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# Tolerance of Yeast to Formic Acid during Ethanol Fermentation

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**Abstract**

This study was carried out to investigate the tolerance of yeasts isolated from some Nigerian traditional fermented foods to formic acid during laboratory-scale fermentation of ethanol.

A total of 27 yeast strains were isolated from burukutu, ogi, kunu and palm wine. The yeasts were screened for formic acid tolerance using spot plate technique on two culture media. One strain was selected based on its ability to tolerate up to 15 mM concentration of formic acid on Yeast Extract Peptone Dextrose Agar and was further identified as *Candida tropicalis* strain IFM 63517. *C. tropicalis* was used for fermentation of ethanol with varying concentrations of formic acid, ethanol and residual glucose concentrations which were monitored at intervals. The total viable cell count was determined using plate count technique. The highest ethanol yield of 8.36% (v/v) with a residual glucose concentration of 0.33 g/L was obtained from 0 mM formic acid (control fermentation vessel) with a total viable cell count of $8.7 \times 10^9$ cfu/ml, while the lowest ethanol yield of 8.00% (v/v) with a residual glucose concentration of 0.14g/L was obtained from 15 mM concentration of formic acid with a total viable cell count of $6.1 \times 10^9$ cfu/ml. The yeast strain used in this work exhibited a high ethanol yield despite the presence of an inhibitory compound (formic acid) when comparing the ethanol yield at its tolerance threshold (15 mM of formic acid) to the control fermentation vessel without formic acid.

**Keywords**: yeast, formic acid, ethanol fermentation, residual sugar, *Candida tropicalis*.

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**Резюме**

Настоящата разработка е насочена към проучване толерантността на дрожди, изолирани от някои традиционни нигерийски ферментирали храни към мравчена киселина по време на ферментация на етанол в лабораторни условия.

От бурукуту, оги, куну и палмово вино са изолирани общо 27 щама дрожди. Проучена е резистентността на тези изолати към мравчена киселина върху две агарови културални среди. Един от тест-щамовете, идентифициран като *Candida tropicalis* IFM 63517, показва резистентност към мравчена киселина в концентрация до 15 mM при култивиране върху среда с декстрозо-пептонен агар и дрождев екстракт. *C. tropicalis* се използва за ферментация на етанол при различни концентрации мравчена киселина, етанол и глюкоза, които се измерват на интервали. Общият брой на жизнеспособнте клетки се определя чрез броене на образуваните колонии в агарова среда в петриеви блюда. Най-високият добив на етанол от 8.36% (обем/обем) с концентрация на остатъчна глюкоза 0.33 г/л се получава от 0 mM мравчена киселина (контролен ферментационен съд) с общ брой жизнеспособни клетки $8.7 \times 10^9$ бр. клетки/мл, докато най-ниският добив на етанол - 8.00% (обем/обем) с концентрация на остатъчна глюкоза от 0.14 г/л се получава от концентрация на мравчена киселина 15 mM с общ брой жизнени клетки $6.1 \times 10^9$ бр. клетки/мл. При сравняване резултатите се установява, че щам *C. tropicalis* IFM 63517 показва висок добив на етанол, въпреки наличието на инхибиращото вещество (мравчена киселина) дори в условията на праговата толерантност на щама (15 mM), в с сравнение с този от контролния ферментор (без наличие на мравчена киселина).

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## Introduction

Yeasts, mostly strains of *Saccharomyces cerevisiae*, have been widely used for bioethanol production industrially, because of their high fermentative ability, ethanol tolerance and rapid growth under anaerobic conditions. Apart from *Saccharomyces*, other genera of yeasts such as *Candida*, *Kluyveromyces*, and *Schizosaccharomyces* have also been employed in the bioconversion of lignocellulosic substrates to bioethanol (Ivanova *et al*., 2011). These yeasts, however, are susceptible to inhibitory compounds present in lignocellulose-derived hydrolysates (Martin *et al*., 2002). One possible solution is to detoxify the hydrolysate to remove the inhibitors; however, this creates additional costs and a potential loss of sugar (Almeida *et al*., 2007). An alternative approach and long-term solution to overcome this problem is to either screen for high inhibitor tolerant yeast strains or create genetically modified strains with desired tolerance properties.

