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Effect of Intrespecific Hybridization Between *Saccharomyces Cerevisiae* and *Saccharomyces Boulardii* on Utilization of Some Carbohydrates

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Abstract: Protoplast fusion technique has facilitated the creation of new strain of yeast with interesting biotechnological properties. In this work, this technique was used to prepare new yeast strain between *Saccharomyces cerevisiae* and *Saccharomyces boulardii*. The main purpose of this study was enhancing its carbon source utilization and fermentation ability. In protoplast preparation process, Novozym^{TM} 234 enzyme and sorbitol was used as lytic enzyme and osmotic stabilizer reagent, respectively. Protoplast regeneration efficiencies of *Saccharomyces cerevisiae* and *Saccharomyces boulardii* were 53.1% and 68.6%, respectively. Fusogenic reagent-polyethylene glycol 4000 (PEG 4000) was used to induce the fusion of protoplast. Galactose and lactose sugar assimilation and tolerance to copper sulfate, cycloheximide and benomycin were used as selectable markers for fusants selection. Sugar utilization of the fusants showed the mixed pattern between *Saccharomyces cerevisiae* and *Saccharomyces boulardii.* The hybrids, which appeared at a frequency of $1x10^{-1}$ (3.6%), presented characteristics of both parents, such as resistance to certain antifungal agents and the ability to grow with either galactose or lactose as the sole carbon source. Determination of the behaviour growth of selected fusants in comparison with parental strains in utilization of different carbon source gave a good behaviour growth strains as in fusant F_2 and F_4 in case of lactose and in F_3 in case of manitole. On other hand, the fermentation acttivity of fusants F_1,F_2,F_3,F_4 and F_5 was ranged from 325 to 350 min while in parental strains was rangead from zero to 325 min. Comparison with parental strains, products of fusion shoud be stable during storage and shoud not revert to their primary forms. These hybrids may have important industrial applications as good fermenting strains.

Key words: *Saccharomyces cerevisiae, Saccharomyces boulardii,* protoplast fusion,carbon sources, ferementation.

INTRODUUCTION

Saccharomyces cerevisiae has a great commerccal value in baking, brewing, distillery industries and as a source of enzymes, and many yeast species *Saccharomyces boulardii* showed botentiol benefits of the host as that used as biotherapeutic agents (Vargao, E. and A. Maraz, 2002). Thus, the use of new technologies was necessary, such as protoplast fusion and trasfomation. New genotypes were obtained by protoplast fusion, which showed recombinant features, while the transformed strains showed heterologus genes (Ros, A.H. and J.S. Harrison, 1993). *Saccharomyces cerevisiae* can exist as either a haploid cell of either Mat a or Mat α mating type, a diploid cell or a polyploid cell, but the transfer of genetic material is normally restricted to conjiugtion between haploids of opposide mating type, however, to isolate geneticalle stable yeast hyprids form strains that are unable to mate by using protoplast fusion (Curran, P.G and V.C. Buqeja, 1996)
Protoplast fusion was a method of choice since it has been widely used for genetic improvement of industrial yeasts. This technique has been used extensively for intraspecific and interspecific transfer of nuclear genes of fungi (Farahank, F.T.S., 1986).

Protoplast fusion is versatile technique for inducing genetic recombination in variety of prokaryotic and eukaryotic microorganisms, protoplasts are propared by treating microorganisms with a lytic enzyme such as lysozyme that remove the cell wall. As a result of this treatment, the cell content would be enclosed only by the cell membrane. The protoplast has to be preserved in a hybertonic medium for their osmotic stabilty and survival. Then, in the presence of fungenic agent such as polyethylene glycol (PEG). Protoplast is induced to fuse and form transcend hybrids. During this hybrid state, the genomes may-reassent and genetic recombination can occure (Martins, C.V.B., 2004; Sharaf, A.N., 2009). In (Selebane, E., 1993). found that fusant of *Candida shehatae* and *Pichia stipitis* showed only marginal in cell DNA content when compared with their parents.

