



Studies on Bioethanol Production with Thermo Tolerant Yeast Isolates and their Co-Cultures using African Wild Cocoyam as Feedstock

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Authors' contributions

This work was carried out in collaboration among all authors. Author NCI and NOU designed the study. Author NOU and NIA wrote the protocol and initial draft of the manuscript. Authors NCI, NOU, NIA and AOC managed other aspects of the study including analyses and literature review. All authors read and approved the final manuscript.

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ABSTRACT

In this work different ways of optimally producing bioethanol at various pH with thermotolerant yeasts and their cocultures using a non-human edible starchy food as feedstock was examined. African wild cocoyam, *Xanthosoma roseum*, sourced from abandoned farmlands in Obukpa, Nsukka, Nigeria was used as the substrate, while strains of *Kluyveromyces marxianus* and *Pichia stipitis* were used to ferment them. First the tubers were gelatinized by boiling under pressure above 100°C before hydrolysis with concentrated H₂SO₄. The hydrolysates were then fermented at 35°C with the thermotolerant yeasts for five days at different pH. Results obtained showed that gelatinized sample of the substrate gave optimum glucose yield when hydrolysed with 1M H₂SO₄ for 60 minutes. *Kluyveromyces marxianus* produced more ethanol than *Pichia stipitis* at all the four fermentation pH values tested. However, optimum ethanol production

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was obtained when the two yeast strains were used as coculture at pH 4.5. The peak time for ethanol production was 96 hours for the individual yeast cultures while that of their coculture was 72 hours. The results of the study indicated that wild cocoyam is an excellent feedstock for bioethanol production with many advantages including being non-edible, thereby eliminating concerns for food security, and containing high amount of carbohydrate. The study also revealed that fermenting sugar hydrolysates with a coculture of microorganisms during bioethanol production is a more efficient process than using individual cultures.

Keywords: Bioethanol; yeast strains; feedstock; biofuel; cocoyam.

1. INTRODUCTION

The increasing impact of climate change and its many environmental consequences has caused an intensification in the search for alternatives to fossil fuels. Beyond their environmental challenges, palpable fear exists over the increasingly higher levels of crude oil production needed to meet rising demands. This has been the case over the past few decades and could lead to such terrible depletions as could have fatal consequence on world energy if no credible substitutes are found [1]. One identified plausible alternative is the use of ethanol which is increasingly becoming one of the most preferred energy options available presently. This is because of some advantages it possesses. For instance, ethanol is considered clean and able to contain greenhouse gas emissions by over 50% [2]. Also, compared to available alternatives, ethanol has the least carbon abatement cost, lower even than electricity [3]. Again, it is a renewable energy and therefore sustainable unlike fossil fuels.

However, to produce it at levels that could be maintained ad infinitum to meet world demand, many hurdles still have to be crossed. Among many others is the fact that at present, its cost of production is much higher than those of fossil fuels; additionally, many food crops are required to produce it at large commercial scales currently [4]. Sugarcane which contains the necessary sucrose feedstock for its production is the major raw material mostly used, although other starchy materials like cassava and corn are increasingly been utilized also [5].

However, the use of these food crops as feedstocks for bioethanol production raises concerns about food security as they constitute staple foods in many regions of the world; even their waste products are used to feed livestock [6]. Thus, an ideal feedstock for bioethanol production should be one that is not edible yet

has high carbohydrate content and is widely distributed and easily processed [7].

Therefore, the search for credible options without the disadvantageous mentioned above is necessary. A feedstock that has high potentials of being used for bioethanol production is African wild cocoyam (*Xanthosoma roseum*). This is because it has been found to have a starch composition of 72% plus the four major sugars: sucrose, maltose, glucose and fructose. It is also not usually eaten by humans nor used as livestock feed. Additionally, it has the advantage of being available all through the year in the tropics [8].

In tropical countries where average day-time temperature is usually high all year round, high temperature fermentation is a common process condition for most industrial productions [9]. However, to achieve high temperature fermentation in bioethanol production, it is necessary to use yeast strains that could thrive in such conditions. These are usually referred to as thermotolerant yeasts [10]. Currently, most industrial ethanol production employs mesophilic strains of *Zymomonas mobilis* and *Saccharomyces cerevisiae* [9]. Despite few reports of applications of thermotolerant yeast strains in industrial ethanol production, only a few have been concerned with *Pichia stipitis* and *Kluyveromyces marxianus* [9,11,12]. This study is therefore a report of our attempt to produce bioethanol at different medium pH using strains of *Kluyveromyces marxianus* and *Pichia stipitis* and African wild cocoyam (*Xanthosoma roseum*) as feedstock.

