

Ethanol Production from Cassava Pulp by a Newly Isolated Thermotolerant *Pichia kudriavzevii* LC375240

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Abstract

A total of 500 thermotolerant fermenting yeast isolates (100 from palm-wine and 400 from spoiled fruits) were screened for ethanol production at high temperatures. The best isolate that produced up to 4% ethanol from 10% glucose at 45°C was selected for further experiments. The optimum pH for ethanol production by the isolate was pH 6 at both 30°C and 42°C. The isolate was identified as *Pichia kudriavzevii* base on the 18s ribosomal DNA. Ethanol production from 200 g/L cassava pulp using Simultaneous Saccharification and Fermentation (SSF) method at 30°C and 42°C by the isolate was investigated. At 30°C, an ethanol concentration of 30 g/L was produced. This represents an ethanol yield of 0.15 g/g of cassava pulp and 58.8% of the theoretical yield. However at 42°C, the concentration of ethanol produced increased to 42 g/L representing an ethanol yield of 0.21 g/g of cassava pulp and 82.4% of the theoretical yield. The isolate produced slightly higher ethanol than the two typed strains NCYC 587 and NCYC 2791 at 42°C. This isolate has a good potential to be used for commercial bioethanol production since it can produce ethanol at 45°C without a significant drop in ethanol yield.

Keywords

Bioethanol, Cassava Pulp, Yeast, Thermotolerant, *Pichia kudriavzevii*

1. Introduction

The use of bio-ethanol as an alternative to fossil fuel has been gaining worldwide attention owing to its many advantages (renewability, sustainability and envi-

ronmentally friendliness) over fossil fuel. Despite the many advantages of bio-ethanol, the current costs of production are still much higher than the cost of fossil fuel, thereby limiting its commercialization in many parts of the world. Currently, for large scale production of bio-ethanol, food crops such as corn, sucrose containing feedstock such as sugarcane [1] [2], as well as starchy materials such as cassava [3] are used as raw materials. Aside from direct competition with food, these raw materials are too expensive to compete with the current fossil fuel prices. Lignocellulosic materials on the other hand are cheap and abundant but the cost of converting them to bio-ethanol is high.

Cassava pulp is a promising feedstock for commercial production of bio-ethanol and other value-added bio-products. It is a residue material that is produced as a waste when starch is extracted from cassava. It is cheap and available throughout the year because cassava which is the source of cassava pulp is flexible in terms of planting and harvesting seasons [4] [5] [6] [7]. Also, cassava can grow on arid and semiarid land with moderate yield [8], does not require much input of fertilizer and has little application in food industry. Cassava pulp contains about 40% - 70% starch on a dry weight basis, 20% - 30% cellulose fiber and other non-starch polysaccharides, with moisture content of 70% - 80% [5] [9] [10]. Due to its high starch and moisture content, it spoils rapidly and causes environmental problems including strong and offensive odour and contamination of local water supply [11] [12]. Currently, Nigeria is the largest producer of cassava in the world [13]. The waste generated from cassava presently is already posing disposal and environmental problems. With the quest to expand cassava production and processing in Nigeria, a lot of cassava pulps will be generated which if not utilized will result in a lot of environmental pollution. Besides, converting this waste to wealth will greatly improve the economic feasibility of cassava processing industries as well as provide jobs for the many unemployed.

Hydrolysis is the first step in conversion of cassava pulp to bio-ethanol. Due to environmental problems associated with acid/base hydrolysis, attention is now focused on biological hydrolysis using purified or crude enzymes, or hydrolytic enzyme-producing microorganisms. The optimal temperatures for most of the carbohydrate hydrolyzing enzymes are usually higher than the optimal temperatures for the conventional yeast strains used to convert the products of hydrolysis to ethanol. Although conversion of cassava pulp to ethanol can be done in two sequential steps of hydrolysis followed by fermentation, many reports have demonstrated that simultaneous hydrolysis and fermentation leads to higher productivities and yields [14]. However, the use of Simultaneous Saccharification and Fermentation process requires a yeast strain capable of growing, fermenting sugars as well as producing high concentration of ethanol at temperatures close to the temperature of hydrolysis [15]. Also, the use of thermo-tolerant yeast will save cost of cooling especially in tropical climates where ambient temperature can easily exceed 40°C [16]. There have been documentations on thermotolerant yeast strains capable of producing ethanol at a temperature up to 42°C [14] [17] [18] [19] among others. However, these yeasts have

not yet been reported for commercial bioethanol production and they are not as robust as *Saccharomyces cerevisiae* in terms of ethanol production. Thus, there is need to screen for more thermotolerant yeast strains for industrial production of bioethanol. The aim of this work is to screen for thermotolerant ethanol fermenting yeasts and evaluate their potentials for bioethanol production from cassava pulp.

2. Materials and Methods

2.1. Samples

Palm wine was collected from wine tappers at Enugu-Ezike while spoilt fruits (mostly soursop) were collected from the middle of compost heaps in and around University of Nigeria Nsukka. All samples were collected into sterile containers and transported to the Microbiology laboratory for treatment and isolation of the yeast strains. Cassava pulp was obtained from Nigeria Starch Mill in Iheala, Anambra state Nigeria. The pulps were sun-dried to constant weight, milled, sieved and stored in a refrigerator until used.

