Novel Research in Microbiology Journal (2022), 6(1): 1494-1514
Research Article

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/
DOI: 10.21608/nrmj.2022.217437

Utilization of Date palm (*Phoenix dactylifera* L.) wastes for bioethanol production using *Pichia kudriavzevii* strains

Folake T. Afolabi[1*]; Ibukun E. Ola[1]

[1]Industrial Biotechnology Unit, Department of Microbiology, University of Ibadan, Ibadan, Nigeria

[*]Corresponding author E-mail: folakeojo1@yahoo.com

Received: 3 January, 2022; Accepted: 4 February, 2022; Published online: 6 February, 2022

Abstract

Tons of Date palm (*Phoenix dactylifera* L.) fruit wastes are discarded daily by the date palm processing industries thus leading to serious environmental problems. This study aimed to investigate the potential of date palm fruit wastes as sugary feedstock for bioethanol production using yeasts. Proximate analysis of the date fruit wastes revealed a moisture content of 8.98 %; crude protein (4.39 %), ash (2.35 %), fat (0.2 %), fiber (0.8 %) and carbohydrate (84.28 %). Sulphuric acid was used for pre-treatment of the date palm fruit substrate. Fermentation was carried out under shaking and static conditions using *Pichia kudriavzevii* yeast strains isolated from date palm fruit wastes. Greater bioethanol yield was observed when the substrates were fermented under shaking condition. Optimization of the physical conditions improved the fermentation process faster, and significantly enhanced the production of bioethanol. An appropriate temperature of 30^{o}C and pH 5 produced high yield of ethanol (5 %) by *Pichia kudriavzevii* SGD21, whereas pH 6 for *Pichia kudriavzevii* SGD30 recorded a higher ethanol yield of 6 %. Under the optimal physical conditions, the fermentation process resulted in the production of 4 % ethanol after an incubation period of 96 h. Moreover, the *Pichia kudriavzevii* strains could be recommended for bioethanol production at 30 % inoculum size, on using sucrose as a carbon source and yeast extract as a nitrogen source. On using the Fourier-Transform Infrared (FTI) spectroscopy, the detected functional group of the produced bioethanol was O-H group. Finally, utilization of date palm fruit wastes and the yeasts strains of *Pichia kudriavzevii* SGD21 and *Pichia kudriavzevii* SGD30 can be exploited for bioethanol production and this could be an effective way for management and utilization of date palm fruit wastes.

Keywords: Date palm fruit wastes, *Pichia kudriavzevii*, Fermentation, Bioethanol production

1. Introduction

The amounts of greenhouse gases present in the atmosphere have increased significantly, due to the high level of pollution that emanated from the use of fossil fuels (Ballesteros *et al*., 2006). Several fossil energy sources (i.e. coal, oil and natural gas) are used for the production of fuel and electricity (Uihlein and Schbek, 2009). Due to the instability in the oil markets and the increase in air pollution, it is imperative to point out other options for renewable and sustainable energy sources. Bioethanol produced from bioenergy feed stocks is one of the sustainable, economic and ecologic solutions for these concerns. It can be produced from reduced sugars, stiff biomass and also from the lignocellulosic biomasses (Sims *et al*., 2008).

Bioethanol derived from biological origins has gained much attention in light of the growing energy crisis in recent years. Bioethanol is now being produced from different sources around the world; the most common are sugar-based plants (Sivakumar *et al*., 2010). Most of the conventional ethanol fermentation feedstock depends on the agricultural crops, such as sugar-based crops; starch-based crops, fruits and vegetables (Balat and Balat, 2009). Several microorganisms are used for the conversion of the different biomasses into ethanol, such as *Saccharomyces cerevisiae* yeast that can be used effectively to produce bioethanol.

Changes in the land-use patterns for bioethanol production that deviate from the food crops could be a threat to the global food security. For this reason, the use of a second generation of bioethanol produced from agricultural wastes such lignocellulosic materials including grasses, crop residues, leaves, sawdust, sludge woodchips, municipal solid wastes and livestock manure is increasing (Sun and Cheng, 2002; Wen *et al*., 2004; Staniszewski *et al*., 2007; Hossain *et al*., 2009).

Agricultural wastes are used because of their high sugar contents, as they provide abundant natural resources for the second generation of bioethanol production. Moreover, the use of these wastes does not compete with the rising food demand from the food crops (Rastogi and Shrivastava, 2017; Robak and Balcerek, 2018; Carrillo-Nieves *et al*., 2019). Fruit wastes including damaged fruits; peels and seeds are potential cheap alternatives used as feedstock for biofuel production (Cheng *et al*., 2009). The previous study conducted by Dalibard, (2018) reported that palms are one of the most useful sugary feedstock plants, because they can be used as sugar generating plants throughout the tropical world since the earliest times. According to Sriroth and Piyachomkwan, (2013), palmyra palm (*Borassus flabellifer*); coconut palm (*Cocos nucifera*), sugar palm (*Arenga pinnata*), wild date palm (*Phoenix sylvestris*), nipa palm (*Nypa fructicans*) and commercial date palm (*Phoenix dactylifera*), are all acceptable plants for sugary feedstock of palms sap.

The wild date palm (*Phoenix sylvestris* Roxb.) is a perennial tall tree plant, which is a monocotyledonous, dioecious and belongs to the Arecaceae family (Krueger, 2011). Date palm juicy fruit is edible, although most of the time it recognized as wastes under the tree (Islam *et al*., 2014). Bioethanol production from date palm fruit and sap could give a lasting solution to vast difficulties of stable energy diversification. Therefore, the objectives of the present study were to investigate the effectiveness of date palm fruit wastes as sugary feedstock for bioethanol production using yeast strains, and to explore the optimum growth conditions for these strains to potentially yield high levels of bioethanol.

2. Materials and methods

2.1. Samples collection, preparation and composition of date palm fruit wastes

Date palm fruits wastes were obtained from the local markets of Agbowo, Bodija, Sango and Beere in

Ibadan, Oyo State, Nigeria. The fruits used were sorting wastes of dates including; fruit with texture defects, very wet fruits, fruits spoiled by microorganisms and insects. They were brought in sterile polythene bags and then transported to the Microbiology laboratory for further use.

The methods described by A.O.A.C. (2005) were used for proximate analysis of the composition of date palm fruit wastes, including moisture content; crude protein, fat, ash and total carbohydrates. The assays were carried out in duplicates.

2.2. Isolation of the yeast isolates

The yeast isolates were obtained from the date palm fruit wastes collected from Agbowo, Bere, Bodija and Sango. The collected date palm fruits were rinsed to remove sand and debris, the seeds were removed and then the flesh was grinded using a sterile mortar and pestle. About 1 g of the grinded fruit was introduced aseptically into 9 ml sterile water and then serially diluted till the dilution of 10^{-6}. An aliquot of 1 ml of each appropriate dilution was dispensed into yeast extract agar (YEA) Petri plates, and then the plates were incubated at 28 ± 25^{o}C for 72 h. After incubation, the growing yeasts were selected, purified and then stored onto YEA slants at 4°C till further use (Kreger-van Rij, 1984).

2.3. Identification of the yeast isolates

2.3.1. Biochemical and microscopic identification

The yeast isolates showing remarkable characteristics were selected, and then identified according to their cultural, microscopic and biochemical characteristics; using several biochemical assays and through detecting the fermentation of 10 different sugars, in reference to Kurtzman and Fell, (1998).

2.3.2. Molecular identification of the yeast isolates

The DNA of the selected yeast isolates was extracted according to the procedure described by Zymo Research kit, USA. Pure culture of each yeast isolate was inoculated into yeast extract glucose broth and then incubated at 28°C for 72 h. After incubation, the broth containing the growing cells was then centrifuged to obtain the cell pellet. The cell pallet of the yeast was lysed using 50 µl of the lysis buffer, vortexed for 10 sec and then left to stand at room temperature for 5 min. The mixture was transferred to a Zymo-spin column in a collection tube, centrifuged for 1 min. at 10,000 rpm, and then the supernatant was discarded. The Zymo-spin column was transferred to a new collection tube and 200 µl of the DNA pre-wash buffer was added to the spin column, and then centrifuged for 1 min. at 10,000 rpm. About 500 µl of the DNA wash buffer was added to the spin column, centrifuged for 1 min., and the spin column was then transferred to a clean microcentrifuge tube. 50 µl of the DNA elution buffer was added, incubated for 5 min. at room temperature, and then centrifuged for 30 sec to elute the DNA (Angelov *et al*., 2015).

