

Molecular Genetic Identification and Phylogeny of New Bio-Ethanol Producing Yeast Isolated from Cheese Whey

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Bio-ethanol has been promoted as alternative fuel because it is renewable fuel and environmental friendly compared with petroleum products. In this study ethanol production by a yeast isolate, designated as YB2 isolated from Kenyan dairy industry, in whey waste was examined. The isolate was initially identified as *Pichia cactophila* using RFLP of PCR-amplified internal transcribed spacers of rDNA (ITS1-5.8S-ITS2). Where size of the PCR products (450bp), and the restriction analyses with two restriction enzymes *HaeIII* and *HinfI* yielded fragments with 450 bp and 200 + 250 bp respectively. The variable D1/D2 domain of the 26S rDNA of the isolate was amplified by PCR and sequenced. The sequences were compared with known 26S rDNA sequences in the GenBank database. Results of 26S rRNA gene confirmed that the isolate was highly related to *P. cactophila* with similarity 100%. Phylogenetic analysis shows that YB2 shared a one cluster with *P. cactophila*. The fermentative performance of the strain YB2 on cheese whey to produce ethanol was evaluated at different parameters such as incubation temperature, initial pH, whey sugar concentrations, and yeast concentrations. Maximum ethanol produced by strain YB2 was achieved at pH 4.5 and 35°C. To our knowledge, there are no reports in literature on utilization of cheese whey by *P. cactophila* for ethanol production. This was therefore, the first time the *P. cactophila* was used to produce ethanol from cheese whey.

Key words: Whey, 26S rRNA gene, 5.8S-ITS rDNA, Phylogenetic analysis, Yeast; Bio-ethanol.

Yeasts are important components of the microflora of many food products. Where they are usually detected in high numbers in various dairy products and environment, they are able to grow on a substrate rich on proteins, lipids, sugars and organic acids. Their broad distribution is due to the proteolytic and lipolytic activity, as well as the ability to ferment / utilize lactose the main sugar

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found in dairy products and to utilize citric, lactic and succinic acids. Yeasts have a great advantage due to their robustness with a wide a range of physiochemical tolerances. Such as the ability to grow in substrates with high salt concentration, low temperatures, and also low pH due to their ability to grow on complex substrates, yeasts may play a beneficial role in the dairy production²⁶. For dairy products, yeasts are not only used in production of cheese but may also be involved in the production of fermented milk products such as

yoghurt. They can also be involved in the spoilage of foods^{11, 32}.

Deak and Beuchat⁶ have shown that the genus *Pichia*, which have a broad distribution in natural habitats and contaminants in a variety of foods, beverages and fermented products produce desired effects in food; contributing to the early stages of wine fermentation.

Studies by Cai *et al.*,⁵, James *et al.*,²⁰, Kurtzman²¹ and Kurtzman²², 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. This is due to their ability to exhibit far greater interspecific differences than the 18S and 25S rRNA genes. Ribosomal regions evolve in a concerted fashion and hence show a low intraspecific polymorphism and a high interspecific variability²⁵. This has proved very useful in the classification of yeast species^{3,19,29,33,36}.

The aims of this study were to use the restriction-fragment length polymorphism (RFLPs) of 5.8S-ITS rDNA region and sequencing of the domains D1 and D2 of the 26S rRNA gene for the identification of yeast isolated from Kenyan dairy products. The ability of two selected isolates for ethanol production from whey was examined.

MATERIALS AND METHODS

Sampling, isolation and conventional identification of yeast strains

The raw material whey was obtained from Brown's cheese industry in Nairobi-Kenya, and the yeast strains were isolated from the dairy environment using YEPD (yeast extract peptone dextrose) agar medium containing, yeast extract 1.0 %, peptone 2.0 %, dextrose 2.0 %, pH 5.5 and PDA (potato dextrose agar) media supplemented with 50 mg antibiotic Chloramphenicol to inhibit growth of bacteria. The isolates were identified 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.

DNA extraction for PCR

DNA extraction was carried out using GenraPuregene[®] 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 GAACCT GCG G-3'), 2.5 µl ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3')³⁵, 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 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).

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 *Hin*FI (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')[17,26]. 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.

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 performed¹⁸.

