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D-XYLOSE FERMENTATION TO ETHANOL BY HYBRIDS OBTAINED THROUGH PROTOPLAST FUSION, BETWEEN *Saccharomyces cerevisiae* AND *Pichia stipitis*

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ABSTRACT

Two *Saccharomyces cerevisiae* auxotrophic mutants (GT160-34B and XJ133-1B) were fused with two *Pichia stiptis* auxotrophic mutants, spontaneous mutant M1 and M2 which resulted from a wild type strain (Y-7124) by UV treatment. Growth behavior of 17 hybrid fusant yeast strains and their parents were tested for growth on different carbon sources (xylose, glucose, lactose, cellobiose, raffinose and starch) indicated that hybrid fusants acquired some characters of *Pichia stiptis* parental strains *via* intergeneric protoplast fusion technique. Results showed that hybrid fusants and parental strains of *Pichia stiptis*, M1 and M2 fermented 5% D-xylose, aerobically, at 30ºC directly to ethanol within 48 hours. The two parents *Saccharomyces cerevisiae*,GT160-34B and XJ133-1B did not show any xylose fermentation efficiency or ethanol production. The two mutants M1 and M2 were 6% and 12% less than its parent *Pichia stiptis* (Y-7124), in xylose fermentation efficiency respectively. On the other hand, M1 showed 17% and 12% decrease in ethanol production and conversion coefficient, respectively than its parent, but M2 reached the same amount of ethanol production and showed 11% increase in conversion coefficient than its parent. Results showed that the tested 17 hybrid fusants showed increasing average in xylose fermentation efficiency ranged from 13% to 40% than its active parent M1 or M2. However, results also, showed that ethanol production from xylose sugar varied and 11 hybrid fusants showed increasing average ranged from 5% to 56% more than the active parent, but the other 6 hybrid fusants showed decreasing average in ethanol production ranged from 5% to 21% less than the active parent. On the other hand, most of the tested hybrid fusants showed decrease in conversion coefficient ranged from 2% to 43% of the active parent, but F1, F2 and F16 showed 16%, 16% and 30% ,respectively, increase in conversion coefficient than the active parent. Highly significant positive correlation between sugar consummation and ethanol production was indicated at (P>0.01).

Keywords: D-xylose, fermentation, ethanol production, protoplast fusion, mutation, UV treatment, carbon sources, conversion coefficient, *Saccharomyces cerevisiae*, *Pichia stipitis*.

INTRODUCTION

The utilization of xylose, a major constituent of hemicellulose which comprises up to 30-40 % of the renewable biomass in nature, has been studied intensively for the production of valuable products such as SCP (Feliu *et al.,*1990), ethanol (Delgenes *et al.,*1990), xylitol (Furlan *et al.*,1991), and hydrogen (Heyndrickx *et al.,*1991). Most studies have focused on ethanol production by natural and mutated xylose-fermenting yeasts (Du

Preez & Prior,1985), as well as by genetically improved *Saccharomyces* strains (Sarthy *et al.,*1987 and Eliasson *et al.,*2001) which obtain xylose-utilizing capability.

The first step in xylose metabolism by yeasts and mycelial fungi is reduction of xylose to xylitol, a reaction catalyzed by [Nicotine amid adenine dinucleotidephospho hydrogenate] NADPH-linked D-xylose reductase. This step is followed by the oxidation of xylitol to xylulose, which is catalyzed by a [Nicotine amid adenine dinucleatide] NAD-linked xylitol dehydrogenase. Most xylose-assimilating yeasts convert xylose to xylulose *via* NADPH- and / or NADH-dependent xylose reductase and NAD-dependent xylitol dehydrogenase, whereas a single enzymatic conversion of xylose by xylose isomerase is performed in bacteria.

In the case of *Saccharomyces cerevisiae* fermentation of xylose cannot proceed because of insufficient enzyme levels (Batt *et al.,*1986) and an imbalance in the NAD / NADH redo system which is involved in xylose metabolism (Bruinenberg *et al.,*1984). In view of the redox balance in xylose metabolism, xylose isomerase genes from several bacteria have been cloned and transformed into *Saccharomyces cerevisiae*. The enzymes produced in *Saccharomyces cerevisiae* were, however, found to be inactive (Amore *et al.,* 1989). The cloning and expression of the xylose reductase (Takuma *et al.,* 1991 and Lee *et al.,* 2000) and xylitol dehydrogenase genes (Kötter *et al.,*1990) isolated from *Pichia stipitis*, possessing the dual cofactor (NADPH / NADH) specific xylose reductase have been investigated.

Four researcher groups discovered the direct conversion of xylose to ethanol almost simultaneously. Two groups observed ethanol production directly from xylose following screens of yeasts for anaerobic xylose metabolism (Schneider *et al.,* 1981 and Slininger *et al.,*1982). One observation came from mutation and selection studies on a yeast strain known to assimilate xylose (Gong *et al.,*1981b) and one came from chance observations of ethanol production from xylose / xylulose mixtures under aerobic conditions (Jeffries, 1981).

Sugars obtained by hydrolysis of lignocellulosic material represent an interesting substrate for ethanol production in terms of availability and cost. Depending on the hydrolysis process, two-stages or single-stage, glucose and xylose derived from lignocellulose can be converted into ethanol by separate fermentation or a co-culture process using in both cases a glucose-fermenting micro-organism and a xylose-fermenting yeast (Du Preez *et al.,* 1986; Leathers & Dien, 2000 and Nigam, 2001).