2. MATERIALS AND METHODS

2.1 Sample Sourcing and Pre-Treatment

The wild cocoyam samples used in the study were obtained from some abandoned farmlands located at Obukpa town in Nsukka LGA of Enugu

State, Nigeria. The wild cocoyam corms were first washed, peeled, rinsed in clean water and sundried for 48 hours. Thereafter, 4 sets of 10 kg samples of the cocoyam corms were weighed out in duplicates and soaked in clean water for 24 hours to dilute out any resident impurities. They were then placed on a clean tray and left to dry in the sun for 4 hours before being transferred to the laboratory and dried in the oven at 100°C for another 4 hours. After this dewatering process, the cocoyam corms were cut into small pieces and transferred to an electric blender and milled into powdery flours.

2.2 Gelatinization of Cocoyam Sample

Exactly 200 g of the powdered cocoyam sample were weighed into a beaker containing 100 mL of water and the contents thoroughly homogenized by stirring. The beaker with its content was covered with aluminium foil and cooked in a pressure cooker for 15 minutes at a pressure of 10 psi and at a temperature of 108.9°C [13]. This treatment gelatinized the sample.

2.3 Optimization of Acid Concentration for Hydrolysis

Afterwards, 15 g of the gelatinized cocoyam sample was weighed into three different 500 mL conical flasks labelled A1, A2 and A3. Then 100 mL of sulphuric acid (H₂SO₄) solution with concentrations; 0.5 M, 1 M and 2 M; were added to conical flasks A1, A2 and A3 respectively. The flasks were then homogenized and incubated at temperature 100°C for 30 minutes (using a water bath). Afterwards, samples were collected and the sugar concentration of each hydrolysate determined. The acid concentration that yielded the highest percentage of sugar in the cocoyam samples was selected as the optimum concentration of acid.

2.4 Optimization of Hydrolysis Time

Fresh 15 g of the gelatinized cocoyam sample was again weighed into two 500 mL conical flasks, labelled B1 and B2. Thereafter, 100 mL of the acid concentration obtained after optimization above was added, with flask B1 incubated for 30 minutes while B2 was incubated for 60 minutes, both at temperature 100°C. The concentration of glucose from hydrolysates B1 and B2 were determined.

2.5 Determination of Sugar Concentration

The method described by Lau and Luk [14] was used. One (1) mL of the sample filtrate was taken

into a test tube. Then, 2 mL of DNSA (dinitrosalicylic acid) was added. A blank was prepared containing 1 mL distilled water and 2 mL DNSA. The test tubes were incubated in boiling water for 10 minutes and cooled. The mixture was made up to 10 mL. A Spectronic 21D (Milton Roy, Made in USA) UV spectrophotometer. was used to check absorbance at 540 nm. A standard curve of glucose was prepared and used to calculate the percentage reducing sugar in the hydrolysate.

2.6 Microorganisms used

Two thermotolerant yeasts (*Kluyveromyces marxianus* and *Pichia stipitis*) were used in this study. A stock culture of molecularly characterised *Pichia stipitis* was kindly provided by Mrs Ndubuisi, I. A. of the Department of Microbiology, University of Nigeria, Nsukka. The *Kluyveromyces marxianus* was isolated from rotten soursop fruit obtained from Ogige market in Nsukka, Enugu state, Nigeria.

2.7 Thermotolerant Yeast Isolation and Identification

Pure cultures of the thermotolerant yeast used in this study were isolated using an enrichment media comprising: YPG agar; containing 1% Yeast extract, 2% Peptone, 2% Glucose and 2% agar. For isolation, a loopful of rotten soursop sample was inoculated on the YPG agar and incubated at 43-45°C for 48 hours. Thereafter, discrete colonies from the agar were serially diluted and sub-cultured on YPG broth (without agar) and incubated for 48 hours at 43-45°C. A loopful of the broth sample was then serially diluted and plated out on YPG agar. The pure isolates obtained afterwards were stored on agar slants at 4°C for subsequent use. The yeasts isolates were then characterized and identified on the basis of their colony morphology and biochemical characteristics using Mycology Atlas [15] as guide.

