BIOETHANOL PRODUCTION FROM DECAYING ORANGES AND PINEAPPLE JUICE USING ETHANOL TOLERANT- YEAST

ABSTRACT

Scientists across the globe ought to harness ways of getting alternative sources of energy which will be renewable, sustainable, efficient and cost effective as a result of the global energy crises owing to the cost of production, transportation and distribution of the products. In Nigeria, decaying fruits always constitute a major environmental pollution during the harvesting season. This study screened, selected the best starter and produced bioethanol from the juice obtained from decaying oranges and pineapple through the process of fermentation and distillation. Samples were collected from different locations within Ile-Ife and transported aseptically to the Laboratory. Microbiological and physicochemical assessment of the isolated strains were on yeast maintenance media. The cell biomass, pH, temperature, brix level, titratable acidity, specific gravity and ethanol yield were monitored during fermentation from day zero to day fourteen. Screening of the isolates obtained from a previous study were carried out to select the best starter for the production of bioethanol.
*S.cerevisae*and *K. marxianus*and showed efficient physico-chemical attributes from the screening of the yeast isolates; a temperature of 30^{o}C and pH 6 was the optimum for the growth of isolates tolerating 15% w/v of Nacl and 20% v/v absolute ethanol; production of catalase,nitrate reduction and fermentation of different sugars. Cultures were inoculated singly and in combination, *S. cerevisiae* gave the highest reduction in brix level from 2.2^{o} at the onset and it reduced to 0.3^{o} at the 21st day of fermentation while the least reduction was seen in *K. marxianus*. Mixed culture of *S. cerevisiae* and *K. marxianus* gave the highest reduction in brix level from 2.0^{o} at the onset to 0.1^{o}, pH reduced from 4.7- 3.3 while the cell biomass increased and the temperature increased from 30^{o}C to 34.5^{o}C at the end of fermentation. Titratable acidity in the fermenting fruits juice increased from 0.23 to 1.76, the specific gravity reduced while the alcohol content increased from zero to 25.63 as the fermentation progressed and a reduction on day 21 (1.67).
Ability of the organisms to grow in changing environmental conditions and ethanol tolerance are attributesessential production while *Saccharomyces cerevisiae*showed the highest attribute followed by *Kluveromycesmarxinus*. This study concluded that *Saccharomyces cerevisiae* can be employed as starter in the industry for the production of bioethanol and in the conversion of agricultural waste to wealth.
Keywords: Bioethanol production and yield, Ethanol-tolerant, fermentation, distillation, waste conversion

1.1 INTRODUCTION

In view of the global rise in energy crisis the world is facing, predictions have been made that the global crude oil production is going to decline five times below its current level by 2050. According to the World Energy Council (WEC) calculations, the world-wide primary energy consumption is approximately 12 billion tons coal equivalent per year. Furthermore, United Nations calculations have shown that the world's population will increase to about 10 billion people by 2050 which will in turn increase energy demands to at least 24 billion tons of coal equivalent per year (twice of what we consume today) depending on economic, social and political developments (United Nations, 2007; Schiffer, 2008).

The increasing demand for fossil fuels caused by burgeoning anthropogenic activities and rapid economic growth provoked wicked environmental issues and resource depletion (Solomon *et al*., 2022 and Thurston., 2022), which is a direct boost to reconstruct the energy

structure, develop and industrialize renewable biofuels [Eswaran *et al*., 2021; Abid*et al*., 2022 andGolroudbary*et al*., 2022).

Continuous depletion of conventional fossil fuel reserves with increasing energy demands and climate change (Agbor*et al*., 2011; Nigam and Singh, 2011) have led to a move towards alternative, renewable, sustainable, efficient and cost-effective energy sources with smaller emissions (Nigam and Singh, 2011). Renewable energy is one of the most efficient ways to achieve sustainable development. Increasing its share in the world matrix will help prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply on a global scale (Chiranjeevi *et al*., 2013).

Numerous potential alternative fuels have been proposed, including bioethanol, biobutanol, biodiesel, methanol, hydrogen, CNG, biogas, Fischer–Tropsch fuel, electricity, and solar fuel (Limayem and Ricke, 2012). Biofuels produce in response to the proper time and conditions coping with world environmental concerns and the exhaustion of non-renewable fossil-based fuels (Sharma *et al*., 2020, Liu *et al*., 2021; Hasan *et al*., 2023).

Biofuel originate from processing of plant oils, sugar beet, cereal, organic waste and processing of biomass. Among liquid biofuels,bioethanol is particularly attractive, having the potential to accelerate sustainable use of resources and change the global economy toward a greener future (Bai *et al*., 2008; Gary *et al*., 2006 amd Singh*et al*., 2022). Continuous biotechnology innovation strongly promotes the upgrading and mass production of biofuels represented by bioethanol. Bio-fermentation based on important model microorganisms is a technology with great development potential beyond all doubt for biofuel production at present and in the future (Chen and Liu., 2021; Shi*et al*., 2011).

As few yeast strains have been found to possess appreciable characteristics for ethanol production, there is a dire need to explore the potential of indigenous strains of yeasts to meet the national requirements for bio-fuel (Qureshi *et al*., 2007).

Yeasts are important microorganisms in food manufacturing and fermentation. Yeast is widely spread in different habitats and these include terrestrial, aquatic and aerial environmental. However, yeasts are considered as an important group of microorganisms in the biosphere. They have been isolated from natural substances like leaves, flowers, sweet fruits, grains, freshly fungi, exudates of trees, insect, dung and soil (Tournas, 2005; Li *et al*., 2008; Zerihun, 2016). Yeasts, being sugar-loving microorganism have been isolated from sugar-rich materials. One of such is fruits. Fruits contain high sugar concentration and hence yeast species are naturally present on these and can be easily isolated from fruits. Distinct wild yeast species are supposed to be present and associated with different fruits in natural environments (Zerihun, 2016). Because of yeast unique fermentative characteristic, there is always a need for yeast strains with better features of fermentation especially high ethanol tolerance for production of ethanol as bio fuel on commercial scale (Colin *et al*., 2006).

Since ancient times, *S. cerevisiae* has had a long historical standing in human civilization and social development, mainly reflected in food production and fermentation such as bread, beer, and wine (Arranz*et al*., 2018 and Ting *et a*l., 2023).

