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Supplementation of Fermentation Media Using Magnesium Ion and Vitamin E for Enhance Bioethanol Production

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ABSTRACT

This study aimed to evaluate the effectiveness of media supplementation in enhancing ethanol tolerance and bioethanol production from yeasts isolated from palm wine. Eighteen yeast isolates were screened for ethanol tolerance by culturing them in YEPDA medium supplemented with magnesium ions and vitamin E. The results revealed that most isolates could tolerate 10% ethanol, with some tolerating up to 15% ethanol. Notably, three isolates—Pichia sporocuriosa 08, Cyberlindnera fabianii and Candida tropicalis demonstrated exceptional tolerance, thriving in 20% ethanol medium. Further investigation into the impact of media supplementation on bioethanol production involved using a controlled fermentation setup with 40% magnesium ions and 20% vitamin E. The findings showed a significant increase in bioethanol yield and fermentation rate compared to the control, with the highest yield of 88.60% achieved using Cyberlindnera fabianii after three days of fermentation. The reducing sugar of the control reduced form 40mg/dLfrom day 1 to 28.87mg/dL on day 5. The lowest reduction was recorded when magnesium ion and vitamin E was used to supplement fermentation using candida tropicalis as starter from 37.05 to 29.79). This suggests that the individual and combined supplementation of magnesium ions and vitamin E significantly enhances both ethanol tolerance and bioethanol production, offering a promising approach for optimizing fermentation processes.

1.0 INTRODUCTION

As a domestically producible fuel, ethanol enhances energy security by reducing reliance on imported oil. This can contribute to a more stable and resilient energy supply. Ethanol production however is likely to remain the foremost biotechnological, yeast-derived commodity for many years to come (Demain and Adrio, 2008). In particular, bioethanol production can make a significant contribution towards securing the long-term supply of renewable fuels and the containment of greenhouse gas emissions, providing local employment and new markets for the agricultural industry and reduced security concerns over national energy supplies (Demain and Adrio, 2008).

Ethanol fermentations in a high concentration of substrate containing a mixture of sugars in the form of glucose and fructose using *Saccharomyces cerevisiae* usually occurs not completely. At the end of the fermentation, the sugar, especially fructose, remains in the medium because it could not be completely converted into ethanol. In the wine-making industry, it is referred as stuck fermentation or sluggish fermentation by which the product has an unexpected sweet taste. In the production of bioethanol, the incomplete conversion from sugar into ethanol has caused lower productivity of fermentation. Such imperfect conversion may occur because yeasts are more capable in converting glucose into ethanol than that of fructose into ethanol (Ventorino *et al*., 2016).

Yeast, like any other living cell, is affected by natural factors such as gene type, environment and nutrition. Overexposure of yeast cells to any of these factors may result in the yeast cells struggling to maintain their metabolic processes. In industrial situation, yeast stress often results from its metabolic by-products (ethanol, higher alcohols) and the nature of the brew system (Gibson *et al.*, 2007).

Yeast strains have been selectively bred for ethanol production, ensuring consistency and efficiency in the fermentation process, maximizing ethanol yield. Some yeast strains have a higher tolerance to ethanol, allowing them to survive and continue fermenting in the presence of elevated ethanol concentrations, which is advantageous for ethanol production. In summary, yeast is instrumental in ethanol fermentation due to its ability to efficiently convert sugars into ethanol, operate in anaerobic conditions, and its adaptability for industrial-scale production Ventorino *et al*., 2016).

The best circumstances for maximizing ethanol yield in the presence of magnesium ions and vitamin E are still unknown, which presents optimization issues. Comprehending the influence of these constituents on ethanol yield is imperative in order to optimize fermentation procedures on an industrial scale, augment production efficiency, and accomplish sustainability objectives.

The increasing need for biofuels has led to investigate green additives, such as vitamin E, to increase production efficiency while reducing resource usage and environmental impact. By examining the impacts of magnesium ion and vitamin E on ethanol yield, this study seeks to close these knowledge gaps and advance our knowledge of sustainability programs and the processes involved in the manufacture of biofuels.

This study is to enhance bioethanol production using supplementation and fermentation media by palm wine yeast isolates.

2.0 METHODOLOGY

2.1 Sources and Collection of Microorganism

The stock culture of yeast isolated from fermenting palm wine was obtained from the Microbiology laboratory, Department of Science Technology.

2.2 Confirmation of Isolates

The collected yeast isolates were confirmed by observing the texture, colour, surface, elevation and margin of the isolates. Cellular morphology was determined by taking a portion of the yeast colony into drops of lacto-phenol cotton blue on a clean glass slide. The slides would then be examined under the microscope using the X40 objective lens (Amoa-Awua *et al.,* 2007). Yeast isolates will further be confirmed with reference to Antia *et al*. (2018).

2.3 Preparation of Inoculum

The yeast strain was transferred into 10 ml Peptone water and shake well for proper mixing. All the culture was adjusted and compared with 0.5 Mc Farland Standard to achieve the same initial cellular concentration at $1.5x10^8$ cells /ml ($1.5 X 10^8$ equivalent to 0.5 Mc Farland Standard) (Shafkat, 2013).

2.4 Ethanol Tolerance Screening Test

The yeast isolates screened for their tolerance to high alcohol concentrations according to the method of Osho (2005). The yeast strains were transferred into 10 ml of YEPDB broth containing different concentrations of ethanol (10%, 15% and 20) (v/v). All the cultures were conducted with same initial cellular concentration at $1.5x10^8$ cells /ml ($1.5 X 10^8$ equivalent to 0.5 Mc Farland Standard) and will be further incubated at 30°C for 48 hours. The cell concentration was determined by measurement of the optical density at 590 nm using spectrophotometer which will be correlated with the cell count. The increase in optical density in tubes was recorded as evidence of growth (Shafkat, 2013).

