J. of Bio. Res & Biotech. 1, (1),53-62 (Jan/June, 2003)

Hybridization of Palm Wine Yeasts (*Saccharomyces Cerevisiae)* with Brewers Yeast by Protoplast Fusion

O.U. Ezeronye
Department of Biological Sciences, Michael Okpara University of Agriculture, P.M.B. 7267, Umudike, Nigeria

Abstract
Haploid auxotrophic strains of *Saccharomyces cerevisiae* were selected from palm wine and propagated by protoplast fusion with Brewers yeast. Fusion resulted in an increase in both ethanol production and tolerance against exogenous ethanol. Mean fusion frequencies obtained for **a** mating types ranged between 8×10^{-6} to 3.0×10^{-5}. Intraspecific fusion between yeast strains gave a higher fusion frequency than interspecific fusion. High efficiency fusion was obtained between α mating types with fusion frequency ranging between 1.4×10^{-6} to 8.6×10^{-4}. The fusion hybrids were uninucleated and stable showing actual karyogamy and prototrophy within six months of storage. Percentage regeneration of protoplast to viable, vegetative cells was between 52% to 77%. Minimal back mutations (reversions) were observed.

Key words: Protoplast fusion, palm wine *Saccharomyces*, ethanol

Introduction

Palm Wine is a widely consumed alcoholic beverage in the tropics particularly in West Africa, Asia and South America. It is probably the most popular naturally fermented alcoholic beverage in West Africa and in Nigeria, its production has developed into small scale industries. It contains a wide variety a microorganisms especially yeasts (Owuama and Saunders 1990) which are usually consumed along with the wine. Fermentation of the sugar and other nutrients present in the juice by the endogenous microflora of the palm sap leads to ethanol and organic acid production. The wine looses its sweetness as the fermentation continues and the original colorless juice becomes milky-white. If not consumed or bottled within 24hrs of its production it gets sour due to excessive fermentation and a sublime Malo-lactic/acetic acid fermentation by the bacterial microflora (Okafor, 1978).

Palm wine has been reported to have a low percentage ethanol ranging from 0.5 - 7.1% (Bassir 1962 Okafor, 1978) and its endogenous yeast has been know to have a low ethanol tolerance (Van Pee and Swings, 1971). With the intense research efforts and several papers in the literature on the microbiology, biochemistry and preservation of palm wine. (Faparusi 1973, Okafor 1978) there has not been a corresponding effort to understand the genetics of yeasts involved in ethanol fermentation in palm wine. The ability of yeast to tolerate high ethanol concentration is known to be controlled by a large number of genes (Casey and Ingedew 1986 Jimenez and Benitez 1987, D' Amore and Stewart 1987 Pretorius, 2000). More than 250 genes are involved in the complex process of control of ethanol tolerance in yeast (Boulton et al 1996). Ethanol production is also reported in the literature to be under polygenic control (Del Castillo, 1985, Miklos and Sipiczk; 1991). High ethanol tolerance is however not necessarily associated with high productivity of alcohol (Sipiczki et al 1988).

As alcohol accumulates in the yeast cells there is a progressive decline in fermentation. Boulton et al (1996) and Miklos and Sipiczki (1991) explained the decline in the fermentation ability of yeast to be due to the combined action of ethanol on the yeast growth rate and fermentation capacity. Yeast cell viability associated with membrane lipid alterations and solute uptake are also involved(Kajiwara et al, 2000). As the genetic basis for ethanol tolerance and productivity is not yet well understood in *Saccharomyces species,* it is difficult to plan genetic breeding programmes. Brown and Oliver (1982) however claimed the use of continuous culture of yeast in a feedback system to select viable mutants with improved ethanol tolerance and fermentation capabilities... Protoplast fusion on the other hand has produced very useful results in the genetic improvement of industrial yeast strains (Barney et al 1980; Johnasson and Sjostrum 1984). It has practical applications in yeast strain development especially as the natural sexual process is circumvented (Pretoruis and Van der Westhuizen 1991, Hammond, 1996). In palm wine however protoplast fusion has not been extensively used as a tool for strain improvement. Our previous communication reports on the use of protoplast fusion recombinants from palm wine yeast for the treatment of effluent from Nigerian paper recycling plant (Ezeronye and Okerentugba, 1999). We believe that protoplast fusion could be applied for genetic improvement of palm wine yeast

The quality of palm wine, ethanol productivity and tolerance, wine bouquet and general acceptability could be improved by this process. This papers reports on the development of protoplast fusion hybrids in Saccharomyces yeast isolated from palm wine and brewers yeast.