The biodiversity of microorganisms on the substrate depends always on the pH of the substrate. Since fruits are acidic in nature they are predominantly inhabited by yeasts (Zerihun, 2016). Yeast strains found on fruit surfaces are capable of converting wide range of sugars into alcohol. Successful fermentations of biomass to produce ethanol require tolerance to high concentrations ethanol, sugar and invertase activities. These cellular characteristics are important because of high gravity (VHG) fermentations, which are common in the

ethanol industry, give rise to high sugar concentrations, at the beginning of the process, and high ethanol concentration at the end of the fermentation.

The enormity of fruits wastage during the harvesting season in Nigeria constitutes environmental problems. However, little effort has been made in order to explore conversation of sugar present in these decaying fruits waste juice for potential application in bioethanol industry. This study seeks to utilize the waste generated from fruits as low-cost raw material for the production of renewable energy (bioethanol).

2. MATERIAL AND METHODS

2.0 Collection of Samples

Decaying orange and pineapple wastes were collected at various markets in Ile-Ife and its environs as well as decaying fruits dumpsite within Obafemi Awolowo University Staff Quarters. It was collected into sterile Ziplocs material and was transported immediately to the laboratory for microbiological analysis.

2.1 Isolation and Screening of Ethanol-tolerant Yeasts

Some pieces of decayed oranges and pineapples were taken and crushed into fine paste. One (1gm) of the sample mixture was serially diluted 10-fold in Maximum Recovery Diluent (MRD) which make up of 0.1 g of peptone and 0.85 g of NaCl in 100 ml of water. Aliquot (100 µl) of appropriately diluted sample was inoculated into Yeast Maintenance Media (YMM) using spread plate method (Kreger-van Rij, 1984). The YMM plates were incubated aerobically in an incubator (DSI300D) at 30 °C for 3 days. Single colony formed was picked and the cells were observed under microscope.

2.2 MICROSCOPY

Microscopic examination of the isolated yeasts were carried out and these include; direct mount, Gram's staining and lactophenol mount. Physicochemical characterization of the isolate includes sugar fermentation, carbon assimilation and growth in 1% actidine(Omoloruna*etal*., 2023a).

2.3 PRODUCTION OF BIOETHANOL FROM DECAYING FRUITS JUICE

2.3.1 Fermentation media preparation

Decaying fruits waste of oranges and pineapple was used as a fermentation media for the study. The fruits waste was collected from local markets in Ile-Ife, Osun State.

2.3.2 Composition of fermentation media for yeast:

The fruits juice consists of a mixture of oranges and pineapple in ratio 1:1 to make 250g. Urea was added (0 .10 g), Conc. H_2SO_4 (0 .30 Ml) for bioethanol hydrolysis and sucrose (7.5 % (w/v)).The composition was added up to 1000 mL with distilled water. The pH was adjusted with a pH meter to 6.0 and it was autoclaved at 121 °C for 15 mins.

2.3.4 Preparation of yeast cell suspension:

A 48 hours old culture of yeast cell was added aseptically to autoclaved fermentation broth media (10 mL) singly (yeast only) and in combination (yeast and yeast) and the tube was shaken gently to form a homogeneous suspension.

2.4 Fermentation of fruits juice for bioethanol production

Fermentation was carried out in Erlenmeyer conical flasks. Two hundred and fifty millilitres (250 mL) fermentation media were taken into 500 mL Erlenmeyer flasks and homogenous suspension of yeast was inoculated into the media in an aseptic condition. The flask was cotton plugged and incubated at 30 ^{o}C for 21 days. Samples was taken at intervals of day zero, three, seven, ten, fourteen, and twenty one for bioethanol production to monitor the following parameters: pH, temperature, optical density, total titratable acidity, brix (sugar content), specific gravity, alcohol content.

2.5 Physicochemical Analysis to Monitor the Progress of the Fermentation of Decaying Oranges and Pineapple Juice for Bioethanol Production

The physicochemical parameters carried out on the sample during fermentation included optical density, temperature, titratable acidity, brix level (total sugar), pH, alcohol content and yield.

2.6 Determination of pH of ethanol

The pH of the fruits juice sample was read from a pH meter (Hanna instruments 8021) standardized with buffer solutions (4 and 7) (A.O.A.C, 2000).

2.7 Determination of yeast cell growth

The yeast growth determination was carried out using spectrophotometer by the method of (Olutiola*et al*., 1991).

2.8 Determination of titratable acidity (TTA) % of ethanol

It was expressed as percent acidity and analyzed using the method of (Wilson *et al*., 2012). TTA was determined by titrating known quantity of the sample against standardized 0.1N NaOH using a few drops of phenolphthalein solution as indicator to achieve pink colour end point which should persist for 15 seconds as shown in equation 1

$$\%\text{ethanol} = \frac{\text{mL of 0.1M NaOH (titre)} \times \text{normality of NaOH} \times 6 \times 100}{\text{Ml of sample}} \quad \text{equation 1}$$

2.9 Brix level (total soluble sugar) determination of ethanol

Sugar content was determined as Brix using a refractometer (Bs eclipse, Belllingham Stanley 45-02 company UK). A clean dry applicator was used to place two drops of the sample on the prism of the refractometer and the value (original gravity of the refractive index) was read (Wilson *et al*., 2012).

2.10 Specific gravity determination of ethanol

The specific gravity was estimated using hydrometer as outlined by Ilande*t al*. (2000).

The hydrometer was slowly inserted into a test jar filled with the banana must, spanned in the liquid to dislodge any air bubbles clinging to the glass, which could cause a test error. At eye level, the specific gravity figures on the glass stem was read where the surface of the liquid cuts across it at 20 ℃**.**

2.11 Determination of alcohol

The alcohol content was measured in percentage volume by volume (%v/v) also by refractometry method as described by Nwachukwu (2010). A clean dry applicator was used to place 2 drops of the sample (must i.e., before fermentation) on the prism of the refractometer and the value (original gravity) of the refractive index was taken. Two drops of the sample collected at 24 hours interval was applied on the prism of the refractometer and the value (final gravity) was taken.

The percentage alcohol content was calculated using the formula:

$$\text{Alcohol by volume} = \frac{(76.08) \times (O.g - Fg)}{1.775 - O.g} \times (F.g / 0.794)$$

Where O.g is the original gravity

F.g is the final gravity.

2.12 Determination of Bioethanol Yield:

The ethanol yield was estimated according to AOAC (1990) by calculation using the formula:

$$\text{Ethanol yield} = \frac{\text{Ethanol produced}}{\text{Sugar consumed}} \times 100$$

3. RESULTS AND DISCUSSION

In this study, a total number of fifteen (15) yeast isolates were isolated from the decaying oranges and pineapple. The culture was identified as yeast based on colony morphology, microscopic examination, budding formation and biochemical tests.

