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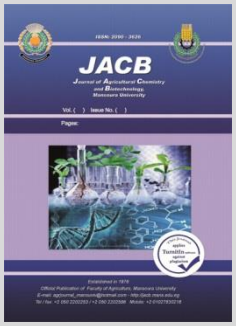
Correlation between Ethanol Productivity and Biomass Formation from Sugarcane Juice Industrial Wastes by Genetically Recombinant *Saccharomyces Cerevisiae*

Mervat I. Kamal* ; A. H. Abd El-Hadi ; K . A . Zaied and Manar E. El-Baz

Department of Genetics , Faculty of Agriculture , Mansoura University.



ABSTRACT

Sugarcane juice sub- products was the main source of carbohydrates for ethanol productivity and biomass formation . It contains sugars as a good alternative material for ethanol production from yeast to meet the large scale consumption of petroleum fuels . Therefore, the main objective of this study was undertaken to determine the correlation between ethanol productivity and biomass formation using different concentrations of sugarcane juice industrial by - products as a main source of carbon .15 hybrids of *Saccharomyces cerevisiae* derived from three crosses conducted between five parental strains were used in this study . The results of fermentation process on bioethanol production were graphically diagrammatic. The data showed in most cases negative correlations between ethanol production and biomass formation as shown from genotypes of crosses P_1 x P_4 and P_3 x P_5 . This indicated that high productivity of ethanol was associated with a lower biomass formation due to ethanol toxicity on the viability of yeast cells . On the contrary , genotypes resulted from cross between P_2 x P_5 showed positive correlation between both parameters at 0.02 and 0.06 g of sugarcane juice sub - products . Therefore, it is possible that genotypes derived from this cross may be more tolerant to ethanol inhibitor. In addition , the greater use of sugars for growing yeast cells resulting in a lower ethanol productivity and vice – versa . The results indicated that genome shuffling via hybridization is a powerful tool for inducing genetic recombinants in yeast for desirable industrial phenotypes.

Keywords *: Saccharomyces cerevisiae* , ethanol , correlation , sugarcane juice.

INTRODUCTION

Bioethanol production from lingocellulosic substances by *Saccharomyces cerevisiae* is becoming an important alternative to petroleum fuel . The bioconvertion of lingocellulosic materials or starch into ethanol indicating a two – step process. The first one is saccharification , where the material is converted into sugar using cellulosic or amylolytic microorganisms or enzymes such as cellulase or α – amylase. The second step was fermentation , where sugar is transferred into ethanol using *Saccharamyces cerevisiae* (Nakamure *et al.* 1997) . This process needs high ethanol tolerant strains . Thus , almost all studies in this field of biotechnology were focused on the development of genetically modified yeast strains harboring cellulase or α – amylase encoding genes from different organisms expressing and excreting these enzymes (Ulgen *et al.* 2002). Genome shuffling is a powerful strategy for quickly inducing recombinant genotypes in yeast to be used in industrial process . The starting population could generated by hybridization to improve ethanol tolerance and ethanol productivity of yeast (Shi *et al.* 2009) .

Classical methods of improving strains had succeeded in originating many industrial strains , but they are time – consuming and laborious because of many rounds of random mutation and selection techniques . Recently , an efficient biotechnology strategy named genome shuffling gained a major interest in the development of hybrids with superior improved phenotype (Stephanopoulos 2002). Genome shuffling allows many strains recombined through recursive hybridization and marking a shuffled strains with genetic exchange which is achieved by the repetition of this process . This offers the advantages of genetic changes at different locations through the entire genome without the necessity of genotype sequence information .

During fermentation, yeast converts simple carbohydrates into bioethanol and carbon dioxide . In addition to these primary metabolites, they also induce smaller volumes of other metabolites that have a marked effect on the quality of the products . The secondary metabolites include higher alcohols, aldehydes, sulfur – containing substance , esters, phenols, carbonyl substances and organic acids , all of which leading to the product aroma (Steensels *et al.* 2014). *Saccharomyces cerevisiae* still remains as the prime species in ethanol production (Shi *et al.* 2009). The main factors in industrial fermentation are ethanol tolerance and the efficiency to produce wine without residual sugars (D' Amore *et al.* 1990). Sugarcane stems remaining in the market after the extraction of juice containing residual sugars , it is without cost , environment friendly if it was used as a raw material for many industries in many African world regions.