Polymerase Chain Reaction (PCR) amplification

The Internal transcribed spacer (ITS) universal primers sets that flanked the ITS1 and ITS2 regions were used to characterize the yeast isolates, including; ITS 1: 5'TCC GTA GGT GAA CCT GCG G 3' and ITS 2: 5'TCC TCC GCT TAT TGA TAT GC 3'. The PCR assay was carried out in a Gene Amp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94^{0}C for 30 sec; annealing of primer at 55^{0}C for 30 sec, at 72^{0}C for 1 min., and a final termination at 72^{0}C for 10 min., after which the primer was finally maintained at 4^{0}C. The amplified fragments were sequenced using a Genetic Analyzer 3130×1 sequencer (Applied Biosystems, USA), according to the manufacturers' manual. The used sequencing kit was that of Big Dye terminator v3.1 cycle sequencing kit, while Bio-Edit software and Mega6 were used for all the genetic analysis, in reference to Josepa *et al*., (2002). The obtained sequences were compared to the National Centre for Biotechnology information (NCBI) Database by using the Basic local Alignment search tool (BLASTIN 2.2.29+); with the default settings used to find the

most closely similar sequences that were sorted by the E score. A representative sequence of 10 of the most similar neighbors was aligned for multiple alignments with the default settings (CLUSTAL W2) (Centre for Evolution Genomics, Arizona).

2.4. Estimation of the total reducing sugar

The fruit palm wastes total glucose content was determined according to the method adopted by Miller, (1959). About 3 ml of 3-5-dinitrosalicylic acid (DNS) reagent was added to 3 ml of glucose sample in a lightly capped test tube, and then the mixture was incubated in a water bath for 5-15 min. at 90^{o}C until the red-brown color disappeared. After cooling, 4 ml of dist. water was added to the samples and then the absorbance was measured at 540 nm using a spectrophotometer (721G Visible Spectrophotometer, China). The reducing sugar concentration was determined from the standard curve prepared using glucose, and then multiplied by the dilution factor.

Different concentrations of glucose were prepared ranging from 1 mg/ ml to 10 mg/ ml. About 1 ml of DNS was added to 1 ml of each glucose concentration in the test tube, and then the mixtures were heated in a boiling water path for 10 min. The test tubes were cooled rapidly after which the optimum density of the sample was read against the blank using the spectrophotometer at 540 nm. A graph of absorbance against the concentration was then plotted (Miller, 1959).

2.5. Pre-treatment of date palm fruit wastes

2.5.1. Pre-treatment by steam explosion (Auto hydrolysis)

The date palm fruit wastes were washed; oven dried, blended into powder using a mortar and then kept in zip-lock bags for further use. About 100 g dry powder of date palm fruit wastes were pre-treated by subjecting them to a high pressure 121^{o}C for 30 min. using an autoclave, according to Tutt *et al*., (2014).

2.5.2. Acidic pre-treatment

With reference to Yousif and Abdulhay, (2017), approximately 100 g dry powder of date palm fruit wastes were pre-treated with 0.6 % H_2SO_4, and then heated at 121^{o}C for 15 min. The pH was adjusted to pH 5 using NaOH.

2.6. Preparation of yeasts cell suspension

The yeast inoculum was prepared in Yeast extract potato dextrose (YEPD) broth (LAB M). A 24 h old culture of each yeast isolate was added individually to 10 ml of autoclaved molasses fermentation medium, and then the tubes were shaken gently to form homogeneous suspensions (Taouda *et al*., 2017).

2.7. Fermentation of hydrolyzed date palm wastes

An aliquot of l g of the blended date palm fruit wastes sample were aseptically added into sterile 2 l Erlenmeyer flask, covered with an aluminum foil, and then allowed to ferment for 5 d on a rotary shaker (MON 106 MODEL, MON Scientific). Samples of 100 ml were withdrawn aseptically at 24 h interval, and estimated for determination of the residual sugar and ethanol contents in the fermentation media (Yousif and Abdulhay, 2017).

2.8. Recovery of bioethanol and determination of its content

The distillation process was used to separate the bioethanol from the fermentation broths. Each fermented broth was placed on the electric heating mantle machine (LAB Heating Mantle 51 Model) at 78^{o}C, and then water flow was connected. The distillation product was collected in a round bottom flask (Wakil *et al*., 2013).

Ethanol content of the fermented samples was determined using a gravimetric method in reference to Wakil *et al*., (2013). The specific gravity was determined using a density bottle. An aliquot of 100 ml of the fermentation samples was distilled individually using an electric heating mantle (LAB Heating Mantle 51 Model), which was used to collect the distillate through a glass condenser. The dry

weight of the empty density bottle was determined. The distillate was poured into a 25 ml volume dry density bottle and then weighed. Equal volume of dist. water was poured into a dry density bottle and then weighed. The weight of the empty dry density bottle was subtracted from both of the weight of density bottle containing the fermented sample distillate and that of dist. water. The specific gravity was determined by dividing the weight of the distillate of the fermented sample by the weight of the dist. water, according to the following equation of Wakil *et al*., (2013):

Specific Gravity = (w1-w)/ (w2-w)

Where; W1=weight of the density bottle plus weight of the distillate of the fermented sample;

W2=Weight of the density bottle plus weight of the dist. water;

W = Weight of the empty density bottle

The specific gravity was used to determine the concentration of bioethanol using the Ethyl Alcohol Conversion Table (Wakil *et al*., 2013).

2.9. Determination of the effects of various physical parameters on bioethanol production

The fermentation process was carried out using the selected two yeast strains, where their activities were known to vary with respect to the inoculum concentration; pH, nitrogen and carbon sources, and the incubation temperature. It is therefore imperative to optimize the fermentation conditions for the yeast cells so that the bioethanol production efficiency increases. Various factors were investigated that affect the bioethanol production from molasses.

2.9.1. Effect of initial pH

In this assay, the effects of different pH values were studied, while the other physical parameters were kept constant of (10 %, 30°C and 4 d); respectively. The different pH values used were; 4.0, 4.5, 5.0, 5.5 and 6.0. The assay was conducted according to the method adopted by Arslan *et al*., (2021).

2.9.2. Inoculum concentration effect

In reference to Mojovic *et al*., (2006), various inoculum concentrations such as 10 %, 20 %, 30 % and 40 % were prepared using diluted YEPD broth medium, while the other physical parameters including substrate content; temperature and the time were kept constant at 10 %, 30°C and 4 d; respectively. The fermentation process was carried out as described earlier.

2.9.3. Effect of co-culture of the selected yeast strains on bioethanol yield

According to Eladpum *et al*., (2012), an aliquot of 500 ml of the fermentation medium was used. A 48 h old culture (5×10^8 cfu/ ml) inoculum of the 2 selected yeast isolates were inoculated simultaneously, and then incubated at 30°C on a shaker at 100 rpm as described before. Samples were taken aseptically at 24 h interval for 5d, and estimated for determination of the bioethanol content in the fermentation media.

2.9.4. Impact of different carbon and nitrogen sources on bioethanol production

Different carbon sources such as glucose; sucrose and fructose were used, while the yeast concentration and the other physical parameters including substrate content; temperature and the incubation time were kept constant of at 10 %, 30°C and 4 d; respectively, in reference to Hosny *et al*., (2016). Similarly, different nitrogen sources including yeast extract; malt extract, urea and peptone were used individually, and the fermentation was processed as described before at substrate content, temperature and the time of 10 %, 30°C and 4 d; respectively (Hosny *et al*., 2016).