Inhibitory compounds capable of inhibiting fermenting yeast fall into specific groups such as weak acids, furan derivatives and phenolic compounds (Sun and Tao, 2010). The types of toxic compounds generated, and their concentrations in lignocellulosic hydrolysates, depend on both the raw material and the operational conditions employed for hydrolysis (Taherzadeh *et al*., 2000). Toxic compounds can act to stress fermentative organisms to a point beyond which the efficient utilization of sugars is possible, ultimately leading to reduced product formation (Palmqvist and Hahn-Hagerdal, 2000b). Formic acid (methanoic acid) is one of the weak acid inhibitors present in lignocellulosic hydrolysates, with a typical concentration of approximately 1.4 g/L (30 mM) (Almeida *et al*., 2007; Greetham *et al*., 2014). The majority of the investigators who have observed the presence of this acid in fermented liquids, however, believed it to play some role in the fermentations of sugar by yeast. The inhibitory effect of formic acid has been ascribed to both uncoupling and intracellular anion accumulation and the reduction of the uptake of aromatic amino acids (Verduyn *et al*., 1992; Pampulha and Loureiro, 2000; Almeida *et al*., 2007). Formic acid is more toxic to yeast strains than either acetic acid or levulinic acid (Almeida *et al*., 2007; Hasunuma *et al*., 2011a), due to a lower pKa value (3.75 at 20°C) than acetic (4.75 at 25°C) and levulinic acid (4.66 at 25°C).

The undissociated form of weak acids can diffuse from the fermentation medium across the plasma membrane and dissociate due to higher intracellular pH, thus decreasing the cytosolic pH (Verduyn *et al*., 1992; Pampulha and Loureiro, 2000). The decrease in intracellular pH is compensated by the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. Consequently, less ATP is available for biomass formation. Inhibition of cytochrome oxidase also increases the production of cytotoxic reactive oxygen species (ROS), leading to cell death due to damage in ROS in cell compartments (Richter *et al*., 1995). For industrial and commercial purposes, low concentrations of formic acid are widely used as one of the major ingredients of antiseptics. According to the intracellular anion accumulation theory, the anionic form of the acid is captured inside the cell and the undissociated acid will diffuse out of the cell until equilibrium is reached. Weak acids have also been shown to inhibit yeast growth by reducing the uptake of aromatic amino acids from the medium, probably as a consequence of strong inhibition of the enzyme permease (Almeida *et al*., 2007).

On the other hand, formic acid has been shown to be a toxic metabolite of methanol, and is a commonly used organic solvent that has been long known to be a selective human neurotoxin (Roe, 1955). Formic acid causes both metabolic acidosis and ocular toxicity by affecting the retina and optic nerve cells, ultimately leading to blindness, a common and permanent consequence of methanol intoxication (Roe, 1955). Formic acid has been demonstrated in vitro to induce mammalian cell death by inhibiting the activity of cytochrome oxidase, the terminal electron acceptor of the electron transport chain that is involved in ATP synthesis, resulting in depletion of ATP and subsequent cell death due to reduction of energy levels so that essential cell functions cannot be maintained (Nicholls, 1975; Nicholls *et al*., 1976). Antioxidants such as catalase and glutathione/glutathione peroxidase may play a role in the protection of ocular cells from formic acid toxicity (Treichel *et al*., 2004).This work, therefore, aimed at investigating the tolerance of yeasts isolated from some Nigerian traditional fermented foods to formic acid during laboratory-scale fermentation of ethanol.

## Materials and Methods

*Microorganisms*

All yeast used for this work were isolated from various fermented beverages (palm wine, burukutu, kunu) purchased from Ibadan metropolis in Oyo State, using standard microbiological isolation

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procedures. Stock of each strain was stored in malt extract agar (MEA) slant at 4°C until required.