Table 1: Biochemical Characteristic of Yeast Associated with Decaying Oranges and Pineapple

Isolate code	Glucose	Sucrose	Xylose	Lactose	Mannitol	Raffinose	Maltose	Meliobiose	Mannose	Galactose	Growth in 0.1% Actinide	Nitrate Reduction Test	Probable identity of isolate
1	++	++	++	++	++	-	++	+	+	++	+	+	***Trichosporonasahii***
2	++	++	++	++	++	+	+	+	+	+	+	+	***Trichosporonaesteroides***
3	++	++	++	++	++	-	-	-	+	+	+	+	***Rhodotorulamucilaginosa***
4	+	+	+	-	-	-	-	+	+	+	+	+	***Pichiameri***
5	++	+	++	++	+	+	-	+	+	+	+	+	***Trichosporonmucoides***
6	++	++	+	-	++	-	-	-	++	++	+	+	***Candida fructus***
7	+	+	+	++	-	-	++	+	+	++	+	+	***Trichosporoncutaneum***
8	+	++	++	++	++	+	+	+	+	-	+	+	***Candida albica***
9	-	-	++	-	-	-	-	-	+	-	+	+	***Candida catemulata***
10	+	+	+	-	-	-	-	-	+	+	+	+	***Candida parapsilosi***
11	++	++	++	++	++	-	-	-	+	+	+	+	***Kluyveromycesmarxianus***
12	++	+	++	-	-	-	+	+	++	+	+	+	***Saccharomyces cerevisiae***
13	++	++	++	-	++	-	-	-	-	-	+	+	***Candida albican***
14	+	-	+	-	-	-	-	-	-	-	+	+	***Kluyveromycesfragilis***
15	-	-	-	-	-	-	-	-	-	-	+	+	***Candida valida***

KEY: ++ Positive and can produce gas, + positive and cannot produce gas, - Negative

Table 2: Carbon Assimilation Table for Two Yeasts used in Bioethanol Production

Probable identity of Organisms	Glucose	Mannose	Xylose	Sucrose	Maltose	Lactose	Raffinose	Galactose	Meliobiose
S. cerevisiae	+	-	-	+	+	-	+	+	-
K.marxianus	+	-	-	-	-	+	-	-	-

Key: + Positive; - Negative

The temperature changes in fermenting fruits juice inoculated with different yeast strains and a mixed culture of the isolated yeast strains for bioethanol production is shown in figure1. In general, fermenting fruits juice with single yeast strains culture resulted in the normal growth of the organism which is 30 °C. The mixed culture of the isolated yeast strains which are S. *cerevisiae*and *Kluvyeromycesmarxianus*have a higher temperature on day 21 which is almost the same with the control.

The changes in cell biomass in the fermenting fruits juice is shown in the figure 2. Samples were inoculated with different yeasts strains and a mixed culture of the isolated yeast strains for bioethanol production. There was an increase in cell growth at the beginning of the fermentation and it decreases as fermentation progresses.

The pH changes in fermenting fruits juice inoculated with different yeast strains and a mixed culture of the isolated yeast strains for bioethanol production is shown in figure 3. Fruits juice with single yeast strain culture shows a decrease in pH of the fermenting medium the onset of fermentation to day 14. It is worthy to note that a sudden changed in pH occur on day 21 with a little increase from the value recorded on day 14.

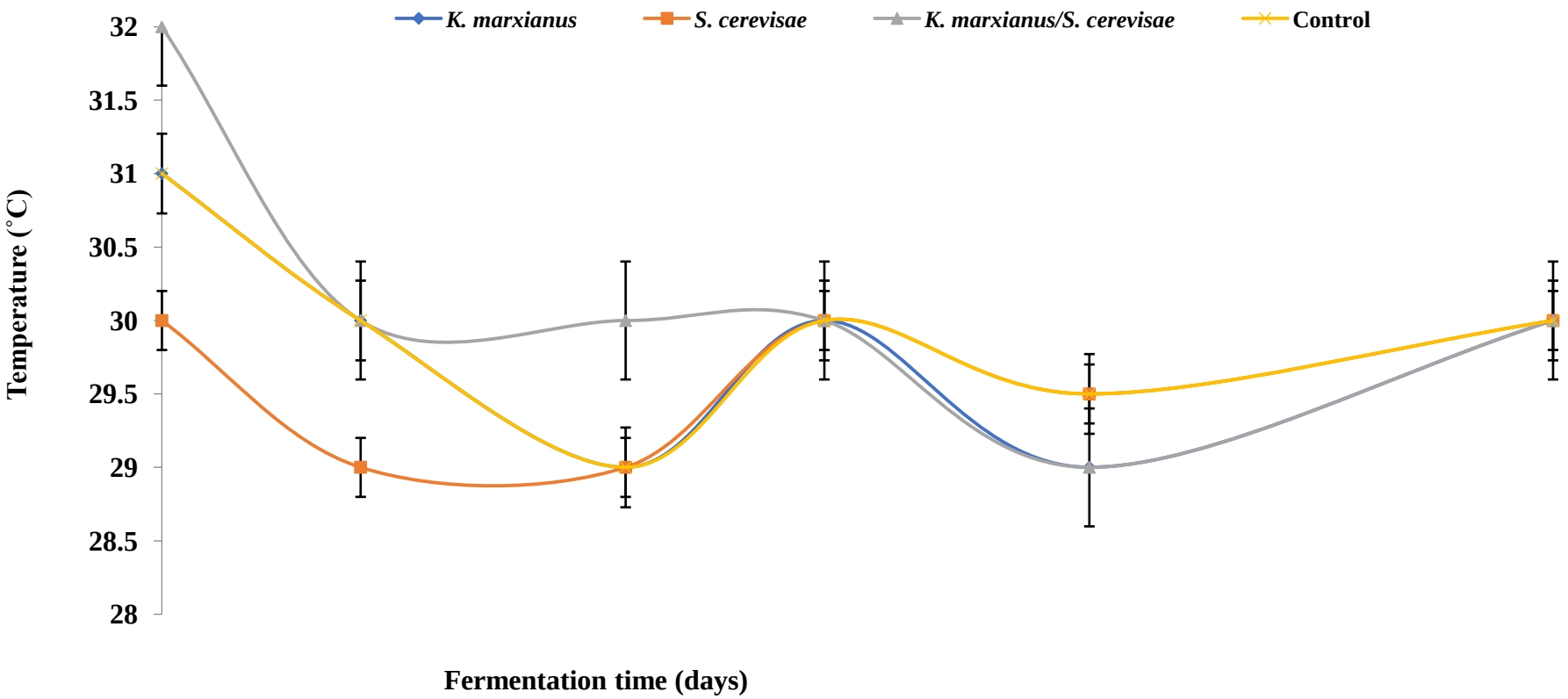

Figure1: Changes in the temperature with time in fermenting fruits juice inoculated with different yeasts and a mixed culture of yeasts for bioethanol production