2.9.5. Effect of agitation on bioethanol yield

To study the impact of agitation on bioethanol production; the fermentation process was carried out at pH 5.0 and a temperature of 30°C in a volume of 100 ml medium, in reference to the method conducted

by Armanul, (2014). A 48 h old inoculum of each yeast strain was added individually to the fermentation medium, and then the flasks containing the same sugar concentration were kept both under shaking at 120 rpm and at a static condition, and thereby the bioethanol content was determined after incubation.

2.10. Determination of the functional group of bioethanol

The functional group of bioethanol was determined using the Fourier-Transform Infrared Spectroscopy (FTIR) (ALPHA). The infrared spectrum analysis was used to identify the vibration signs of the bioethanol recovered from the wastes of date palm. The infrared spectrum of the bioethanol was recorded by passing a beam of infrared light through the sample. When the frequency of the IR was the same as the vibrational frequency of a bond or collection of bonds, absorption took place. Examination of the transmitted light revealed how much energy was absorbed at each frequency (or wavelength), according to Arslan *et al*., (2021).

2.11. Statistical analysis

The experimental data were analysed using Analysis of Variance (ANOVA) to determine the means with SPSS version 23 and the level of significance was set at $p \leq 0.05$. A bar chart was also used to compare the effects of the physico-chemical parameters on bioethanol yield.

3. Results

3.1. Yeast isolates obtained from the date palm wastes

A total of 61 yeasts isolates were recovered from the date palm fruit wastes on YEA medium. All of the isolates were cream colored, with circular and irregular shapes, dull and shiny appearances and entire margins. The cell morphology of the isolates observed under the light microscope showed that the majority of cells were ovoid, apiculate and elongated with budding.

3.2. Biochemical identification of the yeast isolates

Results recorded in Table (1) showed that most of the selected yeast isolates expressed variation in their fermentation of the 10 tested sugars. Almost all isolates utilized glucose; sucrose, dextrose, maltose, fructose, mannitol, sorbitol and inulin. However, none of the isolates fermented galactose.

3.3. Molecular identification

According to results of the PCR amplification, both of the selected yeasts isolates were identified as *Pichia kudrivavzevii* SGD21 and *Pichia kudrivavzevii* SGD30. They were assigned accession numbers of OK33443 and OK334444, respectively. The phylogenetic tree demonstrated that both selected strains were not from the same ancestor, although there was a noticeable similarity between them (Fig. 1).

3.4. Proximate analysis of date palm fruit waste

Results of the proximate analysis of date palm fruit wastes showed the presence of high amounts of carbohydrate content of (84.28 %), moisture content (8.98 %), crude protein (4.39 %), crude fat (0.2 %), fiber (0.80 %) and ash (2.35 %), which made them suitable substrates for the production of bioethanol. The values were recorded in means of duplicates.

3.5. Production of bioethanol from date palm fruit wastes through steam explosion and acid pre-treatments

Results of the production of bioethanol by *Pichia kudrivavzevii* SGD21 and *Pichia kudrivavzevii* SGD30 through steam explosion and acid pre-treatment of date palm fruit wastes demonstrated different bioethanol concentrations, initial and final pH values, initial and final reducing sugars, as presented in Tables (2 and 3), respectively. The bioethanol concentrations varied with the different fermentation days.

Table 1: Biochemical identification of the selected yeast isolates recovered from the date palm fruit wastes

Isolate code	Glucose	Mannitol	Fructose	Maltose	Sorbitol	Sucrose	Galactose	Dextrose	Lactose	Inulin	Urease	1% Acetic acid	Gelatin hydrolysis	Acid from glucose	Probable isolate
AGD3	AG+	-	AG+	AG+	A+	A+	-	AG+	-	-	-	+	-	-	*Dekkera bruxellensis*
AGD11	AG+	A+	AG+	AG+	A+	A+	-	AG+	-	A+	+	-	-	+	*Pichia caribbica*
BGD 1	A+	-	A+	-	-	A+	-	AG+	-	A+	+	+	-	+	*Pichia ohmeri*
BGD 4	A+	-	A+	AG+	-	A+	-	-	A+	-	-	+	-	+	*Schizosccharomyces pombe*
BGD 7	A+	-	A+	A+	-	A+	-	AG+	A+	-	-	+	-	+	*Schizosaccharomyces japonicas*
BRD 14	A+	A+	A+	AG+	-	AG+	-	AG+	-	A+	-	-	+	+	*Candida krusei*
BRD 18	AG+	-	AG+	AG+	-	AG+	-	AG+	-	A+	+	-	-	+	*Candida* parapsilosis
SGD 16	AG+	A+	A+	AG+	-	-	-	AG+	-	A+	-	+	-	+	*Candida casteli*
SGD 17	A+	A+	AG+	AG+	-	A+	-	AG+	-	A+	-	-	-	+	*Rhodotorula mucilagonisa*
SGD 21	AG+	-	AG+	A+	-	-	-	A+	A+	-	-	+	-	-	*Pichia kudrivavzevii*
SGD 25	AG+	A+	A+	AG+	-	A+	-	AG+	-	A+		-	-	-	*Debaryomes hansenii*
SGD 30	A+	A+	A+	AG+	-	AG+	-	AG+	-	A+	-	-	-	+	*Pichia kudrivavzevii*

Where; A: Acid production; AG: Acid and gas production; G: Gas production; -: No growth; +: Growth. All assays were carried out in duplicates

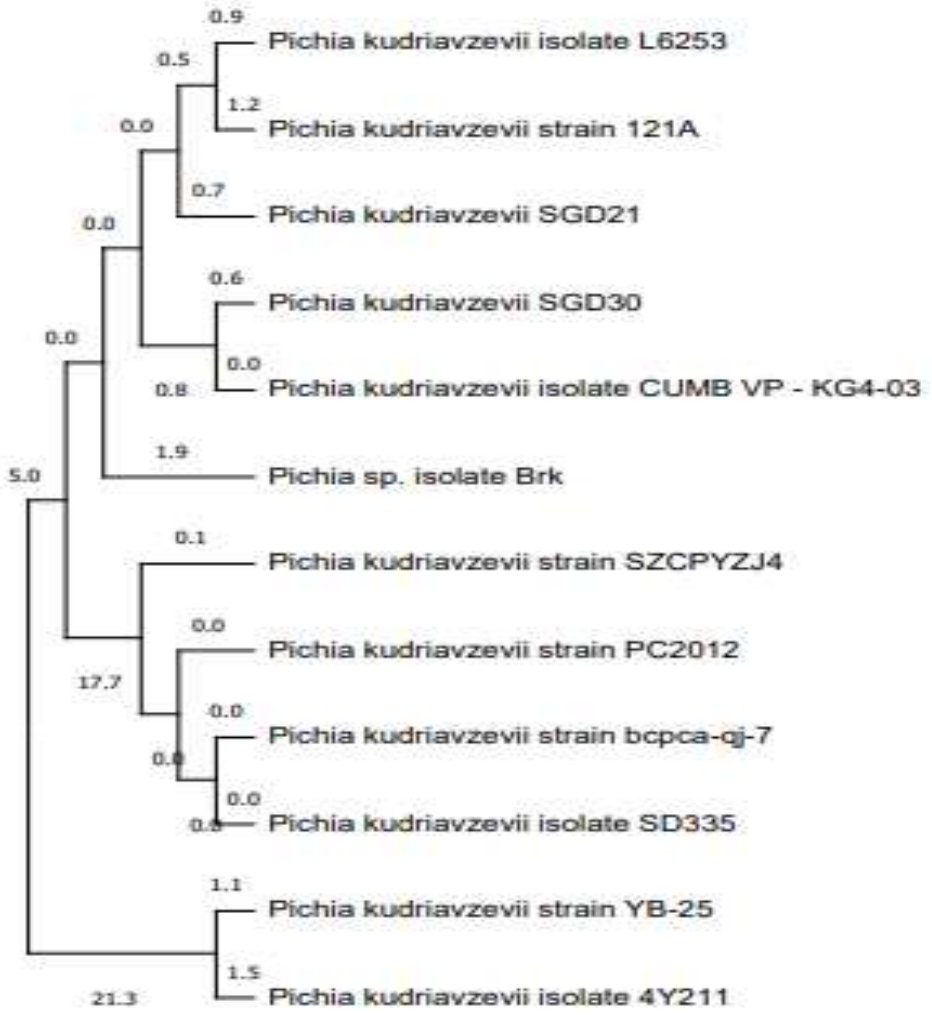

Fig. 1: Phylogenetic tree of the both selected yeast strains recovered from date palm fruit wastes, showing a noticeable similarity between them

