Assessment of the viability of *Saccharomyces cerevisiae* in response to synergetic inhibition during bioethanol production

Corli de Klerk[1], Elvis Fosso-Kankeu[1,*], L. Du Plessis[2] and S. Marx[1]

[1]School of Chemical and Minerals Engineering, and
[2]Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2531, South Africa

Second-generation biofuels, fuels produced from lignocellulosic materials, including wood, agricultural residues and biomass waste include bioethanol, biodiesel and biogas. These fuel sources have great potential as useful substitutes to conventional fossil fuels. Biomass sources are also non-toxic and biodegradable energy sources that can be produced from a wide range of organic materials resulting in economic and renewable energy source. Pretreatment of lingocellulosic biomass is required to reduce physicochemical restrictions that hinder the accessibility of sugars necessary for hydrolysis and fermentation. Various pretreatment processes exist, but all of them produce inhibitory compounds that ultimately reduce ethanol production and cell viability of the fermenting microorganism, *Saccharomyces cerevisiae*. In this study different combinations of inhibitors (acetic acid, formic acid and vanillin) were considered to mimic realistic fermentation conditions during bioethanol production; ethanol yield and cell viability were then concurrently measured over a period of 48 h. The combination of acetic acid and formic acid exhibited ethanol reduction up to 11 ± 3.74%, while cell viability decreased by 23 ± 6.61%. Acetic acid and vanillin reduced ethanol production by 25 ± 1.77% and cell viability by 4 ± 4.38%. Formic acid and vanillin inhibited ethanol production by 31 ± 3.14% and cell viability 16 ± 7.54%. Finally, the synergistic effect of all three inhibitors reduced the final ethanol production by 58 ± 5.09% and cell viability by 27 ± 5.44%, indicating the toxic effect of the synergistic combination.

Keywords: Bioethanol production, cell viability, flow cytometry, *Saccharomyces cerevisiae*, synergetic inhibition.

THE concerns of rapid growth of human population, the ever-depleting natural resources, environmental pollution and industrialization lead to the critical need for alternative sources for fuel and energy production[1–3]. Additionally, the combustion of fossil-fuel sources contributes to greenhouse gas emissions, leading to atmospheric pollution and climate change[4,5]. The global production of large-scale bioethanol is increasing as it can be used as an octane booster or fuel additive, even as a neat fuel source. Compared to commercial fuel sources, bioethanol has multiple advantages ranging from a higher octane number to lower emissions of sulphur dioxide and carbon dioxide[2]. The reduced carbon dioxide concentration in fuel emissions is also an advantage in the development of bioethanol as an alternative fuel source[6]. According to Licht[7], bioethanol has been considered as the most prominent alternative for fossil fuels as it presented a sharp increase in production from 2000, with numbers ranging from 49 billion litres in 2006 to up to 115 billion litres in 2015. It is important to mention that bioethanol production must be cost effective to be implemented at large scale[8,9]. The production cost for bioethanol from food crops is extremely high as the cost of raw materials accounts for approximately 40–70% of production costs, therefore, it is crucial to utilize second-generation feedstocks, not used for human consumption[10,11].

Bioethanol is a natural product produced by fermentation of plant materials that contains sugar and starch (first-generation feedstock), such as sugarcane and grains, the two most common feedstock used for anhydrous bioethanol fermentation[12,13]. Second-generation feedstock known as lignocellulosic materials, such as grasses, woody crops and organic waste materials is popular, because it does not diminish any source of food for human consumption. Lignocellulosic waste materials are predominantly derived from wood or agricultural remains[14]. Up to 90% dry weight of plant material consists of lignin, cellulose, hemicellulose and a small percentage of pectin[15]. Lignin, the protective barrier in lignocellulose, prevents breakdown of macromolecules by bacteria and fungi that will lead to the production of biofuels. This cellular structure must be broken down to the necessary monomer sugars in order for microorganisms to utilize it as a fermenting source. Cellulose is characterized by a rigid crystalline structure that requires harsh treatment to successfully break down[16,17]. Hemicellulose is constructed of short and linear structures, with a large amount of branched chains of sugars[8,18].

Pretreatment processes are required to separate the various biomass constituents, increase lignocellulose

*For correspondence. (e-mail: kaelpfr@yahoo.fr)

porosity and reduce the chemical destruction of fermentable sugars that must be utilized for the production of bioethanol. This is achieved by macroscopic and microscopic alterations of size and structure[8,19]. Various pretreatment procedures are available that can render carbohydrates accessible and allow sufficient biomass breakdown[20]. Physical methods include pyrolysis, mechanical comminution and ozonolysis; physico-chemical procedures consist of steam explosion and CO_2 explosion; chemical treatment entails economically feasible acid hydrolysis and biological pretreatment procedures are conducted by fungal disinfection and enzyme hydrolysis[21,22]. One of the foremost disadvantages of pretreatment processes is the production of inhibitors that obstruct the growth of fermenting microorganisms, *Saccharomyces cerevisiae*[9,19]. The pretreatment process plays a significant role in bioethanol production as it releases inhibitors that will influence bioethanol formation[14].

Compounds that inhibit yeast growth during fermentation include substances such as furans, phenolic compounds and weak acids; no pretreatment method has been developed to meet all the necessary requirements that can remove all inhibitors to allow maximum fermentation capabilities. Aromatics, also known as phenolic compounds, originate from the hydrolysis of lignin components that ultimately partition into cell membranes of the biomass, therefore reducing cell integrity and capability to act as a selective barrier[9,23]. The weak acids are produced from the treatment of hemicellulose biomass[24]. According to Almeida *et al.*[9], the combined effect of inhibitors on cell viability will be greater than the individual effect. Therefore, it is necessary to determine and evaluate the viability of *S. cerevisiae* during the fermentation process as well as the overall effect of ethanol yield. In order to overcome the strong inhibitory effects from the released pretreatment compounds, chemical and biological detoxification procedures are frequently considered[20].