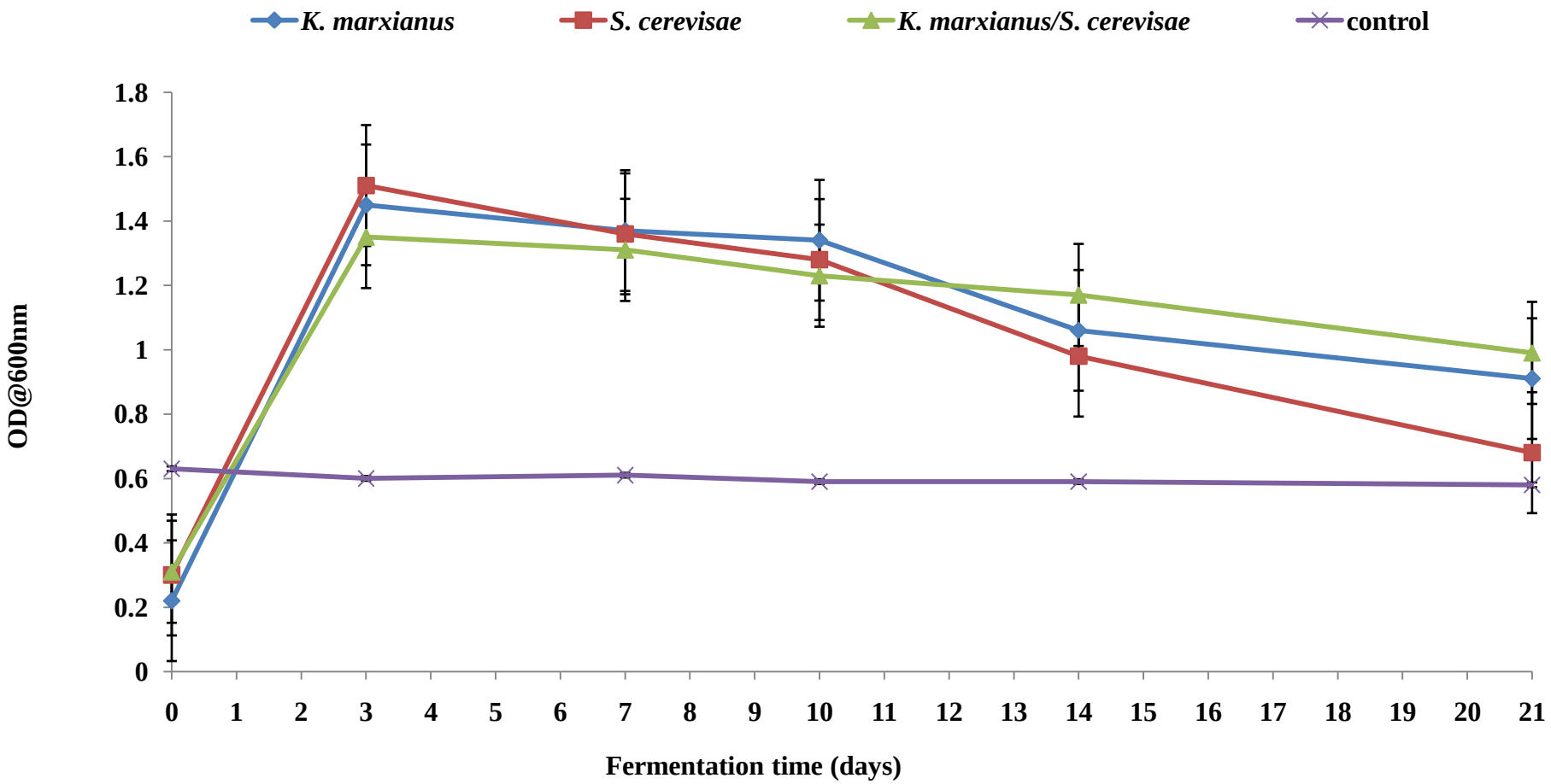

Figure2: Changes in the cell biomass with time in fermenting fruits juice inoculated with different yeasts and a mixed culture of yeasts for bioethanol production