Table 2: Production of bioethanol from date palm fruit waste by *Pichia kudriavzevii* SGD21

Days of incubation	pH		Reducing sugar (mg/ ml)		Ethanol produced (%)
	Initial	Final	Initial	Final	
0	5.00± 0.00	4.05± 0.02	94.37± 0.50	71.29± 2.55	1.0
1	5.00± 0.00	3.81± 0.01	94.37± 0.50	76.04± 0.29	1.0
2	5.00± 0.00	3.72± 0.01	94.37± 0.50	64.79± 0.43	2.0
3	5.00± 0.00	3.61± 0.05	94.37± 0.50	64.46± 1.20	2.5
4	5.00± 0.00	3.70± 0.01	94.37± 0.50	41.96± 0.53	4.0
5	5.00± 0.00	3.31± 0.03	94.37± 0.50	37.46± 0.37	2.5

Where; Values are means of duplicates; (±): represent the standard deviation, $p \leq 0.05$

Table 3: Production of bioethanol from date palm fruit waste by *Pichia kudriavzevii* SGD30

Days of incubation	pH		Reducing sugar (mg/ ml)		Ethanol produced (%)
	Initial	Final	Initial	Final	
0	5.00± 0.00	4.05± 0.00	111.41 ± 0.15	94.21± 0.94	1.0
1	5.00± 0.00	4.26± 0.16	111.41± 0.15	93.71± 0.50	1.0
2	5.00± 0.00	4.06± 0.08	111.41± 0.15	80.21± 0.35	2.5
3	5.00± 0.00	3.70± 0.00	111.41± 0.15	69.87± 0.27	3.0
4	5.00± 0.00	3.72± 0.51	111.41± 0.15	31.46± 0.63	4.0
5	5.00± 0.00	3.44± 0.08	111.41±0.15	27.46±0.14	1.5

Where; values are means of duplicates; (±): represent the standard deviation, $p \leq 0.05$

3.6. Impact of initial pH value on bioethanol yield

The fermentation reaction was sensitive to changes in pH value. The optimum pH recorded for *Pichia kudriavzevii* SGD21 was pH 5, which corresponded to alcohol production level of 5 %. On the other hand, the optimum pH for *Pichia kudriavzevii* SGD30 was pH 6, which recorded the highest alcohol production of 6 %, as demonstrated in Fig. (2).

3.7. Effect of inoculum concentration on bioethanol production

Results of the production of bioethanol using different inoculum concentrations are presented in Fig. (3). Ethanol production was at 0 level when no inoculum was used; however, it was significantly improved as the inoculum concentration increased from 10 % to 30 % for *P. kudriavzevii* SGD21 and *P. kudriavzevii* SGD30. On the other hand, when the used inoculum concentration rose to 40%, the level of bioethanol production significantly decreased.

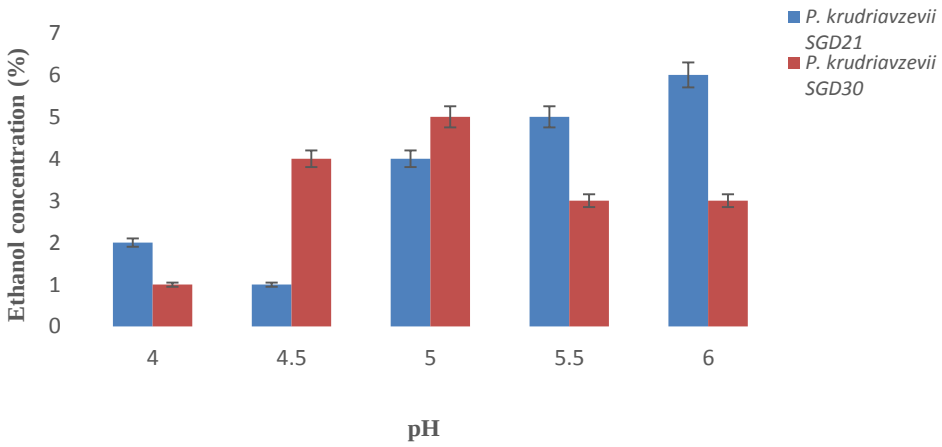

Fig. 2. Effect of initial pH on bioethanol yield obtained from date palm fruit wastes treated individually with both yeast strains. The error bars represent the mean standard deviation ± SD, $p \leq 0.05$

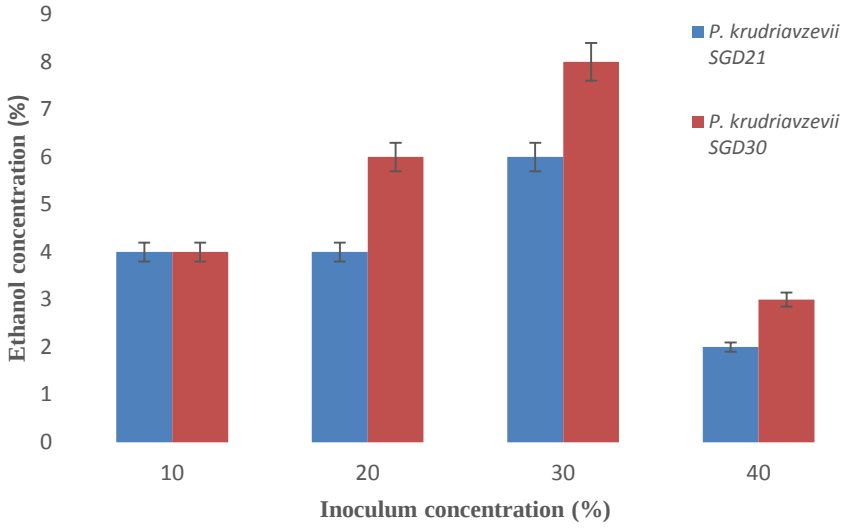

Fig. 3: Effect of inoculum concentration on bioethanol yield obtained from date palm fruit waste treated individually with both yeast strains. The error bars represent the mean standard deviation ± SD, $p \leq 0.05$

3.8. Effect of co-culture of both yeasts strains on bioethanol yield

On co-fermentation, both yeasts strains were inoculated simultaneously. Results presented in Table (4) show that different bioethanol concentrations; initial and final pH, initial and final reducing sugars were recorded on co-inoculation of both yeast strains, with the highest productivity (4 %) of bioethanol content observed when a substrate concentration of 10 % and initial pH 5.0 were employed.

3.9. Impact of different carbon and nitrogen sources on bioethanol production

As demonstrated in Fig. (4), fructose was the best carbon source for bioethanol production, with maximum yield of 12 % recorded by *Pichia kudriavzevii* SGD21, and a yield of 9 % exhibited by *Pichia kudriavzevii* SGD30. Compared to fructose, glucose and sucrose expressed lower bioethanol yields by both yeast strains. On the other hand, Fig. (5) shows that yeast extract was the best nitrogen source for bioethanol production by both yeast strains with a maximum yield of 7 %; compared to peptone, malt extract and urea.

3.10. Effect of agitation on bioethanol level

Fermentation was carried out under shaking and static conditions. Greater bioethanol yield was recorded in the fermentation medium when fermented under a shaking condition, as shown in Fig. (6).

3.11. Evaluation of the functional group of bioethanol

The Fourier Transform Infrared (FTIR) spectrum of the produced bioethanol is demonstrated in Fig. (7a-b). The recorded results showed the presence of peaks ranging between 3810-3500 cm^{-1}, which were indicative of the presence of O-H stretching free alcohol groups that were not inter molecularly bonded. Additional peaks ranging between 3500-3200 cm^{-1} also indicated the presence of O-H groups; however, they were inter-molecularly bonded. A single peak at 2369 cm^{-1} indicated the presence of a strong carbon dioxide group.