2.2. Isolation of Thermotolerant Fermenting Yeasts

The samples for yeast isolation were inoculated into an enrichment medium containing 10% glucose, 2% peptone and 1% yeast extract (10% YPD) and incubated at 42°C for 24 hours. A 100 µL aliquot of the 24 hours old enrichment culture was spread on agar plates containing 2% glucose, 2% peptone, 2% agar-agar and 1% yeast extract. The plates were incubated at 42°C for 24 hours. After incubation, discrete colonies were picked and inoculated into tubes containing 3 ml of 10% YPD and incubated at 30°C. Isolates showing signs of fermentation after 24 hours of incubation were sub-cultured into a fresh 10% YPD and incubated at 42°C for 24 hours. Isolates showing signs of fermentation after incubation at 42°C were sub-cultured into a fresh 10% YPD in a 96 culture well plate, incubated at 30°C for 24 hours and stored at 4°C as stock culture for further use. Prior to use, the stock culture from 4°C was left at room temperature for 2 hours. A 10 µL aliquot of each isolate from the stock culture was inoculated into 180 µL of 10% YPD in a 96 culture well plate using a multichannel pipette. The plates were incubated at 30°C for 16 hours.

2.3. Screening the Isolates for Ethanol Production at High Temperatures

A 10 µL aliquot of each isolate from 16 hours old pre-cultures was inoculated into 180 µL of 10% YPD in a 96 culture well plate using a multichannel pipette. The plates were incubated at 42°C for 24 hours. After incubation, the concentration of ethanol produced by each isolate was determined.

2.4. Rough Screening of the Isolates by NBT/PMS

This was done using a colorimetric method called Nitroblue Tetrazolium/Phenazine

Methosulfate (NBT/PMS) according to Johannes *et al.* [20]. After incubation, the culture was diluted 50 times using distilled water in a 96 culture well plate. Ethanol standards (0.005%, 0.01%, 0.02%, 0.05% and 0.1%) were also prepared and also placed in different wells. A 100 μL of 0.1 M Na-Phosphate buffer, pH 7.5 containing 0.1% Triton X100, 20 μL of 0.4% NAD^+ and 20 μL of Alcohol Dehydrogenase (10 U/mL) were added in each well. The content of each well was mixed well by repeated pipetting and dispensing, and then the plates were incubated at room temperature for 5 mins. After incubation, 20 μL of 0.01% PMS/0.1% NBT mixture was added to each well and the plates were incubated at room temperature for 5 mins. The color intensity was compared with that of the different ethanol standards used. The screening was done at 42°C and the best 10 stains were isolated.

2.5. Confirmation of the Ethanol Production Abilities of the Isolates

The isolates (S26 and P27) were activated in YPD medium for 16 hours, and inoculated into a fresh 3 mL 10% YPD to an optical density (OD) of 0.2 at 600 nm. The tubes were incubated at 42°C or 45°C. The percentage concentration of ethanol produced by each strain was measured using High Performance Liquid Chromatography (HPLC) after 16 and then 24 hours. The best isolate was then used for subsequent experiments.

2.6. Identification of the Isolate

Genome DNA was extracted from 24 hours old pure culture of the isolate according to Dhaliwal *et al.* [17]. About 15 mL of 24 hours old culture in YPD broth was centrifuged at 3000 g for 10 minutes and the supernatant was discarded. The precipitate was washed severally by re-suspending in sterile distilled water, centrifuging at 3000 g and discarding the supernatant. After washing, the precipitate was re-suspended in 2 mL of set buffer (20 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 10 mM EDTA) and 100 μL of zymolyase (1.0 mg/mL) was added. The mixture was incubated at 30°C for 30 minutes. After incubation, 200 μL of 10% SDS (Sodium Dodecyl Sulfate) was added to the mixture, followed by the addition of 2 mL phenol-chloroform isoamylalcohol (NIPPON GENE, Tokyo, Japan). The mixture was centrifuged at 6000 g for 15 minutes after vigorous agitation. The clear aqua phase of the supernatant was transferred into a new tube and 10 μL of RNase was added to it. It was incubated at 37°C for 2 hours. After incubation, 1 mL of phenol-chloroform was added to the mixture followed by centrifugation at 6000 g for 15 minutes. The clear aqua phase of the supernatant was transferred into a clean tube where one-tenth volume (150 μL) of 3 M sodium acetate and 4.5 mL of 100% ethanol were added. The mixture was centrifuged at 6000 g and supernatant was discarded. To the DNA precipitate, 2 mL of cold 80% ethanol was added, it was centrifuged for 5 minutes and the supernatant was discarded. The DNA was air-dried at room temperature for 10 minutes

and re-suspended in sterile distillate water. Polymerase Chain Reaction (PCR) amplification of 18s ribosomal Ribonucleic Acid (rRNA) gene was carried out on the isolated DNA using Internally Transcribed Spacer (ITS) primer. Both ITS4 (5'TCCTCCGCTTATTGATATGC3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') were used for the amplification of appropriate DNA region. The sequence of DNA fragment was decided, and yeast strain was identified by comparison with the sequences of other strains. The evolutionary history of the isolates was inferred using the Neighbor-Joining method according to Saitou and Nei [21]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method of Tamura *et al.* [22] and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 500 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 software according to Tamura *et al.* [23].