*Spot plate analysis*

Progressive sub-culturing of each isolate from a lower formic acid concentration to a higher concentration (0, 5, 10, 15 and 20 mM) using both MEA and yeast extract peptone dextrose agar (YEPDA) was carried out for the selection of the most tolerant strain. Yeast inoculum was prepared by taking a loopful of stock culture to 10 mL of demineralized water and the optical density was compared with MacFarlan standard number 0.5 containing approximately $1.5 \times 10^8$ cells/mL of yeast culture then 5 µL samples of each dilution of the yeast cultures were spotted on MEA and YEPDA plates. The plates were incubated anaerobically at 30°C for 48 h and visible growth differences were recorded (Homann *et al*., 2005).

*Molecular identification of the tolerant yeast strain*

PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, annealing of primer at 55°C and 72°C for 1.5 min and a final termination at 72°C for 10 min.

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Bio systems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. BioEdit software and MEGA 6 were used for all genetic analyses.

*Lab-scale fermentation*

YEPD broth with the addition of formic acid at 0, 5, 10, 15, 20 mM was used in the laboratory-scale fermentations. The pH of the media was adjusted to 4.5 using phosphoric acid under aseptic conditions. From the broth, 100 mL was transferred into mini fermentation vessels (FVs). One mL of the prepared inoculum size of the most tolerant yeast strain was aseptically transferred into each of the bottles. All bottles were incubated at 30°C with shaking at 200 rpm for 24 hours. Samples were collected at specific time intervals to determine the total cell count, and concentrations of glucose.

*Total viable cell count*

One mL of appropriate dilution factor of the suspension of the tolerant yeast strain was plated out using the spread plate method for the determination of the total viable cell of the yeast strain. Plate count technique was employed for the total viable cell of the tolerant strain based on the number of colony forming.

*HPLC analysis*

At intervals of 24 and 48 h, 10 mL samples from the fermentation broth were aseptically collected for determination of the residual glucose concentration using High Performance Liquid Chromatography according to the method of Falque-Lopez and Fernandez-Gomez (1996). Glucose concentration was determined using Agilent 1200 HPLC system composed of: Detector: Refractive Index Detector (RID); Column: Grace-Davison Prevail Carbohydrate ES 5µ column (150mm x 4.6mm); Mobile Phase: 75% Acetonitrile LC Grade: 25% deionised water; Injection volume: 5.0 µl; Flow rate: 1.0 mL/min, and temperature: 25°C.

*GC analysis*

At intervals of 24 and 48 h, 10 mL samples from the fermentation broth were aseptically collected for the determination of the ethanol concentration using Gas Chromatography model HP 6890 Powered with HP ChemStation rev. A 09 01 (1206) software according to the method of Vianna and Elber (2001).

## Results

*Spot plate characteristics (screening)*

All twenty-seven isolates were screened using the spot plate technique with different concentrations of formic acid. Two media, namely MEA and YEPDA for yeast propagation, were used in the screening process to access the tolerance of the various isolates to formic acid (Table 1).

On MEA, all the twenty-seven (100%) isolates were able to grow at 0 mM of formic acid, 11 (40.74%) isolates were able to grow at 5 mM of formic acid, 6 (26.22%) isolates were able to grow at 10 mM of formic acid, none (0%) was able to grow at 15 and 20 mM of formic acid, respectively. Whereas, on YEPD all (100%) the isolates were able to grow at both 0 and 5 mM concentration of formic acid, 10 (37.04%) were able to grow at 10 mM concentration of formic acid, 1 (3.70 %) was able to grow at 15 mM concentration of formic acid and none was able to grow at 20 mM of formic acid concentration.

From the results obtained from the spot plate screening of the isolates, it was observed that YEPDA was the best medium that could support the growth of the isolates as it was able to support the growth of one isolate (P8) up to 15 mM concentration of formic acid.