2.8 Screening of the Yeast Cultures for Ethanol Production at Different pH

First, overnight pre-cultures of the two thermotolerant yeasts were prepared as described by Apiradee [16] by inoculating a loopful of the yeast cultures into YPG (Yeast Extract–Peptone–Glycerol (YPG) *Medium*) broth and incubating at 43°C for 24 hours. Thereafter, the optimum acid and sugar concentrations as determined above, were used to hydrolyse and

evaluate the amount of ethanol produced by the cocoyam samples. The following detailed method was used. Exactly 50 ml of the optimized hydrolysates were dispensed into 3 sets of 4 separate 500 ml conical flasks (making it 12 altogether). To the first two set of flasks were inoculated 1 ml each of the two yeast cells while the third set was inoculated with 0.5 ml of each of them (bringing the total to 1 ml) hence the labelling K1, K2, K3 and K4; P1, P2, P3 and P4; K-P1, K-P2, K-P3 and K-P4 for each of the 12 successive flasks. But before inoculation, the pH of the hydrolysate in the 12 conical flasks were adjusted with 0.4M NaOH in the following way: pH of K1, P1 and KP1 were adjusted to 4.0; that of K2, P2 and KP2 were adjusted to 4.5; then K3, P3 and KP3 to 5.0 and K4, P4 and KP4 to 5.5. All 12 conical flasks were then appropriately covered and incubated at room temperature (35°C) for 120 hours (5 days). The flasks were shaken at interval to produce a homogenous solution and ensure even distribution of the organisms in the substrate mixture. The mixtures were separately filtered after 5 days using Whatman No 1 filter paper.

2.9 Determination of the Ethanol Produced

The ethanol produced at the end of incubation were assessed using the method of Caputi et al. [17] as modified by Oyeleke et al. [18]. This involved the following. First, the fermented liquids in each flask were transferred into round bottom flasks and placed on a heating apparatus whose temperature have been adjusted to 78°C (standard temperature for ethanol production). The flasks were then fixed to a distillation column enclosed in running water. Fresh conical flasks were used to collect the distillates at 78°C. The distillates collected were quantified with a measuring cylinder and first expressed as quantity of ethanol produced in g/l by multiplying the volume of the distillate by the density of ethanol (0.8033g/cm³) before being adjusted to the percentage of ethanol produced by each microorganism or their coculture. This was done by comparing or fitting them to a standard ethanol density curve. Standard ethanol curve was obtained by taking series of percentage (v/v) ethanol (10%, 20%, 30%, 40% and 50%) solution which were prepared in a 100ml volumetric flask and the weights measured.

2.10 Statistical Analysis

Triplicate analyses of all the samples were carried out. Appropriate means and error sources

were thereafter worked out and used during analyses.

3. RESULTS AND DISCUSSION

Table 1 shows some morphological characteristics of the different *Kluyveromyces* species isolated in the course of this work. A few interesting observations from the table shows that it is a yeast and belongs to the genus *Kluyveromyces*. These include the fact that its pigmentation is white and creamy and that it is raised, smooth and clustered with cell length of between 5.0-10.5 cm. These are believed to be distinctive indicators of the yeast *Kluyveromyces marxianus* [19].

3.1 Optimal Acid Concentration for Hydrolysis

The plant-based feedstocks typically used for bioethanol production comprise mostly of cellulose, a very resistant and hard crystalline structure overlaid by the physical barrier formed by the equally hard lignin and hemicellulose [20]. This results in a low or almost nil conversion of these carbohydrate macromolecules to fermentable sugars especially glucose, which should then serve as the base for ethanol production via fermentation by yeast cells. It is therefore usually necessary to pre-treat the plant materials to make them able to hydrolyse to the sugars and acid hydrolysis is one of the best pre-treatment options because it goes all the way to converting hemicellulose into fermentable sugars at very high reaction rates and at very low costs [21]. This explains why we chose to pre-treat the cocoyam feedstock with dilute sulphuric acid in this study. Fig. 1 shows the results obtained when different molar concentrations of dilute H₂SO₄ were used to hydrolyse the gelatinized cocoyam samples. The result shows that the optimum result was obtained when 1M concentration of the dilute acid was used for hydrolysis followed by the 2M concentration while the least concentration (0.5M) gave the least result in terms of quantity of sugar made available for yeast fermentation. Different authors have carried out similar works. Abidin et al. [22] in their work with cassava peel wastes found that the highest amount of fermentable sugar was given by an acid concentration of 0.5M, followed by 1M concentration while the lowest amount of sugar was given by the least acid concentration they used. Obviously, the difference in composition between the cocoyam samples used in the present work and the cassava wastes they