* **Corresponding author.**
E-mail address: mervat _ y2007@yahoo.com/ 01008665560
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Sugarcane stem is one of the most promising renewable feedstock , not only for bioconversion into fuels and biochemical products , but also as a forage for ruminants (Anderson and Akin 2008), as well as , as a source of fibers for manufacturing paper (Reddy and Yang 2005). A one gram of glucose is converted into 0.511 gram of ethanol (Maiorella *et al.* 1981) . The formula of glucose conversion into bioethanol by yeast cells was demonstrated before by Benarji and Ayyanna (2016) as follows :

$$C_6H_{12}O_6 (180\ g.\ mol) \longrightarrow 2C_2H_5OH (92\ g.\ mol) + 2\ CO_2 (88\ g.\ mole)$$

The limited resources of energy call for search about renewable resources . The prices of oil and natural gas increased from day to day. Though, the production of bioethanol from renewable carbohydrate substances such as the residual sugars in sugarcane juice sub - products was an alternative liquid fuel has been attracting interest over the African world (Roukas 1994) . Recently , agriculture crops had considerable interest such as sugarcane juice wastes after the juice was extracted to be used for bioethanol production. It is used as a cheap carbohydrate source for ethanol productivity . This study focus on these cheap carbohydrate sources for the production of ethanol. Sugarcane , as well as , sweet sorghum are attractive sources for ethanol production . The juice excreted from the fresh stalks is composed of sucrose , glucose and fructose and can be readily fermented into alcohol (Ratnavathi *et al.* 2010). Sugarcane molasses are also the traditional raw substrate for ethanol productivity with a promising future.

This study aimed to investigate the correlation between ethanol productivity and biomass formation using the sugars excreted from sugarcane juice sub-products through the fermentation process by different recombinant genotypes of *Saccharomyces cerevisiae* .

MATERIALS AND METHODS

Microbial strains

Five yeast strains were used in this study. These strains, as well as, their references or sources are listed in Table (1).

Table 1. Yeast strains used in this study.

Yeast strains	Source	Designation
Saccharomyces cerevisiae	Baker's 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	Fermented grape juice	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

Sugarcane juice sub - products

Sugarcane juice wastes were used in this study with its residual sugars being used as a sole source of carbon instead of glucose added to the fermentation medium .It was collected from the local market of sugarcane juice industry in Mansoura city through October 2018. The white fibers inside the stem were collected after removal of the outer 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 with 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 used for ethanol production was consisted 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 marking 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 yeast strains.

Agents Antifungal	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 of 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 were 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 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 the 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 related with each concentration of ethanol prepared to be used as a standard (Ciani and Ferraro 1998).

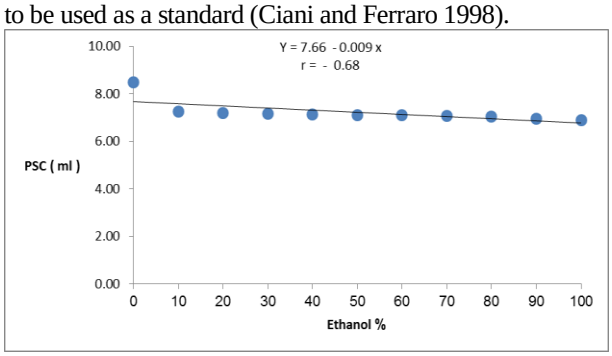

Figure 1. Standard curve for ethanol determination.

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).

Statistical analysis

One of statistical modeling is regression analysis . It was used in this study as a set of statistical estimation of the relationship between two variables such as ethanol yield as a dependent variable and biomass production as the independent variable . It helps to show the dependent variable changes for each one of the independent variable (Fox 1997) . In addition , correlation was also estimated between the two variables to measure the association between them . Its value can vary from minus one to plus one . Minus one reflected the negative correlation which indicated that as the value of ethanol yield increases then the value of biomass production decreases , and vise versa . These reflected that the variables move together (Lindley 1987).

