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Genotypic Variation in Ethanolic Fermentation Using New Genetically Recombinants of *Saccharomyces cerevisiae*

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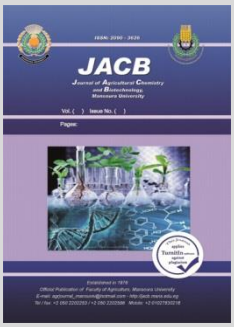

ABSTRACT

This study aimed to investigate metabolic changed related to glucose – repression by the analysis of ethanol productivity which is mainly affected by the glucose effect. Five parental isolates and 15 genotypes derived from three crosses were used in this study to be evaluated for heterosis of ethanol productivity. Bioethanol produced can contribute to a cleaner environment using a cheap substrates such as sugarcane juice sub – products used in this study . Mating between yeast isolates gave different recombinant genotypes some of them showed glucose – repressed or depressed to higher concentrations of sugarcane juice sub – products. Sugar repression is one of the major limitations in the fermentation of cost – effective ethanol productivity . When the hybrid genotypes were grown in a culture containing 0.02 sub – products they are exhibited positive hybrid vigor for ethanol production overall the genotypes compared to the mid – parents. All hybrids exhibited negative heterosis when they were cultivated in the culture containing 0.04 sub – products, indicating that they were glucose – sensitive due to glucose pulses at this concentration. In addition, when the hybrids resulted from the cross between p_1 x p_4 were grown in a fermentation culture containing 0.06 sub – products they are also exhibited negative heterosis for ethanol productivity. In contrast, all the hybrids derived from the crosses between p_2 x p_5 and p_3 x p_5 overcoming glucose repression of 0.06 sub – products containing medium because heterosis seemed to appear at this level of carbohydrates.

***Keywords**: Saccharomyces* hybrids, heterosis, ethanol productivity, sugar repression, hybrid biodiversity.

INTRODUCTION

Fossil fuels had been used in the world for many years to supply primarily the energy and organic chemicals requirements. Meanwhile, the energy sector was faced with the problem of petroleum fuel reduction. In 2050 , the world society is expected to increase up to 10 billion , thus will increase the consumption to fuels in the world (Germec *et al.* 2016). Therefore , efforts are needed for enhanced the production of biofuels from biomass materials to decreasing petroleum cost and to supply the energy requirements of the world (Fatehi 2013) .

Renewable resources are the most low cost materials in the environment , which generally exist in the field of pre – and post – harvest agricultural losses , agro – industrial wastes and the effluent of food processing industry (Galbe and Zacchi 2012). Sugarcane refuse was also a kind of renewable resource released in nature as a post harvest waste of sugarcane juice factory. Sugarcane is a major commercial sugar crop in the tropical and subtropical environments around the world .

Ethanol was an organic solvent named as "Bioethanol" , produced through fermentation process using microorganisms. Sugars converted into ethanol is one of the earliest anaerobic organic reaction. Many authors decided that the attention must be devoted to the conversion of various materials of starchy and sugar crops such as sorghum, molasses, potato, cassava, sugarcane, fruit juices, corn and cashew apple juice using yeast and bacterial cells.

The industrial production of bioethanol using yeast cells was started at the end of 19th century after the yeast was isolated and identified by Louis Pasteur . During 1908 , Henry Ford designed a fuel with a mixed of gasoline and alcohol , it was referred to as " future fuel " in the year of 1925 (Ravindra 2007). Ethanol (C_2H_5OH) has a molecular weight of 46.07 and boiling point at 78° C . The oxygenated biofuels such as bioethanol was an effective alternative for renewable fuels (KrishnaSwamy *et al* . 2012).

Production of bioethanol from sugarcane juice wastes for use as a transportation fuel is a good biotechnology. It was first introduced in the United States of America in the early 1900's. Fermentation had been used since thousands of years as an effective and low – cost resource to manufacture safety of foods . Yeast are the main group of microorganisms that has been used in fermentation and production of alcoholic beverages such as beer and wine (Belloch *et al.* 2008) . Most works on ethanol productivity from sweet crops juice have been done using free cells of *saccharomyces cerevisiae* in batch process (Bvochora *et al* .2000).

To achieve high fermentation efficiency with specific properties , new and genetically improved yeast strains are needed (Romano *et al.* 1985). Yeast selection through breeding programs can by crossing the spores (Winge and laustsen 1938) , crossing of cells (Lindegren and Lindegen 1943), crossing the cells with spores (Takano and Oshima 1970) and protoplast fusion (Russell and Stewart 1979) .

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Yeasts are often diploid strains and homothallic (Thornton and Eschenbruch 1976), never haploid. The single spores can form diploid by self diploidization. This revealed that breeding by spore conjugation is a good method for inducing genetically recombinant hybrids in yeast. A suitable isolation or collection of diploids , homozygous strains with a high sporulation efficiency and high viability of spores was required (Romano *et al*. 1985). Genome shuffling allows many yeast strains recombined through hybridization to carry certain genotypic improvements especially in alcoholic fermentation . This offers the advantage of genetic changes at different locations throughout heterozygosity to exhibit heterosis in yeast without the necessity for genome sequence information (Shi *et al*. 2009) . When the nutrients are abundant for *saccharomyces cerevisiae* they are reproduced via mitosis as diploid cells. In addition , when the diploid cells undergo starvation to the nutrients they are sporulate to form haploid spores through meiosis (Herskowitz, 1988).