During fermentation where glucose is consumed to produce ethanol, the secondary inhibitors commonly formed are acetic acid and lactic acid. The mechanisms of these inhibitors in the presence of initial inhibitors are not yet fully understood. According to Fu *et al.*[25], the fermentation inhibition of certain compounds can be enhanced by a combination of other compounds present in the mixture. It has also been well-established that ethanol inhibits the growth of most microorganisms, including *S. cerevisiae*. Once ethanol has reached a concentration that will inhibit the growth of the yeast, no further fermentation is possible without the aid of external influencers[26].

The viability of *S. cerevisiae* is influenced by the increasing concentration of inhibitors during pretreatment process[1]. During this time, secondary inhibitors are also formed. Furthermore, ethanol is also considered as an additional inhibitor that reduces cell performance. According to Kubota *et al.*[27], the ethanol produced is toxic to yeast cells as it induces stress and reduced growth. However, the impact of synergetic inhibition on yeast viability as well as bioethanol production has not been well established; most of the previous studies have considered only the impact of individual inhibitors and cell viability has been determined mainly using plate counts[28]. The use of plate counts could provide some important information; however, growth on agar media is influenced by various factors, including cultivation media, incubation temperature as well as natural selectivity of yeast cells to the media. Moreover, the plate count method is time-consuming as it requires up to 7 days for the results to be indicated. Therefore, a niche arises for development of rapid, accurate methods that can quantify the viability of yeast cells in the presence of naturally occurring inhibitors. The development of fluorescent staining methods facilitates accurate measurement of total cell concentration (TCC), damaged cells and dead cells with the aid of flow cytometry, thus allowing the ability to enumerate the complete amount of yeast cells as well as indicate the effect of the inhibitors on the growth of yeast over time. This novel method to determine the effect of yeast cell viability can help distinguish between various methods to maximize fermentation capabilities for bioethanol production.

In this study, the synergetic effect of inhibitors on the viability of *S. cerevisiae* during ethanol production was evaluated to better correlate the susceptibility of yeast strain to pretreatment-derived inhibitors and ethanol production rate. The plate counts were compared with flow cytometry TCC and the damaged cell measured at various inhibitory concentrations. All the methods used the same yeast cell samples over a constant period of time, from which the results could be determined and correlated.

Methodology

Materials

The weak acids, acetic acid (95.5%) and formic acid (99.9%), were purchased from Associated Chemicals Enterprises (ACE, Johannesburg, South Africa) and the phenol, vanillin purchased from Merck (Darmstadt, Germany). Commercial Anchor yeast was purchased as the source of *S. cerevisiae*. Furthermore, the LIVE/DEAD® FungaLight™ Yeast Viability Kit was purchased from ThermoFisher Scientific (Massachusetts, USA). Buffer tablets were purchased from Sigma-Aldrich (St Louis, Missouri, USA) for flow cytometry analysis preparation. Additionally, ethanol (99.9%) and sodium hydroxide (>98%) were purchased from Sigma-Aldrich (South Africa) and Rochelle Chemicals, South Africa respectively. Deionized water was used to make up the volumes of the respective solutions.

Growth of S. cerevisiae

First, the dried yeast cells were restored with the inoculation of the sterilized YPD nutrient broth. The pH of the solution was adjusted to 6.5 by adding 0.1 M NaOH. The broth and agar solutions were autoclaved at 121°C for 20 min to ensure sterility and prevent initial contamination.

The yeast cells were fermented in sterile 500 ml GL 45 laboratory glass bottles with blue PP screw caps and pouring rings. The inoculum of 0.05 g dry yeast cells was added to 1000 ml sterilized broth to be incubated overnight at 30°C in a shaking incubator with a speed of 120 rpm to ensure sufficient growth conditions of cell cultures and reduction in the lag time of yeast growth. One millilitre of broth was transferred to 20 g/l glucose in deionized water in 100 ml GL 45 laboratory glass bottles with blue PP screw caps and pouring rings. The new inoculum was further incubated at 30°C for 48 h.

Determination of minimum inhibitory concentration

The YPD aerobically grown yeast cultures were inoculated in broth solutions that were dosed with various pretreatment concentrations and combinations of acetic acid, formic acid and vanillin. All experiments were carried out in 100 ml GL 45 laboratory glass bottles with blue PP screw caps and pouring rings, each containing 50 ml solution, shaking speed of 120 rpm and incubation temperature of 30°C. In order to ensure that pH does not limit the growth of yeast, the former was adjusted to 6.5 with NaOH, prior to the addition of any inhibitor or fermentation. The samples were analysed at set time intervals to determine the minimum inhibitory concentration (MIC); the lowest concentration at which no visible growth of the organism can be observed, with the exception of one or two small colony formations[29]. Experiments were done in duplicate. Table 1 shows the various inhibitory combinations.

Determination of synergistic effects on the growth and ethanol yield of yeast cells

A 4 ml aliquot yeast culture was added to 46 ml of glucose (20 g/l) solution in deionized water contained in 100 ml GL 45 laboratory glass bottles with blue PP screw caps and pouring rings. The MIC of different inhibitors was added to the solutions to ensure that the final concentration would correspond to that of pretreatment concentration and the samples were analysed at set intervals for 48 h. After fermentation, the samples were filtered through a 0.2 µm micro pore syringe filter and all sugars, ethanol and secondary inhibitors (acetic and lactic acid) were quantified with an Agilent series 1200 HPLC fitted with a HPX-87H Aminex column at 55°C RID and 30°C column temperature. The mobile phase used was 0.005 M H_2SO_4 at a flow rate of 0.6 ml min^{-1}, with an injection volume of 5 µl.