Table 4: Effect of co-culture of *Pichia kudriavzevii* SGD21 and *Pichia kudriavzevii* SGD30 in the fermentation medium on bioethanol yield

Days of incubation	pH		Reducing sugar (mg/ ml)		Ethanol produced (%)
	Initial	Final	Initial	Final	
0	5.0± 0.00	4.57± 0.00	124.73 ± 0.15	105.21± 0.94	1.0
1	5.0± 0.00	4.68± 0.00	124.73 ± 0.15	98.09± 0.50	2.0
2	5.0± 0.00	4.48± 0.00	124.73 ± 0.15	71.21± 0.35	4.0
3	5.0± 0.00	4.42± 0.00	124.73 ± 0.15	69.87± 0.27	1.0
4	5.0± 0.00	4.26± 0.25	124.73 ± 0.15	67.46± 0.63	1.0
5	5.0± 0.00	4.03± 0.04	124.73 ± 0.15	69.46± 0.14	0.0

Where; values are means of duplicates; (±): represent the standard deviation, $p \leq 0.05$

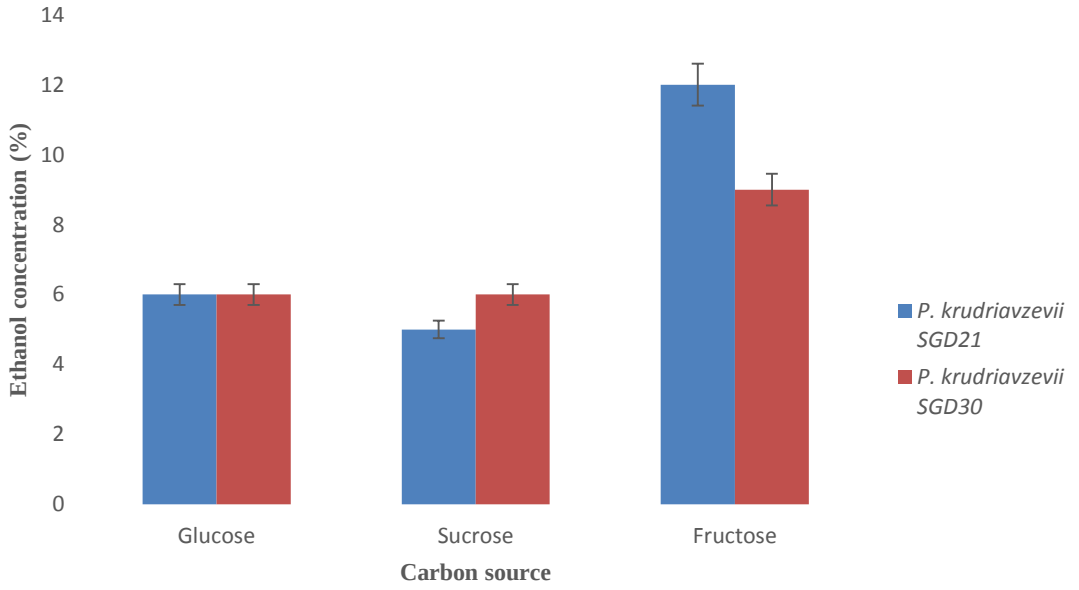

Fig. 4. Effect of different carbon sources on bioethanol yield obtained from date palm fruit waste treated individually with both yeast strains. The error bars represent the mean standard deviation ± SD, $p \leq 0.05$

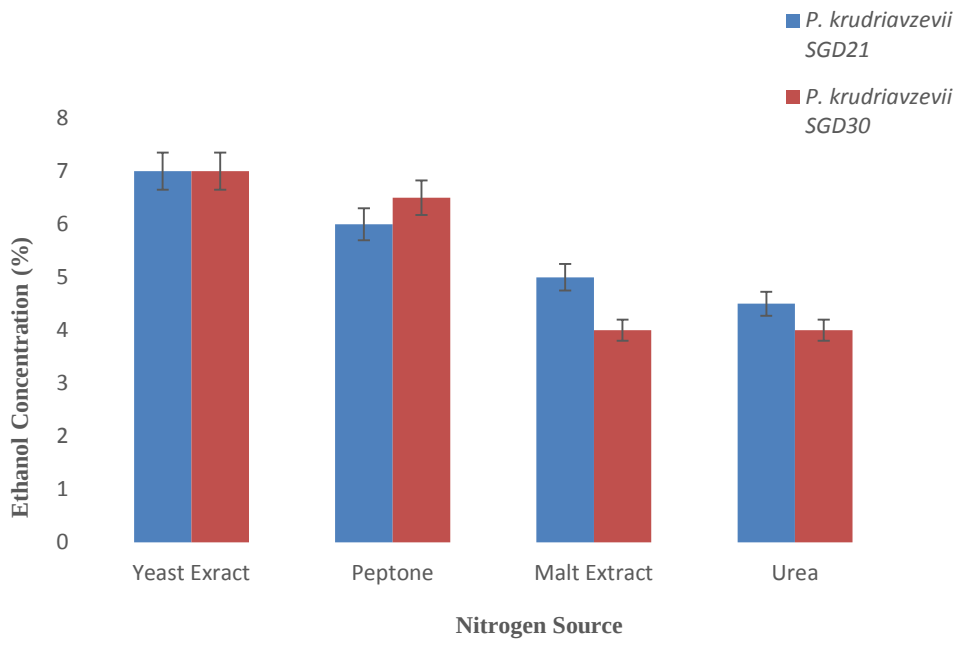

Fig. 5. Effect of different nitrogen sources on bioethanol yield obtained from date palm fruit waste treated individually with both yeast strains. The error bars represent the mean standard deviation ± SD, $p \leq 0.05$

