Full Length Research Paper

Simulated inhibitory effects of typical byproducts of biomass pretreatment process on the viability of *Saccharomyces cerevisiae* and bioethanol production yield

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The abundance of second generation feedstock reinforces the consideration of biofuel over fossil fuel, as bioethanol can be produced from lignocellulosic materials. However, the pretreatment required for oxidation of lignocellulose into hexose often results in the production of inhibitors likely to impede the activity of *Saccharomyces cerevisiae* during bioethanol production. This study aimed to investigate the comparative inhibitory effects of acetic acid and vanillin on the viability of *S. cerevisiae* and the production yield of bioethanol. Different concentrations of inhibitors were spiked in the fermentation broth then the production of bioethanol monitored overtime and correlated with cell viability. The results showed that the inhibition of *S. cerevisiae* by vanillin is more potent compared to acetic acid; however the reduction of bioethanol yield after 12 h was more pronounced with acetic acid (42.8% reduction) than with vanillin (33.3% reduction) which was ascribed to the simultaneous production of weak acids during the fermentation process. The viability test has shown that in the presence of lower concentrations of inhibitors, *S. cerevisiae* can adapt for the first 12 h of fermentation and then may improve ethanol production yield overtime. At lower concentrations (2 g/l vanillin and 4 g/l acetic acid) the effect of inhibitors on the viability of *S. cerevisiae* and ethanol productivity does not last and can be overcome by the adaptation of the yeast. However, the presence of higher concentrations (4 g/l vanillin and 6 g/l acetic acid) results to nearly total inhibition of bioethanol production and the remediation of such effect may therefore require a detoxification process.

Key words: Bioethanol production, *Saccharomyces cerevisiae*, inhibition, acetic acid, vanillin, cell viability.

INTRODUCTION

Globally bioethanol technology is rapidly expanding due to progressive depletion of non-renewable fuel reserves and the potential for carbon neutral processes to contribute in the reduction of emission rate of polluting

gasses to the atmosphere. Bioethanol production is also sustainable, reasonably cost effective, and easy to add into fuel distribution systems (Tomas-Pejoet al., 2008). Currently, first and second generation feed stocks are available for the production of bioethanol. First generation feedstock includes food crops and is therefore likely to negatively impact on the bio diverse regions which are destroyed to avail land required to grow crops. The main disadvantage of this approach is the increased cost of food as crops are used to produce bioethanol (Naik et al., 2010). Second generation feedstock mainly consists of lignocellulosic materials which are widely abundant, comprising about 50% of the biomass on earth and are available as industrial, agricultural, forestry and municipal residues (Almeida et al., 2007). Lignocellulosic materials for ethanol production have been classified into six groups by Sanchez and Cardona (2008). Herbaceous biomass, crop residues, cellulose wastes, softwood, hardwood and municipal solid wastes. 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 setup, as the process of the conversion of lignocellulosic materials into bioethanol is not yet optimized. However, this approach does not affect the food crops,therefore minimizing the overall impacted cost of the second generation biofuel compared to the first generation biofuel (Naik et al., 2010).

The challenge is that second generation feedstock has a very complex structure as they are made of hemicellulose, lignin and cellulose. The production of bioethanol from this feedstock therefore requires a preliminary step of pre-treatment to release digestible sugar monomers; the problem with the pre-treatment is the formation of inhibitors which inhibit the growth of fermenting organisms. Some of these inhibitors generally found in the hydrolysates include aromatic compounds (that is, phenolics), furans (furfurals and 5-hydroxymethylfurfural), weak acids (acetic, levulinic and formic acids), raw material extractives (acidic resins, tannic, and terpene acids), and heavy metals (iron, chromium, nickel and copper) (Chandel et al., 2011). The formation of these components can lead to the inhibition of the growth of microorganisms by affecting the rate of the sugar uptake with simultaneous decay in the product formation (Palmqvist and Hahn-Hagerdal, 2000). The effect of such inhibitors on the production of biofuel has been intensively studied; several authors (Cao et al., 2010; Veeravalli et al., 2013; Liu et al., 2015) have reported the inhibition of hydrogen production as well as a shift in microbial community caused by furan derivatives present in the hydrolysate. The inhibition of the fermentation process by inhibitors in the lignocellulosic hydrolysates has also been alluded to (Delgenes et al. 1996; Bellido et al. 2011; Huang et al. 2011). Using the hydrolysate derived from wheat straw

pretreatment with steam explosion for ethanol fermentation by *Pichiastipitis*, Bellido et al. (2011) observed a considerable reduction of the ethanol productivity. On the other hand, Huang et al. (2011) observed that weak acids such as acetic acid and formic acid were more potent inhibitors of yeast during bioethanol production as compared to phenols and aldehyde.

To overcome the effects of inhibitors from lignocellulosic hydrolysates on the fermentation process, physical, chemical and biological detoxification methods are often considered (Klinke et al., 2004). However, consideration of a detoxification step in the fermentation process may increase the cost as well as the production time. Although, *Saccharomyces cerevisiae* can tolerate the presence of inhibitors for a short while, this is often done at the cost of an extended lag phase and reduces ethanol productivity (Palmqvist et al., 1999; Larsson et al., 2000; Almeida et al., 2007; Landaeta et al., 2013). There is therefore a need to further investigate the behaviour of *S. cerevisiae* in the presence of inhibitors from lignocellulosic hydrolysates as well as the impact on bioethanol production yield.The inhibition may therefore lead to ineffective use of lignocellulosic biomass and insufficient yield for the commercialization of the process. Identifying the effects that the inhibitors, specifically acetic acid and vanillin (phenol), have on the growth of *S.cerevisiae* will then be correlated with the reduction in bioethanol yield.

METHODOLOGY

Chemicals

Acetic acid (95.5%) and vanillin (>99%) which act as the main inhibitors during bioethanol production from lignocellulosic biomass where purchased from Associated Chemical Enterprises (ACE) and MERCK, respectively. Chemical ingredients for the preparation of growth media included peptone, yeast extract which were purchased from SIGMA-ALDRICH, while Glucose and agar powder were obtained from ACE. Other common chemicals used included ethanol (99.9%) (SIGMA-ALDRICH) and sodium hydroxide (>98%)(ROCHELLE CHEMICALS).

Preparation of media

The growth supporting broth medium for yeast was prepared using Yeast extract, Peptone and Dextrose (YPD). YPD broth medium contained 10, 20 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 min.