Many authors were applied the protoplast intergeneric fusion as a powerful tool for ethanol yield improvement (Johannsen *et al.,* 1985; Vallin & Ferenczy, 1985; Abramova & Kapultsevich, 1986; Gupthar & Garnett, 1987; Selebane *et al.*, 1993).

This work is aiming to obtain *Saccharomyces cerevisiae* strain(s) having the ability to produce ethanol alcohol from xylose sugar through protoplast fusion with *Pichia stipitis.*

MATERIALS AND METHODS

Strains:

The identities and sources of the different haploid yeasts used throughout this investigation are presented in Table (1).

Table (1): Yeast strains, genotypes and their sources.

Yeast strains	Genotype	Source
Saccharomyces cerevisiae GT160 – 34 B	a- ad -1, leu - 2, his - 6, met -14 and lys - 9	YGSC[a]
Saccharomyces cerevisiae XJ133 – 1B	∝- met - 4 and gal -1	YGSC[a]
Pichia stipitis Y – 7124	Wild type	NRRL[b]

[a]**YGSC: Yeast Genetic Stock Center, Univ. of California, Berkeley, CA 94-720, USA. Kindly supplied through Prof. Dr. / A. M. M. Ali, Microbial Genetics Dept., NRC, Cairo, Egypt.**
[b]**NRRL: Northern Regional Research Center, 61604, USA. Kindly supplied through Prof. Dr. / M. S. Abd El-Salam, Microbial Genetics Dept., NRC, Cairo,** Egypt.

Media:

1. Basal Medium (BM) (Widdel, 1980): It was used for testing the ability of *S. cerevisiae* and *P. stipitis* to grow with 5 % D-xylose or D-glucose as a sole carbon source.
2. Complete Medium (CM) **(**Cox & Bevan, 1962): This medium was utilized for growing and maintenance of the yeast strains. And also was used for pre-growing strains to protoplast formation and as a fermentation medium supplemented with 5 % Xylose as a sole carbon source for ethanol production.
3. Vogel's Minimal Medium (MM) (Vogel,1955): This medium was used for the identification of the original *Pichia stipitis* yeast strain and its induced auxotrophic mutants.
4. Sporulation Medium (SM) (McClary *et al.,* 1959): This medium was used to test the ability of *Pichia stipitis* yeast strain to convert the vegetative cells to spores.
5. Minimal Medium for Regeneration (MMR) **(**Sipiczki & Ferenczy, 1977): It consists of; 0.67% Yeast nitrogen base (YNB), 3% Glycerol or Sorbitol, 0.1% Glucose or Xylose, 0.6% KCL and 3% Agar.
6. Regeneration Minimal Medium (MMRI): It is similar to MMR but without KCL and agar reduced to 1.8 %. This medium was used for the stabilization of fusion products.

N.B. All media were sterilized by autoclaving at 121°C for 25 minutes under pressure of 15 Lb / square inch. But in case of xylose containing media, the sugar was sterilized separately for 10 minutes only.

Methods:

1. Testing the original yeasts abilities to utilize xylose and glucose:

   The ability of *Saccharomyces cerevisiae* and *Pichia stipitis y*east strains to grow with D-xylose or D-glucose as a sole carbon source was tested. Liquid BM was used in this experiment and the growth behavior of the

tested strains was scored as optical density (OD) measurements at 600 nm, using UV-1201 shimadzu spectrophotometer, every 24 hours for 3 days. And also, cell dry weight was scored for each yeast strain.

2.Sporulation ability of *Pichia stipitis* :

The original yeast strain *Pichia stipitis* was examined for its sporulation ability on SM medium after incubation at 30°C for up to 17 days and then tested using a microscope.

3.Mutation induction and mutants isolation:

The original yeast strain *Pichia stipitis* when tested on MM with 2 % xylose as a sole carbon source and incubated at 30°C for 3 days, it showed growth similar to wild type (W.T.) yeast strain. To obtain auxotrophic mutants, mutation induction in *Pichia stipitis* was carried out according to Laplace *et al.* (1992), using the physical mutagen Ultra Violet irradiation (UV), for variable times; 1, 1.5, 2, 2.5 and 3 minutes under continuous stirring at a distance of 25 cm between the dishes and the UV source (UV-20 W, 100 V Lamp). The survived colonies were counted, picked up and plated on both MM and CM with 2% xylose. The colonies which showed growth on CM but not on MM, were considered as auxotrophs (mutants) and then recultured on CM with 2% xylose slants and kept for further experiments.

4.Identification of *Pichia stipitis* auxotrophic mutants:

Nutritional requirements of each mutant obtained were identified according to Holliday, (1960).

5.Sugar fermentation efficiency:

Yeast sugar consumption was measured as the decrease in xylose concentration in the fermentation media by refractometry (Quemener & Mercier, 1980). The amount of consumed sugar per unit of original sugar was calculated as sugar fermentation efficiency.