2.7. Characterization of the Isolate

The isolate was characterized by checking its sugar assimilation profile, temperature tolerance, pH, ethanol and Acetic acid tolerance.

2.8. Sugar Assimilation Profile

This was carried out using the Api 20C AUX sugar assimilation kit (Analytab products, Div. of Ayerst Laboratories, Plainview, New York). The isolate was inoculated in 10% YPD and incubated at 30°C on a rotary shaker at 120 rpm for 8 hours. The OD of the isolates at 600 nm was adjusted to 0.4 in 0.8% NaCl. A 100 µL aliquot of the NaCl-diluted sample was transferred into a basal medium containing all the nutrients required for growth except the sugar, and subsequently, a 200 µL of this was transferred into each sugar well. The kit was incubated at 30°C for 72 hours and the ability of the isolates to assimilate a sugar was inferred by comparing the turbidity in each sugar well with the control.

2.9. Temperature Tolerance

The temperature tolerance ability of the isolate was measured by incubating it at different temperatures (30°C, 37°C, 40°C, 42°C, 45°C and 47°C) in 10% YPD. The isolate was first pre-cultured at 30°C for 16 hours. It was inoculated into a fresh 10% YPD to an OD of 0.2 at 600 nm. The concentration of ethanol produced by the isolate was measured after 24 and 48 hours using HPLC.

2.10. pH Tolerance

The pH tolerance ability of the isolate was measured by inoculating them in 10% YPD adjusted to different pH values (2, 4, 6, 8 and 10) using either hydrochloric

acid (for acidic pH) or sodium hydroxide (for the alkaline pH). This was incubated at 30°C or 42°C and cultivated for 48 hours. Measurement of concentration of ethanol produced by the isolate was also done using HPLC.

2.11. Ethanol and Acetic Acid Stress Tolerance

The growth phenotypes of the isolated strain and two typed strains (NCYC 587 and NCYC 2791) were compared in media containing ethanol and acetic acid. Approximately $2.0 - 6.0 \times 10^4$ cells ($A_{600} = 1.0$) of each strain (dilution factor 10^0) and those of serially diluted ($10^{-1} - 10^{-4}$) were spotted onto YPD containing 6% (w/v) ethanol, and varied concentrations (50 mM, 60 mM, and 70 mM) of acetic acid. These plates were incubated for 22 hours at 40°C.

2.12. Analysis of Starch and Cellulose Contents of the Cassava Pulp

The starch content was analyzed using the iodine method. One gram (1 g) of the cassava pulp was homogenized in 70 mL of distilled water. It was boiled for 30 minutes and allowed to cool. The volume was adjusted to 100 mL using distilled water. This was centrifuged and 0.1 mL supernatant was added into a test tube. A 5 mL of 1:100 dilution (1 mL of iodine solution: 100 mL of distilled water) of iodine solution was then added to the 0.1 mL of the supernatant. The volume of the mixture was adjusted to 10 mL using distilled water and the absorbance was taken at 680 nm. Analysis of cellulose was done according to the methods of Updegraff [24].

2.13. Production of Koji Enzyme Used for Hydrolysis

A 30 g portion of rice and 20 g of rice bran were steamed together. After steaming, it was allowed to cool to room temperature. Spores of the *Aspergillus fumigatus* on potato dextrose agar slants were harvested using normal saline and inoculated into the mixture of rice and rice bran. This was covered with muslin cloth and incubated at 30°C for 72 hours with daily stirring for effective enzyme production. A 2.5 g of this *koji* was used as the source of enzyme throughout the experiment.

2.14. Pretreatment and Hydrolysis of Cassava Pulp and Glucose Assay

A 20% (w/v) Cassava pulp was pre-treated by autoclaving at 121°C, and pressure of 15 psi for 10 minutes. It was cooled to room temperature before hydrolysis. A 2.5 g of the *koji* enzyme from *Aspergillus fumigatus* was added to the pre-treated cassava pulp as enzyme for starch hydrolysis throughout the experiment. Cassava pulp without pretreatment (20% w/v) was used as the control. The pre-treated cassava pulp, as well as the control was incubated with *koji* at 42°C for 48 hours. After 48 hours, the concentration of the reducing sugar (glucose) released was measured using HPLC.

2.15. Ethanol Production from Cassava Pulp by *Pichia kudriavzevii* S26, *K. marxianus* NCYC 587, and NCYC2791

The pretreated cassava pulp (20% w/v) was supplemented with 2% polypeptone and 1% yeast extract before fermentation. Yeast strains (S26, NCYC587, and NCYC2791) were pre-cultured in YPD. The pre-cultured yeasts were inoculated into the pretreated cassava pulp (20% w/v) to a final OD of 0.6 at 600 nm. These cassava pulps were incubated at 30°C and 42°C. Samples for ethanol detection were taken at every 24 hour, and the concentration of ethanol was analyzed by HPLC (Prominence, Shimazu Seisakusho, Kyoto, Japan), using Aminex HPX87H (300 × 7.8 mm) (BioRad, California, USA) as the column.

2.16. Statistical Analysis

All the experiments were performed three times and the results were presented as mean ± SD. The data were subjected to Analysis of Variance (single classification) and where significance differences were observed, Least Significance Difference (LSD) was used to separate the means.

