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Full Length Research Paper

Selection and characterisation of high ethanol tolerant *Saccharomyces* yeasts from orchard soil

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Six yeast strains with different levels of ethanol tolerance were isolated from orchard soil. Out of the six isolates, isolate Orc 6 showed the highest ethanol tolerance (20%) while isolates Orc 2 and Orc 11 had 15% ethanol tolerance. High level ethanol tolerant *Saccharomyces* yeast, Orc 6, was investigated for its potential application in ethanologenic fermentations. Data presented in this study revealed that Orc 6 yeast isolate tolerated osmotic stress above 12% (w/v) sorbitol and 15% (w/v) sucrose equivalent of osmotic pressure thus exhibiting superior osmotolerance than the reference production wine yeast strain. Invertase activity was also higher for Orc 6 yeast when grown in both sorbitol and sucrose media. Sorbitol increased yeast sedimentation rate in contrast to sucrose. Generally, the new yeast strain, Orc 6, showed superior fermentative performance compared to the reference production yeast strain.

Key words: *Saccharomyces* yeasts, osmotic pressure, invertase activity, sedimentation rate, sucrose.

INTRODUCTION

Over the past two decades most bioethanol related researches in Nigeria and many other developing tropical countries have focused primarily on the isolation of local *Saccharomyces* yeasts and their use for industrial ethanol production (Bulawayo et al., 1996; Ezeogu and Emeruwa, 1993; Ezeogu and Okolo, 1994a, b; Okafor, 1987; Sefa-Dedeh et al., 1999). Yeasts have been isolated from many sources for industrial purposes. Such sources include cashew, apple juice (Osho, 2005), palm wine (Bechem et al., 2007; Nwachukwu et al., 2006) and fermenting cassava tubers (Oyewole and Odunfa, 1988) among others. Despite the evolving trend of using bacteria for ethanol production, yeast is still the primary choice for fermentation (Chandra and Panchal, 2003). Yeasts are used in the fermentative production of ethanol, alcoholic beverages, baking products, protein and vitamin supplements in human and animal diets as well as in the production of single cell proteins. However, efforts to characterize these yeasts have fallen short of expectation.

In the assessment of yeasts of the genus *Saccharomyces* for economic and efficient ethanologenic processes, certain specific physiological properties are important and required. These include good tolerance to high concentrations of ethanol, sugars and acids as well as high osmotic pressure (Ansanay-Galeote et al., 2001; Benitez et al., 1983; Ezeogu and Okolo, 1984a; Okolo et al., 1990; Okolo et al., 2004 and Stewart et al., 1984). Also good flocculation/sedimentation ability depending on process requirements as well as good invertase activity and excellent specific ethanol productivity are important characteristics of yeasts capable of converting sucrose to ethanol (Jameonoz and Benitez, 1986).

This study therefore, was aimed at selecting *Saccharomyces* yeast strains from Nsukka orchard soil with potentials for industrial ethanol production.

METHODS

Yeast strains and culture conditions

Yeast strains were isolated from University of Nigeria, Nsukka orchard soil. Isolation was performed on glucose–yeast extract–

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Table 1. Ethanol tolerance of orchard soil isolates of *Saccharomyces* yeasts.

Yeast designation	Mean maximum ethanol tolerance (% v/v)	Relative ethanol tolerance (%)
Orc 1	12.6	63.0
Orc 2	15.0	75.0
Orc 3	13.2	66.0
Orc 6	20.0	100.0
Orc 11	15.0	75.0
Orc 10	14.4	72.0

mineral salts agar medium (Okafor, 1987) following a 24 h enrichment step in 25 ml YPD broth (Hayashida et al., 1975) containing chloramphenicol to inhibit bacterial growth. Cultures were routinely maintained by regular subculture on YPD medium. The conventional methods described by Lodder (1970) and Kreger van Rij (1984) were used in identifying the isolates as *Saccharomyces* species. The medium described by Bajpai et al. (1988) was used for determination of ethanol tolerance. This medium was supplemented with 35 gl^{-1} soybean meal. Media sterilization was by autoclaving at 121°C for 15 min at 15 psi.