Materials and Methods

Yeasts Strains and Media

Haploid auxotrophic yeast strains (ON-21, RA- 03, RA - 07, RA - 01) were isolated from palm wine and characterized. BR - 13 and BY - 15 were obtained from Golden Guinea Breweries, Umuahia, Abia, Nigeria. The auxotrophic strains were *Saccharomyces* species. Mating type

a (MAT a) include ON - 21 *Saccharomyces cerevisiae* (his met), BR - 13 *Saccharomyces* uvarum (trp). Mating type α (MAT α) strains were RA - 01 *Saccharomyces cerevisiae* (phe) BY - 15 *Saccharomyces cerevisiae* (arg). The methods of Mortimer and Hawthrone (1969) were used for selection of haploid auxotrophic mutants. Yeasts strains were maintained in YPD agar which contained yeast extract 1% Peptone 1% Dextrose and 2% Agar. Cultures for fermentation and ethanol tolerance tests were grown in erlenmeyer flasks containing YPS broth (yeast extract 0.5%, Peptone 1%, Sucrose 30% at pH 4.5). Each Erlenmeyer flask contained 5ml of YPS and was incubated with slow agitation in a shaker incubator. The complete medium (CM) was also used as the presporulation medium and contained 1% yeast extract 2% Peptone, 2% Dextrose and 2% agar. Sporulation medium (SPA) contained 1% potassium acetate, 0.1% yeast extract, 0.05% Dextrose and 2% agar. The minimal vitamin medium (MVM) contained 0.67% yeast nitrogen base (Difco) 2.0% glucose, 2.0% agar.

Preparation of Auxotrophic strains

Haploid auxotrophic yeast cell grown overnight in complete medium were harvested, washed twice and resuspended in protoplast buffer, 0.1M phosphate buffer (pH 7.2) containing 0.1% (v/v) of β - mercaptoethanol and 0,8M sorbitol. Crude snail gut enzyme extract from the giant African snail (Achatina achatina) was added to a final concentration of 4mg/ml (Ezeronye and Okerentugba, 2001) The mixture was incubated with gentle shaking at low speed (200 x g) for 90 minutes at 30°C. The resulting protoplasts were rinsed five times in protoplasting buffer supplemented with 0.01M $CaC1_2$ and then resuspended in the same buffer at a concentration of 2×10^8 protoplast/ml and placed on ice before use.

Construction of Protoplast fusion hybrids

Equal amounts of protoplast (2×10^8/ml) prepared from different auxotrophic strains were mixed and washed gently in protoplast buffer. The washed cells were re-suspended in 1ml of prewarmed (30°C) solution 30% (w/v) polyethylene glycol (PEG4000 BDH Chemical, England) sterilized by membrane filtration, (Millipore 0.45) containing $CaC1_2$ (100mM), 1M sorbitol and 0.1M phosphate buffer (pH 7.5). After 30min at room temperature, the protoplasts were centrifuged and appropriate dilutions were made in protoplasting buffer and mixed with osmotically stabilized melted agar (MVM, 0.6M NaC1, 0.8M sorbitol, 2% glucose and 2% agar) kept at 45°C and poured as a thin top layer on osmotically stabilized minimal vitamin medium. The cells were mass-mated by incubating at 28°C for 4 days. The frequency of protoplast fusion was estimated by comparing the number of colonies appearing on selective MVM agar plate (MVM supplemented with selected amino acids, purines and pyrimidines at a concentration of 100mg/ml) and on MVM agar plates supplemented with all the nutrients required for growth of each original parental strain. Fusants (hybrids) were produced and stored in refrigerator at 4°C before use.

Nuclear Staining

The nuclei in the fusants were counted under fluorescence microscope (Type Biolar FR, KOVO, Praha Czech) after staining the cells with 4,6 - diamino - 2 - phenylindole (DAPI) by the methods of Williamson and Fennell (1975) to ascertain nuclear fusion (karyogamy) in the fusants.

Growth & Ethanol Productivity of Palm wine Yeast

The ability of wild types, and fusants, to produces ethanol and also grow in various concentrations of ethanol was tested using the methods of Miklos and Sipizki (1991). Ethanol tolerance was tested in samples of stationary - phase cultures inoculated into 300 ml erlenmeyer flasks containing 200 ml of YPS
(10^6 cells/ml) and supplemented with various amounts of ethanol (0, 3, 6, 9, 12, 15%). The flasks were closed with traps to allow CO_2 to leave and incubated at 30°C in a shaker incubator 200g per min). Samples were collected after 72h and the number of yeast cells was determined using a haemocytometer.

Ethanol production in batch culture was conducted in erlenmeyer flasks as described for the tolerance test described above. Samples of stationary - phase cultures were inoculated into 300ml erlenmeyer flasks containing 200ml of YPS (10^6cells/ml). Fermentation lasted for 7 days at 30°C (Miklos and Sipizki, 1991). Ethanol concentrations were determined by the specific gravity methods (AOAC, 1984) and with a gas chromatograph (Model 5710A, Hewlett Packard, Atlanta, GA, U.S.A) equipped with a glass column (1.5m x 1.5mm ID) packed with 80 - 100 mesh Porapak N (Waters Associates) and with 50ml nitrogen carrier gas min^{-1} at an oven temperature of 165°C. Growth was monitored with a Klett-Summerson Colorimeter (Klett Afg Co. Inc, New York, U.S.A.) using a no 66 red filter.

