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Mutagenic Strength of Ethanol Tolerant Saccharomyces Species Isolated from Palmwine Sap (*Raphia Sudanica*)

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I. Introduction

Nowadays, manufacturers have begun to show unprecedented interest in innovations by introducing benefits from re-engineering and genetic manipulation. These innovations were ushered in because of certain constraints intrinsic to the organisms being used for the respective manufacturing processes. These include the need to improve yeast resistance to ethanol, mutagens, 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 (Aunstrup,2007). In order to obtain strains showing more suitable properties, genetic manipulation methods have been used (Dubey, 2009). 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 classical mutagenesis, protoplast fusion and transformation necessary (Arnord and Jose, 2008).

However, classical mutagenesis which involves the production of mutants by the exposure of microbial strains to mutagenic chemicals or ultraviolet rays to induce changes in their genomes leading to production of Improved strains would be the main focused of this research work. Seven days (168 hours) ethanol tolerance Saccharomyces species isolated from palmwine sap would be subjected to mutagenic effect of acridine orange (a basic fluorescent dye that selectively binds to genetic material and can differentiate between deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)), Such mutants may, however, show undesirable secondary mutations which can influence the behaviour of cultures during fermentation. Saccharomyces appear microscopically as globose or ovoidal cells with multilateral budding and possibly pseudohyphae. The yeast forms one to four ascospores, which are smooth and ellipsoidal. Colonies appear smooth, usually flat, and occasionally raised and opaque (Martini and Martini, 1998). The two primary species found in wines, S. bayanus and S. cerevisiae (anamorph: Candida robusta), ferment glucose, sucrose, and raffinose and assimilate glucose, sucrose, maltose, raffinose, and ethanol but not nitrate. Saccharomyces cannot utilize five carbon sugars (e.g., pentoses) (Kudo et al., 1988). On a dry weight basis, Saccharomyces contains 3% to 5% phosphate, 2.5% potassium, 0.3% to 0.4% magnesium, 0.5% sulfur, and trace amounts of calcium, chlorine, copper, iron, zinc, and manganese (Monk, 1994; Walker, 1998). Yeast must be supplied with a source of phosphate, which is incorporated into nucleic acids, phospholipids, adenosine-5′-triphosphate (ATP), and other compounds. Potassium is necessary for uptake of phosphate, and a deficiency may be linked to sluggish alcoholic fermentations (Kudo et al., 1988). Other minerals needed by Saccharomyces during fermentation have a variety of functions but are used primarily as enzyme activators. Besides minerals, yeasts require various vitamins such as thiamin, riboflavin, pantothenic acid, pyridoxine, nicotinamide, biotin, and inositol depending on species and specific growing conditions (Monk, 1994; Ough et al., 1989). In general, practically all strains of Saccharomyces require biotin

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and pantothenic acid while some also need inositol and/or thiamin for synthesis of nucleic acid, protein and fatty acids (Walker, 1998).

Palm wine is a traditional wine extracted from palm trees Elaeis guineensis and Raphia hookeri. Palm wine is consumed for its nutritional effect because of its probiotic content (Ezeronye 2004; Lourens-Hattingh and Wiljoen 2001; Heller 2001). Palm wine is consumed throughout the tropics and appears as a whitish liquid produced by natural fermentation of the sap of Elaeis guineensis and Raphia hookeri (Uzogara et al., 1990; Uzochukwu et al., 1991). The unfermented sap is clean, sweet, colourless syrup containing about 10 - 12% sugar, which is mainly sucrose (Bassir, 1962; Okafor, 1975a). Upon fermentation by the natural microbial flora, the sugar level decreases rapidly as it is converted to alcohol and other products (Obire, 2005) whereas, the sap becomes milky-white due to the increased microbial suspension resulting from the prolific growth of the fermenting organism (Okafor, 1975).

Palm wine is characterized by an effervescence of gas resulting from the fermentation of the sucrose content, by the fermenting organisms (Bassir, 1962). Previous studies on the microbiology of E guineensis and R. hookeri have incriminated several bacterial and yeast flora to be involved in the fermentation process (Fapa-runsi and Bassir, 1972; Okafor, 1972; Okafor, 1975; Eze and Ogan, 1987; Amanchukuru et al., 1989; Ejiofor, 1994; Orimaiye, 1997; Nester et al., 2004). These organisms have also been reported to originate from several sources, which include tapping equipment, containers, the environment, etc (Faparunsi and Bassir, 1972; Eapen, 1979).