Ethanol tolerance assay

Yeast ethanol tolerance was assayed according to the maximum ethanol production method of Hayashida et al. *(*1975). Aliquots of culture inocula cultivated without soybean meal supplementation and containing 1 x 10^8 cells were used to inoculate 100 ml sterile fermentation medium in 250 ml Erhlenmeyer flasks. Fermentation lasted 16 days at 30°C under static culture conditions. Flasks were fitted with fermentation bungs partially filed with concentrated H_2SO_4 to trap vapourised ethanol and water. Fermentation rate was calculated by measuring daily decreases in whole culture weight. During the fermentation, sucrose was added stepwise to maintain a concentration of 12 - 20% (w/v) in the middle stages of the fermentation and 3.5% (w/v) in the final stages (Hayashida et al., 1975). Sucrose level was calculated using the Gay Lussac's equation described by Hayashida et al., 1974). Ethanol was estimated as described previously by Ezeogu and Emeruwa (1993).

Effect of osmotic stress on yeast growth

Portions of YPD cultures containing 1 x 10^8 cells and grown for 24h at 30°C were used to inoculate 100ml volumes of YPD media supplemented with sorbitol in the range 0 - 25% (w/v) or 0 – 40% (w/v) sucrose. To evaluate the effect of osmotic pressure on yeast growth rate, samples were withdrawn from the fermenting culture at 4 h intervals and yeast cell mass measured at 660 mm using a spectrophotometer (Spectronic-20). The effect of osmotic stress on yeast growth rate was calculated as the logarithmic growth constant (k) in the particular medium as described by Ezeogu and Okolo (1994b).

Effect of osmotic stress on yeast invertase activity

Yeasts were grown for 48 h as described above and then harvested by centrifugation at 5,000 g for 10 min. Cells were then washed three times in phosphate buffered saline (pH 7.0) before resuspension in the same buffer to an optical density of 0.13 at 660 nm using a spectrophotometer (Spectronic-20). 1 ml of the cell suspension was used to assay for invertase activity according to the method described by Ezeogu and Okolo (1994a) using 2 ml of 4% (w/v) sucrose solution in a 0.1 M sodium acetate buffer, pH 5.6. Reducing sugar was determined by the method of Nelson and Somogyi (1952). One unit of invertase activity was defined as any amount of enzyme capable of producing 1 µmol glucose equivalent per min under the assay conditions.

Effect of osmotic stress on sedimentation rate

Yeast isolates were grown for 24 h at 30°C on MYGP broth with or without sorbitol in the range 5 - 25% (w/v) or 0 - 4% (w/v) sucrose. Cells were harvested by high-speed centrifugation at 16,000 g for 10 min at 4°C and used for preparing standard cell suspensions of about 1 x 10^8 cells/ml in 0.9% saline. These suspensions were then used to measure the reduction/decrease in optical density reading over a period of 2 h at 660 mm (Ezeogu and Okolo, 1994b) using a Corning Colorimeter Model 253. The sedimentation rate was calculated using the following formula:

$$\% SR = \frac{\text{Total drop in colorimeter reading}}{\text{Colorimeter reading at 0 h}} \times 100$$

Where SR = sedimentation rate.

Effect of sucrose level on yeast ethanol productivity

Yeast isolates were grown on YPD medium with or without sucrose in the range 5 – 45% (w/v) for 72 h. Cells were harvested by centrifugation at 400 g for 10 min. The cell free supernatant was assayed for ethanol using the method described by Ezeogu and Emeruwa (1993). Ethanol productivity was calculated as a percentage of total medium sugar converted to ethanol.

Statistical analyses

Statistical analyses were by the Student t-test. Correlation analyses were performed according to procedures described by Cohen (1988).

RESULTS

Ethanol tolerance of yeast isolates

Ethanol tolerance of six yeast isolates form Nsukka orchard soil identifies as *Saccharomyces* yeasts according to the method of Lodder (1970) and Kreger Van Rij (1984) were studied using maximum ethanol production method of Hayashida et al. (1975). A total of six morphologically different yeast strains Orc 1, Orc 2, Orc 3, Orc 6, Orc 10 and Orc 11 were isolated from orchard soil. Data presented on Table 1 indicate that the yeast isolate orc 6, at a maximum ethanol production level of 20% (w/v), was the most ethanol tolerant of all yeasts examined. The isolates orc 11, orc 12, orc 10, orc 3 and orc 1 at 15, 14, 13 and 12% (v/v) ethanol respectively were between 20 and 37% less tolerant to ethanol than orc 6. The result re-