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 one selected yeast designated as YB2 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 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 H₂SO₄ 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 YB2 reported in this study had been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession Nos. KC512909.

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 YB2 was selected. The yeast strain was cultured for the identification on the basis of conventional methods summarized by an identification key². Results show that strain YB2 could able to grow best at pH 4.5 and at pH 5.5 and at temperatures 30 and 37°C . The strain could able to assimilate the sugars glucose, glycogen, calcium 2-ceto gluconate, sorbitol, methyl-d-glucopyranoside, n-acetyl-glucosamine, cellobiose, D-maltose, D-saccharose, D-trehalose and raffinose, but was not able to assimilate D-melezitose, xylitol, adonitol and xylitol. Morphological traits and physiological abilities, on which identification and

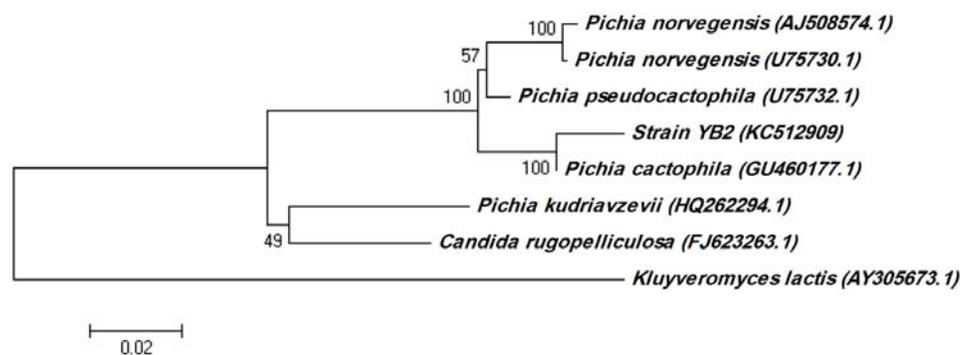


Fig.1. Phylogenetic relationship between *Pichia cactophila* strain YB2 and other 26S rRNA sequences of published strains. In the phylogenetic tree, YB2 and *Pichia cactophila* were clustered together as one clade segments corresponding to an evolutionary distance of 0.02 are shown with bars. Accession numbers for sequences are as shown in the phylogenetic tree

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 YB2 was found to have 100% similarity with the D1/D2 26S rRNA sequence of strain *Pichia cactophila* (fig.2).

To confirm the position of the isolated strain YB2 in phylogeny, a number of sequences were selected from Genbank database for the construction of a phylogenetic tree using MEGA4 program. As shown in (Fig. 1), the phylogenetic tree indicated that strain YB2 and *P. cactophila* were in the same clade cluster. Therefore, the isolate was identified as *P. cactophila*.

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^{23,8,9,15,16,17,18,34}.

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, 4.5mg/l iron, it had calcium content of 28.37 mg/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 *P. cactophila* strain YB2

Ethanol production by *P. cactophila* strain YB2 in cheese whey was examined in batch