6.Induction of Yeast Protoplasts:

Yeast cells were converted to protoplasts following Farahnak *et al.* (1986) method with a slight modification. Yeast strains were aerobically grown to early stationary phase in 25 ml Erlenmeyer flasks containing 10 ml aliquots of CM medium with 2 % xylose for 20 h at 30°C, then the cells were harvested by centrifugation at 3000 rpm for 20 min. The cell pellets were washed three times with distilled water and resuspended in hypertonic buffer (0.1 M Tris-HCL, pH 7.5, 2 mM EDTA, 50 mM 2-Mercaptoethanol and 0.45 M KCl) and incubated at 30°C for 10 minutes with gentle agitation. The cells were again centrifuged and resuspended in lytic buffer (0.7 M KCl, 1% 2-Mercaptoethanol and 0.1 % Snail enzyme) and incubated at 30°C for 17 h. The cells were periodically checked under the microscope for the formation of protoplasts by using iodine solution (2g Potassium iodide, 1g Iodine, up to 300 ml Distilled water). Protoplasts were then collected by centrifugation at 3000 rpm for 10 minutes, washed at least three times with the washing buffer (0.1 M Phosphate buffer, pH 7.5 and 0.8 M Sorbitol) and resuspended in the same buffer.

7.Protoplast Fusion:

Attempts to fuse each of *S. cerevisiae* strains; G T 160-34B and XJ 133-1B with each of *Pichia stipitis* mutants; M1 and M2, which were selected in the present study, were done according to the method of Farahnak *et al*. (1986) with a slight modifications as follows: Protoplast suspension of both parental yeast strains were mixed in 1:1 ratio, centrifuged at 3000 rpm for 10 minutes. The pellets were resuspended in fusion buffer (10 mM Tris-HCL , pH 8.1, 10 mM $CaCl_2$ and 35% PEG with M.W.4000). The suspensions were incubated at 30ºC for 30 minutes. 0.1 ml of the suspension was mixed with 10 ml of melted regeneration minimal medium (MMRI) and poured into plates containing a thin bottom layer of the same medium supplemented with carbon source (glucose or xylose). Plates were sealed and incubated at 30ºC for 5 days. For fusion control, parental protoplasts were submitted to the same treatment as described for protoplast fusion but in the absence of Ca^{-2} ions. For the morphological analysis of fusion products, the fused cells were propagated on the above layer (containing MMR).

8.Growth behaviour of some selected fusants and their parental yeast strains on different carbon sources:

Growth behaviour of fusant strains was compared with that of their parents on medium containing 0.67% yeast nitrogen base (YNB) supplemented with one of the following carbon sources; Xylose (2%), Raffinose (1.5 %), Cellobiose (0.2 %), Starch (0.2 %), Glucose (2%) or Lactose (1 %), after incubation period at 30ºC for seven days.

9.Determination of ethanol production:

Conical flasks (250 ml in volume) contained 90 ml fermentation medium [1% yeast extract, 2% Peptone with 5% D-xylose] were inoculated with 10 ml liquid culture of the tested yeast strains grown on CM plus xylose at 30ºC for 18 hours. Then the flasks were shaken on a rotary shaker (200-rpm) at 30ºC for 48 hours. The ethyl alcohol formed was measured using “Apellometer” the apparatus which utilized for measuring alcohol production in Hawamdeia Distillation Factory, Sugar and Integrated Industries Company, accomplished for ethanol production. This apparatus depends on the boiling point of the fermented mash (fermented medium), where opposite relation between the boiling point of the mash and its riches in alcohol is to be detected.

10. Statistical analysis:

The collected data were statistically computed using the software SPSS for Windows. Release 7.5.1 (Dec. 20, 1996), SPSS Inc.

RESULTS AND DISCUSSION

Screening of the original tested yeasts:

The two yeast strains of *Saccharomyces cerevisiae;* (GT160-34B and XJ133-1B), and *Pichia stipitis* (Y-7124) were tested on solid medium containing each of the two different carbon sources, D-glucose or D-xylose.

The results indicated that the two *Saccharomyces cerevisiae* strains (GT160-34B and XJ133-1B) gave good growth on D-glucose sugar as a sole carbon source whereas *Pichia stipitis* strain gave very good growth. On the other hand, when D-xylose sugar was used, the two original strains of *Saccharomyces cerevisiae* did not give any growth but *Pichia stipitis* strain gave very good growth. Also, when the original yeast strains were tested on liquid medium with D-xylose sugar as a sole carbon source, the growth rate of the tested yeast strains measured as optical density (OD) every 24 hours for three days and dry weight / g was determined for each strain as shown in Table (2). With respect to growth rate as OD or dry weight, the results indicated that XJ133-1B strain was higher than GT160-34B strain and that *Pichia stipitis* strain was the best yeast strain compared with the two *Saccharomyces cerevisiae* yeast strains.

In case of *Saccharomyces cerevisiae,* fermentation of xylose cannot proceed because of insufficient enzyme levels (Batt *et al.,*1986), and an imbalance in the NAD/NADH redox system which is involved in xylose metabolism (Bruinenberg *et al.,*1984), or the enzymes that produced in *Saccharomyces cerevisiae* were found to be inactive (Amore *et al.,* 1989). Gong *et al.* (1981a and 1983) reported similar results. They found that *Saccharomyces cerevisiae* was able to produce ethanol from xylulose but were unable to utilize xylose. Also, Gong *et al.* (1983) reported that *Saccharomyces cerevisiae* was poorly grow when D-xylose was used as a carbon and energy source.

Table (2): Growth rate and dry weight / g of the original yeast strains*.

