EXPERIMENTAL RESULTS

(1). Screening of Yeasts:

The ability to utilize of the original two yeast strains of *Saccharomyces cerevisiae;* (GT 160 – 34 B), (ST X 23-58), (XJ133 – 1B), and *Pichia stipitis* (Y – 7124) were tested on two different carbon sources, D-glucose and D-xylose sugars. The basal medium was used in this experiment and results are shown in Table (2).

These results indicate that all yeast strains tested give good growth on D-glucose sugar as a sole carbon source whereas *Pichia stipitis* strain, which gives very good growth. On the other hand, when D-xylose sugar was used, the original strains of *Saccharomyces cerevisiae* do not give any growth but *Pichia stipitis* strain gives very good growth.

(2). Growth Measurements (Growth Rate):

Table (3) shows the growth rate of all original yeast strains measured as optical density (O.D.). In each tube unit of turbidity using Shimadzu UV-1201 spectrophotometer at 600 nm every 24 hours for three days were taken as measurements.

Cells were collected by centrifugation at 3000 rpm for 25 minutes and dried for three hours at 105 ° C. Dry weight/g was determined for each strain. With respect to growth rate the results

showed that *Pichia stipitis* was the best yeast strain compared with other *Saccharomyces cerevisiae* yeast strains.

Table (2): Growth behaviour of yeast strains in the basal medium on two different carbon sources

The original Yeast Strains	**Carbon source in basal medium**	
	D-glucose	**D-xylose**
***Saccharomyces cerevisiae* 1(GT 160 – 34 B)**	++	-
***Saccharomyces cerevisiae* 3(ST X 23-58)**	++	-
***Saccharomyces cerevisiae* 5(XJ133 – 1B)**	++	-
***Pichia stipitis* (Y – 7124)**	+++	+++

Growth of strains scored as follows:

(+++) Very good growth.

(++) Good growth.

(-) No growth

Table (3): Growth rate and dry weight/gm for all original yeast strains studied

Original Yeast Strains	O. D. after*			Dry weight/gm/ 100 ml cell suspension
	24 H	48 H	72 H	
Saccharomyces cerevisiae 1(GT 160 – 34 B)	0.070	0.182	1.227	0.0221
Saccharomyces cerevisiae 3(ST X 23-58)	0.091	0.118	0.124	0.0382
Saccharomyces cerevisiae 5(XJ133 – 1B)	0.136	1.360	1.512	0.1795
Pichia stipitis (Y – 7124)	1.200	2.737	2.872	0.7104

* O.D = Optical density

(3) Sporulation Ability of *Pichia stipitis* Strain:

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

(4) Mutation Induction and Mutants Isolation :

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 at a distance of 25 cm. The treated suspension was kept in dark for two hours. Samples of each dosage were diluted after treatment up to (10^{-6}) and plated on complete medium (CM) supplemented with 5 % D-xylose, then the plates were incubated at 30 ° C for 72 hours.

Increasing the exposure time over 3 minutes affected the survival of the cells drastically.

The developing colonies were counted and tested on both MM and CM supplemented with D-xylose to detect the auxotrophic mutants. Survival cells and auxotrophic mutants were listed in Table (4) and Fig (1).

Table (4): Survivors percentages and Auxotrophic mutants recovered from *Pichia stipitis* strain after different UV treatments.

Time/minute (Dose)	Means of survival cells/10^{-6}	Survival %	Auxotrophic mutants
Control 0	17	100.00	3
1	13	76.47	3
1.5	11	64.71	2
2	7	41.18	4
2.5	5	29.41	1
3	3	17.65	3

