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Full Length Research Paper

Characterization of palm wine yeasts using osmotic, ethanol tolerance and the isozyme polymorphism of alcohol dehydrogenase

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Physiologic characteristics of ten palm-wine yeast isolates obtained from nine localities in four provinces in southern Cameroon were assessed using sensitivity to chloramphenicol, tolerance to acetic acid, ethanol tolerance, osmotolerance as well as protein and alcohol dehydrogenase (ADH) polymorphism. None of these isolates was sensitive to 30 µg/ml chloramphenicol and all were non-tolerant to 1% acetic acid. Some of the isolates (Vip 2 and Vip 10) showed tolerance to both high sucrose and ethanol concentrations, criteria which are useful in fermentation. Results indicated that 80% of the strains were able to grow at 15% alcohol solution and only 20% grew on 40% sucrose solution. The denatured protein pattern (SDS-PAGE) as well as the native protein pattern was similar for all strains. The ADH pattern showed a high diversity based on which isolates were differentiated into three patterns. The electrophoretic patterns showed that the ADH pattern was the best criterion for diversity characterisation because of its specificity and variability.

Key words: *Saccharomyces*, alcohol tolerance, osmotolerance, alcohol dehydrogenase polymorphism, palm wine.

INTRODUCTION

Yeasts are unicellular eucaryotic fungi which can be isolated from various sugar rich sources including palm wine (Okafor, 1972; Okagbue, 1988). Palm wine is a local alcoholic beverage obtained from different kind of palm trees, such as the oil, raffia and date species. The genus *Elaeis* native to western Africa is represented by an important diversity in Cameroon, but the species *Elaeis guineensis* is the most exploited because of its oil and sap, which are widely consumed in southern part of the country. Besides, its sap constitutes a good growth medium for numerous micro-organisms especially for yeast, lactic and acetic acid bacteria. Palm wine is also used locally as a traditional raising agent in dough making and also to produce a local spirit by adding sugar. In Cameroon, palm wine has been produced industrially, bottled and exported for sales abroad. However due to storage problems, this product is actually less developed. Nigerian palm wine yeast has been used to develop baking yeast. The dough made with this yeast tasted better and was of better quality when compared to dough made with a commercial strain of baker's yeast (Ejiofor et al, 1994).

The ease with which yeasts are cultured and the inocuity of many species have made them the most widely used micro-organisms. It is used for fermentation industry (production of alcoholic beverages and bakery products), and also as protein and vitamin supplements in human and animal diets. Brazil produces more than 11 billion litres of alcohol per year by yeast fermentation of sugar cane called "green petrol" (Smith, 1996) but few microbial selection are involved in increasing the yield. Baker's yeast is also interesting as single cell protein because it could provide 53% proteins for *Saccharomyces cervisiae* from molasses substrate and it is also a

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Table 1. Sources of isolates and characteristics of *Saccharomyces* spp. yeast from palm wine (*Elaeis guineensis* Jacq.) in Cameroon.

Strain code	Source of palm wine		Microscopic examination	
	City origin	Province	Gram	Shape
Vip 1	Kumba	South-West	+	Oval
Vip 2	Mamfe	South-West	+	Oval
Vip 3	Buea	South-West	+	Oval
Vip 4	Mamfe	South-West	+	Oval
Vip 5	Limbe	South-West	+	Oval
Vip 6	Bamenda	North-West	+	Oval
Vip 7	Douala	Littoral	+	Oval
Vip 8	Yaoundé	Centre	+	Oval
Vip 9	Bokito	Centre	+	Oval
Vip 10	Lobe	South-West	+	Oval
By1	Saf-instant™	Commercial baker's yeast	+	Oval

source of invertase enzymes and glucoamylase for *S. diastacus* (Wainwright, 1992). Improved knowledge of yeast molecular biology combined with genetic engineering techniques has made feasible the use of yeast for the production of human and animal proteins such as rennin, growth hormone and hepatitis B vaccine (Bourgeois and Leveau, 1995). In order to select yeasts for optimal production of ethanol, certain requirements are very important. These include osmotolerant, ethanol tolerant, acid tolerant and specific properties according to the process requirements (Stewart et al., 1984). To be able to exploit the intrinsic characteristics and new capabilities of yeast, it is necessary to investigate some physiological attributes of yeast isolates. This work aims at screening yeast isolates from palm wine on the basis of specific properties.