3. Results

3.1. Ethanol Production at High Temperatures by the Best Isolates

A total of 500 colonies (100 colonies isolated from palm wine and 400 colonies isolated from spoiled fruits) were obtained. The results of ethanol production by the 10 best isolates, (Table 1) at 42°C and 45°C are shown in Figure 1. With the exception of P71, all the isolates produced higher concentration of ethanol at 42°C than at 45°C. However, in some isolates such as P27 and S26, there was no significant difference in the concentration of ethanol produced at 42°C and 45°C ($p > 0.05$). On the whole, isolates P27 and S26 produced high ethanol concentration (>4%) from 10% glucose. S26 was selected for further studies because it showed more stable performance in terms of the concentrations of ethanol produced from glucose medium under high temperatures than P27 (Figure 2).

Table 1. The 10 best isolates and their isolation source.

ISOLATE	ISOLATION SOURCE
P83	Palm wine
S67	Spoilt fruit (soursop)
P27	Palm residue
P71	Palm wine
S26	Spoilt fruit (soursop)
S65	Spoilt fruit (banana)
S2	Spoilt fruit (soursop)
S86	Spoilt fruit (guava)
S1	Spoilt fruit (soursop)
P5	Spoilt fruit (pineapple)

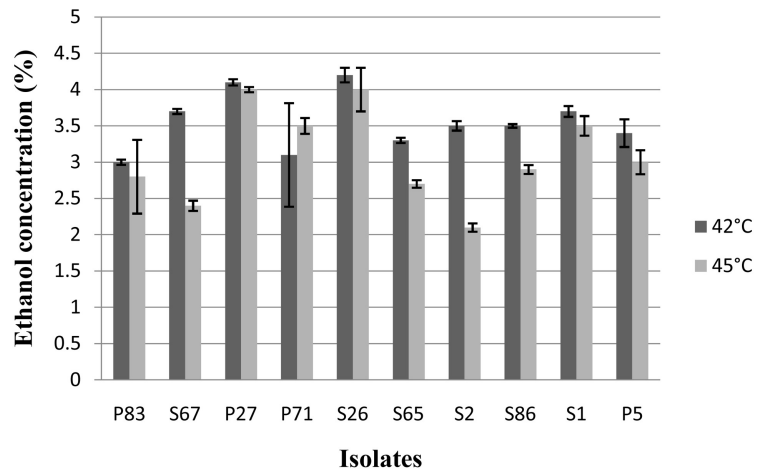


Figure 1. Ethanol production by the 10 best isolates at 42°C and 45°C. The ethanol concentrations were determined after 24 h of cultivation.

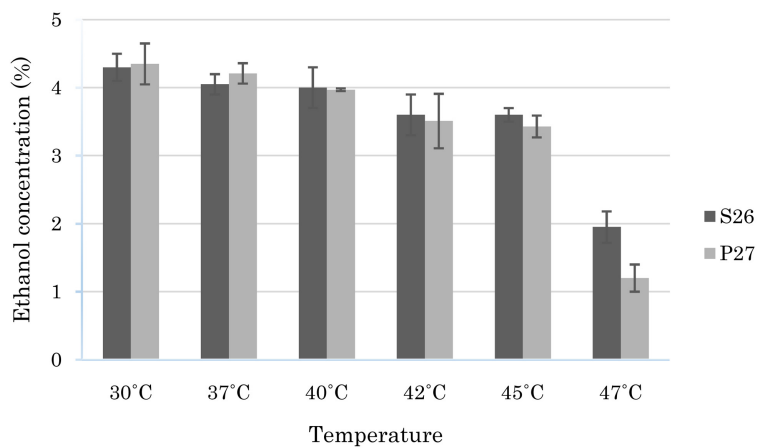


Figure 2. Effect of temperature on ethanol production by isolates S26 and P27. The ethanol concentrations were determined after 24 h of cultivation.

3.2. Characterization and Identification of the Isolate

Isolate S26 was able to assimilate up to 10 sugars (**Table 2**). It assimilated glycerol as carbon source but was not able to assimilate xylose (**Table 2**). This sugar assimilation pattern is a characteristic of *Pichia kudriavzevii* [25].

Time courses of ethanol production by isolate S26 at various temperatures are shown in **Figure 3**. It produced the highest concentration of ethanol (4.3% w/v) at 30°C and the concentration of ethanol decreased gradually as temperature increased. It is important to note that it was able to produce high concentration of ethanol (3.6% w/v) even at 45°C, corresponding to ethanol yield of 70.6%. There was a significant decrease in the concentrations of ethanol produced by the isolate at 47°C ($p < 0.05$).

The effects of pH on ethanol production by the isolate at 30°C and 42°C are shown in **Figure 4**. As shown in **Figure 3**, at a pH of 6, the concentrations of ethanol produced at 30°C and 42°C were 4.3% and 3.7%, which correspond to ethanol yields of 84.3%, and 72.5%, respectively. At extreme pH values of 2 and

Table 2. Sugar assimilation profile of the isolate S26.