*Molecular identity of the tolerant strain*

Gene sequence from the characterized isolate showed 99% identity similar to *Candida tropicalis*

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**Table 1.** Spot plate screening of isolates using different concentrations of formic acid (mM)

| S/N | Isolate code | Formic acid concentrations of (mM) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MEA | | | | | YEPDA | | | | |
| | | 0 | 5 | 10 | 15 | 20 | 0 | 5 | 10 | 15 | 20 |
| 1 | P1 | + | - | - | - | - | + | + | - | - | - |
| 2 | P2 | + | - | - | - | - | + | + | - | - | - |
| 3 | P3 | + | - | - | - | - | + | + | - | - | - |
| 4 | P4 | + | - | - | - | - | + | + | - | - | - |
| 5 | P6 | + | + | + | - | - | + | + | + | - | - |
| 6 | P7 | + | + | - | - | - | + | + | + | - | - |
| 7 | P8 | + | + | + | - | - | + | + | + | + | - |
| 8 | P9 | + | - | - | - | - | + | + | + | - | - |
| 9 | P10 | + | - | - | - | - | + | + | - | - | - |
| 10 | P11 | + | + | - | - | - | + | + | - | - | - |
| 11 | K1 | + | - | - | - | - | + | + | - | - | - |
| 12 | K4 | + | + | + | - | - | + | + | + | - | - |
| 13 | K5 | + | + | - | - | - | + | + | - | - | - |
| 14 | K6 | + | + | + | - | - | + | + | + | - | - |
| 15 | K7 | + | - | - | - | - | + | + | - | - | - |
| 16 | K8 | + | + | + | - | - | + | + | - | - | - |
| 17 | K9 | + | + | + | - | - | + | + | + | - | - |
| 18 | K10 | + | - | - | - | - | + | + | - | - | - |
| 18 | K10 | + | - | - | - | - | + | + | - | - | - |
| 20 | B1 | + | - | - | - | - | + | + | - | - | - |
| 21 | B2 | + | - | - | - | - | + | + | + | - | - |
| 22 | B3 | + | - | - | - | - | + | + | + | - | - |
| 23 | O1 | + | - | - | - | - | + | + | - | - | - |
| 24 | O2 | + | + | - | - | - | + | + | - | - | - |
| 25 | O3 | + | - | - | - | - | + | + | + | - | - |
| 26 | O4 | + | - | - | - | - | + | + | - | - | - |
| 27 | O5 | + | - | - | - | - | + | + | - | - | - |

Key: + = Growth; - = No growth

strain IFM 63517 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene partial sequence.

*Determination of glucose concentration*

The residual glucose concentration from the various mini fermentation vessels containing varying concentrations of formic acid was monitored during the period of fermentation at 24 and 48 h, respectively, using HPLC. With 0 mM of formic acid, residual glucose concentration at 24 h of fermentation was 0.4±0.00 while at 48 h of fermentation there was a decrease in the residual glucose concentration to 0.33±0.01. With 5mM of formic acid, residual glucose concentration at 24 h of fermentation was 0.19±0.01 while at 48 h of fermentation there was a decrease in the residual glucose concentration to 0.10±0.01. With 15 mM of formic acid, residual glucose concentration at 24 h of fermentation was 0.21±0.01 while at 48 h of fermentation; there was a decrease in the residual glucose concentration to 0.14±0.01, as shown in Table 2.

**Table 2.** Residual glucose concentration at 24 and 48 h of glucose fermentation by *C. tropicalis* strain IFM 63517

| Formic acid (mM) | Residual glucose concentration (g/L) | |
|---|---|---|
| | 24 h | 48 h |
| 0 | 0.40±0.00[a] | 0.33±0.01[a] |
| 5 | 0.19±0.01[b] | 0.10±0.01[b] |
| 15 | 0.21±0.01[b] | 0.14±0.01[c] |

Note: Means (results of duplicate) with different superscript letter down the column are statistically significant at $p \leq 0.05$

*Ethanol yield of glucose fermentation by C. tropicalis strain IFM 63517 at 24 and 48 h*

The total ethanol yield from the various mini fermentation vessels containing varying concentrations of formic acid was monitored during the period of fermentation at 24 and 48 h, respectively, using gas chromatography.