George et al. (2009) described the enhancement of the recombinaenic and mutagenic activities of bleomycin in yeast by intercalation of acridine compounds into DNA. Strain D7 of Saccharomyces cerevisiae isolated from palmwine was used to measure the induction by bleomycin (BLM) of mitotic recombination at the trp5 locus and point mutations at ilv1 in the presence and absence of acridine compounds. BLM is a potent mutagen and recombinagen in the D7 assay. The acridines vary, some being mutagenic or recombinagenic and others not. Combined treatments were used to distinguish whether a genetically inactive acridine has no effect on the genetic activity of BLM or modulates its action. When an acridine is itself genetically active, combined treatments were used to determine whether its effects are additive with those of BLM or whether there is interaction between the two compounds. Acridine compounds that share the ability to intercalate between the base pairs of DNA but differ in their mutagenic specificity owing to the presence of different substituent groups were analysed. Clear potentiation and synergistic interactions were detected in combined treatments with BLM and aminoacridines, nitroacridines or an acridine mustard. Potentiation and synergy were also observed in sequential exposures in which the yeast were grown in the presence of acridine compounds and then treated with BLM in the absence of free acridine. The results are consistent with an increase in BLM susceptibility conferred by acridine intercalation. It is likely that the intercalating agents increase the access of BLM to the minor groove of DNA, where it abstracts a hydrogen from the 4' position of deoxyribose, creating a free radical that is processed.

The main focus of this paper is to mutagenically alter the genome (using acridine orange) from the reference Saccharomyces species isolated from palmwine sap (incubated for 168 hours), that coded for metabolic/enzymatic synthesis in fermentation and reassess the mutated yeast strains for fermentative potential to determine the true genetically stable and the degree of persistence of the species in fermentation processes with the objective of targeting the selection and improvement of Saccharomyces species in other to improve the overall process control, yields and efficiency as well as the quality, safety and consistency of bioprocesses products.

II. Materials and Method

a) *Sample Collection*

Fresh palm wine samples from Raphia palm (Raphia sudanica) was collected from main traditional palm wine trappers in Akur village (Kwara state polytechnic), Ilorin, Kwara state whittin 30 minutes of tapping. The freshly tapped samples was collected using pre-sterilized 200ml capacity sample bottle with perforated screw cap. The perforated screw caps were plugged with sterile non-absorbent cotton-wool. The samples were transported to the laboratory in a cooler equipped with packs of freezing mixture of salt and ice-block for analysis within 1 hour of collection.

b) *Laboratory ageing of palmwine sample*

The freshly tapped Palmwine sample collected was stored in opaque 250 ml conical flask plugged with cotton wool for 168 hours (7 days) at room temperature (28±2˚C) on an orbital shaker at 100 rpm to prevent sedimentation. Determination of pH and percentage ethanol content (% v/v) of palm wine sample was carried out aseptically at 0, 24, 36, 48, 72, 96, 120, 144, and 168 hours of fermentation (Ogbulie et al., 2007 and Nwachukwu et al., 2006a and b).

c) *Isolation and identification of ethanol tolerant Saccharomyces spp. from aged palmwine*

Five milliliters of thoroughly mixed palmwine was centrifuged in sterile centrifuge bottles at low speed for 5 minutes. One milliliter of the sediment was transferred using sterile pipette onto sterile Glucose Yeast-extract Agar (GYA), spread using a sterile bent glass rod and incubated at room temperature (28±2˚c)

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for 24 – 48 hours (Okafor, 2008). Chlorophenicol was added to the GYA at 0.05 mg/ml to suppress the growth of bacteria (Nwachukwu , 2008, Bechem et al., 2007). Vaughan-Martini and Martini, (1998) Barnett et al. (1990), Kregger Van-Rij (1987) for yeast identification were employed. The test include morphology, surface characteristics, presence of pseudohyphae (pseudomycellium), ascospore formation and vegetative reproduction, nitrate utilization, growth in 10% NaCl + N50% glucose in yeast extract, growth in°C37and 30° C and growth in 50% w/w glucose yeast extract . Fermentation test include sugars such as lactose, maltose, saccharose, galactose and raffinose.