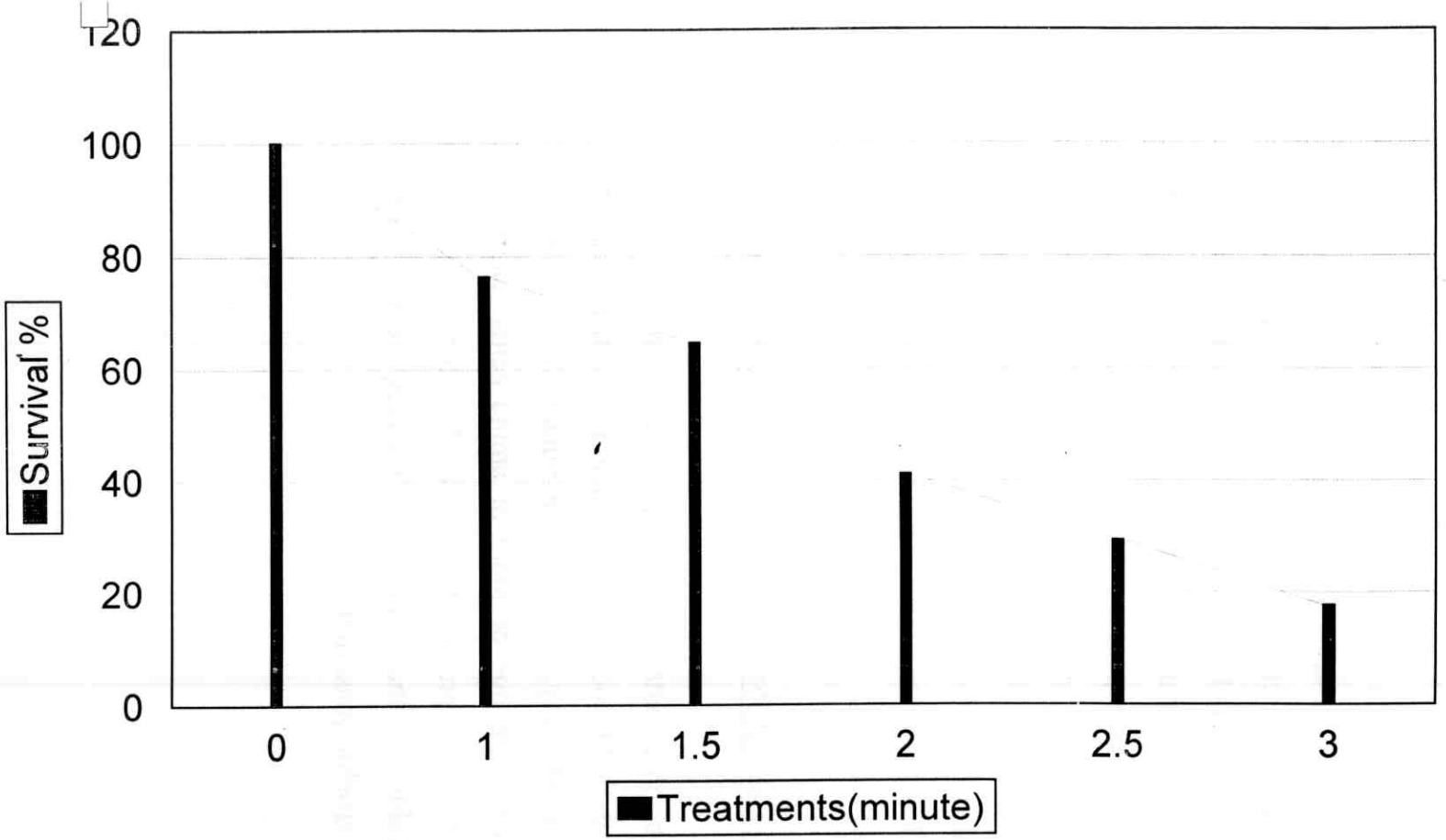

Fig (1): Survivors percentages and Auxotrophic mutants recovered from Pichia stipitis strain after different UV treatments.

Results in Table (4) show that the auxotrophic mutants are induced using UV irradiation. The survival percentages of *Pichia stipitis* after applying different exposure times of UV irradiation are presented in Table (4) and illustrated in Fig (1). The obtained results showed that survival percentages are decreased gradually with increasing the exposure time of UV. It is clear from Table (4) that, when using *Pichia stipitis*, the survival percentage was dropped from 76.47 % at 1 min exposure time to 17.65 % at 3 min exposure time. Auxotrophic analysis of *Pichia stipitis* illustrate that 16 different isolates failed to grow on MM either spontaneous or inducible (Table 4).

