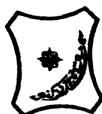
Bajopas Volume 11 Number 1 June, 2018

http://dx.doi.org/10.4314/bajopas.v11i1.1



Bayero Journal of Pure and Applied Sciences, 11(1): 1 - 7
Received: December, 2017
Accepted: January, 2018
ISSN 2006 – 6996

INFLUENCE OF NITROGEN SOURCES ON ETHANOL PRODUCTION BY *Saccharomyces* spp IN THE PRESENCE OF FORMIC ACID

Oshoma, C. E.[1*] and Obueh, Henrietta O.[2]
[1]Department of Microbiology, University of Benin, P.M.B. 1154, Benin City, Nigeria.
[2]Department of Biology, College of Education, Ekiadolor, P.M.B. 1144, Benin City, Nigeria
*Correspondence author:cyprian.oshoma@uniben.edu

ABSTRACT
Formic acid is one of the major inhibitory compounds present in hydrolysates derived from lignocellulosic materials, the presence of which can significantly hamper the efficiency of converting available sugars in the hydrolysates to bioethanol. The influence of different nitrogen sources supplemented media on bioethanol fermentation with Saccharomyces spp in the presence of formic acid was studied. The addition of ammonium sulphate and urea significantly increased cell number, glucose utilization, ethanol and glycerol production when compared with control media. It was observed that supplementation with nitrogen sources contributed assimilable nitrogen for the yeast strains growth when stressed with formic acid. The best concentration of nitrogen to be utilized by yeast was found to be 0.80 gN/L. The maximum cell numbers were 8.17± 0.23x 10^7 and 8.68± 0.16x 10^7 cells/mL for ammonium sulphate medium while control had the least of 4.00± 0.20x 10^7 and 4.97± 0.08x 10^7 cells/mL for S. cerevisiae NCYC2592 and S. arboricolus 2.3319 respectively. Ethanol accumulation increased with the consumption of glucose. The maximum ethanol production were 20.49 ± 1.24 and 19.74± 0.89 g/L using ammonium sulphate for S. cerevisiae NCYC2592 and S. arboricolus 2.3319 respectively. There was significant difference in ethanol production when the nitrogen sources were compared with the control medium ($p< 0.05$). The highest glycerol produced were 2.21 ± 0.04 and 3.11± 0.05 g/L using ammonium sulphate for S. cerevisiae NCYC2592 and S. arboricolus 2.3319 respectively. The conclusion was that yeast tolerance to formic acid and ethanol production could be achieved when fermentation medium is supplemented with nitrogen sources specifically ammonium sulphate.
Keywords: Lignocellulose, inhibitors, Saccharomyces, fermentation, yeast, biofuel.

INTRODUCTION

Bioethanol fermentation from lignocellulose biomass is a clean burning renewable fuel which is increasingly used as an alternative fuel. Ethanol production from the renewable sources has received considerable interest in recent years, despite the fact that ethanol fermentation will require subsidies in order to economically compete with fossil fuel (Thomas and Ingledew, 1990). The raw material, cost, capital, equipment and production processing greatly influence the profitability of ethanol fermentation. Presently, second generation ethanol production using lignocellulosic materials, serve as the possible cheapest raw materials, but the feasible technology for their efficient and economically converted to ethanol is yet to be fully achieved. The use of lignocellulosic materials as the raw substrate for ethanol production have many technical hurdles and most significant is the development of efficient economical pretreatment and hydrolysis steps for the release of fermentable sugars from the biomass (Mosier *et al*., 2005). In addition, a problem is engineering robust, process-relevant industrial microorganisms that are capable of mixed sugar fermentation and tolerant to inhibitors (Hahn-Hageral *et al*., 2007).

Lignocellulosic hydrolysates contain a variety of sugars and inhibitory compounds such as formic acid and various phenolic compounds. Weak acids such as acetic, levulinic and formic acid are commonly formed during hydrolysis (Palmqvist and Hahn-Hagerdal, 2000a; Jonsson and Martins, 2016). Theyinhibit yeast fermentation by reducing ethanol yields. The problem of inhibitory toxicity has been addressed through detoxification of hydrolysate before fermentation ((Palmqvist and Hahn-Hagerdal, 2000b; Kapu *et al*., 2013), which also increases cost of production (Almeida *et al*., 2007). With strains unable to adapt to the inhibitors, pose a primary challenge of developing industrial microorganisms with tolerance to the inhibitors that are formed and released as biomass is broken down in the pretreatment and hydrolysis step (Hahn-Hageral *et al*., 2007; Wimalasena *et al*., 2014). However, the production of economically feasible bioethanol requires optimization of ethanol yield and production rate (Albers *et al*., 1996). The ethanol yield may be improved by suppressing the formation of unwanted biomass and by-products, such as glycerol. The reduction of glycerol production may also make the purification process cheaper (Albers *et al*., 1996).

