Production of high-ethanol-yielding *Saccharomyces cerevisiae* of palm wine origin by protoplast fusion

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Abstract

Two high ethanol-yielding recombinants of *Saccharomyces cerevisiae* were made by protoplast fusion. The parental strains were isolated from 25 day old palm wines (*Raphia raphia* and *Elaensis guineensis*) obtained from South-eastern Nigeria. The parental strains were isolated on Glucose Yeast Agar at 28 ºC. They had ethanol tolerance in the range of 18% – 20% v/v ethanol. Cassava starch hydrolysates produced by both acid and enzyme hydrolysis methods were used as substrates for fermentation to ethanol by the isolates and recombinants. The enzyme hydrolysates enabled production of higher ethanol levels (13.7% v/v max) by the isolates than the acid hydrolysates (8.7% v/v max). Twenty six randomly selected regenerate recombinants were examined after protoplast fusion for the desired markers which were enhanced ethanol tolerance and production. Only two of the recombinants showed the desired recombination. They exhibited an enhanced tolerance of 24% v/v ethanol for designate F_5 and F_{14}. And also gave higher yields of ethanol (16% v/v). One recombinant F16 performed significantly lower than the parental strains yielding only 4.6% v/v ethanol. [Life Science Journal. 2008; 5(4): 64 – 68] (ISSN: 1097 – 8135).

Keywords: palm wine; ethanol tolerance; protoplast fusion

1 Introduction

Industries especially those that make use of yeasts in the production of alcohols are very traditional, reflecting the conservative attitude of most manufacturers[1]. Amongst the options available for an organization pursuing industrial microbiology to help maximize its profits in the face of its "Competitors race" for the same market, strain improvement appears to be one of the single factors that has contributed the greatest profits[2]. Nowadays however, manufacturers have begun to show unprecedented interest in innovations by introducing benefits from re-engineering and genetic manipulation. These innovations were ushered in as a result of certain constraints intrinsic to the organisms being used for the respective manufacturing processes. Of special attention are the yeasts in ethanol fermentations. These include the need to improve yeast resistance to ethanol, temperature, carbon dioxide as well as eliminating production of other compounds which may contaminate the product and of course, improve both yield and product recovery. In order to obtain strains showing more suitable properties, genetic manipulation methods have been used. However due to the euploid, diploid or polyploid nature of most strains of yeast used in ethanol fermentation, traditional crossing techniques have not been very useful. This made the use of other technologies such as protoplast fusion and transformation necessary[1]. Protoplast fusion is an important tool for genetic manipulation of industrial yeast strains[3]. New genotypes having relatively improved qualities have been obtained by protoplast fusion[3,4,5,6]. In protoplast fusion protoplasts are used as starting materials to transfer foreign genes into other cells.

The ethanol fermentation industry in Nigeria is still in infancy. The local industries rely heavily on the fermentation of palm juices by microorganisms

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indigenous to palm wines. Nwachukwu *et al* found a variety of organisms in palm wines with *S. cerevisiae* being the most important in palm juice fermentation[7]. This work was aimed at improving the ethanol yielding capability of *S. cerevisiae* of palm wine origin by protoplast fusion.

2 Materials and Methods

2.1 Isolation and identification

Strains of *S. cerevisiae* were isolated and identified from 25-day old palm wines (*Raphia raphia* and *Elaensis guineensis*) obtained from South-eastern Nigeria and aged at room temperature.

Five millilitres of thoroughly mixed wine was centrifuged in sterile centrifuge bottles at low speed for 5 minutes. One millilitre of the sediment was inoculated by streaking on plates of Glucose Yeast Agar (GYA), and incubated at 28 ºC for 24 hours[8]. Chloramphenicol was added to the GYA at 0.05 mg/ml to discourage growth of bacteria[9]. The yeast colonies that developed were isolated and purified by further streaking on GYA. Standard methods for yeast identification[10,11] were employed.

2.2 Determination of ethanol tolerance

Ethanol tolerance of the isolates and recombinants was determined based on visual assessment of turbidity and viability in a tube of basal medium containing 4 g/L $(NH_4)_2SO_4$; 2 g/L K_2O; 0.7 g/L $MgSO_4 \cdot 7H_2O$; 200 g/L glucose[12]. One hundred and fifty mililitres of prepared sterilized basal media containing known percentages of ethanol was inoculated with actively growing yeast cells to final concentrations of 1.6×10^7 cells/ml. Observations were made after 48 hours incubation at 25 ºC[13,14,15]. Evidence of turbidity/sedimentation indicated growth and consequently tolerance.