d) Fermentation Assessment Test

Batch fermentation was carried out on each of the saccharomyces isolate, by transferring 1ml of the isolate starter culture contanning about 107 cells ml-1 into 100ml of the fermentation medium in 250ml conical flasks. The flasks were incubated at room temperature. Determination of percentage ethanol content (% v/v) produced by each isolate was carried out aseptically at 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 days of fermentation period (Nwachukwu and Ibekwe, 2004; Wellala et al., 2004)

e) Determination of Minimum Inhibitory Concentration (MIC) of Acridine Orange

The minimum inhibitory concentration of the acridine orange was determine according to the method described by Crosa et al. (1994), Akiotoh-E and Reed (1981)

Yeast extract glucose medium was prepared and sterilized using autoclave. One milliliter (1ml) of the prepared broth was dispensed into the test tubes numbered 2 to 12 using sterile pipette. A stock solution containing a concentration of 0.2g of acridine orange in 10ml of deionized water was prepared and 1ml of the solution was dispensed into each of tube number 1 and 2. Subsequently, from tube 2, serial dilution was carried out using 1ml transfer up to tube number 10 and 1ml from tube 10 was discarded.

Also an overnight culture (starter culture) of each of the test isolates were prepared and 1:100 (10^2) dillution of broth were made and from this dillution, 1ml of the inoculum was transferred into each tube 1 to 12 with exception of tube 11, to which another yeast extract glucose medium would be added.

The final volume in each tube was 2ml with exception of tube number 1 that had only 1ml of acridine orange (served as control for acridine orange), Tube 12, which contained 1ml of culture and acridine orange, served for viability of the culture in use and tube 11 served as control for the sterility of the sterile broth.

The final concentration of acridine orange in each of the tube numbered 1 to 10 after dillution were; 20,000, 10,000, 5,000, 2,500, 1,200, 625, 312.5, 156.25, 75.125, and 37.06 ug/ml respectively.

The set-up was incubated at 28±2° C for 24 -48 hours after which the tubes were examined for growth. The last tubes in which growth failed to occur for each isolated species were taken to be the minimum inhibitory concentration (MIC) tube. The concentrations of the MIC and sub-MIC tubes were noted and SUB-MIC concentrations were used for the determination of genetic strength.

The above procedures were repeated for each of the isolated Saccharomyces species to determine the genetic strength and stability of the species.

f) Determination of mutagenic strength in isolated Saccharomyces species

Determination of genetic strength from isolated Saccharomyces species using mutagenic method (acridine orange) was performed according to the method of George et al. (2008), Grinsted and Bennet (1988), Akiotoh-e and Reed (1981).

Sub-MIC concentrations for each species were chosen against such isolate in the determination procedure.

The test Saccharomyces species was inoculated into a sterile yeast glucose medium and allowed to stand for 24 hours. This broth served as inoculum. Another sterile medium broth was prepared and distributed in 1ml volume into four different test tubes and sterilized by autoclaving. A stock solution of acridine orange in sterile deionized water (sub-MIC concentration) was prepared and 1ml of the solution was dispensed into the test tubes containing sterile broth. The content of the tube was vortexed for seconds and 20µl of overnight-incubated inoculum was added with the help of micropipette (each tubes represented different isolates of the Saccharomyces species). The tubes were incubated at room temperature (28±2°C) for 24-48 hours after which the growth obtained were inoculated into the Petri-dishes containing yeast-extract glucose agar + chlorophenicol and incubated under the same conditions.