According to Gibson *et al*. (2007) the physiological state of yeast strain directly influences the fermentability and economic feasibility of bioethanol production process. Relatively some studies have shown the influence of nitrogen sources on yeast growth and product formation (Albers *et al*., 1996).

These studies mainly focused on beer fermentation. Yeasts are capable of utilizing a range of different inorganic and organic sources of nitrogen for incorporation into the structural and functional nitrogenous components of the cell. In industrial fermentation media, available nitrogen is usually in the form of complex mixtures of amino acids, rather than ammonium salts. Nevertheless, media are often supplemented with inexpensive inorganic nitrogen forms, such as ammonium sulphate (Walker, 1998).

Yeast strains mostly *Saccharomyces* species are capable of utilizing a wide range of nitrogen sources for growth. However, not all nitrogen sources equally influences growth (Shure *et al*., 2000). Cell growth on assimilable nitrogen sources has been shown to be essential for cell biomass, thus influences yeast tolerance to fermentation stress (Schure*et al*., 2000; Yue *et al*., 2012). Ammonium compounds have been implicated as a good nitrogen source, reason been that yeast cells easily convert the molecule into glutamate and glutamine. Therefore, media supplementation with nitrogen source is necessary for yeast cell growth during fermentation, this in turn imparts the cell tolerance to stresses such as ethanol and formic acid (Yue *et al*., 2012). Hence, cell survival and metabolism in the presence of inhibitors are the first priority during fermentation (Chen *et al*., 2009). Therefore, in this study, the influence of different nitrogen sources on the growth and product formation of two yeast strains in the presence of formic acid was investigated.

MATERIALS AND METHODS

Yeast strains

The strains used in this study, *Saccharomyces cerevisiae* NCYC2592 and *Saccharomyces arboricolus* 2.3319, were obtained from the National Collection of Yeast Cultures (NCYC), Norwich, UK. These strains were previously investigated to have promising formic acid tolerance abilities (Oshoma*et al*., 2015).Further investigation was carried out in order to improve formic acid tolerance of these two strains, the impact of addition of nitrogen sources on their formic acid tolerance performance. The strains were stored in glycerol stock at -80°C until required. The inoculum was prepared by taking a loopful of stock culture to 10 mL YPD (yeast extract 10 g/L, peptone 20 g/L and glucose 20 g/L) broth and incubating in 30 mL sterlin tube at 30°C, 120 rpm for 48 hr.

Phenotype Microarray (PM) Plates of proline effect on formic acid tolerant yeast cells

Phenotypic microarray of tolerant yeast cells to formic acid was described according to procedure of Greetham *et al*. (2014). Cells of *S. arboricolus*2.3319 and *S. cerevisiae* NCYC2592 were harvested from the 48 hr old culture and washed thrice with sterile deionised water and resuspended in 10 mL of sterile deionised water in a test tube. The transmittance was adjusted to 62% (5.0x 10^{6} cells/mL) using turbidimeter (Biolog, USA). Phenotypic microarray was carried out to study the effect of different nitrogen sources (Urea and Ammonium sulphate) on metabolic profiles of *S. cerevisiae* NCYC2592 and *S. arboricolus* 2.3319 strains in the presence of 40 mM formic acid. The concentrations used ranged from 0.5 to 1.0 gN/L and control (0 gN/L) without nitrogen source. The biolog medium was prepared using 6% (w/v) glucose, 0.67% (w/v) YNB (yeast nitrogen base without amino acid and ammonium sulphate), 0.2 uL of dye D (Biolog, USA), different nitrogen sources (0, 0.5 – 1.0 gN/L) and 40 mM formic acid. Final volume 30 µL was made up using distilled water and transferred into various wells with different concentrations of nitrogen sources (0, 0.5 – 1.0 gN/L). The well also contained 75 µL IFY buffer™ (Biolog, USA), 3.8 µL of yeast (previously adjusted to the 62% transmittance) and 11.2 µL distilled water. The 96 wells plates were loaded into the Omnilog reader (Biolog, USA) and incubated at 30°C for 96 hours under anaerobic condition; data was recorded photographically at 15-min intervals. The conversion of dye intensity was detected and transformed into a signal value that reflected cell metabolic activity and dye conversion. The signal data were compiled upon completion of the incubation and data exported from the Biolog software into Microsoft Excel. Each experiment was performed in triplicate.