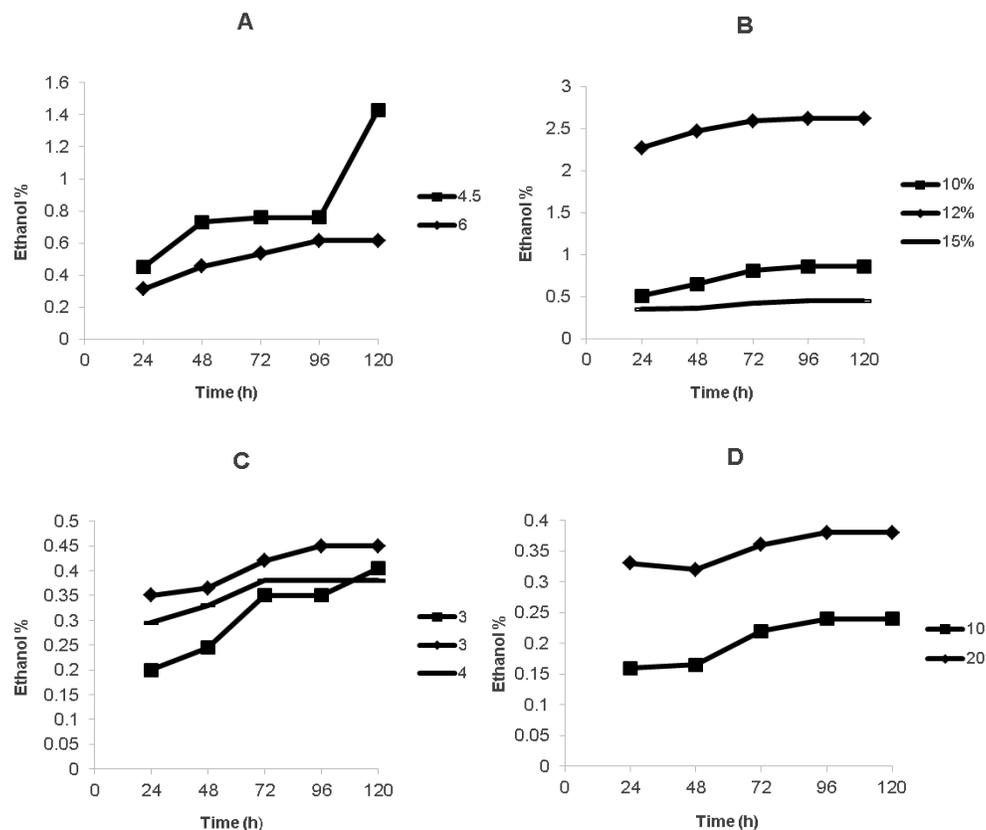


Fig. 3. Effect of different cultural conditions on ethanol production by *Pichia cactophila* strain YB2. (a) initial pH, (b) whey sugar concentrations (brix), (c) temperature and (d) yeast concentrations (w/v)

mode. The fermentative performance of strain YB2 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 *P. cactophila* strain YB2 yielded an ethanol concentration of 1.43% at pH 4.5 and 0.615% at pH 6 (Fig. 3a). The pH of whey reduced from 4.5 to 3.23 after period 120 h, while at pH 6 the pH reduced to 3.705, with total carbohydrate concentration reducing from 2 mg/l to 0.24mg/l at pH 4.5 and to 0.29mg/l at pH 6. The brix value reduced to 0.4 and 0.55 for pH 4.5 and 6 respectively from an initial 5.3. Ethanol yields at different sugar concentrations were as follows; at 10% sugar concentration, ethanol yield concentration was 0.86% with reduced pH of 3.56 from 5.3, total carbohydrates concentration reduced to 2.22mg/l from 19.50 (at 24h), while the brix reduced to 5 from the initial value of 10%. The ethanol yield at 12% Brix was

2.62% with a reduced pH of 3.86 from 5.3, total carbohydrates reduced to 2.46mg/l from 16.03mg/l at 120 h, while the brix reduced to 6.9% from 12%. At 15% brix the ethanol yield was 0.49% with reduced pH of 3.71 and total carbohydrates of 1.54 mg/l from initial of 20.36 mg/l and brix value of 8.3% at 120 h (fig. 3b).

Results obtained when fermentation was carried out at temperature 30°C was as follows; ethanol concentration was 0.35%, pH reduced to 4.32 at 120 h, carbohydrate concentration reduced to 0.75 mg/l from initial 4.56 mg/l in 120 h and a brix value of 2.3% from an initial 5.3%. The ethanol yield was 0.45% and 0.38 % at temperatures of 35°C and 40°C respectively, with a reduced pH of 3.63, total carbohydrates of 2.10 mg/l and 2.6 brix at 120 h, for fermentation carried out at 35°C, when whey was fermented at 40°C the pH reduced to 3.46 with a total carbohydrate of 0.93mg/l and a brix value of 2.8 at 120 h (Fig. 3c),

At 10% and 20% yeast concentration the ethanol yield was 0.24 % and 0.38 % respectively,