2.5 Media Supplementation for Enhanced Ethanol Tolerance

This was done according to the modified method of Zhang *et al.* (2016). The pure cultures of the yeast isolates with highest level of tolerance obtained from ethanol tolerance test ($1.5 X 10^8$ equivalent to 0.5 Mc Farland Standard) were used and conserved on a Yeast Extract Peptone Dextrose Medium (YEPD) agar slant at 4 °C. The organisms were inoculated onto 2% agar slants of YPD and grown at 28 °C for 48 hours. Vitamin E (4mg, 8mg, 12mg, 16mg and 20mg) magnesium ion (10%, 20%, 30%, 40% and 50%) and olive oil (1%, 2%, 3%, 4% and 5%) on the ability of the yeast were used to supplement YPD medium, supplements ranging as indicated above. Each of the supplement were added together with the highest percentage level of ethanol tolerance obtained from the tolerance test (25 and 30%). All were done in triplicates including the control and incubated for 72hours at 30 ^{0}C. Ultraviolet (UV) spectrophotometry (PYE Unicam Sp 9, Cambridge, UK) was used to measure yeast growth.

2.6 Production of Bioethanol using the supplemented medium

One milliliter (1 mL) of yeast inoculum from the already prepared 0.5 MC Farland equivalent standard was introduced into 50 mL of Yeast Extract Peptone Dextrose (YEPD)broth medium supplemented with 100 g/L glucose. Magnesium ion (40%), Vitamin E (20mg/L) both singly and in consortium. These were incubated at 30°C for 72 h. The growth of the isolates in the tubes were observed quantitatively using standard plate count technique (Guimarães *et al.* 2006).

2.7 Spectrophotometric Analysis of Ethanol Concentration

In this method of spectrophotometric analysis, ethanol concentration was determined by solvent extraction and dichromate oxidation reaction as described as follows. Firstly, ethanol in liquid sample was extracted by Tri-n-butyl phosphate (TBP, Sigma Aldrich,

USA). One mL of TBP and 1mL of aqueous sample was mixed in a microtube and then vortexed vigorously for 1 minute. The mixture was centrifuged at 3,420 xg for 5 minutes to separate into two phases. Upper phase, TBP layer, was clear and transparent, while lower phase, water, was turbid. Then, 500 µL of upper phase was transferred to a new microtube and mixed with 500 µL of dichromate reagent (containing 10% w/v of $K_2Cr_2O_7$ in 5 M of H_2SO_4), and vortexed vigorously for 1 minute. The mixture was set still for 10 min at room temperature to allow oxidation product in lower phase develop its color to blue green. One hundred microliters of the oxidation products were diluted with 900 µL of deionized water. The optical density at 595 nm (A595) of tested sample was measured in spectrophotometer (T80+ UV/Vis Spectrometer, PG Instrument Ltd., USA). The ethanol concentration in sample was estimated from the ethanol standard curve representing the relationship between A595 and the concentrations of ethanol (Malinee *et al.*, 2019)

2.8 Determination of Reducing Sugar

The reducing sugar concentration was determined quantitatively using the dinitro-salicylic acid (DNSA) method according to Afolabi and Ayodele, (2020) in triplicates. Approximately, 1mL of dinitrosalicylic acid was added to 1mL of each supernatant (filtrates) in test tubes labeled accordingly, and then the mixtures were heated in boiling water for 10 minutes. The test tubes were cooled rapidly in tap water and the volume was adjusted to 12 ml using distilled water. A blank containing 1mL distilled water and 1mL of DNSA was prepared. The optical density of the samples was measured against the blank using a spectrophotometer (JENWAY: Model 6405, UK) set at 540 nm. The concentration of the reducing sugar in the supernatant was estimated using the glucose standard curve.

2.9 Statistical Data Analysis

Descriptive statistic (mean and standard deviation), Inferential statistic (ANOVA) and Data visualization methods (Histogram, Bar chart, Line Graph and Pie Chart) were used to analyze the data obtained.

3.0 RESULTS, DISCUSSION AND CONCLUSION

3.1 Results

Table 3.1: Effect of ethanol concentration on yeast isolates on YEPDB medium using spectrophotometer

S/N	YEAST ISOLATES	**Ethanol concentration (% v/v)/** percentage of transmittance recorded (Mean±Sd)			
		10	**15**	**20**	**Control**
1.	*Cyberlindnera fabianii*	5.00±0.12	30.50±0.12	35.90±1.13	36.90±0.34
2.	*Candida tropicalis*	15.40±0.16	33.20±0.15	34.60±0.15	36.90±0.34
3.	*Candida tropicalis*	15.30±0.15	30.80±1.70	36.00±0.14	36.90±0.34
4.	*Candida fabianii*	5.10±0.64	25.40±0.48	36.10±0.11	36.90±0.34
5.	*Pichia sporocuriosa*	8.80±0.13	26.90±0.41	33.80±1.09	36.90±0.34
6.	*Candida tropicalis*	10.10±0.15	27.20±0.17	34.60±0.15	36.90±0.34
7.	*Cyberlindnera fabianii*	12.80±0.16	28.30±0.04	35.70±0.30	36.90±0.34
8.	*Pichia sporocuriosa*	5.20±0.10	24.40±0.02	26.70±0.13	36.90±0.34
9.	*Cyberlindnera fabianii*	9.80±0.36	30.20±0.45	35.30±0.34	36.90±0.34
10.	*Candida tropicalis*	15.20±0.17	33.90±0.40	35.80±0.25	36.90±0.34
11.	*Cyberlindnera fabianii*	10.70±0.32	26.60±0.21	27.20±0.26	36.90±0.34
12.	*Candida tropicalis*	12.90±0.15	25.90±0.03	33.20±1.81	36.90±0.34
13.	*Pichia sporocuriosa*	12.20±0.36	22.80±0.45	33.80±0.59	36.90±0.34
14.	*Cyberlindnera fabianii*	6.80±0.27	30.10±0.32	35.20±0.53	36.90±0.34
15.	*Candida tropicalis*	13.20±1.83	31.30±0.28	35.80±0.41	36.90±0.34
16.	*Pichia sporocuriosa*	12.80±0.28	22.90±0.17	23.80±0.29	36.90±0.34
17.	*Candida fabianii*	5.60±0.14	26.90±0.05	35.70±0.51	36.90±0.34
18.	*Candida fabianii*	5.30±0.16	26.60±0.02	36.10±0.16	36.90±0.34