Saccharomyces cerevisiae genome size in about 12.2 Mbp organized on 16 chromosomes and about 92 % of this genome are believed to be functional (Mortimer *et al*. 1994).

Yeasts and bacteria have been used for ethanol production since many thousands of years from pure carbon sources such as; glucose , starch , sucrose , xylose, ect ; industrial plants (sugarcane, sugar beet , sweet sorghum , ect.) or by – products of food and chemical industry (whey , molasses , sugarcane juice refuse , ect). Among these , baker's yeast is the most microorganism from yeasts used from past to present , in addition to *Zymomonas mobilis* and *Pichia stipites* (Atiyeh and Duvnjak 2003).

This study aimed to develop of new recombinant genotypes from *Saccharomyces cerevisiae* via hybridization to improve ethanol productivity through hybrid vigor. Furthermore, evaluate the recombinant genotypes for their efficiency to perform alcoholic fermentation using the residual sugars of sugarcane juice industry refuse as a main source of carbohydrates converted into ethanol.

MATERIALS AND METHODS

Microbial strains

Five yeast strains are the genetic materials used in this study. These strains, as well as, their references or sources are listed in Table 1.

Table1. Yeast strains used in this study.

Yeast strains	Source	Designation
Saccharomyces cerevisiae	Bakers yeast , a block of compressed fresh yeast in its wrapper , The Egyptian Starch, Yeast and Detergents Company.	P_1
Saccharomyces cerevisiae	Microbial Genomics and Bioprocessing Research, United States, Department of Agriculture, USA	P_2
Saccharomyces cerevisiae	grape juice Fermented	P_3
Saccharomyces cerevisiae	Instant yeast supplier silesaffre 59703 marcq France	P_4
Saccharomyces cerevisiae	Fermented wheat flour juice having a popular name " Buzza "	P_5

Sugar juice sub - products

Sugarcane juice wastes were was used in this study because of residual sugars are contained as a sole source of carbon instead of glucose added to the fermentation medium. They were collected from the local market of sugarcane juice in Mansoura city during October 2018. The white fibers inside the stem were collected after removal the outside surface of the stem. It was cut to separate parts three cm long .The parts containing residual sugars were used as a sole source of carbon in the fermentation medium of ethanol productivity at different concentrations including, 2, 4 and 6% .

Media and growth condition

Yeast extract peptone dextrose medium (YEPD) was used as a complete medium for growth and maintenance of yeast strains according to Chung *et al.* (1995). Pre Sporulation medium was used to stimulate the cells to sporulate according to (Bähler *et al.* 1994). Sporulation medium was also used according to Sherman *et al*. (1982). Fermentation medium was used for ethanol production. It consist of (g / L), sugarcane juice industrial wastes with the concentration of , 2 % or 4 % or 6 % ; peptone , 10g ; yeast extract , 2 g and distilled water up to 1000 ml.

Reagents used for determining ethanol productivity

These reagents were prepared according to Plevaka and Bakoshinskaia (1964) . They included Potassium dichromate solution (PS), Ferrous ammonium sulphate solution (Titrate solution) and Diphenylamine solution indicator.

Reagents used for determining reducing sugars

These reagents were prepared according to Nelson (1944). They included Nelson's A, Nelson's B and arseno - molybdate reagent.

Antifungal markers agents

Selectable genetic markers are an important tool in the construction of yeast hybrids. Ideally, the antifungal markers allow efficient selection of yeast hybrids without affecting any cellular functions. Antifungal resistance markers are alternative to auxotrophic markers. Thus, nine antifungal drugs were used in this study with different concentrations (µg/ml) for genetically marking yeast strains as shown in Table 2.

Table 2. Antifungal drugs and their concentrations used for genetic marking of yeast strains.

Antifungal Agents	Concentration (mg/ml)	Abbreviation
Flocazole	0.01	*Floz*
Flucoral	0.01	*Fluc*
Fungican	0.01	*Func*
Treflucan	0.01	*Tref*
Lamisil	0.5	*Lami*
Fungisafe	0.5	*Funs*
Itracon	0.01	*Itrc*
Itranox	0.01	*Itrn*
Trosyd	0.01	*Tros*

Methods

Yeast isolation

Yeast isolates were recovered from four sources including (i) fermented grapes , (ii) baker›s yeast , (iii)

fermented wheat flour juice having a popular name " buzza " and (iv) instant yeast . About one gram of each source was used and further serially diluted in conical flask 100 ml using distilled water. About 100 µl of each of the last two serial dillutions was then spread on the top of YEPD medium. Then, the spread yeast cells were further incubated for 72 hours at 30 °C. Single colonies of the expected yeast isolates were picked up and then purified and screened by using a microscope and selective medium (Bonciu *et al*. 2010).