Cell quantification

The growth of *S. cerevisiae* in the fermentation broth was quantified by the measurement of absorbance using a SHIMADZU spectrophotometer at a wavelength of 600 nm. This measurement of optical density (OD) provided an indication of the number of cells present, whether dead, alive or injured.

Bioethanol production

Bioethanol production is represented as the percentage of ethanol formed from an initial glucose concentration of 20 g/l. The ethanol yield (see eq. (1)) was determined by taking into account the total volume changes as ethanol is produced during the fermentation process, where [*C*] represents the ethanol produced in the fermentation broth (g/l).

$$\text{Ethanol yield (g/g)} = [C] \times \frac{\text{Total volume } (l)}{\text{Sugar weight (g)}}. \quad (1)$$

The concentration of sugar was calculated using eq. (2).

$$[C_s] = \frac{\text{Area of sugar (HPLC)}}{\text{Slope sugar calibration curve}} \times \text{dilution factor}. \quad (2)$$

Cell viability

The viability of yeast cells was determined using both culture method and flow cytometry. The first method entailed the serial dilution of cells with the addition of deionized water, up to a concentration of 10^{-6}. The diluted samples were then spread evenly over the agar plates, followed by an incubation period of 48 h at 30°C.

Table 1. Inhibitor combination matrix indicating the possible combinations at various concentrations to determine minimum inhibitory concentration

Acetic acid (g/l)	Formic acid (g/l)	Vanillin (g/l)
4	0	0
0	2	0
0	0	2
0.262	0.158	0
0.391	0	0.029
0	0.37	0.049
0.250	0.150	0.020

The amount of colonies and average of duplicate plates were represented as colony-forming units (CFUs).

The flow cytometry analysis was done using LIVE/DEAD® FungaLight™ yeast viability kit. All samples were initially collected in micro-centrifuge tubes and centrifuged for 1–3 min to pellet the cells. A cell-killed control sample was prepared using the pellet of the centrifuged culture, added to 1 ml isopropyl-alcohol and incubated at room temperature for 30–60 min, while stirring every 15 min. An untreated control sample and an experimental control sample collected in micro-centrifuge tubes were centrifuged for 1–3 min to pellet the cells and remove the supernatants. All samples, including the cell-killed control, were washed in 1 ml buffer solution, centrifuged for 1–3 min, followed by removal of supernatants. The sample pellet was suspended in 1 ml buffer solution and an aliquot of 200 µl was placed in 10 ml buffer to dilute the suspension to approximately 10^6 cells/ml.

For the staining procedure, triplicate analysis was done for 1 ml of yeast suspension (killed, untreated and experimental) in the respective test tubes. An unstained sample was produced without adding any dye. For single-colour dye controls, 1 µl SYTO 9 dye was added to the tube of untreated samples as well as to one tube of killed cell samples. Propidium iodide (1 µl) was added to the other tube of control cell samples and killed cell samples. For preparation of experimental samples, 1 µl SYTO 9 and 1 µl propidium iodide dye were added to the samples. Following the staining, samples were vortexed gently and incubated at room temperature for 30 min.

Cells were analysed with a FACSVerseTM (BD Biosciences, San Jose, CA, USA) flow cytometer. After incubation, the stained cells were analysed by flow cytometry using 480/500 nm as excitation/emission wavelengths for SYTO-9 and 490/635 nm as excitation/emission wavelengths for propidium iodide respectively. The cells were detected in three separate groups: live cells with green fluorescence, dying cells with both red and green fluorescence and dead cells with red fluorescence. Gates were set on the dot plot FSC and SSC during analysis. The percentage cells with positive fluorescence for all the parameters were calculated from the respective histograms or two-parameter fluorescence dot plots. Each experiment was repeated two times in triplicate ($n = 3$) and results expressed as percentage cells with positive red fluorescence relative to the negative control (cells treated with isopropyl-ethanol).

Results and discussion

Clear differences were found between the effects of different inhibitory compounds when various combinations and concentrations were used. The results of plate counts and flow cytometry were in agreement with one another with small deviations, but there was a strong trend correlation with decrease in combined inhibitory effects.

Individual inhibitory effect on the growth of S. cerevisiae

The MIC of individual inhibitory concentrations has been determined by Fosso-Kankeu *et al.*[1] to be 2 g/l for vanillin and 4 g/l for acetic acid. Further experimentation led to MIC of 2 g/l for formic acid. In glucose-containing media with pH higher than 4.5, yeast cells can activate an adaptive response to the environment and resume growth after a longer lag phase (Figure 1 *a* and *b*).

Synergistic inhibitory effect on the growth of S. cerevisiae

In order to simulate pretreatment concentrations, the average MIC concentration values were determined for experimentation. The concentrations considered were 0.25 g/l for acetic acid, 0.15 g/l formic acid and 0.02 g/l for vanillin. In all cases it was found that an increase in concentration caused stress to the yeast, as seen by reduced growth rates and increased lag times.

Effect of the weak acid combination

The combination of acetic acid and formic acid produced a similar growth trend (Figure 1 *b*), as the individual inhibitors are both classified as weak acids and therefore have similar inhibitory mechanisms on the yeast cells. The short lag phase of 4 h indicates that the yeast cells adapt to the spiked broth and develop normally for the remaining fermentation time. The inhibitors are introduced during the lag phase to obtain maximum contact between them and the yeast cells. It is also observed that the low concentration of weak acids can be beneficial for fermentation, as it acts as a catalyst that delivers the necessary energy required for ATP formation. However, very high acid concentrations will lead to a pH decrease beyond the cell capacity, which will lead to cell disordered fermentation and ultimately cell lysis. This can be clarified by the weak acid theory, where the undissociated molecules can freely diffuse through the cell membrane and dissociate in the cell cytoplasm due to a greater intracellular pH, acidifying the cytoplasm. However, the cell will attempt to uphold the internal pH levels by homeostasis through excretion of the excess H^+ ions by translocating the plasma membrane that commonly utilizes ATP for activity. The interference of weak acids leads to higher ATP requirements for cell preservation and activity. Therefore, the ATP is channelled towards pH maintenance rather than cell growth[30].