Yeast strains	Optical density (OD) at 600 nm after			Dry weight / g / 100 ml cell suspension after 72h incubation
	24 h	48 h	72 h	
GT160-34B	0.070	0.182	1.227	0.0221
XJ133-1B	0.136	1.360	1.512	0.1795
Y-7124	1.200	2.737	2.872	0.7104

***Using CM medium supplemented with D-xylose as sole carbon source.**

Sporulation ability of *Pichia stipitis* Strain:

Pichia stipitis was tested for sporulation by plating on sporulating medium (SM) and examined by microscope. Results indicated that *Pichia stipitis* was not able to form any spores even after incubation for 17 days, which prove that this strain should be haploid.

Mutation induction in *Pichia stipitis*, Mutants Isolation and auxotrophus identification :

UV-light was used as mutagenic agent for the induction of mutation in *Pichia stipitis.* It was used for 1, 1.5, 2, 2.5 and 3 minutes. Samples from each treatment were diluted up to (10^{-6}) and plated on complete medium (CM) supplemented with 5% D-xylose instead of glucose, then the plates were incubated at 30ºC for 72 hours. Ultraviolet light has been widely used in the field of microbial breeding. This may be probably due to its availability, safe in use and mostly due to the fact that the UV-induced mutants proved to be more stable in fermentation industries, (Mohamed, 1990). It is clear from

Table (3) that increasing the exposure time to 3 minutes affected the survival of the cells drastically where the survival percentage was dropped from 76.47% at 1 min. exposure time to 17.65% at 3 min. exposure time. This data are in good agreement with Ibrahim *et al.* (1998). They found that the survival percentages of spores of either *Bacillus thurinigenesis* or *Bacillus subtillus* are decreased gradually with increasing the exposure time of UV. The developing colonies were counted and tested on both MM and CM supplemented with D-xylose instead of glucose to detect the auxotrophic mutants. Auxotrophic analysis of *Pichia stipitis* mutants illustrated that the16 different isolates (spontaneous or induced) failed to grow on MM (Table,3). Auxotrophic mutants were picked according to their failure to grow on MM and tested for their nutritional requirements according to (Holliday, 1960).

Table (3): Effect of different exposure times of UV light on survival percentages and auxotrophic mutants induction from *Pichia stipitis* (Y-7124) yeast strain.

Exposure time (minute)	Mean of survival cell numbers	Survival %	No. of isolated Auxotrophic mutants
Control	$17x10^6$	100	3[a]
1	$13x10^6$	76.47	3[b]
1.2	$11x10^6$	64.71	2 [b]
2	$7x10^6$	41.18	4 [b]
2.5	$5x10^6$	29.41	1 [b]
3	$3x10^6$	17.65	3 [b]

[a] **Spontaneous isolated mutants.**
[b] **UV-induced isolated mutants.**

Table (4) showed the nutritional requirements of some selected *Pichia stipitis* auxotrophic yeast mutants. The two mutants (M2) and (M3) require three amino acids. The first needs leucien, histidine and cysteine while the second needs histidine, cysteine and aspartic acid. Moreover, the mutant (M4) needs both histidine and cysteine while the mutant (M1) (spontaneous mutant) needs only histidine to grow on MM. This results are in good agreement with similar results using UV irradiation for mutation induction in *Pichia spp.* e.g. Sibirnyi *et al.* (1977) on *Pichia guilliermondii,* and Laplace *et al.* (1992) on *Pichia stipitis.*

Table (4): Nutritional requirements of some selected *Pichia stipitis* auxotrophic mutants.

***Pichia stipitis* Auxotrophic mutants.**	**Requirement(s)***
M1[a]	His.$^-$

M2[b]	His.⁻, Cys.⁻ and Leu.⁻
M3[b]	His.⁻, Cys.⁻ and Asp.⁻
M4[b]	His.⁻ and Cys.⁻

[a] Spontaneous isolated mutants.
[b] UV-induced isolated mutants.
***(His.) Histidine, (Cys.) Cysteine, (Leu.) Leucien and (Asp.) Aspartic acid**

Protoplast formation and fusion.

The protoplast fusion technique was done to obtain *Saccharomyces cerevisiae χ Pichia stipitis* fusants, which can grow on xylose and produce good yield of ethanol. Two *Saccharomyces cerevisiae* mutant strains (GT160-34B and XJ133-1B) and two *Pichia stipitis* mutant strains (M1 and M2) which induced spontaneously and after UV treatment, respectively, were used in this study. According to the conditions described under material and methods, microscopic examination with iodine solution showed that the gradual degradation of yeast cell wall was started after the addition of wall lytic enzyme (snail enzyme). Thus, during the preparation of protoplasts, a spheroplast state must be logically anticipated. The total number of survived cells was counted before addition of lytic enzyme by plate count method (Table 5). A similar method involves the use of mechanical or outlytic enzymes. The efficiency of outlytic enzymes in the formation of protoplasts can be greatly affected by age, strain, culture condition (Villanueva, 1966). It has been found that cells pre-treated with mercapto-compounds, such as β-mercaptoethanol or dithiothreitol tend to protoplast easier, since the glycosidic bonds are rendered more susceptible to enzymatic cleavage (Russell & Stewart, 1979). However, protoplast formation may depend on the sensitivity of the different yeast strains to lytic enzymes and on the reaction of different cells of the same culture, the walls of older cells being highly resistant (Anderson & Millbank, 1966). When cells of yeasts were converted into protoplasts, pellets were washed three times with washing buffer and mixed in different combinations as presented in Table (5). Pellets were resuspended in fusion buffer, which contains PEG. Adding the fusion buffer to the protoplast suspension resulted in intensive agglutination, which led to the formation of large aggregates. The number of protoplasts in the aggregates depended mainly on the density of the pellets. After PEG treatment, protoplast mixtures were embedded into solid minimal medium for regeneration (MMR). Some of them have increased in volume. Cell walls were subsequently reconstructed and converted into normal cells. These cells were regarded as revertants of protoplasts, which have arisen, by fusion, as confirmed by a series of control experiments. The efficiency of fusion was expressed as a percentage of reverting protoplasts. Mixture of complementing protoplasts but untreated with fusion buffer (PEG) were used as control. Table (5) show that the fusion between XJ133-1B and M1 had higher frequency of regeneration percentage (2%) when grown on MMR supplemented with sorbitol and glucose than the other fusions which showed regeneration percentage in regeneration media less than 1%. These results are in good agreement with the results of Svoboda (1978). He found that the frequency of hybrid colony formation in regeneration medium was less than