Genetic marking

Antifungal drugs were used in this study for genetic marking yeast strains. Susceptibility to antifungal drugs was measured by plate diffusion method according to Collins and lyne (1985).

Hybridization technique

This technique was done between the cells carrying the opposite genetic markers until the colonies of cells appeared on sporulation medium which formed asci. Then each colony formed asci was picked up and grown on YEPD slant agar medium according to **Grinsted and Bennett (1990)** .

Determination of ethanol

The amount of potassium dichromate solution consumed in the oxidation of ethanol (PSC) determination was calculated according to the following equation:

PSC = 10 – [0. 26 X TS]

Where, PSC: The amount of potassium dichromate solution consumed in oxidation of ethanol.

TS: The amount of titration solution consumed in oxidation of ethanol.

The ethanol of unknown sample was determined using a standard curve of ethanol . The standard curve (Fig. 1) was dependent on the amount of PSC in the reaction which is related with each concentration of ethanol prepared to be used as a standard (Ciani and Ferraro 1998).

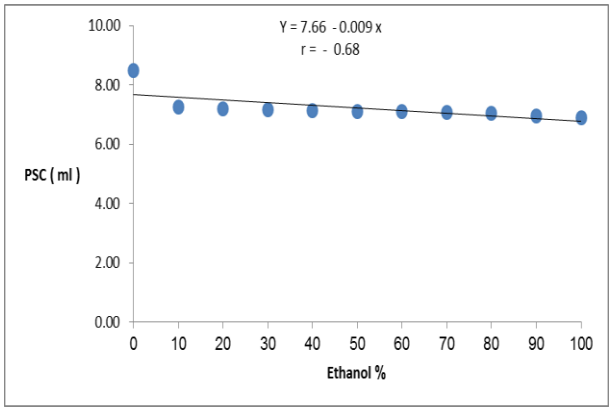

Figure 1. Standard curve for ethanol determination.

Biochemical traits

Biomass formation

After the media were centrifugated at 7000 rpm for 10 min, the supernatants were kept in ice - cold and used for determining consumed reducing sugars. Precipitated yeast cells were then weighted (Walsh *et al*. 1991).

Determination of reducing sugars

Reducing sugars (RS) were determined by the method of Nelson (1944) using sucrose standard curve as shown in Figure (2). The blank was carried out using the fermentation medium without inoculation. The consumed reducing sugars of samples were determined via a standard curve of sucrose (Fig. 2) using the following equation:

Consumed sugars (CS) = Initial concentration of sugars (IC) - Residual sugars in the fermentation medium at the end of fermentation time (RSF).

Bioconversion efficiency (ethanol yield) was expressed as ml ethanol produced per gram sugar (s) consumed (Nichols *et al*. 2001).

The volumetric ethanol productivity (Q_p) and the percentage of bioconversion efficiency or yield efficiency (E_y) were calculated by the following equations:

$$Q_p = P/t \quad \text{and} \quad E_y = P/CS$$

Where, P is the actual ethanol concentration produced (ml / L), t is the fermentation time (h) giving the highest ethanol concentration for batch and fed-batch fermentations.

The standard curve was prepared using different concentrations of sucrose ranging from 0 to 10 mg / ml. However, sucrose is a common disaccharide sugar, composed of two monosaccharides: glucose and fructose which have the same molecular formula ($C_6H_{12}O_6$) but glucose had a six member ring while fructose has a five member ring structure. It has the molecular formula $C_{12}H_{22}O_{11}$.

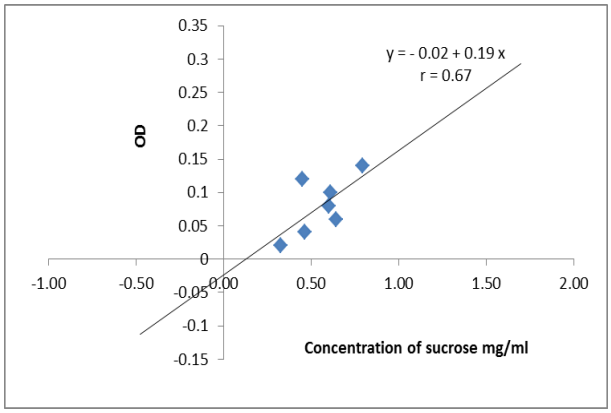

Figure 2. Standard curve for sugars determination.

Statistical analysis

Experimental data were subjected to the analysis of variance using completely randomized design according to Snedecor and Cochran (1955). Least significant difference (L.S.D.) was used to compare between means if the F-test was significant.

RESULTS AND DISCUSSION

Bioethanol produced from 0.02 substrate.

