ORIGINAL ARTICLE

Correlation between ethanol stress and cellular fatty acid composition of alcohol producing non-*Saccharomyces* in comparison with *Saccharomyces cerevisiae* by multivariate techniques

K. M. Archana · R. Ravi · K. A. Anu-Appaiah

Abstract Wine production is a complex process both from biochemical and microbiological point of view in which yeast plays a central role. The use of the wine yeast *Saccharomyces cerevisiae* and non- *Saccharomyces* yeasts as mixed starter cultures for wine fermentations is of increasing interest to enhance the quality of wine.The most common stress, yeast cells encounter during wine fermentation is the increase in ethanol concentration.To enhance ethanol tolerance, alteration in the cellular lipid composition is one of its defence mechanism. Ethanol tolerance and cellular fatty acid composition of alcohol producing non *Saccharomyce*s forms were compared with enological strains of *Sacccharomyces cerevisiae*. *Saccharomyces cerevisiae* used for the study, tolerated 15 % of ethanol and the non *Saccharomyces* strains such as, *Issatchenkia occidentalis and Issatchenkia orientali*s tolerated 10 % of ethanol. On exposure of *Saccharomyces cerevisiae* to ethanol stress, the proportion of monounsaturated fatty acids increased with concomitant decrease in saturated fatty acids. Decrease in monounsaturated fatty acids, exhibited by non-*Saccharomyces* yeasts when exposed to ethanol stress, could be one of the reasons for their inability to withstand more than 10 % of alcohol. Multivariate techniques of data analysis – principal component analysis and linear discriminant analysis were employed in order to establish differentiation criteria as function of yeast strains, alcohol stress and their fatty acid profile. Based on the data, Chemometrics, such as principal component analysis and discriminant function analysis, can be successfully applied to fatty acid data to categorize the yeast.

Keywords Yeasts · Ethanol stress · Lipid composition · Chemometrics

K. M. Archana · K. A. Anu-Appaiah (✉)
Department of Food Microbiology, Council of Scientific and Industrial Research (CSIR)-Central Food Technological Research Institute (CFTRI), Mysore, Karnataka, India
e-mail: anuapps@yahoo.com

R. Ravi
Department of Sensory Science, Council of Scientific and Industrial Research (CSIR)-Central Food Technological Research Institute (CFTRI), Mysore, Karnataka, India

Introduction

Wine is one of the oldest known medicine which protects against cardiovascular disease, atherosclerosis, hypertension, type 2 diabetes, neurological disorders, certain types of cancer and metabolic syndrome.The alcoholic, polyphenolic and lipid components of wine contribute to these beneficial effects (Guilford and Pezzuto 2011; Fragopoulou et al. 2000).The biological process of wine making involves the interactions of enzymes from different microorganisms, especially yeasts (Moreno-Arribas and Polo 2005). Yeast strains are exposed to a variety of biotic and abiotic stresses during fermentation. These stresses, when prevailing over the cellular defence systems, can affect cell viability, with negative consequences on the progression of fermentative process (Mannazzu et al. 2008). One of the most common stress, that yeast cells encounter during alcohol fermentation is the increased ethanol concentration. High levels of ethanol affects the integrity of cell membrane, damage permeability of the membrane to numerous ionic species and decrease the fluidity of plasma membrane (Ma and Liu 2010). Such levels of alcohol leads to the dissipation of transmembrane electrochemical potential and subsequently acidify intracellular

and vacuolar condition (Ma and Liu 2010). At high concentrations, ethanol perturbs protein conformation of key glycolytic enzymes such as pyruvate kinase and hexokinase, causing protein denaturation and dysfunction (Ma and Liu 2010). Increased ethanol concentration affects the uptake of glucose, maltose, ammonium, amino acids and also causes cell leakage of nucleotides, amino acids and potassium ions (Ma and Liu 2010).