Culture propagation for laboratory scale fermentations

*S. arboricolus*2.3319 and *S. cerevisiae* NCYC2592 were cultured in laboratory scale fermentations for the investigation of formic acid tolerance. Strain for inoculation was prepared as follows; yeast culture was streaked on YPD agar plates and incubated at 30°C for 48 hr to have a distinct colony. A single colony of each strain was aseptically inoculated into 10 mL of YPD broth and incubated on an orbital shaker at 120 rpm at 30°C for 48 hr. The 10 mL culture was transferred into a 250 mL shake flask containing 100 mL of YPD broth and cultured for 48 hr at 30°C, and was finally transferred into 1 L of YPD and cultured at 30°C for 48 hr at 120 rpm. At the end of yeast propagation, the culture was transferred into weighed 500 mL centrifuge tubes and centrifuged at 5000 rpm at 4°C for 5 min using Bechman centrifuge (Model-J2-21). The supernatant was discarded and pellets were washed twice with sterile reverse osmosis(RO) water and centrifuged each time of washing. The supernatant was discarded, pellets and tubes weighed. The weight of pellet was taken by subtracting the weight of empty tube from the weight of pellet and tube. This was designated as the wet weight of pellet. From the wet pellet 0.4% (w/v) of the yeast was used for inoculation according to the method of Oshoma*et al*. (2015).

Experimental procedure

After the inoculum preparation of*S. cerevisiae* NCYC2592 and *S. arboricolus* 2.3319 strains as stated above fermentation was carried out in 150 mL (mini) fermentation vessels as described by Greetham*et al*. (2014).The YNB composition of 0.67% (w/v) YNB (yeast nitrogen base without amino acid and ammonium sulphate) and4%(w/v) glucose was prepared and sterilized using membrane filtration. The media investigated were control medium composed of YNB and 40mM formic acid while the different nitrogen sources media composed of YNB, 40mM formic acid and 0.8 gN/L Urea or ammonium sulphate.

The pH of the media was adjusted to 4.5 using phosphoric acid and/or NaOH under aseptic conditions. From the pH adjusted broth, 100 mL was transferred into mini fermentation vessels (FVs). The prepared 0.4% (w/v wet weight) of yeast pellet was aseptically transferred into each of the bottles. Then the bottle was sealed and equipped with a bubbling CO_2 outlet. All bottles were incubated at 30^oC with orbital shaking at 200 rpm for 24 hr. Samples were collected at specific time intervals to determine the total cell number using methylene blue staining method. At the same time samples were withdrawn and centrifuged (4000 rpm for 5 min). The supernatant was transferred into a tube and frozen at -20^oC. These were analyzed for concentrations of glucose, glycerol and ethanol using HPLC. All fermentations were carried out in triplicate.

Determination of Total cell number of yeast

The total cell number was determined with a haemocytometer according to the method of Sami *et al*. (1994). Methylene blue 0.01% (w/v) was dissolved in sodium citrate 2% (w/v) solution. Yeast broth at various sampling time point was diluted using sterile water. The cell suspension was mixed with methylene blue solution in a ratio 1:1. The solution was examined microscopically and total cells counted using Neubauer haemocytometer (depth 0.1 mm, area 0.0025 mm^2, Marienfield, Germany) and compound light microscope (Zeiss, Oberkocken, Germany) at ×40 objective lens.

HPLC analysis

Glucose, glycerol and ethanol concentrations were determined using a JASCO HPLC system composed of a JASCO AS-2055 Intelligent Auto sampler (JASCO, Essex, UK), and a JASCO PU-1580 Intelligent HPLC pump according to modified procedure of Davis *et al*. (2011). The Rezex ROA organic acid H^+ organic acid column (5µm, 7.8mm × 300mm, Phenomenxe, Macclesfield, UK) was used and the mobile phase was 5mN H_2SO_4 with a flow rate 0.5 mL/minute.

Statistical analysis

All fermentation cultures were carried out in triplicate. Mean and standard deviation of triplicate samples were calculated using Excel (Microsoft, USA). The differences between samples were compared using t-test one-way analysis of variance (ANOVA) (Excel Microsoft, USA). Differences were considered statistically significant when $p<0.05$ (Ogbeibu 2015).

RESULTS

Formic acid and proline effect on the metabolic output of yeast cells.

A range of nitrogen concentrations (0.5 - 1.0 gN/L) was screened to determine an optimum concentration that is suitable to increase the formic acid stress tolerant of *S. cerevisiae* NCYC2592 and *S. arboricolus* 2.3319. The metabolic output profile (Fig. 1) of strains in the presence of different nitrogen concentrations (0.5 – 0.9 gN/L) showed an increase in the redox signal intensity. The highest redox signal intensity was found when concentration was 0.8 gN/L for both Urea and ammonium sulphate. However, there was no significant difference ($p>0.05$) when the nitrogen source concentration increased from 0.5 to 0.9 gN/L. The maximum redox signal intensity of 94.33± 1.51 and 114.33± 3.05 for *S. cerevisiae* NCYC2592 and *S. arboricolus* 2.3319 respectively. From the screened concentrations, 0.8 gN/L was selected as the best concentration that can be added to the medium in order to increase tolerance to formic acid stress.

