairy environment using YEPL (yeast extract peptone dextrose) agar medium containing, yeast extract 1.0 %, peptone 2.0 %, lactose 2.0 %, and pH 5.5 and PDA (potato dextrose agar) medium supplemented with 50 mg antibiotic Chloramphenicol to inhibit growth of bacteria. The isolate was identified based on their physiological and morphological properties according to **Barnett *et al*.** (2000). 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.

DNA isolation for PCR

The yeast isolate was sub cultured on YEPL medium for 36 hours at 30°C and then DNA extraction was carried out using GentraPuregene® Yeast/Bacteria kit (Qiagen, USA) According to manufacturer`s instructions.

PCR amplification of the 5.8S-ITS rDNA region

The amplification of the ITS1-5.8S-ITS2 region was carried out in 50 µl reaction mixture containing 25 µl Taq Master mix (Roche, USA), 18 µl PCR water, 2.5 µl each of forward and reverse primers ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3'), and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (**White *et al*., 1990**), and 2.0 µl DNA template. The PCR conditions was: 40 cycles including an initial denaturation at 95°C for 4 min, 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 product 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).

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).

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'–GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5' GGTCCGTGTTTCAAGACGG –3') (**Kurtzman and Robnett, 1998; Hesham *et al*., 2006b**). 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 product 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.

D1/D2 26S rDNA gene sequencing and phylogenetic analysis

DNA for sequencing was amplified with forward and reverse primers (NL1-NL4). The amplified D1/D2 fragments were purified and sequenced using an ABI 3730 automated sequencer. The yeast 26S rDNA sequences obtained were then aligned with known 26S rDNA sequences in 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. A phylogenetic tree was constructed with MEGA version 4.0 using a neighbor-joining algorithm, plus the Jukes-Cantor distance estimation method with bootstrap analyses for 1,000 replicates was performed (**Hesham and Mohamed, 2011**).

Fermentation setup

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 was carried out using the yeast isolate BM9 previously sub cultured on yeast extract peptone lactose (YEPL) medium at 30°C. 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 were examined.

Ethanol production determination

The amount of ethanol in the fermented whey samples was determined in 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 0.005M 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 0.45mm membrane filter prior to injection into the machine with 1, 2, 4, 6 and 10% ethanol (Scharlab S.L., Spain) as control.

Nucleotide sequence accession number

The sequences of the gene coding for 26S rRNA gene of strain BM9 reported in this study has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. KC512907.

RESULTS AND DISCUSSION

Yeast isolation, screening and convention identification

In this study, twenty eight yeast isolates were isolated from Kenyan dairy products and were screened for their ability to ferment cheese whey. Out of them, one isolate designated as BM9 was selected. The yeast strain was cultured for the identification on the basis of conventional methods summarized by an identification key (**Barnett *et al*., 2000**). Results show that strain BM9 was able to grow best at pH 4.5 and 5.5 and at temperatures 25,30, and 35°C. Strain BM9 was able to assimilate the carbon sugars including, glucose, glycogen, calcium 2-ceto gluconate, arabinose, adonitol, xylitol, inositol, methyl-d-glucopyranoside, n-acetyl-glucosamine, cellobiose, D-maltose , D-saccharose, D-trehalose, D-melezitose and raffinose but was not able to assimilate xylitol, inositol, sorbitol and carbon source D-melezitose . 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 (**Barnett *et al*., 2000; Guillamón *et al*., 1998**).

Identification of yeast strain by RFLPs of the 5.8S-ITS rDNA region

Genetic variability revealed by RFLPs of the 5.8SITS DNA region was used to identify the yeast isolate BM9. PCR products of the 5.8S-ITS rDNA region from the strain was 750 bp. The digestion of the PCR products with the enzymes *Hae*III, and *Hinf*I yielded two to four fragments. The digestion for the isolate BM9 were 650 + 80 bp for *Hae*III and 250 +190 + 120 + 80 bp for *Hinf*I . These results allowed to identify the isolate at specie level as *Kluyveromyces marxianus* after comparing the molecular mass of the restriction products with those previously described in the literature (El-**Sharoud *et al*., 2009**), 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 (**Fernandez- Espinar *et al*., 2000; Martin *et al*., 2007**). This method was found valuable and convenient for differentiation at species level of yeasts of importance for surface ripened cheeses (**Petersen *et al*., 2001**).