Statistical Analysis

Ethanol yield in the cultures of the putative fusants was compared with that of the wild type cultures and the results were judged by the student's t-test statistics. The differences in ethanol tolerance between the wild types and the fusants was also subjected to the student's t-test statistical analysis.

Results

Fusion and Regeneration Frequency

Fusion frequency of the protoplasts was in the range of 8.0×10^{-6} to 3.0×10^{-5} for MAT **a** and 1.4×10^{-6} to 8.6×10^{-4} for MAT α (Table 1). Minimal back mutations (reversion) were observed compared to the high fusion frequency of the protoplasts. Therefore colonies growing on regeneration medium were considered as fusants (karyotypes). Intraspecific fusion between *Saccharomyces cerevisiae* strains from palm wine and brewers yeast i.e (RA - 01 and BY - 15) gave the highest fusion frequency. Fusion between *Saccharomyces uvarum* strains (RA - 03 and RA - 07) from palm wine and *Saccharomyces uvarum* from brewer's yeast (BR -

13) gave a lower fusion frequency. Percentage regeneration of protoplasts to normal cells using 0.8m sorbitol as osmotic stabilizer ranged between 55 - 77%.

Stability of Fusants

Colonies of the putative fusants were grown for 5 days on the regeneration medium and twelve colonies were selected at random from the prototrophic fusion product (three from each fusion product) and transferred to fresh selective medium. After 5 days the colonies were transferred to complete medium and replica-plated in minimal medium to determine the frequency of segregants. None of the selected colonies were auxotrophic segregants. The fusion colonies were transferred to fresh selective minimal medium and their prototrophic characters were examined monthly for 6 month. Results show that the putative fusants remained prototrophic with little or no change in cell concentration. All the putative fusants were uninucleated as revealed by the DAPI staining which shows that actual karyogamy had taken place between individual candidate auxotrophic parent strains.

Table 1: Fusion Frequency Between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* from Palm Wine (ON-21, RA-03, RA-01, RA-07) and Brewers Yeast (BR-13, BY-15).

Strain Combination*	FUSION PRODUCT (FUSANT)	FREQUENCY (mean values) Regeneration (%)	Reversion	Fusion
MAT a				
ON - 21 + BR - 13	FP 31	76	2.1×10^{-9}	3.0×10^{-5}
RA - 03 + BR - 13	FP 30	55.6	0	8.0×10^{-6}
MAT α				
RA - 01 + BY - 15	FP 37	75	0	8.6×10^{-4}
RA - 07 + BY - 15	FP 38	77	1.0×10^{-8}	1.4×10^{-6}

* *Saccharomyces cerevisiae:* ON - 21 (HIS MET), BY - 15 (arg.), RA - 01 (cys).

- *Saccharomyces uvarum:* RA - 03 (ade), RA - 07 (phe) BR - 13 (trp)

Growth and Ethanol Production of Putative Fusants and Wild Types

The growth and ethanol production of the putative fusants and wild type parents were monitored in YPS broth containing 30% sucrose. Fig 1A and B shows the time course of the cell growth (OD_{690}) and ethanol yield for both the fusants and wild types at 25°C respectively. The fusants grew more rapidly than the wild types with a higher specific growth rate ranging between 0.3 - 0.42 whereas the wild types grew slowly with specific growth rate ranging between 0.12 - 0.25. The ethanol yield was also higher in the cultures of the putative fusant than the wild type culture ($p > 0.05$). There was a close similarity between the growth patterns

and ethanol yield of the individual putative fusants (Fig. 1A). Result for ethanol tolerance measured as cell density after 3 days in YSP medium plus 5% ethanol is shown in Table 2. The fusants had a better ability to tolerate ethanol (72-80%) than the wild types (28-40%) and the auxotrophic strains (30-45%) from palm wine. The differences in ethanol tolerance between the wild types and fusants was statistically significant (P>0.05).