1% when he studied the possibility of fusion of *Saccharomyces cerevisiae* protoplasts derived from the same and opposite mating type cells. However, the low yield of resultant hybrids could be disruption of some fused protoplasts during washing from PEG and mainly, by insufficient embedding of protoplasts in gel medium which, at least in budding yeast, is the most important requirement for successful regeneration of cell-wall (Necas, 1971). It should also be mentioned that not all micro-colonies manifest themselves into visible colonies. Since PEG-induced fusion of yeast spheroplasts was firstly reported (Van Solingen & Vander Plaat, 1977), the method has grown in popularity. On the other hand, the use of PEG as a spheroplast fusionic agent has a number of limitations, which have been discussed in detail by Zimmermann & Vienken (1982). They concluded that: (a) optimum fusion conditions vary between species, (b) the fusion process cannot be monitored under a microscope, (c) cell number to be fused cannot be pre-selected, (d) the fusion process often extends over a long time, (e) a loss of interacellular material occur, which may decrease hybrid viability, (f) fusigenic compounds also affect cell viability, and finally (g) hybrid yield is low. Necas (1971 and 1980) reported that successful application of protoplast fusion is dependent on the subsequent culture and reversion of protoplasts to the typical vegetative state. This aspect of technology has been established practice for many years. No special medium constituents are necessary other than the inclusion of an osmotic stabilizer. Of all the yeasts, *Saccharomyces cerevisiae* appears to be the most fastidious in that total wall regeneration occurs only on solidified media. Data presented in this study showed that regeneration of protoplasts produced large-sized cell colonies than the parental strains. This may due to the effect of hybrid vigour. These results are in good agreement with the results of Vallin & Ferenczy (1985). They studied protoplast intergeneric fusion of *Candida tropicals* and *Pichia gueilliermondi*i. After complementation and regeneration, the resulting cells were yeast-like, large ovoid in shape and contained only one nucleus per cell and more DNA than the parental type. They also suggested that the hybrids are aneuploids carrying the haploid genome from *Candida tropicals* and only one or more or a few chromosome from *Pichia gueilliermondi*i. Results of intergeneric protoplast fusion between *Saccharomyces cerevisiae* and *Kluyveromyces lactis* induced by PEG were reported by Borun & Jinke (1990). They stated that the cell volume of fusants was about the sum of the cells of the two parents. DNA contents of most fusants were about two to three times higher than that of the parents. On the other hand, Selebane *et al.* (1993) found that fusants of *Candida shehatae* and *Pichia stipitis* showed only marginal increases in cell DNA content when compared with their parents.

Table (5): Effect of different regeneration media on regenerated colonies through protoplast fusion between different yeast strains. Abd El-Sabour, M. S.

Yeast	Hybrids*	Regeneration media	No. of survivals cells / ml	Protoplast formation (No. / ml)	No. of regenerated colonies on MMR	% Regeneration

GT160-34B x M1	Glycerol + Glucose	1.2×10^8	20×10^6	10×10^3	0.05
GT160-34B x M2		0.8×10^8	8×10^6	0.7×10^3	0.01
XJ133-1B x M1		1.4×10^8	10×10^6	20×10^3	0.20
XJ133-1B x M2		1.0×10^8	0.9×10^6	0.1×10^3	0.01
GT160-34B x M1	Glycerol + Xylose	1.2×10^8	0.8×10^7	50×10^2	0.06
GT160-34B x M2		0.8×10^8	0.3×10^7	0.3×10^2	0.001
XJ133-1B x M1		1.4×10^8	0.8×10^7	50×10^2	0.06
XJ133-1B x M2		1.0×10^8	0.3×10^7	200×10^2	0.67
GT160-34B x M1	Sorbitol + Glucose	1.2×10^8	0.4×10^6	2×10^2	0.05
GT160-34B x M2		0.8×10^8	2×10^6	0.1×10^2	0.001
XJ133-1B x M1		1.4×10^8	0.3×10^6	60×10^2	2
XJ133-1B x M2		1.0×10^8	1×10^6	0.1×10^2	0.001
GT160-34B x M1	Sorbitol + xylose	1.2×10^8	0.1×10^6	0.1×10^3	0.10
GT160-34B x M2		0.8×10^8	8×10^6	10×10^3	0.13
XJ133-1B x M1		1.4×10^8	3×10^6	20×10^3	0.67
XJ133-1B x M2		1.0×10^8	10×10^6	2×10^3	0.02

***GT160-34B & XJ133-1B are two different *Saccharomyces cerevisiae* mutants.**
***M1 & M2 are spontaneous and UV-induced mutants, respectively derived from *Pichia stipitis* (Y-7124).**

After the protoplast induction and fusion, the hybrid fusants were selected by their ability to grow on the selective medium supplemented with xylose as a sole carbon source without addition of any amino acids. Complementation must has been occurred since the parental strains were auxotrophs and thus incapable of growing on this medium.

