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EFFECT OF GROWTH PHASES OF *SACCHAROMYCES CERVISIAE* ON THE EFFICIENCY OF EXPRESSION FOREIGN GENE(S)

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ABSTRACT

The direct transfer of genetics materials into yeast cells *Saccharomyces cereviciae* as well as using of amylase, cellulase and Osmo gene(s) as gene(s) marker, for measuring the efficiency of transformations during the growth curve of the cells was investigated. A framework is developed for an adequate comparison of data obtained during different phases of growth. Results indicated that the exponential phase is the best phase of introduce DNA across the cell membrane. Whereas transformation percentage reached its peak at the end of exponential phase.

Results also revealed that the transformation rates of cellulase expression gene(s) were lower than the transformation rates of α-amylase expression gene(s). These results might be due to the different regulatory genes with different locations which are proposed to explain the ability of producing the enzymes by the same yeast transformed isolates in a different rates.

On the contrary, the high concentrations of NaCl decreased gradually the transformation percentage during the exponential period. On the other hand, no transformants can be obtained in the high concentrations of NaCl on the early exponential phase or stationary phase. This may due to a very low survival rates of cells.

The greatest transformation rate which were recorded at the exponential phase (late exponential phase) in particular, may be attributed to the thickness of cell wall of yeast wall than other phases. The results concerning efficiency of transformants to express foreign genes did not only indicate possible transfer the foreign genes but are also instrument in developing a goal directed optimization strategy of the growth rate in the application of direct transformation technique.

INTRODUCTION

Saccharomyces cervisiae has become a reference mode for the understanding of eukaryotic biology at the cellular and molecular level. Transformation of yeast cells is currently performed using chemical fusants such as polyethylene glycol (PEG) and alkylation on intact cells (Ito *et al*., 1983).

The renewed world wide interest is biotechnology and subsequent necessity for efficient production of food is associated with increasing demand for food due to the increasing of the classical food sources.

The production of microorganisms in a large scale for nutrition, depends upon the strain used and raw materials. Different raw materials

such as molasses, whey, wood hydrolyzate, cellulose, amylase and hydrocarbons are usually for propagation and production of single cell protein. Reed and Peppler (1973) mentioned that hydrolysis of the wood deciduous trees and stalks of annual plants (rice halls, tobacco stalks, bagasse pith, saw dust) yield about 25% hexose and 75% pentose.

Microbial enzymes are widely used in food processing. Many new enzymes and enzyme processes acting and nearly all types of organic products (starch, sugars, proteins, fat fibers and flavour compounds) have become into the industry during 1980's. Their application have a major impact on enzyme technology in general. The particular roles of immobilized enzymes and genetic engineering in food enzymology are very important in biotechnology.

Park and Mattoo (1988) produced a transformed hybrid strains of S. cerevisiae capable of simultaneous secretion of glycoamylase and α-amylase. The strains were transformed by plasmid (RuS_{12}) containing mouse salivary α-amylase cDNA in an expression vector containing yeast alcohol dehydrogenase promoter and a segment of yeast 2 μm plasmid.

Objectives of this work were study the recovery curves of the cell populations (growth curves) of yeast *Saccharomyces cerviseae* as well as using of amylase, cellulase and Osmo gene(s) as genes marker, for measuring the efficiency of transformations.

MATERIAL AND METHODS

A. Strains:

1. *Saccharomyces cervisiae* (5) (αmet-6, gal-2) was obtained from the culture collection of Microbial Genetics Depart., N.R.C.. The double point mutations in the strain did not revert at a detectable frequency.

2. a. Donor DNA of amylase gene(s) was isolated and purified from *Aspergillus niger* S(104). This strain was obtained from Depart. of Agric. Microbiology, Fac. of Agric., Ain Shams University.

b. Green algae isolated from marine was used as DNA donor of Osmo gene(s).

c. Donor DNA of cellulase gene(s) was isolated and purified from *Asp. niger*. This strain was obtained from Dept. of Resource Center, Ain Shams University, Faculty of Agric..

DNA was isolated and purified by the method of Bendich and Boltene (1967).

B. Media and cell culture:

Med. (1) was used for propagation and maintenance of yeast strain. It consist of yeast extract peptone (YEP) 2% glucose, 2% peptone, 0.3% yeast extract and 2% agar for plates was used as a complete medium for yeast cells (Barnett *et al*., 1984).

Med. (2) α-amylase medium: in this medium, starch was added instead of glucose. This medium was used as selected medium for amylase transformants.

Med. (3) was used to select cellulase transformants producer. It

consist of (g/l) cellulose powder, 10, $(NH_4)_2 SO_4$, 5; KH_2PO_4, 3; $MgSO_4$, 0.5; Yeast extract, 0.05; agar 20 and tap water up to 1 liter, pH 5.5-6.0 (cellulose was used instead of glucose).