(5) Identification of *Pichia stipitis* Auxotrophic Mutants:

Auxotrophic mutants were picked according to their failure to grow on MM and tested for their nutritional requirements according to (**Holiday, 1960**). These mutants were grown on MM supplemented with one or more of amino acids, vitamins and nitrogen bases as previously described under materials and methods. Table (5) shows the nutritional requirements of *Pichia stipitis* auxotrophic yeast mutants.

Table (5). Nutritional requirements of some *Pichia stipitis* auxotrophic mutants recovered and used in protoplast fusion.

Time/minue	Mutants Number	Requirements (s)
0	*Pichia stipitis* 171 (M2)	His^-.
2	*Pichia stipitis* 73 (M1)	His^-., Cys^-., Leu^-.
3	*Pichia stipitis* 152 (M3)	His^-., Cys^-., Asp^-.
3	*Pichia stipitis* 130 (M4)	His^-., Cys^-.
M = Mutants		

Four auxotrophic mutants are able to grow on MM supplemented with different nutritional requirements. The results show that two mutants (*Pichia stipitis*) 73 (M1) and 152 (M3) require three amino acids. The first needs leucine, histidine and cysteine while the second needs histidine, cysteine and aspartic acid. Moreover, mutant *Pichia stipitis* 130 (M4) needs both histidine and cysteine while mutant *Pichia stipitis* 171, (M2) (spontaneous mutant) needs only histidine to grow (Table 5).

(6) Sugar Fermentation Efficiency Measured as Sugar Consumption:

D-xylose sugar concentration (consumed sugar) was measured for both the original strains and the tested mutants by

refractometry after growing on 5 % xylose and incubation at 30 ° C for three days.

Consumption was measured as the decrease in xylose concentration in the fermentation media. The results are shown in Table (6).

Table (6) showsthat all *Saccharomyces cerevisiae* strains could not utilize xylose. *Pichia stipitis* strain besides its mutants could utilize xylose. It worthwhile saying that each of the mutants consumed double of what has been consumed by its wild type parent.

(7) Induction of Yeast Protoplasts:

The protoplast fusion technique was done to obtain *Saccharomyces cerevisiae* χ *Pichia stipitis* fusants can grow on xylose and produce good yield of ethanol. Two *Saccharomyces cerevisiae* strains (GT 160-34 B and XJ 133- 1B) and two *Pichia stipitis* mutants [(M1 (73) and M2 (171)] were used in this study.

Table (6): D-xylose sugar consumption percentage of all tested strains after three days incubation at 30 °C

Tested strains	Sugar in the medium %					Consumed Sugars % (C - X)
	C	R1	R2	R3	x	
Saccharomyces cerevisiae 1(GT 160 – 34 B)	5	5	5	5	5	0.00
Saccharomyces cerevisiae 3(ST X 23-58)	5	5	5	5	5	0.00
Saccharomyces cerevisiae 5(XJ133 – 1B)	5	5	5	5	5	0.00
Pichia stipitis (Y – 7124)	5	4.8	4.8	4.8	4.8	0.20
Pichia stipitis 73 (M_1)*	5	4.6	4.6	4.6	4.6	0.40
Pichia stipitis 171 (M_2)	5	4.6	4.6	4.6	4.6	0.40
Pichia stipitis 152 (M_3)	5	4.6	4.6	4.6	4.6	0.40
Pichia stipitis 130 (M_4)	5	4.6	4.6	4.6	4.6	0.40

• M = Mutant

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. The counts are represented in Table (7).

Table (7): The mean number of survived cells of the tested yeast strains

Tested strains	Mean number of survival cells
Saccharomyces cerevisiae 1(GT 160 – 34 B)	8.7×10^7
Saccharomyces cerevisiae 5(XJ133 – 1B)	1.2×10^8
Pichia stipitis 73 (M_1)*	7.2×10^7
Pichia stipitis 171 (M_2)*	1.5×10^8

- M_1 and M_2: induced mutant and spontaneous mutant derived from *Pichia stipitis.*

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 were highly resistant [**Anderson and Millbank, 1966**].