Yeast cells have developed appropriate mechanisms to deal with the damages caused by increased ethanol concentration. In response to the damage, yeasts change the membrane compositions to antagonize membrane fluidization and stabilize the plasma membrane. Specifically, the levels of unsaturated fatty acids and ergosterol increased in response to the high concentration of ethanol (Ding et al. 2009). Palmitoleic acid and oleic acid are the key plasma membrane components in S.cerevisiae to compensate the deficits caused by ethanol stress (Ma and Liu 2010). The concentration of these two unsaturated fatty acids in cellular lipids was higher in ethanol tolerant strains (Ma and Liu 2010). Palmitoleic acid and oleic acid were formed by the same catabolic membrane desaturase encoded by OLE1 through oxygen and NADH-dependent desaturation of palmitic acid and stearic acid respectively (Ma and Liu 2010). The role of unstaurated fatty acids in ethanol tolerance was confirmed by synthetic mono-unsaturated fatty acids and expression of insect desaturase TniPVE in yeast (Ma and Liu 2010).

S. cerevisiae, referred to as "the wine yeast" is the most important yeast for wine, responsible for the production of alcohol and secondary metabolites of importance to wine. Non Saccharomyces yeasts are also metabolically active during fermentation and produce a plethora of by-products such as glycerol, acetaldehyde, acetic acid, succinic acid, higher alcohols and fatty acid esters. The use of Saccharomyces and non- Saccharomyces yeasts as mixed starter cultures for wine fermentations is of increasing interest to enhance the quality of wine (Comitini et al. 2011; Jolly et al. 2006).

The Ethanol tolerance of S.cerevisiae is well known (Lee et al. 2011; Mobini-Dehkordi et al. 2006; Moneke et al. 2008). It is widely accepted that, increase in fatty acid composition of yeast is one of the key defence mechanism under ethanol stress (Ding et al. 2009). However, ethanol tolerance studies of alcohol producing non Saccharomyces yeasts is very scarce. Multivariate analysis was used to study the effect of ethanol stress, on the cellular fatty acid composition of alcohol producing non Saccharomyces yeasts, in comparison with enological strains of S.cerevisiae.

Materials and methods

Yeast strains

The yeast strains used in the study were isolated using SDA Chloramphenicol medium.These strains were molecularly identified by amplifying the D1/D2 region of large sub unit 28S rDNA gene. In the first experimental setup, we have carried out ethanol tolerance studies and cellular lipid profile of the yeast isolates. The second experimental setup includes, screening of yeast isolates for the production of alcohol and its analysis. All experiments were conducted in triplicates and the average results have been computed.

Culture medium

The culture medium used was yeast extract peptone dextrose (YEPD) medium supplemented with ethanol ranging in concentration from 0 to 20 %.

Effect of ethanol addition on cell growth

To evaluate the effect of ethanol on yeast cell growth, the ethanol supplemented medium was inoculated with 5 % of inoculum and incubated at 25 °C for 72 h in stationary condition. Growth rate was determined every 24 h (Jimenez and Beneitez 1986; Jimoh et al. 2013; Osho 2005).

Alcohol production by the selected isolates

Alcohol production medium (Yeast Extract [1 %], peptone [2 %], dextrose [20 %]) was inoculated with 10 % of the inoculum, incubated at 25 °C for 72 h and checked for the production of alcohol (Jimenez and Beneitez 1986). The amount of alcohol produced was estimated spectrophotometrically (Caputi et al. 1968). Qualitative analysis of the alcohol produced was analysed by Gas Chromatography (GC). Shimadzu GC 2010 equipped with Flame Ionization detector (FID) and Zebron Wax plus capillary column containing (polyethylene glycol) was used with a temperature programming from 40 °C (1 min hold) to 70 °C at a rate of 5 °C min^{-1} and to 220 °C at a rate of 25 °C min^{-1} for 3 min. Nitrogen was used as the carrier gas with the flow rate of 1 ml min^{-1}.

Fatty acids analysis

Lipids were extracted with chloroform-methanol (2:1) from the biomass, which was collected after 72 h of incubation. The lyophilized yeast biomass was homogenized at room temperature for 15 min using the chloroform methanol mixture [100 mg in 10 ml of solvent mixture]. The homogenate is centrifuged to recover the liquid phase. For methyl esters preparation of fatty acids, 2 ml of the FAME reagent which consists of methanol: acetyl chloride in 9.5:0.5 ratio was added to the dried lipid portion. The mixture was refluxed for 3 h at 80 °C in a water bath. This was followed by the addition of 5 % sodium chloride solution and the fatty acid methyl esters were extracted using hexane. The hexane layer was washed with 5 ml potassium bicarbonate solution and dried over anhydrous sodium sulphate

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(Christie 1982). The fatty acid methyl esters were analysed by Shimadzu GC 2010 equipped with FID using RTx - 2330 capillary column with a temperature programming from 120 °C (2 min hold) to 265 °C (10 min hold) at a rate of 5 °C min^{-1}. Nitrogen was used as the carrier gas with the flow rate of 1 ml min^{-1}. The fatty acid methyl esters were further confirmed through GC-MS analysis.