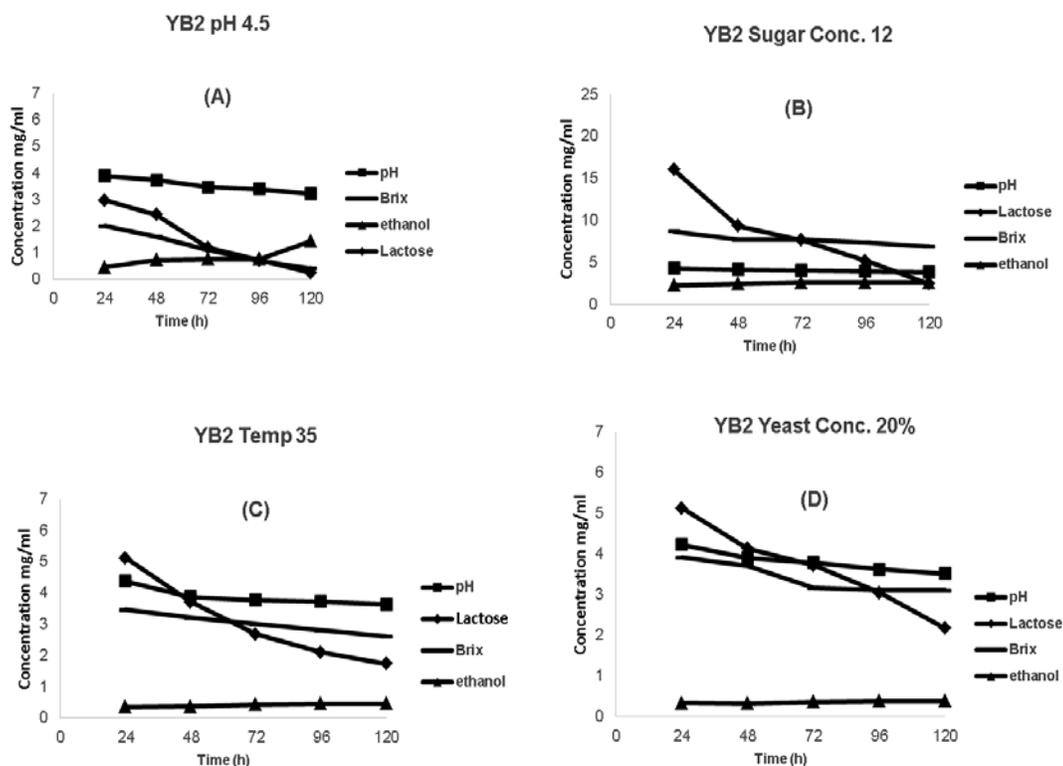


Fig.4. Optimum fermentation condition of strain YB2 *Pichia cactophila* (a) pH 4.5, (b) 12 % whey sugar concentrations (brix), (c) 35°C temperature and (d) 20% yeast concentrations (w/v)

at 10% yeast concentration the pH reduced to 3.75 while the total carbohydrate was 0.62mg/l and brix was 3.8. At 20% yeast concentration, the pH reduced to 3.51 while the total carbohydrate reduced to 2.16mg/l and a brix value of 3.1% in 120 h (Fig. 3d).

The optimum conditions of fermentation for the strain YB2 in the four variables was at pH 4.5, sugar concentration 12%, temperature 35 °C and a yeast concentration of 20% with an ethanol production 1.43 % (w/v) at 120 h (Fig. 4 a-d).

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^{12,14,30}. 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^{4,7,31,27,24,37}. Several yeasts belonging to the genus *Pichia* such as *Pichia farinosa*, *Pichia fermentans* and *Pichia stipitis* are known to produce ethanol from glucose¹. The most promising yeasts that have the ability to use both pentose and hexose sugars are *Pichia stipites*. There are no reports in literature on utilization of cheese whey by *P. cactophila* for ethanol production. This was therefore the first time the *P. cactophila* was used to produce ethanol from cheese whey.

CONCLUSION

In summary, this study using various molecular techniques, we could able to identify isolated yeast at specie level. Data obtained from sequencing of the domains D1 and D2 of the 26SrRNA gene confirmed the findings concerning the restriction-fragment length polymorphism (RFLPs) of 5.8S-ITSrDNA region. An ethanol production was achieved from cheese whey by yeast isolate YB2. Maximum ethanol produced by

strain YB2 was achieved at pH 4.5 and 35°C. There are no reports in literature on utilization of cheese whey by *P. cactophila* for ethanol production. This was therefore, the first time the *P. cactophila* was used to produce ethanol from cheese whey.

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