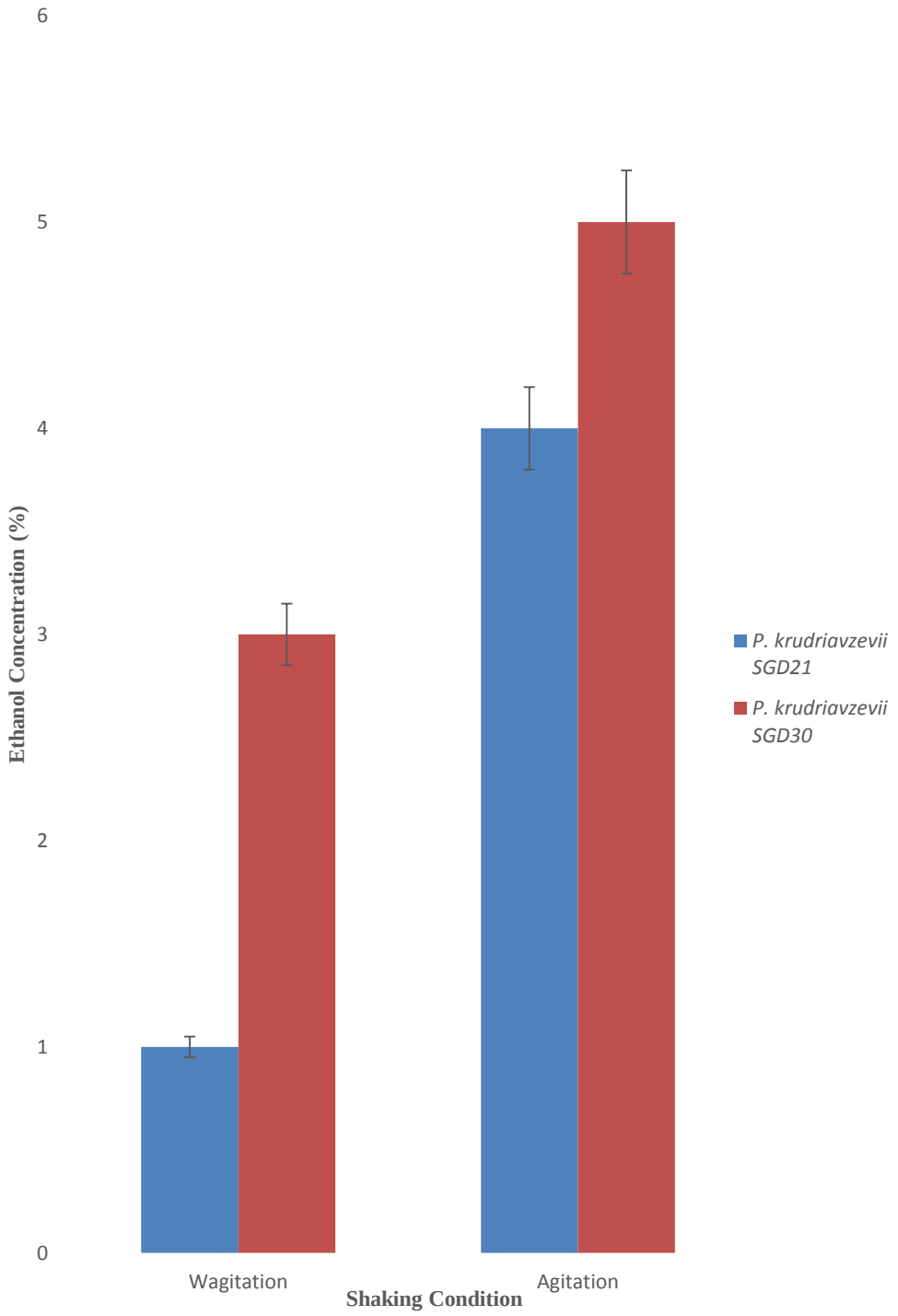

Fig. 6: Effect of agitation on bioethanol level obtained from date palm fruit waste treated individually with both yeast strains. The error bars represent the mean standard deviation ± SD, $p \leq 0.05$

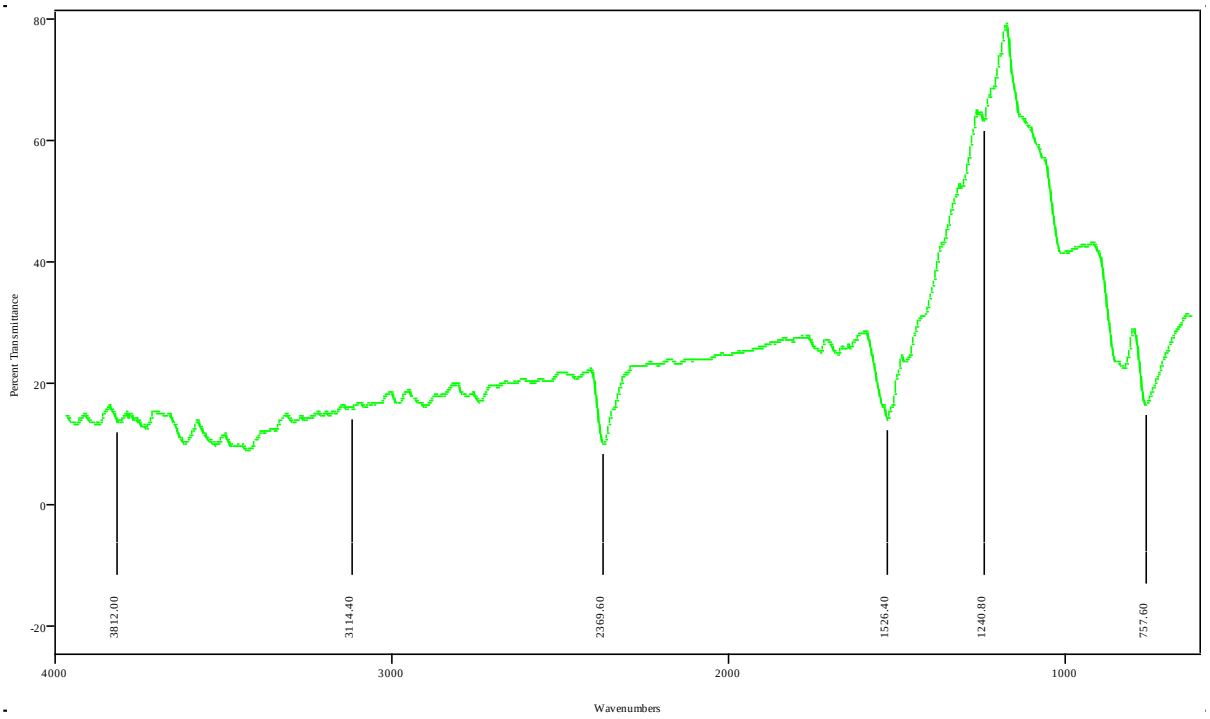

Fig.7a: Fourier Transform Infrared (FTIR) spectrometric graph of produced bioethanol obtained from date palm fruit wastes fermented by *P. krudriavzevii* SGD21strain

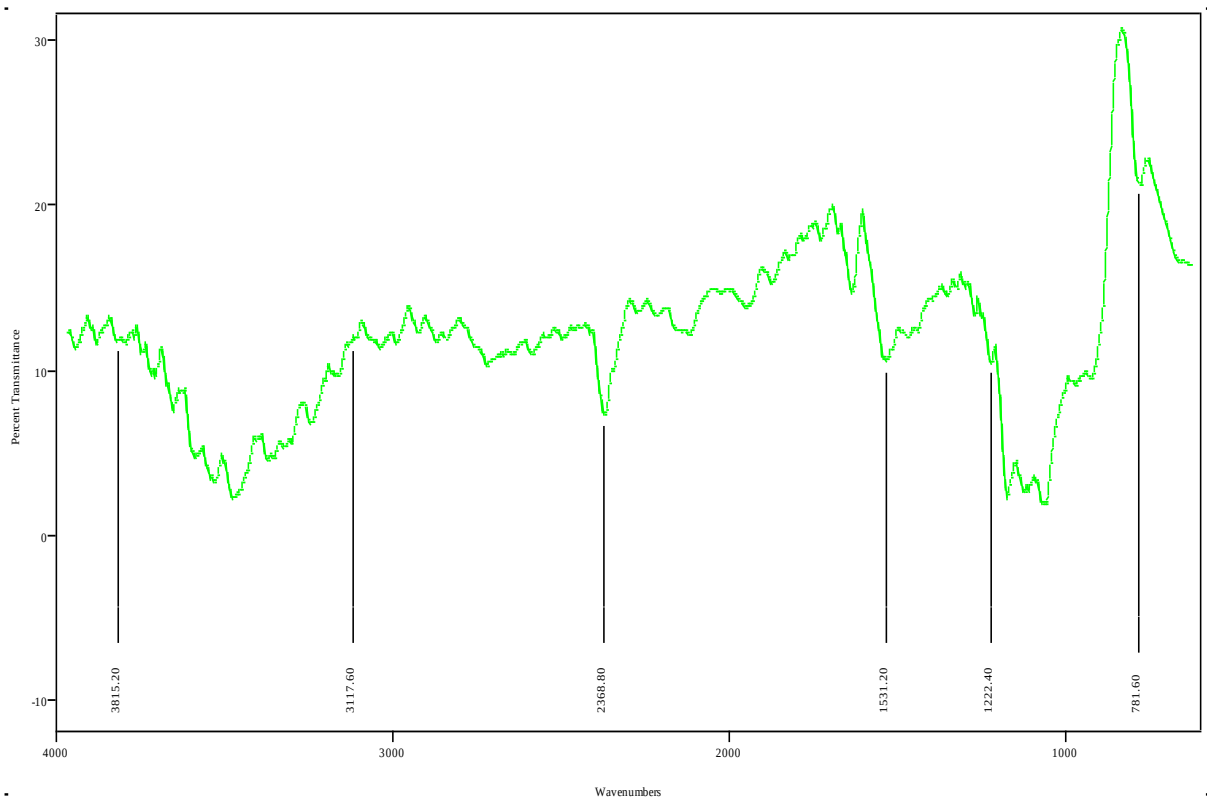

Fig. 7b: Fourier Transform Infrared (FTIR) spectrometric graph of produced bioethanol obtained from date palm fruit wastes fermented by *P. krudriavzevii* SGD30 strain

4. Discussion

For effective bioethanol production, the efficiency of delignification depends mainly on the type of pre-treatment. In this study, sulphuric acid (H_2SO_4) was used for pre-treatment of the fruit waste substrates. This is in accordance with the previous work of Praveen Kumar *et al*., (2017), who used agricultural waste materials as alternative sources of cellulosic materials feedstock during bioethanol production.