(8) Protoplast Fusion:

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 (8). Pellets were resuspended in fusion buffer, which contains poly ethylene glycole (PEG) (4000) as described under materials and methods.

Adding to 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. 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 controls.

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 acid. Complementation must has been occurred since

the parental strains were auxotrophs and thus incapable of growing on this medium.

Results show that regeneration of protoplasts produced large-sized cell colonies than the parental strains. Results in Table (8) also, show that the frequencies of fusion were low [0.01, 0.05, 0.01. 0.20, 0.001, 0.06, 0.67, 0.06, 0.001, 0.05, 0.001, 0.13, 0.10, 0.02 and 0.67 respectively] except in the fusion between *Saccharomyces cerevisiae* XJ133 – 1B and M2 (171) which had higher frequency (2%) when grown on MMR supplemented with sorbitol and glucose than in other fusion.

However, the low yield of resulted hybrids could be caused by 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 the cell wall (**Necas, 1971**). It should be also mentioned that not all microcolonies manifest themselves into visible colonies.

Table (8): Protoplast fusion of *Saccaromyces cerevisiae* and *Pichia stipitis* using four different regenerations media

Strains hybridized	Protoplast formation (No./ml)	No. of regenerized colonies on MMR	Regeneration %	Regeneration media
S. c. GT 160-34 B x M_1 (73) S. c. GT 160-34 B x M_2 (171) S. c. XJ 133-1 B x M_1 (73) S. c. XJ 133-1 B x M_2 (171)	0.8×10^7 0.2×10^8 0.9×10^6 0.1×10^8	0.7×10^3 0.1×10^5 0.1×10^3 0.2×10^5	0.01 0.05 0.01 0.20	Glycerol + Glucose
S. c. GT 160-34 B x M_1 (73) S. c. GT 160-34 B x M_2 (171) S. c. XJ 133-1 B x M_1 (73) S. c. XJ 133-1 B x M_2 (171)	0.3×10^7 0.8×10^7 0.3×10^7 0.8×10^7	0.3×10^2 0.5×10^4 0.2×10^5 0.5×10^4	0.001 0.06 0.67 0.06	Glycerol + Xylose
S. c. GT 160-34 B x M_1 (73) S. c. GT 160-34 B x M_2 (171) S. c. XJ 133-1 B x M_1 (73) S. c. XJ 133-1 B x M_2 (171)	0.2×10^7 0.4×10^6 0.1×10^7 0.3×10^6	0.1×10^2 0.2×10^3 0.1×10^2 0.6×10^4	0.001 0.05 0.001 2..00	Sorbitol + Glucose
S. c. GT 160-34 B x M_1 (73) S. c. GT 160-34 B x M_2 (171) S. c. XJ 133-1 B x M_1 (73) S. c. XJ 133-1 B x M_2 (171)	0.8×10^7 0.1×10^6 0.1×10^8 0.3×10^7	0.1×10^5 0.1×10^3 0.2×10^4 0.2×10^5	0.13 0.10 0.02 0.67	Sorbitol + Xylose

* M_1 (73) and M_2 (171) Induced mutant and spontaneous mutant derived from *Pichia stipitis* strain

(9) Sugar Consumption in Hybrid Fusants:

Ninty-one hybrid fusants were obtained after protoplast fusion experiments. All of which were able on xylose sugar as mentioned in materials and methods.

These fusants besides their parental strains were tested for their xylose fermentation efficiency measured as sugar consumption after growing on 5 % xylose and incubation for 72 hours at 30 ° C under steady state. Results are represented in Table (9).

Results in Table (9) show that *Saccharomyces cerevisiae* strains GT 160 – 34B and XJ 133 - 1B could not utilize xylose, while the two *Pichia stipitis* mutants (M_1 and M_2) were higher in their xylose sugar consumption (0.4 %) compared with their parental strain which consumed 0.2 % (Table 9). Most fusants gave sugar consumption values similar to that of M_1 and M_2 (0.4 %). Some fusants gave higher sugar consumption than their parental strains (0.6 %) showing that there was hybrid vigour effect. Some other fusants gave sugar consumption values like the parent *Pichia stipitis* (0.2 %), whilst the rest of the fusants were not able to utilize xylose (Table 9).