Sugars	Degree of assimilation
GLUCOSE	+++
GLYCEROL	+++
2KG	---
ARABINOSE	---+
XYLOSE	---
ADOLOSE	---+
XYLITOL	---+
GALACTOSE	+++
INOSITOL	---
SORBITOL	---+
MDG	---
NAG	---
CEL	---
LACTOSE	---+
MALTOSE	---+
SACROSE	+++
TRE	---
MLZ	---
RAF	---+

+++ = high assimilation; ---+ = moderate assimilation; ---+ = low assimilation, and --- = no assimilation.

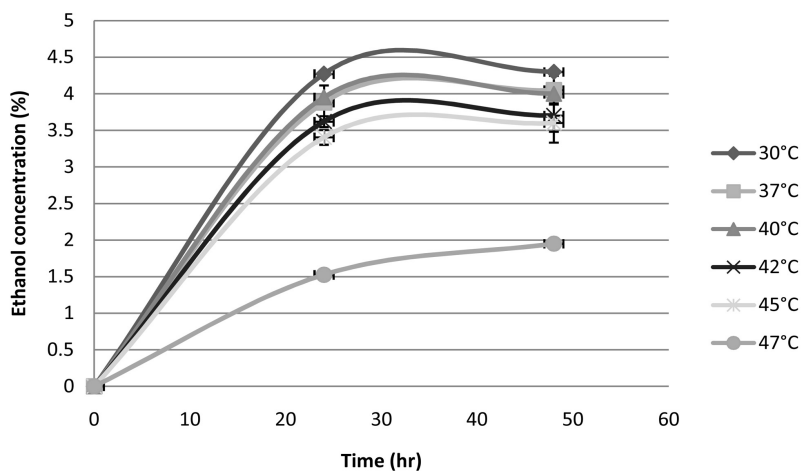


Figure 3. Effect of temperature on ethanol production from 10% YPD by isolate S26.

10, S26 was not able to produce ethanol.

The effect of ethanol and acetic acid on the growth of the isolate is shown in **Figure 5**. The isolate was able to grow on YPD supplemented with 6% ethanol as well as the one supplemented with 70 mM acetic acid. It also exhibited higher tolerance to acetic acid and ethanol than NCYC587 and NCYC2791 (**Figure 5**).

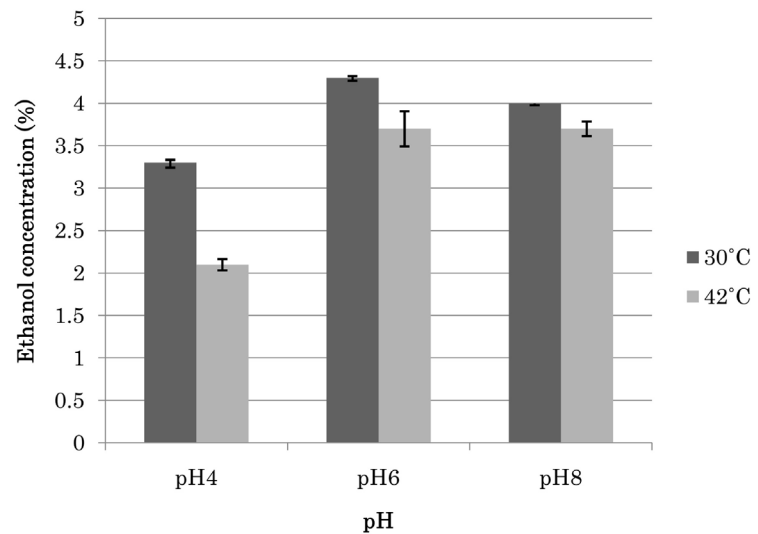


Figure 4. Effect of pH on ethanol production by the isolate S26 at 30°C and 42°C. The ethanol concentrations were determined after 24 h of cultivation.

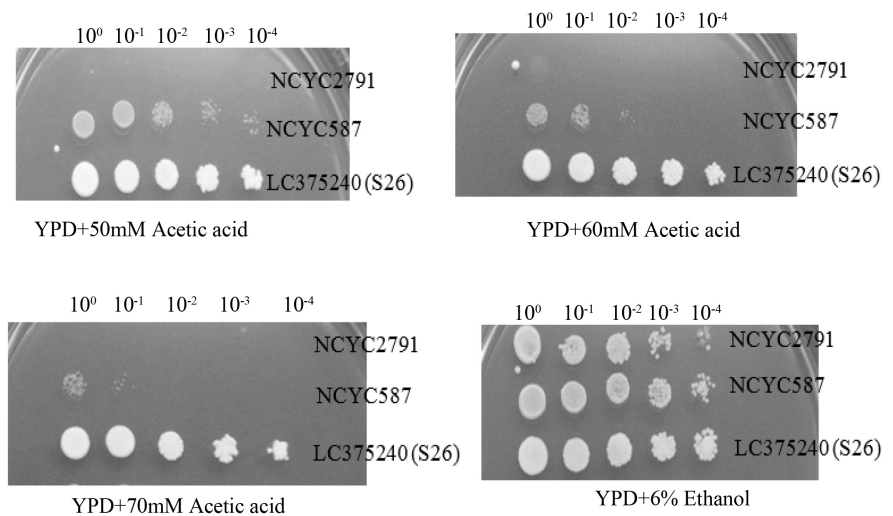


Figure 5. Effects of acetic acid and ethanol on the colony growth of the isolate, LC375240(S26), NCYC 2791 and NCYC 587 on YPD plates. The undiluted (10^0) and serially diluted yeast cells were plated YPD plates containing various concentrations of acetic acid or 6% ethanol and incubated at 40°C for 24 h. The results show that NCYC2791 was very sensitive to acetic acid and was completely inhibited even when undiluted broth (10^0) was plated. On the other hand, LC375240 (S26) was resistant to acetic acid and grew well in the presence of 70 mM acetic acid and 6% ethanol.