The colonies appeared were then subjected to fermentative re-assessment as described to determine the genetic strength and stability

III. Result and Discussion

The two most important physiochemical parameters determined in this research work clearly show that the percentage content of ethanol of freshly tapped palmwine was 2.0%v/v with pH of 6.0. The ethanol content increased with time of ageing to a peak of 5.1% v/v at 72hours , after which it diminish gradually to a level of 3.0% v/v after seven (7) days of ageing (figure 1). The decline in the ethanol level of the sample with respect to age is an indication of preference of the microorganisms for metabolism especially the yeast for fermentation (Priest and Campell, 1996). This result could be as result of some of the alcohol being

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converted into more organic acids by the fermenting yeasts and bacteria (Ogbulie et al., 2007; Kenneth and Charles, 2007; Robert, 2006; Nwachukwu et al., 2006a; Ogbonna, 1984; Okafor, 1972). The steadily reduction of pH of the wine sample from 6.0 to 1.5 after 7 days of fermentation is probably due to the accumulation of acidity, because of formation of organic acid with time. This result is correlated with the finding of Nwachukwu et al. (2006a), Oyagade (2004), Okafor (1972).

Microscopic examination of the 7-days aged palm wine sample showed that palm wine serves as a good medium for the growth of numerous microorganisms especially yeast cells. However, the following ethanol tolerant Saccharomyces species; S. cerevisiae, S. uvarun, S. lactis, S. flagilis and Hansenulla species were isolated and identified . All Saccharomyces species isolates showed resistance to chloramphenicol. The chloramphenicol inhibits the growth of most bacteria while allowing the yeast to grow. The ethanol tolerance results indicated that with no exogenous ethanol added, the saccharomyces yeast isolated after 7-days clearly tolerated the physico-chemical conditions imposed by the palm wine (Nwachukwu et al., 2006c). This indicated that they were better adapted to the conditions in the wines than some of the other organisms involved in palm wine fermentation. The occurrence of these ethanol tolerant saccharomyces isolates in the palm wine samples, however, supports the reports made by Amoa-Awua (2007), Nwachukwu et al., (2006a), Oyagade (2004), Ejiofor (1994) Ikenebomeh and Omayuli (1988), Casey and Igledew (1989), Ogbonna (1984), Okafor, (1980), Faparunsi and Bassir (1971) and Okafor (1975).

Nine (9) days Fermentation assessment test indicating the rate of production of ethanol on 15% glucose culture medium by the isolated Saccharomyces species as presented in figure 2 indicated the initial rate of ethanol production was higher in S. cerevisiae (4.1% v/v) than S. uvarum, and S. flagilis (1.4% v/v) While S. lactis shown zero (0% v/v) production until after 48 hours. However, the percentage of ethanol produced after 9 days of assessment was highest in S. uvarum, and S. flagilis (10.8%v/v) and lowest in S. lactis (8.1%v/v) while S. cerevisiae recorded 10.4% v/v.

A clear relationship in the fermentation pattern was evidence between the four isolates, where the initial rate of fermentation was the highest in S. cerevisiae followed by S. uvarum S. flagilis and S. lactis. However, the percentage of ethanol by each isolate on 15% glucose medium after reaching the contant specific gravity varies from 10.4 and 10.8 to 8.1 indicating that the final ethanol yield was not significant difference among the four Saccharomyces species. These result was in agreements with the finding of Wellalla et al. (2004) Tabera et al. (1985) and Ogbonna (1984) in which one Saccharomyces species that showed a higher rate of fermentation than another at one stage of fermentation was not always found to be the highest ethanol producer. However, the increased production and tolerance of ethanol could be as a result of duplication/mutation of the genes responsible for both ethanol production and tolerance, these characters have been known to be polygenic (Nwachukwu, 2008).

The determination of the minimum inhibitory concentration (MIC) of the mutagenic agent, acridine orange (BDH Chemical Ltd, Poole, England) was to provide a minimum concentration (sub-MIC) of the agent that would selectively bind to the yeast chromosomal DNA molecule for easier mutational changes (George et al., 2009; Grinsted and Bennet, 1988). The minimum inhibitory concentration (MIC) of the acridine orange (mutagenic agent) to the reference Saccharomyces species was determine after 48 hours of specific incubation (figure 3). After three (3) successive experimental trials for each isolate, both S. lactis and S. uvarum had the highest (MIC) value of 4166.67μg and 3333.33μg while S. cerevisiae and S. fragilis recorded 2083.33μg and 1250μg respectively. Therefore, the sub-MIC values recorded are 1666.67, 2083.33, 1041.67 and 625 for S. uvarum, S. lactis, S. cerevisiae and S. fragilis respectively.