MATERIALS AND METHODS

Sample collection

Palm wine samples were collected from the centre, littoral, south-west and north-west provinces of Cameroon (Table 1). 20 ml of palm wine was collected into 10 ml of 10% glycerol solution. Each sample was labelled with the place, date and time of collection. The samples were then stored at 4°C until required for use. Strains of interest were then selected and kept on malt extract agar (MEA) at 4°C.

Check-up of palm wine samples

Before the isolation of yeast, the palm wine samples were observed and described under compound microscope following Gram staining at high magnification (x 1000) according to Collins et al. (1989).

Micro-organisms

The yeast isolates used in this study were isolated by streaking a loopful of palm wine on MEA (5% w/v malt extract, 0.5% w/v mycological peptone and 1.5% w/v agar, Fluka). Following Gram staining and microscopic examination, yeast colonies were picked up and sub-cultured on MEA. A commercial strain of baker's yeast from Saf-instant™ was included as control. The isolates were kept on MEA plates at 4°C and sub-cultured every fortnight.

Culture media

A synthetic medium YEPS containing the following (g/l): yeast extract, 10; potassium dihydrogen phosphate, 2; sucrose, 150, was used (Larpent and Larpent-Gourgaud, 1990). All pre-cultures used in the study were carried out in yeast/peptone/glucose (YPG) broth (Difco). The final pH of the media used was adjusted to 4.5 using 0.5 M H_2SO_4 before autoclaving for 15 min at 121°C.

Screening of yeast diversity

Microscopic examination was done on air-dried slides following Gram staining. Morphology of the yeast as well as any visible internal or budding cells structure was noted and tentative identification was made according to Lodder (1971) and Barnett et al. (1990). Sensitivity to chloramphenicol was evaluated by growing isolates in MEA in the presence of 30 µg/ml chloramphenicol discs, using the method of Kirby et al. (1966). Incubation was at the room temperature for 3 - 5 days followed by observation for growth. Tolerance to 1% acetic acid solution was conducted by growing strains in a medium consisting of 10 g glucose, 1 g trypton, 1 g yeast extract, 2 g agar, all in a volume of 100 ml into which 1 ml of acetic acid was added after autoclaving. A control was done in strains which were grown in this same medium, without acetic acid. Incubation was performed at room temperature (20 - 6°C) for 3 -5 days, followed by observation for growth. Ethanol tolerance was done by growing isolates in YEPS containing ethanol at 5, 10, 15 and 20% (v/v). Samples were collected every two hours and the optical density measured at 595 nm against a medium blank. The specific growth rate µ was calculated from the growth curve. Osmotolerance experiments were conducted in YEPS containing the following sucrose concentration (g/l): 100, 150, 300, 400 and 500 (Leveau and Bouix, 1979). Samples were collected every two hours and the optical density measured at 595 nm against a medium blank. The specific growth rate µ was calculated from the growth curve. Fermentation was done using 2 ml of the selected 24 h precultures of isolated strains inoculated into 250 ml of YEPS, in a conical flask. Incubation was at room temperature with shaking at 100 rpm for 72 h. Samples were collected every twelve hours and