While NCYC2791 was not able to grow on YPD containing 70 mM acetic acid, our isolate (S26) grew very well even when diluted to 10^{-4} (Figure 5).

Phylogenetic tree for the isolate was constructed from the sequence and blast search result by using MEGA6 software (Figure 6). The isolate (S26) was grouped with the clade of *Pichia kudriavzevii*. This result agreed with the data on sugar assimilation (Table 2). Consequently, the isolate, S26, was identified as *Pichia kudriavzevii*, and it was registered as LC375240 at Genebank.

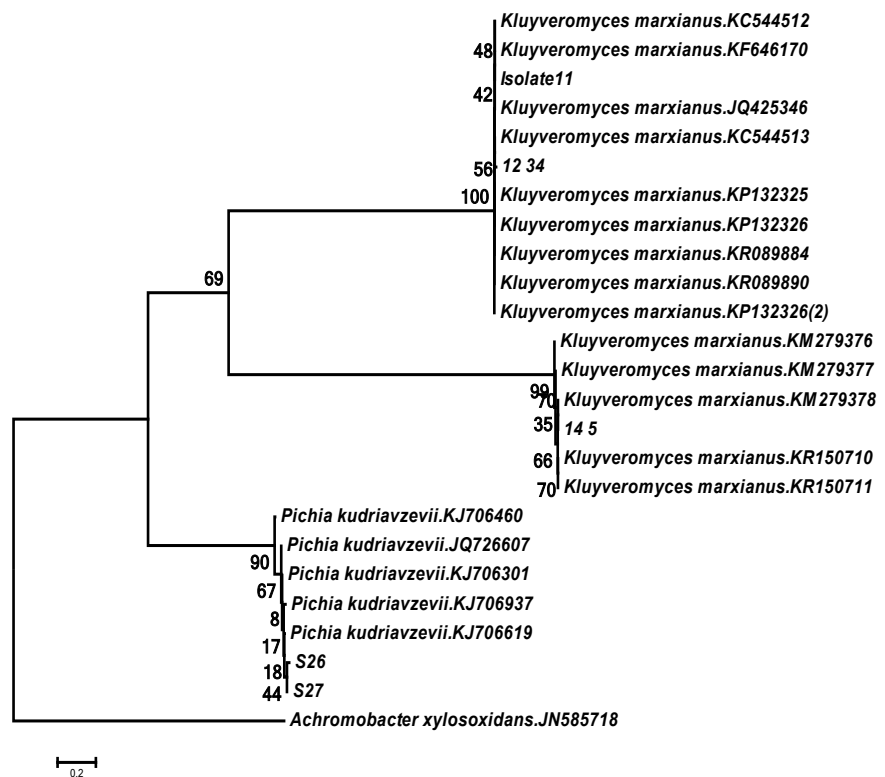


Figure 6. Phylogenetic tree of the isolates.