Effect of acetic acid and vanillin combination

The effect of acetic acid and vanillin is more pronounced than the individual effects of the inhibitors as they exhibit

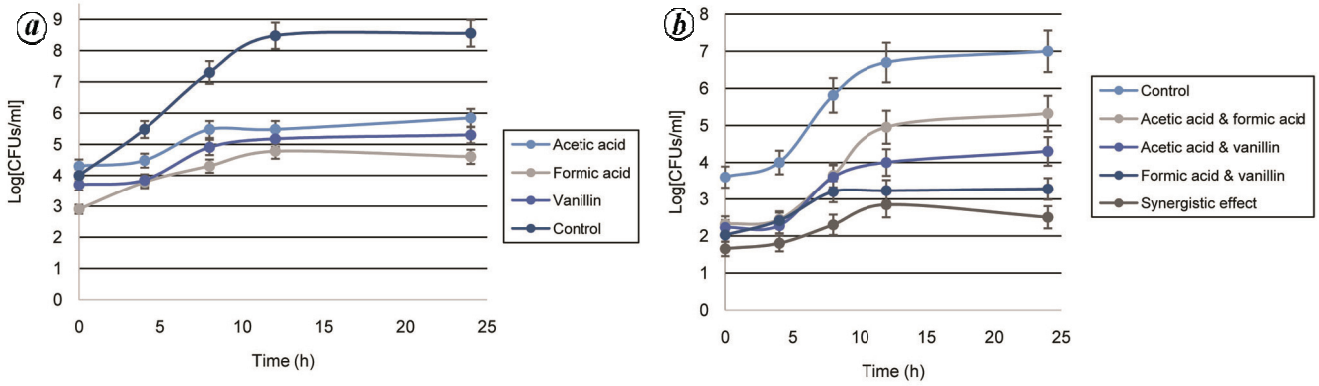

Figure 1. Effect of inhibitors on the growth of *Saccharomyces cerevisiae* expresses as log (CFUs)/ml over a period of 24 h. ***a***, Individual inhibitors at MIC and. Acetic acid at 4 g/l, formic acid at 2 g/l and vanillin at 2 g/l. ***b***, The combination of various inhibitors at MIC concentrations. Results are presented as the mean of 4 duplicate runs.

slower cell growth from 8 h onwards (Figure 1 *b*). This is due to strong inhibitory effects of vanillin, even at low concentrations, where the compound destroys cell membranes which leads to integrity loss and decrease in fermentation capabilities. Furthermore, the weak acid causes delay in microbial growth and the vanillin exhibits a strong toxic effect early on during fermentation[31].

Effect of formic acid and vanillin combination

Combination of formic acid and vanillin has shown to increase the inhibitory potential of two chemical compounds, resulting in a strong synergistic effect on cell growth as well as cell viability (Figure 1 *b*). From 8 h onwards, viability also decreases as indicated by the colonies forming on nutrient agar. This lethal effect is ascribed to formic acid and vanillin being classified as some of the most lethal inhibitors that are released during the pretreatment processes[32]. According to Maiorella *et al.*[33], the toxic effect of formic acid is due to chemical interference with cell maintenance functions that disrupts the normal cell metabolic processes.

Effect of three inhibitors in combination

The synergistic effect of all three inhibitors at the pretreatment MIC exhibits slow growth from 12 h onwards grounded on OD values; however, when the cell viability was tested, there was no evident growth from 12 h up to 48 h. This is due to the synergistic effect of the three inhibitors in combination, as well as the formation of secondary inhibitors during fermentation. As the amount of weak acids increases and decreases pH, the cytoplasm is acidified by dissociation into acid anions and protons. Furthermore, lethal concentrations induce reactive oxygen species (ROS) accumulation in the cells, ultimately leading to cell lysis[34].

During destruction of hemicellulose, weak acids are released and during breakdown of lignin, phenolic compounds are released. The individual MIC of acetic acid is determined as 4 g/l, while similar values are obtained for the more toxic formic acid and vanillin at 2 g/l. It is also observed that at higher concentrations, there is a decrease in cell viability due to cell damage and decrease in activity.

In the presence of both weak acids, there is a long lag phase up to 24 h, followed by considerable amount of ethanol formation. This is due to the similar inhibitory mechanism of weak acids, rather than dual effects, resulting in 88.77 ± 3.73% ethanol yield. All the glucose in growth medium is consumed in both fermentation as well as in the presence of combined weak acids, even though the rate of consumption is lower for the control. This is credited to the fact that acetic acid may act as an initial catalyst for fermentation and formation of secondary inhibitor earlier in the process.

The combination of acetic acid and vanillin indicates that the initial ethanol formation is rather significant, but it becomes stagnant after 36 h with almost no further fermentation taking place. This is coupled with slow glucose consumption with only a slight decrease and significant amounts available for further fermentation. This is ascribed to the toxic effect of vanillin as it degrades the cell structure resulting in a decrease in ethanol yield to 75.06 ± 1.78%. The dual effect of formic acid and vanillin is strong, it takes up to 24 h for the cells to become accustomed to the inhibited environment and only produces ethanol up to 68.53 ± 1.64%. The synergistic effect of all three inhibitors causes a strong effect on fermentation capabilities, where only 58.47% ethanol is formed after 48 h of fermentation. The low ethanol production potential is due to the powerful effect of all three inhibitors acting simultaneously on the yeast cells.