Statistical processing

Multivariate statistical techniques have been employed to extract information from complex data and also to interpret data obtained by instrumental techniques (Arvantoyannis et al. 1999; Cordella et al. 2002; Penza and Cassano 2004). Multivariate techniques of data analysis – principal component analysis (PCA) and Linear discriminant analysis (LDA) – were employed in order to establish differentiation criteria as a function of the yeast strains, as function of alcohol stress and their fatty acid profile. The application of multivariate analysis such as principal component analysis or discriminant analysis provides the possibility to use and understand the data generated based on the properties of the sample for classification among them. Principal component analysis is a popular, most commonly used multivariate technique for dimensionality reduction of the data. It is a mathematical procedure for resolving sets of data into orthogonal components whose linear combinations approximate the original data to any desired degree of accuracy. In this study, PCA was used to extract the first three principal components from the fatty acid data and to examine the possibility of grouping the samples. On the other hand, discriminant function analysis (DFA) is a supervised classification technique, where the number of categories and the sample that belong to each category are pre-defined. This method provides a number of orthogonal linear discriminant functions, equal to the number of categories minus one, that allow the samples to be classified in one or another category (Adams 1995; Martens and Martens 2001; Naes et al. 2002). PCA and DFA was performed using a statistical software - Statistica (v 5.5 StatSoft, Tulsa, OH, USA) on the PCA sample scores on components 1 and 2, which provides the maximum level of separation in models developed.

Result and discussion

Identification of the yeast strains

The rDNA region is variable, to allow reliable separation of all the yeast strains (Kurtzman and Robnett 1997). Based on the amplification of the D1/D2 region of large sub unit 28S rDNA gene, yeast strains were identified as *S.cerevisiae* designated as ITB with the accession *No.* FN393977.1; *S.cerevisiae* AAV2, accession *No.* KF551990; *Issatchenkia occidentalis*

Table 1 Effect of ethanol stress on growth rate after 72 h of incubation 1) *S.cerevisiae ITB* 2) *S.cerevisiae 101,* 3) *S.cerevisiae* AAV2 4) *Issatchenkia orientalis CL1132* 5) *Issatchenkia occidentalis Ap C*

Sl.No	Yeast strain	Maximum growth attained (Log CFU/ml)			
		Control	5 %	10 %	15 %
1	ITB	9.212b	8.914b	8.380c	7.556b
2	101	9.230b	9.009b	8.778c	7.301b
3	AAV2	9.232b	8.954b	8.740c	7.505b
4	CL1132	7.477a	7.468a	6.929b	0.000a
5	Ap C	7.477a	7.270a	5.398a	0.000a

Values in a column with different letters differ significantly at $P < 0.05$ by LSD test (Least Significant Difference)

ApC, accession *No. KF551991; Issatchenkia orientali*s CL1132, accession *No. KF551992;* and reference CFTRI strain *S.cerevisiae* designated as *101*.