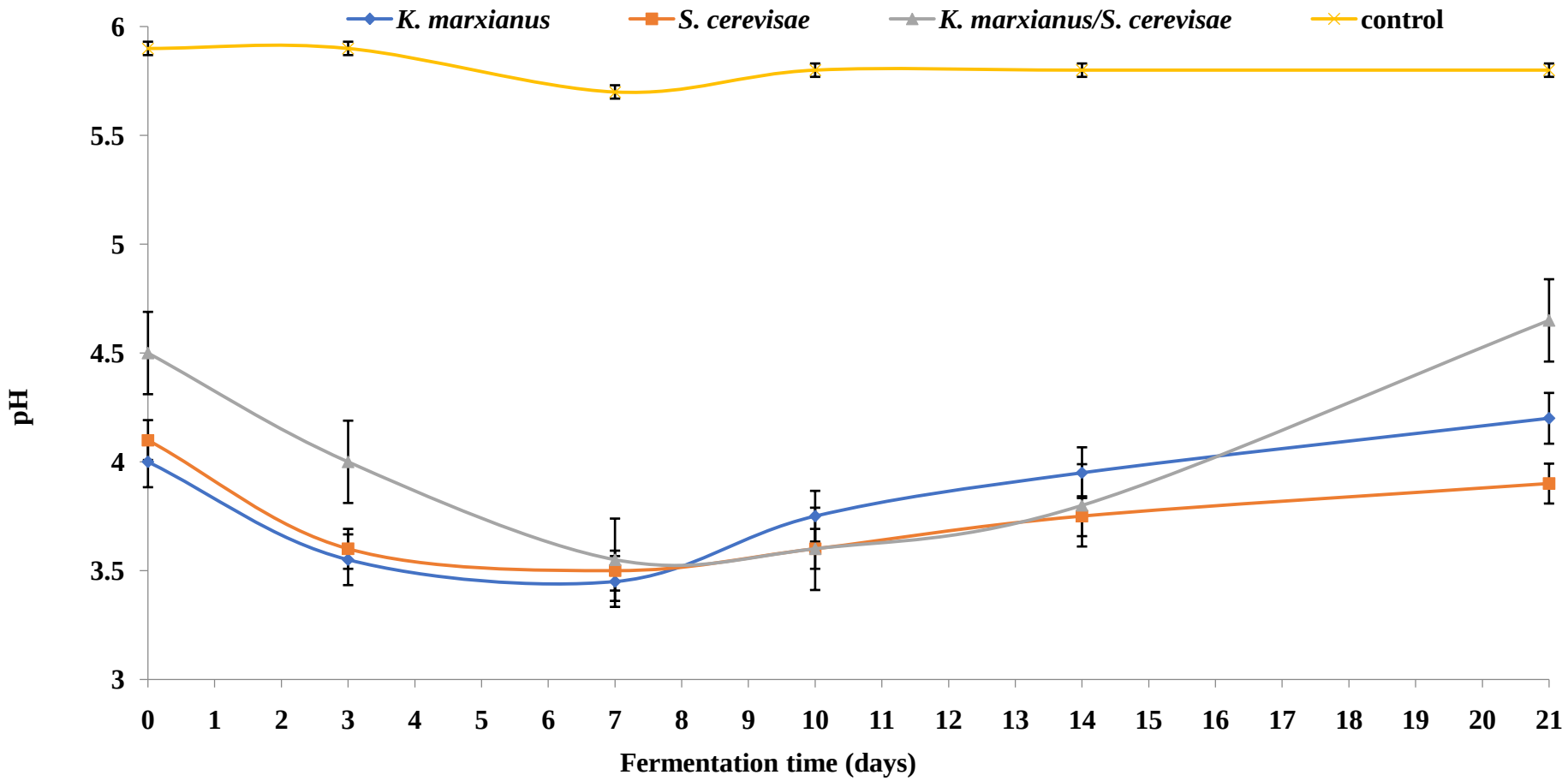

Figure3: Changes in the pH with time in fermenting fruits juice inoculated with different yeasts and a mixed culture of yeasts for bioethanol production

The changes in brix level in fermenting fruits juice inoculated with different yeast strains and a mixed culture of *S. cerevisiae* and *K. marxianus* for bioethanol production is shown in Figure 4 below. In all the fermentation sets, the brix level decreased as fermentation progresses from a starting brix of 2.2^0 at the onset of fermentation to a range of between 0.1^0, 0.3^0 and 0.45^0 respectively at the end of the 21 days' fermentation process. In the single strains yeast series, *S. cerevisiae* gave the highest reduction in brix level (from 2.2^0 at the onset to 0.3^0 at the 21 day of fermentation) while the least reduction was seen in *K. marxianus*. Mixed culture of *S. cerevisiae* and *K. marxianus* gave the highest reduction in brix level (from 2.0^0 at the onset (0 day) to 0.1^0 at the end of the 21 days fermentation) which was significantly different from fermentation between the single yeast strains.

Titratable acidity in the fermenting fruits juice inoculated with single yeast strains increases as fermentation progressed. The fermenting fruits juice inoculated with *S. cerevisiae* gave the highest level of titratable acidity to day 14 of the fermentation process (Figure 5). The titratable level of *K. marxianus* also increase but not at the same rate with the *S. cerevisiae*. The titratable acidity of the mixed yeast isolates increases with little significance in growth different up to the 14 days of fermentation and then remained stable.

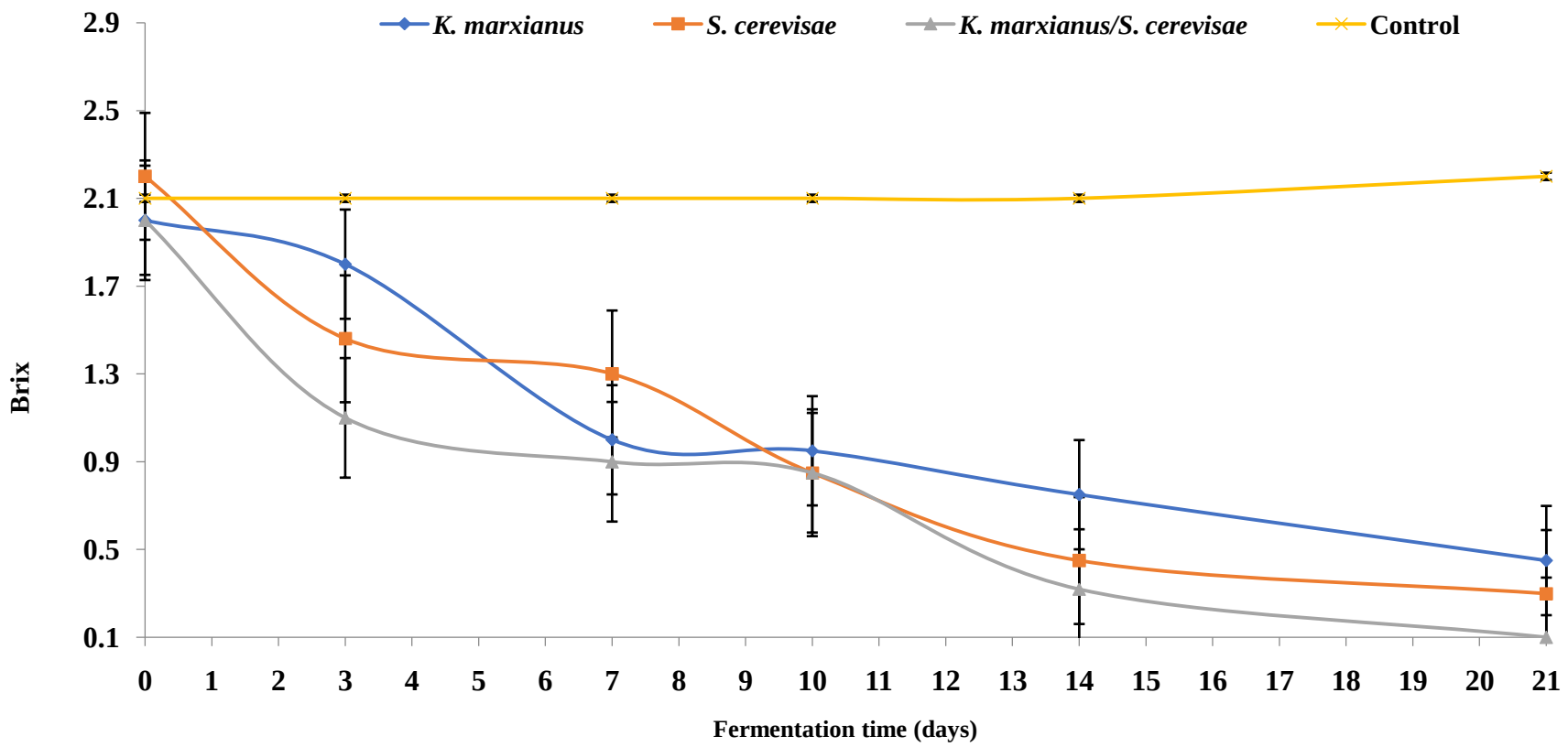

Figure 4: Changes in the level of brix with time in fermenting fruits juice inoculated with different yeasts and a mixed culture of yeasts for bioethanol production