2.3 Preparation of substrates for fermentation

Substrates used for fermentations in this work were reducing sugars obtained by acid and enzyme hydrolysis of a high yielding cassava cultiver IITA98/0581. The tubers used were obtained from The Agricultural Development Project (ADP) Imo State, Nigeria. The fresh cassava tubers were peeled by removing the two outer most layers. The starchy layer was then washed and pulverized using a manual grater. The slurry was subsequently dried by spreading in thin layers on aluminum foils in an oven at 60 ºC. The slurry was occasionally stirred to ensure even drying over a period of 2 days. On drying, it was milled in a manual blender. The powdery starch was sieved through a 60 mm mesh and stored dry for subsequent use.

2.2.1 Enzyme hydrolysis of *Cassava Starch*. Crude enzymes used for the hydrolysis was obtained by malting sorghum grains (Zm-Dandam) developed from Sk5192 variety (obtained from Institute of Agricultural Research Zaria, Nigeria) for 3 days on a mat. The grains were then dried at 60 ºC in an oven for 48 hours and subsequently dry milled in a blender to form a coarse mill. The upward infusion mashing procedure used by Hug and Pfenninger[16] was used. The optimium condition for the enzyme hydrolysis of *Cassava starch* by the crude enzyme was determined after several trials.

Cassava starch (100 g) was suspended in 400 ml of distilled water and gelatinized at 90 ºC for 15 minutes. This was immediately cooled under tap water and 50 g of the ground malt was added and stirred vigorously. Another 600 ml of distilled water was added to bring the final volume of 1 L. The mixture was again stirred. This gave a 15% total solid mash. The pH of the mash was adjusted to 5.3 with 1.0 N HCl. The temperature was raised and kept at 50 ºC for 30 minutes. The temperature was further gradually raised to 65 ºC and held for another 60 minutes. The temperature was finally raised to 80 ºC and held for 10 minutes to stop any further enzyme action. The amount of reducing sugar produced was determined using the Di-Ntro Salicylic Acid (DNS) method[17].

2.2.2 Acid hydrolysis of *Cassava Starch*. One gram cassava starch powder was dissolved in 10 ml of distilled water. The mixture was then gelatinized at 90 ºC for 15 minutes in a water bath. The gel was cooled under tap water immediately (optimum conditions for acid hydrolysis using 1.0 N HCl was determined after several trials). To this aliquot of 10 ml starch solution 30 ml of 1.0 N HCl was added making an acid-gelatinized starch ratio of 3 : 1. The tubes in replicate were subjected to 121 ºC in an autoclave for 30 minutes. The reaction was terminated by adding appropriate amounts of NaOH to neutralize the acid. Again the level of reducing sugar was determined by DNS method.

2.2.3 Preparation of fermentation medium. The enzyme hydrolysed starch substrate slurry was boiled for 1 hour to precipitate any available proteins. Afterwards, the slurry was centrifuged at 2500 rpm for 15 minutes to remove any precipitate. The supernatant containing the soluble reducing sugars was dispensed in 300 ml volumes into 500 ml conical flasks. Nitrogen and phosphorus sources were added in the form 4 g/L $(NH_4)_2SO_4$, 1 g/L K_3PO_4 and 0.7 g/L $MgSO_4 \cdot 7H_2O$ respectively. Yeast extract at 2 g/L was also added.

The pH was adjusted to 4.5 – 5.0 using 1 N HCl. The flasks were properly plugged with sterile cotton wool and autoclaved at 121 ºC for 15 minutes. The reducing sugar content was adjusted to 20%. The flasks were subsequently allowed to cool and left overnight to ensure sterility before inoculation. The same procedure was also used in the preparation of the fermentation medium with the acid hydrolysate except that the pH was adjusted to 4.5 – 5.0 using in 1 N NaOH before autoclaving

2.4 Preparation of yeast inoculum for fermentation

Starter cultures were prepared by reviving the most ethanol tolerant strains previously stored as slant cultures on Sabouraud Dextrose Agar (SDA) overlayed with mineral oil and refrigerated. The cultures were transferred into sterile 100 ml Sabouraud Dextrose Broth (SDB) contained in 250 ml conical flasks plugged with sterile cotton wool. This was incubated at 30 ºC in an orbital shaker at 125 rpm. After 24 hours 10 ml of the cultures were subsequently and respectively transferred into 100 ml of sterile media for adaptation at 35 ºC (adaptation medium: hydrolysed *cassava starch* adjusted to give final conc. of 12% w/v reducing sugar, ammonium sulphate 0.085%, and ammonium hydrogen phosphate 0.12%, pH of 4.4 – 5.0).