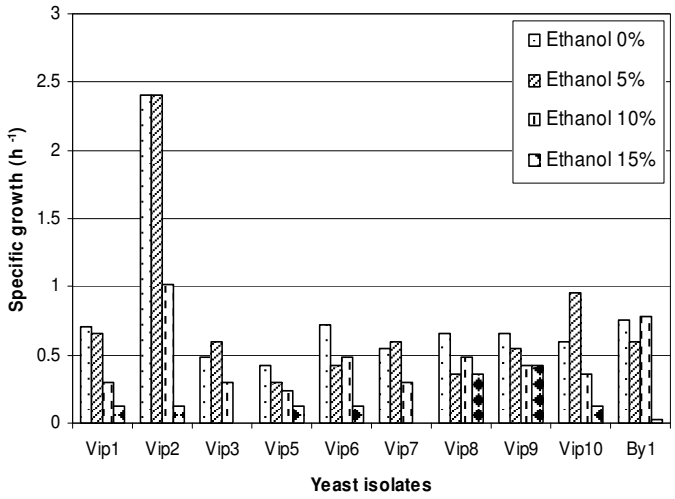

Figure 1. Ethanol tolerance of different isolates of *Saccharomyces* spp. isolated from palm wine and a Baker's yeast (By1).

centrifuged at 4°C for 10 min at 4500 rpm. Supernatant was collected and stored at -20°C until required for ethanol analysis. Fermentation in molasses was the same as that in YEPS, but the medium consisted of 250 g molasses, 1.6 g urea and distilled water was added to give 1000 ml. For ethanol analysis, sample was first passed through a filter containing anhy-drous sodium sulfate. After, 4 µl of the filtrate was then added to 6 µl of butanol. Then, 0.3 µl of this mixture was then injected into the column at intervals and a chromatogram was obtained. The injection needle was rinsed with butanol between injections. The chromatogram gave the concentration of ethanol in each mixture. Prior to electrophoresis analysis, protein extraction was by the method of Nwaga et al. (1990), in which wet cells were ground in the presence of alumina and polyvinyl poly-pyrrolidone, at 4°C. This was followed by acetone treatment at –20°C and then centrifugation. The pellet was lyophilised, and then ground at 4°C. Recovery was by addition of extraction buffer followed by centrifu-gation. Supernatant was immediately used for electrophoresis studies. For the study of the denatured membrane proteins, SDS-PAGE sample buffer was added to pellet obtain above and heated in a hot water bath for 10 min. Polyacrylamid gel electrophoresis (PAGE) was by the method of Hames and Rickwood (1985) using a 7.5% resolving gel. The run was at 4°C for 2 h. Revelation of ADH was according to the method of Siciliano and Shaw (1976) in which the gel was incubated in a solution containing the enzyme's substrate for 30 - 60 min at room temperature. Revelation of native proteins was by Fairbanks et al. (1971) which involved fixation of proteins followed by staining using Coomassie Brilliant blue R-250 (CBB) dye. This was accomplished in about 45 - 60 min with gentle agitation. SDS-PAGE was according to the method of Hames and Rickwood (1985) using a 12.5% polyacrylamide gel at room temperature. Revelation of the denatured proteins was as for native proteins above, using the method of Fairbanks et al. (1971).

RESULTS AND DISCUSSION

Microscopic examination of palm wine samples showed that palm wine serves as a good medium for the growth of numerous micro-organisms (at least ten bacterial morphotypes) which included gram positive and gram negative bacteria mostly in chains and in clusters (rods and cocci) as well as gram positive yeast (about ten times larger than the bacteria). Examination following isolation showed a pure culture of gram positive yeast, most of which were in clusters with the same oval shape.

All yeast isolates showed resistance to chloramphenicol since they could grow in the presence of the discs. This result goes to confirm the reason for the addition of chloramphenicol at low concentrations to the growth medium during the primary isolation of yeast from a mixed culture (Linné and Ringsrud, 1992). The chloramphenicol inhibits the growth of most bacteria while allowing the yeast to grow.

All the isolates were non tolerant to 1% acetic acid solution, since they grew well in the control medium but did not grow at all in the test medium. The pH of the test medium prior to the addition of 1% acetic acid was 6.8, but upon addition of the acid the pH dropped to 3.7. This result confirms the findings of Nwaga et al. (1998) that *S. cerevisiae* grows at pH 4.5 to 6.5 with optimum around 6.0. In palm wine, lactic acid and acetic may reach 0.1 - 0.3% and 0.2 - 0.4% respectively, whilst the alcohol contents of samples collected within the day were between 1.4 and 2.82%; palm wine which had accumulated over night, 3.24 to 4.75%; and palm wine held for 24 h, over 7.0% (Amoa-Awua et al., 2007). This also shows that as the pH of the palm wine decreases with increase in age and acetic acid production, the population of yeast decreases while that of acidophilic bacteria increases.