RESULTS AND DISCUSSION

Correlation between ethanol productivity and biomass formation by the hybrids of P_1 x P_4

As shown from the genome shuffling between strains P_1 x P_4 , the correlation between biomass yield and alcohol production was negative at 0.02 , 0.04 and 0.06 concentration of sugarcane juice sub - products (Figures 2, 3 , 4 , 5 , 6 and 7) . The results indicated that at the different concentrations of sugars, the hybrid strains grown slowed markedly produced more ethanol. This indicated that under semi aerobic condition the yeast cells took the direction of ethanol production above their growth rates . Though, yeast genotypes converted more levels of sugars to be produce a higher rate of ethanol .It was noticed that large amounts of ethanol production was associated with a lower biomass growth rate . This study successfully improved ethanol production via genome shuffling technique , which is a powerful means to rapidly improve the complex phenotypes of microorganisms . The hybrid genotypes could reduce their growth rate to be more efficient in converting sugars into ethanol due to the negative correlation obtained in this study between both parameters. The high ethanol concentrations produced at almost all cases was associated with a lower growth rate . This could be attributed to the yeast cells response to the physical effects of high ethanol production which had a toxic effect leading to decrease of growth rate . These results agreed with You *et al.* (2003) who reported that ethanol tolerance is also strictly related with lipid composition of cell membrane . This indicated the role of membrane lipids on ethanol tolerance and production. D' Amore *et al.* (1989) reported that yeast strain completely converted 0.15 glucose to 6.4 % ethanol, although when the glucose concentration was increased to 0.20 , the same strain could not completely metabolize the sugar and yet produced 7.00 % ethanol . It can be noticed that genome shuffling technique could improve ethanol tolerance and ethanol productivity. Further investigations are needed under large scale conditions to evaluate the suitability and sustainability of yeast genotypes for practical use in alcohol distilleries.

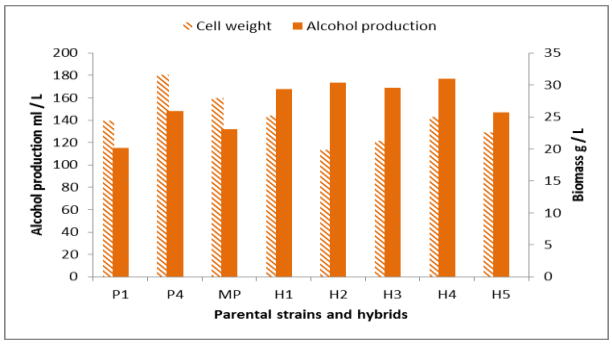

Figure 2 . Fermentation diagram of 2 % sugarcane juice sub – products by the hybrids resulted from the mating between P_1 X P_4 illustrating the relation between ethanol productivity and biomass formation.

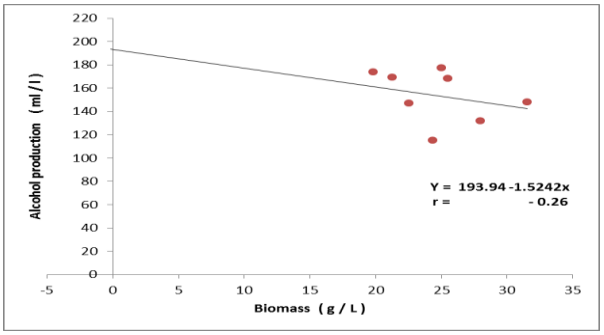

Figure 3. Correlation between ethanol productivity and biomass formation in the fermentation of 2 g sugarcane juice sub - products by the hybrids resulted from the mating between P_1 X P_4 .

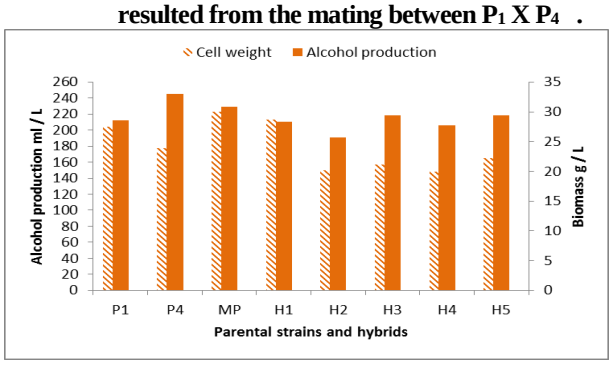

Figure 4. Fermentation diagram of 6 % sugarcane juice sub - products by the hybrids resulted from the mating between P_1 X P_4 illustrating the relation between ethanol productivity and biomass formation.