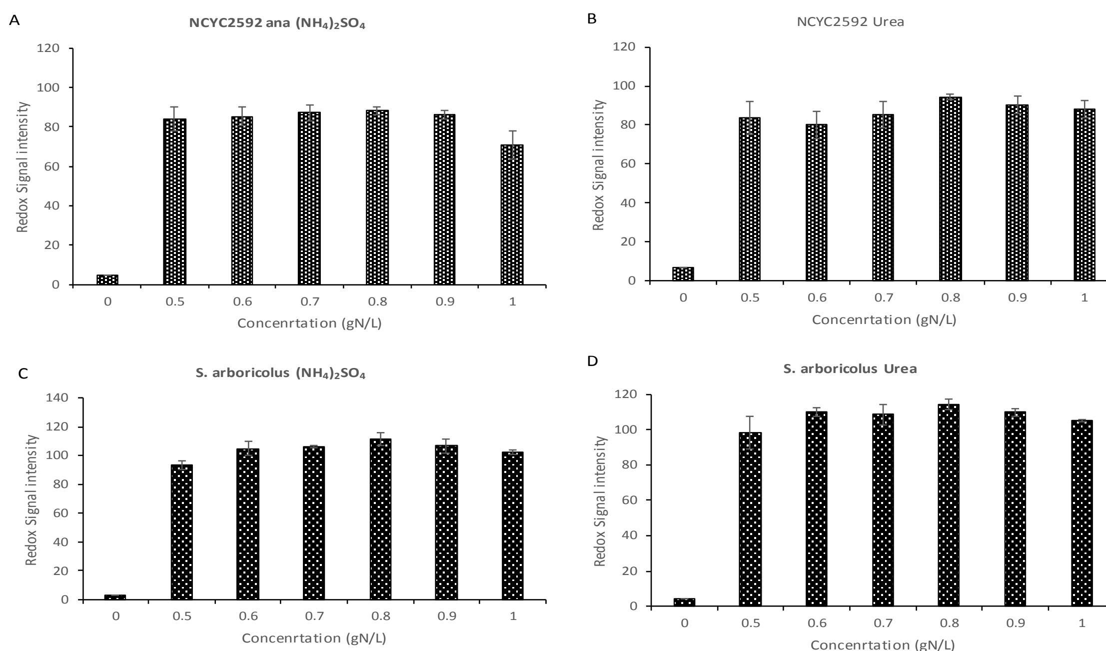


Figure 1: Metabolic output of *S. cerevisiae* NCYC2592 (A and B) and *S. arboricolus* 2.3319 (C and D) on media containing different nitrogen sources (0, 0.5– 1.00 gN/L) and formic acid 40 mM incubated at 30^oC under anaerobic conditions. (Biolog unit = Redox signal intensity). Values indicate the means of three replicates and error bar represents standard deviation.

The impact of formic acid on the fermentation profile of *Saccharomyces* spp

The progress of fermentation was measure routinely by the observation of yeast cell number (Fig. 2). The maximum cell number was 8.17± 0.23x $10^7$and 8.03± 0.42x 10^7 cells/mL for *S. cerevisiae* NCYC2592 when the media were supplemented with ammonium sulphate and urea as nitrogen sources respectively, after 24hr of fermentation.The maximum cell numbers for *S. arboricolus* 2.3319were 8.37± 0.35x 10^7 and 8.68± 0.16x 10^7 cells/mL for media supplemented with ammonium sulphate and urea as nitrogen sources after 12hr of fermentation respectively. By contrast, yeast cell numbers reached maximum of4.00± 0.20x 10^7 and 4.97± 0.08x 10^7 cells/mL in the control media of *S. cerevisiae* NCYC2592 and *S. arboricolus* 2.3319 respectively. Statistically, there was significantly different when comparing the nitrogen sources media to the control medium ($p<0.05$).

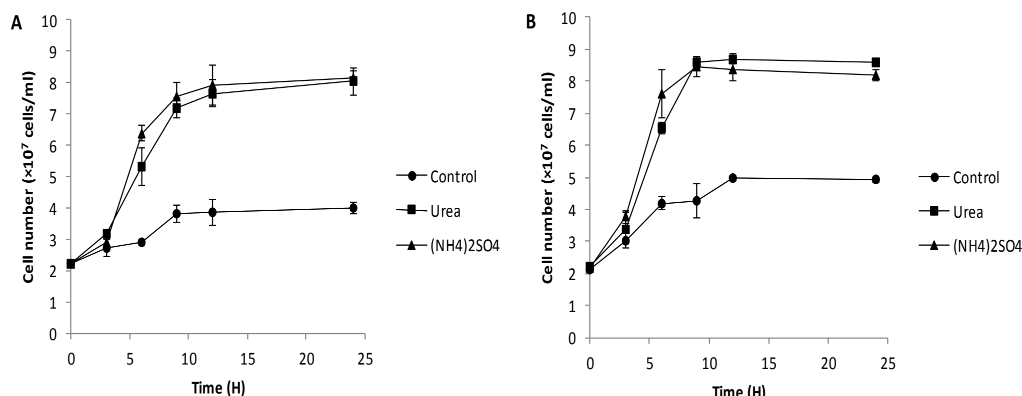


Figure 2: Growth profile during fermentation using *S. cerevisiae* NCYC2592 (A) and *S. arboricolus* 2.3319 (B) with or without nitrogen sources incubated under anaerobic condition at 30 ^{o}C for 24 hrs. Fermentations were carried out in the presence of control (without nitrogen) or 0.80 gN/L and 40 mM formic acid using YNB medium. Values indicate the means of three replicates and error bar represents standard deviation.