However, hybridization in yeast strains may improve ethanol production from carbohydrate sources such as sugarcane sucrose used in this study. The results presented in Table 3 showed that all hybrid genotypes resulted from the cross between p_1 x p_4 expressed hybrid vigor for ethanol production from 0.02 sugarcane juice sub – products in relation to the mid – parents. Transaldolase (TAL) genes and formate dehydrogenase (FDH) genes activities conferred resistance to weak acids in recombinant sucrose – fermenting yeast (Sanda *et al*. 2011). Similarly, FDH genes in recombinant *Saccharomyces cerevisiae* may be up – regulated in the yeast hybrids (Hasunuma *et al*. 2011). Baker's yeast encodes two *FDH* genes (*FDH_1* and *FDH_2*) used for converting weak acids into carbon dioxide (Overkamp *et al*. 2002) to detoxify weak acids (Hasunuma

et al. 2011). The observed hybrid vigor in this study may be due to up – regulated of *TAL* and *FDH* genes in recombinant yeast hybrids.

Table 3 . Hybrid vigor and kinetic parameters of ethanol productivity from 2 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_1 x p_4.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield †	Q_P	E_Y	CS
P_1	73.19	-	1	0.44	3.70	19.77
P_4	167.56	-	1	0.99	8.62	19.43
MP	120.37	120.37	1	0.72	6.16	19.60
H_1	225.33	0.87	1.87	1.34	10.80	20.86
H_2	241.71	1.01	2.01	1.44	12.66	19.09
H_3	228.22	0.90	1.90	1.72	11.36	20.09
H_4	252.30	1.10	2.10	1.50	12.51	20.17
H_5	164.67	0.37	1.37	0.98	8.37	19.68
F – test	**	NS	**	NS	NS	**
L.S.D 0.05	90.740		0.482			6.03
L.S.D 0.01	125.942		0.669			8.38

**** = Significant at 0.01 level of probability.**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity , Y_{ps} : ethanol yield related to sugar consuming , E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

The results summarized in Table 4 and 5 clearly showed hybrid vigor for ethanol production by all hybrids genotypes fermented sugarcane juice sub – products. The hybrids resulted from the cross between p_2 x p_5 generated marked heterosis ranged between 84% to 216 % above the mid – parents. However, the hybrid genotypes resulting from the cross between p_3 x p_5 showed heterosis profile ranged from 117 % to 227 % . The results obtained herein demonstrated that efficient ethanol production from residual sugarcane juice sub- products was possible at 0.02 concentration in terms of ethanol concentration and product yield. However, some hybrids genotypes showed the maximum ethanol productivity in relation to the mid – parents. Recently, Oner *et al*. (2005) found that ethanol productivity from starch as the substrate by petite mutant reached 16 % higher than the wild type strain of *Saccharomyces cerevisiae*. To improve and use the petite mutant strain for future industrial applications, it should integrated which α – amylase gene to convert starch into ethanol. These results indicated that all the hybrid genotypes derived from three cross were efficienting converting of 0.02 sub – products of sugarcane juice to ethanol, carbon dioxide and other secondary metabolites (Pretorius 2000). In fact, during fermentation the yeast cells exposed to continuous mix of several stress conditions. The hybrid genotypes may be tolerance to four environmental stress as, pH, high glucose concentration, temperature and ethanol than the parental genotypes (Cardona *et al*. 2007). In addition, the genes related to bioconversion of sucrose into ethanol may be naturaly expressed and better adapted to stress conditions of fermentation than in the parental strains. These results agreed with Belloch *et al*. (2008), who found that all hybrids among species of *Saccharomyces* were able to grow at 37° C, and the hybrids also might have inherited the capability to grow at high concentration of alcohol. As a general conclusion, yeast hybrid genotypes may be better adaptated to fermentation stress conditions than the parental genotypes, therefore they must be prefered in industrial application. This is in harmony with Gonzalez *et al*. (2007), who found that natural hybrids of wine yeasts are not only well adapted to fermentation environment, but also they produce higher values of aromatic compounds than the parental genotypes. The results also agreed with Belloch *et al*. (2008), who found that hybrid yeast strains were able to resist high concentration of glucose and low pH in grape musts, as well as, better adapted to grow at low and higher temperatures than the parental strains. Genome recombination used in this study generated a novel population of yeast with further improvements in ethanol productivity. Successive improvement of yeast populations revealed the importance of heterosis in the improvement process of industrial yeasts. This because heterosis may show superior qualities than those of either parent.