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On other hand we observed the wild types strains and their fusants grow normally on all tested sugare excepted. The results obtained in numerous studies seem to confirm the significance of protoplast fusion as a method of improving industrial yeast strains, despite difficulties in introducing specific genes and low stability of recombinants (Dziuba, E. and J. Chmielewska, 2002). (Reed, G. and T.W. Nagodawithana, 1991) obtained an optimal qualtiy control policy for the overall specific growth rate of baker's yeast which maximizes the ferementation activity in the making of bread by direct searching based on the mathematical model.

In view of the importance and the need for development of genetically constructed yeast strain which is capable of growing on some important carbohydrates and with high efficiency in the process of fermentation, we have undertaken this study. The aimes of these ivestigation to study the impact of protoplast fusion between *Saccharomyces cerevisiae* and *Saccharomyces boulardii* on some activeties such as utilization of carbohydrate and ferementation ability.

MATERIAL AND METHODS

Yeast Strains:

Two yeast strains were used in this study, *Saccharomyces cerevisiae* and *Saccharomyces baularodii*. They were obtained from Microbial Genetic Department, NRC, Dokki, Giza, Egypt.

Media:

-Yeast extract peptone (YEP): Both yeast strains were grown and maintained on YEP medium which contained 1% yeast extract, 2% peptone and 2% agar (Cox, B.S. and E.A. Bevan, 1962). Stocks were maintained on this medium at 5^0C.

-Regeneration minimal medium (MMR): It consists of 3% agar, 0.7% YNB medium without amino acids, 0.8 M sorbitol, 0.1% glucose and 0.6% KCl (Sipiczi, M. and L. Fereczy, 1977).

-Regeneration minimal medium (MMR1) [Top layer medium]: It is similar to MMR but without KCl and agar was reduced to 1.8 %. It was used for stabilization of fusion products.

Buffers and Solution:

1. Lytic enzyme buffer: KCl (0.6 - 0.7 M) lytic enzyme (Novozyme from *Tricohoderma harazinum* TM 234).
2. Washing buffer (osmotic stabilization buffer) Sorbitol 0.8M Phosphate buffer 0.1M PH 7.5.
3. Fusion buffer : 35% PEG (MW. 4000 or 6000) $CaCl_2$ (filter sterilization) 10 mM.

Selection Markers:

In order to differentiate hybrids from parent cultures, two different set of parameters were taken into account and used as selectable markers to screen for the fusant strains.. The first one is the tolerance to anti-fungal in both parental cultures. Five antifungal agents were used according to (Canilo-Mufloz, 1996; Carrillo-Mufloz, A.J., 1999; Padmini, R., 2009; Vilchezet, Regis A., 2002) with final concentrations as follows: Copper sulfate (16 mg/ml), Cycloheximide (20 mg/ml), Benomycin (0.75 mg/ml), Miconazole (25 µg/ml) and Nystatin (20 µg/ml). the Kirby-Bauer disc diffusion method for antimicrobial susceptibility test was used (NCCLS., 1992). The other character was sugar assimilation to examine the basic sugar assimilation profiles of parent strains. Strains were examined for their capability of utilizing galactose, glucose and lactose as carbon sources. Carbohydrate utilization patterns were performed with each strain and each carbohydrate in YEP broth containing 1% (w/v) portions of the corresponding sugars after incubation period of 24 h at 37°C under aerobic conditions (Lin, C.C.L. and D.Y.C. Fung, 1985).

Protoplast Preparation and Fusion:

Saccharomyces cerevisiae and *Saccharomyces baularodii* cells from the exponential phase were subjected to protoplasts formation according to (Farahank, F.T.S., 1986). Portions (5 ml) of each culture were collected and centrifuged at 500 x g for 5 min. Cells were then washed three times with sterile distilled water and were suspended in the protoplasting solution containing 0.6 M KCl, 10 mM 2- mercaptoethanol, 50 mM phosphate buffer (pH 7.5), and 6mg of Novozym^{TM} 234(*Trichoderma harzianum*) per ml. The suspension was incubated at 37°C with occasional shaking, and it was checked periodically under the microscope for the formation of protoplasts. Practically all of the cells were converted into protoplasts within 1 h. These protoplasts were lyses when exposed to the hypotonic environment, as was confirmed by the microscopic examination. Protoplasts were collected by centrifugation at 6000 rpm for 10 min and washed at least three times with protoplasting buffer (0.1 M phosphate buffer [pH 7.5], 0.8 M sorbitol) and resuspended in 1 ml of same buffer.