Batch fermentation

Batch fermentation was carried out in 250 ml Erlenmeyer flask, mainly using glucose as substrate for the yeast *S. cerviseae*. The inoculum was prepared by adding 0.005 g of dry *S. cerevisiae* cells
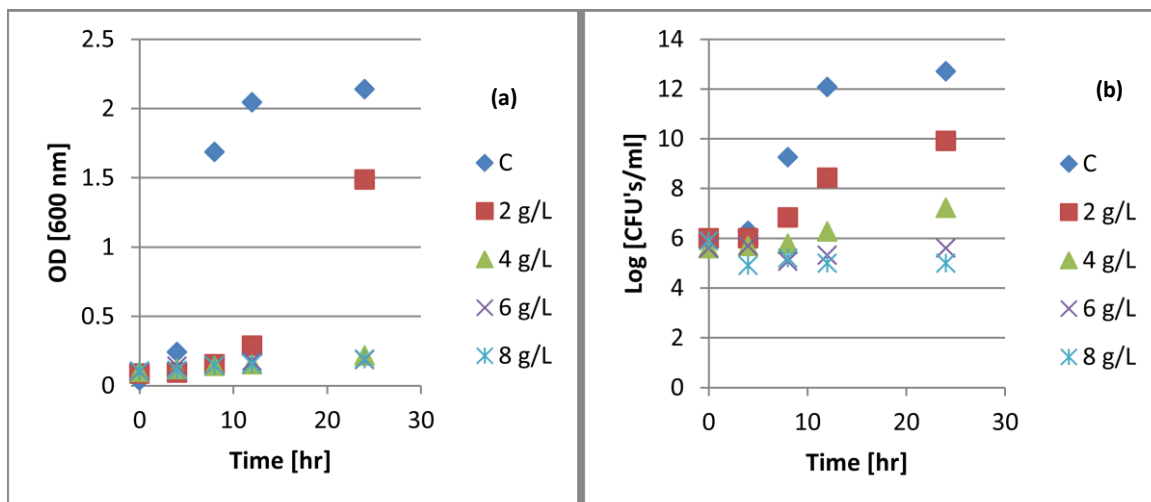
Figure 1. Inhibition of *S. cerevisiae* growth in presence of various concentrations of vanillin: (a) expression of growth by absorbance; (b) expression of growth by colonies count.

to one litre of sterilized broth and incubated overnight at 30°C in a shaking incubator (120 rpm). The culture was inoculated in 20% glucose solution contained in 100 mL GL 45 laboratory glass bottles with blue PP screw caps and pouring rings then incubated at 30°C for 48 h.

Determination of minimum inhibitory concentration

Yeast grown aerobically for 24 h in YPD broth was inoculated in broth spiked with different concentrations of acetic acid and vanillin (2, 4, 6 and 8 gper liter of broth). All experiments were conducted in Erlenmeyer flasks containing 50 mL broth, pH 6, 120 rpm shaking speed and incubated at 30°C. Samples were analyzed at set time intervals (3, 6, 8, 12 and 24 h) to determine the minimum inhibitory concentration.

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 or 6 g.L^{-1} and 2 or 4 g.L^{-1}, respectively. Samples were analysed at set time intervals over a period of 48 h.

Quantification and viability of yeast cells

The growth of *S. cerevisiae* in the fermentation broth in the absence and presence of inhibitors was quantified through measurement of the absorbance. The total *S. cerevisiae* cells were measured at a wavelength of 600 nm using a spectrophotometer (Shimadzu). This measurement of the optical density (OD) gave an indication of the total cells (alive, injured or dead) present. The amount of viable yeast cells was determined using culture method. The culture was serially diluted with sterilized de-ionized water. Diluted cells were plated on agar medium (30 g/L glucose, 5 g/L yeast extract, 2 g/L NH$_4$Cl, 1 g/L KH$_2$PO$_4$, and 0.3 g/L MgSO$_4$, 7H$_2$O, 20 g/L agar) in Petri dishes then incubated at 30°C for 48 h. The number of colonies counted and the average of duplicate plates was expressed as colony forming units (CFUs).

Analytical method

The fermentation liquor was filtered through a 0.2 µmmicro pore syringe filter and the ethanol was quantified in the filtrate using a high performance liquid chromatograph (HPLC). An Agilent 1200 HPLC fitted with a refractive index detector was used with an isocratic mobile phase of 0.005 M H$_2$SO$_4$.

RESULTS

Vanillin and acetic acid belong to the groups of phenolic compounds and weak acid respectively; they 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 a large variety of phenols and acids formed during pre-treatment, vanillin and acetic acid were chosen in this study as they occur in the largest quantities. 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 and also to delineate the factors contributing to the decrease of ethanol yield in the presence of inhibitors.

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

Effect of vanillin

Figure 1a and b show that the inhibition effect of vanillin increased with the concentration and exposure time. The minimum inhibitory concentration (MIC) could be estimated as 2 g/l. When the inhibitor was present at
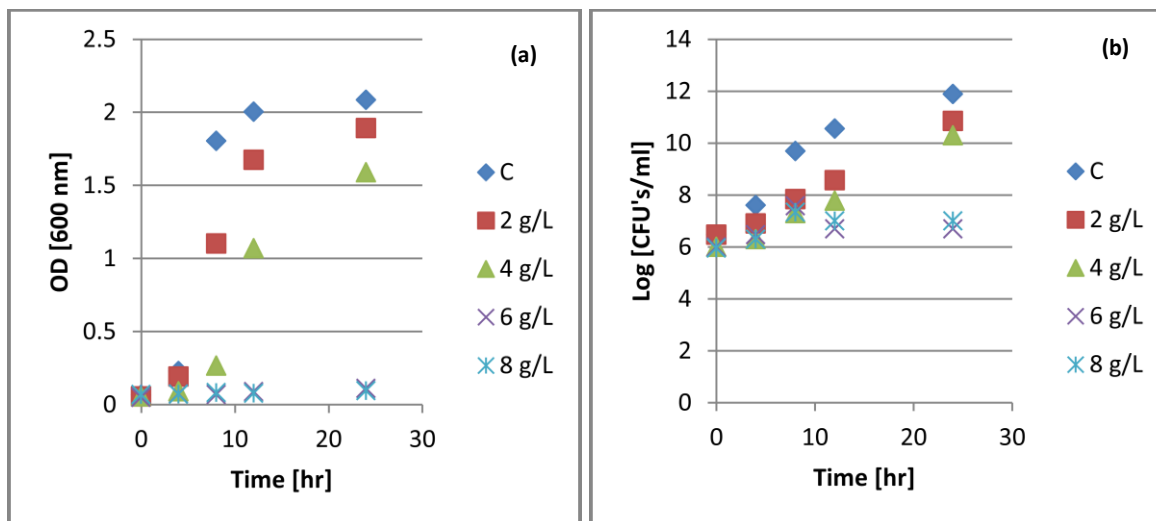
Figure 2. Inhibition of *S. cerevisiae* growth in presence of various concentrations of acetic acid: (a) expression of growth by absorbance; (b) expression of growth by colonies count.

concentrations of 2, 4 or 6 g/l, there was a similar trend between the OD measurement and colony count; however a dissimilarity was observed after 8 h incubation and in the presence of 8 g/l vanillin, as the cell count indicated no growth while the OD value of 0.2 was recorded; this implies that the cells were no longer viable after 8 h incubation in the presence of 8 g/l vanillin.