Ribosomal DNA sequence analysis

In order to confirm the correct affiliation of the isolate BM9, we sequenced the D1/D2 domain of the 26S rRNA gene of this isolate and compared with the sequence of 26S rRNA regions in GenBank by means of BLAST search of the National Center for Biotechnology Information (NCBI) databases. Alignment results of the 26S rRNA gene sequences of the isolate show that the D1/D2 26S rRNA sequence of BM9 was found to have 99% similarity with the D1/D2 26S rRNA sequence of strain *K. marxianus* (**Figure 1**).

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BM9        4    ACCGGGCATTGCCTTAGT-ACGGCGAGTG-AGCGGCAAAAGCTCAAATTTGAAATCTGGC  61
                ||||||  ||||||||||| |||||||||| ||||||||||||||||||||||||||||||
K.marx.   31    ACCGGG-ATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGC  89

BM9        62   GTCTTCGACGTCCGAGTTGTAATTTGAAGAAGGCGACTTTGTAGCTGGTCCTTGTCTATG  121
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.    90   GTCTTCGACGTCCGAGTTGTAATTTGAAGAAGGCGACTTTGTAGCTGGTCCTTGTCTATG  149

BM9        122  TTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGATCCCAGTTATT  181
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.    150  TTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGATCCCAGTTATT  209

BM9        182  TGTAAAGTGCTTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAAT  241
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.    210  TGTAAAGTGCTTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAAT  269

BM9        242  TCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGA  301
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.    270  TCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGA  329

BM9        302  TGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCAT  361
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.    330  TGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCAT  389
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BM9       362  TTGATCAGACATGGCGTTTGCTTCGGCTTTCGCTGGGCCAGCATCAGTTTTAGCGGTTGG  421
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.   390  TTGATCAGACATGGCGTTTGCTTCGGCTTTCGCTGGGCCAGCATCAGTTTTAGCGGTTGG  449

BM9       422  ATAAATCCTCGGGAATGTGGCTCTGCTTCGGTAGAGTGTTATAGCCCGTGGGAATACAGC  481
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.   450  ATAAATCCTCGGGAATGTGGCTCTGCTTCGGTAGAGTGTTATAGCCCGTGGGAATACAGC  509

BM9       482  CAGCTGGGACTGAGGATTGCGACTTTTGTCAAGGATGCTGGCGTAATGGTTAAATGCCGC  541
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K.marx.   510  CAGCTGGGACTGAGGATTGCGACTTTTGTCAAGGATGCTGGCGTAATGGTTAAATGCCGC  569

BM9       542  CCGTCTTGACCAACCGGAC  560
               ||||||||||||||| ||||
K.marx.   570  CCGTCTTGACCAAC-GGAC  587
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Figure 1 Sequence alignment of the isolate BM9 against 26S rDNA sequence data of *Kluyveromyces marxianus* showing four base substitutions

To confirm the position of the isolated strain BM9 in phylogeny, a number of sequences were selected from Genbank database for the construction of a phylogenetic tree using MEGA4 program. As shown in (**Figure 2**), the phylogenetic tree indicated that strain BM9 and *K. marxianus* were in the same clade cluster. Therefore, the isolate was identified as *K. marxianus*.
The molecular methods based on the PCR-RFLP of the ITS region of the rDNA and the partial sequencing of 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 (**Kurtzman and Robnett, 1998; Esteve – Zarzoso *et al*., 1999; Hesham *et al*., 2006 a,b; Villa-Carvajal *et al*., 2006; Hesham *et al*., 2009; Hesham and Mohamed, 2011**).

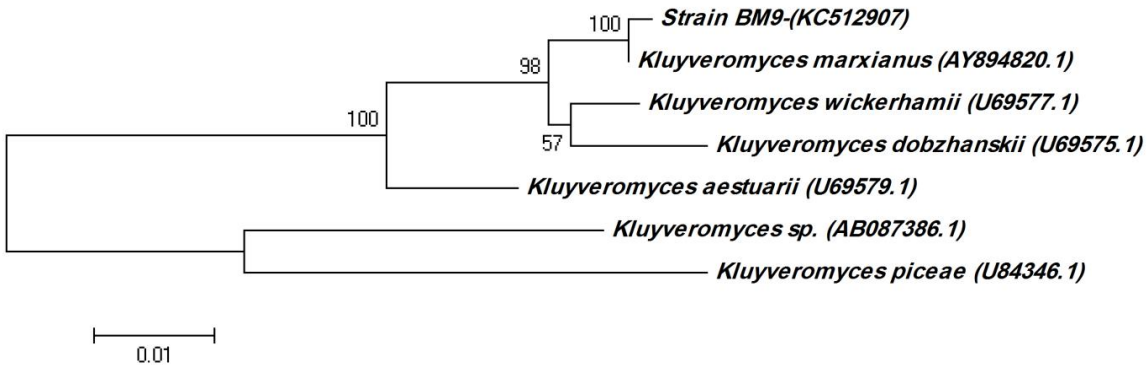


Figure 2 Phylogenetic relationship between *Kluyveromyces marxianus* strain BM9and other 26S rRNA sequences of published strains. In the phylogenetic tree, BM9and *Kluyveromyces marxianus* were clustered together as one clade segments corresponding to an evolutionary distance of 0.01 are shown with bars. Accession numbers for sequences are as shown in the phylogenetic tree.