Ethanol stress and cell growth

The results indicated that, when exposed to ethanol stress, *S.cerevisiae* strains 101, ITB and AAV2 tolerated 15 % of ethanol, when compared to the non *Saccharomyces* which resisted upto 10 % (Table 1). Except *S.cerevisiae* strain *ITB,* all other yeast isolates used for the study exhibited maximum growth after 72 h of incubation. Growth was not observed, when all these yeast strains were exposed to 20 % of alcohol. Although *S. cerevisiae* cells adapt to the ethanol stress, increasing ethanol concentration resulting from fermentation eventually lead to growth inhibition and cell death. When the adaptive responses fail, the ultimate consequence of ethanol toxicity is cell death (Carmona-Gutierrez et al. 2012). Earlier Osho (2005) had reported tolerance of *S.cerevisiae* strains up to 12 % of alcohol. Jolly et al. (2006) observed that, *C. stellata* tolerated 12 % of ethanol and produced elevated glycerol concentrations of 10–14 g/l. However, Lee et al. (2011) reported, *S.cerevisiae* and *Pichia anamola* to resist 15 % of alcohol.
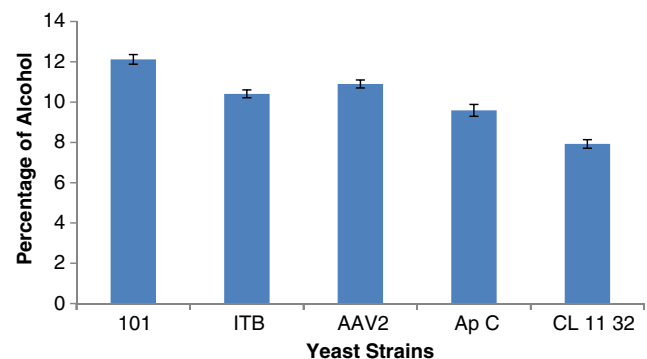

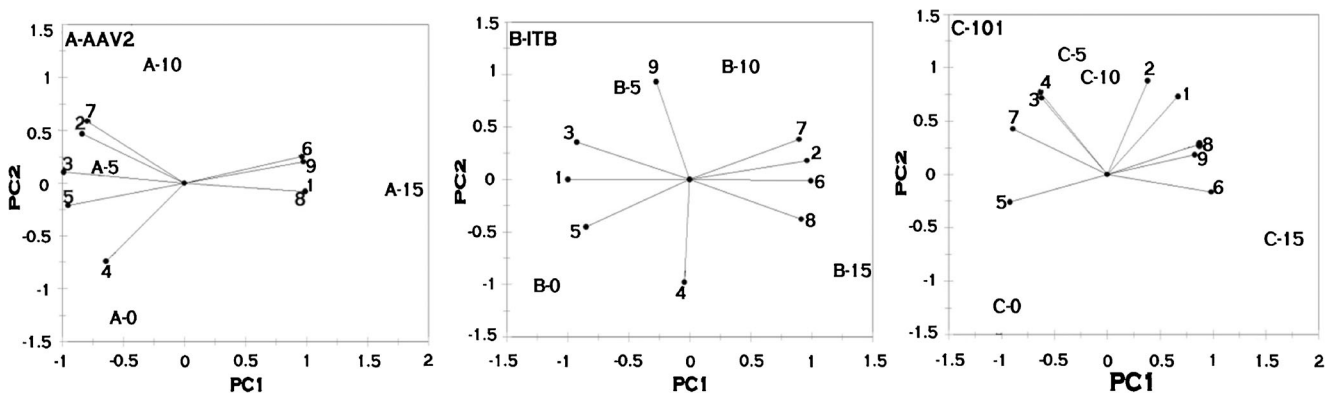**Fig. 1** Production of alcohol by the yeast isolates

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Fig. 2 Principal component analysis of cellular fatty acid profile of *Saccharomyces cerevisiae* strains when exposed to ethanol stress. AAV2 - *Saccharomyces cerevisiae*, ITB - *Saccharomyces cerevisiae*, 101 - *Saccharomyces cerevisiae* [0,5,10,15 written adjacent to letters A,B,C,D,E refers to the percentage of ethanol. Fatty acids are designated as follows: 1: Caprylic., 2: Capric., 3: Lauric., 4: Myristic., 5: Palmitic., 6: Palmitoleic., 7: Stearic., 8: Oleic., 9: Linoleic., 10: Linolenic

Alcohol production by the selected strains

The identified selected yeast isolates were grown in the alcohol production medium, incubated at 25 °C for 72 h. In our study, the yeast strains such as *Saccharomyces* 101, ITB, AAV2 produced 12, 10.6, 10.7 % and *Issatchenkia* ApC, CL1132 produced 9.3 and 7.7 % of alcohol respectively (Fig. 1). Qualitative analysis of the alcohol produced by the selected isolates was carried out by Gas Chromatography. The results indicated, ethyl alcohol to be the major alcohol produced by all the isolates with an exception of *S.cerevisiae* strain ITB, which produced Iso- propyl alcohol in addition to ethyl alcohol. *S.cerevisiae* strains produced 10.47 % and *Hanseniaspora spp.* produced 5.02–8.72 % of alcohol (Ndip et al. 2001; Jolly et al. 2006). In our earlier studies with *S. cerevisiae* (standard culture S-170) we had recorded that the available glucose and the growth rate of the organism (Somshekar and Anu-Appaiah 2013).