The results for ethanol tolerance are shown on Figure 1. They show the specific growth rates (h^{-1}) of yeast isolates at various concentrations of ethanol (%, v/v) and 15% (w/v) sucrose at room temperature in YEPS medium. The ethanol tolerance results indicated that with no exogenous ethanol added, the generation times were less than 10 h for all strains studied, an observation which is similar to that of Bulawayo et al. (1996). In 15% (v/v) ethanol the lowest generation was 9.9 h for strain Vip 9, followed by 11.6 h for Vip 8, then 34.4 h for isolates Vip 10, Vip 6, Vip 5, Vip 1 and Vip 2; strain By1 had a generation time of 173.3 h. It is interesting that Vip 3 and Vip 7 showed no growth at 15% ethanol concentration. Similar inhibition of growth at 15% ethanol was observed for yeast grown on YEPS by Bulawayo et al. (1996). The isolates Vip 8 and Vip 9 were the most alcohol tolerant at 15% (v/v) ethanol. Contrary to the findings of Bulawayo et al. (1996) all the isolates used in this study showed growth at 15% (v/v) ethanol except strains Vip 3 and Vip 7.

The results for osmotolerance are shown on Figure 2. The histogram shows the specific growth rates (h^{-1}) of yeast isolates at various sucrose concentrations (% w/v) at room temperature in YEPS medium. The isolates By1, Vip 3 Vip, 5 Vip 6, Vip 7 and Vip 9 had maximum specific growth rates in 10% (v/v) sucrose, while Vip 1 Vip 2 and Vip 8 had maximum specific growth rates in 15% (w/v) sucrose. Vip 10 had maximum specific growth rate at 30% (w/v) sucrose. Only Vip 2 and Vip 10 showed growth

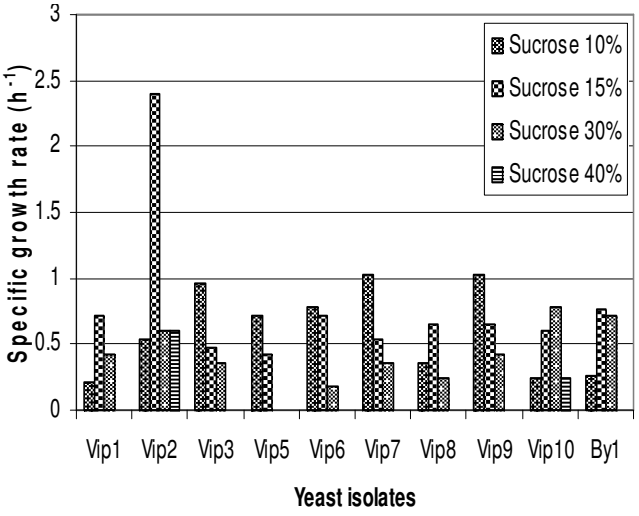

Figure 2. Osmotolerance of different isolates of *Saccharomyces* spp. isolated from palm wine and the Baker's yeast (By1).

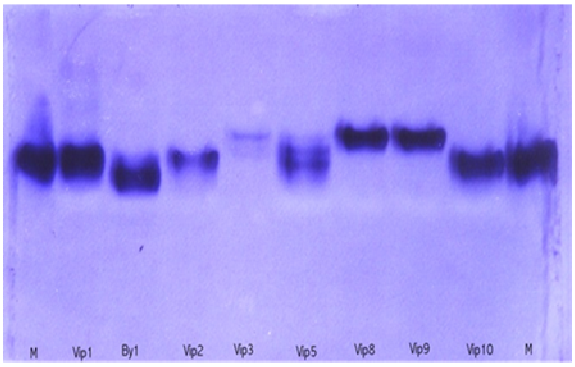

Figure 3. Alcohol dehydrogenase electrophoretic profiles of different isolates of *Saccharomyces* spp from palm wine, a Baker's yeast (By1) strain and a Sigma ADH purified enzyme (M).