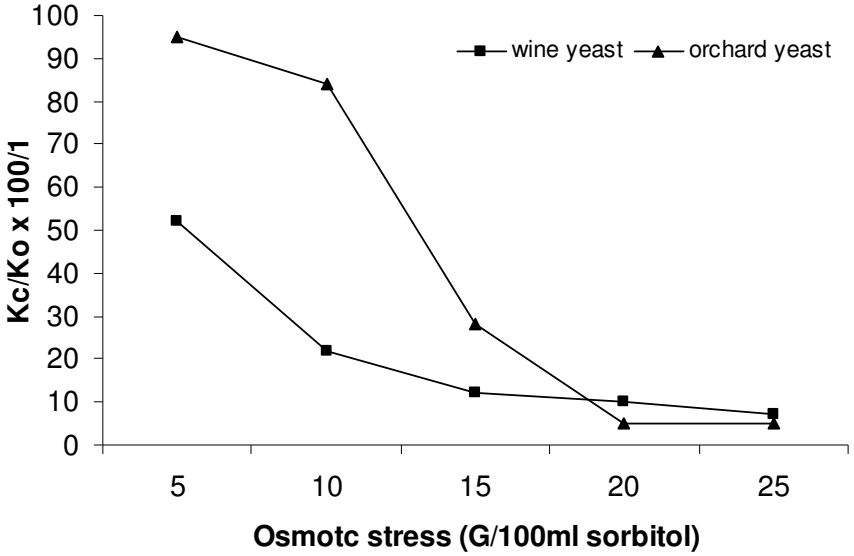

Figure 1. Effect of sorbitol level on the mean log growth rate (Kc) expressed in % of rates in basal medium.

vealed that Orc 1 had the least ethanol tolerance compared to other yeast strains.

Effects of osmotic stress on yeast growth

The effects of osmotic stress on the growth of yeast orc 6 were investigated in view of the osmotic pressure build up in fermentation systems designed to favour high volumetric ethanol productivity. An industrial wine yeast *Wy*, obtained from the Department of Microbiology, University of Nigeria, Nsukka culture collection was used as reference yeast. The non-metabolizable sugar, sorbitol at 0 - 25% (w/v) was added to the basal medium to build up the osmotic pressure. The results are depicted in Figure 1 where it can be seen that yeast growth rate declined progressively as medium osmotic pressure was increased above 5% (w/v) sorbitol elicited nearly 50% decrease in yeast specific growth rate compared to control values. Subsequent increase in medium sorbitol up to 25% (w/v) caused a progressive reduction in the logarithmic growth rate constant of the yeast up to 96% at 25% (w/v) sorbitol. The wine reference yeast showed better tolerance to osmotic pressure than the test Orc 6 yeast strain up to 15% (w/v) sorbitol equivalent of osmotic pressure. Beyond this level however, the reference yeast exhibited greater sensitivity to osmotic pressure effects compared to Orc 6.

Effect of sucrose on yeast growth kinetics was evaluated. Varying concentrations of sucrose were added to the basal medium in place of sorbitol. As shown in Figure 2, the logarithmic growth rate constant (k) for both yeast strains was reduced progressively as the level of sucrose in the medium was increased. However, beyond 12% (w/v) medium sucrose concentration, the orchard yeast isolate orc 6, was significantly more tolerant of the osmotic stress induced by the sugar than did the control yeast strain. Furthermore, sucrose appeared to elicit lower inhibitory effects on the yeasts than sorbitol when applied at equal concentration presumably indicating differences in osmotic properties of the different sugars.

Effect of osmotic stress on invertase activity

The effects of increasing osmotic pressure on the activity of Invertase, a major enzyme in yeast ethanologenic conversion of sucrose, were therefore investigated. Figure 3 summarizes the results obtained form fermentations conducted with various concentrations of sorbitol. Yeast invertase activity increased as the concentration of the medium sorbitol was increased for both test and reference yeast strains. Growth in media of osmotic pressure range 0 - 25% (w/v) sorbitol equivalent elicited 87 to 175% and 39 to 74% enhancements in yeast invertase activity for *orc* 6 and the reference wine yeast respectively. Nevertheless, the reference yeast expressed significantly higher invertase activity than the orchard soil yeast at virtually all the sorbitol levels studied except at the 10% sorbitol concentration where identical values of invertase activity were recorded for both yeasts.
Peak invertase activity was attained at the 20% (w/v) sorbitol equivalent of osmotic pressure for both yeasts.