Ethanol stress and variation in cellular fatty acid composition

S.cerevisiae strains ITB, 101 and AAV2 produced increased quantities of oleic and palmitoleic acids with a concomitant decrease in lauric and palmitic acids when exposed to extraneously added ethanol. Linolenic acid increased only in strain AAV2. The concentration of the other fatty acids detected, varied in all the three strains when exposed to ethanol stress. The presence and absence of these fatty acids were confirmed by GC-MS analysis.

In this study, we have compared the fatty acid profile of *S.cerevisiae* strains 101, ITB and AAV2 with alcohol producing non *Saccharomyces* forms like *Issatchenkia orientalis* CL1132 and *Issatchenkia occidentalis* ApC. Both ApC and CL1132 showed increase in caprylic and stearic acids followed by decrease in oleic acid. Myristic and palmitoleic acids increased in ApC with the concomitant decrease of these two fatty acids in CL1132. The concentration of the other fatty acids varied between the non *Saccharomyces* strains.

The ability of cells to increase the concentration of unsaturated fatty acids in plasma membrane is the principal mechanism used by yeasts to adapt to the presence of ethanol stress. The addition of increasing concentrations of ethanol to the culture medium raises the proportion of monounsaturated fatty acids of phospholipids such as oleic acid (18:1) with concomitant decrease in saturated fatty acids such as palmitic acid
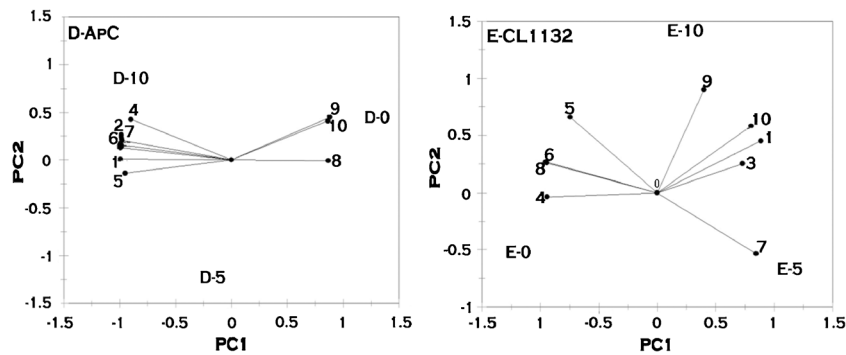

Fig. 3 Principal component analysis of cellular fatty acid profile of non *Saccharomyces* strains when exposed to ethanol stress. Ap C - *Issatchenkia occidentalis*, CL1132- *Issatchenkia orientalis*. [0,5,10,15 written adjacent to letters A,B,C,D,E refers to the percentage of ethanol. Fatty acids are designated as follows: 1: Caprylic., 2: Capric., 3: Lauric., 4: Myristic., 5: Palmitic., 6: Palmitoleic., 7: Stearic., 8: Oleic., 9: Linoleic., 10: Linolenic
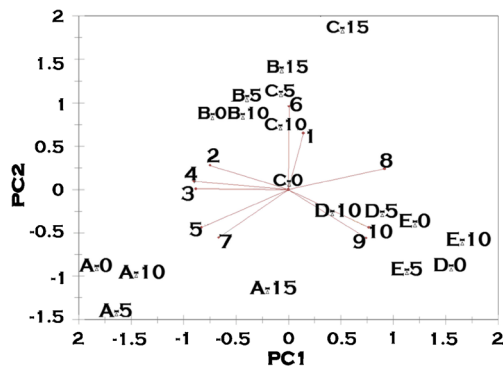
Fig. 4 Principal component analysis of cellular fatty acid profile of the yeast strains in the study when exposed to ethanol stress. A: AAV2-*Saccharomyces cerevisiae*., B: ITB- *Saccharomyces cerevisiae*., C: 101-*Saccharomyces cerevisiae*., D: Ap C-*Issatchenkia occidentalis*., E: CL1132- *Issatchenkia orientalis* [0,5,10,15 written adjacent to letters A,B,C,D,E refers to the percentage of ethanol]. Fatty acids are designated as follows: 1: Caprylic., 2: Capric., 3: Lauric., 4: Myristic., 5: Palmitic., 6: Palmitoleic., 7: Stearic., 8: Oleic., 9: Linoleic., 10: Linolenic