The fusion of protoplasts of yeasts was induced using a solution of 35% polyethylene glycol (PEG 4000) in 10mM $CaCl_2$. This mixture was incubated in room temperature for 20 min, followed by centrifugation at 6000 rpm for 5 min and pellet was resuspended on 1 ml of protoplast buffer. Fused protoplasts in one ml of this buffer were transferred to 10 ml of regeneration medium taken in conical flask and incubated at 30°C for 72 h. The rate of protoplasts formation [%] and regeneration of the protoplasts [%] were determined. Screening of recombinant fused cultures was achieved by streaking the slant cultures on the YEP medium supplemented with markers (Kawa-Rygielska, J., 2004).

Determination of Behavior Growth of the Two Parents and Their Selected Fusants on Different Carbon Sources:

To determine the biological effect of mitochondrial activity by using different types of carbohydrates as carbon sources, yeast parent strains and their selected fusants were tested by growing in medium containing agar, yeast nitrogen base in proportion of 2.0, 0.67 g/ml, respectively. Extra carbon source; glucose, galactose, fructose, lactose, sucrose, mannitol, sorbitol at concentration of 2% and starch at concentration of 1.5% were added whenever indicated the tested plates were incubated at 30ºC for 3 days. The growth was scored as: (+++) good stimulation, (++) intermediate stimulation, (+) poor stimulation and (-) no growth (Nevien A., 2011).

Wheat Meal Fermentation:

The yeast strains were grown in liquid YEP medium. The cells were collected, washed three times by sterile distilled water and recollected by centrifugation. Four grams flour and 2.25 ml yeast suspension containing 0.5 g .wet yeast cells, from each strain, were mixed using stirring rod in 150 ml low-form beakers. The results was transferred to palm of hand and knead into coherent round meal ball. Each meal ball was replaced in beaker and covered with 80 ml water. The beakers were incubated at 30 ºC. The time for the meal ball to start disintegration from the time of meal ball immersion was recorded. Triplicates were used for each yeast strain (Ali, A.M.M., 1978).

RESULT AND DISCUSSION

Selection Markers:

In order to differentiate the hybrids from parent cultures, two different set of parameters were taken into account as shown in Table (1and 2) which represents a comparison of parental strains. Results in Table (1) revealed that *S. boulardii* was able to consume galactose and lactose and thus showed good growth while *S.cerevisiae* failed to show growth. In contrast, both strains were able to consume glucose. The second marker is the anti-fungal resistance character which was important selective and differentiating factor (Lin, C.C.L. and D.Y.C. Fung, 1985). The two parental strains were examined for their antifungal resistance on five different plates each of them was supplemented with one of the antifungal agent and results was summarized in (Table 2). It has been found that *S.cerevisiae* was resistance to copper sulfate and cycloheximide compared to *S. boulardii* which was sensitive and this was agreement with results obtained by (Tahoun, M.K., 2002).

Table 1: The ability to consume galactose, glucose and lactose by yeast parental strains.

Strains	Sugar assimilation		
	Galactose	Glucose	Lactose
S.cerevisiae	-	+	-
S. boulardii	+	+	+

(+) growth, (-) no growth

Results also revealed that *S.cerevisiae* was sensitive to benomycin while *S. boulardii* was resistance. On the other hand, both strains were resistance to miconazole and sensitive to nystatin. Based on results obtained, medium supplemented with these selectable markers was used for selection of interspecific hybrids. Each new fusants strains combined the antifungal and sugar assimilation properties of their corresponding parental strains.

Protoplast Fusion:

Interspecific transfer of nuclear genes of yeast has recently been achieved by fusion of protoplasts. Protoplast fusion has made a significant contribution to our understanding of the genetics and biochemistry of the nonconventional yeasts. It has facilitated the creation of novel strains of yeast that display enhanced biotechnological potential.

Table 2: The antifungal resistant pattern of yeast parental strains.

