Susceptibility of *Saccharomyces Cerevisiae* to Inhibitors and Impact on Bioethanol Production Yield

Elvis Fosso-Kankeu, Sanette Marx, and Anton Meyer

Abstract**—**There have been increasing concerns in developing countries over the competition between food and energy resulting from the production of bioethanol from edible biomass. Second generation lignocellulose feedstock is an attractive alternative, as bioethanol can be produced from non-edible materials. However, the pretreatment required for hydrolysis of lignocellulose into pentose and hexose sugars often results in the production of inhibitors likely to impede the activity of Saccharomyces cerevisiae during bioethanol production. This study aims to investigate the comparative inhibitory effects of acetic acid and vanillin on the viability of S. cerevisiae and the production yield of bioethanol. The fermentation broth was spiked with different concentrations of vanillin or acetic acid were and the bioethanol concentration were monitored over time and correlated with cell viability. The results showed that although S. cerevisiae was mostly susceptible by vanillin compared to acetic acid, the inhibitory effect of acetic acid on S cerevisiae had a more severe influence on the final bioethanol yield after 12 h (42.8% reduction) than vanillin (33.3%). The latter was ascribed to the simultaneous production of weak acids during the fermentation process. The viability test has shown that S. cerevisiae can adapt to the presence of inhibitors over 12 h and at lower concentrations (2 g/l vanillin and 4 g/l acetic acid) the effect of inhibitors on S. cerevisiae and ethanol production yield can be overcome by the adaptation of the yeast.

Keywords**—**Bioethanol production, *S. cerevisiae*, inhibition, acetic acid, vanillin, cell viability.

I. Introduction

GLOBALLY bioethanol technology is rapidly expanding due to progressive depletion of non-renewable fuel reserves and the potential of biofuels to reduce the emission of polluting gasses to the atmosphere [1]. Bioethanol is also sustainable, reasonable cost effective and easy to add into fuel distribution systems [2]. Currently first and second generation feedstock are available for the production of bioethanol. The latter contains lignocellulose materials which are the most abundant material on earth and is comprised of hemicellulose, lignin and cellulose [3].

First generation feedstock includes food crops which receive criticism as bio-diverse regions are destroyed to produce land to grow crops. Large scale bioethanol production also has a negative impact on the cost of food crops [4].

Second generation feedstock consists of lignocellulose materials which include industrial, municipal, agricultural and forestry residues [5]. These materials are inexpensive and abundant as they consist of the non-eatable parts of plants. Currently the production of second generation bioethanol is an expensive process which does not make it a viable commercial process. Advantages are that food crops are not affected. If second generation biofuels can be commercialized it can become a cost effective process when compared to first generation bioethanol [4].

However, the use of second generation feedstock for bioethanol production requires a preliminary pre-treatment step to liberate digestible sugar monomers; the problem with the pre-treatment is the formation of inhibitors which inhibit the growth of fermenting organisms. The yeast *Saccharomyces cerevisiae* is commonly used as ethanol fermenting organism, because it has a high tolerance to these inhibitors in comparison to other fermentation organisms [5]. Inhibitory effects are escalated when up-scaling fermentation processes for large scale production and it is therefore vital that its impact on the yeast and expected yields are well understood.

In this paper, the inhibitory effect of acetic acid and vanillin on *S cerevisiae* viability and ethanol yield is investigated. The inhibitory effect on S cerevisiae is then correlated with the reduction in ethanol yield.

II. Methodology

A. Chemicals

Acetic acid (weak acid) (95.5%) and vanillin (a phenol) (≥99%) which act as the main inhibitors during bioethanol production from lignocellulose biomass where purchased from Associated Chemical Enterprises (ACE) and MERCK respectively. Chemicals for culture such as agar media, peptone and yeast extract where purchased from Sigma Aldrich chemical company. Glucose and Agar powder were obtained from ACE chemical company. Other chemicals used

Elvis Fosso-Kankeu is with the School of Chemical and Minerals Engineering of the North West University, Bult area-Potchefstroom-South Africa (Tel:+2718 299 1659; fax:+2718 299 1535; e-mail:24838616@nwu.ac.za).

Sanette Marx is with the School of Chemical and Minerals Engineering of the North West University, Bult area-Potchefstroom-South Africa

Anton Meyer is with the School of Chemical and Minerals Engineering of the North West University, Bult area-Potchefstroom-South Africa

included ethanol (99.9%) (Sigma Aldrich chemical company) and sodium hydroxide (NaOH) (≥98%) (Rochelle chemicals). All chemicals were used as received from the supplier without purification or modification.