Effect of acetic acid

Data plotted in Figures 2a and b clearly indicate the inhibition of *S. cerevisiae* in the presence of acetic acid; it was observed that the inhibition effect also increased with the concentration and time. The MIC was found to be 2 g/l with only little effect on the growth of the yeast. There was no perfect correlation between the adsorbance and the cell counts as shown by the behaviour of the yeast at 6 and 8 g/l of acetic acid. This implies that at those concentrations, although the cells multiply in the first 8 h, metabolic rearrangement may also take place resulting in the decrease of the yeast's biomass (Yousef and Uneja, 2002). Exposing yeast to various environmental stress conditions, Tibayrenc et al. (2010) also found that there was an increase of population of significantly smaller cells size.Comparing the effects of the two inhibitors, it can be observed that in general vanillin has a pronounced inhibitory effect than acetic acid; for the same MIC (2 g/l), vanillin caused more reduction of growth than acetic acid; and at 8 g/l, vanillin had a lethal effect while acetic acid only had a static effect. It has been reported (Klinke et al., 2003; Almeida et al., 2007) 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 (Russel, 1992).

### **Bioethanol yield influenced by MIC level of inhibitors**

The minimum inhibitory concentrations of 2 g/l of vanillin and 4 g/l of acetic acid were chosen to determine their effect on the production of ethanol by *S. cerevisiae*. It is important to use relatively low concentrations to mimic the level produced following pretreatment of biomass.

Inhibitory effect of vanillin

The impact of vanillin on the production of ethanol in the first 36 h was quite obvious as shown in Figure 3, the constant reduction of bioethanol production compared to the control not exposed to the vanillin; however after 36 h, the yeast seem to recover and perform better in the presence of vanillin resulting in higher production of ethanol; this could be explained by the cell count as an increase was recorded while the OD remained lower than the control values, implying that the cells may have lost weight but remained more active after longer exposure to vanillin. The simultaneous production of weak acids during ethanol production may have also played a role in the stabilization of ethanol production rate after 36 h, as discussed later. Figure 4a and b both express the growth of *S. cerevisiae* during bioethanol production, the results clearly show that OD values could not be strictly corroborated to the number of cells, as the trend of the
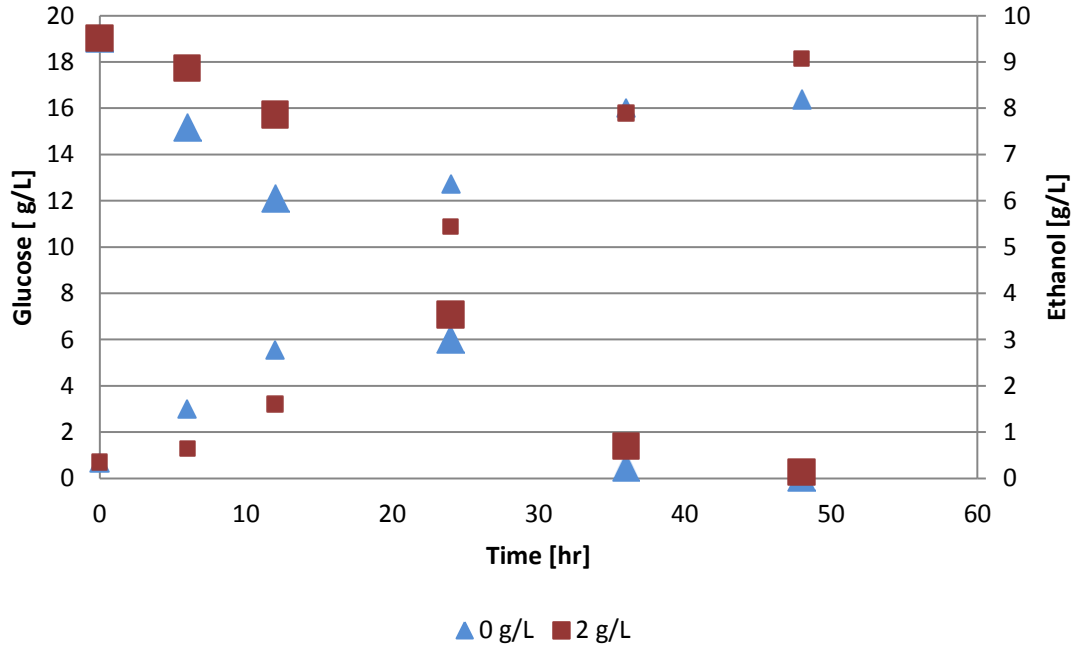
Figure 3. Glucose consumption and ethanol production in the presence of 2 g vanillin. Large symbols (glucose), small symbols (ethanol).
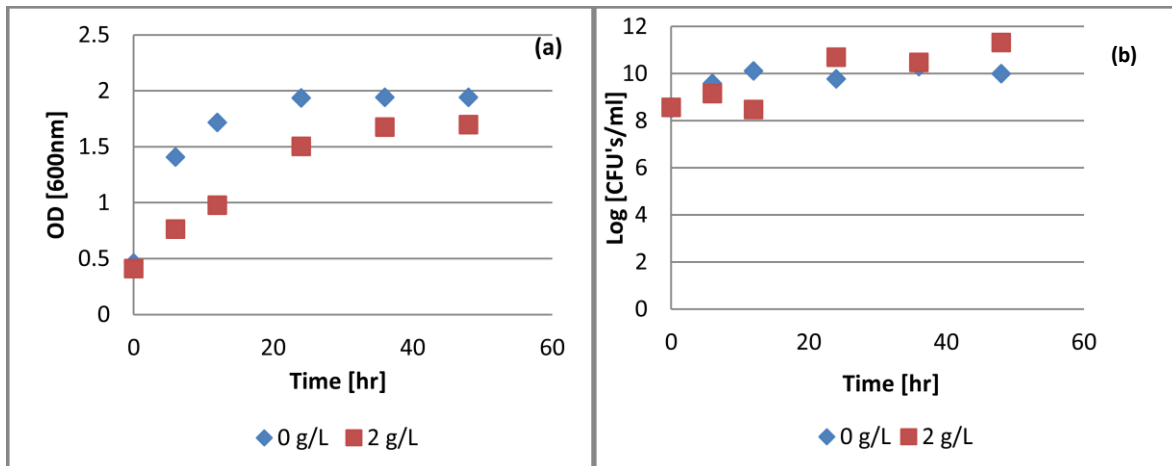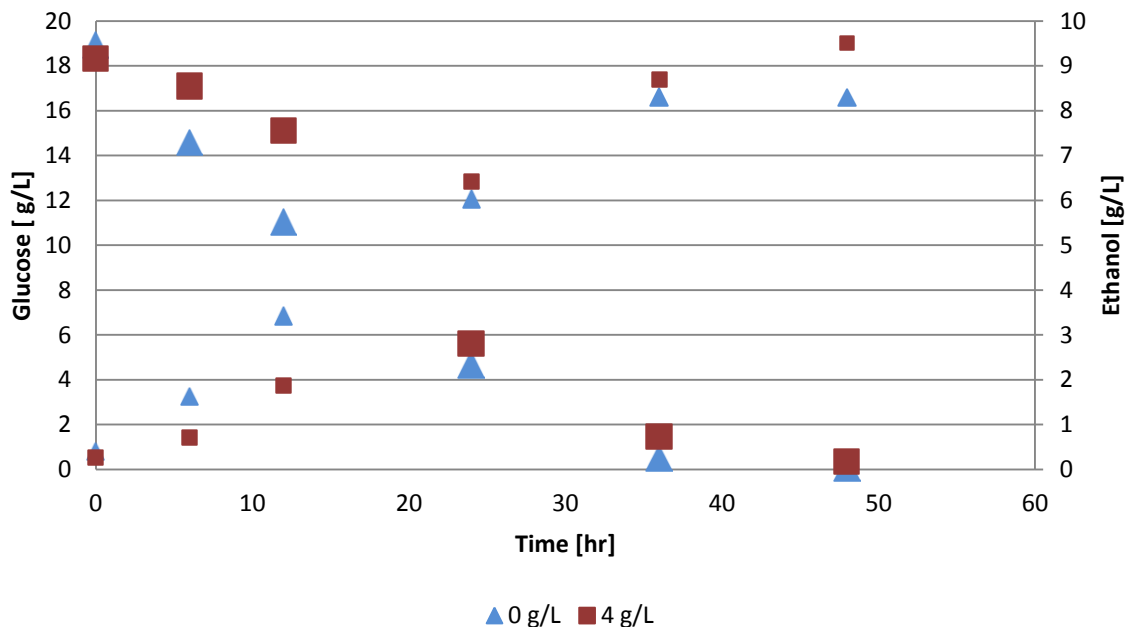