Secondary inhibitor formation

During the degradation of glucose for fermentation, there is a clear formation of acetic acid as well as lactic acid. However, in the presence of primary inhibitors, the

concentration of secondary inhibitors decreased. This is ascribed to reduced cell activity in the presence of higher initial inhibitory concentrations[1] and synergistic combinations.

Acetic acid

Acetic acid is a cell stress and death-inducing agent produced during fermentation of yeast. At industrial level, the presence of acetic acid can have a negative effect on fermentation, as it can affect product quality and fermentation capabilities. At low pH values, the increased antimicrobial action of acetic acid leads to cytoplasmic acidification, inhibiting the metabolic process.

It is perceived that secondary acetic acid formation (Figure 2) produces a significant effect on *S. cerevisiae* by preventing adequate metabolic activities for proper fermentation. The long lag phase is due to the adaption of yeast to the inhibited environment; however, the final acetic acid concentration is lower compared to the reference fermentation. It is clear that the lack of fermentation at higher concentrations results in lower secondary inhibitor formation as the amount of acetic acid drastically decreases at lower cell growth rates. There is only a slight increase from 1 to 2 g/l in the presence of all three pretreatment inhibitors (Figure 2), where the combined presence of acetic acid and vanillin produces a significant increase from 0.5 to 6.2 g/l (Figure 2). The combined effect of formic acid and vanillin at MIC level is similar, with an increase in 0.3 and 0.5 g/l respectively (Figure 2). The combination of two weak acids leads to substantial reduction compared to the control, as the cells are damaged by the same mechanism from acid combination. In the presence of acetic acid in the medium, the pH decreases, leading to increased inhibitory activity and reduced cell viability which cause a delay in glucose usage and ethanol production. This is also supported by Rolland *et al.*[35] who showed that *S. cerevisiae* cells grown on it are not able to metabolize glucose in the presence of acetic acid.

Lactic acid

The formation of lactic acid is significant in the control fermentation with a final concentration of 2.32 g/l (Figure 3). The concentration decreases periodically with the addition of inhibitors, yielding a final concentration of 1.82 g/l (Figure 3). Acetic acid and vanillin as well as formic acid and vanillin yield lactic acid concentration of 1.65 g/l. With an increase in synergistic concentrations, the production of lactic acid as secondary inhibitor will halt completely (Figure 3). As lactic acid is formed and pH lowered, the microorganism is at risk to be antagonized and stressed, leading to a decrease in cell metabolic activity. The effect of lactic acid appears to be different from that of acetic acid; the production of lactic acid halts the production of ethanol and therefore glucose consumption is stagnant. Large-scale effects of lactic acid are relevant as it is easily produced as a by-product from fermentation and the occurrence of lactic acid bacteria at industrial scale could result in the competing for nutrients.

The specific growth rates of yeast follow an exponential decrease in growth as the lactic acid concentration increases, proving that the inhibitory effect of secondary inhibitors is also significant and highly synergistic. It is also noted that as ethanol production increases, ethanol formation rate decreases, indicating that it acts as an additional inhibitor to the batch. Table 2 shows pH values of the fermenting product with the formation of acetic acid and lactic acid.

Productivity and ethanol formation

The formation of bioethanol in the presence of more than one toxic compound is clearly inhibited as the synergistic effect leads to multiple inhibitory mechanisms (Figure 4). The decrease of ethanol after 36 h is due to the lack of fermentation capabilities as a result of decrease in cell activity as it continues from stationary phase to the death phase. The volumetric productivity of each of the inhibitors has been examined and a clear reduction is visible

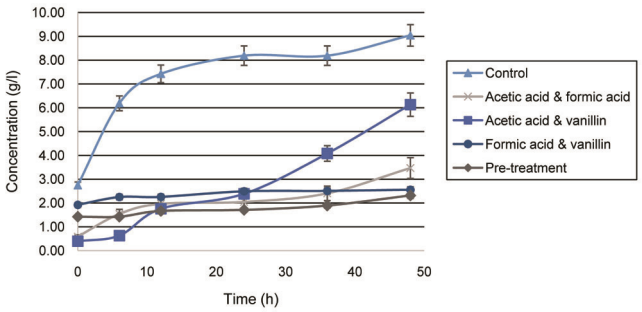

Figure 2. Effect of the presence of inhibitors on the concentration of secondary acetic acid. Results are presented as mean ± standard deviation (n = 3).

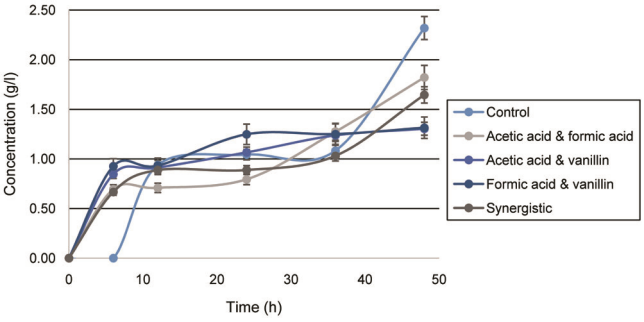

Figure 3. Variation of lactic acid content of fermentation broth in the presence of inhibitory combinations. Results are presented as mean ± standard deviation (n = 3).