The use of sucrose as sole carbon substrate and the effects of different concentrations of this sugar on the elaboration of yeast invertase activity was observed as

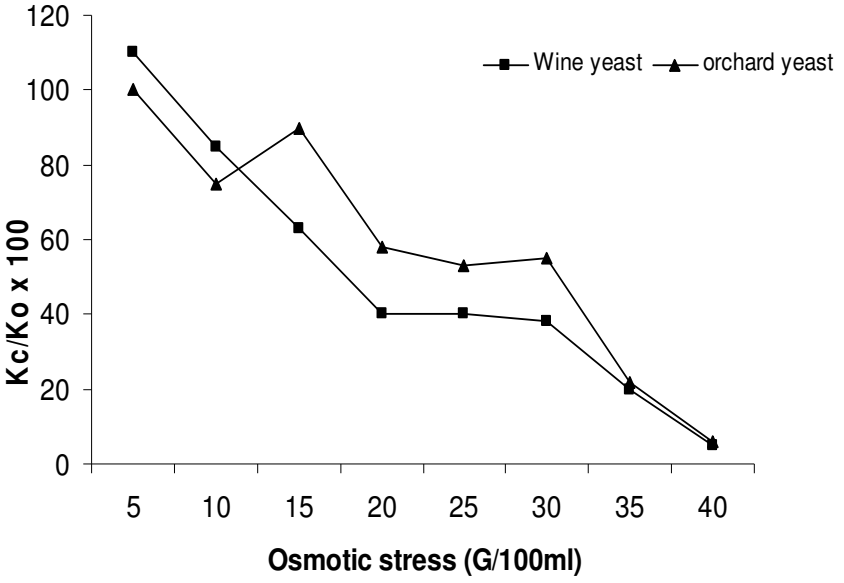

Figure 2. Effect of sucrose level on the mean log growth rate (Kc) expressed in % of rates in basal medium.

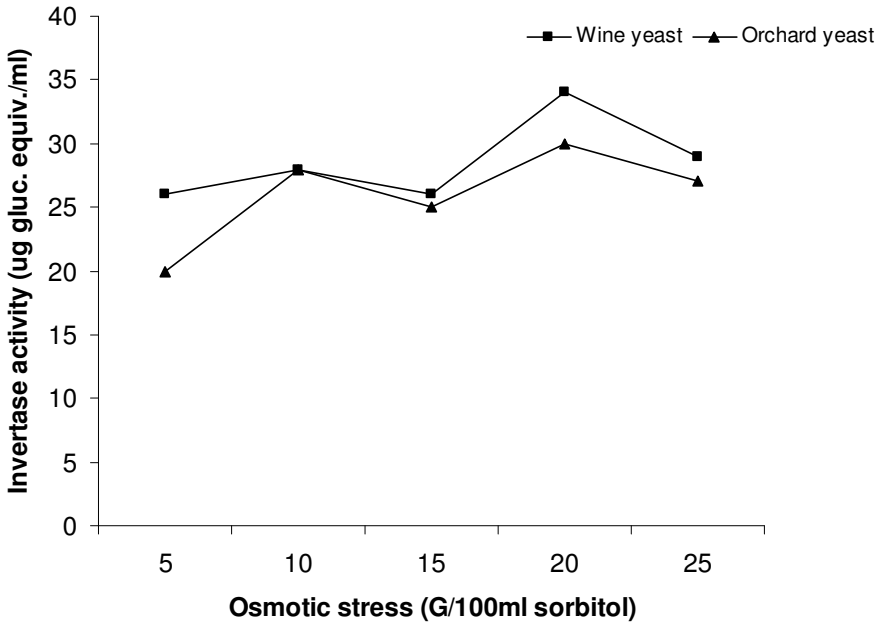

Figure 3. Effect of medium sorbitol on yeast invertase activity.

increasing levels of sucrose was applied. However, as shown in Figure 4, sucrose concentrations ranging from 0 to 40% (w/v) generally caused repression of invertase activity for both yeasts. Although a slight increase in the enzyme activity was observed at 15% (w/v) medium sucrose supplementation, enzyme activity decreased by 10 – 58% and 6 – 30% for orc 6 and reference yeast respectively due to sucrose supplementation.