Table 3.2: Viability of the Three (3) selected yeast isolates on YEPDB medium with increasing ethanol concentrations using spectrophotometer

Isolates	Concentration of Ethanol used/percentage of transmittance recorded (Mean±Sd)				
	10%	20%	25%	30%	Control
Pichia sporocuriosa 08	5.00±0.76	26.40±0.45	24.50±0.24	27.00±0.29	36.90±0.14
Cyberlindnera fabianii 11	11.30±0.71	27.10±0.32	28.10±1.12	29.10±0.15	36.90±0.14
Candida tropicalis 15	12.10±0.31	23.60±0.30	27.40±1.35	29.20±0.13	36.90±0.14

Table 3.3: Protective effect of Magnesium ions on viability of ethanol stressed cells of yeasts

Isolates	Magnesium ions %concentration	Concentration of Ethanol used/percentage of transmittance recorded (Mean±Sd)		
		25%	30%	Control
Pichia sporocuriosa	10	78.20±0.44	81.90±0.04	86.90±0.15
	20	81.80±0.45	86.30±0.19	87.70±0.30
	30	83.40±0.60	86.10±0.02	88.00±0.13
	40	25.80±0.43	27.90±0.23	84.20±0.34
	50	84.10±0.64	86.20±0.14	89.60±0.25
Cyberlindnera fabianii	10	83.50±0.56	81.10±1.70	86.90±0.15
	20	84.10±2.11	86.10±0.48	87.70±0.30
	30	78.10±0.89	85.40±0.48	88.00±0.13
	40	9.50±0.89	2.80±0.17	84.20±0.34
	50	76.20±0.58	87.20±0.04	89.60±0.25
Candida tropicalis	10	79.20±0.43	83.20±0.45	86.90±0.15
	20	71.80±0.86	84.90±0.40	87.70±0.30
	30	77.90±0.81	84.10±0.01	88.00±0.13
	40	22.50±0.42	2.30±0.03	84.20±0.34
	50	81.30±0.44	85.80±0.43	89.60±0.25

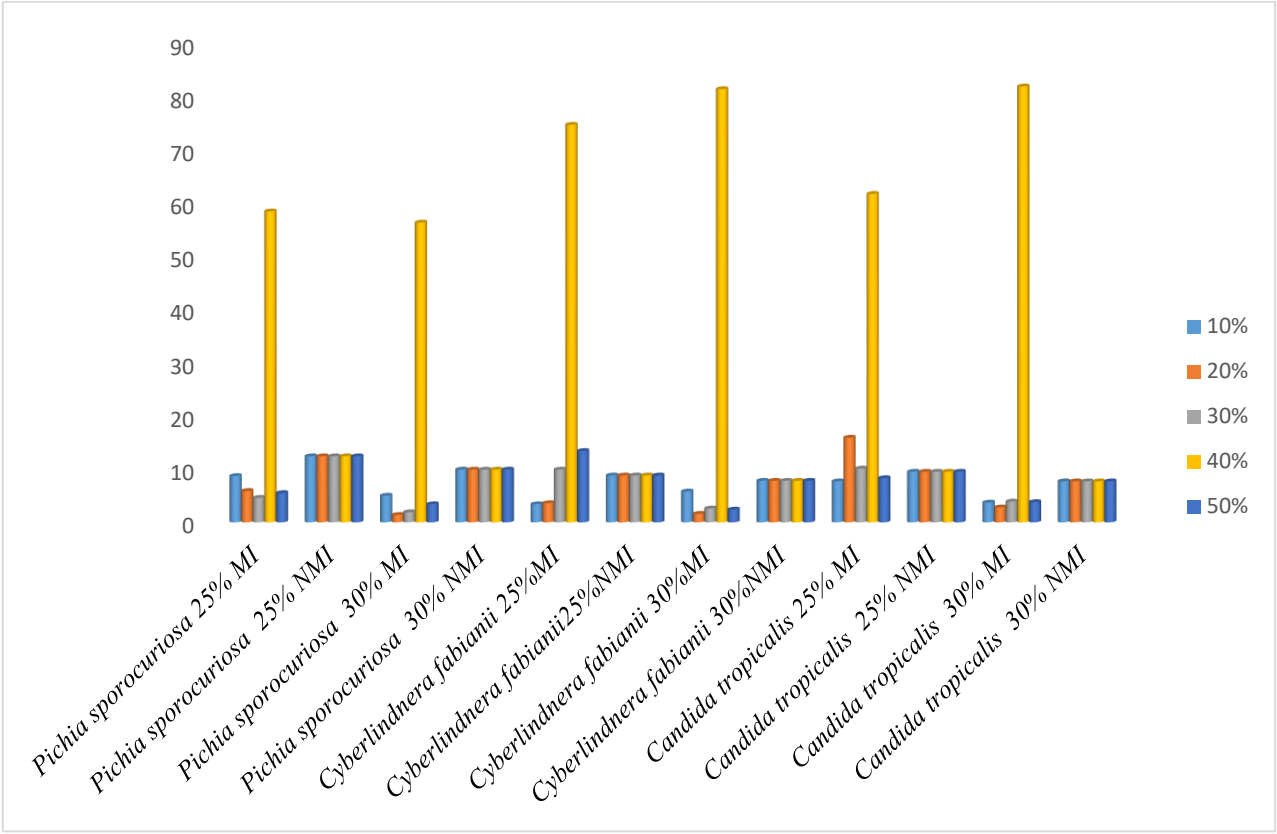

Fig. 3.1: Viability of the yeast isolates in increasing ethanol concentrations with and without magnesium ions.