3.3. Ethanol Production from Cassava Pulp

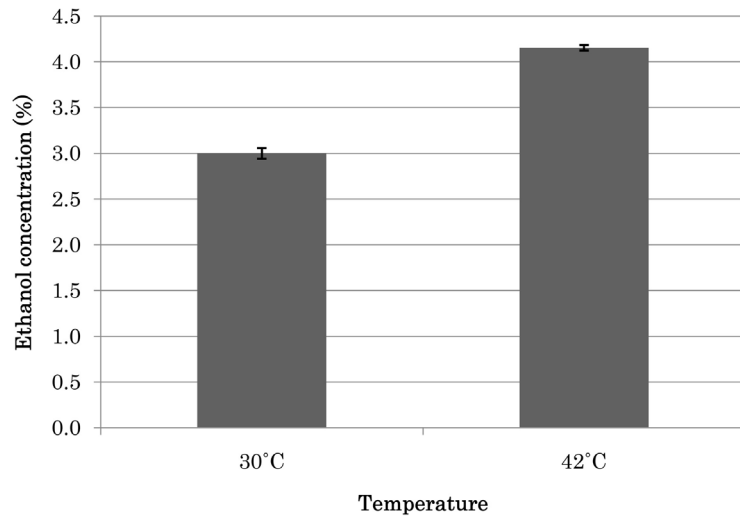
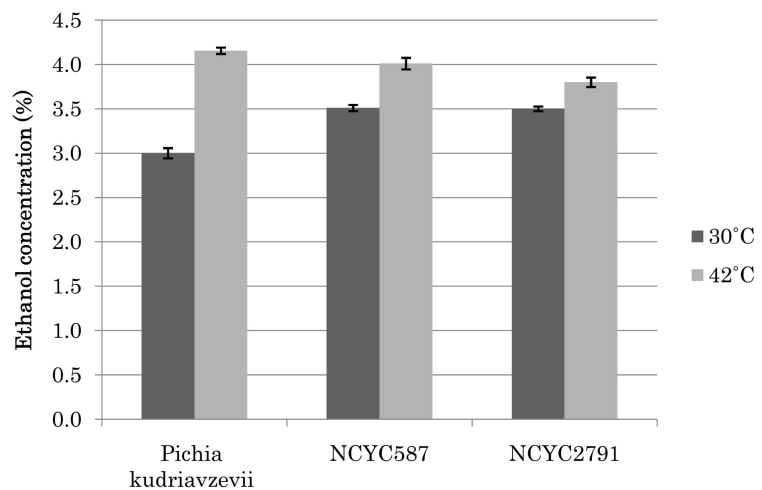
The starch and cellulose contents of dried cassava pulp from Nigeria Starch Mill, Iheala were $48.6\% \pm 4.0\%$ and $21.5\% \pm 2.0\%$ respectively. The cassava pulp (20% w/v) was pre-treated by autoclaving at 121°C for 20 minutes and hydrolyzed using *Aspergillus fumigatus* koji. After 24 hours of hydrolysis, the concentration of glucose increased from 0.69 ± 0.05 to 10.09 ± 0.05 while the concentration of starch decreased from 9.66 ± 0.2 to 0.56 ± 0.02 (Table 3). Besides, little sugar was detected in cassava pulp without pretreatment (data not shown). The results of Simultaneous Saccharification and Fermentation of 20% cassava pulp using koji and each of the yeast strains (S26, NCYC587, NCYC2791) are shown in Figure 7. S26 produced higher concentration of ethanol at 42°C ($4.2\% \pm 0.03\%$) than at 30°C ($3.0\% \pm 0.06\%$). A comparison of ethanol production ability of S26 with those of known thermotolerant strains of *Kluyveromyces marxianus* is shown in Figure 8. All the three strains (S26, *K. marxianus* NCYC587 and NCYC2791) produced higher ethanol concentrations of 4.2%, 4.0% and 3.7%, respectively, at 42°C than at 30°C . These correspond to ethanol yields of 82.3%, 78.4% and 72.5%, respectively (Figure 8). These results demonstrated that the new isolate (S26) has a great potential for ethanol production from cassava pulp at high temperatures.

4. Discussion

Pichia kudriavzevii which is able to grow and produce ethanol within a temperature

Table 3. Effect of autoclaving for 10 minutes and hydrolysis using *Aspergillus fumigatus* koji on the composition of 20% (w/v) cassava pulp.

Components	Initial	24 h after hydrolysis
Starch	9.66 ± 0.2	0.56 ± 0.02
Cellulose	1.64 ± 0.03	0.49 ± 0.01
Glucose	0.69 ± 0.05	10.09 ± 0.05

**Figure 7.** Ethanol production from cassava pulp at 30°C and 42°C by the isolated *Pichia kudriavzevii* S26 (LC375240) using SSF. The ethanol concentrations were determined after 24 h of fermentation.**Figure 8.** Comparison of ethanol production abilities of the *P. kudriavzevii* S26 (LC375240) and some typed strains from cassava pulp using SSF at 30°C and 42°C (*K. marxianus* NCYC587 and NCYC2791). The ethanol concentrations were determined after 24 h of fermentation.

range of 30°C and 47°C was isolated in this study. This conforms the previous reports that many thermo-tolerant strains of this yeast grow well at temperatures as high as 45°C to 52°C and can efficiently ferment and produce ethanol at tem-

peratures of between 38°C and 45°C [14] [18] [26] [27] [28].

Using 20% cassava pulp supplemented with 1% yeast extract and 2% peptone at 42°C, the isolate produced 42 g/l ethanol representing an ethanol yield of 0.21 g-ethanol/g-cassava pulp using Simultaneous Saccharification and Fermentation method. This was 82.4% of theoretical ethanol yield. At 30°C under the same conditions, the isolate produced 30 g/l ethanol representing an ethanol yield of 0.15 g/g of cassava pulp and 58.8% theoretical ethanol yield. It is interesting to note that the ethanol yield was even higher at 42°C than at 30°C. This could be because 42°C is closer to the optima temperature for the saccharifying and hydrolytic enzymes and highlights the significance of this study. The temperature inside fermentation tank increases gradually during fermentation but even without temperature control, it does not usually rise above 45°C. Thus with a thermotolerant yeast which can efficiently produce ethanol within a wide range of temperature (between 35°C and 45°C) means that ethanol production can be done without temperature control. In other words, the *P. kudriavzevii*, S26 (LC375240) isolated in this study has a great potential for commercial ethanol production. This strain produced even higher concentration of ethanol at 42°C than at 30°C (Figure 3 and Figure 7). When conventional yeast, *Saccharomyces cerevisiae*, was cultivated consecutively at high temperature, the morphological shape of the cells became elongated [29]. This morphological alteration was observed also observed in *P. kudriavzevii* when it was used for continuous ethanol fermentation under high temperature [30]. The sporadic filamentous cells might be a requirement for tolerance or acquired acclimation in the fermentation system at high temperatures.