Table 2: Ethanol Tolerance of Some Wild Types, Auxotrophic Strains and Fusants

Strains	Markers	Ethanol Tolerance (%)
OW - 12	Wild type*	40
ON - 21	His met	38
BR - 13	Trp	45
RA - 03	Ade	45
OK - 10	Wild type	35
RA - 01	Cys	32
RA - 07	Phe	30
BY - 15	Arg	35
OG - 12	Wild type	28
Fp - 30 (Fusant)**	+	80
FP 31	+	72
FP 37	+	75
FP 38	+	80

- Wild type and auxotrophic mutants are *Saccharomyces cerevisiae* strains isolated from palm wine

** Fusant (FP) and wild types are prototrophic.

*** Cell density after 3 days in yeast extract, sucrose, peptone medium (YSP) plus 5% ethanol compared to that in YSP without ethanol (YSP alone)

Discussion

Protoplast fusion hybrids of *Saccharomyces* yeast from palm wine were constructed with brewers yeast with the aim of improving its genome with favourable alleles from the genome of the brewers yeast. Earlier reports have shown that palm wine yeast is a poor fermenter producing alcohol in the range of 0.5 - 7% in palm sap (Bassir 1962). Results obtained from this study confirms this report as the wild type yeasts from palm wine produced only a maximum of 5% ethanol in sugar substrate. Protoplast fusion between candidate auxotrophic strains from palm wine and brewers yeast resulted in enhanced ethanol productivity and tolerance. The putative fusants produced a maximum of 15% ethanol in sugar substrate. The improvement may have been due to an increase in ploidy. The ability of yeast to tolerate high concentration of alcohol is reported to be under polygenic control (Pretorius, 2000). It is also

believed that ethanol productivity is not necessarily associated with high alcohol tolerance (Sipiczki et al 1988). The high alcohol productivity of the putative fusants in this study agree with the reports of Miklos and Sipiczki (1991) who bred a distillers yeast by hybridization with a wine yeast. Intraspecific protoplast fusion between *Saccharomyces cerevisiae* strains from palm wine and brewers yeast gave a higher fusion frequency than interspecific fusion frequency between palm wine and brewers yeast. Interspecific protoplast fusion is generally believed to produce unstable hybrids with low fusion frequency (Kobori et al 1991). The high fusion frequency obtained in this study is attributed to the use of membrane filtration for the sterilization of PEG. The use of membrane filter sterilized PEG as the fusogenic agent rather than the standard autoclaved PEG method was reported by Kobori et al (1991) to improve fusion frequency between *Candida tropicalis and Candida biodinii* which agrees with results obtained in this study. A possible explanation is that filtration removes contaminating high molecular weight substances that interfere with the functions of PEG as a fusogenic agent. The fusants were uninucleated showing actual karyogamy and remained stable showing prototrophy even after six months on selective minimal and complete media in the absence of nutritional pressure. That the fusants showed these properties meant that effective complementation and gene recombination had occurred between candidate fusion partners.

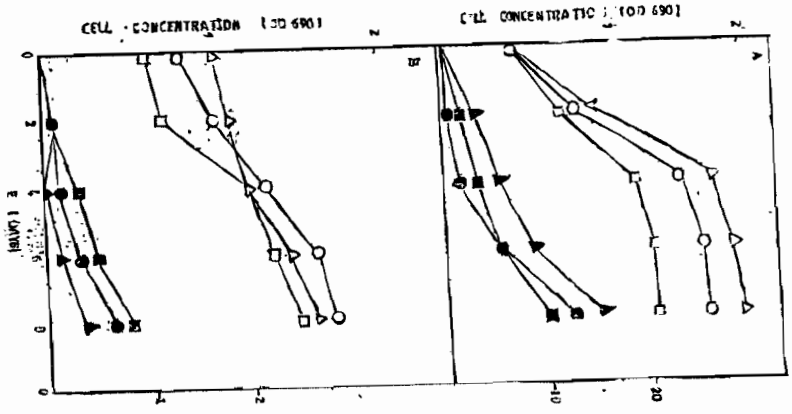

Fig 1: Growth and ethanol *production* of S. *cerevisiae* wild type *strains* and fusants from *palm* wine during ethanol fermentation in batch culture at 25°C

A- Fusant (Fp 31, Fp 30, Fp 37). Cell concentration (OD 690) (open *Symbol)* Fp 31 0, Fp 30 □ Fp 37 △

Ethanol concentration (mg/ml) (Closed symbol) Fp 31 • Fp 30 ■ Fp37 ▲

Wild type (Ow-12, 0G-12, OK-10). Cell concentration (OD 690) (Open symbol) *OW-12* □ OG-12 0; OK-10 △

Ethanol concentration (mg/ml) (Closed symbol) *Ow-12* ■ ; OG-12 • OK-10 ▲

The observed percentage regenerated colonies from the crude snail gut enzyme treated cells (55 - 77%) were higher than values obtained by Christensen (1979) and Okerentugba (1984). Results obtained from this study however compares with values obtained for percentage regenerated colonies by Johanseen and Sjostrom (1984). Low percentage regenerated colonies has been explained to be due to aggregation of protoplasts or chain formation resulting from lack of separation of protoplasts or chain in some yeast strains. The result highlights a possible improvement in ethanol yield in palm wine yeast isolates using the protoplast fusion technique.

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