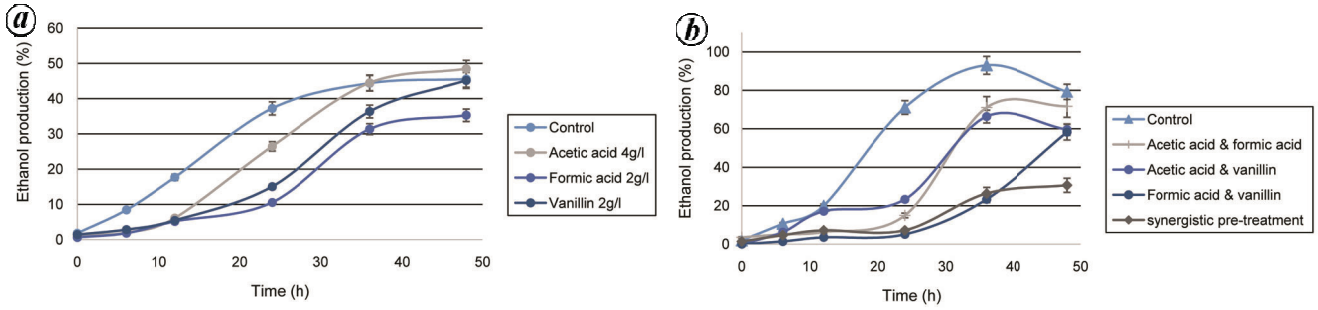

Figure 4. Ethanol production in the presence of (***a***) individual inhibitors at the MIC of acetic acid, formic acid and vanillin, and in the presence of (***b***) combined inhibitors in the system. The data are presented as mean values with a standard deviation from duplicate experiments over a period of 48 h of fermentation.

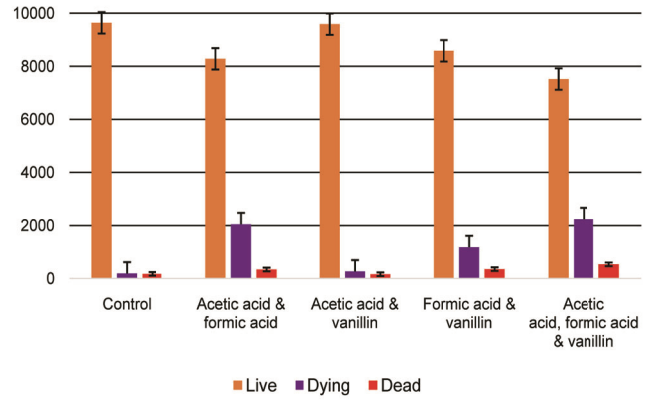

Figure 5. Flow cytometry results for cell viability after 24 h of fermentation represented by the amount per 10,000 events.

Table 2. pH values at various inhibitor combinations, $n = 4$

Inhibitory combination	pH
Acetic acid	4.13 ± 0.15
Formic acid	4.10 ± 0.10
Vanillin	5.64 ± 0.09
Acetic acid and formic acid	3.41 ± 0.06
Acetic acid and vanillin	3.97 ± 0.11
Formic acid and vanillin	3.75 ± 0.18
Acetic acid, formic acid and vanillin	2.81 ± 0.12

from productivity (2.12 ± 0.23 g/l) obtained with the control (Table 3). The sugar present in the final product indicates that incomplete fermentation has taken place and the inhibitors clearly affect cell viability to sufficiently ferment glucose to produce ethanol.

Cell viability studies

According to the plots (figure not shown) of cell viability studies from flow cytometry analysis. The control had a TCC of 10,000 events with 96.37% live cells, 1.77% dead cells and 1.91% dying cells after a period of 48 h of incubation.

The following combinations were used at pretreatment MIC to mimic authentic inhibitory possibilities.

1. *Acetic acid and formic acid*

Results of cell viability obtained in the presence of combined weak acids help delineate three categories of cells, namely living cells (82.83%), dead cells and dying cells; shows that some cells are still active after 48 h of incubation. However an increase in dying cells (20.48%) indicates a decrease of cell activity and viability due to weak acid accumulation damaging the cell wall and altering the intracellular pH to reduce activity. The presence of dead cells (3.42%) indicates that the synergistic effect of weak acids leads to more toxic effects and decrease in cell viability. Figure 5 shows a clear shift.

2. *Acetic acid and vanillin*

Acetic acid coupled with vanillin (Figure 5) results in low values for dead cells (1.63%); as acetic acid initially acts as a fermentation catalyst that can increase cell activity prior to cell lysis. The damaged cells that will ultimately die (2.67%) are also more significant compared to those of the control. However, the lower value of weak acids is due to the fact that vanillin acts on resilient cells capable of tolerating inhibition. Thus the damaging effect would be slower, but ultimately inhibition will occur as DNA synthesis of cells is compromised and no replication can occur. This effect is strongly exhibited by plate counts, implying that results into the transformation of *S. cerevisiae* mostly to non-reproduciblebut viable cells; such a type of inhibition is considered as fungistatic.

3. *Formic acid and vanillin*

The combination of formic acid and vanillin results in a trend, similar to that of weak acid combination. This is ascribed to the fact that a larger number of cells are injured and the reduced activity results in lower ethanol

Table 3. Summary of productivity results. The results are presented as mean and standard deviation from two duplicate analysis

Process	Final sugar concentration (%)	Ethanol yield (%)	Productivity (g/l.h)
Control	0.00	96.85 ± 3.79	2.12 ± 0.23
Acetic acid and formic acid	0.00	88.77 ± 3.73	1.79 ± 0.15
Acetic acid and vanillin	59.60 ± 1.44	75.06 ± 1.78	1.51 ± 0.0.24
Formic acid and vanillin	71.96 ± 1.215	68.53 ± 1.64	1.38 ± 0.06
Pre-treatment synergistic combination	53.14 ± 2.01	41.53 ± 3.40	0.84 ± 0.11