KEYS: 25%MI= Viability of yeast at 25% ethanol with magnesium ion.
25%NMI = Viability of yeast at 25% ethanol without magnesium ion.
30%MI= Viability of yeast at 30% ethanol with magnesium ion.
30%NMI= Viability of yeast at 30% ethanol without magnesium ion.

Table 3.4: Protective effect of Vitamin E on viability of ethanol stressed cells of yeasts

Isolates	Vitamin E in mg/L	Concentration of Ethanol used/Percentage of transmittance recorded (Mean±Sd)		
		25%	30%	Control
Pichia sporocuriosa	4	38.70±0.14	78.70±0.12	80.10±0.29
	8	39.40±0.16	77.90±0.14	78.40±0.31
	12	65.60±0.15	81.50±0.13	82.10±0.30
	16	60.10±0.14	77.80±0.12	79.30±0.29
	20	42.80±0.15	78.30±0.13	79.10±0.30
Cyberlindnera fabianii	4	60.00±0.16	68.40±0.14	80.10±0.29
	8	78.70±0.16	59.60±0.14	78.40±0.31
	12	77.60±0.16	78.70±0.14	82.10±0.30
	16	69.80±0.17	74.10±0.15	79.30±0.29
	20	59.00±0.32	73.80±0.30	79.10±0.30
Candida tropicalis	4	69.30±0.36	64.10±0.34	80.10±0.29
	8	46.60±0.27	81.10±0.25	78.40±0.31
	12	71.80±0.28	76.60±0.12	82.10±0.30
	16	75.20±1.83	65.40±0.14	79.30±0.29
	20	38.90±0.61	33.30±0.13	79.10±0.30

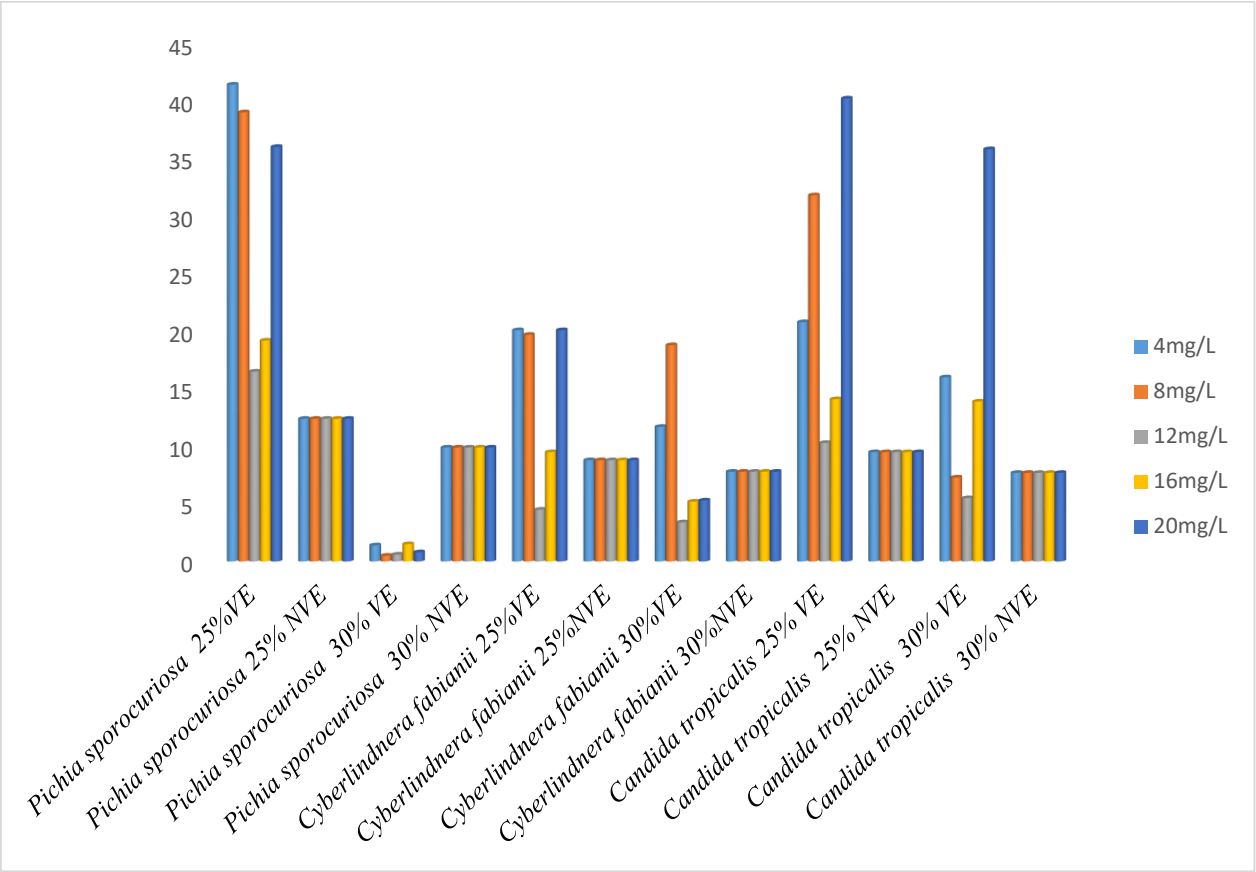

Fig. 3.2: Viability of the yeast isolates with increasing ethanol concentrations with and without Vitamin E.