Fermentation re-assessment test conducted after mutagenic experiment to determine the genetic strength of the isolated species using sub-MIC values are presented in figure 4. The result show that S. lactis had zero percentage (0% v/v) ethanol content after 9 days of fermentation while S. flagilis produced 2.6 %v/v ethanol concentration after 4 days of fermentation period and 2.8 %v/v was observed in S. uvarum after 3 days of fermentation. The highest rate of ethanol concentration was detected in S. cerevisiae with 10.3 %v/v ethanol content recorded after 5 days of fermentation period. However, amount of ethanol produced was terminated in S. cerevisiae, S. flagilis and S. uvarum from 5, 4 and 3 days and constant throughout the duration of fermentation period as shown in figure 4.

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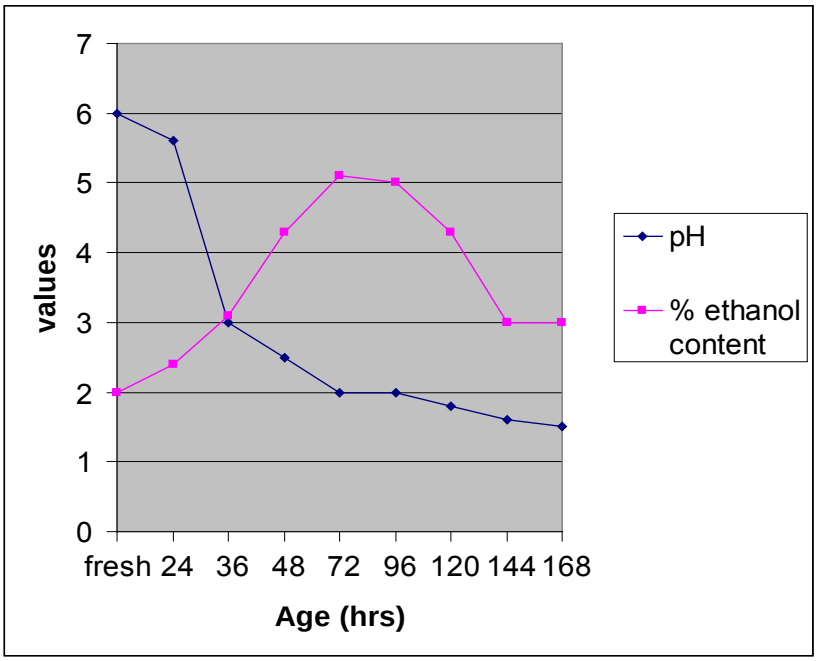

Figure 1 : Changes in the physiological parameters of aged palmwine

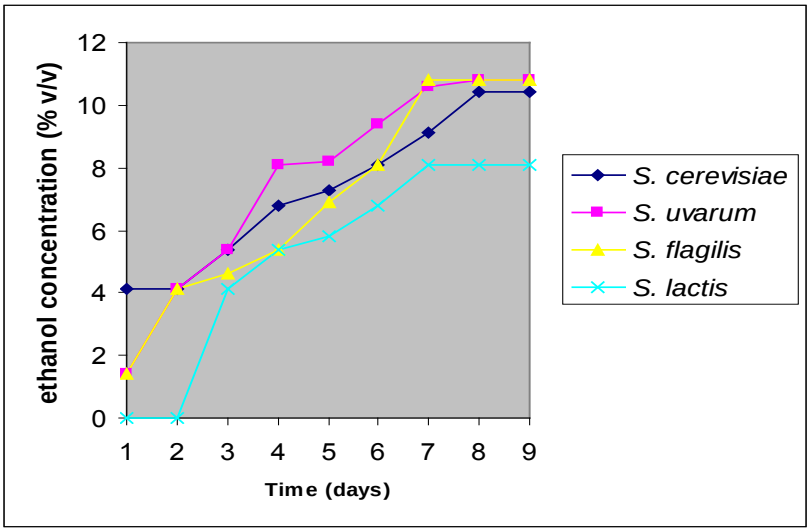

Figure 2 : Rate of ethanol production by isolated Saccharomyces species on 15% glucose culture medium (Fermentation assessment test).