Table 1. Morphological and biochemical characterization of yeast isolates

Characteristics	Y1	Y2	Y3	Y4	Y5
Pigmentation	Creamy;White	Creamy;White	Creamy;White	Creamy;White	Creamy;White
Colony Morphology	Ovoidal;Globose;Single or pairs	Raised;Smooth;Cylindricalto elongated	Flat;Ellipsoidal;Oblong toeclipse	Convex;Wrinkled;Undulating	Ovoidal Rough Undulating
Ascospore	-	+	-	+	-
Pseudohyphae	+	+	-	+	-
Glucose	+	+	+	+	+
Galactose	+	+	-	+	-
Lactose	+	V	+	-	-
Dextrose	-	+	-	-	-
Xylose	-	-	+	-	-
Sucrose	-	+	+	+	+
Maltose	+	-	-	+	-
Urease	-	-	-	-	-

Key: += Positive; - = Negative; V = Variable

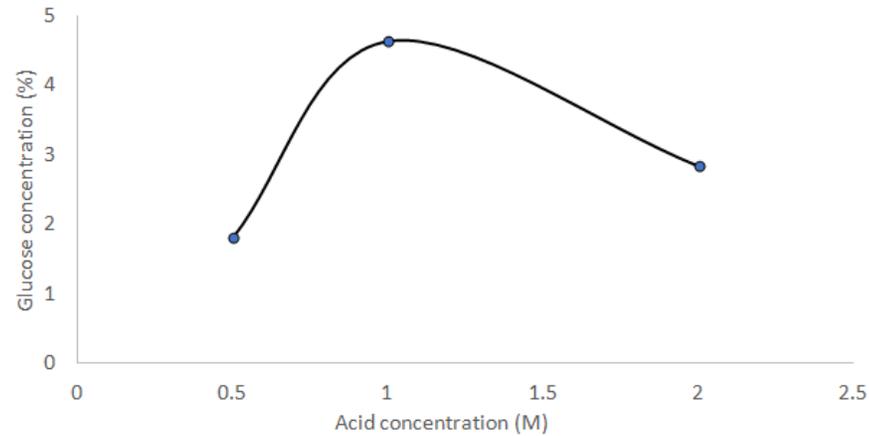


Fig. 1. Percentage Glucose yield at different acid concentrations

used may have played some part in the differences observed in the two results. Delcampo et al. [23] found that the presence of dilute acid facilitated the release of more sugars compared to those without added acids. All of these go to show the importance of dilute acids especially dilute sulphuric acids as good pre-treatment alternatives in the hydrolysis of plant-based carbohydrate materials for ethanol production by yeasts. Apart from use of dilute acids which extend beyond sulphur acid to HCl among others, other pre-treatment options include use of bases and alkaline and also different aspects of physical and heat-based options including very high temperatures among others [24].

3.2 Optimal pH and Time for Ethanol Production

A key factor in the production of ethanol from cellulosic plant-based materials is pH. This is because the pH of the fermentation broth solution determines to a large extent, the process

of sugar conversion to bioethanol as very acidic or highly alkaline fermentation solutions can inhibit the fermentation process, thus reducing the amount of bioethanol produced [25].

Results obtained in our work showed that the pH played an important role during the course of the work. This is readily shown in Figs 2 to 5. Fig 2 shows the time course of ethanol production over the 120 hours experimental period when incubated at pH 4. The figure shows that the optimal ethanol yield was obtained after 72 hours. Of the three experimental cultures used, the best ethanol yield was given by the coculture which combined the two yeasts.