Table 4 . Hybrid vigor and kinetic parameters of ethanol productivity from 2 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_2 x p_5 in batch fermentation.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield †	Q_P	E_Y	CS
P_2	76.07	-	1	0.45	3.86	19.69
P_5	94.37	-	1	0.56	4.76	19.84
MP	85.22	85.22	1	0.51	4.31	19.77
H_6	156.96	0.84	1.84	0.93	8.93	17.58
H_7	238.82	1.80	2.80	1.42	11.21	21.30
H_8	222.44	1.61	2.61	1.32	13.27	16.76
H_9	269.63	2.16	3.16	1.60	13.66	19.73
H_{10}	257.11	2.02	3.02	1.53	12.73	20.19
F – test	**	*	**	**	**	NS
L.S.D 0.05	61.864	0.693	0.563	6.90	2.22	
L.S.D 0.01	85.864	0.986	0.783	9.58	3.08	

***, ** = Significant at 0.01 level of probability.**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity , Y_{ps} : ethanol yield related to sugar consuming , E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

Table 5 . Hybrid vigor and kinetic parameters of ethanol productivity from 2 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_3 x p_5 in batch fermentation.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield †	Q_P	E_Y	CS
P_3	96.30	-	1	0.57	4.86	19.80
P_5	94.37	-	1	0.56	4.76	19.84
MP	95.33	95.33	1	0.57	4.81	19.82
H_{11}	271.55	1.85	2.85	1.62	14.99	18.11
H_{12}	274.44	1.88	2.88	1.63	15.13	18.14
H_{13}	291.78	2.06	3.06	1.74	14.45	20.19
H_{14}	207.04	1.17	2.17	1.23	10.37	19.96
H_{15}	312.00	2.27	3.27	1.86	16.64	18.75
F – test	**	*	**	**	**	NS
L.S.D 0.05	90.740	0.693	0.926	1.78	6.83	
L.S.D 0.01	125.942	0.986	1.286	2.47	9.48	

***, ** = Significant at 0.01 level of probability.**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity , Y_{ps} : ethanol yield related to sugar consuming , E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

Bioethanol produced from 0.04 substrate

Table 6, 7 and 8 showed negative heterosis for ethanol production from 0.04 sugarcane juice sub – products. All genotypes derived from the crosses between p_1 x p_4 , p_2 x p_5 gave negative heterosis at 0.04 substrate. These results indicated that yeast hybrids have different sugar metabolism characteristics at different concentrations of sugars. The hybrid genotypes were able to utilize the sugars at lower concentrations to be converted into ethanol, whereas the high concentrations are in general unable to ferment by the hybrid genomes, resulting in a lower fermentations rates (Magalhaes *et al.* 2016). Walther *et al.* (2014) demonstrated that the hybrid genomes of *Saccharomyces cerevisiae* were unable to ferment maltotriose might be because they lost significant portions of their genomes after hybridization, and possibly lost genes needed for maltotriose utilization during this reorganization of the genome. In general, the outperform of genotypic hybrids in relation to the parental strains may be due to possibly lost genes needed for tolerance to high sugar concentrations. The hybrids were clearly superior to ferment the lower concentrations of sugars than the higher concentrations. There was a clear link between the fermentation performance of hybrid genotypes and the content of sugars in the fermentations medium. This may be due to suppression of fermentation capacity at high sugar concentrations. Ethanol productivity by the hybrid genotypes was affected by high glucose concentration, this stress condition occur simultaneously at the beginning of fermentation, consequently, yeast cells can not tolerate sugar stress condition. These results appeared that the hybrid genomes showed reduced tolerance to high sugar concentration, indicating that the yeast hybrids may have not inherited the capability genes related to tolerate high sugar concentration or it may have inherited these genes but they are suppressed at the higher concentrations of sugars. The results agreed with Ferreira and Lucas (2005), who found that the molecular nature of glucose – repression phenomenon remains obscure, but it can speculate about the possible change in nuclear localization of factors like Cat 8 .

Table 6 . Hybrid vigor and kinetic parameters of ethanol productivity from 4 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_1 x p_4.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield †	Q_P	E_Y	CS
P_1	353.41	-	1	2.10	14.63	24.16
P_4	447.78	-	1	2.66	23.99	18.66
MP	400.59	400.59	1	2.38	19.31	21.41
H_1	348.59	-0.13	0.87	2.07	13.98	24.92
H_2	291.78	-0.27	0.73	1.74	11.94	24.43
H_3	370.74	-0.07	0.93	2.21	29.99	24.37
H_4	336.07	-0.16	0.84	2.00	15.72	21.38
H_5	371.70	-0.07	0.93	2.21	15.05	24.69
F – test	NS	NS	NS	NS	NS	NS
L.S.D 0.05						
L.S.D 0.01						

NS = Insignificant differences
Q_p : Volumetric ethanol productivity ,
Y_{ps} : ethanol yield related to sugar consuming , E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

Table 7 . Hybrid vigor and kinetic parameters of ethanol productivity from 4 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_2 x p_5.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield †	Q_P	E_Y	CS
P_2	318.74	-	1	1.89	16.18	19.70
P_5	330.29	-	1	1.96	13.79	23.95
MP	324.52	324.52	1	1.93	14.99	21.83
H_6	251.33	- 0.23	0.77	1.49	12.65	19.87
H_7	216.67	- 0.33	0.67	1.29	10.72	20.22
H_8	302.37	- 0.07	0.93	1.79	13.86	21.81
H_9	269.63	-0.17	0.83	1.60	12.78	21.10
H_{10}	235.93	-0.27	0.73	1.40	11.22	21.03
F – test	NS	NS	*	**	**	NS
L.S.D 0.05			0.198	4.77	5.31	
L.S.D 0.01			0.275	6.62	7.37	