KEYS: 25%VE= Viability of yeast at 25% ethanol with Vitamin E
25%NVE = Viability of yeast at 25% ethanol without Vitamin E.
30%VE= Viability of yeast at 30% ethanol with Vitamin E.
30%NVE= Viability of yeast at 30% ethanol without Vitamin E.

Table 3.5: Determination of Protective Effect of Supplementations on Percentage ethanol yield, reducing sugar conversion rate and the starter yeast survival rate

Supplements	Fermentation	Yeast Isolates								
		Pichia sporocuriosa			*Cyberlindnerafabianii*			*Candida tropicalis*		
		RS (mg/dL)	% BEY	YD (cfu/ml)	RS (mg/dL)	% BEY	YD (cfu/ml)	RS (mg/dL)	% BEY	YD (cfu/ml)
MI	D1	53.05^{c}	ND	1.5 x 10^{8} ^{c}	53.05^{d}	ND	1.5x10^{8} ^{b}	53.05^{d}	ND	1.5x10^{8} ^{a}
	D2	50.25^{c}	23.33 ^{a}	3.3 x 10^{7} ^{b}	50.24^{d}	28.58 ^{a}	2.3x10^{7} ^{b}	50.43^{d}	24.22^{a}	3.5x10^{7} ^{a}
	D3	42.42^{b}	54.62 ^{d}	2.6 x 10^{6} ^{b}	37.02^{b}	88.60 ^{a}	2.8x10^{6} ^{a}	42.84^{c}	77.11^{f}	2.7x10^{6} ^{a}
	D4	38.50^{ab}	48.57^{c} ^{d}	8.0 x 10^{4} ^{a}	30.03^{ab}	50.50 ^{a}	8.0x10^{5} ^{a}	30.35^{c}	48.13^{c}	3.9x10^{5} ^{a}
	D5	33.15^{a}	21.27 ^{a}	4.0 x 10 ^{4} ^{a}	27.96^{a}	20.81 ^{a}	3.0x10^{5} ^{a}	28.34^{a}	29.12 ^{ab}	3.0x 0^{5} ^{a}
VE	D1	33.82^{a}	ND	1.5 x 10^{8} ^{c}	33.82^{ab}	ND	1.5x10^{8} ^{b}	33.82^{b}	ND	1.5x10^{8} ^{a}
	D2	33.70^{a}	9.60^{a}	3.8 x 10^{7} ^{b}	31.33^{ab}	23.22 ^{a}	4.8x10^{7} ^{b}	31.44^{ab}	10.12^{a}	8.8x10^{7} ^{a}
	D3	31.95^{a}	51.16 ^{d}	2.9 x 10^{7} ^{b}	24.26^{a}	61.64 ^{a}	3. x10^{6} ^{a}	29.48^{ab}	44.76^{c}	6.6x10^{6} ^{a}

	D4	29.12 ^{a}	30.60 ^{b}	2.0 x 10^{7} ^{b}	23.56 ^{a}	45.54 ^{a}	2.6x10^{6} ^{a}	27.89 ^{a}	30.22 ^{ab}	6.1x10^{6} ^{a}
	D5	26.56 ^{a}	23.20 ^{a}	1.16 x 10^{7b}	22.38 ^{a}	23.94 ^{a}	2.4x10^{6} ^{a}	22.68 ^{a}	22.52 ^{a}	5.7x10^{6} ^{a}
MI + VE	D1	37.05 ^{ab}	ND	1.5 x 10^{8} ^{c}	37.05 ^{bc}	ND	1.5x10^{8} ^{b}	37.05 ^{b}	ND	1.5x10^{8} ^{a}
	D2	36.73 ^{a}	19.43 ^{a}	9.3 x 10^{7} ^{b}	35.78 ^{b}	18.88 ^{a}	9.1x10^{7} ^{b}	35.28 ^{b}	20.19 ^{a}	8.3x10^{7} ^{a}
	D3	35.86 ^{a}	37.13 ^{b}	8.7 x 10^{6} ^{b}	33.52 ^{b}	36.72 ^{a}	7.8x10^{6} ^{a}	31.54 ^{ab}	38.45 ^{b}	4.8x10^{6} ^{a}
	D4	25.34 ^{a}	30.29 ^{ab}	3.1 x 10^{5} ^{a}	31.22 ^{b}	33.56 ^{a}	6.1x10^{5} ^{a}	30.11 ^{ab}	31.28 ^{ab}	8.1x10^{5} ^{a}
	D5	23.30 ^{a}	29.46 ^{ab}	2.0 x 10^{5} ^{a}	25.21 ^{a}	27.81 ^{a}	6.0x10^{5} ^{a}	29.79 ^{ab}	27.13 ^{a}	5.0x10^{5} ^{a}
	D1	40.52 ^{b}	ND	1.5 x 10^{8c}	40.52 ^{d}	ND	1.5x10^{8b}	40.52 ^{c}	ND	1.5x10^{8} ^{a}
CONTROL	D2	38.88 ^{ab}	18.98 ^{a}	9.9 x 10^{7b}	37.42 ^{a}	15.55 ^{a}	9.3x10^{7b}	38.27 ^{b}	13.32 ^{a}	9.3x10^{7} ^{a}
	D3	36.05 ^{a}	33.46 ^{b}	5.8 x 10^{7b}	33.11 ^{a}	36.52 ^{b}	4.8x10^{6a}	36.53 ^{b}	34.02 ^{b}	8.8x10^{6} ^{a}
	D4	30.22 ^{a}	28.99 ^{a}	3.1 x 10^{7b}	32.18 ^{a}	33.29 ^{b}	3.1x10^{6a}	31.22 ^{ab}	33.43 ^{b}	8.1x10^{6} ^{a}
	D5	28.87 ^{a}	23.09 ^{a}	1.2 x 10^{7b}	30.25 ^{a}	22.75 ^{a}	2.7x10^{6a}	30.02 ^{ab}	20.25 ^{a}	4.1x10^{6} ^{a}

Keys: D = Day; MI = Magnesium ion; VE = Vitamin E; OL = Olive oil; RD = Reducing Sugar; BEY = Bioethanol yield; YD = Yeast Density; ND = Not Determined.