The effect of formic acid on glucose consumption

The fermentation profiles of *S. arboricolus* 2.3319 and *S. cerevisiae* NCYC2592 for glucose consumption were compared under different nitrogen sources in the presence of formic acid stress (40 mM). The cellular capacity of yeast strain to utilize glucose was assessed. An assessment of glucose consumption revealed that *S. arboricolus* 2.3319 exhausted the sugar within 8 hr of fermentation while *S. cerevisiae* NCYC2592 completed the utilization after 12 hr (Fig. 3). Glucose consumption rate was observed not statistically significant to the different nitrogen sources. In the control medium, glucose concentration of 5.09± 1.24and 4.95 ± 0.04g/L was observed after 24 hr of fermentation for *S. cerevisiae* NCYC2592 and *S. rboricolus* 2.3319 respectively. This was statistically significant comparing the control medium to the different nitrogen sources media ($p < 0.05$).

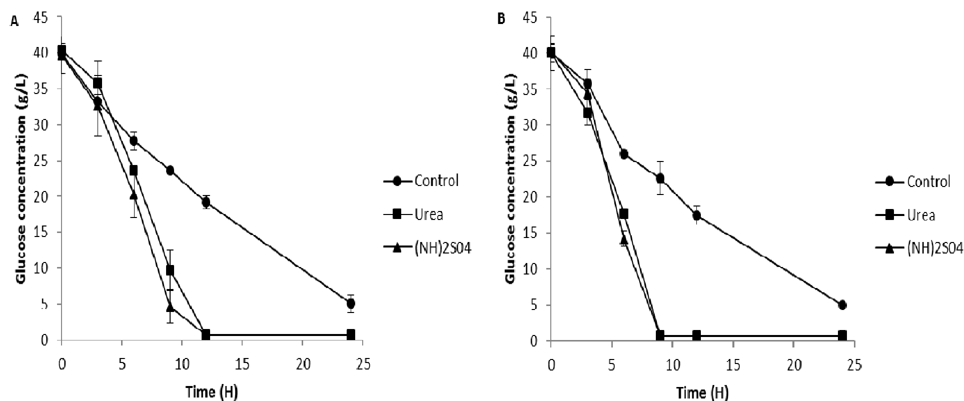


Figure 3: Glucose utilization of *S. cerevisiae* NCYC2592 (A) and *S. arboricolus* 2.3319 (B) during fermentation with or without nitrogen sources. Fermentations were conducted using YNB medium supplemented with 40 mM formic acid and either 0gN/L (control) or 0.80 gN/L nitrogen source. Each fermentation was maintained under anaerobic conditions at 30°C and 200 rpm for 24 hr. Values indicate the mean of three replicates and errors bar represents the standard deviation.

The effect of formic acid on ethanol production by yeast strains

Ethanol accumulation was simultaneously high with the consumption of glucose (Fig 4). Therefore, addition of nitrogen sources to the media increased ethanol production than without nitrogen source by both strains after 24 hr fermentation. Fermentative performance for ethanol production showed some variations among the nitrogen sources. The maximum ethanol production were 20.49± 1.24and 19.74± 0.89 g/L using ammonium sulphate for *S. cerevisiae* NCYC2592 and *S. arboricolus*2.3319 respectively. There was significant difference in ethanol production when compared the nitrogen sources for *S. cerevisiae* NCYC2592 ($p < 0.05$). The production of ethanol by *S. arboricolus*2.3319 showed no much significant difference with the nitrogen sources at $p > 0.05$.The result indicated that addition of ammonium sulphate confirmed tolerance to 40 mM formic acid stress on the yeast cells.