With 0 mM of formic acid ethanol yield at 24 h of fermentation was 5.99±0.02 while at 48 h of fermentation, there was an increase in the ethanol yield to 8.36±0.05. With 5 mM of formic acid, ethanol yield at 24 h of fermentation was 5.68±0.01 while at 48 h of fermentation there was an increase in the ethanol yield to 8.24±0.01. With 15 mM of formic acid, ethanol yield at 24 h of fermentation was 5.01±0.02 while at 48 h of fermentation there was an increase in the ethanol yield to 8.00±0.02, as shown in Table 3.

**Table 3.** Ethanol yield at 24 and 48 h of glucose fermentation by *C. tropicalis* strain IFM 63517

| Formic acid (mM) | Ethanol yield (%v/v) | |
|---|---|---|
| | 24 h | 48 h |
| 0 | 5.99±0.02[a] | 8.36±0.05[a] |
| 5 | 5.68±0.01[b] | 8.24±0.01[b] |
| 15 | 5.01±0.02[c] | 8.00±0.02[c] |

Values are means of triplicate plate count

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*Total viable cell count determination*

The total viable cell count of *C. tropicalis* strain IFM 63517 used in the fermentation process was determined using the plate count technique at intervals of 12, 24, 36 and 48 h. With 0 mM of formic acid, there was a total cell count of 5.9x10$^9$, 6.8x10$^9$, 7.4x10$^9$ and 8.7x10$^9$ cfu/ml at 12, 24, 36 and 48 h of fermentation, respectively. This is shown in Table 4. With 5 mM of formic acid, there was a total cell count at 48 hours 4.2x10$^9$, 5.1x10$^9$, 6.6x10$^9$ and 6.9x10$^9$ cfu/ml at 12, 24, 36 and 48 h of fermentation, respectively, while with 15 mM of formic acid, there was a total cell count of 3.3 x10$^9$, 4.7 x10$^9$, 5.3 x10$^9$ and 6.1 x10$^9$ cfu/ml at 12, 24, 36 and 48 h of fermentation, respectively.

**Table 4.** Total viable cell count of *C. tropicalis* strain IFM 63517 throughout the period of glucose fermentation with various concentrations of formic acid

| Time (h) | Formic acid concentration (mM) | | |
|---|---|---|---|
| | 0 | 5 | 15 |
| 0 | 1.5x10$^8$ | 1.5x10$^8$ | 1.5x10$^8$ |
| 12 | 5.9x10$^9$ | 4.2x10$^9$ | 3.3x10$^9$ |
| 24 | 6.8x10$^9$ | 5.1x10$^9$ | 4.7x10$^9$ |
| 36 | 7.4x10$^9$ | 6.6x10$^9$ | 5.3x10$^9$ |
| 48 | 8.7x10$^9$ | 6.9x10$^9$ | 6.1x10$^9$ |

Values are means of triplicate plate count

**Discussion**

Despite the fact that yeasts are readily capable of utilizing monosaccharides as their energy source, this ability can easily be hampered by the presence of inhibitory substances such as formic acid, which is a typical weak organic acid formed as a by-product in the anaerobic breakdown of glucose by yeast. Yeast isolates used in this work were obtained from various indigenous fermented beverages of Nigeria (palm wine, ogi kunu, burukutu). Serial dilution and appropriate dilution factors of x10$^3$ and x10$^6$ of each of these beverages were plated out for yeast isolation. According to Banwo *et al*. (2015), the ready availability of yeast in such food products is a result of the high sugar content hence leading to the addition of desirable flavour to fermented foods.