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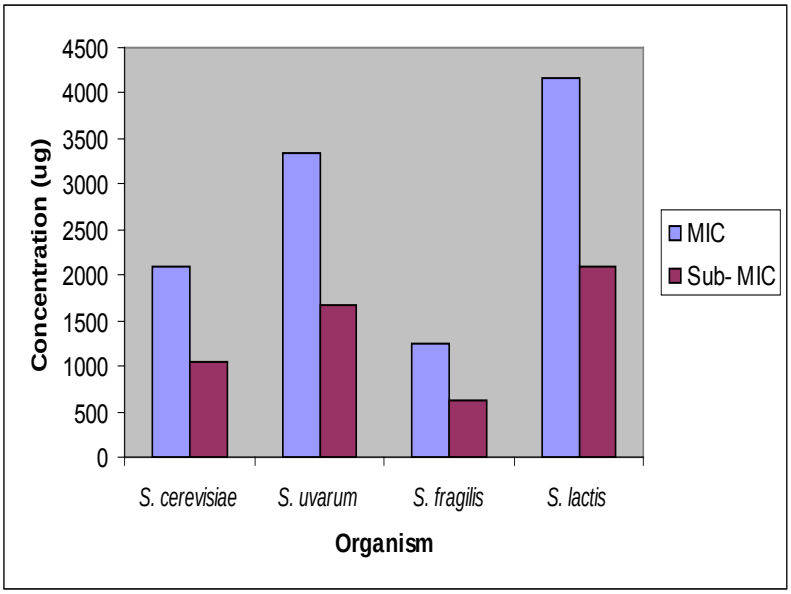

Figure 3 : Minimum and sub-Minimum inhibitory concentration of ACRIDINE ORANGE (mutagens) on isolated Saccharomyces species

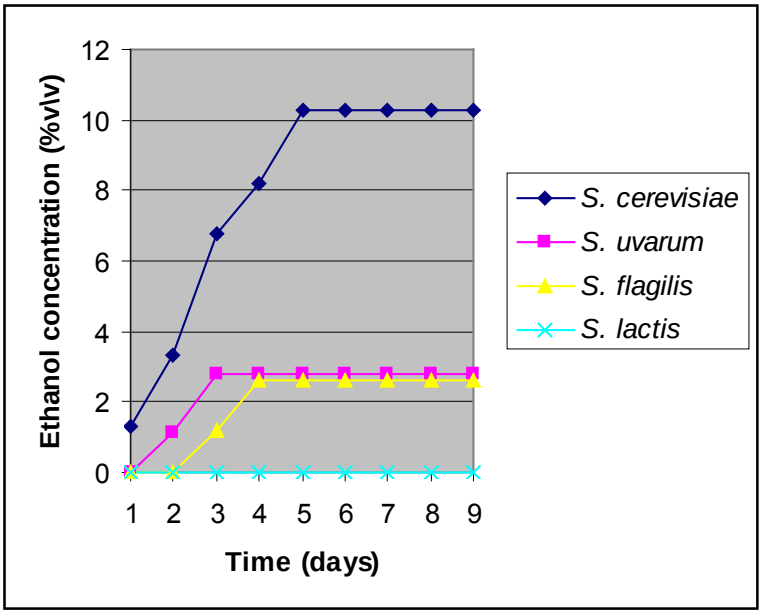

Figure 4 : Ethanol production by mutagenic treated saccharomyces isolates on 15 % glucose culture medium. (fermentation re-assessement test)

IV. Conclusion

Mutagenesis experiment conducted indicated that the four isolated Saccharomyces species actually harbours genetic materials encoded for fermentative property, that is, the fermentative ability of the isolates are partially and completely control by genetic properties embedded in the nuclear material. This was confirmed after fermentation re-assessment test at which all isolates recorded negative values compared to earlier fermentation assessment test except S. cerevisiae that show relative genetic stability.

This research work has provided ideas about the mutagenic capability and effectiveness of fermentative abilities of the Saccharomyces species and raises a very strong hope for the industries ethanol fermentation industries of reduced production costs. The application of their stability is that, these yeast mutants could be promising as industrial organisms for use in ethanol and other fermentation industries.

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