Effect of sugar levels on sedimentation rates

The effects of medium sugar concentration on sedimentation rates of our orc 6 yeast isolate were evaluated

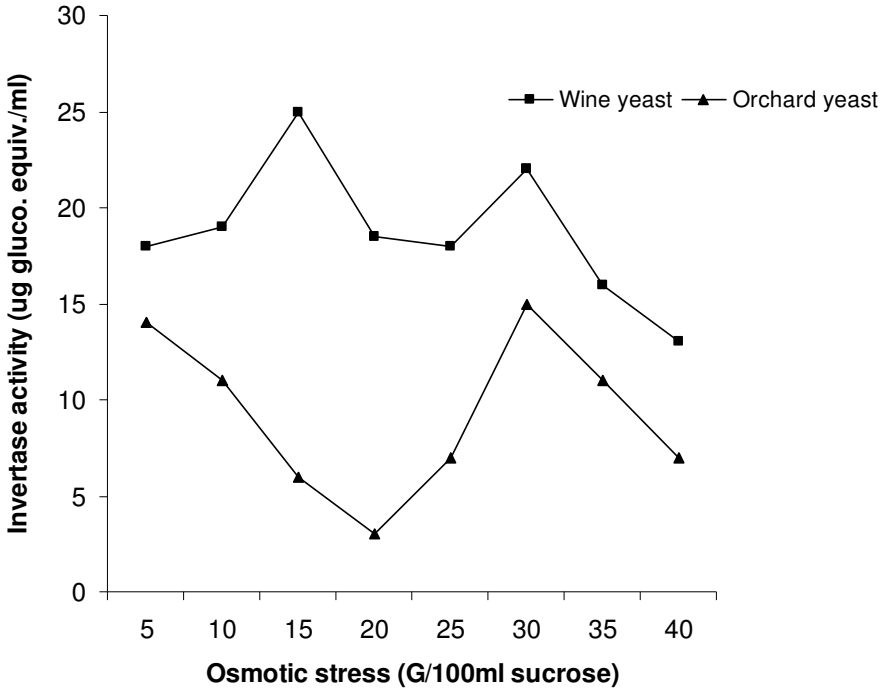

Figure 4. Effect of medium sucrose on yeast invertase activity.

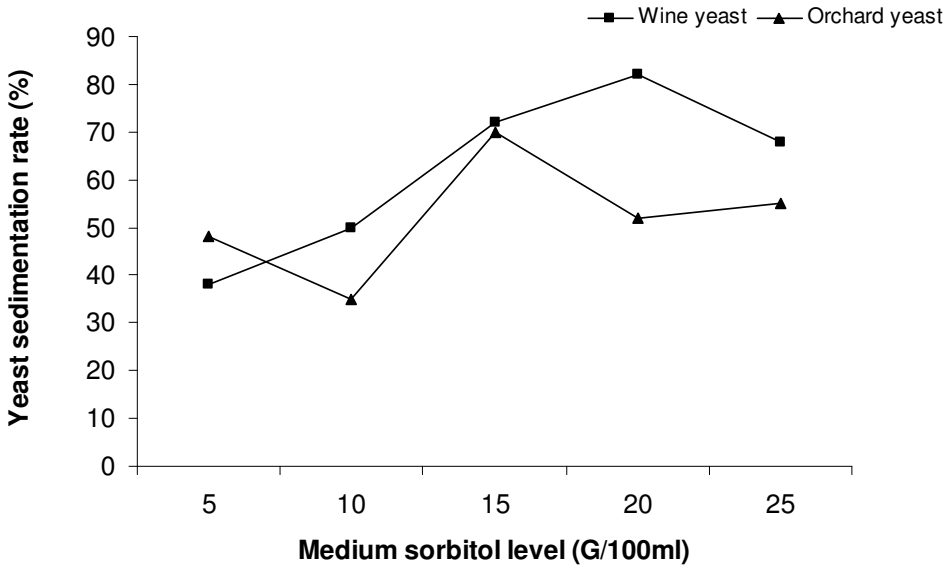

Figure 5. Effect of medium sorbitol level on yeast sedimentation rate.