***,** = Significant at 0.01 level of probability,**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity , Y_{ps} : ethanol yield related to sugar consuming , E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

Table 8 . Hybrid vigor and kinetic parameters of ethanol productivity from 4 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_3 x p_5 in batch fermentation.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield †	Q_P	E_Y	CS
P_3	645.1	-	1	3.84	30.40	21.22
P_5	330.29	-	1	1.96	13.79	23.95
MP	487.74	487.74	1	2.90	21.60	22.58
H_{11}	344.74	-0.29	0.72	2.05	15.63	22.05
H_{12}	303.33	-0.38	0.63	1.81	13.60	22.30
H_{13}	351.48	-0.28	0.73	2.09	16.57	21.21
H_{14}	421.78	-0.14	0.88	2.51	20.62	20.45
H_{15}	333.23	-0.32	0.70	1.98	15.85	21.02
F - test	**	NS	**	**	**	**
L.S.D 0.05	116.847		0.143	1.96	3.94	4.03
L.S.D 0.01	162.177		0.199	2.72	5.48	5.60

**** = Significant at 0.01 level of probability,**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity ,
Y_{ps} : ethanol yield related to sugar consuming , E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

Then the hybrid genotypes were simultaneously acclimated to high concentration of sugars. Therefore, the acclimation of hybrid yeast genotypes to high concentration of sugars could lead to simultaneous metabolism of fructose and glucose for the production of ethanol from sugarcane juice sub – products. Cho *et al.* (2014) found that acclimation of yeasts to specific sugar such as galactose reduced the glucose – induced repression on the transport of galactose. Ozcan *et al.* (1997) found that expression of *SUC_2* gene in *saccharomyces cerevisiae* was induced via a low levels of glucose. The problem of glucose repression in genotypic hybrids of yeast may lead to future acclimation of the yeast cells to high sugar concentrations. On the other hand , Gancedo (1992) demonstrated that the addition of glucose inhibits the transcription of glucose repressible genes , e.g. *SUC_2* (encoding invertase), *HXK_1* gene (encoding hexokinase isoenzyme I) and the *GAL* gene (encoding the enzymes of

the Leloir pathway. The long term repression of enzyme synthesis is known as catabolic repression. The data presented above showed that the hybrid genotypes were sugar – sensitive under the high concentrations of the sub – products containing sugars yield a remarkable insight into these phenomenon referred to as glucose repression. This is in harmony with Petrik *et al*. (1983), who decided that all possibilities of metabolic regulation needs to demonstrate the particular behavior of high glucose – sensitive yeast genotypes.

Bio ethanol production from 0.06 sub – products

The results obtained in Table (9) showed that most of the hybrid genotypes resulted from the cross between p_1 x p_4 showed negative heterosis for ethanol production from 0.06 sub – products which ranged between – 0.08 to 0.25 . The negative heterosis obtained herein may be due to glucose repression which various attempt to explain these phenomenon in *S . cerevisiae*, such as evolutionary engineering (Madhavan *et al*. 2009) and deletion of key genes involved in glucose repression such as MIG_1 and MIG_2 (Klein *et al*. 1999) . This agreed with Sijin *et al*. (2010), who reported that glucose repression in mixed sugars of fermentation medium improved the overall sugar metabolism efficiency and bioethanol production by *S. cerevisiae*. Glucose repression observed in this study may be due to semi – aerobic fermentation because aerobic fermentation is a consequence of glucose repression. When high concentrations of glucose was present the expression of genes involved in tricarboxylic acid cycle, oxidative phosphorylation, glyoxylate cycle, gluconeogensis and utilization of sugars other than glucose were repressed (Gancedo 1998). In addition , the expression of alcohol fermentation genes was induced. This mechanism resulted in preferential use of glucose over the other carbon sources. When baker's yeast grown in a mixture of glucose and other carbon sources like galactose, sucrose , maltose , fructose ect. glucose was utilized first , whereas the other sources of carbon were not utilized until glucose was exhausted (Leonie *et al*. 2001). Birky and JR (1975) demonstrated that repressed cells in relative to derepressed cells showing the following: high reduced the activity of mitochondrial enzymes related to electron transport correlated with decreased oxygen uptake, decrease in mitochondrial DNA molecules per cell and a small mitochondrial numbers. These alterations seems to change the physiology of mitochondria and the cell which affect the expression of mitochondrial genes. The resulting zygote from hybridization containing numerous copies of mitochondrial genomes and will be heteroplasmic for mitochondrial genes while the parents carried different alleles, Through ten generation, or less, the somatic segregation of mitochondarial genophores resulted in homoplasmic diploid cells (homozygous) at all mitochondarial gene location. In addition, mitochondrial genes loci undergos recombination in heteroplasmic cells (Thomas and Wilkie 1968). It is essential to show the output of heterozygosity influenced by the high concentration of the carbon source used for bioconversion into etanol which exhibited negative heterosis as a consequence of glucose repression modifies the direction of positive heterosis obtained at the lower concentration of sugarcane juice sub- products.