It was observed that date palm fruit wastes have a high amounts of carbohydrate content of (84.28 %), moisture content (8.98 %), crude protein (4.39 %), crude fat (0.2 %), fiber (0.80 %) and ash (2.35 %), which made them suitable substrates for the production of bioethanol. It is known that date fruit contains less amount of fat, more fiber and high ash content. The higher ash content expresses the presence of high mineral contents, and thus date palm is also a good source of several nutrients. These nutrients support the microbial growth during the fermentation process for bioethanol production (Awan *et a*l., 2018).

The pH of date palm fruit filtrates decreased as the fermentation days increased. This is in line with the study conducted by Wakil and Onilude, (2011), where a decrease in pH was observed in fermented weaning blends with the increase in the fermentation period. Moreover, the decrease in pH was also recorded by Wakil and Osamwonyi, (2012); to be one of the characteristics of the process of fermentation.

The reducing sugar of fermenting filtrates significantly decreased as the fermentation days increased, in agreement with the previous study of Teck-Yuan *et al*., (2011). The recorded maximum yield of alcohol observed with the increase in sugar fermentation is supported with the findings of Bhatti *et al*., (2019), who stated that more sugar consumption increases the bioethanol yield. Similarly, Rani *et al*., (2006) also reported a decrease in the level of reducing sugar as the fermentation progressed.

The effect of co-culture of *P. kudriavzevii* SGD21 and *P. kudriavzevii* SGD30 in the fermentation medium was observed, where the highest bioethanol yield was recorded after 48 h of fermentation. These low results obtained when both yeasts strains were inoculated simultaneously in the fermentation medium may be attributed to the competition for nutrients between them. Similar results had been made on co-culture of *Zymomonas mobilis* with *Pichia stipitis* during the previous study conducted by Fu *et al*., (2009), which may be attributed to oxygen deprivation in the *Z. mobilis* cells. The effect of initial pH on bioethanol yield was observed. The optimum recorded pH value was 5.0, which corresponds to the highest alcohol production. Each microorganism has its optimum specific pH that enhances its specific enzymes to catalyse the required reactions. *P. kudriavzevii* SGD21 had the maximum recorded bioethanol concentration of 6 % at pH 6, while *P. kudriavzevii* SGD30 had the maximum bioethanol level of 5 % at pH 5. It is generally known that yeasts favor slightly acidic environment (Hosny *et al*., 2016). According to Mansi and Mita, (2016), maximum specific alcohol was produced at pH 5.0. Moreover, the same authors reported that an initial pH of 5.0 of the fermentation media had great influence on bioethanol production. However, any increase or decrease in the initial pH of the fermentation medium from 5.5 causes marked decreases in the bioethanol yield.

In this study, ethanol production significantly improved as the quantity of inoculum increased from 10 % to 30 % in the fermentation media inoculated with *P. kudriavzevii* SGD21 and *P. kudriavzevii* SGD30. On the other hand, when the inoculum amount was raised to 40 %; the bioethanol production was significantly decreased. Currently, an inoculum concentration of 30 % was recorded as an appropriate concentration. In contrast to this study, Arslan *et al*., (2021) predicted that production of bioethanol from date palm fruit wastes can be increased by using 25 % as an initial inoculum concentration. Addition of

sucrose to the fermentation medium as a carbon source had the highest bioethanol yield compared to glucose and fructose, in agreement with Bhatti *et al*., (2019). On the other hand, yeast extract was the best nitrogen source for bioethanol production with a yield of 7 %; followed by peptone, malt extract and urea, in accordance with the previous study conducted by Hosny *et al*., (2016). Greater bioethanol yield was observed when the substrate was fermented under shaking conditions, which may be attributed to the proper distribution of the nutrients available for utilization by the fermenting yeasts. This correlates with the study of Armanul *et al*., (2014) who recorded that all yeast isolates produced better bioethanol yield under shaking conditions; during fermentation of pineapple peels as substrate.

The Fourier Transform Infrared Spectrophotometer (FTIR) analysis of the produced bioethanol confirmed that the ethanol group (C_2H_5OH) was present. According to Manzoor *et al*., (2020), who stated that during FTIR analysis; if the beak region is observed between 3.200-3.600 cm^{-1}; this confirms the presence of an ethanol group. This result is similar to the recent work of Arslan *et al*., (2021), who recorded the presence of an alcoholic functional group in the bioethanol produced from fermentation of wastes of the date palm fruit. All the optimized physical conditions improved the fermentation process and significantly enhanced the production of bioethanol. An appropriate temperature of 30^{o}C, pH of 5 and 6 for *P. kudriavzevii* SGD21 and *P. kudriavzevii* SGD30; respectively, fermentation time of 96 h, inoculum size of 30 %, sucrose as C source, yeast extract as N source, and fermentation under agitation; all resulted in maximum bioethanol production by the tested yeast strains.

Conclusion

In this study, it was revealed that both *P. kudriavzevii* SGD21 and *P. kudriavzevii* SGD30 strains were tolerant to 20 % ethanol concentration, mesophilic (30^{o}C) and can survive at various pH ranges (2, 4, 6, 8 and 10). The highest bioethanol concentration was obtained when the physical factors of the fermentation process were optimized. It was observed that 30 % inoculum concentration was the best concentration for high bioethanol production. Finally, these currently tested yeast strains could be used at the industrial level to obtain an increased yield of bioethanol on fermentation. Accordingly, fermentation may be considered as an effective way of date palm fruit wastes management and utilization.

Acknowledgement

The authors acknowledge the Department of Microbiology, University of Ibadan, Nigeria, for providing some of the materials used in this work.

Conflict of interest

The authors declare that they have no conflict of interests.

Funding source

There was no external funding source for this research work.

Ethical approval

Not applicable.

5. References

A.O.A.C. (2005). Official method of analysis of AOAC International, 18th Ed. Maryland, USA.

Angelov, A.D.; Stefanova, P. and Angelov, A.I. (2015). Molecular identification of yeast using amplification and sequencing of ITS1-5.8S-ITS2 rDNA region. Isham Journal. 54: 47-51.

Armanul, N.; Rahman, S.S. and Choudhury, S. (2014). Isolation of *Saccharomyces cerevisiae* from Pineapple and Orange and Study of Metal's Effectiveness on Ethanol Production. European Journal of Microbiology and Immunology. 7: 76-91. https://doi.org/10.1556/1886.2016.00035

Arslan, A.; Summar, A.N.; Muhammad, J.J.; Muhammad, W.; Ehsan, A.; Iqrar, A.K.; Muhammad, F.M.; Azhari, S. and Rana, M.A. (2021). Efficient utilization of date palm waste for bioethanol production through *Saccharomyces cerevisiae* strain. Food Science and Nutrition. 9: 2066-2074. https://doi.org/10.1002/fsn3.2175

Awan, K.A.; Butt, M.S.; Sharif, M.K. and Hussain, F.J.P. (2018). Compositional Profiling of selected Pakistani date cultivars. Pakistan Journal of Agricultural Sciences. 55(3): 575-581. https://doi.org/10.21162/PAKJAS/18.669

Balat, M. and Balat, H. (2009). Recent trends in global production and utilization of bio-ethanol fuel. Applied Energy. 86(11): 2273-2282. https://doi.org/10.1016/j.apenergy.2009.03.015

Ballesteros, I.; Negro, M.J.; Oliva, J.M.; Cabanas, A.; Manzanares, P. and Ballesteros, M. (2006). Ethanol production from steam-explosion pre-treated wheat straw. Applied Biochemistry and Biotechnology. 130: 496 -508. https://doi.org/10.1385/ABAB:130:1:496

Bhatti, Z.A.; Rajput, M.U.H.; Maitlo, G.; Solangi, Z.A. and Shaikh, G.S. (2019). Impact of storage time, rain and quality of molasses in the production of bioethanol. Mehran University Research Journal of Engineering and Technology. 38(4):1021-1032.