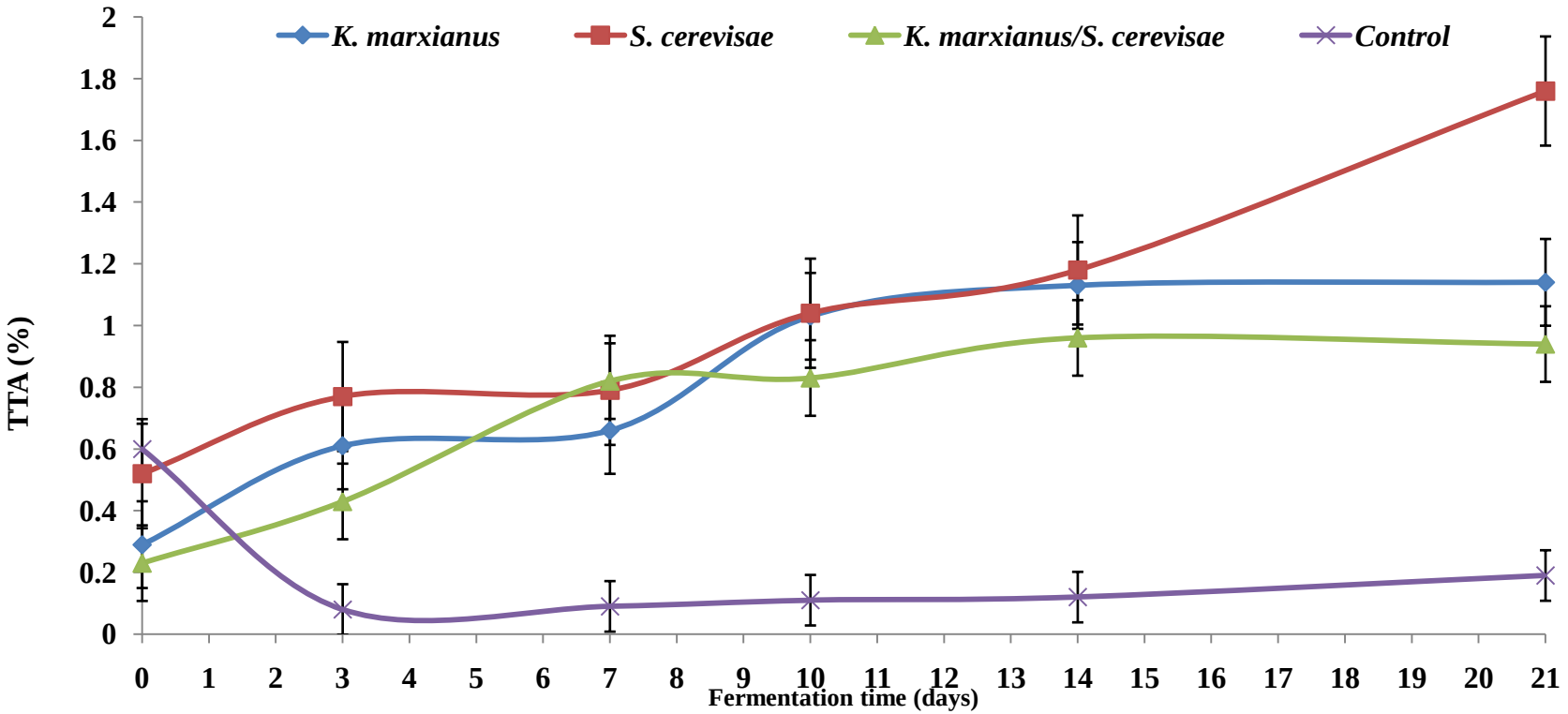

Figure 5: Changes in the titratable acidity with time in fermenting fruits juice inoculated with different yeasts and a mixed culture of yeasts for bioethanol production.

The changes in specific gravity of the fermenting fruits juice inoculated with single and mixed strains of yeast respectively is shown in figure 6. The changes in specific gravity in single and mixed strains of yeast fermentation culture showed the same trend of gradual decrease as fermentation progressed. Mixed culture of *S. cerevisiae* and *K. marxianus* gave the lowest specific gravity value (1.001) as fermentation progressed. *S. cerevisiae* gave the highest specific gravity value. It is significant to note that the mixed culture of yeast strains significantly reduced the specific gravity of the fermented product compared to the value obtained for the single strains of yeast series involving in the fermentation.

The changes in alcohol content follow the same trend in all the single and mixed yeast strains fermentation has shown in figure seven. They all showed a gradual increase between zero to 14 and a decrease on day21 of fermentation, with fermentation involving *S. cerevisiae* as a single yeast strain giving the highest alcohol content value of 5.05 in day 14 and a sharp decrease on day 21.