Note: The superscript indicates the result of the ANOVA (with post hoc test ranking). The values with different superscript are significantly different while those with similar superscript were similar at p=0.05

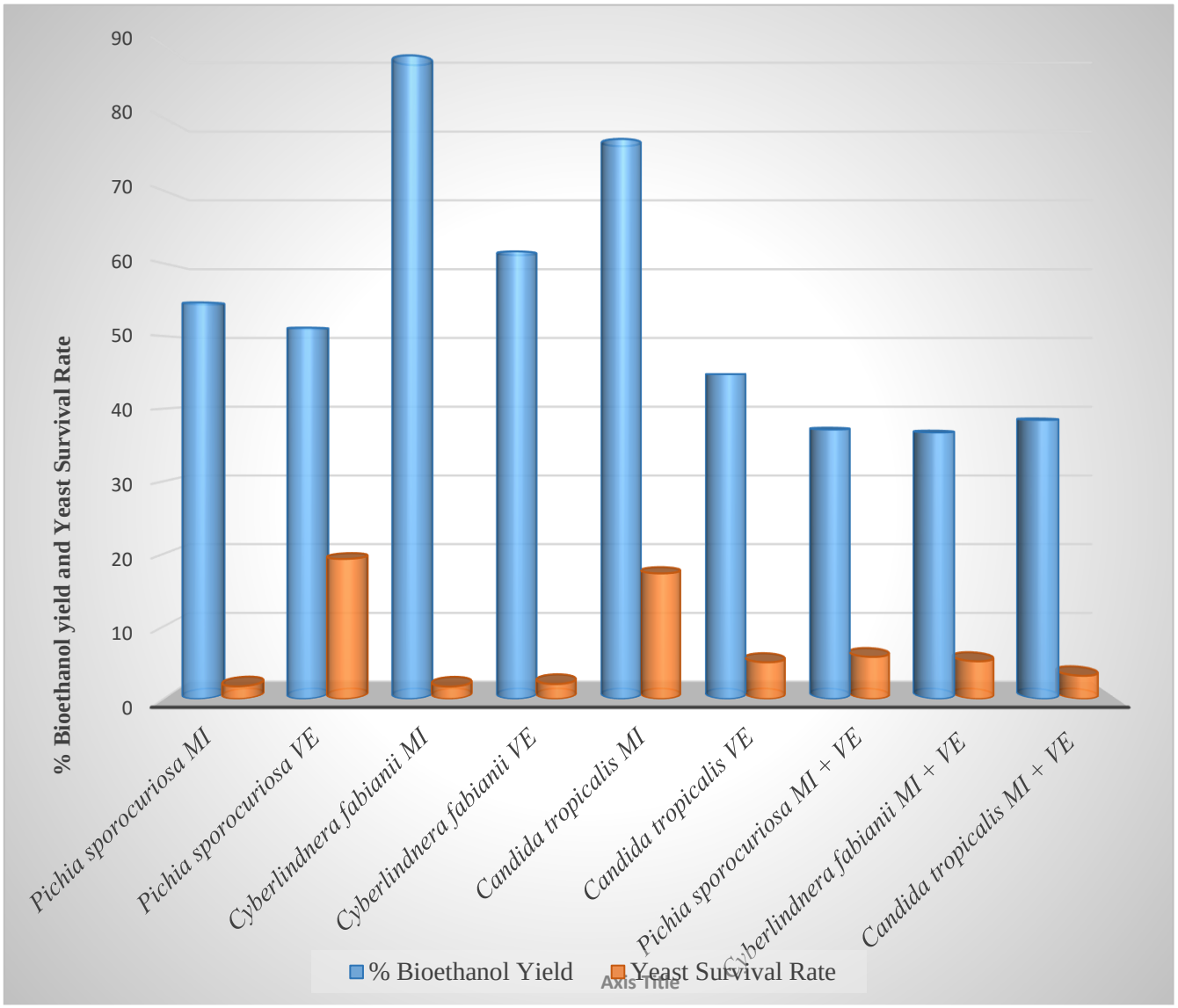

Fig. 3.3: Percentage (%) Bioethanol Yield and the Yeast Survival Rate after Day 3 fermentation using individual and combined Supplements

3.2 Discussion

The ethanol tolerance test results are displayed in Table 3.1. All the 18 isolates grew well and could withstand a 10% ethanol medium; 15 of the isolates could also survive a 15% ethanol medium, while some of them had trouble. Due to their ability to withstand and thrive on a 20% ethanol medium, three (3) of the isolates— *Pichia sporocuriosa 08*, *Cyberlindnera fabianii 11*, and *Candida tropicalis 15*, were selected.

Following their selection, the three isolates that could tolerate 20% ethanol were exposed to 25% and 30% ethanol concentrations (Table 3.2). Even though the development slows down at 25% and 30%, the isolates still managed to survive.

Table 3.3 displays the outcome of the impact of magnesium ions on the ethanol tolerance level of yeast that was isolated from fermenting palm wine. The outcome demonstrates that in all of the isolates examined, the largest growths were noted at high dosages of magnesium ion concentration supplements. It was evident that the isolates grew well at 25% and 30% ethanol media with 40% magnesium ion supplementation; *Cyberlindnera fabianii* exhibited the best ethanol tolerance. Figure 3.1 further illustrates the long-term defense of cells against ethanol stress by magnesium ions, demonstrating a 40% boost in cell proliferation.