Spot plate screening of the 27 yeast isolates from various fermented foods using both MEA and YEPDA showed that the highest tolerance threshold of the isolates to formic acid on MEA was 10 mM, where 6 (26.22%) isolates were able to grow whereas there was an increase in the tolerance threshold on YEPDA up to 15 mM of formic acid with 1 (3.70%) isolate being able to grow. This result agrees with the work of Keating *et al*. (2006), who utilized Yeast Nitrogen Base agar (YNBA) and YEPDA for the spot plate screening of yeast isolate to various inhibitory weak organic acids and found that yeast could tolerate higher inhibitory concentrations on YEPDA compared to YNBA or MEA, which are mere minimal media for yeast isolation with reasons being that YEPDA as an enriched medium may have a higher buffering capacity against weak organic acids, of which formic acid is an example. Similarly, Oshoma *et al*. (2015) subjected various strains of non-*Saccharomyces cerevisiae* to various concentrations of formic acid and observed that *S. paradoxus* DBVPG6466, *S. kudriavzeii* IFO1802, *S. arboricolus* 2.3319 *S. cerevisiae* NCYC2592 exhibited tolerance to 35 mM and 20 mM formic acid on YPD and YNB media, respectively, while other strains did not grow, thus showing that yeast can tolerate higher concentrations of inhibitors in YEPD medium than in other minimal growth media.

Molecular characterization of the most tolerant and selected yeast isolate revealed the isolate to have 99% identity to *C. tropicalis* strain IFM 63517. Similar results where *Candida* species were isolated and characterized from fermented foods such as kunu, pito, ogi and palm wine were reported in the works of Sanni and Lonner (1993); Ikpoh *et al*. (2013); Banwo *et al*. (2015).

Based on the results obtained from spot plate screening, *C. tropicalis* IFM 63517 strain was selected and tested for formic acid tolerance. Compared with the controlled fermentation vessel without formic acid where cell growth was unaffected by any inhibitory compound, cell growth of *C. tropicalis* IFM 63517 strain at both 5 mM and 15 mM concentrations of formic was still maintained above 75%. This result conforms to what was obtained by Huang *et al*. (2011), who studied the inhibitory effect of organic acids on strains of Saccharomyces species. He stated that in the fermentation of glucose with different concentrations of formic acid, it was observed that the cell concentration decreased with an increase in formic acid concentration in the fermentation vessels. The reasons behind this have not been well established but a potential reason could be a result of the diversion of metabolic energy (ATP) in the cell to pump out excess proton from the cytoplasm of the cell for a balanced osmotic pressure at the expense of cell biomass pro-

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duction and accumulation (Wikandari *et al*., 2010).

At 24 h, there was a statistically significant difference in the ethanol yield at 0, 5 and 15 mM concentrations of formic acid. The same was observed at 48 h, although there was a significantly higher ethanol yield at 48 h. In summary, there was more ethanol yield at 48 h and observations revealed that as the formic acid concentration increased, there was lesser yield of ethanol, thereby signifying the repressing effect of increased concentration of formic acid on ethanol yield by the yeast isolate.

The overall glucose consumption of *C. tropicalis* IFM 63517 was not affected by formic acid. At 24 h of fermentation, the highest residual glucose (0.40±0.00) was observed at 0 mM of formic acid while it was least (0.19±0.01) at 5 mM formic acid. At 48 h of fermentation, the same trend of residual glucose was observed (0.33±0.01) and (0.10±0.01) for both 0 mM and 5 mM concentrations of formic acid, respectively. A similar result was reported in yeast fermentations with acetic acid by Keating *et al*. (2006) and this supports the assumption that in the presence of formic acid, metabolic activity is increased for the pumping out of protons from the cell thus leading to quick utilization of available glucose in the fermentation wort.

## Conclusion

The yeast strain used in this work exhibited a high ethanol yield despite the presence of an inhibitory compound (formic acid) when comparing the ethanol yield at its tolerance threshold (15 mM of formic acid) to the control fermentation vessel without formic acid.

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