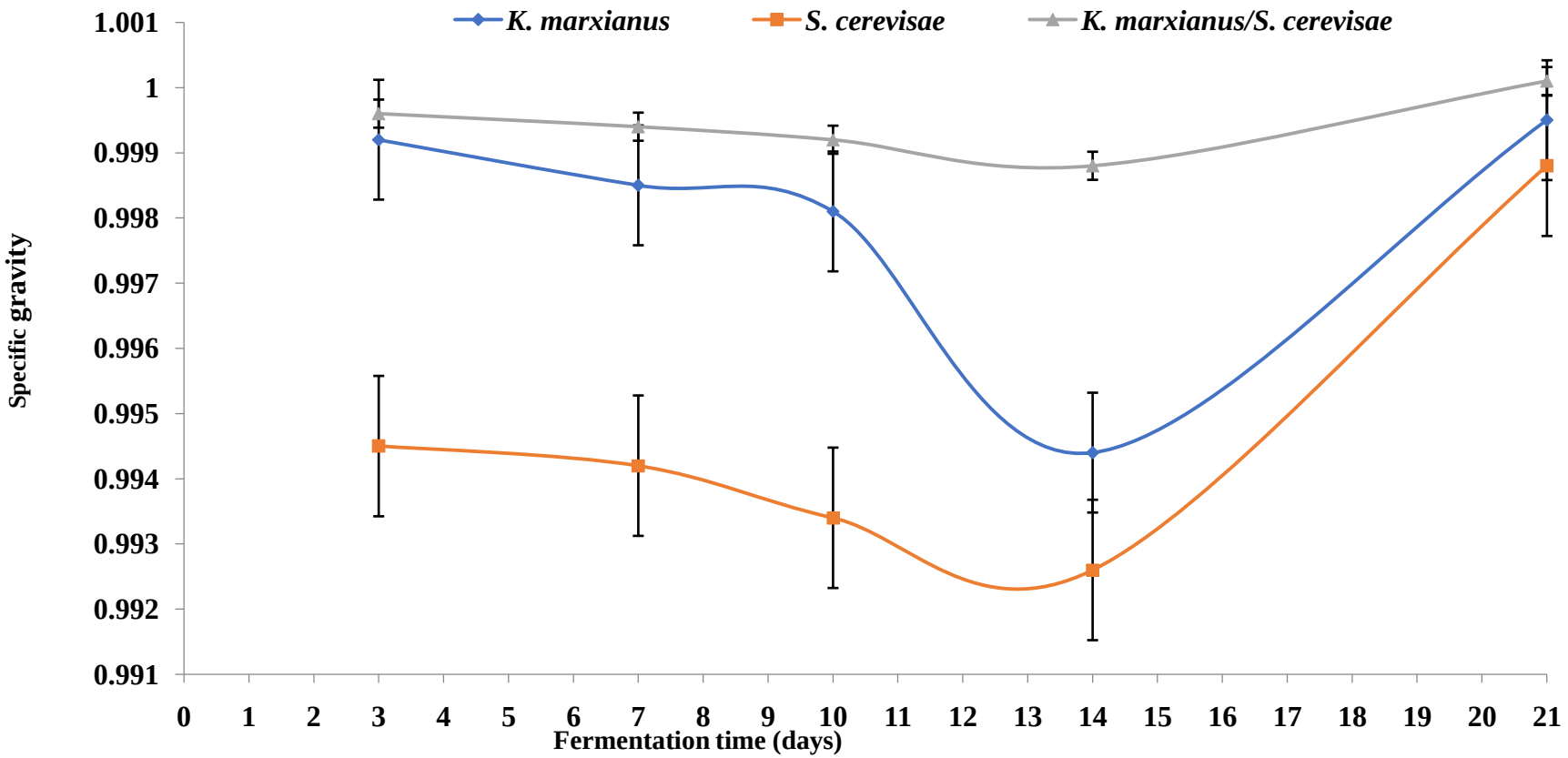

Figure 6: Changes in the Specific Gravity with time in fermenting fruits juice inoculated with different yeasts and a mixed culture of yeasts for bioethanol production

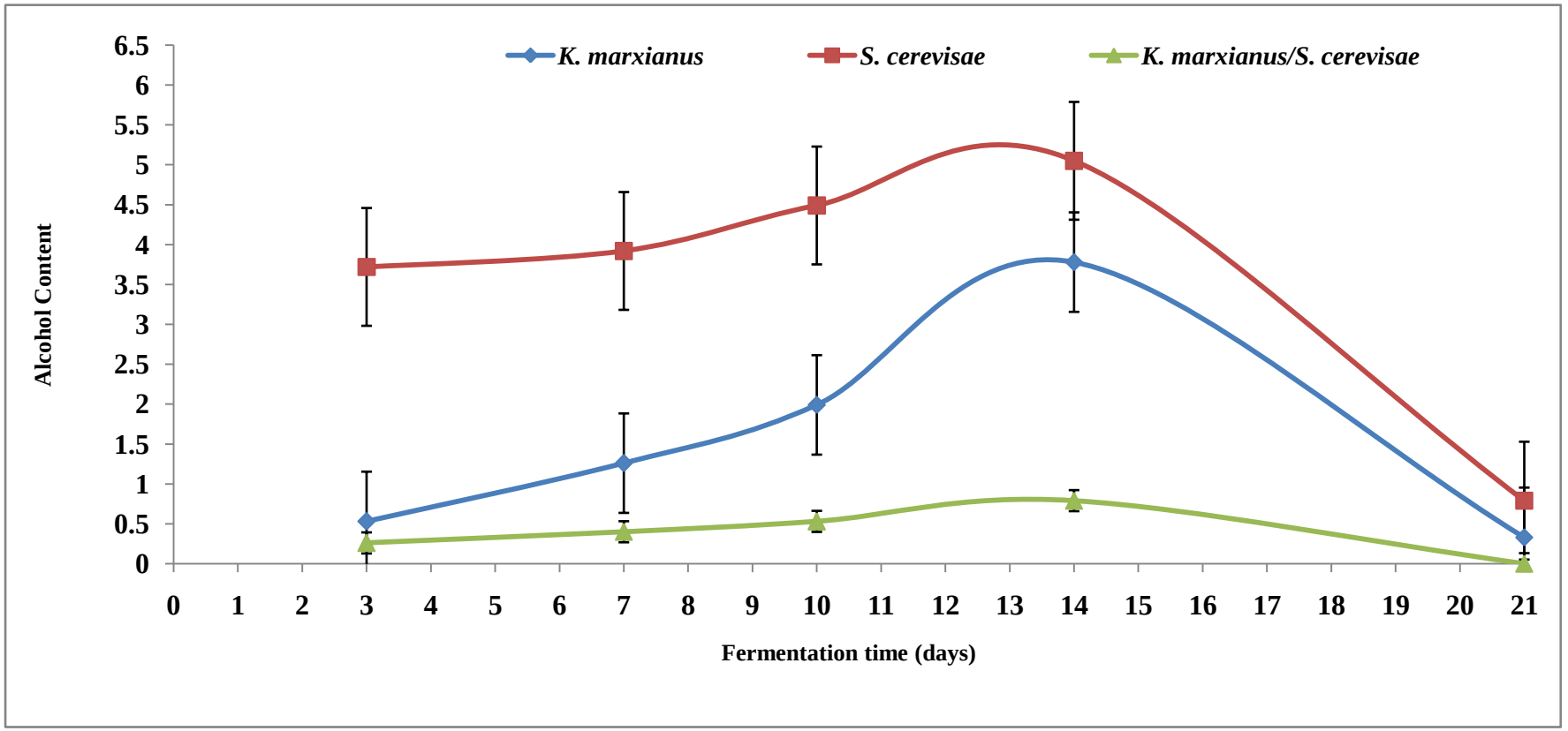

Figure 7: Changes in the alcohol content with time in fermenting fruits juice inoculated with different yeasts and a mixed culture of yeasts for bioethanol production.