Figure 4. Growth expression of *S. cerevisiae* during fermentation and in the presence of vanillin (2 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.

OD plots do not express clearly the rapid multiplication of cells in the presence of the inhibitor after 10 h; the cells probably lose weight during adaptation to the presence of the inhibitor, but continue to grow rapidly compared to the control.

Inhibitory effect of acetic acid

Figure 5 shows that there was a decrease of glucose

concentration as the ethanol was formed, clearly indicating that ethanol production results from the use of glucose by *S. cerevisiae*; however, the rate of glucose breakdown was slow at the beginning and therefore lower production of ethanol for the first 12 h in the presence of acetic acid; the trend changed after 12 h as more ethanol was produced in the flask containing the acetic acid. This could have merely been ascribed to the adaptation of *S. cerevisiae*, but the patterns of OD values and cell count (Figure 6a and b) do not confirm this, further discussion

Figure 5. Glucose consumption and ethanol production in the presence of 4 g acetic acid: Large symbols (glucose), small symbols (ethanol).
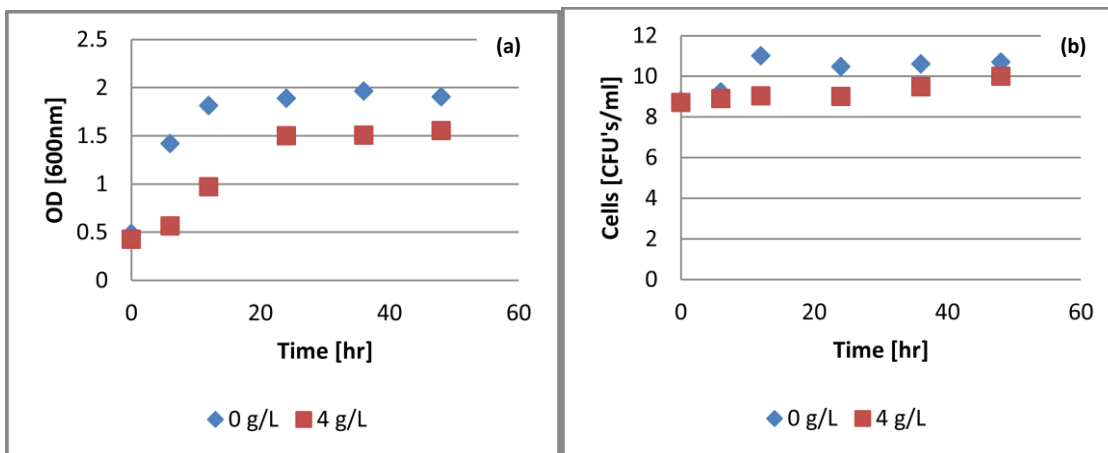
Figure 6. Growth expression of *S. cerevisiae* during fermentation and in the presence of acetic acid (4 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.

will be done in the following sections. The plots of optical density and cell count in Figure 6a and b indicate an extended lag phase and more sluggish exponential growth phase in the presence of the inhibitor. However, after 48 h there was as much cells in the control sample as in the sample with the inhibitor, implying that the yeast adapted overtime.

Bioethanol yield influenced by higher concentrations of inhibitors

The inhibitory effects at relatively higher concentration of

vanillin (4 g/l) and acetic acid (6 g/L) on the growth of *S. cerevisiae* was observed in Figures 1 and 2, respectively. A significant effect on the bioethanol production yield could therefore be expected at higher concentrations of inhibitors.