against values for the reference wine yeast. As shown in Figure 5, sedimentation rate was enhanced by sorbitol at all levels of supplementation for both yeasts. Sedimentation rates were improved 3- to 5- folds when the yeasts were cultivated in media containing various levels of sorbitol compared to values for unsupplemented basal medium for orc 6 and *Wy* reference yeasts, respectively. The effects of sucrose levels on sedimentation rates were also studied for the two yeasts. Data illustrated in Figure 6 revealed that sedimentation rate increased as sucrose concentration was increased at all levels of sucrose in the medium relative to the basal medium.

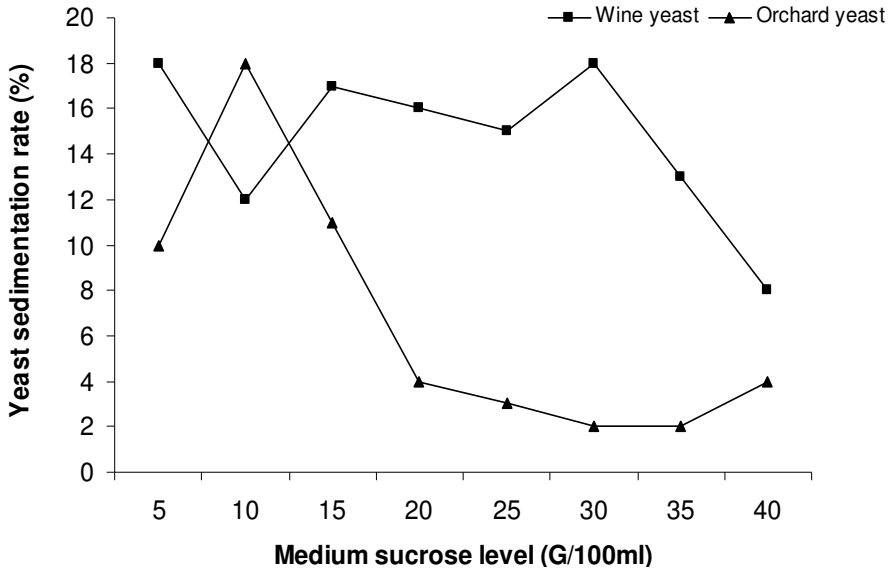

Figure 6. Effect of medium sucrose level on yeast sedimentation rate.

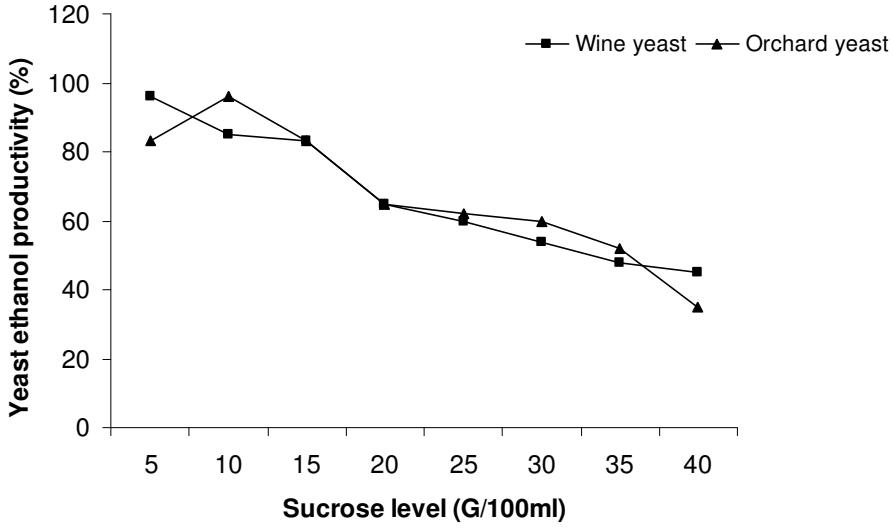

Figure 7. Effect of sucrose level on yeast ethanol productivity.