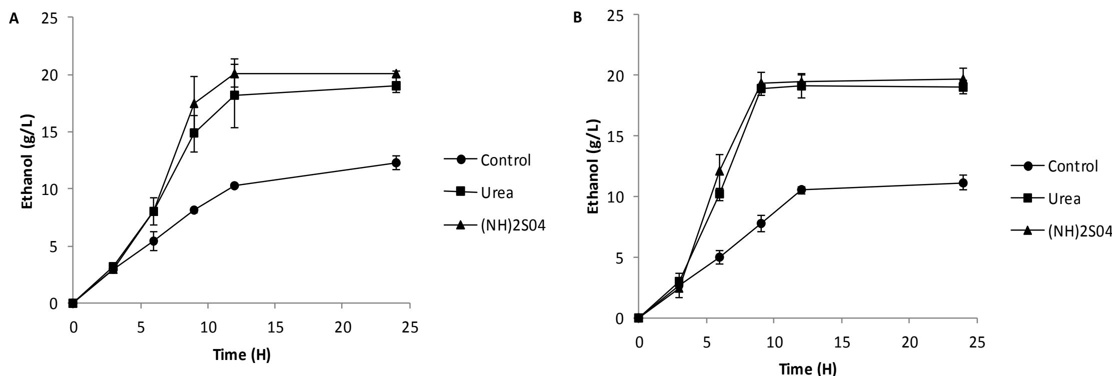


Figure 4: Ethanol production of *S. cerevisiae* NCYC2592 (A) and *S. arboricolus* 2.3319 (B) during fermentation with or without nitrogen sources. Fermentations were conducted using YNB medium supplemented with 40 mM formic acid and either 0gN/L (control) or 0.80 gN/L nitrogen source. Each fermentation was maintained under anaerobic conditions at 30°C and 200 rpm for 24 hr. Values indicate the mean of three replicates and errors bar represents the standard deviation.

The effect of formic acid on glycerol production byyeast strains

Glycerol, one of the metabolites formed during fermentation, was also determined (Fig 5). Nitrogen sources added to the media increased glycerol production than without nitrogen source by both strains after 24 hr fermentation. Production of glycerol showed some variations among the nitrogen sources. The highest glycerol produced were 2.21± 0.04 and 3.11± 0.05 g/Lusing ammonium sulphate for *S. cerevisiae* NCYC2592 and *S. arboricolus*2.3319 respectively. Statistically, there was significant difference in glycerol production when comparing the nitrogen sources for *S. cerevisiae* NCYC2592 ($p < 0.05$). The production of glycerol by *S. arboricolus* 2.3319 showed no significant difference with the nitrogen sources at $p < 0.05$.The result indicated that addition of ammonium sulphate confirmed tolerance to 40 mM formic acid stress on the yeast cells.

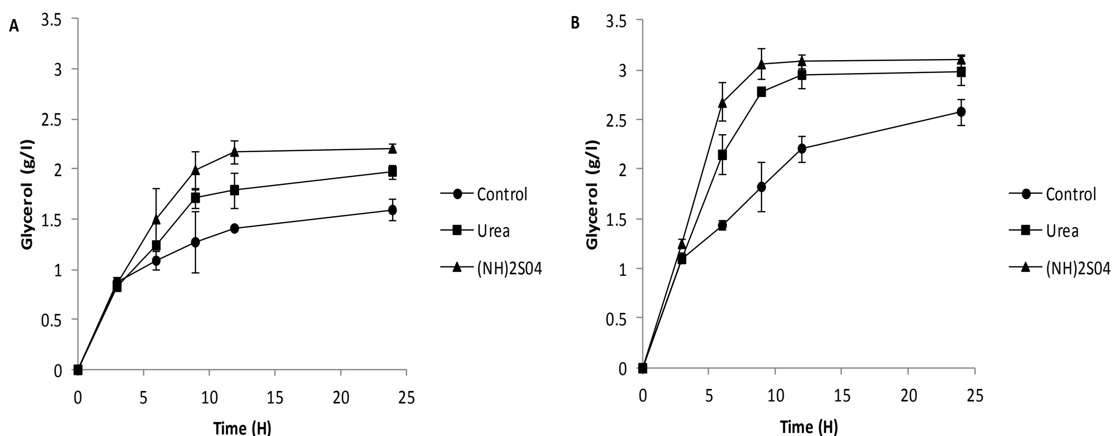


Figure 5: Glycerol production of *S. cerevisiae* NCYC2592 (A) and *S. arboricolus* 2.3319 (B) during fermentation with or without nitrogen sources. Fermentations were conducted using YNB medium supplemented with 40 mM formic acid and either 0gN/L (control) or 0.80 gN/L nitrogen source. Each fermentation was maintained under anaerobic conditions at 30°C and 200 rpm for 24 hrs. Values indicate the mean of three replicates and errors bar represents the standard deviation.

DISCUSSION

This work was investigated through media supplementation with the aim that ammonium sulphate and urea will supply the assimilable nitrogen for cell tolerant to formic acid during ethanol production. The result showed that increase in metabolic output when the nitrogen source was increased to 0.8 gN/L and gradual decreased after this concentration. This collaborates with previous work that increased nitrogen sources concentration to 0.8 gN/L improved ethanol production during fermentation stress (Yue *et al.*, 2012).