A similar trend was also observed in Fig 3 as the optimal ethanol yield was obtained after 72 hours of fermentation at pH 4.5. However, the ethanol yield obtained at pH of 4.5 was 25.13% which was over 6-fold higher than that obtained when the pH was 4 (4.21%). This is an obvious indication of the great impact hydrogen ion concentration has in ethanol production during

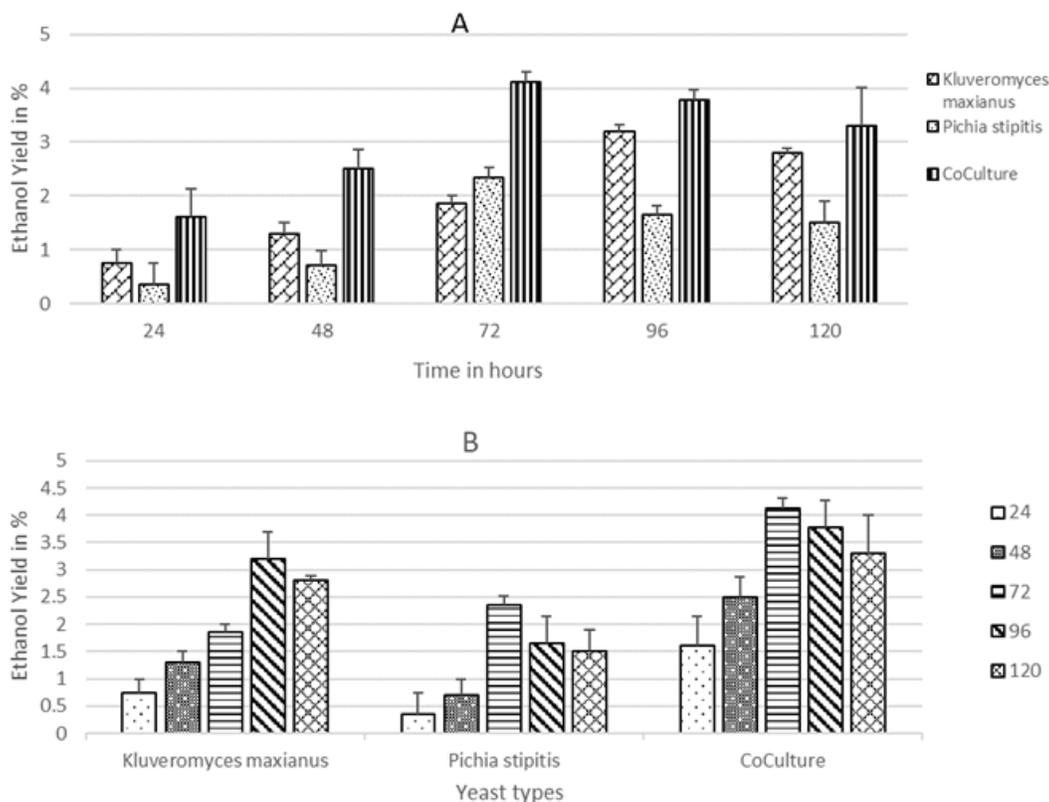


Fig. 2. Time course of ethanol yield percentage by yeast cultures at pH 4.0 in terms of A, Time and B, Yeast cells