Effect of sucrose concentration on yeast ethanol productivity

The effects of increasing medium sucrose concentration and the proportion of total medium sugar converted to ethanol were studied for our orchard yeast and the reference yeast. As depicted in Figure 7, both yeasts exhibited similar sucrose to ethanol conversion ability under all conditions evaluated. However, orc 6 yeast isolate showed slightly superior capacity to convert sucrose to ethanol up to 30% (w/v) sucrose above which the reference yeast exhibited better ethanol productivity.

DISCUSSION

High level of ethanol production by non-industrial *Saccharomyces* yeasts isolates from palm wine juice had been reported by Ezeogu and Emeruwa (1993) in a sake-type fermentation, but none from yeasts isolated from orchard soil. Like the palm wine yeast, orc 6 yeast isolate exhibited remarkably high ethanol tolerance comparable

to industrial yeasts such as sake and distiller's yeasts associated with high level ethanol tolerance (Casey and Ingledew, 1986; Flor and Hayashida, 1983; Hayashida et al., 1974). The level of ethanol tolerance of 20% by yeast strain Orc 6 is in agreement with the report of Nwachukwu et al. (2006) on *S. cerevisiae* isolated from raffia palm wine but differed with 15 and 10% ethanol concentration observed for yeast isolates Vip 8 and Vip 9 by Bechem et al. (2007) and fermenting cassava tuber yeasts by Ekunsanmi and Odunfa (1990), respectively. It is interesting to note strains Orc 1, Orc 3 and Orc 10 showed no growth at 15% ethanol concentration. Similar inhibitions of growth at 15% ethanol have been observed for other yeasts (Bulawago et al., 1996 and Bechem et al., 2007).

The wine reference yeast showed better tolerance to osmotic pressure than the test orc 6 yeast strain up to 15% (w/v) sorbitol equivalent of osmotic pressure. Beyond this level however, the reference yeast exhibited greater sensitivity to osmotic pressure effects compared to orc 6. Some workers (D' Amore et al., 1988; Dombek and Ingram, 1986) have reported changes in the growth dynamics of yeasts upon exposure to various osmotic stress conditions. The decrease in logarithmic growth rate constants of the test yeasts in relation to increasing osmotic pressure is therefore consistent with the views expressed by these workers (Osho, 2005).

Furthermore, sucrose appeared to elicit lower inhibitory effects on the yeasts than sorbitol when applied at equal concentration presumably indicating difference since osmotic properties of different sugars. These observations are in reasonable agreement with the observations of Jacobson and Piper (1989) who advanced that equal concentrations of different sugar elicited different inhibitory actions on yeast growth rates and this may be related to differences in osmotic properties of the sugars. Our results on sucrose tolerance is consistent with the observation of Bechem et al. (2007) and Nwaga et al. (1998) with regard to optimum sugar concentration for specific growth rate of yeast strain Vip 10 and alcohol production from starch breakdown by amylases, respectively.

Differences in the pattern of yeast sensitivity to increasing substrate concentration were also observed with the two sugars. For instance, the reference yeast showed higher sensitivity to increasing sucrose concentration at 15% (w/v) and above contrary to results obtained with sorbitol (Figure 1). It is possible that in addition to osmotic pressure effects, factors such as ethanol production and formation of other inhibitory by-products such as higher alcohols, acetic acid etc. (Okolo et al., 1987; Verdyum et al., 1990; Blieck et al., 2007) could have been responsible for potentiation of osmotic stress in the sucrose medium.

Physiochemical conditions operating during ethanologenic fermentations are believed to affect the activities of major enzymes involved in the process (Dombek and Ingram, 1986; Nagodawithana and Steinkraus, 1976). Correlation analyses confirmed a highly significant positive relationship between yeast invertase activity and medium osmotic pressure for both yeasts at $r = 0.83$ for orc 6 and $r = 0.80$ for the reference yeast. The significant positive displacement of the intercept in favour of the reference yeast indicates that this yeast possessed greater capacity to elaborate invertase activity than orc 6. Significant differences in slopes of the regression lines for both yeasts indicate marked differences in the capacities of the yeasts to respond positively to every unit increase in medium osmotic pressure.