Evaluating the effect of ammonium sulphate and urea on yeast tolerant to formic acid for ethanol production showed that ammonium sulphate improved yeast tolerance and produced more ethanol than urea. Previous work also investigated that nitrogen supplementation influences yeast strains tolerance to fermentation stress (Albers *et al.*, 1996; Greetham *et al.*, 2014). It was observed that the nitrogen sources enable the yeast strains to utilize the glucose faster and increased ethanol production than the control medium. Probable reason may be that the strains were able to use the nitrogen sources to meet their protein and nucleic acid requirement (Akponah *et al.*, 2012). The control medium was deficient of nitrogen source, hence lower cell growth, poor glucose utilization and lower ethanol yield. Based on the data presented, it is important to supplement media with nitrogen sources in order to overcome formic acid stress. The presence of formic acid, as one of the weak acids in the hydrolysate from lignocellulose biomass cannot be avoided (Palmqvist and Hahn-Hagerdal 2000b; Almeida *et al.*, 2007). During pretreatment and hydrolysis processes, fermentable sugars are liberated and generated weak acids as inhibitors (Almeida *et al.*, 2007). This paper demonstrated that nitrogen sources enable yeast cells to cope with formic acid and fermentation stresses during ethanol production. It was observed that in the control medium, yeast cells were not able to utilize the glucose completely after 24 hr hence lower yield of ethanol.

Ammonium sulphate and urea supplementation was used in high sugar fermentation under controlled conditions that led to the contribution of assimilable nitrogen (Bely *et al.,* 2003). Therefore, lignocellulosic hydrolysates are found to be low in nutrient and nitrogen with Coffee Pulp waste pretreatment contributed only about 0.056% nitrogen content (Hamadi *et al.*, 2014). The addition of nitrogen sources into the hydrolysate deficient of nitrogen may be economically useful during ethanol production from lignocellulose biomass. Greetham *et al.* (2014) observed that proline, a nitrogen source, addition improved yeast tolerance to acetic acid and furfural stress during fermentation.

Higher amount of glycerol was produced with the nitrogen sources than the control media. Glycerol is one of the major by-products produced by yeast cells during fermentation (Chen *et al* 2014). The consequence of glycerol accumulation was to improved cell biomass when stressed in order to increase their tolerance level (Tomas-Pejo *et al.*, 2010). Glycerol production demonstrated that certain amount of carbon was diverted for other product formation instead of ethanol (Keating *et al.*, 2006). Glycerol plays the role of compactible solute when cells are stressed (Chen *et al.*, 2014). The control medium produced lower amount of glycerol compared to the nitrogen sources media. Martin *et al.* (2002) suggested that production of lesser amount of glycerol could be the consequence of low cell number in the control medium due to poor availability of assimilabe nitrogen. Ammonium sulphate accumulated more glycerol than Urea. *S. arboricolus* 2.3319 accumulated more glycerol than *S. cerevisiae* NCYC2592. This implies that production of glycerol in ammonium sulphate medium than urea may act as a determinant of acid tolerance. The glycerol is acting as a polyol to the cells which enhances strain tolerance to formic acid (Walker, 1998). This study agreed with the report of Tomas-Pejo*et al.* (2010) that increase in glycerol production contributed to strain tolerance to inhibitory compounds found in lignocellulose hydrolysate which resulted to better cell growth.

CONCLUSION

The results of the investigation showed that formic acid as an inhibitory compound in the lignocellulose hydrolysate negatively impact yeast fermentative performance. The inhibitory effect was seen in cell number, glucose consumption and ethanol production in the control (without nitrogen) medium. It could be concluded that nitrogen sources such as ammonium sulphate influences yeast cells tolerance to formic acid and fermentation stress thus, producing higher amount of ethanol.

Acknowledgement

The authors thank the financial support provided by Tertiary Educational Trust Fund from the University of Benin, Benin City, Nigeria. We acknowledged the support of Bioenergy and Brewing Science building, University of Nottingham, Nottingham, UK, for providing the research laboratory.

REFERENCES

Almeida, J.R.M., Modig, T., Peterson, A., Hahn-Hagerdal, B., Liden, G. andGorwa-Grauslund M.F. (2007) Increase tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *Journal of Chemistry, Technology andBiotechnology*82(4): 340 – 349.

Akponah, E. and Akpomie, O. O. (2012). Optimization of bio-ethanol production from cassava effluent using *Saccharomyces cerevisiae*. *African Journal of Biotechnology*11(32): 8110-8116.

Albers, E., Larsson,C., Liden, G., Niklasson,C. andGustafsson, L. (1996).Influence of the Nitrogen Source on *Saccharomyces cerevisiae* anaerobic growth and product formation *Applied and Environmental Microbiology*62 (9): 3187–3195.

Bely, M., Rinaldi, A. and Dubourdieu, D. (2003). Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation, *Journal of Bioscience and Bioengineering*96(6): 507–512.