B. Microorganisms and media

S. cerevisiae (Anchor yeast) used in this study was obtained from a local supermarket. YPD broth media contained 10 g.L^{-1}, 20 g.L^{-1} and 10 g.L^{-1} of yeast extract, peptone and dextrose in de-ionized water. Agar medium contained 10 g.L^{-1} yeast extract, 20 g.L^{-1} peptone, 10 g.L^{-1} dextrose and 15 g.L^{-1} agar in de-ionized water. The pH was adjusted to 6.5 using 0.1 M NaOH. Sterilization of the broth and agar media where done at 121°C for 20°C.

The broth was inoculated with 0.05 g.L^{-1} dried yeast cells and allowed to grow for 20 hours at a shaking speed of 120 rpm at 30°C.

C. Determination of minimum inhibitory concentration

Yeast grown aerobically for 24 hours in YPD broth (50 mL) was inoculated in broth spiked with different concentrations of acetic acid and vanillin (2, 4, 6 and 8 gram per liter of broth). All experiments were conducted in Erlenmeyer flasks and samples were analyzed at set time intervals (3, 6, 8, 12 and 24 hours) to determine the minimum inhibitory concentration.

D. Determination of the effect of inhibitors on bioethanol yield

An aliquot of 4 ml of yeast culture was added to glucose (46 mL, 20 g.L^{-1}) in 100 mL GL 45 laboratory glass bottles with blue PP screw caps and pouring rings. Adequate volume of acetic acid and vanillin was added to the glucose mixtures to make a final concentration of 4 g.L^{-1} and 2 g.L^{-1}, respectively. Samples were analysed at set time intervals over a period of 48 hours.

E. Quantification of yeast cells

The total biomass was measured at a wavelength of 600 nm using a spectrophotometer (Shimadzu). The amount of viable yeast cells was determined using the serial dilution methods with sterilized de-ionized water. Diluted cells where evenly distributed across agar plates and incubated at 30°C for 48 hours. The number of colonies was expressed as colony forming units (CFU's).

III. Results And Discussion

Vanillin and acetic acid belong to the groups of phenolic compounds and weak acid respectively and are generated during pre-treatment and hydrolysis of second generation feedstock used for the production of bioethanol. Vanillin is a phenolic compound derived from lignin breakdown and acetic acid is a derivative from hemicellulose breakdown during pre-treatment. Although there are various phenols and acids formed during pre-treatment, vanillin and acetic acid were chosen for investigation in this study because they are dominants. Few studies have been previously carried out to determine the inhibitory effect of these compounds. The particularity of this study is to correlate the inhibitory effect to the viability of the yeast.

A. Effect of inhibitors concentration on the growth of S. cerevisiae over time

Effect of vanillin

Figures 1a and b show that the inhibitory effect of vanillin on the growth of *S. cerevisiae* becomes more severe with increasing concentrations of vanillin in the broth. The minimum inhibitory concentration was found to be 2 g.L^{-1} as expressed by both the OD and the colony count. The colony count showed that the cells present in the flask containing 8 g.L^{-1} of vanillin were not viable after 8 h incubation, implying that there is a total microbicidal effect under such conditions.

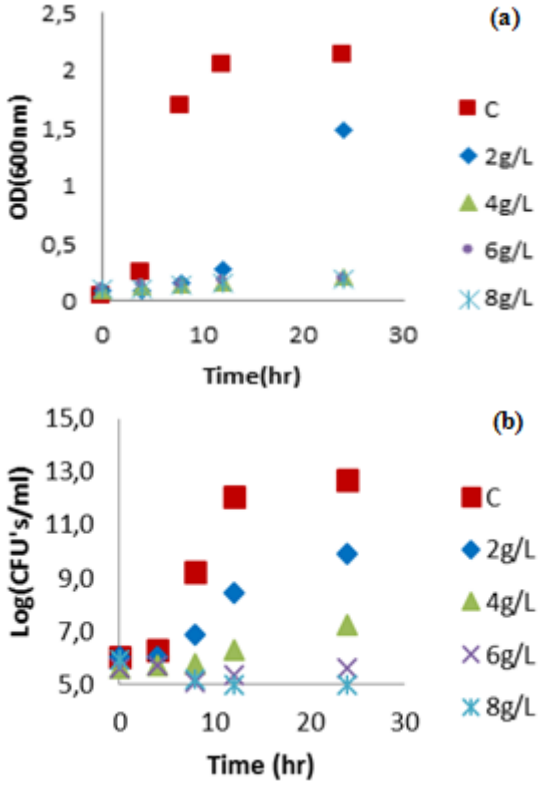

Fig. 1 Inhibition of *S. cerevisiae* growth in presence of various concentrations of vanillin: (a) expression of growth by absorbance; (b) expression of growth by colonies count