2.5 Fermentation

Three hundred millilitres of sterile medium for fermentation was inoculated with 3.3×10^6 pre-adapted actively growing cells per millilitre of media. Each flask was properly corked with sterile cork connected to a fermentation tube containing concentrated sulphuric acid. This was to enable CO_2 leave the flask but will trap water vapour or any volatile alcohols. The flasks were then incubated at 28 ºC for 158 hours. Distillation at 88 ºC and recovery through a thick layer of calcium oxide determined percentage levels of ethanol produced.

2.6 Protoplast fusion of the isolates

The methods described by Van-Soligen and Van-Derplatt[18], Farahnak *et al*[5] and Priest and Campbell[19] were adopted.

2.6.1 Preparation of protoplasts. The most ethanol resistant isolate of *S. cerevisiae* designated Re, Pa and Pd were grown aerobically to early stationary phase in 250 ml flasks containing 50 ml YPD medium with shaking. Harvest was by centrifuging 5 ml cultures at 500 rpm for five minutes. The cells were subsequently washed 3 times with sterile distilled water. The cells were then suspended in the zymolase protoplasting solution (2-mercapto-ethanol, 50 mM phosphate buffer (pH 7.5) and 0.5 mg zymolase 60000 per ml) from Sigma Co. UK. The suspension was incubated at 30 ºC for 1 hour with occasional shaking. It was periodically examined under microscope for formation of protoplasts. Protoplasts were harvested by centrifugation at 1000 rpm for ten minutes. They were then washed 3 times with the protoplasting buffer and 0.08 M sorbitol.

2.6.2 Protoplast fusion. The protoplasts from the three *S. cerevisiae* isolates (Re, Pa and Pd) were mixed and suspended carefully in polyethylene glycol (PEG) solution containing 35% PEG (Mol wt 3350), 10 mM $CaCl_2$ and 0.8 M sorbitol. The suspension was incubated at room temperature (28 ºC – 30 ºC) for 30 minutes under U.V. light. Subsequently, the fused cells were washed with the protoplasting buffer. One ml of the suspension was mixed with 10 ml of the regeneration medium (3% agar, 0.7% YPD and 0.8 M sorbitol). This was poured into plates containing a thin bottom layer of agar with the same medium composition. The plates thereafter were incubated at 30 ºC for 3 – 7 days until visible regenerated colonies emerged. Colonies that emerged were purified and assayed for the desired recombination, which was enhanced ethanol tolerance and production. The effect of ground soy beans (Glycine max) on ethanol tolerance of the regenerated recombinant yeasts were also evaluated. This was achieved by the addition of ground food grade soy beans at 2 g/L of the media used for ethanol tolerance. The media was filtered aseptically after sterilization before inoculation.

3 Results

3.1 Ethanol tolerance of isolates and hydrolysis of *Cassava starch*

After aging the palm wines for 25 days, only nine *S. cerevisiae* isolates showed appreciable tolerance to ethanol. Six of the isolates were recovered from Raphia palm wine (Ra – Rf), while three were from oil palm wine (Pa, Pb and Pd). Ethanol tolerance of the isolates ranged from 12% – 20% v/v ethanol. *S. cerevisiae* designate Re exhibited the highest tolerance of 20% v/v ethanol, while designate Rd was the least ethanol tolerant (11% v/v). The results were shown in Table 1. Reducing sugar yield from cassava hydrolysis experiments showed that hydrolysis by the two step acid and enzyme hydrolysis process yielded the highest level of reducing sugar (93 mg/ml). This value was followed closely by the yield obtained from the enzyme process (87 mg/ml). The acid hydrolysates gave the least yield (83 mg/ml). Fermentation by the isolates showed that the

yeast designates Re and Pa produced the highest level of ethanol (13.7% v/v) using the enzyme hydrolysate as substrate. Fermentation of the acid hydrolysates always yielded less ethanol than the acid + enzyme hydrolysates which in turn gave lower yields than the enzyme hydrolysate. ANOVA showed no significant difference in the yields of Re and Pa at $P = 0.05$. However the ethanol yields for the different hydrolysates was significantly different at $P = 0.05$ (Table 2).