Inhibitory effect of vanillin

Figures 7 and 8 clearly indicate the effects of higher concentrations of vanillin on the ethanol production yield and the viability of *S. cerevisiae.*For the total duration of the fermentation process the ethanol yield in the
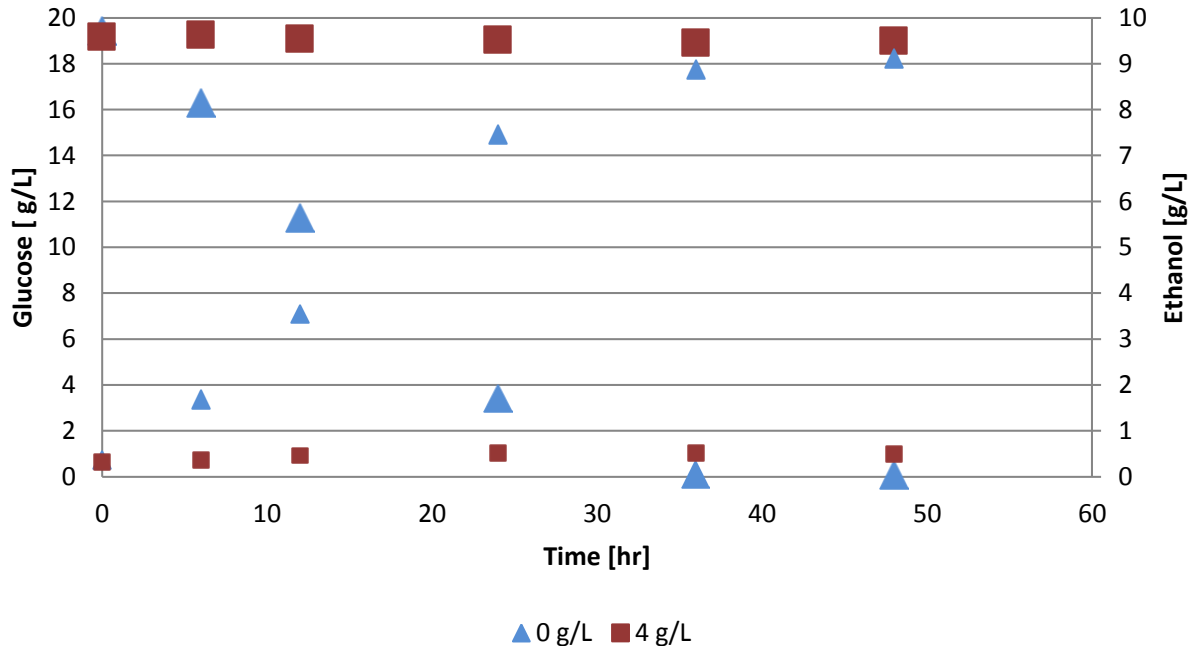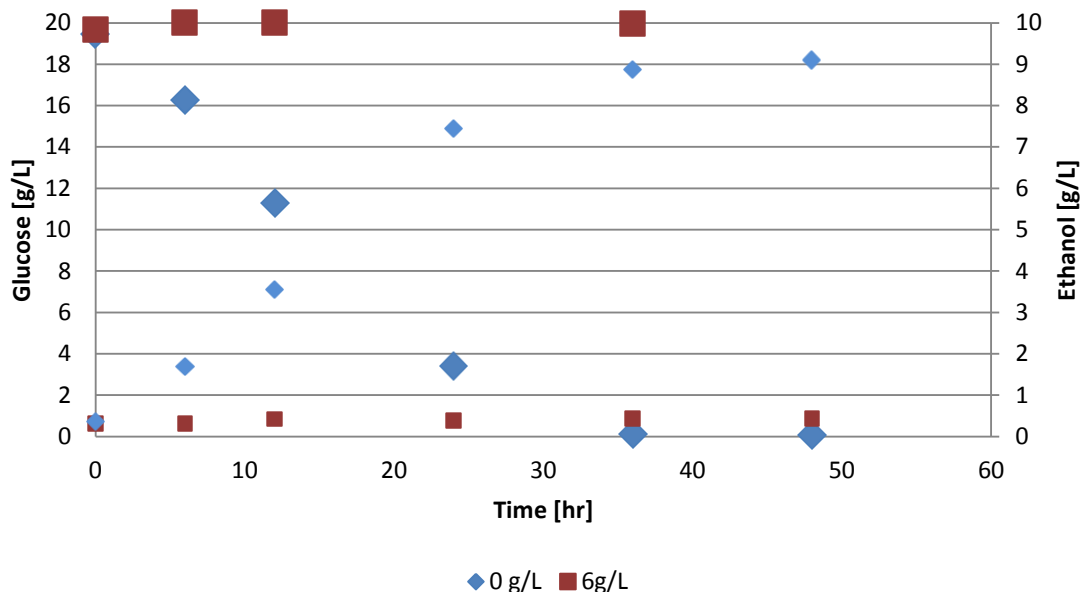
Figure 7. Glucose consumption and ethanol production in the presence of 4 g/L vanillin: large symbols (glucose), small symbols (ethanol).
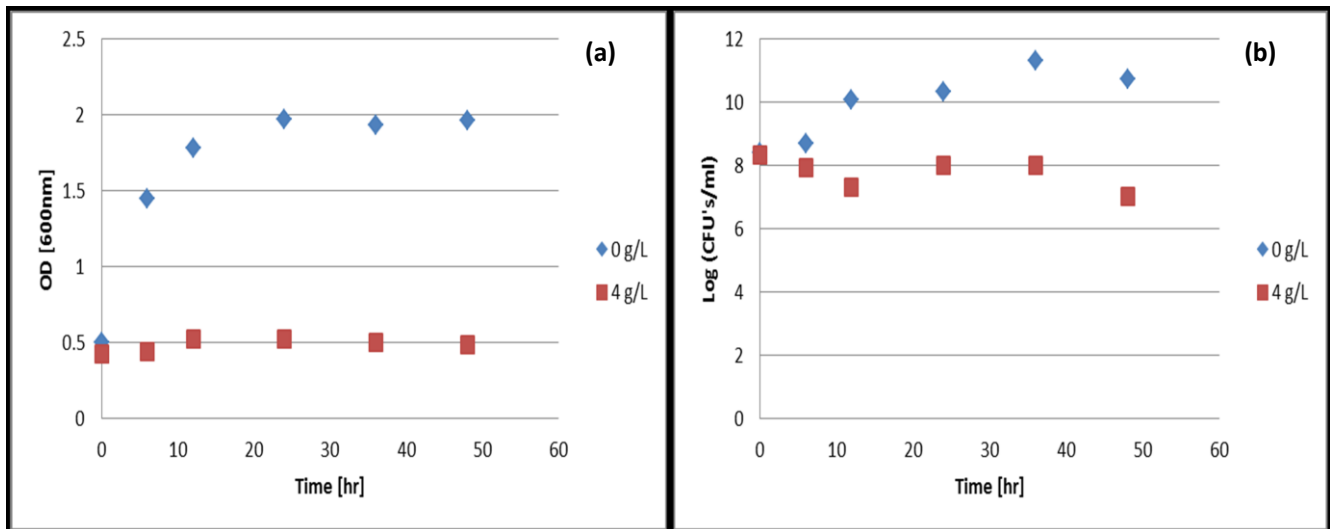
Figure 8. Growth expression of *S. cerevisiae* during fermentation and in the presence of vanillin (4 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.

presence of 4 g/L vanillin (Figure 7) remained constant throughout the 48 h. Glucose concentration also remained constant at about 20 g/L in the presence of the inhibitor. When comparing these results to that of the effect of the MIC of vanillin (2 g/L) it can be observed that the final ethanol concentration decreases from 9 g/L in the presence of lower (2 g/L) of inhibitor to 0.5 g/L at higher (4 g/L) concentration of the inhibitor, respectively.