From the highest consumers of the above ninety-one fusants, seventeen fusants as well as the parental strains were selected and tested for their sugar consumption after different time intervals (i.e.

Table (9): Sugar fermentation efficiency measured as xylose consumption % of some hybrid fusants isolated from two different regeneration media and their parental strains after growing on 5 % xylose for 72 hours at 30 °C

Hybrid fusants numbers and parental strains	Type of Media (regeneration media)								
	Glycerol + Xylose				Sorbitol + Xylose				
	Strains hybridized				Strains hybridized				
	A x M_1 *	A x M_2 *	B x M_1 *	B x M_2 *	A x M_1 *	A x M_2 *	B x M_1 *	B x M_2 *	Parental yeast strains
1	0.6	0.4	0.2	0.2	0.6		0.4		
2	0.4	0.4	0.0	0.4	0.2	0.4	0.6		
3		0.4	0.0	0.2	0.6		0.6		
4		0.4		0.2	0.6		0.4		
5		0.4		0.0	0.4			0.4	
6		0.4		0.0	0.4		0.4	0.4	
7		0.4		0.2	0.6		0.6		
8		0.4		0.0	0.6		0.4	0.4	
9		0.4		0.2	0.6	0.4	0.4	0.4	
10		0.4		0.2	0.6	0.4	0.4	0.4	
11		0.6		0.2	0.4			0.6	
12		0.4		0.0	0.6		0.4		
13		0.4		0.0	0.4				
14		0.4		0.0	0.4		0.4		
15		0.4		0.0	0.4		0.4	0.4	
16		0.4		0.2	0.2		0.4	0.4	
17		0.4		0.0	0.4			0.4	
18		0.4		0.2	0.4			0.4	
19		0.4					0.4	0.4	
20		0.4						0.4	
21		0.4							
P. stipitis									0.2
M1 (73)									0.4
M2 171									0.4
GT 160-34B	(A) *								0.0
XT 133-1B	(B) *								0.0

- M1 and M2 → Induced mutant and spontaneous mutant derived from *P. stipitis* strain
- A → *S. cerevisiae* GT 160-34B
- B → *S. cerevisiae* XT 133-1B

24, 48 and 72 hours) to reveal the ability of each of them for xylose consumption after each incubation period and the results are represented in Table (10).

Table (10) and Fig. (2) showed that *Pichia* stipitis, on liquid medium with xylose M1, M2 and all the fusants reached their maximum consumption after 48 hours except fusants Nos.3, 2, 7 and 1 in the regeneration media sorbitol + xylose which reached their maximum after 72 hours.

(10) Determination of Ethanol Production:

Ethanol production and growth rate were determined as described in materials and methods.

Yeast strains were grown aerobically on 5 % xylose sugar with shaking (200 rpm) for 48 hours at 30 ° C. Ethonal production, sugar consumption and growth rate for the 17 selected fusants and their parents *Saccharomyces cerevisiae* GT 160-34 B, *Saccharomyces cerevisiae* XJ 133-1B, M_1, M_2 and *Pichia stipitis* Y-7124 are illustrated in Table (11) and Fig. (3).

The results obtained show that ethanol production was found to be varied in different strains used except the two *Saccharomyces cerevisiae* strains (GT 160-34 B and XJ 133-1B) which did not produce any ethanol. The fusant No. 20 (on regeneration media

Table (10): Sugar fermentation efficiency of 17 fusants and their parental strains measured as sugar consumption % after different incubation periods at 30 °C.

Regeneration media	Strains hybridized	Selected fusants number	Consumed sugar % (fermentation efficiency after		
			24 hours	48 hours	72 hours
Sorbitol + Xylose	A x M_1 *	1	0.2	0.4	0.6
	A x M_1	12	0.2	0.6	0.6
	A x M_2	2	0.2	0.4	0.4
	A x M_2	10	0.2	0.6	0.6
	B x M_1	2	0.2	0.4	0.6
	B x M_1	3	0.2	0.4	0.6
	B x M_1	7	0.2	0.4	0.6
	B x M_1	9	0.2	0.4	0.4
	B x M_2	11	0.2	0.6	0.6
	B x M_2	15	0.2	0.4	0.4
	B x M_2	19	0.2	0.4	0.4
Glycerol + Xylose	A x M_1 *	1	0.2	0.6	0.6
	A x M_1	2	0.2	0.4	0.4
	A x M_2	11	0.2	0.6	0.6
	A x M_2	15	0.2	0.4	0.4
	A x M_2	16	0.2	0.4	0.4
	A x M_2	20	0.2	0.4	0.4
		Pichia stipitis	0.2	0.2	0.2
		M_1 (73)	0.2	0.4	0.4
		M_2 (171)	0.2	0.4	0.4