Table 1. Ethanol tolerance of isolated *S. cerevisiae*

Isolate designation	source	Ethanol tolerance % (v/v)
Ra	Raffia palm wine	15.0
Rb	Raffia palm wine	12.0
Rc	Raffia palm wine	12.0
Rd	Raffia palm wine	11.0
Re	Raffia palm wine	20.0
Rf	Raffia palm wine	12.0
Ra	oil palm wine	19.0
Rb	oil palm wine	17.0
Rc	oil palm wine	18.0

Mean of 3 assays.

Table 2. Ethanol production from *Cassava starch* hydrolysate by the parental strains and recombinants

Yeast designate		Ethanol production in % (v/v)		
		A	A + E	E
Parental strains	Re	9.7	9.7	13.7
	Ra	8.7	10.3	13.7
	Rd	9.0	10.3	13.3
Recombinants	F_5	–	–	16
	F_{14}	–	–	16
	F_{16}	–	–	4.6

3.2 Effect of protoplast fusion on the recombinants

A total of 26 randomly selected recombinants from the regeneration medium were purified and screened for enhanced ethanol tolerance which served as primary marker for the desired recombination. Only two of the regenerated yeasts designated F_5 and F_{14} showed significant enhancement of their tolerance to ethanol (24% v/v) compared with the tolerance of the parental strains (18%, 19%, 20% v/v). One of the recombinants F_{16} proved to have diminished significantly in its ethanol tolerance capability to 12% v/v (see Table 3). Supplementation of the medium with soybean enhanced the tolerance of F_5 and F_{14}, while the tolerance of the F_{16} was not affected. The other screened regenerated recombinants maintained an ethanol tolerance profile similar to that of the parental strains. Protoplast fusion also enhanced the ethanol production ability of the recombinants F_5 and F_{14} to 16% v/v ethanol. This value is higher than that of the parental strains (13.7, 13.3). The yeast designate F_{16} however performed less than any of the parental strains (see Table 2).

4 Dicussion and Conclusion

The yeast isolated after 25 days clearly tolerated the physico-chemical conditions imposed by the palm wine[7]. They were better adapted to the conditions in the wines than some of the other organisms involved in palm wine fermentation. The level of ethanol tolerance between 15% and 20% (v/v) compared favorable with brewing, sake and distillers yeasts[20]. The initial trials for ethanol production ability of the isolates revealed that keeping the fermentation at pH 4.5 – 5.0 by ammonia water helped keep away contamination and extended fermentation time and consequently higher ethanol yields. Fermentation of the cassava hydrolysates gave varying ethanol yields which depended on the method of hydrolysis. The levels of ethanol produced by the isolates used in this study produced higher levels than that obtained by Robinson and Kutianwala[21], Ameh and Okagbue[22]. Thus ethanol yield depends on the organism used, the fermentation process, the fermentation wort and the recovery process.

The general low yield of ethanol from acid hydrolysed cassava could be due to unfermentable sugars such as hydroxy methyl furfural and hydroxyl-methyl-furans. Keim[23,24] have reported that the use of traditional methods of acid hydrolysis formed large amounts of unfermentables and thus leads to low ethanol yields.

Amongst the 26 regenerated recombinant yeasts screened, only 2 exhibited possession of the desired recombination genes. These 2 recombinants also remained stable over the 152 hour fermentation period. The increased tolerance and production of ethanol could be as a result of duplication/mutation of the genes responsible for both ethanol tolerance and production. These characters have been known to be polygenic. The implication of their stability is that these recombinants could be promising as industrial organisms for use in ethanol fermentation using cassava enzyme hydrolysates as substrates. This study also shows that the *Saccharomyces cerevisiae* yeasts of plam wine origin are amenable to genetic manipulation, which could be used to better their efficiency as industrial organisms.