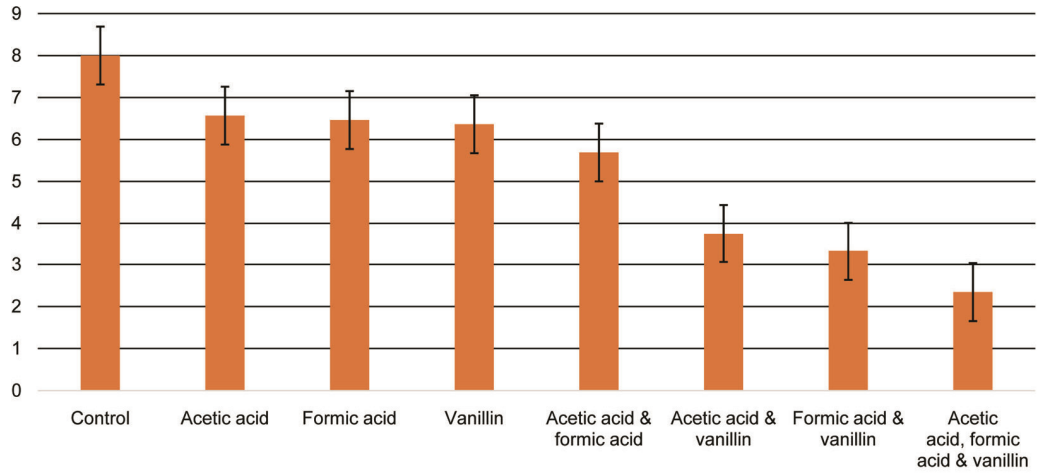

Figure 6. The amount of viable (live) cells, expressed as log (CFUs/ml) as determined by plate counts. Results are presented as the mean and the standard deviation of double triplicate analysis

production. The live cells, at 86.29%, are accompanied by 12.12% dying cells and 3.57% dead cells. The presence of vanillin in the batch leads to yeast lysis, where constituents are broken down as formic acid damages the rigid cell wall responsible for the shape of the cell.

4. *Synergistic effect*

The effect of the three inhibitors follows a trend similar to that of the plate counts (Figures 5 and 6) as cell viability is found to decrease with a noteworthy amount of cells classified as dying or injured, thus allowing no further fermentation or cell growth. This correlates with the plate counts as cell growth decreases from 36 h onwards in the presence of all three inhibitors. The number of dying cells also reduces and an increase in dead cells is observed as the inhibitors influence cell viability early during fermentation. Figure 6 shows the final cell counts in the presence of inhibitors.

Conclusion

Alcoholic fermentation by *S. cerevisiae*, a robust yeast strain, is significantly affected by the presence of pretreatment process products. The results indicate that the degree of inhibition of toxic compounds is primarily dependent on the nature, classification and concentration of the inhibitor compound as well as the growth phase of the microorganism. Furthermore, the YPD broth offers some protection against external stresses as it is rich in yeast extract and encourages yeast growth.

For the three inhibitors used, vanillin and formic acid display the strongest inhibitory effects, whereas acetic acid acts as a catalyst for cell growth at low concentrations. Single presence of inhibitors also indicates that cells have the capability to metabolize the inhibitor and overcome the inhibitory effect. However, the cumulative effects of inhibitors enhance incapacitation of microorganisms to overcome the toxic effect, lowering cell activity and bioethanol productivity.

Furthermore, flow cytometric sorting with a combination of PI and SYTO-9 proves to be an effective method for the rapid determination of viable cells and a correlation method to plate counts. Even though the number of cells differs for the two methods, flow cytometry provides information on damaged cells that the plate counts does not include, as dying cells do not reproduce on the nutrient medium. This, therefore provides us information about the mechanism of inhibition which is found to be fungistatic.

1. Fosso-Kankeu, E., Marx, S. and Meyer, A., Simulated inhibitory effects of typical byproducts of biomass pretreatment process on