The ethanol yields obtained in this study are higher than those reported in some previous works. For example, Kaewkrajay *et al.* [19] reported that *P. kudriavzevii* PBB511-1 produced 37.0 g/l ethanol with 40% of theoretical yield in a cassava starch hydrolysate medium containing reducing sugar (180 g/l), $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/l), KH_2PO_4 (0.5 g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g/l), and yeast extract (1.0 g/l) at 45°C. Yuangsaard *et al.* [18] reported that *P. kudriavzevii* DMKU 3-ET15 (isolated from traditional fermented pork sausage) could produce ethanol from cassava starch hydrolysate medium composed of 18% glucose, 0.05% $(\text{NH}_4)_2\text{SO}_4$, 0.09% yeast extract, 0.05% KH_2PO_4 , and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ but at 45°C, only 4% (w/v) ethanol, (42% of theoretical yield) was produced. Dhaliwal *et al.* [17] reported isolation of *P. kudriavzevii* from sugarcane juice which at 40°C, produced 71.9 g/l of ethanol and a productivity of 4.0 (g/l-h) from sugarcane juice composed of 14% (w/v) sucrose, 2% (w/v) glucose, and 1% (w/v) fructose. Oberoi *et al.* [28] reported isolation of a thermo-tolerant *P. kudriavzevii* HOP-1 which produced 24.25 g/l of ethanol, corresponding to 82% of the theoretical ethanol yield on glucan basis from 1% sodium hydroxide-treated rice straw via simultaneous saccharification and fermentation at 40°C. However, some reports have also shown that higher ethanol concentrations and yields can be obtained with *P. kudriavzevii* species. For example, Yuangsaard *et al.* [18] re-

ported an ethanol theoretical yield of 85.4% using *P. kudriavzevii* DMKU 3-ET15 (isolated from traditional fermented pork sausage) at 40°C. On the whole, the final ethanol concentrations and yields depend, not only on the strain, but also on fermentation conditions, especially the nature of substrate. Cassava pulp, which is generated in large quantities in Nigeria and poses a lot of environmental problem was used in this study. The starch content of the pulp (48%) was lower than those reported in other works [5] [9] [11] [31]. However, the cellulose content (21.5%) was higher than the value reported by Rattana-chomsri *et al.* [9] and Virunanon *et al.* [31]. They reported a cellulose contents of 15.6% and 4.11% respectively. Generally, the starch and cellulose contents of cassava pulp depend on the variety of cassava, the age of the cassava and the processing method. Nevertheless, the present study has demonstrated that cassava pulp is a good substrate for ethanol production.

Nigeria remains the world largest producer of cassava and in 2016, she produced a total of 57,134,478 tons. With the technology currently used to produce cassava starch in Nigeria, 400 kg of pulp is produced per ton of cassava tubers (on wet weight basis) and assuming 60% water content of the pulp, 160 kg of dry pulp is produced per ton of cassava tubers. If only 20% of cassava tubers (11,426,895 tons of cassava tubers) is processed into starch, 1,828,303 tons of dry pulp will be produced. With the current yield of 0.21 g-ethanol/g-pulp and assuming ethanol density of 0.789 mL/g, 266 L of anhydrous ethanol will be produced per ton of dry pulp. Thus, it is estimated that 486,328,598 L of ethanol will be produced from the pulp generated from 20% of cassava produced in Nigeria. Currently, Nigerian local refineries are producing only about 8 million liters of Premium motor spirit (PMS) per day. This requires only 800,000 liters of ethanol per day (292,000,000 L per annum) to make E10 (90% gasoline: 10% ethanol) and can be supplied by converting pulp from only 12% of cassava produced in Nigeria. Cassava pulp, being a waste, is usually exposed to many kinds of microorganisms and thus contains high levels of organic acids (lactic acid or acetic acid). These organic acids inhibit ethanol fermentation [32]. However, our isolated *P. kudriavezevii* can grow on YPD supplemented with 70 mM acetic acid even at 10^{-4} dilution. It has also been known that *P. kudriavezevii* has high tolerance to organic acids, and it was able to produce ethanol from YPD medium containing 50 mM acetic acid [33]. Therefore, *P. kudriavzevii*, S26 a robust yeast for ethanol production without proper sterilization of cassava pulp.

It is also important to note that the isolate obtained in the present study gave higher ethanol yield than those of the typed ethanol-fermenting thermo-tolerant strains of *Kluveromyces maxianus* from the National Collection of Yeast Cultures Institute in the United Kingdom (NCYC 2791 and NCYC 587) under the same culture conditions. This yeast strain thus hold a good potential for large scale bio-ethanol production in tropical areas like Nigeria as they will help reduce the cost of cooling during fermentation, save time, reduce contamination and thus reduce the cost of producing bio-ethanol.

5. Conclusion

A thermo-tolerant ethanol-fermenting strain of *Pichia kudriavzevii* S26 (LC375240) was isolated from decaying fruits in Nigeria. The potential of the isolate in producing ethanol from cassava pulp was evaluated. Under optimum temperature of 42°C, the isolate was able to produce 0.21 g-ethanol/g-cassava pulp. It is estimated that with this strain, the amount of ethanol needed to produce E10 from all the PMS locally refined in Nigeria can be produced from cassava pulp generated from only 12% of cassava tubers produced in Nigeria by Starch Mills. Higher ethanol yields can be obtained by optimizing the pretreatment of cassava pulps to enhance effective conversion of all the starch and non-starch contents of the pulp to fermentable sugars.

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Conflicts of Interest

There is no conflict of interests.

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