Whey characterizations

From the study it was found out that the chemical oxygen demand (COD) of whey was 68,000 mg/l, its protein content on wet basis was 0.7% while on a dry basis it was 1.02 %, and the ash content was 0.55 %. Whey sample also had a lactic acid content of 0.69 %. Whey was found to have mineral boron of 40 mg/l, 4.5 mg/l iron, it had calcium content of 28.37mg/ml, zinc content of 1.52 mg/l while cobalt was not detected. The initial total soluble solids were 5.3% with a pH of 5.2.

Ethanol production by *K. marxianus* strain BM9

Ethanol production by *K. marxianus* strain BM9 in cheese whey was examined in batch mode. The fermentative performance of the strain on cheese whey to produce ethanol was evaluated at different 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). The results showed that the fermentation using *K. marxianus* strain BM9, yielded an ethanol concentration of 4.92% at pH 4.5 and 1.28% at pH 6 (**Figure 3a**).

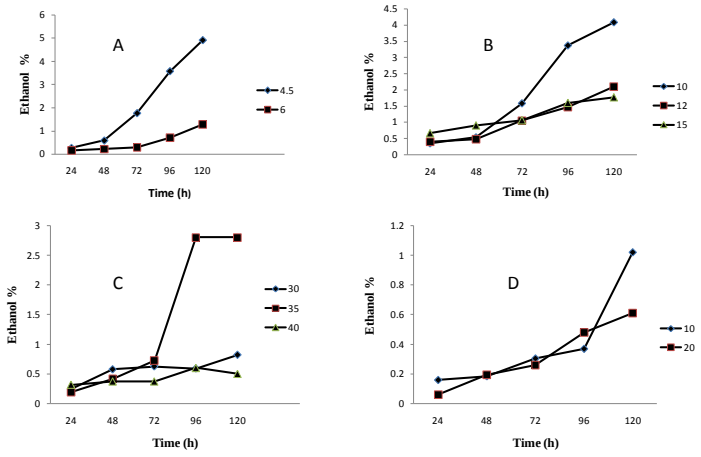


Figure 3 Effect of different cultural conditions on ethanol production by *Kluyveromyces marxianus* strain BM9. (a) initial pH, (b) whey sugar concentrations (brix), (c) temperature and (d) yeast concentrations (w/v).

The pH of whey reduced from 4.5 to 3.24 after period 120 h, while at pH 6, the pH reduced to 3.68, with total carbohydrate concentration reducing from 2 mg/l (in 24 h) to 0.27 mg/l (in 120 h) at pH 4.5 and 2.61 mg/l (in 24 h) to 0.41 mg/l (in 120 h) at pH 6. Total soluble solids (brix) was reduced from the initial concentration of 5.3 mg/l to 0.3 and 0.68 mg/l, during fermentation carried out at 4.5 and pH 6 respectively. Ethanol concentration at different sugar concentrations