Table 3.4 shows the results of the effect of vitamin E on the level of ethanol tolerance of yeast that was isolated from palm wine. Compared to the media lacking vitamin E, all three species exhibit noticeably faster growth in the 25% ethanol medium (Fig. 3.2). Using 20 mg of vitamin E, *Candida tropicalis* showed the greatest growth. With the exception of *Candida tropicalis*, it was evident that the majority of the organisms failed to thrive in 30% ethanol medium, even at all vitamin E concentrations. In a 30% ethanol medium enhanced with 20 mg of vitamin E, the isolate develops nicely (Fig. 3.2).

The results show that yeast isolated from palm wine has high degree of ethanol tolerance, which was also confirmed by Chandrasena *et al.* (2016). Three (3) of the isolates (*Pichia sporocuriosa*, *Cyberlindnera fabianii* and *Candida tropicalis*) showed unique characteristics as they were able to tolerate and grow well in 20% ethanol medium.

Ethanol tolerance is one of the criteria for selection of strains and unique properties of the yeast that makes it exploitable for industrial ethanol production. The ethanol tolerance of the yeast isolated from palm wine could depend on their ability to tolerate the physicochemical conditions and inherent genetic make-up. The use of efficient yeast strains with higher ethanol tolerance to improve ethanol yields in the fermentation product (palm wine) would reduce distillation costs and hence the profitability of the overall process (Chandrasena *et al.*, 2016). The ethanol tolerant yeast from palm wine between 15 and 20% has been favorably used in brewing (Wolf *et al.*, 2018). Narendranath *et al.* (2017) and Thanonkeo *et al.* (2017) reported that 13% and 7% ethanol concentrations respectively for yeast growing in toddy. Similarly, ethanol tolerance of selected strains of yeast had also been confirmed by Nwanchukwu *et al.* (2010) who reported 15% and 10% ethanol concentrations in a separate study. The isolates struggled to grow at both 25% and 30%, as the growth reduces but they survive well.

The effect of magnesium ions on the ethanol tolerance level of yeast isolated from fermenting palm wine shows that highest growths were observed at high dose of magnesium ion concentration supplements in all the isolates tested. It clearly observed that at 40% magnesium ion supplementation, the isolates grow well at both 25% and 30% ethanol media, *Cyberlindnera fabianii* showed the highest ethanol tolerance. The long-term protection of cells from ethanol stress by magnesium ions shows improvement in growth of cells at 40%. These results suggest that magnesium exerts a protective role for yeast cells toward ethanol tolerances, enabling cells to remain viable at high levels for relatively long periods. This study revealed that magnesium ion has certain effect in increasing the growth level at higher percentage of ethanol. This study corroborates the study of Zhao and Bai, (2019) who reported significant growth increases when magnesium ion was used in alcohol tolerance test. This agrees with the study of Birch *et al.* (2014) who reported that most of the ethanol producing yeast strains isolated from palm wine could tolerate ethanol concentration at varying concentration. In yeast, magnesium has been shown to increase the effect of ethanol tolerances, maintaining viability levels over 50% (Birch and Walker, 2000).

Magnesium ion is poorly soluble in organic solvent such as ethanol, so this evidence that the magnesium ion does not increase or decrease the concentration of ethanol when added to the medium as supplement. The increase in the number of viable yeast cell may be as a result of magnesium ion is the most abundant divalent metal ion involved in wide range of biological process and prolonged exponential growth, resulting in increased yeast cell mass. This finding confirms the claim of Gidado *et al.* (2017). This supports the theory that magnesium is of crucial importance in linking ATPase to the yeast membrane (Gibson *et al.*, 2007). When the rate of phosphate absorption in yeast is decreased, there is an accompanying decrease in glycolysis. The addition of magnesium ions in this situation reversed this effect, suggesting that the decrease in phosphate absorption was closely linked to a lack of free magnesium ions in the yeast cell (Mira *et al.*, 2010).

Effect of vitamin E on the ethanol tolerance level of yeast isolated from palm wine revealed that all the three organisms showed remarkable increased growth in 25% ethanol medium when compare with the medium without Vitamin E. The highest growth was recorded by *Candida tropicalis* using 20mg of vitamin E. it was clearly observed that most of the organism struggled to survive 30% ethanol medium even at all concentration of vitamin E used with exception of *Candida tropicalis*. The isolate grows well in 30% ethanol medium supplemented with 20mg of vitamin E.

This study corroborates with the study of Nwachukwu *et al*. (2010) who reported significant ethanol tolerance when vitamin E were used in alcohol tolerance test. Zhang *et al.* (2016) also confirm that ethanol tolerance was significantly improved by vitamin E supplementation, which correlated with the increased level of polyunsaturated fatty acids.

Vitamin E dissolve in ethanol but does not reduce the concentration of the ethanol, this may be attributed to the fact that the best concentration was the highest concentration (20mg/L). In this study, when the yeast was grown in increasing ethanol concentrations, the cell viability levels were slowly reduced in the treatment without vitamin E addition. Vitamin E supplementation improved ethanol tolerance and the yield of yeast. Zhang *et al.* (2016) showed that the increase in vitamin E concentration affected the total amount of the yeast membrane phospholipids in terms of the amount of PUFA and polyunsaturated fatty acid/saturated fatty acid ratio. The results suggest that vitamin E supplementation can improve the cell membrane fatty acid composition under stress conditions. Yeast often encounter low or high temperature, acids heavy metals and other abiotic stresses, sometimes simultaneously. These stresses can reduce the productivity of the fermentation. Fatty acids are a key component of the plasma membrane. The saturated/unsaturated fatty acids ratio can improve membrane fluidity (Mizoguchi and Hara, 1996), thus inducing stress resistance by changing membrane fluidity (Navarro *et al.,* 2009; Aguilera *et al.,* 2016)

It has been reported that ethanol tolerance is associated with multiple membrane components. The ethanol tolerance of yeast is very complicated, and the comprehensive mechanisms are not yet very clear. It is assumed that vitamin E may prevent lipid peroxidation of the plasma membrane, thus the relationship between cell viability levels and vitamin E is of significance. Zhang *et al.* (2016) confirmed that the PUFAs ratio in the membrane could be adjusted by vitamin E addition, thus improving the cell viability levels, and that yeast ethanol production performance can be improved by supplementing vitamin E.