Thus, the fermenting organism is very sensitive to the slight increase of the concentration of vanillin, the two fold increase led to almost 95% reduction of the bio-ethanol yield, showing the impact of inhibitor when using pre-treatment and hydrolysis methods that produce more than two gram per litre of vanillin from second generation feedstock. Figure 8 shows a total inhibition of cells growth as expressed by the absorbance (a) and the viability test

Figure 9. Glucose consumption and ethanol production in the presence of 6g/L acetic acid: Large symbols (glucose), small symbols (ethanol).

expressed by CFU values (b) confirming the inhibitory effect of 4 g/L vanillin. According to the colonies counts there is attempt by the cells to adapt to the presence of the inhibitor in the interval time between 25 to 35 h; the inhibition effect is however persistent because of the cumulative effects of other inhibitors such as lactic and acetic acids produced during fermentation.

Inhibitory effect of acetic acid

During the 48 h of fermentation, the ethanol yield in the presence of 6 g/L acetic acid (Figure 9) remained constant. Glucose concentration also remained constant in the presence of the inhibitor. It is quite evident that increasing the concentration of acetic acid from 4 to 6 g/L has resulted to a more pronounced inhibitory effect on the yeast, preventing adequate organization of the metabolic activities required for the fermentation of glucose to bioethanol; hence the concentration of glucose remaining constant throughout the 48 h. The inhibitory effect of 6 g/L of acetic acid on *S. cerevisiae* growth could be observed in Figure 10a and b, as the OD values did not increase during the 48 h of incubation; this implies that there was no growth as the cells were exposed to the inhibitor, but the cells grow well in the absence of inhibitor. The effect related to increased concentration of acetic acid could be noted when comparing the OD values at 4 and 6 g/L of the inhibitor. The count of colonies, provide information about the viability of the cells; it is observed in Figure 10b that the cells number decreases overtime indicating a microbicidal effect of 6 g/L of acetic acid; this effect is more pronounced than

with 4 g/L acetic acid. This therefore explains the drastic drop of 95% of bioethanol yield.

Formation of week acids during bioethanol production

In this study the formation of weak acids during the fermentation of glucose was monitored to determine their contribution in the inhibition of *S. cereviseae* and subsequently the effect on the yield of bioethanol. It was observed that the amount of weak acids formed varied with the initial concentration of the inhibitors in the fermentation broth.

In the presence of MIC level of inhibitors

Figure 11a and b below show that there was formation of acetic and lactic acids during the degradation of glucose and formation of ethanol by *S. cerevisiae*; it can however be seen that in the presence of the inhibitor (vanillin) the production of weak acids is lowered. The accumulation of these weak acids has contributed to significantly reduce after 12 h, the performance of the yeast not previously exposed to inhibitors (Figure 3).The formation of weak acids including lactic and acetic acids was observed during the production of ethanol in the absence and presence of acetic acid (4 g/L) (Figure 12a and b). However, in the presence of acetic acid the inhibition effect led to the reduction of the amount of lactic acid formed while the increase of the amount of acetic acid was likely due to the combination with the residual
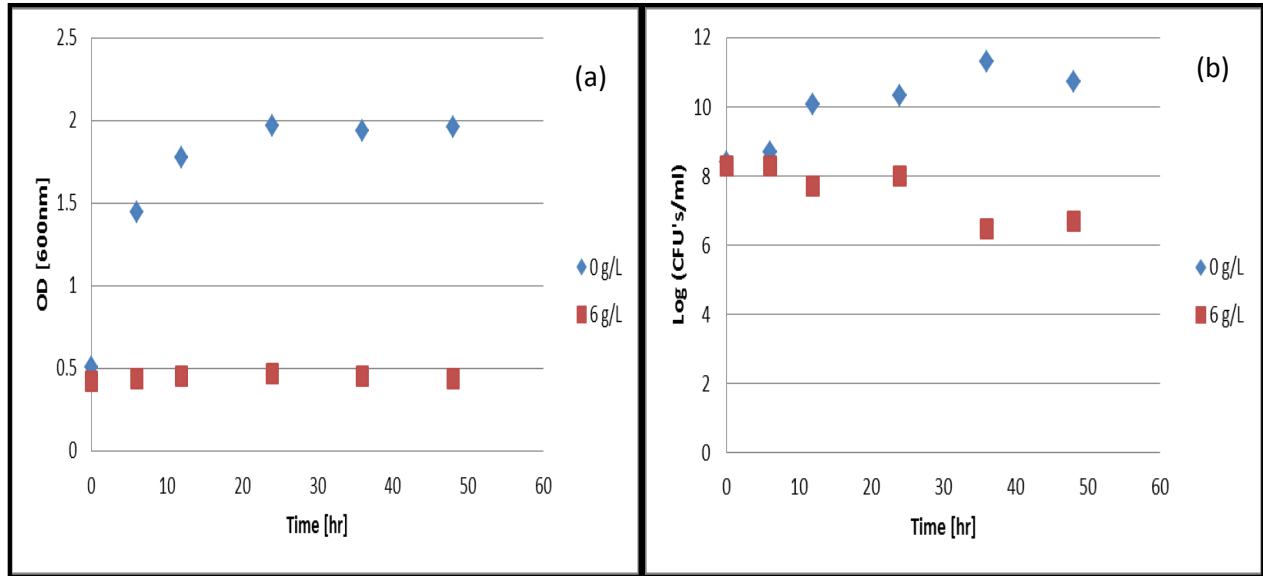
Figure 10. Growth expression of *S. cerevisiae* during fermentation and in the presence of acetic acid (6 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.
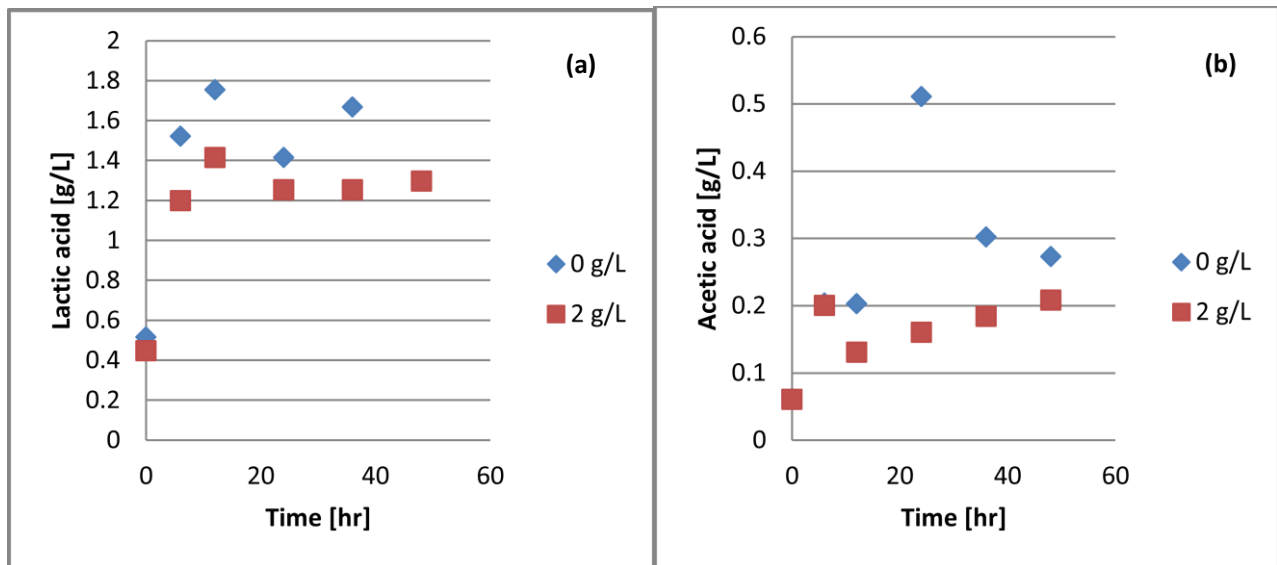
Figure 11. Formation of acetic acid and lactic acid during fermentation and in presence of vanillin (2 g/L): (a) Lactic acid formation, (b) acetic acid formation.