were 4.08 %, 2.10 %, and 1.77 % ethanol (**Figure 3b**) at 10 %, 12 %, and 15 % brix sugar concentration, respectively. When whey sugar concentration was adjusted to 10 %, 12 %, and 15 % brix, the pH reduced from 5.3 to 3.55, 3.68 and 3.42 at 120 h, respectively. The total carbohydrate concentration reduced from 19.50 mg/l (at 24 h) to 1.58, 1.68 and 1.04 mg/l (at 120 h), with 10, 12 and 15 % brix concentration, respectively. On the other hand, the brix value at 10 % reduced to 3.65 % ,while at 12 %, it reduced to 7.25 % and at 15 % it reduced to 7.5 %. As shown in **Figure 3c**, at temperature 30 ^{o}C, the ethanol concentration was 0.82 %, while at 35 ^{o}C it was 2.80 % and at 40 ^{o}C it was 0.5 %. The pH reduced from 5.3 to 4.29 at 30 ^{o}C, 3.69 at 35 ^{o}C, and 3.41 at 40 ^{o}C, with total carbohydrates to 0.75 mg/l for fermentation carried out at 30 ^{o}C, 0.93 mg/l at 35 ^{o}C and 0.47 mg/l at 40 ^{o}C. The brix amount reduced to 3.05, 2.75, and 3.15 % for temperatures 30, 35 and 40 ^{o}C, respectively from an intial brix of 5.3 % . On the other hand ethanol production was 1.02 % and 0.61 % at yeast concentration 10 % and 20 %, respectively (**Figure 3d**) with reduced brix of 3.15 and 2.55, respectively. The pH value reduced to 3.67 and 3.46 for 10 and 20 %, respectively, while the total carbohydrates value reduced to 1.26 mg/l and 1.19 mg/l for 10 and 20 % yeast concentration, respectively. The optimum conditions of fermentation for the strain BM9 in the four variables were at pH 4.5, 10 % sugar concentration, temperature 35 ^{o}C and yeast concentration of 10 %, (**Figure 4a-d**) however the best condition for fermentation using this strain of *K. marxianus* was at pH 4.5 which produced an ethanol concentration of 4.92 % with a reduced brix of 0.3% at 120 h from an original brix of 5.3 % at 0 h.

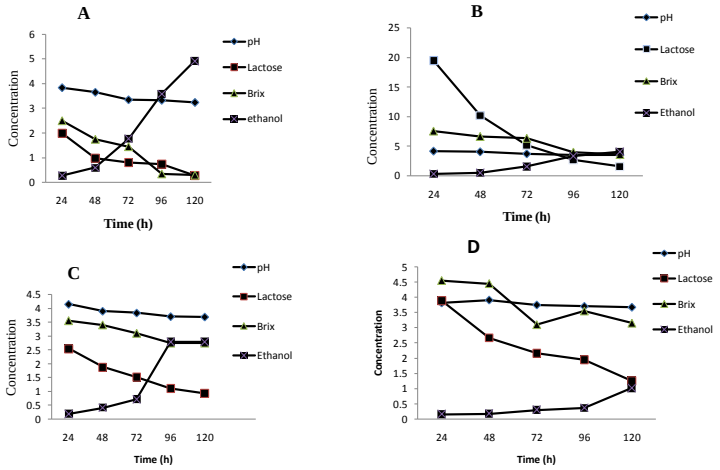


Figure 4 optimum fermentation condition of strain BM9 *Kluyveromyces marxianus* (a) pH 4.5, (b) 10 % whey sugar concentrations (brix), (c) 35^{o}C temperature and (d) 10% yeast concentrations (w/v)

Compared to fossil fuels ethanol has the advantages of produced from renewable sources, providing cleaner burning and producing low greenhouse gases. Waste biomass has been the most widely used raw material for production of ethanol (**Hari *et al*., 2001; Nigam, 2000; Gong *et al*., 1999**). However, ethanol production from waste biomass is expensive since the process requires separation of lignin from cellulose, hydrolysis of cellulose to sugars, fermentation of sugar solution to ethanol and separation of ethanol from water, so inexpensive way is required for ethanol production. Among the inexpensive and highly available raw materials for ethanol production is cheese whey which is the waste by-product of dairy industries.

Ethanol production by several yeasts from whey has been studied (**Ozmihci and Kargi, 2007; Lukondeh *et al*., 2005; Lark *et al*., 1997; Zhang and Lynd , 2007**), and *K. marxianus* strains are usually used for commercial production |(**Ling, 2008**). **Grba *et al.*** (**2002**), 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 30-37 °C, lactose concentration was between 5% and 15 % and pH varied between 4.5-5.0. He reported that the optimal temperature was 34 °C for ethanol production with *K. marxianus* which was agreement with our finding. High temperature alcoholic fermentation of whey also was carried out by **Kourkoutas *et al.*** (**2002**) using *K. marxianus* MB3 yeast. **Zoppellari and Bardi,** (**2012**) found that the best performances for ethanol production by *K. marxianus* were reached at low temperatures (28^{o}C); and high temperatures are also compatible with good ethanol yields in whey fermentations.

CONCLUSION

In summary, this study by using various molecular techniques, we were able to identify the yeast isolate at species level. Data obtained from sequencing of the domains D1 and D2 of the 26S rRNA gene confirmed the findings concerning the restriction-fragment length polymorphism (RFLPs) of 5.8S-ITS rDNA region . High ethanol productivity was achieved from cheese whey by yeast isolate. Maximum ethanol produced by strain BM9 was achieved at pH 4.5 and 35 ^{o}C.

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