Seventeen hybrid fusants were selected after protoplast fusion experiments. All of them were able to grow on D-xylose sugar (Table, 6). The selected hybrid fusants and their parental yeast strains were tested for growth behavior on different carbon sources (Table, 7), and ethanol production from xylose (Table, 8).

Table (6): Growth behavior of some selected hybrid fusants and their parental yeast strains on D-xylose.

Hybrid fusants*	Parental strains	Regeneration media	Growth on D-xlose
F1	GT160-34-B[a] x M1[c]	Xylose +	+
F2	GT160-34-B[a] x M1[c]		+
F3	GT160-34-B[a] x M2[d]		+
F4	GT160-34-B[a] x M2[d]		+

F5	XJ133-1B[b] x M1[c]	Sorbitol	+
F6	XJ133-1B[b] x M1[c]		+
F7	XJ133-1B[b] x M1[c]		+
F8	XJ133-1B[b] x M2[d]		+
F9	XJ133-1B[b] x M2[d]		+
F10	XJ133-1B[b] x M2[d]		+
F11	XJ133-1B[b] x M2[d]		+
F12	GT160-34-B[a] x M1[c]		+
F13	GT160-34-B[a] x M1[c]	Xylose	+
F14	GT160-34-B[a] x M1[c]	+	+
F15	GT160-34-B[a] x M1[c]	Glycerol	+
F16	GT160-34-B[a] x M2[d]		+
F17	GT160-34-B[a] x M2[d]		+

*** isolated from different regeneration media supplemented with xylose.**
[a & b] GT160-34B & XJ133-1B are two different *Saccharomyces cerevisiae* mutants.
[c & d] M1 & M2 are spontaneous and UV-induced mutants respectively derived from *Pichia stipitis* (Y-7124).
(+) refer to good growth on D-xylose as sole carbon source.

Growth behaviour of the selected fusants and their parental yeast strains on different carbon sources:

Seventeen fusant yeast strains and their parents were tested for growth on xylose (pentose), glucose (hexsose), cellobiose and lactose (disaccharides), raffinose (trisaccharide) and starch (polysaccharide) to elucidate the sugar utilization control by genome. The results in Table (7) indicated that the hybrid fusant yeast strains and their parental strains grow normally on D-glucose and D-xylose except the two parent *Saccharomyces cerevisiae* yeast strains (GT160-34B and XJ133-1B) which did not show any growth on xylose. Reed & Nagodawithana, (1991) reported that many number of yeast species including *Saccharomyces cerevisiae* can be grown on hexoses but cannot be grown on pentoses so that they can not grow on wood sugars. Using polysaccharide as carbon source revealed that all tested fusant strains did not show any growth on starch as *Saccharomyces cerevisiae*. These results may be due to the lake of the genes (STA and / or DEX) that code for the enzyme amyloglucosidase, which hydrolyzes starch and dextrin to yield glucose. Errat & Nasim (1986) reported that the enzyme amyloglucosidase is used for hydrolyzing starch or dextrin, at the α-1-4,α-1-6 linkages and to some degree, at 1-3 linkages to yield glucose. The starch or dextrin degradation capability was due to the presence of any one of STA genes (STA1, STA2 and STA3). Sheetz & Dickson, (1981) stated that baker's yeast, *Saccharomyces cerevisiae* does not have the genes (LAC4 and LAC12) that code for the required enzyme system (i.e. β-galactosidase and lactose permease) to assimilate lactose as carbon source. So the two fusant strains F10 and F11 which gave normal growth on lactose may be constructed as new strains to utilize lactose by intergeneric protoplast fusion techniques. These fusants should be new strains, which may have all the characteristics of *Pichia stipitis* parental strain (M1). The present results also showed that all tested hybrid fusants and their parents did not give any growth on raffinose. Lampen (1968) reported that there are at least six unlinked polymeric genes (Suc1-5 and Suc7) can be identified in yeast.

Table (7): Growth behavior of some selected hybrid fusants and their parental yeast strains on different carbon sources.