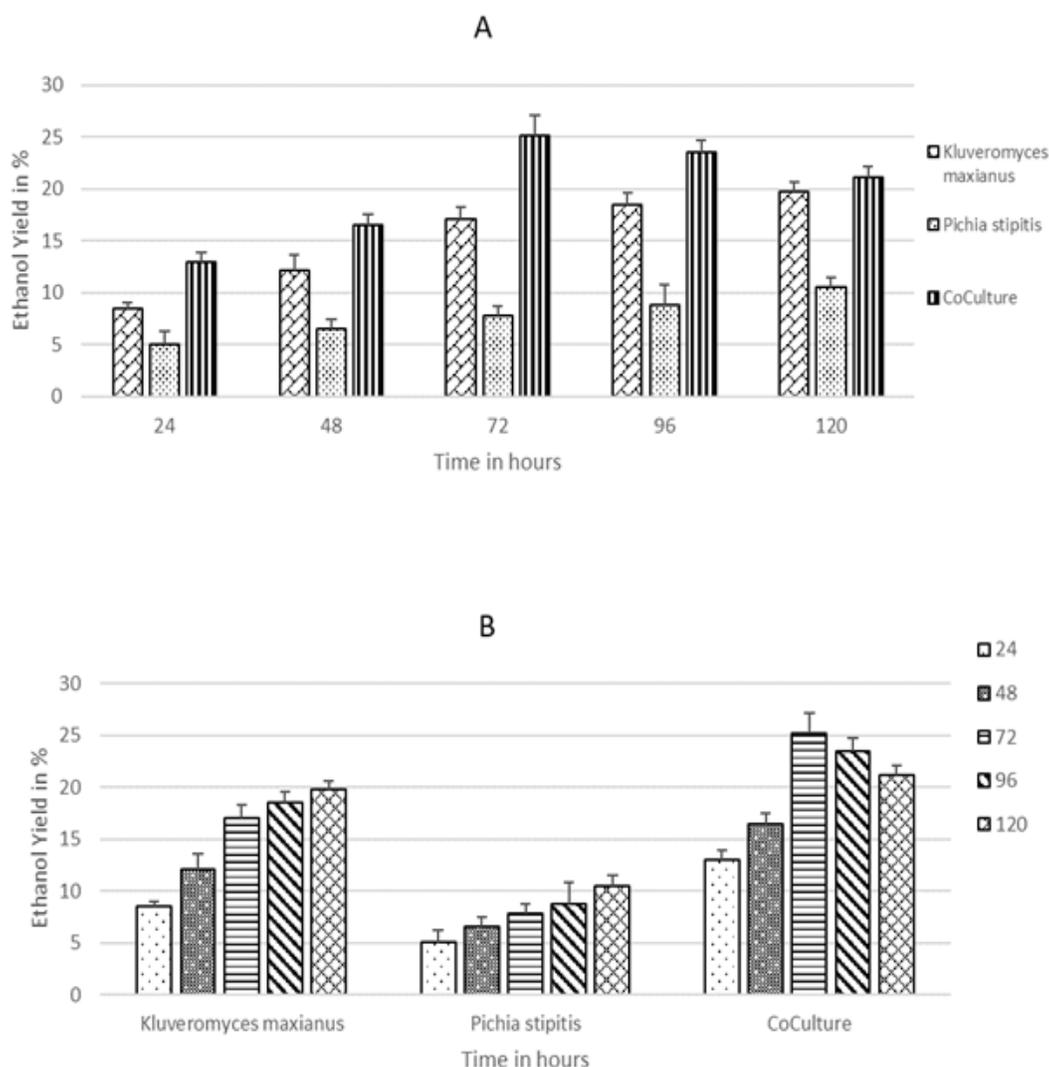


Fig. 3. Time course of ethanol yield percentage by yeast cultures at pH 4.5 in terms of A, Time and B, Yeast cells

the experiment. From the figure it was observed that the yeast species *Kluyveromyces maxianus* consistently gave a higher ethanol yield than *Pichia kudriavzevii*. However, as in the previous figure, the coculture of the two microorganisms gave the optimum ethanol yield at that incubation condition. In their work, Hashem et al. [9] found consistently, that is, with all the yeast strains they used, that the highest ethanol yield was usually obtained after 72 hours incubation time. However, this optimal yield was obtained at a higher pH of 6.5 unlike in our case. In their work, the peak ethanol yield of about 11.7% was given by *Kluyveromyces maxianus* strain GU133331 which is considerably less than the over 15%

obtained by the *Kluyveromyces* sp. isolated and used in the present study and even much less so than that (over 25%) given by the coculture.

Figs 4 and 5 which represent the time course of ethanol yield with the three yeast treatments at pH(s) 5 and 5.5 showed a similar trend as the two previous ones in terms of giving their peak ethanol yield at 72 hours. However, the amount of ethanol produced at the two pHs were considerably less than that produced at pH 4.5. Similar to previous observations, they also produced their optimal ethanol levels when the two yeast strains were used together as coculture. Also, consistent with the previous