Chen, X., Li, Z., Zhang, X., Hu, F., Ryu, D.D.Y. andBao, J. (2009) Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. *Applied Biochemistry and Biotechnology*159: 591 – 504.

Chen, D., Chia, J.Y. and Liu, S.Q. (2014). Impact of addition of aromatic amino acids on non-volatile and volatile compounds in lychee wine fermented with *Saccharomyces cerevisiae* MERIT. ferm. *International Journal of Food Microbiology* 170:12–20

Davies, S.M., Linforth, R.S., Wilkinson, S.J., Smart, K.A. and Cook, D.J. (2011). Rapid analysis of formic acid, acetic acid, and furfural in pretreated wheat straw hydrolysates and ethanol in a bioethanol fermentation using atmospheric pressure chemical ionisation mass spectrometry. *Biotechnology and Biofuels*4:28

Gibson, B.R., Lawrence, S.J., Leclaire, J.P., Powell, C.D. and Smart K.A. (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Review* 31: 535 – 569.

Greetham, D., Takagi, H. andPhister, T.P. (2014). Presence of proline has a protective effect on weak acid stressed *Saccharomyces cerevisiae*. *Antonie vanLeeuwen*. DOI 10.1007/s10482.

Hahn-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I. and Gorwa- Grauslund, M.F. (2007). Towards industrial pentose-fermenting yeast strains. *Applied Microbiology andBiotechnoogy.*74: 937-953.

Hamadi, S., Muruke, M.H. and Hosea, K.M.M. (2014). Optimization of fermentation parameters for production of ethanol from Coffee Pulp Waste using *Pichia anomala* M4 Yeast isolated from Coffee environment in Tanzania. *International Journal of Environmental Sciences* 3 (4): 255-262.

Jonsson, L.J. and Martins, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*199: 103 – 112.

Kapu, N.S., Piddocke, M. and Saddler, J.N. (2013). High gravity and high cell density mitigate some of the fermentation inhibitory effects of softwood hydrolysates. *AMB Express* 3: 15 – 23.

Keating, J.D., Panganiban, C. and Mansfield, S.D. (2006). Tolerance and adaptation of ethanologenic yeasts to lignocellulosic inhibitory compounds. *Biotechnology and Bioengineering* 93: 1196- 1206.

Martin, C., Fernandez, T., Garcia, R., Carrillo, E., Marcet, M., Galbe, M. and Jonsson, L.J. (2002). Preparation of hydrolysates from tobacco stalks and ethanolic fermentation by *Saccharomyces cerevisiae. World Journal of Microbiology andBiotechnology*18: 857 -862.

Moiser, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresourxe Technology*96: 673-686.

Ogbeibu, A.E. (2015). Biostatistics: *A Practical Approach to Research and Data Handling* 2nd edition. Mindex Publishing Company Lagos, Nigeria. 264pp.

Oshoma, C.E., Greetham, D., Louis, E.J., Smart, K.A., Phister, T.G., Powell, C. and Du, C.(2015). Screening of non-*Saccharomyces cerevisiae* strains for tolerance to formic acid in bioethanol fermentation *PLoS One* 10(8):e0135626.

Palmqvist, E. and Hahn-Hagerdal, B. (2000a). Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. *Bioresource Technology* 74: 25–33.

Palmqvist, E. and Hahn-Hagerdal, B. (2000b). Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresource Technology*74: 17–24.

Sami, M., Ikeda, M. and Yabuuchi,S. (1994). Evaluation of the alkaline methylene blue staining method for yeast activity determination. *Journal of Fermentation andBioengineering*78: 212-216.

Schure, E.G., van Riel, N.A.W. and Verrips, C.T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae. FEMS Microbiology Reviews*24: 67-83.

Thomas, K.C. and Ingledew, W.M. (1990). Fuel alcohol production: effects of free amino acid nitrogen on fermentation of very-high-gravity wheat mashes. *Applied and Environmental Microbiology*56: 2046 – 2050.

Tomas-Pejo, E., Ballesteros, M., Oliva, J.M. and Olsson, L. (2010). Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes. *Journal of Industrial Microbiology and Biotechnology*37: 1211 – 1220.

Wimalasena, T.T., Greetham, D., Marvin, M.E., Liti, G., Chandelia, Y., Hart, A., Louis, E.J., Phister, T.G., Tucker, G.A. and Smart, K.A. (2014). Phenotypic characterization of *Saccharomyces* spp. yeast for tolerance to stresses encountered during fermentation of lignocellulosic residues to produce bioethanol. *Microbial Cell Factories* 13:47.

Yue, G., Yu, J., Zhang, X and Tan, T. (2012). The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice. *Biomass and Bioenergy*39**:** 48 – 52.

Walker, G.M. (1998).*Yeast Physiology and Biotechnology*. John Wiley and Sons, Chichester, New York, 350pp.