Hybrid fusants and its parental yeast strains	Carbon source					
	Pentose	Hexsose	Disaccharide		Trisaccharide	Poly-saccharide
	Xylose	Glucose	Cellobiose	Lactose	Raffinose	Starch
GT160-34B[a]	-	+	-	-	-	-
XJ133-1B[a]	-	+	-	-	-	-
Y-7124[b]	+	+	+	-	-	-
M1[c]	+	+	+	+	-	-
M2[c]	+	+	+	-	-	-
F1[d]	+	+	+	-	-	-
F2[d]	+	+	+	-	-	-
F3[d]	+	+	+	-	-	-
F4[d]	+	+	+	-	-	-
F5[d]	+	+	+	-	-	-
F6[d]	+	+	+	-	-	-
F7[d]	+	+	+	-	-	-
F8[d]	+	+	+	-	-	-
F9[d]	+	+	+	-	-	-
F10[d]	+	+	+	+	-	-
F11[d]	+	+	+	+	-	-
F12[d]	+	+	+	-	-	-
F13[d]	+	+	+	-	-	-
F14[d]	+	+	+	-	-	-
F15[d]	+	+	+	-	-	-
F16[d]	+	+	+	-	-	-
F17[d]	+	+	+	-	-	-

[a]GT160-34B & XJ133-1B are two different *Saccharomyces cerevisiae* mutants.
[b]*Pichia stipitis* yeast strain.
[c]M1 & M2 are spontaneous and UV-induced mutants, respectively derived from *Pichia stipitis* (Y-7124).
[d] Hybrid fusants.
(+) growth & (-) non growth.

Only one of them is required for the fermentation of the sucrose or one-third of raffinose molecule, e.g. β-fructosidase (invertase) that hydrolyzes the sucrose to glucose and fructose as well as can degrade the raffinose to fructose and melibiose. On the other hand, baker's yeast is unable to utilize the disaccharide melibiose due to the lake of enzyme α-galactosidase (melibiase) to cleavage it to glucose and galactose. With cellobiose, all tested hybrid fusant strains and *Pichia stipitis* parental yeast strain showed good growth except both of *Saccharomyces cerevisiae* strains (GT160-34B and XJ133-1B) which do not give any growth on cellobiose.

Ethanol production from xylose:

Ethanol production and xylose fermentation efficiency measured as sugar consumption were tested for 17 selected hybrid fusants and their parental yeast strains (GT160-34B, XJ133-1B, M1, M2, and Y-7124) after growing aerobically for 48 hours at 30ºC were illustrated in Table (8).

The two parent strains of *Saccharomyces cerevisiae* (GT160-34B and XJ133-1B) did not show any xylose fermentation efficiency or ethanol production. Similar result was reported by Gong *et al.* (1981a) and (1983), they found that *Saccharomyces cerevisiae* was able to produce ethanol from

xylulose but were unable to utilize xylose. The two mutants M1 and M2 were 6% and 12% less than its parent *Pichia stipitis* yeast strain (Y-7124) in xylose fermentation efficiency, respectively. On the other hand, M2 reached the same amount of ethanol production and showed 11% increase in conversion coefficient than its parent. But M1 showed 17% and 12% decrease in ethanol production and conversion coefficient than its parent, respectively. Results also showed that the tested hybrid fusants varied and some of them were more than its active parent (M1 or M2) in xylose fermentation efficiency. The hybrid fusants; F1, F2, F12, F13, F14 and F15, which derived from the fusion between the two parents; GT160-34B and M1, showed increase in xylose fermentation efficiency ranging from 13 to 38% more than the active parent (M1). The hybrid fusants; F3, F4, F16 and F17, which derived from the fusion, between the two parents; GT160-34B and M2, showed increase in xylose fermentation efficiency ranging from 20 to 40% more than the active parent (M2). The hybrid fusants; F5, F6 and F7, which derived from the cross between the two parents XJ133-1B and M1, showed increase in xylose fermentation efficiency ranging from 31 to 38% more than the active parent (M1). The hybrid fusants; F8, F9, F10 and F11, which derived from the fusion between the two parents; XJ133-1B and M2, showed increase in xylose fermentation efficiency ranging from 20 to 27% more than the active parent (M2). However, results showed that ethanol production from xylose sugar was varied among the different hybrid fusants and some of them exceeded the active parent (M1 or M2). The results also showed that there was an increase in ethanol production ranged from 5% (F12 and F13) to 56% (F16), in most hybrid fusants tested. But, F4, F8, F9, F11, F14 and F15 showed decrease in ethanol production reached to 17%, 14%, 14%, 7%, 21% and 5%, respectively less than the active parent (M1 or M2). On the other hand, most of the tested hybrid fusants showed decrease in conversion coefficient ranged from 2% (F3) to 43% (F14) less than the active parent (M1 or M2). But, F1, F2 and F16 showed 16%, 16% and 30% increase in conversion coefficient, respectively than the active parent (M1 or M2). However, this fact has led to a novel approach, which consists of employing microbial isomerases to transform xylose to xylulose, which can then be fermented. Results in this study showed that hybrid fusants and parental strains of *Pichia stipitis*, M1 and M2 fermented 5% D-xylose at 30ºC directly to ethanol within 48 hours. These results were in agreement with those obtained by Du Preez *et al.* (1986). Also, Ligthelm *et al.* (1988) reported that the highest ethanol yield coefficient of 0.47 h^{-1} was obtained on D-xylose with *Pichia stipitis.* Highly significant positive correlation between sugar consummation and ethanol product was indicated at (P>0.01).

Table (8): Ethanol production of some hybrid fusant yeast strains and their parents.