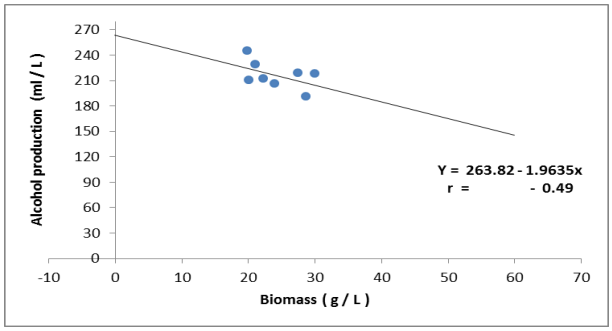

Figure 5. Correlation between ethanol productivity and biomass formation in the fermentation of 6 % sugarcane juice sub - products by the hybrids resulted from the mating between P_1 X P_4 .

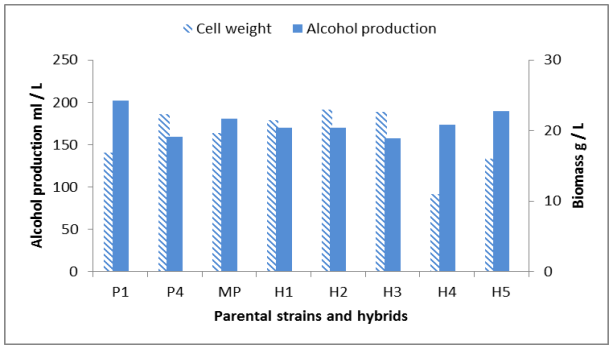

Figure 6. Fermentation of 4 % sugarcane juice sub - products by the hybrids resulted from the mating between P_1 X P_4 illustrating the relation between ethanol productivity and biomass formation.

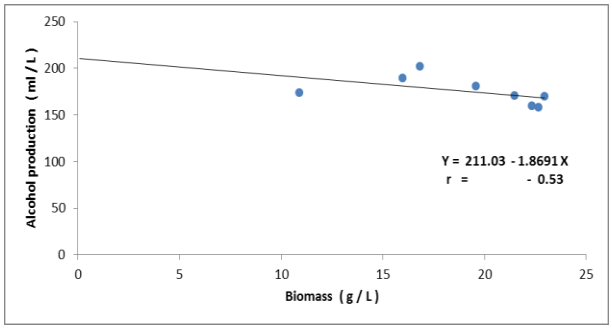

Figure 7. Correlation between ethanol productivity and biomass formation in the fermentation of 4 % sugarcane juice sub - products by the hybrids resulted from the mating between P_1 X P_4 .

Correlation between ethanol productivity and biomass formation by the hybrids of P_2 x P_5

As shown from the results in Figures 8, 9, 10 and 11 , the high ethanol concentrations produced by the hybrids resulted from the mating between P_2 x P_5 was associated with high growth rate . This indicated the potential advantages of these genotypes to be used in industrial ethanol production , as well as, of higher biomass formation . The results represent a positive correlation between ethanol yield and biomass production at 0.02 and 0.06 g. This correlation was achieved by the liner regression obtained between biomass formation with ethanol yielding . The positive correlation between both parameters indicated that the genotypes derived from this cross may be more tolerant to ethanol productivity at lower concentrations of sugars . These results are in harmony with Garay – Arrovo *et al.* (2004) who found that haploid strains produced in the laboratory if compared with industrial diploid or polyploidy strains are difficult to use for bioethanol production , because they have lower tolerance to acid , ethanol and other fermentation inhibitors. However , hybridization as shown by Yamada *et al.* (2010) is one of the most effective ways to improve and combine the traits of parental haploids for industrial purposes. In addition , crossbreeding is a classical method of mating strains from opposite mating types to produce new heterozygous diploid cells(Pretorius and Bauer 2002). Therefore , it is thus possible to generate recombinant genotypes tolerance to inhibitor factors such as ethanol by hybridization between strains harboring the opposite markers.

On the other hand , at the concentration of 0.04 sugarcane juice sub – products, the correlation between biomass formation and ethanol productivity was negative as shown in Figures (12 and 13) . This indicated that at the concentration of 0.04 lower biomass formation was associated with higher volumetric ethanol productivity, in contrast with the results obtained at 0.02 and 0.06 concentrations of sugarcane juice sub - products. These results may be due to higher alcohol production at the concentration of 0.04 substrate which showed toxicity on the viability of yeast cells leading to the negative correlation obtained herein. This indicated that the number of viable cells increased during the begining of fermentation , after which ethanol productivity begins to increase in the fermentation medium it decreased slowly . The decline in the biomass concentration could be due to the toxicity of ethanol , as well as, to the reduced of sugars availability which was converted to ethanol , which showd the inhibitory effected on yeast genotypes (Kamini and Gunasekaran 1989). The results obtained in this study are in agreement with Roukas (1994) , who obtained a lower ethanol concentration with a higher inoculum amount , which may resulted from greater use of sugars for growing the yeast cells and maintenance of a high biomass concentration , resulting in a lower ethanol yielding .