Carrillo-Nieves, D.; Alanís, M.J.R.; de la Cruz Quiroz, R.; Ruiz, H.A.; Lqbal, H.M.N. and Parra-Saldívar, R. (2019). Current status and future trends of bioethanol production from agro-industrial wastes in Mexico. Renewable and Sustainable Energy Review. 102: 63-74. https://doi.org/10.1016/j.rser.2018.11.031

Cheng, N.G.; Hasan, M. and Kumoro, A.C. (2009). Production of ethanol by fed-batch fermentation. Pertanika Journal of Science and Technology. 17: 399-408.

Dalibard, C. (2018). The Potential of Tapping Palm Trees for Animal Production. UN Food Agricultural Organization. http://www.fao.org/ag/aga/AGAP/

Eladpum, A.; Limtong, S. and Pisalaphong, M. (2012). High-temperature ethanol fermentation by immobilized coculture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering 114 (3): 325-329.

Fu, N.; Peiris, P.; Markham, J. and Bavor, J. (2009). A novel co-culture process with *Zymomonas mobilis* and *Pichia stipites* for efficient ethanol production on glucose/xylose mixtures. Enzyme and Microbial Technology. 45(3): 210-217. https://doi.org/10.1016/j.enzmictec.2009.04.006

Hosny, M.; Mervat, A.A.; Seham, A.E. and Hussein, H.E. (2016). Factors affecting bioethanol production from hydrolysed bagasse. International Journal of Advanced Research in Biological Sciences. 3(9): 130-138. http://dx.doi.org/10.22192/ijarbs.2016.03.09.019

Hossain, S.M.; Anantharaman, N. and Das, M. (2009). Anaerobic biogas generation from sugar industry wastewaters in Three-phase Fluidized-bed bioreactor. Indian Journal of Chemical Technology. 16(1): 58-64.

Islam, S.A.; Miah, M.A.Q.; Habib, M.A. and Moula, M.G. (2014). Performance of some mainland trees and palm species planted in the coastal islands of Bangladesh. Journal of Asiatic Society of Bangladesh. 40: 9-15.

Josepa, S.; Josep, C.; Braulio, E. and Jose, M.G. (2002). Isolation and identification of yeast associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. Microbiology Review. 157: 267-274.

Krejer-van Rij, N.J.W. (1984). The Yeasts: A taxonomic study. Elsevier Science Publishers. Journal of Basic Microbiology. 3: 45-104.

Krueger, R.R. (2011). Date palm germplasm. In: Jain, S.M. (Ed.), Date Palm Media B.V., 12: 371-406.

Kurtzman, C.P. and Fell, J.W. (1998). The yeasts - a taxonomic study, 4th Edition. Elsevier Science, Amsterdam, The Netherlands.

Mansi, V. and Mita, V. (2016). Optimization of carbon, nitrogen concentration and pH value for high ethanol production by yeast. KBS Commerce and Nataraj Professional Sciences College. 3: 2321-2705.

Manzoor, M.F.; Ahmed, Z.; Ahmad, N.; Aadil, R.M.; Rahaman, A. and Siddeeg, A. (2020). Novel processing techniques and spinach juice: Quality and safety improvements. Journal of Food Science. 85(4): 1018-1026. https://doi.org/10.1111/1750-3841.15107

Miller, G.L. (1959). Use of dinitro-salicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31**:** 426-428.

Mojovic, L.; Nikolic, S.; Rakin, M. and Vukasinovic, M. (2006). Production of bioethanol from corn meal hydrolysates. Fuel. 85: 1750-1755.

Praveen Kumar, M.K.; Shyama, S.K.; Shamim, K.; Dubey, S.K.; Avelyno, D.; Sonaye, B.H.; Kadam Samit, B. and Chaubey, R.C. (2017). Effects of gamma radiation on the early developmental stages of Zebrafish (*Danio rerio*). 142: 95-101. https://doi.org/10.1016/j.ecoenv.2017.03.054

Rani, P.; Garg, F.C.; Sharma, S.S. and Wati, L. (2006). Ethanol production from potato starch. Industrial Food Packer. 63(4): 63-68.

Rastogi, M. and Shrivastava, S. (2017). Recent advances in second generation bioethanol production: an insight to pre-treatment, saccharification and fermentation processes. Renewable and Sustainable Energy Review. 80: 330-340. https://doi.org/10.1016/j.rser.2017.05.225

Robak, K. and Balcerek, M. (2018). Review of second-generation bioethanol production from residual biomass. Food Technology and Biotechnology. 56: 174-187. https://doi.org/10.17113/ftb.56.02.18.5428

Sims, R.; Taylor, M.; Saddler, J. and Mabee, W. (2008). From 1st to 2nd generation biofuel technologies. IEA.

Sivakumar, G.; Vail, D.R.; Xu, J.; Burner, D.M.; Lay, J.O.; Ge, X. and Weathers, P.J. (2010). Bioethanol and biodiesel: Alternative liquid fuels for future generations. Engineering in Life Sciences. 10(1): 8-18. https://doi.org/10.1002/elsc.200900061

Sriroth, K. and Piyachomkwan, K. (2013). The outlook of sugar and starch crops in biorefinery. Bioresource Technology. 83: 1-11.

Staniszewski, M.; Kujawski, W. and Lewandowska, M. (2007). Ethanol production from whey in bioreactor with co-immobilized enzyme and yeast cells followed by evaporative recovery of product - Kinetic model predictions. Journal of Food Engineering 82(4): 618-625. https://doi.org/10.1016/j.jfoodeng.2007.03.031

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technology. 83: 1-11. https://doi.org/10.1016/S0960-8524(01)00212-7

Taouda, H.; Chabir, R.; Aarab, L.; Miyah,Y. and Errachidi, F. (2017). Biomass and bio-ethanol production from date extract. Journal of Materials and Environmental Sciences 8(9): 3391-3396.

Teck-Yuan, D.; Siew-Ling, H.; Lisa-Gaik-Ai, O. and Tau-Chuan, L. (2011). Investigating the potential of using coconut husk as substrate for bioethanol production. International Conference on Biotechnology and Environment Management. 18(6): 29-32.

Tutt, M.; Kikas, T.; Kahr, H.; Pointner, M.; Kuttner, P. and Olt, J. (2014). Using steam explosion pre-treatment method for bioethanol production from floodplain meadow hay. Agronomy Research (Tartu). 12(2): 417-424.

Uihlein, A. and Schbek, L. (2009). Environmental impacts of a lignocellulosic feedstock biorefinery system: An assessment. Biomass and Bioenergy. 33: 793-802. http://dx.doi.org/10.1016/j.biombioe.2008.12.001

Wakil, S.; Fasiku, S.; Adelabu, B. and Onilude, A. (2013). Production of bioethanol from spontaneous fermentation of palm oil mill effluent (POME). Researcher. 5(2): 28-35.

Wakil, S.M. and Osamwonyi, U.O. (2012). Isolation and screening of antimicrobial producing lactic acid bacteria from fermented millet gruel. International Research Journal of Microbiology. 3(2): 72-79.

Wakil, S.M. and Onilude, A.A. (2011). Time related total lactic acid bacteria population diversity and dominance in cowpea-fortified fermented cereal-weaning food. African Journal of Biotechnology. 10(6): 887-895. https://doi.org/10.5897/AJB10.1488

Wen, Z.Y.; Liao, W. and Chen, S.L. (2004). Hydrolysis of animal manure lignocellulosic for reducing sugar production. Bioresource Technology. 91(1): 31-39. https://doi.org/10.1016/s0960-8524(03)00166-4

Yousif, Y. and Abdulhay, H. (2017). Bioethanol production from banana peels using different pre-treatments. Asian Journal of Biological and Life Sciences. 6(3): 408-412.