Hybrid fusants and their parental yeast strains	Consumed sugar (g / 100 ml)	EOH* (g / 100 ml)	Conversion coefficient
GT160-34B[a]	0.0	0.00	0.00

XJ133-1B[a]	0.0	0.00	0.00
Y-7124[b]	3.4	2.37	69.71
M1[c]	3.0	2.37	79.00
M2[c]	3.2	1.96	61.25
F1[d]	3.6	2.55	70.83
F2[d]	3.6	2.55	70.83
F3[d]	3.6	2.78	77.22
F4[d]	4.2	1.96	46.67
F5[d]	4.4	2.14	48.64
F6[d]	4.2	1.96	46.67
F7[d]	4.2	2.09	49.76
F8[d]	3.8	2.05	53.95
F9[d]	3.8	2.05	53.95
F10[d]	3.8	2.59	68.16
F11[d]	3.6	2.14	59.44
F12[d]	3.6	2.05	56.94
F13[d]	3.6	2.05	56.94
F14[d]	4.4	1.55	35.23
F15[d]	4.4	1.87	42.50
F16[d]	3.6	3.69	102.50
F17[d]	3.8	2.78	73.16

[a]GT160-34B & XJ133-1B are two different *Saccharomyces cerevisiae* mutants.
[b]*Pichia stipitis* yeast strain.
[c]M1 & M2 are spontaneous and UV-induced mutants, respectively derived from *Pichia stipitis* (Y-7124).
[d] Hybrid fusants. ***EOH = Ethanol (CH3-CH2OH).**

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تخمير سكر الزيلوز الخماسي إلي الكحول الاثيلي بواسطة بعض الهجن المتحصل عليها من خلال الدمج البروتوبلاستي بين خميرتي سكارواميسيس سيريفيسيا و بيتشيا ستيبتيس .

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2 **قسم الوراثة الميكروبية – شعبة بحوث الهندسة الوراثية والبيوتكنولوجيا - ا لمركز القومي للبحوث – الدقي - مصر.**

يهدف البحث الحالي إلي إكساب خميرة سكارومايسيس سيريفيسيا صفة استعمال وتخمير سكر الزيلوز الخماسي (D-xylose) وإنتاج الكحول الاثيلي (Ethanol). وقد تم إنجاز ذلك من خلال دمج المادة الوراثية لكل من خميرتي سكارومايسيس سيريفيسيا *Saccharomyces cerevisiae* وبيتشيا ستيبتيس *Pichia stipitis* بواسطة الدمج البروتوبلاستي Protoplast fusion. حيث دمجت طافرتي العوز الغذائي-GT160 34B و XJ133-1B لخميرة سكارومايسيس سيريفيسيا مع طافرتي العوز الغذائي M1 و M2 المشتقتين من السلالة البرية Y-7124 لخميرة بيتشيا ستيبتيس تلقائيا و بعد المعاملة التطفيرية بالأشعة فوق البنفسجية UV، علي التوالي. عند دراسة سلوك النمو لعدد ١٧ عزلة هجين بالإضافة لآبائها الأصلية علي مصادر مختلفة من الكربون اشتملت علي: الزيلوز والجلوكوز واللاكتوز والسيلوبيوز والرافينوز بالإضافة للنشا. أشارت النتائج إلي أن العزلات الهجين قد اكتسبت بعض صفات الأب الأصلي بيتشيا ستيبتيس عن طريق التهجين البروتوبلاستي بين الجنسي خصوصا القدرة علي استعمال سكر الزيلوز. كما أظهرت النتائج أن العزلات الهجين بالإضافة لآبائها الأصلية (السلالة البرية Y- 7124 وM1 وM2) قاموا بتخمير ٥% زيلوز هوائيا عند ٣٠ درجة مئوية خلال ٤٨ ساعة. لم يظهر الأبوين الأصليين GT160-34B وXJ133-1B لخميرة سكارومايسيس سيريفيسيا أي مقدرة تخميرية لسكر الزيلوز أو إنتاجية للايثانول. الطفرتين M1 وM2 كانتا اقل كفاءة تخميرية لسكر الزيلوز عن السلالة البرية Y-7124 بنسبة ١٢% و ٦%، علي التوالي. الطافرة M1 أظهرت ١٧% و ١٢% نقص في إنتاجية الايثانول ومعامل التحويل، علي التوالي عن السلالة البرية. الطافرة M2 أعطت نفس الإنتاجية من الايثانول بالإضافة إلي إنها قد أظهرت زيادة في معامل التحويل مقدارها ١١% عن السلالة البرية. العزلات الهجين كانت متابينة في كفاءتها التخميرية لسكر الزيلوز وإنتاجيتها للايثانول. وقد أظهرت هذه العزلات الهجين مدي كفاءة تخميرية لسكر الزيلوز تراوح مابين ١٣% إلي ٤٠% زيادة عن الأب الأصلي النشيط "من حيث الكفاءة التخميرية لسكر الزيلوز" (M1 أو M2). وقد أظهرت ١١ عزلة هجين زيادة في إنتاجية الايثانول عن الأب النشيط تراوحت ما بين ٥% و ٥٦%. بينما أظهرت الستة عزلات الهجين الأخرى تناقصا في إنتاجية الايثانول تراوحت ما بين ٥% و ٢١% عن الأب النشيط. معظم العزلات الهجين أظهرت تناقصا في معامل التحويل تراوح ما بين ٢% و ٤٣% عن الأب النشيط. بينما أظهرت العزلات الهجين F1 وF2 وF16 زيادة في معامل التحويل بمعدل ١٦% و ١٦% و ٣٠%، علي التوالي عن الأب النشيط. وقد أوضح التحليل الإحصائي وجود علاقة ارتباطية موجبة عالية المعنوية عند (P>0.001) بين السكر المستهلك (Consumed sugar) والايثانول المنتج (Ethanol production) .