the viability of *Saccharomyces cerevisiae* and bioethanol production yield. *Afr. J. Biotechnol.*, 2015, **14**, 2383–2394.
2. Chen, H. and Fu, X., Industrial technologies for bioethanol production from lignocellulosic biomass. *Renew. Sustain. Energ. Rev.*, 2016, **57**, 468–478.
3. Sarkar, N., Ghosh, S. K., Bannerjee, S. and Aikat, K., Bioethanol production from agricultural wastes: an overview. *Renew. Energ.*, 2011, **37**, 19–27.
4. Ahmia, A. C., Danane, F., Bessah, R. and Boumesbah, I., Raw material for biodiesel production. Volarization of used edible oil. *Rev. Energ. Renou.,* 2014, **17**, 335–343.
5. Samuel, T. M., Bioethanol fermentation of corn cob using immobilized yeast cells. Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, South Africa, 2001.
6. Sheenan, J., Aden, A., Paustian, K., Killian, K., Brenner, J., Walsh, M. and Nelson, R., Energy and environmental aspects of using corn stover for fuel ethanol. *J. Ind. Ecol*., 2004, **7**, 117–146.
7. Licht, F. O., World ethanol markets: the outlook to 2015. Tunbridge Wells, UK, 2006.
8. Chandel, A. K., Chan, E. S., Rudravaram, R., Narasu, L. M., Rao, L. V. and Ravindra, P., Economics and environmental impact of bioethanol production technology: an appraisal. *Biotechnol. Mol. Biol. Rev.*, 2007, **2**(1), 14–32.
9. Almeida, J. R. M., Modig, T., Petersson, A., Hahn-Hagerdal, B., Liden, G. and Gorwa-Grauslund, M. F., Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J. Chem. Technol. Biotechnol*., 2007, **82**, 340–349.
10. Tye, Y. Y., Lee, K. T., Abdullah, W. N. W. and Leh, C. P., The world availability of non-wood lignocellulosic biomass for the production of cellulosic ethanol and potential pretreatments of enzymatic saccharification. *Renew. Sust. Energ. Rev.*, 2016, **60**, 155–172.
11. Naik, S. N., Goud, V. V., Rout, P. K. and Dalai, A. K., Production of first and second-generation biofuels: a comprehensive review. *Renew. Sust. Energ. Rev.*, 2010, **14**, 578–597.
12. Wallace-Salinas, V., Improving stress tolerance in industrial *Saccharomyces cerevisiae* strains for ethanol production from lignocellulosic biomass. Doctoral thesis, Faculty of Engineering, Lund University, Sweden, 2014.
13. Dias, M. O. S., Modesto, M., Ensinas, A. V., Nebra, S. A., Filho, R. M. and Rossel, C. E. V., Improving bioethanol production form sugarcane: evaluation of distillation, thermal integration and cogeneration systems. *Energy*, 2010, **36**(6), 1–13.
14. Talebnia, F., Karakashev, D. and Angelidaki, I., Production of bioethanol from wheat straw: an overview on pretreatment hydrolysis and fermentation. *Bioresour. Technol.*, 2010, **101**, 4744–4753.
15. Kumar, P., Barrett, D., Delwiche, M. J. and Stroeve, P., Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind. Eng. Chem. Res*., 2009, **48**, 3713–3729.
16. Gray, K. A., Zhao, L. and Emptage, M., *Bioethanol. Biocatal. Biotransform.*, 2006, **10**, 141–146.
17. Olsson, L. and Hahn-Hagerdal, B., Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb. Technol.*, 1996, **18**, 312–331.
18. Zheng, Y., Pan, Z. and Zhang, R., Overview of biomass pretreatment for cellulosic ethanol production. *Int. J. Agric. Biol. Eng.*, 2009, **2**, 51–68.
19. Chandel, A. K., Da Silva, S. S. and Singh, O. V., Detoxification of lignocellulosic hydrolysates for improved bioethanol production. In *Biofuel Production for Improved Bioethanol Production*, In Tech, 2011, vol. 10, pp. 225–246.
20. Klinke, H. B., Thomsen, A. B. and Ahring, B. K., Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl. Microbiol. Biotechnol.*, 2004, **66**, 10–26.
21. Sanchez, O. J. and Cardona, C. A., Trends in biotechnological production of fuel ethanol. *Bioresour. Technol.*, 2008, **99**, 5270–5295.
22. Kuloyo, O. O., Ethanol production by yeast fermentation of an *Optuntia ficusindica* biomass hydrolysate. Master's dissertation. Faculty of Natural and Agricultural Sciences, University of the Free State, Bloemfontein, South Africa, 2010.
23. Jayakody, L. N., Hayashi, N. and Kitagaki, H., Molecular mechanisms for detoxification of major aldehyde inhibitors for production of bioethanol by *Saccharomyces cerevisiae* from hot-compressed water-treated lignocellulose. In *Stress Biology of Yeasts and Fungi: Applications for Industrial Brewing and Fermentation* (eds Takagi, H. and Kitagaki, H.), Springer, 2015.
24. Huang, H., Guo, X., Li, D, Liu, M., Wu, J. and Ren, R., Identification of crucial yeast inhibitors in bio-ethanol and improvement of fermentation at high pH and high total solids. *Bioresour. Technol.*, 2011, **102**, 7486–7493.
25. Fu, S., Hu, J. and Liu, H., Inhibitory effects of biomass degradation products on ethanol fermentation and a strategy to overcome them. *BioResources*, 2014, **9**, 4323–4335.
26. Bauer, F. F. and Pretorius, I. S., Yeast stress response and fermentation efficiency: how to survive the making of wine. *S. Afr. J. Enol. Viticult.*, 2000, **21**, 27–51.
27. Kubota, S. *et al.*, Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol. *Biosci. Biotechnol. Biochem.*, 2014, **68**, 968–972.
28. Wallace-Salinas, V. and Gorwa-Grauslund, M. F., Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. *Biotechnol. Biofuels*, 2013, **6**, 1–9.
29. Andrews, J. M., Determination of minimum inhibitory concentrations. *J. Antimicrob. Chem.*, 2001, **48**, 5–16.
30. Narendranath, N. V., Thomas, K. C. and Ingledew, W. M., Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. *J. Indust. Microbiol. Biotechnol.*, 2001, **26**(3), 171–177.
31. Piper, P., Calderon, C. O., Hatzixanthis, K. and Mollapour, M., Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology*, 2001, **147**, 2635–2642.
32. Westman, J. O., Ethanol production from lignocellulose using high local cell density cultures. Investigations of flocculating and encapsulated *Saccharomyces cerevisiae*, Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden, 2014.
33. Maiorella, B., Blanch, H. W. and Wilke, C. R., By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.*, 1983, **25**, 103–121.
34. Guaragnella, N., Antonacci, L., Passarella, S., Marra, E. and Giannattasio, S., Hydrogen peroxide and superoxide anion production during acetic acid-induced yeast programmed cell death. *Folia Microbiol.*, 2007, **7**, 237–240.
35. Rolland, F., Winderickx, J. and Thevelein, J. M., Glucose-sensing and signalling mechanisms in yeast. *FEMS Yeast Res.*, 2002, **2**, 183–201.

ACKNOWLEDGEMENT. We thank Mr G. van Rensburg, North-West University, Potchefstroom for assisting in the laboratory.

Received 20 August 2017; accepted 9 May 2018

doi: 10.18520/cs/v115/i6/1124-1132