(16:0). The proportion of 18:2 acid was higher in the less ethanol tolerant strains (Aguilera et al. 2006). Oleic acid seems to be the most important unsaturated fatty acid in counteracting the toxic nature of ethanol through its effect on plasma membrane fluidity (You et al. 2003). The hydrophobic core of the membranes may allocate ethanol molecules in the presence of (C16) monounsaturated fatty acids, which leads to tolerance (Mannazzu et al. 2008). Our results pertaining to the variation of cellular fatty acid profile, when exposed to ethanol stress were mostly consistent with those previously studied. In our study, we have observed that, *S. cerevisiae* strains increased mono unsaturated fatty acids when exposed to ethanol stress, which is depicted in Fig. 2. The non-*Saccharomyces* strains used for the study produced 8 % of alcohol and also tolerated

up to 10 % without manipulating the cultivation conditions. Non *Saccharomyces* strain*s* exhibited a decrease in mono unsaturated fatty acids when exposed to ethanol stress (Fig. 3). This could be one of the reasons for their inability to withstand more than 10 % of alcohol.

The role of non *Saccharomyces* in fermentation is usually limited by their inability to tolerate ethanol. The results of the study by Pina et al. (2004) show that manipulating the culture conditions prior to ethanol challenge, the ethanol tolerance of some non-*Saccharomyces* strains like *H. guilliermondii* can be increased. In our study we have the non-*Saccharomyces* strains which can produce alcohol of 8 % and also withstand 10 % of alcohol without manipulating the cultivation conditions. Alexandre et al. (1994) reported that a significant reduction in the level of phospholipid content was observed when 4 % (v/v) ethanol was added to the growth medium of *Kloeckera apiculata*, while *S.cerevisiae* grown in the YPDE maintained the same level of phospholipids as the control cells. High ethanol tolerance is linked to the ability to maintain a high rate of phospholipid biosynthesis.

Statistical study

Principal component analysis

The correlation between the alcohol stress and the fatty acid profile of individual yeast strains are depicted in Fig. 2. In sample A (AAV2), B (ITB) and C (101), it was observed that the fatty acids such as palmitoleic (6) and oleic (8) acids increased with a concomitant decrease in lauric (3) and palmitic (5) acids. The fatty acids which have increased in all the three *Saccharomyces* strains were present on the positive side of the

Fig. 5 Discriminant function analysis
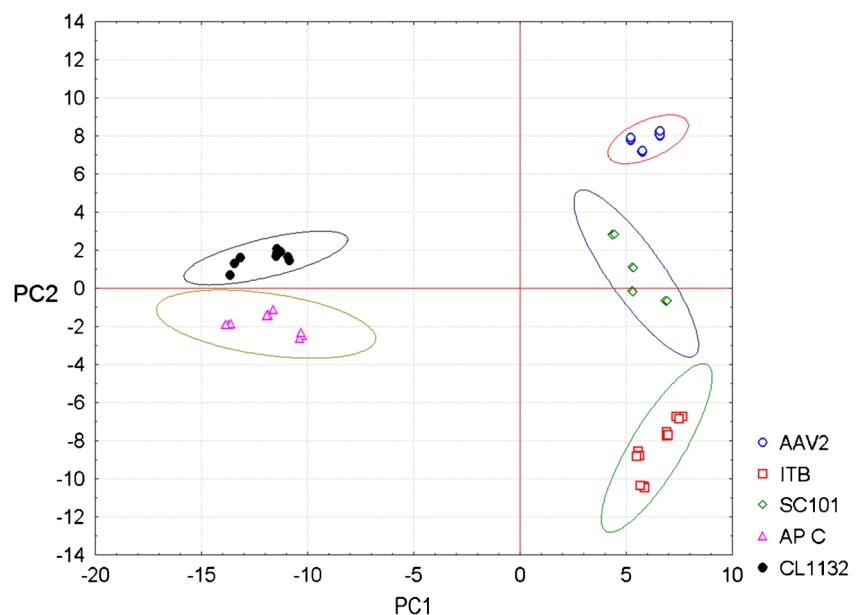
principal axis and are mostly associated with the samples exposed to 15 % of alcohol.