acid. The formation of weak acids in the control samples after 24 h probably led to the inhibition of *S. cerevisiae*, this explains why the performance of the yeast exposed to inhibitors from the first hour was better than the control after 24 h.

In the presence of higher concentration of inhibitors

Figure 13a and b show the formation of lactic acid and

acetic acid during fermentation, the amounts of these acids is relatively low compared to that obtained during fermentation in the presence of lower (2 g/L) concentration of vanillin. Acetic acid final concentration was halved from 0.2 to 0.1g/L, certainly as a result of reduced cell activity. The amount of cells is directly related to the amount of acetic acid and lactic acid produced. In Figure 14a and b lactic and acetic acids formation is reduced drastically in the presence of 6 g/L acetic acid; this translates to the significant reduction of activity.
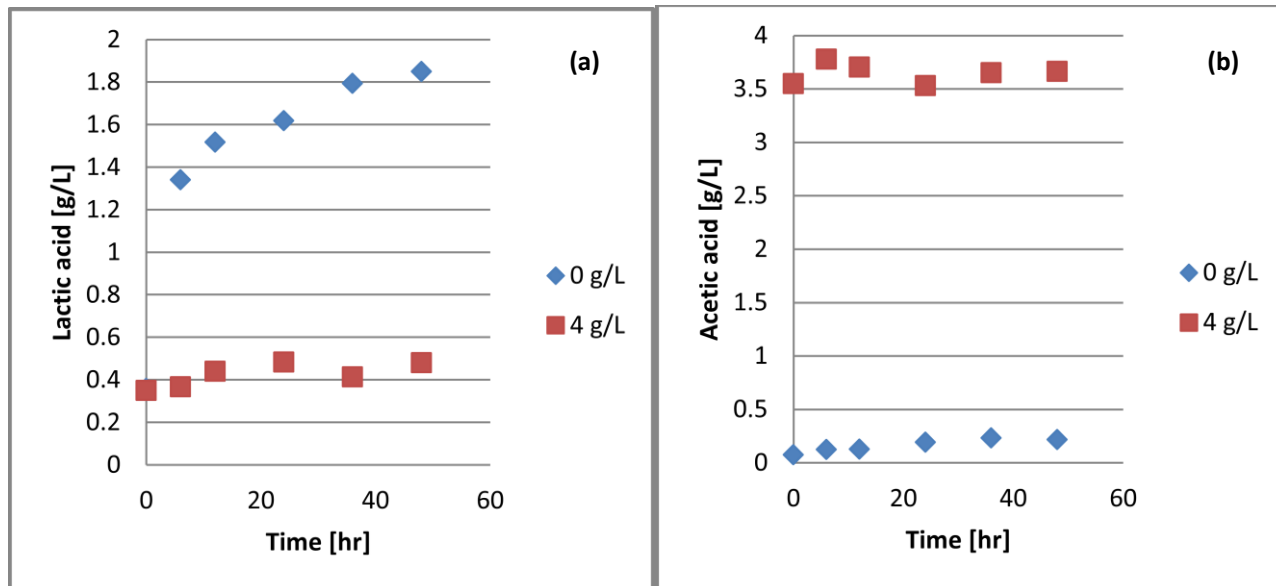
Figure 12. Formation of acetic acid and lactic acid during fermentation and in presence of acetic acid: (a) lactic acid formation (b) Acetic acid formation.
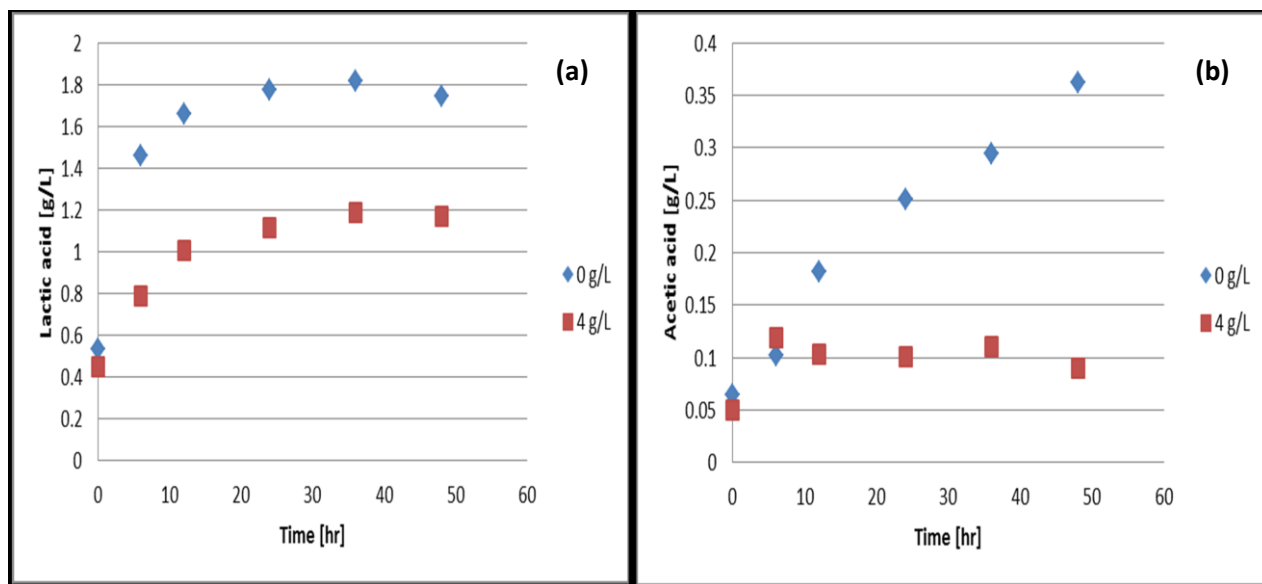