at 40% (w/v) sucrose. The isolate Vip 2 was isolated from an alcohol fermentation plant and that might be an adaptation to alcohol and osmotolerance. Our results on yeast growth also confirmed those of Nwaga et al. (1998) concerning the optimum sugar concentration for alcohol production from starch breakdown by amylases. All the isolates used in this study were unable to grow at 50% (w/v) sucrose unlike the isolates used by Bulawayo et al. (1996) which all showed growth at 50% sucrose. However, the strains used by these authors were isolated from red wine and sweet stem sorghum juice which are plant with high sugar content. Some isolates like Vip 2 and Vip 10 had both good tolerance to 10% ethanol and relatively good tolerance to high sugar concentrations as well.

Changes in ethanol content during the fermentation in YEPS were assessed for most yeast isolates studied both in YEPS and molasses. The ethanol levels rose sharply after 24 h as in the observations of Bulawayo et al. (1996) in which the ethanol levels rose sharply after 30 h. This suggests that another sugar source was used after the first one.

Upon incubation of the gel in the substrate of alcohol dehydrogenase an electrophoretic pattern was obtained, which enables one to classify the yeast isolates into 3 groups (Figure 3). Isolates Vip 1, Vip 2, Vip 5 and Vip10 which showed a migration pattern very similar to the standard ADH from Sigma company constituted a group. They had a frontal ration (Rf) value of 0.39 to 0.41.

The isolates Vip3, Vip 8 and Vip 9 constituted another group. Their migration rate was slower as compared to the previous group. They had a Rf value of 0.35. The industrial strain By1 which had a Rf value of 0.44 constituted a third group. Its migration was faster compared to the other two groups. Migration here was based on difference of charge therefore it does not imply that all strains in a group have identical molecular weight. Some yeast isolates (Vip1, Vip 2 and Vip 10) showed an ADH pattern similar to the purified Sigma ADH pattern and different from the Saf-instant™ commercial yeast one. Is there any relation between ADH polymorphism and alcohol tolerance activity of the yeast? The pattern of denatured proteins shows at least 30 polypeptide bands present in all isolates.

The pattern of native proteins shows 8 to 10 bands, present in all isolates. The ADH pattern is shown on Figure 3. This pattern shows a good diversity between the yeast studied and one will immediately choose the enzyme pattern for characterisation because of its specificity and variability. Electrophoretic protein patterns have been used to analyse wine yeast and polymorphism of esterases to differentiate natural populations of wine yeast (Wöhrmann and Lange, 1980).

Comparing the results of this study to those of Bulawayo et al. (1996), it seems as if palm-wine yeast isolates are more ethanol tolerant than red wine or sweet stem sorghum juice ones, while sweet stem sorghum juice and red wine strains seems to be much more osmotolerant. Concentrations of 5% alcohol and 15% sucrose concentration seem to offer the best conditions for an optimum growth rate of the 10 yeast isolates used in this study. But according to Nwachukwu et al. (2006), palm wine yeasts isolates may show a range of 10 - 20% alcohol tolerance. Since alcohol toxicity is more severe when it is stored inside the yeast cell, it seems useful to assess not only 15% added alcohol but also during fermentation. Application of yeast to traditional biotechnologies such as baking, brewing, distiller's fermentations, and wine making need physiological and genetic improvements of their environmental stress properties according to Attfield (1997).

Conclusion

The results obtained with these *Saccharomyces* yeast isolated from samples of palm wine from 4 provinces

where palm trees are naturally growing or planted for oil production have shown a small morphological diversity. An important functional diversity has been found for alcohol tolerance, osmotolerance, ADH profile pattern from electrophoretic polymorphism as well. This indicates that the use of wider range of yeast isolates could be outlined and further developed for valorisation. These results could be applied for optimisation of alcohol production, brewery and baking industries as well.

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