- A and B › Two *S. cervisiae* strains (GT160-34B and XJ 133-1B)
  M_1(73) and M_2 (171)› Induced mutant and spontaneous mutant drived from *P. stipitis* strain

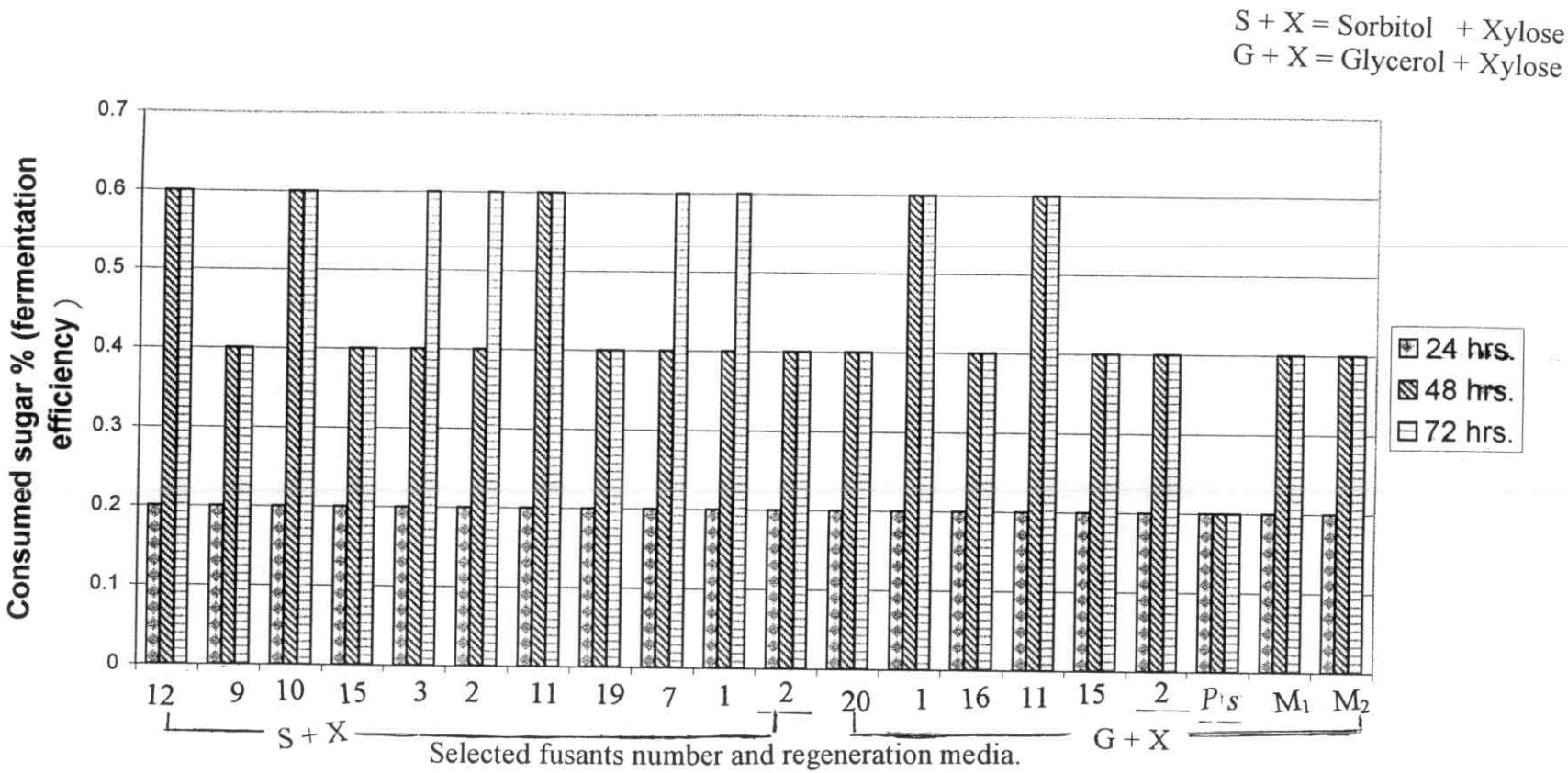

Fig (2): Diagram to compare xylose sugar fermentation efficiency of 17 fusants on two different regeneration media and their parental strains measured as sugar consumption % after different incubation times at 30 °C

Table (11): Ethanol production, sugar consumption and growth rate of each of 17 fusants and their parent yeast strains under aerobic conditions.

Regeneration media	Strains hybridized	Selected fusants number and their parents	Consumed sugar %	Growth rate	EoH* G/100ml
Sorbitol + Xylose		A	0.00	1.144	0.00