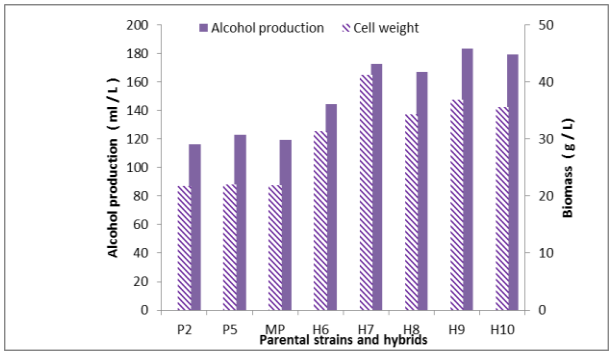

Figure 8. Fermentation diagram of 2 % sugarcane juice sub - products by the hybrids resulted from the mating between P_2 X P_5 illustrating the relation between ethanol productivity and biomass formation.

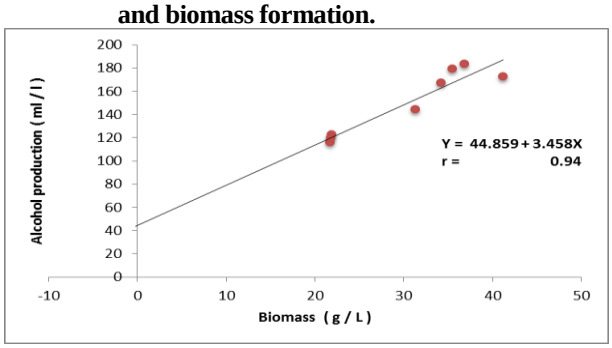

Figure 9. Correlation between ethanol produtivity and biomass formation in the fermentation of 2 % sugarcane juice sub - products by the hybrids resulted from the mating between P_2 X P_5 .

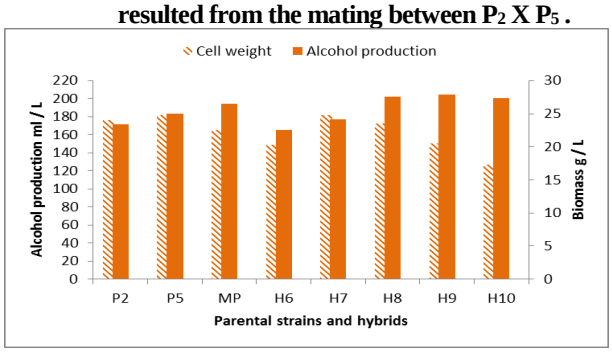

Figure 10. Fermentation of 6 % sugarcane juice sub - products by the hybrids resulted from the mating between P_2 X P_5 illustrating the relation between ethanol productivity and biomass formation.

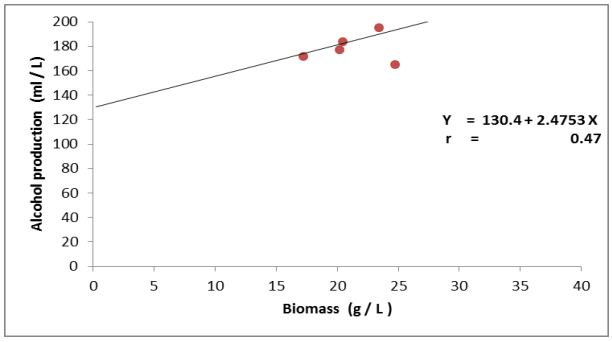

Figure 11. Correlation between ethanol productivity and biomass formation in the fermentation of 6 % sugarcane juice sub - products by the hybrids resulted from the mating between P_2 X P_5 .