Figure 13. Formation of acetic acid and lactic acid during fermentation and in the presence of vanillin (4 g/L): (a) Lactic acid formation, (b) Acetic acid formation.

Therefore, the inhibition during the 48 h period results predominantly from the activity of the acetic acid introduced at the beginning of the fermentation.

DISCUSSION

By exposing the yeast to lower and higher concentrations of inhibitors it was possible to better understand its fer-

mentability behaviour; the inhibition of yeast at lower concentration of inhibitors brought about two scenarios. A deceleration phase was observed during the adaptation of yeast in the first 12 h, resulting in lower consumption rate of glucose and lower ethanol productivity.

The ethanol productivity value dropped from around 0.26 g/L h in the control sample to about 0.121 and 0.137 g/L h in the presence of vanillin (2 g/L) and acetic acid (4 g/L), respectively, representing approximately 50%
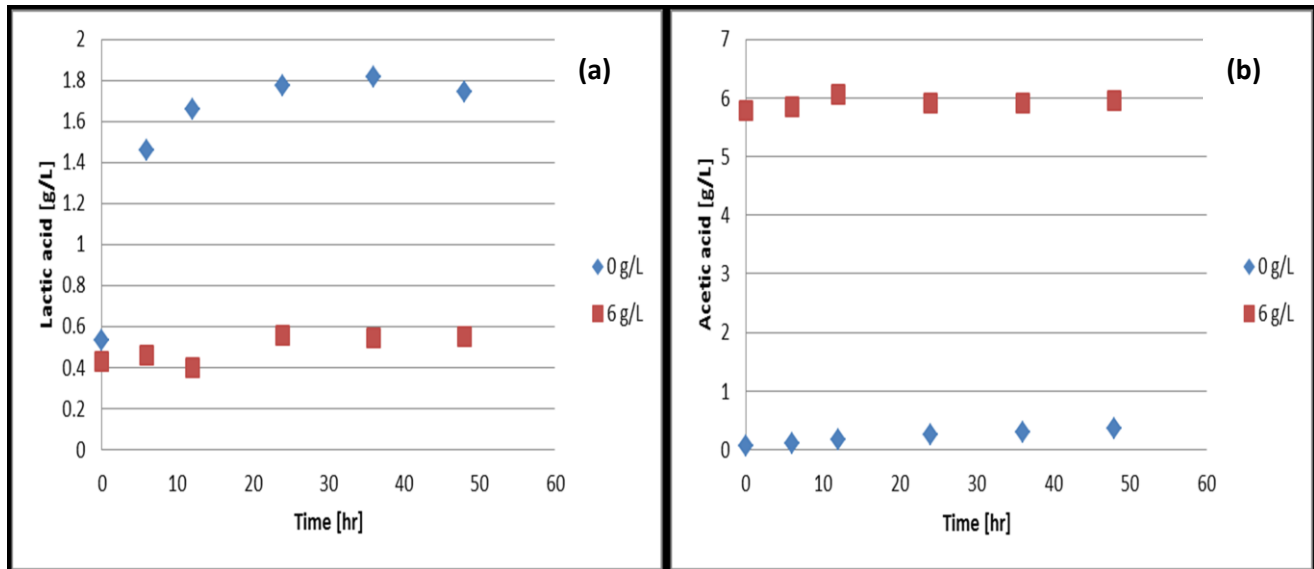
Figure 14. Formation of acetic acid and lactic acid during fermentation and in the presence of acetic acid (6 g/L): (a) lactic acid formation, (b) acetic acid formation.

reduction. In the second phase the yeast had adapted and the cells were very active, judging by the higher productivity values 0.213 and 0.236 g/L h in presence of vanillin (2 g/L) and acetic acid (4 g/L), respectively; these values were equal or higher than the control value of 0.219 g/L h. It is however important to mention that the acetic acid and lactic acid formed during fermentation in the control sample, were much likely to inhibit the non-adapted yeast.

The recorded changes in bioethanol productivity in the presence of inhibitors were not always correlated with the OD values, but reflected the growth pattern expressed as cell plate count or viability which translates into the ability of cells to grow and replicate. After consumption of almost all the glucose, it was found that at 48 h the inhibitory effects on the yeast's growth did not affect the bioethanol yield, but rather increased the yield from 0.412 g/g in the control sample to 0.454 and 0.476 g/g in the presence of vanillin (2 g/L) and acetic acid (4 g/L), respectively. Similar results have also been previously reported by researchers studying the inhibitory effect on the fermentation (Moreno et al., 2013; Klinke et al., 2004; Palmqvist and Hahn-Hagerdal, 2000).

In the presence of higher concentrations of vanillin (4 g/L) and acetic acid (6 g/L) the trend of bioethanol productivity was almost constant from the first hour till 48 h, as the yeast consumed very little glucose. The bioethanol yield was very low 0.0243 and 0.0216 g/g in the presence of vanillin (4 g/L) and acetic acid (6 g/L), respectively, while a high yield 0.455 g/g was recorded in the control sample. The optical density was constant in the presence of inhibitors not giving an exact indication of the physiological state of the yeast; however the cell

count showed a decrease of cell viability as there was reduction of the number of cell from 0 to 48 h. The OD measurement must therefore be complemented by the cell count to have an indication of the yeast physiological response to inhibition during fermentation.

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. (2011). It was observed that at the minimum inhibitory concentrations, the inhibitors could reduce the bioethanol productivity only in the first 12 h of fermentation, which may therefore not be a serious problem if the fermentation process takes longer than 24 h. However, relatively higher concentrations have been found totally inhibitory of the yeast activity, preventing the use of glucose and reducing the bioethanol yield by approximately 95%. For such concentrations of inhibitors the inhibition may be overcome by the use of detoxification methods to avoid a significant drop of the ethanol yield.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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