		B	0.00	0.166	0.00
		P. stipitis Y-7124	3.40	0.848	2.37
		M1 (73)	3.00	0.676	2.37
		M2 (171)	3.20	0.630	1.96
	A x M_1 *	12	3.60	0.527	2.78
	B x M_1	9	4.20	0.706	1.96
	A x M_2	10	3.60	0.888	2.55
	B x M_2	15	3.60	0.887	2.55
	B x M_1	3	3.80	0.660	2.05
	B x M_1	2	3.80	0.884	2.05
	B x M_2	11	3.80	0.781	2.59
	B x M_2	19	3.60	0.916	2.14
	B x M_1	7	4.40	0.758	2.14
	A x M_1	1	4.20	0.407	1.96
	A x M_2	2	4.20	0.835	2.09
Glycerol + Xylose	A x M_2 *	20	3.6	0.864	3.69
	A x M_1	1	3.8	1.060	2.78
	A x M_2	16	3.6	0.329	2.05
	A x M_2	11	3.6	0.100	2.05
	A x M_2	15	4.4	0.290	1.55
	A x M_1	2	4.4	0.759	1.87

* EoH = Ethanol (CH_3-CH_2-OH)

- A and B › Two *S. cervisiae* (GT160-34B and XJ 133-1B)
- M_1 : Induced mutant.
- M_2 : Spontaneous mutant