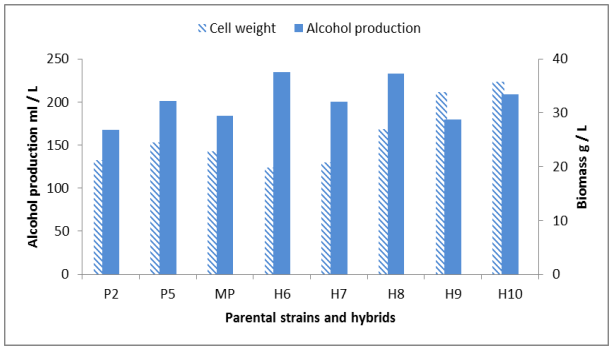

Figure 12. Fermentation of 4 % sugarcane juice sub - products by the hybrids resulted from the mating between P_2 X P_5 illustrating the relation between ethanol productivity and biomass formation.

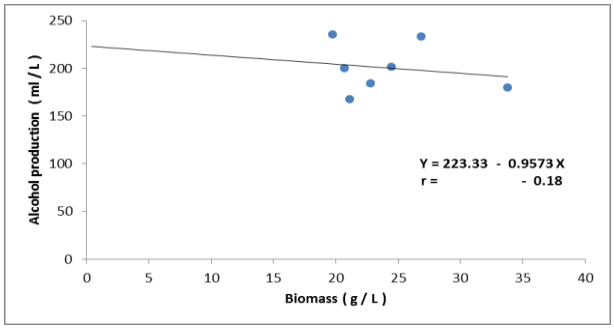

Figure 13|. Correlation between ethanol productivity and biomass formation in the fermentation of 4 % sugarcane juice sub - products by the hybrids resulted from the mating between P_2 X P_5 .

Correlation between ethanol productivity and biomass formation by the hybrids of P_3 x P_5

As expected , the results presented in Figures (14 , 15 , 16 , 17 , 18 and 19) showed negative correlation between biomass yield and ethanol productivity at all concentrations of sugarcane juice sub – products fermented process by the genotypes . These results revealed that the maximum concentrations of ethanol was achieved correlated with a decreased in biomass formation . The data of lower biomass formation with higher ethanol yield are in good agreement with the observations reported earlier by Hutter and oliver (1998) and Panoutsopoulou *et al.* (2001) for nuclear petite *S. cerevisiae* nuclear petite using glucose as a carbon source , and by Shi *et al.* (1999) for a nuclear petite of *Pichia stipites* yeast utilizing xylose for ethanol productivity . The decreased in biomass formation could be due to the inhibitory effect of ethanol on yeast cells (Rosa *et al.* 1986) . The effect of induced negative correlation between both parameters may be due to efficient sugars converted directly into ethanol than using it for growing the cells. In addition, the hybrid genotypes may be less tolerant to ethanol productivity which inhibit the growing of cells. These hybrids had technological values for decreased biomass formation and increased ethanol productivity as well . This suggests that loci determining alcohol production are dominant in this genetic background. This may be explained by recessive deleterious alleles present in the defective parent that are easily counterbalanced by a simple cross with the efficient parent . More interestingly , heterosis effects were

observed by Marullo *et al.* (2006) for ethanol productivity . The same authors suggested that commercial yeast strains carried deleterious mutations , some of which were removed by sporulation .

On the other hand , the final superiority of this hybrid probably resulted from the combination of positive alleles by the effect of dominance (Zeyl and Bell 1997) or over dominance (Hall and Wills 1987). Cell maintenance or final biomass production may explain these phenomena and may thus be of particular interest for controlling the conversion of sugars into ethanol , especially at high sugar concentrations . For other means , the hybrid exhibited intermediate properties if compared to the parental strains . Thus , fermentation properties such as ethanol productivity were improved by the mixing of two heterogeneous genomes. Genetic effects such as dominance and heterosis explain why good values from both parents are found in the hybrid. This results agreed with Marullo *et al.* (2006) , who found that few progeny of yeast clones (13 %) presented as a good trait value as the best parent . In this case , the absence of transgressive progeny values indicated that all of the enhancer loci were located in the better parent and silencer loci in the other one (Marullo *et al.* 2006) . Therefore , Marullo *et al.* (2006) confirmed that breeding strategies are an excellent way of combining numerous technological properties in a single hybrid genotype.