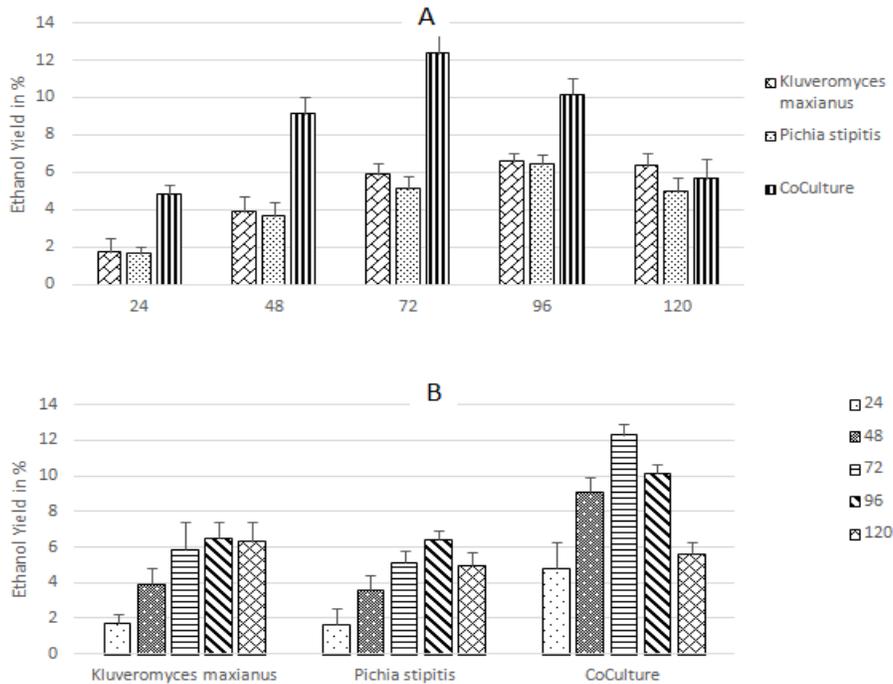


Fig. 4. Time course of ethanol yield percentage by yeast cultures at pH 5.0 in terms of A, Time and B, Yeast cells

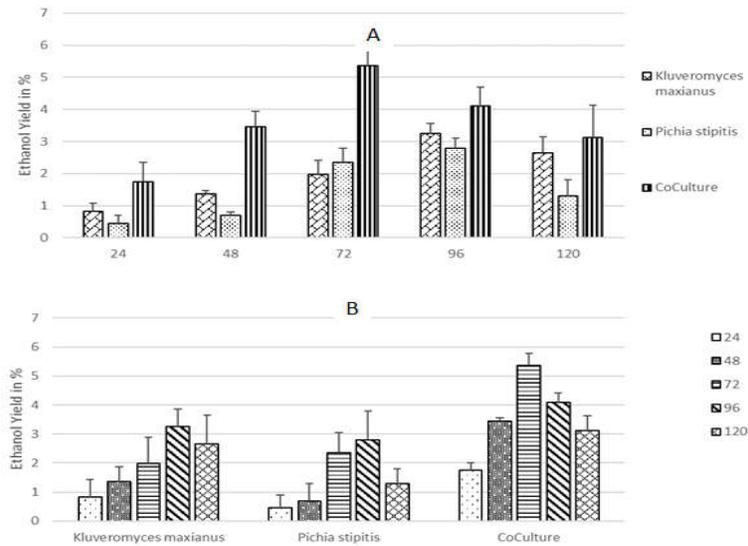


Fig. 5. Time course of ethanol yield percentage by yeast cultures at pH 5.5 in terms of A, Time and B, Yeast cells

observations, the figures shows that *Kluveromyces maxianus* produced higher amount of ethanol than the *Pichia* strain.

Several possible reasons could be given for the better performance observed with the coculture.

In the first place, they mimic the fact that most microbial species naturally coexist and interact with each other. Typically, these microorganisms show their greatest effectiveness at those periods when they are present in association with

other microbial groups [26]. For instance, it is believed that cocultures perform much better than monocultures in the production of enzymes necessary for substrate utilization and conversion to useful by-products [27]. Also, monocultures mostly use carbon and similar substrates for growth and increased biomass yield, whereas cocultures tend to convert these compounds to intermediate compounds including ethanol [26].

4. CONCLUSION

Results obtained in the experiment reported in this paper revealed that African wild cocoyam, a non-human edible starchy food has the potential to be used as feedstock in the production of bioethanol using yeast cells at different pH levels. The yeast cells used which were *Kluyveromyces marxianus* and *Pichia stipitis* were able to ferment the carbohydrate feedstock used. Of the two yeasts used, *Kluyveromyces marxianus* produced more ethanol than *Pichia stipitis*. However, the use of the two different yeast types together as a coculture resulted in the production of more bioethanol (25%) compared to when the microorganisms were used alone. This was so at all the pH levels tested for. This higher ethanol production was also achieved at considerable lesser time.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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