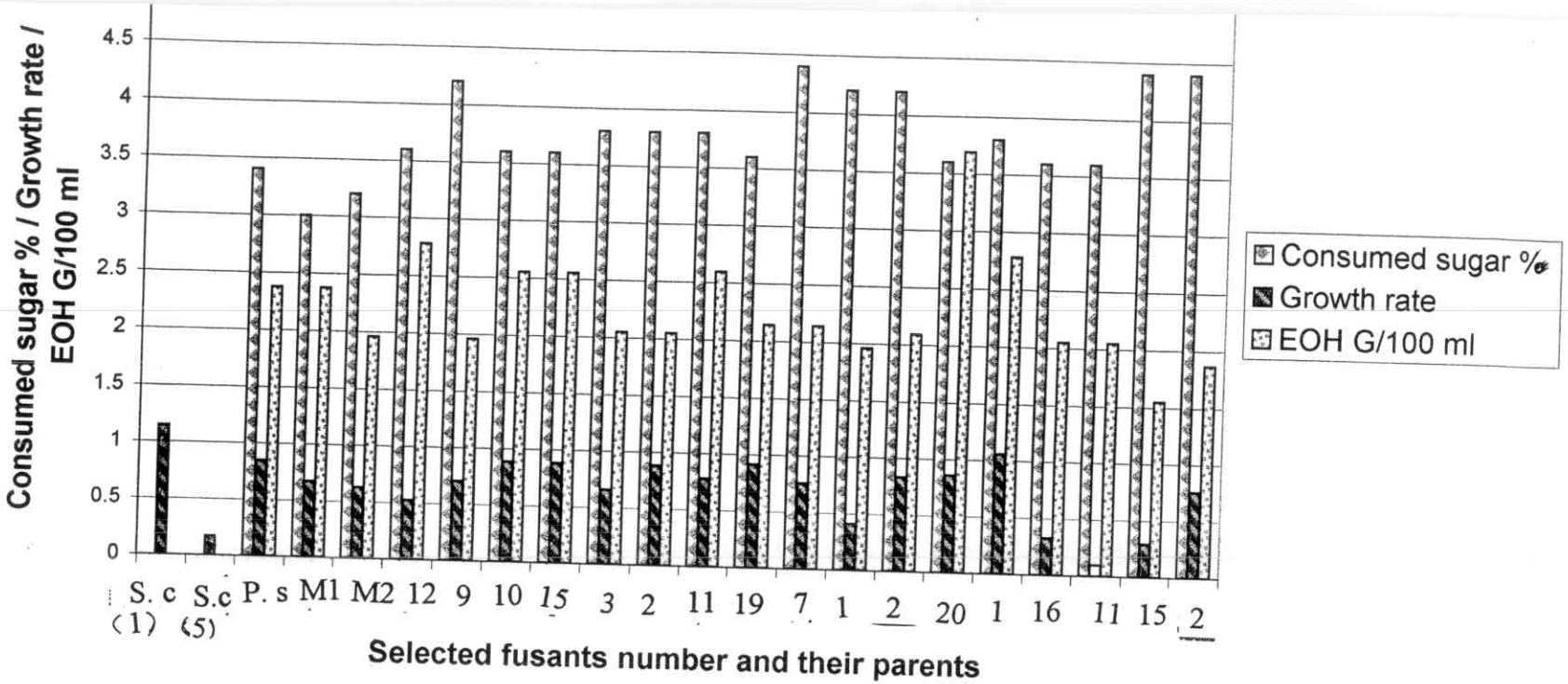

Fig (3): Diagram to compare ethanol production, sugar consumption and growth rate of each of 17 fusants and their parent yeast under aerobic conditions

glycerol + xylose) gave the highest ethanol (3.69 g/100 ml) production, followed by the hybrid fusant No. 12 (on regeneration media sorbitol + xylose) and No. 1 (on regeneration media glycerol + xylose) which yielded 2.78 g/100ml, then the fusant No. 11 (in regeneration media sorbitol + xylose) which produced 2.59 g/100 ml ethanol (Table, 11).

However, two other fusant Nos. 10 and 15 (on regeneration media sorbitol + xylose) produced the same amount (2.55 g/100 ml) of ethanol. All, the above gave fusants higher values of ethanol compared with their parental strains (Table, 11).

The rest of the fusants and M_2 produced amounts of ethanol less than of the parent *Pichia stipitis* and M_1 which produced the same amount (2.37 g/100 ml) of ethanol, (Table, 11).

Results (Table, 11) also reveal that no relationship was detected between ethanol production and growthrequirements of the tested fusants.

(11) 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 different carbon sources. The results are represented in Table (12). It can be seen that while original strains and

Table (12): Growth behavior of the selected fusants and their parents on different carbon source.

Carbohydrate or Sugar	Type	Parents					Code number of selected fusants															
		* A	* B	* PS	* M_1 73	* M_2 171	12	9	10	15	3	2	11	19	7	1	2	20	1	16	11	15	2
Glucose	Hexose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	Disaccharide	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	Polysacchride	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	Disacchride	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Raffinose	Trisacchride	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* A: *S. cerevisiae* GT 160-34B
* B: *S. cerevisiae* XJ 133-1 B
* P. s.: *Pichia stipitis* Y-7124
* M_1 and M_2: Two mutants derived from *Pichia stipitis* strain.

* Growth of strains scored as follows:
(+) growth
(-) no growth

their derived fusants grow normally on glucose as a carbon source, none of them gives any growth on raffinose or starch. On cellobiose all of them grow normally except both *Saccharomyces cerevisiae* strains (GT 160-34 B or XJ 133-1B) but on lactose sugar only two fusants besides M_2 give normal growth while the rest fusants and their parents showed no growth, (Table 12).