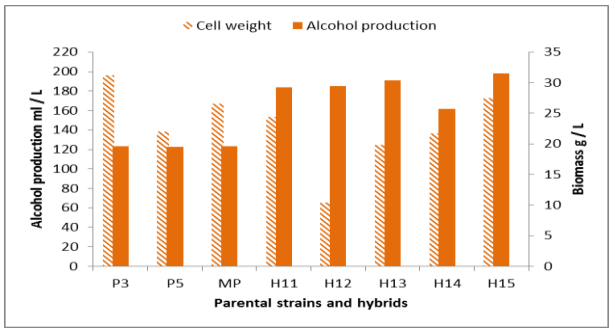

Figure 14. Fermentation diagram of 2 % sugarcane juice sub - products by the hybrids resulted from the mating between P_3 X P_5 illustrating the relation between ethanol productivity and biomass formation.

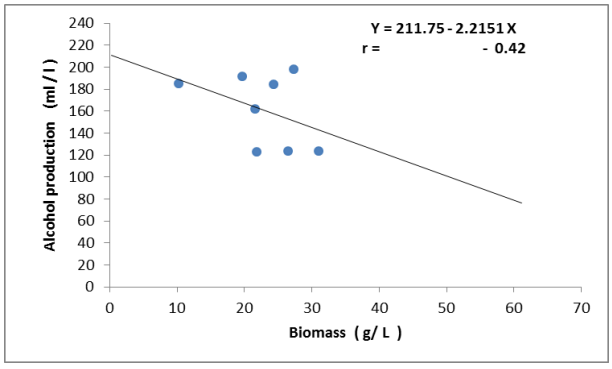

Figure 15. Correlation between ethanol productivity and biomass formation in the fermentation of 2 % sugarcane juice sub - products by the hybrids resulted from the mating between P_3 X P_5 .

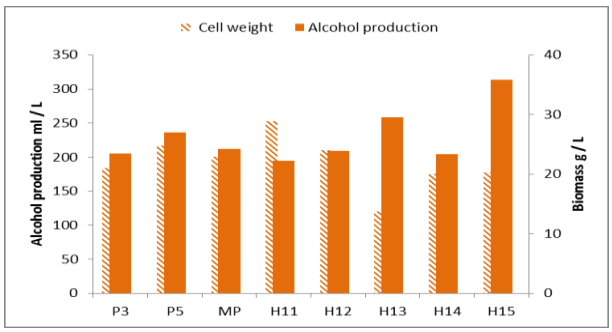

Figure 16. Fermentation of 6 % sugarcane juice sub - products by the hybrids resulted from the mating between P_3 X P_5 illustrating the relation between ethanol productivity and biomass formation.

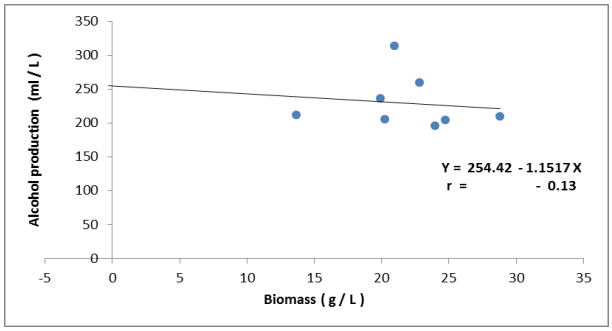

Figure 17. Correlation between ethanol productivity and biomass formation in the fermentation of 6 % sugarcane juice sub - products by the hybrids resulted from the mating between P_3 X P_5 .

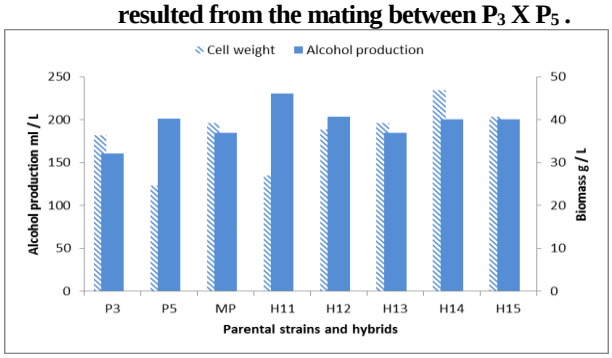

Figure 18. Fermentation of 4 % sugarcane juice sub - products by the hybrids resulted from the mating between P_3 X P_5 illustrating the relation between ethanol productivity and biomass formation.