Strains	The antifungal agent				
	Benomycin	Copper sulfate	Cycloheximide	Miconazole	Nystatin
S.cerevisiae	-	+	+	+	-
S. boulardii	+	-	-	+	-

(+): resistant, (-) sensitive.

Table 3: Protoplast formation.

Parent strains	Number of cells/ml	Intact yeast cells*		Protoplast formation**	
		Number /ml	%	Number /ml	%
S. boulardii	3.4×10^7	3550	0.0104	0.75×10^7	2.22
S.cerevisiae	2.5×10^7	2332	0.0093	0.45×10^7	1.8

*growing on CM - **according to the microscopic examination of the protoplast suspensions.

Results in Table (3) showed that the rate of protoplasts formation of the yeast cells in the set conditions exceeded 22.2 % and 1.8 % in case of *saccharomyces boulardii* and *Saccharomyces cerevisiae*, respectively. It should be noticed that rate of protoplasts formation of both yeast strains was noted as soon as after 20 minutes of incubatioin in lytic solution (Sharaf, N.E., 2008). (Javaderkar, V., 1995) reported a characteristic susceptibility to Novozym^{TM} 234. After incubation of these strains with 4mg/cm^3 concentration of Novozym, they obtained 95% of protoplasts in 15 minutes. Obtaining the same results of protoplasts formation of *S. cerevisiae* in the conditions presented above required longer time of reactions.The regenration percentage of the induced protoplast was determined by mixing 0.1 ml of prptoplasts suspension with the toplayer medium (MMR1) and powred onto plates containing a thin bottom layer of regeneration medium (MMR) supplemented with 0.75 mg or 16mg/ml of Benomycin or Copper sulfate, respectively. Results in (Table 4) showed that after indubation at 37 ^{0}C for 4-7 days, the rate of protoplasts regeneration varied from 53.1% in case of *S.cerevisiae* to 68.6% in *S. boulardii*.

Table 4: Regneration colonies on regeneration medium.

Parent strains	Number of survivals colonies /ml *	No of regeneration colonies /ml **	% Regeneration
S. boulardii	3.4×10^7	23327×10^5	68.6
S.cerevisiae	2.5×10^7	13253×10^5	53.1

*growing on CM -**growing on regeneration medium.

Table (5) shows that after seven days of incubation at 37^{0}C, 0.1×10^{10} hybrid fusants colonies were found per mixed protoplasts, (*S.cerevisiae & S. boulardii*). The fusion between (S.b:: S.c) had regeneration (Protoplast fusion) percentage (3.6%) when grown on MMR medium supplemented with 0.75 mg and 16 mg/ml of Benomycin and Copper sulfate respectively, incubation up to 1 h at 37^{0}C. 0.6 M Kcl was used as osmotic stabilizer. In these investigation the fusant percentage between *Saccharomyces cerevisiae* and *Saccharomyces boulardii* showed that a higher frequency of regeneration percentage (3.60%). These results are in a good agreement with the results of (Selebane, E., 1993). He found that fusant of *Candida shehatae* and *Pichia stipitis* showed only marginal in cell DNA content when compared with their parents.

Table 5: Formation of hybrid fusant colonies.

Yeast hybrid	Number of hybrid fusants colonies/ml	% Protoplast fusion
S. boulardii × *S.cerevisiae*	0.1×10^{10}	3.60

A major limitation to the results that could be achieved using traditional methods in the study of microbial genetic has been the barriers to fusion that exist both between isolates belonging to different species and also often between different isolates to the same species. Thus the scientific warker may have obtaned to varieties of fungi, each with their own desirable characteristics, out have been unablle to combine the characterics in a single organism (Elliot, B. Gingold, 2008).

In protoplast fusion, it is important that the cell wall of plant and microoganisms is degraded, so various enzymes used for this process such as cellulase and pectinase or macerozyme acting on plant cell wall are degraded by the action of lysozyme. Fungal wall degraded by Novozym^{TM} 234 which iculudes glucanose and chitniase. These results are in a good agreement with the results of (Narayanswamy, S., 1994). The use of PEG as a spheroplast fusion agent has a number of limitations, which have been discussed in detail by (Nivien, A. Abosereh, 2007).