Effect of acetic acid

Data plotted in Figures 2a and b clearly indicate that there was inhibition of *S. cerevisiae* in the presence of acetic acid which increased with an increase in acetic acid concentration and time. The minimum inhibitory concentration was found to be 2 g.L^{-1}. The trend of the OD plot does not totally correlate with the trend of colony count as shown by the behaviour of the yeast at 6 g.L^{-1} of acetic acid. This implies that at a concentration of 6 g.L^{-1}, the inhibitory effect of acetic acid may lead to endogenous metabolism between 6

and 8 h resulting in the reduction of cells biomass.

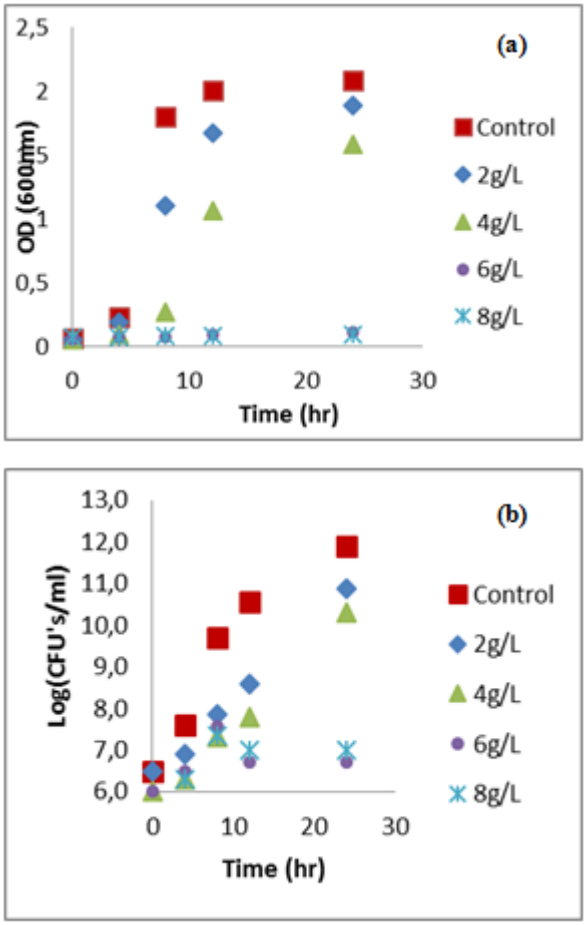

Fig. 2 Inhibition of *S. cerevisiae* growth in presence of various concentrations of acetic acid: (a) expression of growth by absorbance; (b) expression of growth by colonies count

Comparing the effects of the two inhibitors, it can be observed that in general vanillin has a more pronounced inhibitory effect than acetic acid. For the same minimum inhibitory concentration (2 $g.L^{-1}$), vanillin caused a larger reduction of growth than acetic acid and at 8 $g.L^{-1}$, vanillin had a lethal effect while acetic acid only had a static effect. It has been reported [5, 6] that phenolic compounds are stronger inhibitors than acids because of their aldehyde and ketone groups. It is suggested that phenolic compounds act on biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices; while the inhibitory effect of acetic acids has been ascribed to uncoupling and intracellular anion accumulation [7].

B. Impact of inhibition on bioethanol yield

Concentrations of 2 $g.L^{-1}$ of vanillin and 4 $g.L^{-1}$ of acetic acid were chosen to determine the effect of these inhibitors on the production of ethanol by *S. cerevisiae*. It is important to use lower concentrations to mimic the amount produced during pretreatment of lignocellulose.

Impact of vanillin on bioethanol production

The utilization rate of glucose was slower in the presence of vanillin during the first 36 h of fermentation (see Figure 3). The final ethanol concentration was however higher in the presence of vanillin compared to the control sample where no vanillin was present. The latter was confirmed only by the cell count and not the OD values, implying that the cells may have lost weight but remain more active after longer exposure to vanillin.