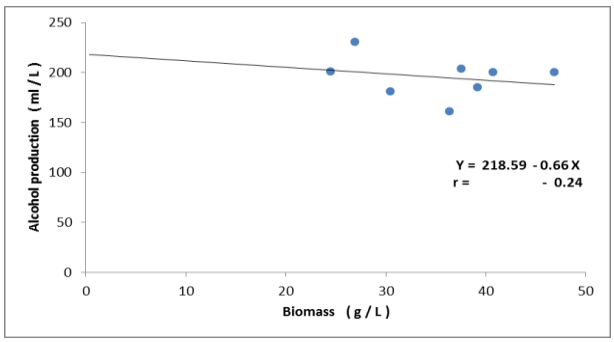

Figure 19. Correlation between ethanol productivity and biomass formation in the fermentation of 4 % sugarcane juice sub - products by the hybrids resulted from the mating between P_3 X P_5 .

In conclusion, the acidic pH almost exhibit inhibitory properties on fermentation process leading may be responsible for decreasing ethanol production. The acidity of fermentation medium may be due to secondary metabolities formed during the fermentation . Higher ethanol productivity causes increase in pH values of the fermentation medium and showed a negative effect on biomass formation. Moreover, the hybrid genotype expressed increased ethanol productivity. New recombinant genotypes in yeast are essential to bioethanol business. Optimization of ethanol fermentation must be considered. Therefore, sugarcane juice industrial by – products are proved as a suitable substrate for ethanol production because of feasible economic bioprocess, to meet the required fuel quantity, bioethanol. Acidic pH can inhibit the activity of baker's yeast. The inhibition due to pH is more effective on the biomass yield than on ethanol synthesis. Furthermore, similar strategy can be used to select heterozygous genotype able to produce large amounts of ethanol than biomass synthesis, in addition to ethanol tolerance and weak organic acids in the fermentation medium, as well as, other compounds responsible for extracellular acidification.

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العلاقة بين إنتاج الإيثانول وتكوين المادة الخلوية الحية من مخلفات صناعة عصير قصب السكر بإستخدام تراكيب وراثية جديدة من خميرة الخباز

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

قسم الوراثة – كلية الزراعة – جامعة المنصورة

تعتبر مخلفات صناعة عصير قصب السكر مادة رئيسية للمركبات الكربوهيدراتية بالنسبة لإنتاج الإيثانول . تحتوى هذه المخلفات على السكريات التى تعتبر مادة بديلة جيدة لإنتاج الإيثانول من الخميرة لمواجهة معدل الإستهلاك المرتفع للوقود البترولى . لذا تهدف هذه الدراسة إلى توضيح العلاقة بين إنتاج الإيثانول وتكوين المادة الخلوية الحية بإستخدام تركيزات مختلفة من مخلفات صناعة عصير قصب السكر لإستغلال السكريات الموجودة بها كمصدر رئيسى للكربون . تم فى هذه الدراسة إستخدام خمس سلالات من الخميرة وخمسة عشر تركيب وراثى هجين ناتجة عن ثلاث تهجينات . أظهرت نتائج عمليات التخمر لإنتاج الإيثانول فى معظم الحالات علاقات سالبة بين إنتاج الإيثانول وتكوين المادة الخلوية الحية كما إتضح من التهجين بين العزلات $P_1 \times P_4$ ، $P_3 \times P_5$. تعكس هذه النتائج أن إنتاج الإيثانول بواسطة هذه التراكيب الوراثية كان مصحوباً بمعدل نمو منخفض بسبب سمية الإيثانول على حيوية خلايا الخميرة . وعلى العكس من ذلك أظهرت نتائج التراكيب الوراثية الناتجة عن التهجين بين العزلات $P_2 \times P_5$ علاقة موجبة بين كلتا الصفتين عند التركيزات (2% ، 6%) من هذه المخلفات . ولذا فإنه من المحتمل أن التراكيب الوراثية الناتجة عن هذا التهجين قد تكون أكثر تحملاً للعوامل المثبطة للنمو مثل وجود الإيثانول فى البيئة . بالإضافة إلى ذلك فإن المعدل المرتفع لإستهلاك خلايا الخميرة من السكر فى النمو سوف يترتب عليه إنتاج منخفض من الإيثانول والعكس بالعكس . تعتبر عملية التهجين بين التراكيب الوراثية لعزلات مختلفة آداة فعالة لإنتاج تراكيب وراثية جديدة فى سلالات الخميرة قد تكون لها خصائص مميزة فى مجال الصناعة .