Table 9 . Hybrid vigor and kinetic parameters of ethanol productivity from 6 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_1 x p_4.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield †	Q_P	E_Y	CS
P_1	322.59	-	1	1.92	12.37	26.08
P_4	200.30	-	1	1.19	8.25	24.28
MP	261.45	261.45	1	1.55	10.32	25.18
H_1	232.07	- 0.11	0.89	1.38	9.83	23.60
H_2	231.11	- 0.12	0.88	1.37	9.84	23.49
H_3	195.48	-0.25	0.75	1.16	8.44	23.16
H_4	241.71	-0.08	0.92	1.44	10.86	22.25
H_5	287.92	0.10	1.10	1.71	12.55	22.94
F – test	NS	NS	NS	NS	**	NS
L.S.D 0.05					6.39	
L.S.D 0.01					8.78	

**** = Significant at 0.01 level of probability,**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity ,
Y_{ps}: ethanol yield related to sugar consuming , E_y: yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

Table (10) and (11) showed that all the hybrids genotypes resulted from the crosses between p_2 x p_5 and p_3 x p_5 showed positive heterosis at 0.06 concentration of sub – products, except for one genotypic hybrid resulting from the cross between p_2 x p_5 . The results indicated that the hybrid genotypes derived from these crosses derepressed the high concentration of sugars leading to heterosis for ethanol production ranged from – 0.04 to 0.54 and 0.05 to 0.55 by the hybrids derived from p_2 x p_5 and p_3 x p_5 , respectively. This agreed with Grimes *et al*. (1974), who found that glucose repressed and derepressed diploid cells containing the same value of mitochondrial DNA packaged into approximately 4- 5 organelles in repressed cells or about 22 organelles in derepressed cells. The same trend was also observed by Hoffman and Avers (1973), who found in another strain of yeast that there was only single large mitochondrion under repressing and derepressing environment. Mitochondrial DNA is another important factor affecting glucose repression. Moreover, the genotypes of the parental strains of the crosses p_2 x p_5 and p_3 x p_5 may contribute with high amount of mitochondrial DNA in heterozyotic diploids. These results emphasize the mitochondrial transmission genetics on the physiological and biochemical state of yeast cells. Randez – Gil *et al*. (1998) reported that hexokinase II encoded by HXK_2 may enter the nucleus participated nuclear protein as DNA – protein comples which regulated the repression of at least the SUC_2 gene by glucose. The phosphorylation of this enzyme was necessary to enter the nucleus and initiate glucose repression (Herrero *et al*. 1998). This mechanism may not occurre in the catabolite repression shown herein, where hexokinase II does not entered the nucleus leading to derepressed of at least SUC_2 gene encoding invertase. Leonie *et al.* (2001) reported that glucose fermentation into ethanol yields two ATPs per glucose consumed, meanwhile complete oxidation of glucose into carbon dioxide and water yielding roughly 20 ATPs.

As shown in this study catabolite – repression altered glucose fermentation leading to heterosis appeared at higher sugar concentration. This phenomenon may be useful for

fermentation process that can use sugar crops by – products as a feed stock for ethanol production. However, the high concentration of sugars in the fermentation medium was sensed by the cell, the hybrid genotypes derived from the crosses between p_2 x p_5 and p_3 x p_5 showed de- repressed utilizing the high sugar concentration, as a consequence the heterosis was appeared, whereas the hybrids resulted from the cross between p_1 x p_4 showed negative heterosis. The data discussed above show that heterosis at 0.06 concentration of the substrate may be due to glucose – tolerance genotypic hybrids yields a remarkable heterosis phenomenon generally referred to glucose – derepressed. Fermentation medium containing extracellular high sugar concentration did not produce a signal for glucose repression of the genotypes exhibiting heterosis.

Table 10 . Hybrid vigor and kinetic parameters of ethanol productivity from 6 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_2 x p_5 in batch fermentation.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield†	Q_P	E_Y	CS
P_2	223.41	-	1	1.33	8.52	26.22
P_5	320.67	-	1	1.91	12.63	25.38
MP	272.04	272.04	1	1.62	10.54	25.80
H_6	418.89	0.54	1.54	2.49	17.67	23.70
H_7	317.78	0.17	1.17	1.89	14.81	21.45
H_8	414.07	0.52	1.52	2.46	16.59	24.96
H_9	260.00	-0.04	0.96	1.55	12.22	21.27
H_{10}	344.74	0.27	1.27	2.05	14.05	24.54
F - test	**	*	**	NS	NS	NS
L.S.D 0.05	94.548	0.381	0.311			
L.S.D 0.01	131.228	0.542	0.431			

***, ** = Significant at 0.01 level of probability,**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity ,
Y_{ps} : ethanol yield related to sugar consuming ,
E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

Table 11 . Hybrid vigor and kinetic parameters of ethanol productivity from 6 % sugarcane refuse fermented by *Saccharomyces cerevisiae* hybrids resulted from hybridization between p_3 x p_5 in batch fermentation.