Table 3: Percentage Yield of Bioethanol Produced from Decayed Fruits Juice

DAYS	*K. marxianus*	*S. cerevisiae*	*K. marxianus* and *S. cerevisiae*
3	2.69	18.88	1.67
7	6.39	19.89	2.69
10	10.10	22.79	6.30
14	19.18	25.6 3	10.10
21	1.67	4.01	0
Total Yield	**40.03**	**91.20**	**20.76**

Saccharomyces cerevisae and *Kluvyreomycesmarxianus* were selected based on their ability to tolerate high concentration of ethanol which is one of the essential attributes necessary for the production of bioethanol.

One of the parameters monitored during fermentation of fruits juice by single strains of yeast and mixed culture for bioethanol production is temperature. Temperature plays an important role in the production of ethanol, since the rate of alcoholic fermentation increases with the increase in temperature. The optimum temperature of ethanol ranges between 25°C to 40°C which depend on the room temperature. When temperature goes below the optimal range, their ability to catalyse the intended reaction slows down. In this study, the change in temperature observed during fermentation of fruits juice for bioethanol production ranges from 29-32°C. The result is similar to the work of Reddy and Reddy (2006) who recommended that the fermentation temperature for ethanol production up to 30°C should be considered. It also agreed with the results of Maysa (2010) who reported that the highest ethanol levels by two *Saccharomyces cerevisiae* strains at 30°C.

The observed changes in cell biomass of the optical density within the period of fermentation could be due to increase in microbial load arising from microbial succession with changes in fermentation end products. These results agree with reports of previous workers (Amerine and Kunkee, 2005; Robinson, 2006; Okafor, 2007).

The pH value has significant influence on alcoholic fermentation. Enhanced ethanol production through fermentation can be obtained by controlling pH of the broth as it is one of the key factors for ethanol production having direct influence on organisms as well as on their cellular processes (Kasemets*et al*, 2007. In general, hydrogen ion concentration in fermentation broth can change the total charge of plasma membrane affecting the permeability of some essential nutrients into the cells. The pH values of ethanol produced by the process of fermentation ranges from 4 to 6. In this study, the pH of the fermenting medium decreases as fermentation progressed to day 14^{th}. A sudden increase in pH was observed at the end of 21^{st} day of the fermentation. The mixed culture of *K. marxianus* and *S. cerevisiae* gave the highest sudden increase in pH value of 4.65 while S. cerevisiae gave the lowest pH value of 3.9 on 21 day. The result of this study agrees with Chanprasartsuk*et al*. (2012) who reported final pH value of 3.9. The final pH value of *K. marxianus*is 4.2. The final pH value obtained was similar with results reported by Chanprasartsuk*et al*. (2012) who obtained final pH value of 3.9. This value was however high compared those of (3.4 to 3.5) obtained by Idise (2012) at the end of pineapple juice fermentation but was concordant with the pH of the wines after fermentation which is generally 2.0 to 4.0 (Perrin, 2008).

In addition, titratable acidity is an important characteristic during fermentation process and it depends on the biochemical composition of fruit juice used in the alcoholic fermentation and process parameters of fermentation. The titratable acidity increases throughout the fermentation process. Similar observations were made by Chowdhery and Roy (2007) when they reported an increase in titratable acidity (from 0.51 to 3.30%) during the alcoholic fermentation. This result does not agree with Vaidya *et al*. (2009) who reported decrease in titratable acidity (from 1.07 to 0.52%) after fermentation of kiwi from fruits juice.

The brix level is the sugar content of the fermenting fruits juice. The brix level decreases from 2 to 0.1 throughout the fermentation process. The result of this study does not agree with Akubor*et al.* (2003) observed the decrease in TSS of banana juice from 18 to 4.8^{o} brix at the end of 14 days fermentation at 30 ± 2 °C temperature.

In addition, specific gravity is used to measure the sugar and alcohol content. As the fermentation progressed, the specific gravity considerably decreased and reached a value. The decrease in specific gravity is clear indication of yeast fermenting the sugar resulting in ethanol and vinegar production. There is an inverse relationship between specific gravity and alcohol content. The lower the specific gravity, the higher the alcohol content. The increase in acidity may be due to the activities of the microorganisms breaking down sugars to produce both alcohol and carbon dioxide. This study agrees with the work of Duarte *et al*. (2010) who reported higher alcohol inoculated with *S. cerevisiae* UFLA CA 1162 isolated from fermented fruits

The ethanol yield has mentioned above shows that *S. cerevisiae* gave the highest ethanol yield of 91. 20 at 30 ^{o} C at the end of the fermentation process. The result of this study shows does not agree with the work of Lin and Shen *et al*. (2008) who reported ethanol yield of 75.79% at 28^{o}C and 89.89% at 30 ^{o}C from sweet sorghum juice using immobilized yeast cell.

4. CONCLUSION

In conclusion, the result of this study has revealed the usefulness of waste. It can be used in the production of bioethanol. Bioethanol is an eco-friendly fuel that can be used in unmodified petrol engines (Hansen *et al*., 2005). Combustion of ethanol results in relatively low emission of volatile organic compounds, carbon monoxide and nitrogen oxides. This reduce greenhouse gases thereby leading to a clean environment. Lignocellulosic biomass has been projected to be one of the main resources for economically attractive bioethanol production. One of such biomass is agricultural wastes which are renewable, less costly and abundantly available in nature. Agricultural wastes do not demand separate land, water, and energy requirements. Effort should be made in converting this waste to wealth.

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COMPETING INTERESTS

"Authors have declared that no competing interests exist.".

AUTHORS' CONTRIBUTIONS

'Author D' designed the study, performed the statistical analysis and wrote the protocol, authors A and D carried out the practical aspect of the research, author D wrote the first draft of the manuscript. 'Authors B, C, D' managed the analyses ofthe study and were involved in writing the manuscript. 'Author D' managed the literature searches. All authors read and approved the final manuscript."

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