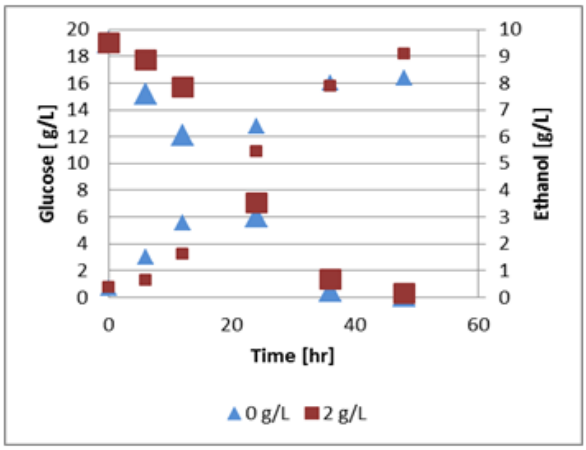

Fig. 3 Glucose consumption and ethanol production in the presence of vanillin. Large symbols (glucose), small symbols (ethanol)

Impact of acetic acid on bioethanol production

Figure 4 shows that there was a decrease in glucose concentration as the ethanol was formed; clearly indicating that ethanol production results from the use of glucose by *S. cerevisiae*, but glucose was utilized at a slower rate in the presence of acetic acid compared to the control. As was the case with vanillin, a higher final ethanol concentration was obtained in the presence of acetic acid compared to the control. This can be ascribed to the adaptation of *S. cerevisiae*, but the patterns of OD values and cell count (Figures not shown) did not corroborate what was initially ascribed to the decrease of cells mass.

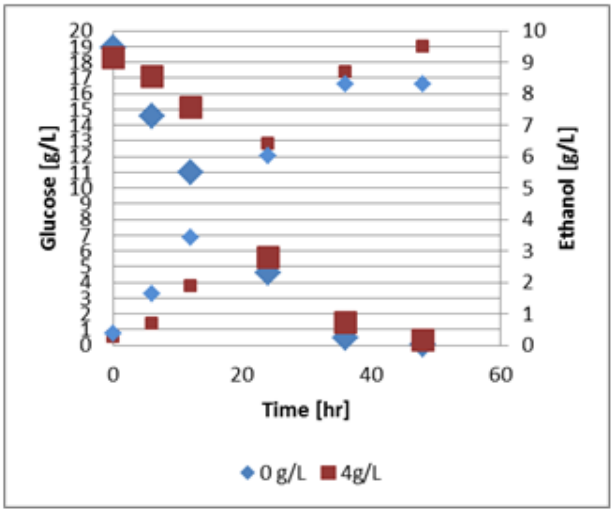

Fig. 4 Glucose consumption and ethanol production in the presence of acetic acid: Large symbols (glucose), small symbols (ethanol)

Optical density and cell count (data not shown) indicate an extended lag time and more sluggish exponential growth phase in the presence of inhibitor.

IV. Conclusion

In this study, the behaviour of *S. cerevisiae* in the presence of inhibitors is enlighten by the viability test, showing that in the process of adaptation the cell biomass is reduced, but the yeast continues to grow and produce ethanol. Vanillin is found to be more toxic to the fermenting organism *S. cerevisiae*. The potency of vanillin has also been reported by Chandel *et al.* [3] who ascribed this to the smaller molecular weight of vanillin. It was observed that the inhibitors can reduce the bioethanol yield only in the first 12 h of fermentation, and may not be a serious problem at the concentrations considered in this study if the fermentation process takes longer than 24 h. For shorter fermentation times however, the inhibition may be overcome by the use of detoxification methods to avoid a reduction in ethanol yield.

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The corresponding author is currently a Senior Lecturer in the School of Chemical and Minerals Engineering at the North-West University (Potchefstroom). He has published a couple of articles in accredited journals including: "The health implication of relationships between bacterial endotoxin, cyanobacteria, coliforms and water stored in domestic containers of rural households in South Africa. Journal of Water and Health", Vol 8 (4), 2010, pp 601-610; "A comprehensive study of physical and physiological parameters that affect biosorption of metal pollutants from aqueous solutions". Journal of Physics and Chemistry of the Earth, Vol 35, 2010, pp 672-678 and "Prediction of metal-adsorption behaviour in the remediation of water contamination using indigenous microorganisms". Journal of Environmental Management, 92 (10), pp 2786-2793. Examples of book chapters published include: Metal derived complexes for improved fight against bacteria. In: Mishra A.K., Tiwari A. and Mishra S.B. (Eds). Smart Biomolecules in Medicine. VBRI Press, India. ISBN 978-81-920068-01, 2010, pp. 199-226 and "High-technology therapy using biomolecules or synthetic compounds for HIV inhibition". In: Mishra A.K (Eds). Nanomedecine for Drug Delivery and Therapeutics. Wiley, Scrivener Publisher. ISBN: 978-1-1184-1409-5. 2013, pp 3-38. His main research focus has been on the monitoring of water quality and bioremediation of polluted water, minerals processing and, biofuel.

Dr Elvis Fosso-Kankeu has been the recipient of several merit awards.