Strains	Ethanol production (ml / L)	Hybrid vigor	Yield†	Q_P	E_Y	CS
P_3	204.15	-	1	1.22	8.42	24.25
P_5	320.67	-	1	1.91	12.63	25.38
MP	262.41	262.41	1	1.57	10.52	24.82
H_{11}	406.37	0.55	1.55	2.42	16.02	25.37
H_{12}	327.40	0.25	1.25	1.94	12.65	25.87
H_{13}	274.44	0.05	1.05	1.63	10.16	27.02
H_{14}	318.74	0.21	1.21	1.89	12.02	26.51
H_{15}	318.74	0.21	1.21	1.89	12.28	25.96
F - test	NS	NS	NS	NS	NS	**
L.S.D 0.05						3.38
L.S.D 0.01						4.69

**** = Significant at 0.01 level of probability,**
NS = Insignificant differences
Q_p : Volumetric ethanol productivity ,
Y_{ps} : ethanol yield related to sugar consuming ,
E_y : yield efficiency,
CS : consumed sugar and † Yield relative to the mid – parent .

In conclusion, the cells of *Saccharomyces cerevisiae* subjected to acetate medium were converted into haploid ascospores from diploid cells. Hybridization is a powerful technique for rapid generation new genotypes of recombinants in yeast for desirable industrial phenotypes. This technique was used in this study to improve ethanol productivity through genome shuffling in yeast. Some hybrid genotypes were tolerant to glucose effect and revealed heterosis for ethanol productivity from sugarcane juice industrial sub – products which may be suitable for practical use in alcohol distilleries. In addition, the suitability of hybridization technique and the availability of yeast isolates for generating new hybrids has the potential to greatly increase the genotypic and phenotypic diversity in yeast to select the efficient genotypes produced alcohol without recourse to genetic modification. This technique may offer advantageous traits of interest in the resulting hybrids with a greater genetic stability.

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التباين الوراثى فى التخمر الإيثانولى بواسطة إستخدام تراكيب وراثية جديدة من خميرة الخباز.

ميرفت إبراهيم كمال ، خليفه عبد المقصود زايد ، أشرف حسين عبد الهادى و منار الشربينى الباز

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تهدف هذه الدراسة إلى دراسة التغير فى العمليات الكيموحيوية المرتبطة بقمع الجلوكوز من خلال تحليل إنتاج الإيثانول والذى يتأثر بشكل أساسى بتركيز الجلوكوز فى بيئة التخمر . تم فى هذا البحث إستخدام خمس عزلات أبوية وخمسة عشرة تركيب وراثى هجين ناتجة عن ثلاث تهجينات وذلك لتقييم قوة الهجين بها بالنسبة لإنتاج الإيثانول . خاصة وأن الإيثانول المستهدف إنتاجه من هذه الدراسة سوف يساهم فى نظافة البيئة من خلال إستخدام مادة تفاعل رخيصة الثمن ناتجة عن صناعة عصير قصب السكر . أعطى التهجين بين عزلات مختلفة من الخميرة تراكيب وراثية جديدة بعض خلاياها حدث لها قمع الجلوكوز والبعض الآخر تغلب على قمع التركيزات العالية من السكر الموجود فى البيئة . حيث يعتبر قمع السكر هو أحد العوامل الرئيسية المحددة والمؤثرة على عملية التخمر بإستخدام مواد تفاعل غير مكلفة إقتصادياً فى إنتاج الإيثانول . عندما تم تنمية التراكيب الوراثية الهجينة فى وسط التخمر المحتوى على 0,02 جرام من مادة التفاعل أظهرت كل التراكيب الوراثية قوة هجين موجبة بالنسبة لمتوسط الأبوين فى إنتاج الكحول . بينما أظهرت كل التراكيب الوراثية الهجينة قوة هجين سالبة عندما إحتوت بيئة التخمر 0,04 جرام من مادة التفاعل ، مما يعكس حساسية كل التراكيب الوراثية لهذا التركيز من مادة التفاعل والذى أدى إلى زيادة تركيز الجلوكوز فى البيئة . كما أظهرت التراكيب الوراثية الهجينة الناتجة عن التزاوج بين العزلات $p_1 \times p_4$ قوة هجين سالبة أيضاً بالنسبة لإنتاج الإيثانول عند تنميتها فى بيئة نمو تحتوى على 0,06 جرام من مادة التفاعل المستخدمة. وعلى النقيض من ذلك أظهرت كل الهجن الناتجة عن التهجينات التالية : $p_2 \times p_5$ ، $p_3 \times p_5$ قمع لكبت الجلوكوز عند تركيز 0,06 جرام من مادة التفاعل المستخدمة ويعكس ذلك قوة الهجين الموجبة التى أظهرتها التراكيب الوراثية الهجينة عند هذا المستوى من المواد الكربوهيدراتية .