The results presented in Table 3.5 provide insights into the protective effects of the supplements (singly and in Consortium) on the percentage ethanol yield, reducing sugar conversion rate, and starter yeast survival rate during fermentation with different yeast isolates (*Pichia sporocuriosa*, *Cyberlindnera fabianii*, *Candida tropicalis*).

Over the fermentation period from Day 1 (D1) to Day 5 (D5) using magnesium ion as supplement, the reducing sugar levels (RS) decreased, while bioethanol yield (BEY) which was not determined on day 1, increased for all yeast isolates from day 2 to day 3. For example, on Day 3 with *Cyberlindnera fabianii*, at D2 showed a bioethanol yield of 28.58%, which improved to 88.60% at D3 and later reduced to 20.81% on the fifth day of fermentation, Yi-Huang *et al*. (2018) confirmed that 72hours is the best fermentation time for ethanol production by yeast. The yeast density (YD) exhibited decreasing trend, reaching 3.0 x 10^4cfu/ml on D5 from 1.5 x 10^8 on D1. These trends align with previous research (Henry and Tsietsie, 2013), emphasizing the positive impact of magnesium ions on yeast fermentation. The results suggest that magnesium ions play a crucial role in enhancing bioethanol production and maintaining yeast viability.

Throughout the fermentation period, vitamin E (VE) supplementation positively influenced bioethanol yield although not as good as magnesium ion. For instance, with *Cyberlindnera fabianii* at day 3, the bioethanol yield increased from 23.22% to 61.64% (Table 3.5), and yeast density reduced from 4.8 x 10^7cfu/ml to 3.1 x 10^6cfu/ml. Zhang *et al*. (2016), studied the influence of vitamin E supplementation on yeast fermentation had similar result which confirmed that the outcomes are consistent with the known antioxidant properties of vitamin E, supporting its role in protecting yeast cells during fermentation.

These results indicate that the combined supplementation did not consistently outperform individual supplements, emphasizing the complexity of interactions between magnesium ion and vitamin E in the fermentation process. The control group without any supplementation serves as a baseline for this research to check the protective effect of the supplements on the yeast viability for improving percentage bioethanol yield.

The Percentage (%) bioethanol yield and the yeast survival rate after Day 3 fermentation using individual Supplements**.** *Pichia sporocuriosa* when magnesium ion was used as supplement, has a high bioethanol yield (54.62%) but a low yeast survival rate (1.73%). This suggests efficient bioethanol production but poor yeast viability. Vitamin E supplementation has a moderate bioethanol yield (51.16%) and a higher yeast survival rate (19.3%) (Fig. 3.3). This indicates a balance between bioethanol production and yeast viability.

Cyberlindnera fabianii shows a very high bioethanol yield (88.6%) but a low yeast survival rate (1.87%) when the fermentation medium was supplemented with magnesium ion. Similar to *Pichia sporocuriosa*, it indicates efficient bioethanol production at the expense of yeast viability. Vitamin E supplement has a decent bioethanol yield (61.64%) and a moderate yeast survival rate (2.06%). This strain strikes a better balance between bioethanol production and yeast viability compared to Magnesium ion.

When Magnesium ion was used as supplement with *Candida tropicalis*, high bioethanol yield (77.11%) and a moderate yeast survival rate (17.3%) were observed. This strain demonstrates efficient bioethanol production with relatively better yeast viability, Henry and Tsietsie (2013) had similar results. Vitamin E supplementation has a lower bioethanol yield (44.76%) and a moderate yeast survival rate (5.06%). It shows a trade-off between bioethanol production and yeast viability compared to magnesium ion. Also it was clearly observed that high percentage bioethanol productions were recorded when the supplements were used singly than when in consortium (Fig 3.3).

3.3 Conclusion

In conclusion, yeast isolates from palm wine show good ethanol tolerance, making them suitable for bioethanol production. Supplementing the growth media with magnesium ions, Vitamin E, and olive oil enhances yeast growth and ethanol tolerance. However, the effectiveness of these supplements varies among different yeast strains, with some strains exhibiting a trade-off between higher ethanol yields and lower survival rates. The combined use of supplements does not consistently outperform individual supplements, highlighting the need for tailored approaches to optimize both yeast performance and bioethanol production.

3.4 Recommendation

1. To further investigate the molecular mechanisms underlying the synergistic effects of magnesium ion, vitamin E, and olive oil, potentially through gene expression studies and membrane composition analysis.
2. Exploration of additional supplements or combinations to enhance yeast viability and bioethanol production, considering different concentrations and formulations to optimize the protective effects.
3. Continuous monitoring of fermentation processes to understand the temporal dynamics of yeast viability and bioethanol yield, contributing to the development of more efficient and controlled industrial fermentation strategies.
4. Extend the research to assess the long-term stability and efficiency of the supplemented fermentation processes, contributing to the development of robust and sustainable industrial applications.
5. Consider conducting scale-up experiments to evaluate the feasibility of implementing the optimized supplement combination in larger fermentation systems

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