References

1. Martins CVB, Horii J, Pizzirani-Kleiner AA. Characterization of fusion products from protoplasts of yeasts and their segregants by electrophoretic karyotyping and RAPD. Rev Microbiol (serial on the Internet). 1999(cited 2008 Dec 23); 30(1): 71 – 6. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0001-37141999000100014&lng=en. doi: 10.1590/S0001-37141999000100014.
2. Okafor N. Industrial Microbiology. University of Ile Ife Press Ltd. Ile Ife. 1987; 435.
3. Isteretant D, Spiegle K. The influence of recombinant systems on the protoplast fusion of *Saccharomyces cerevisiae*. Journal of Am Soc of Brew Chemists 2006; 46(3): 60 – 4.
4. Hopwood D, Wrigh H. Bacterial protoplast fusions recombination in fused protoplasts of *Streptomyces coelicolor*. Mol Gen Genet 1978; 162(3): 307 – 17.
5. Farahnak F, Seki T, Dewey D, Ryu Y, Ogrydziak D. Construction of Lactose assimilating and high ethanol producing yeast by protoplast fusion. Appl Envt Microbiol 1995; 51(2): 362 – 7.
6. Lorant H, Laszlo M, Laszlo K, Csaba B. Production of Trichoderma strains with pesticide poly resistance by mutagensis and protoplast fusion. Antonino van Leeuwenhoek 2006; 89(3 – 4): 387 – 93.
7. Nwachukwu I, Ibekwe V, Anyanwu B. Investigation of some physico-chemical and microbial succession parameters of palm wine. Journal of Food Technology 2006; 4(4): 308 – 12.
8. Okafor N. Palm wine yeasts from parts of Nigeria. J Sc Fd Agric 1972; 23: 1399 – 407.
9. Nwachukwu I. Effects of Nicotiana tabacum extracts on selected dermatophytes. Namoda Tech Scope Journal of Sc and Tech 2001; 5(1): 241 – 4.
10. Phaff HJ, Starmer WT. Yeasts associated with plants. In: Kreger-van Rij NJW (Ed). The Yeasts, A Taxonomic Study. 3rd Edition. Elsevier, Amsterdam. 1987; 890 – 2.
11. Barneth J, Payne R,Yarrow D. Yeast: Characteristics and Identification. 2nd Edition. Cambridge Univ Press. 1990; 999.
12. Bhajpai D, Sharma A, Raghuran N, Bhapai P. Rapid production of ethanol in high concentration by immobilized cells of *S. cerevisiae* through soy flour supplantation. Biotechnol Lett 1988; 10(3): 217 – 20.
13. Skinner C, Emmons C, Tsuciya. Hennicis Moulds Yeasts and Actinomycetes. John Wiley and Sons NY. 1961; 87 – 99.
14. Nwachukwu I, Ibekwe V, Nwabueze R, Anyanwu B. Characterization of palm wine yeast isolates for industrial utilization. African Journal of Biotechnology 2006; 5(19): 1725 – 8.
15. Nwachukwu I, Ewelike C, Ibekwe V. Isolation and screening of local yeast strains for attributes important for ethanol production. Journal of Raw Materials Research 2006; 3(1): 55 – 62.
16. Hug H, Pfeoninger J. Brewing trials using infusion and decoction mashing methods. Proceedings of Eur Conv Brew 1979; 355 – 65.
17. Miller G. Dinitrosaliclic acid (DNS) reagent for determination of reducing sugars. Analytical Chemistry 1959; 31: 429 – 31.
18. Van-Soligen P, Vander-Platt C. Fusion of yeast spheroplasts. Journal of Bacteriol 1977; 130(2): 946 – 7.
19. Priest FG, Campbell I (Eds). Brewing Microbiology. Chapman and Hall London Publishers. New York 1996; 196.
20. Cassey G, Ingledew W. Ethanol tolerance in yeasts. Crit Rev Microbiol 1986; 13: 219 – 90.
21. Robinson B, Kutianwala J. Cassava: its potentials as an industrial crop. In: World Crops. London U.K Press. 1982; 59 – 78.
22. Ameh JB, Okagbue RN. Isolation and selection of local yeast strains for ethanol production. Bull Biotech Soc of Nigeria 1987; 3: 23.
23. Keim CR. Preparation of acid hydrolysed starch substrate and their fermentability. 11th Int. Carbohydrate Symposium Canada August. 1982; 22 – 8 .
24. Keim CR. Technology and economics of fermentation alcohol – an update. Enzyme Microbial Technology 1983; 5(2): 103 – 14.