As depicted in the Fig. 3, sample D (Ap C) indicated the slight increase in fatty acids such as capric (2), caprylic (1) myristic (4) palmitic (5) palmitoleic (6) and stearic (7) acids when exposed to ethanol stress. These fatty acids were present on the negative side of the principal axis 1 and were associated with 10 % alcohol. Linoleic (9), linolenic (10) and oleic (8) acids decreased when exposed to ethanol stress and were present on positive side of the principal axis 1. In sample E (CL1132), it was observed that the fatty acids such as linoleic (9), linolenic (10), caprylic (1), lauric (3), stearic (7) acids were present on the positive side of the principal axis1. These fatty acids increased when exposed to ethanol stress, followed by the concomitant decrease in palmitic (5), palmitoleic (6) and myristic (4) and oleic acids which were present on negative side of the principal axis. From the PCA analysis carried out, it was observed that the fatty acids present on the positive side are oppositely correlated to the fatty acids present on the negative side of the principal axis 1.

The principal axis 1 accounted 78.91 % of the variance while principal axis 2 for 13.45 % and together accounted for 92.36 % of the total variance of the data matrix. The high variance of the axis also indicates the PCA model developed is capable of explaining the variables and their relationships and also the validity of the model. The PCA resulting plot is shown in Fig. 4. Sample A (AAV2) occupied the third quadrant and associated more with palmitic acid (5) and stearic acid (7). On the other hand, sample B (ITB) that occupied the second quadrant are closely associated with each other and also with palmitoleic acid (6) followed by caprylic(1). Sample C (101) occupied the second quadrant and is associated with Palmitoleic (6) and caprylic (1) acids. Sample D (Ap C), occupied the fourth quadrant and associated more with oleic (8) linoleic (9) and linolenic (10) acids. Sample E (CL1132) showed variations in their positions as the function of alcohol stress, in fourth quadrant and is associated with linoleic (9) and linolenic (10) acids. The above results suggest that PCA is capable of classifying fatty acids with respect to their sample origins and indicated their associations with other variables and isolates.

Multivariate statistical analysis of the data performed using partial least square linear regression modeling indicated a strong correlation between the overall lipid composition and the final ethanol concentration, as an indicator of ethanol tolerance (Henderson et al. 2013). From the Principal Component Analysis (PCA) carried out by Aguilera et al. (2006), it was reported that the best parameters in discriminating the yeast strains found to be the percentages in oleic acid, palmitoleic acid and ATP ase activity. Marullo et al. (2004) reported PCA analysis of the three technological traits such as ethanol tolerance, volatile acidity, H_2S production on a yeast population of 48 strains. For the three traits analysed maximal projection conserves 83 % of the information with the two components axis, 1 and 2 that explain 50 % and 33 % of the total inertia.

Discriminat function analysis (DFA)

Discriminat function analysis (DFA) was also performed on the fatty acid data against five different yeast strains as a function of alcohol stress at various concentrations (Fig. 5). The alcohol concentration was increased from 0 to 15 % at four levels (0, 5, 10 and 15). The results of DFA clearly demonstrated its classification power by segregating yeast strains into five distinct clusters. Among the clusters formed, S.cerevisiae strains lie on the positive side of the axis and non Saccharomyces strains ApC and CL1132 lie on the negative side of principal axis.

Conclusions

Our results emphasize the comparison of cellular fatty acid composition in the alcohol producing non-Saccharomyces yeasts and enological strains of S.cerevisiae, when subjected to ethanol stress. The fatty acid profile pattern varied between the alcohol producing non Saccharomyces and Saccharomyces yeasts. Analysis of the fatty acid profiles indicate the different clustering of these forms. This signifies that Chemometrics, such as Principal component analysis (PCA) or Discriminant Function analysis (DFA), can be successfully applied to fatty acid data to categorize the yeast strains. The results of our finding can be more appropriate while studying the ecology of mixed culture fermentation.

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