The pattern of response of yeast invertase activity to increased osmotic pressure due to sorbitol was similar indicating identical responses to sorbitol. High medium sugar levels and elevated osmotic stress along with high temperature and ethanol concentration (Larue et al., 1984) are among several physiochemical conditions associated with reduction of yeast membrane stability leading to suppression of the activity of membrane bound enzymes including invertase. It is therefore striking that as illustrated in Figure 3, application of such high concentrations of sorbitol caused significant improvements in yeast invertase activity. While an authoritative explanation for this behaviour cannot be immediately proffered, it is plausible that the addition of sugar up to a certain break point/threshold value would cause improved yeast invertase activity. Such a value may vary with type of sugar. However, high invertase activity is essential when yeast is to be used in the production of ethanol from sucrose-based substrates (Ekunsanmi and Odunfa, 1990; Harrison and Graham, 1970).

These observations were confirmed by correlation analyses data that revealed a negative relationship between yeast invertase activity and medium sucrose supplementation. The reference yeast, however, showed a better correlation at $r = 0.6$ compared to $r = 0.31$ for orc 6 thus supporting the view that growth on sucrose medium produced no distinct pattern for yeast invertase development. Data presentation in this study further suggests that sucrose and sorbitol differ in their modes of influence on yeast invertase development under high osmotic stress conditions. Sucrose is a fermentable sugar, thus the inhibition of invertase development by sucrose; its natural substrate at all level of supplementation is particularly remarkable considering that the substrate of most catabolic enzymes acts as their inducers. The high invertase activity obtained in this study is consistent with the observation of Osho (2005) who reported high invertase activity with *S. cerevisiae* 0271 isolated from fermenting cashew apple juice.

The growth of yeast on this sugar results in the formation of ethanol and several other stoichiometrically minor products including short chain organic acids (Palmpulha and Loureiro, 1989). The destabilizing effects which

these products have on yeast plasma membrane through the displacement of annular phospholipids from integral proteins (Conrad and Singer, 1981) and their induction of cell membrane lipolysis (Casey and Ingledew, 1986; Millar et al., 1982) have long been known. Besides, ethanol is believed to act in synergy with osmotic stress to cause disruptions in membrane integrity (Loureiro et al., 1984). Most membrane bound enzymes are sensitive to perturbations in membrane integrity and lipid composition. It is therefore possible that the growth of yeast in sucrose medium with the resultant ethanologenesis would have induced changes in membrane integrity with concomitant depression of invertase activity. Laure et al. (1984) reported changes in enzyme activity due to ethanol-induced conformational changes in yeast plasma membrane.

Yeast which posses good sedimentation properties make product recovery easier due to their ability to settle out of fermentation medium. Among several factors believed to influence yeast sedimentation rates are the physiological state of the cell (Rhymes and Smart, 1996) and temperature (Gonzalez et al., 1996) as well as the ionic composition of the growth medium (George et al., 1994). The sedimentation and flocculation properties of yeast are important characteristics considered in choosing yeasts for use in industrial fermentations (Stewart et al., 1993; Van der Aar, 1996).

Correlation analysis indicated a highly significant positive relationship between medium sorbitol concentration and sedimentation rates for both yeasts at r = 0.74 and r = 0.85 for *Wy*. Growth of *orc* 6 yeast isolate in sucrose medium elicited between 46 – 80% decreases in its sedimentation rate compared to the unsupplemented medium while a decrease of 10 – 93% was observed for reference yeast relative to the basal medium. These observations indicate that the level and nature of sugar used in cultivating the yeast are additional important factors that influence yeast sedimentation. The reference yeast exhibited better sedimentation rates than orc 6 in most of the assays indicating superior flocculation property. The existence of strain specific differenced in yeast sedimentation properties had been reported (Rhymes and Smart, 1996).

Increase in medium sugar level is believed to affect the relative proportion of total medium sugar converted to alcohol (D' Amore et al., 1988; Jimenez and Benitez, 1986; Nagodawithana and Steinkraus, 1976). The decline in yeast ethanol productivity at high medium sucrose levels as observed in this study is in close agreement with the finding of several other researchers of the *Saccharomyces* genus in medium of high osmotic pressures (D'Amore et al, 1988; Dombek and Ingram, 1986).

Conclusion

In this study, we have isolated and characterized a high level ethanol and sugar tolerant *Saccharomyces* yeast from Nsukka orchard soil. The results obtained from this study reveal a strong indication of the yeast's great potential in the production of ethanol using locally available substrates.

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