Our results as to obtaind fusants of *S. boulardii* and *S. cerevisiae,* the used of natural marker, such as the growth of strains in medium with antifungal and the growth of strains in medium with different carbon source. Protoplast is induced to fuse and from transint hybrids. During this hybrid state, the genomes may-re-assort the genetic rcomination can occure (Martins, 2004).

Determination of behavior growth of both parents and their selected fusants on different carbon sources: To determine the biological effect of mitochondrial activity by using different types of carbohydrates as carbon sources, yeast parent strains and their selected fusants were tested by growing in medium containing agar, yeast nitrogen base in proportion of 2.0, 0.67 g/ml, respectively and results was summarized in Table (6). Extra carbon source; glucose, galactose, fructose, lactose, sucrose, mannitol, sorbitol were added at concentration of 2%, and starch at concentration of 1.5% whenever indicated the tested plates were incubated at 30ºC for 3 days. The growth was scored as: (+++) good stimulation, (++) intermediate stimulation, (+) poor stimulation and (-) no growth (Nevien A., 2011).

Table 6: Behavior Growth of Both Parents and Their Selected Fusants on Different Carbon Sources.

Carbon source		Strains						
Suger	Type	S.c.*	S.b.*	F1**	F2**	F3**	F4**	F5**
Glucose	Hexose	+++	+++	+++	+++	++	++	+++
Galactose		++	-	+	++	++	+	+
Fructose		++	+++	++	+++	++	++	++
Lactose	Disaccharide	++	-	+	++	+	++	+
Sucrose		++	+++	+++	++	+++	++	++
Mannitol	Sugeralcohol	++	-	+	+	++	+	+
Sorbitol		-	-	-	-	-	-	-
Starch	Polysaccharide	++	-	-	-	-	-	-

* Parental strains (S. *cerevisiae* and *S. boulardii*) - ** selected fusants

Growth scored as follows; (+++) good, (++) intermediate, (+) poor and (-) no growth.

ResuIts showed that while parental strains and their selected fusants can grow normally on glucose, fructose and sucrose with different abilities as a carbon source,they cannot give any growth on sorbitol. In contrast, *S.cerevisiae* grow normally on galactose, lactose, mannitol and starch while *S. boulardii* cannot. All selected fusants can grow normally on all carbon sources except sorbitol and starch. Growth behavuior of parental strains and their fusants derived from it was tested on different carbon source to elucidate the sugar utilzation control by mitochndrial genome and whether different fusants differ in genetic lesion on their mitochondrial DNA. (Reed, G. and T.W. Nagodawithana, 1991) repoted that any number of yeast species including *S. cerevisiae* can be grown on hexoses but canot be grown on wood sugars.

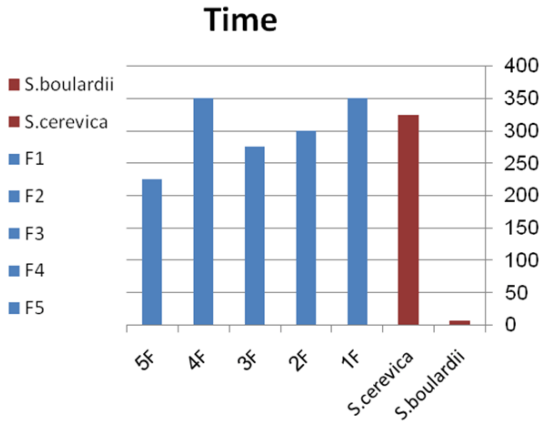

Fig. 1: Illustrate fermentation activity of parental strains and their fusants.

Wheat Meal Fermentation:

The fermentation activity in parental strains of Sacchromyces [S. baularodi & S. cerevisiae] and theer fusant were shown in diagram 1. Data concerning S. baularodi & S. cerevisiae (w.t strains) ranged from (0 - 325 min) and F_1 , F_2 , F_3 , F_4 , F_5 ranged from (350-225 min). The results obtained had attributed to maltigenic nature of growth rate. Growth rate is a complex character controlled with various genes in one or more pathways. It is the end product of their expressions.

These gene are affected with a large number of regulatory genes and probably different operators. This conclusion may be accaunted for, if one cosiders the work of (Imbault, P., 2007; Nivien A. Abosereh, 1992).

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