Med. (4) Salt medium: glucose peptone yeast extract medium was supplemented with different concentrations of NaCl; 3%, 6%, 8% and 10%. NaCl free medium was used to determine the components cells.

C. Yeast cultivation and growth curve:

Conical flasks (250 ml) containing 100ml of (YEP) medium were inoculated with 5ml of yeast suspension (2.4×10^6 cells/ml), on a reciprocal shaker (150 strike.min.) at 30°C for 55 hours. Therefore, samples were taken periodically from the cultures under aseptic conditions at 0,4,8,21,28,44 and 55 hours to determine the optical density spectrophotometrically at 540 nm. The growth density against the time was plotted on semi-log paper, to draw growth curve of the organism. This curve was used to calculate growth parameters as follow:

1. Specific growth rate (μ) Standburg and Whitaker, 1984): This parameter was calculated from the exponential phase of growth using the following equation:

$$\mu = (\log A_1 - \text{Log } A_o)\ 2.303\ (t1-t_o)$$

where:

μ: specific growth rate A_1: growth density at t_1 time

A_o: growth density at t_o time

2. Doubting time (t_d):

The time required for doubling the growth density was calculated from the following equation:

$$t_d = \text{Ln2} - \mu - 1$$

where:

t_d: doubling time μ: specific growth rate

3. Multiplication rate (MR):

The number of growth multiplication per unit of time was calculated from the following equation:

$$MR = \mu(\text{Ln2})^{-1}$$

where:

MR: multiplication rate μ: specific growth rate

4. Number of generations (N): this parameter was calculated from the following equation:

$$N = (\text{Log } A_n - \text{Log } A_o)\ (\text{Log } 2)^{-1}$$

where

N: Number of generation

A_o: growth density at the beginning of experimental phase

A_1: growth density at the end of experimental phase

D. Transformation protocols:

Yeast strains *Saccharomyces cervisiae* was transformed by the Hideaki *et al*. (1985). Yeast strain was grown in (YEP) medium at 30°C for 55 hours incubation period to detect different growth phases (growth curve). After every phase, the cells were harvested and washed in distilled water at room temperature, then they were responded in distilled water with 0.5ml of 0.3% lithium acetate and frozen at 7°C for 30 minutes (freezing enhances the transformation efficiency). After thawing, the cells were mixed with 1 µg DNA of the donor strains.

E. Selection of yeast transformants:

For the selection of trasnsformant yeast cells should be capable to produce:

1. **The enzyme (α-amylase)**, 0.1 ml of the cell suspension was spread on the selective medium (med. 2) in Petri dishes. The plates were incubated at (30-32°C) for (3-4) days and the appearing colonies were tested by the iodine solution. The yeast appearing clear zone were counted as yeast transformants.

2. **Transformants** which can assimilate cellulose were isolated. samples of treated cells were plated on medium supplemented with 1% pure cellulose. Samples with suitable dilution of the components were also plated on (YEP) medium free from cellulose. Growth examinations of the original untransformed yeast strain was done on plates supplemented with pure cellulose 1%.

3. **Salt tolerant transformants** were selected on (YEP) medium supplemented with different concentrations of NaCl, 0%, 2%, 4%, 6%, 8% and 10% (osmotic medium). The plates were incubated for 7 days at (30-32°C) in order to determine the lethal level and tolerance ranges.

The growing cells in the final cell preparations after transformation steps were counted by spreading dilutions of cell suspension on (YEP) agar medium (med 1).

RESULTS AND DISCUSSION

1. Growth curve of *S. cerevisiae* (5):

Fig. (1) and table (1) show the growth curve and growth parameters of *S. cerevisiae* during 55 hours time course. Results clearly showed that the tested yeast strains grew exponentially during the first 21 hours of incubation followed by a stationary phase up to the end of incubation. The exponential growth exhibited 3 stages being: 1. the early exponential phase; 2. the medium exponential phase and 3. the late exponential phase. The specific growth rates of these stages were 0.3346 h^{-1}, 0.2497 h^{-1} and 0.119 h^{-1} respectively. It means that the growth rate of the yeast was decreased

gradually during the exponential phase. On the other hand, increase of doubling time led to decreasing the specific growth rate, while the other parameters, multiplication rate and number of generations gave higher values at early stages of exponential growth as compared with that observed at the latter stages. These results are in line with those observed by Rose and Harrison (1970) and Reed and Peppler (1973).

Due to the presence of different stages of growth, growth parameters were highly affected by this early phase and consequently the biological activities and the thickness of cell wall were also affected. So, it is valuable to study the effect of different growth stages on the producing transformants.

2. Effect of different growth phases on improving transformation:

In contrast to classical transformation methods which give poor and unreproducible results, the optimizing growth phases improved the efficiency of introducing DNA to yeast cells.

Results in table (2) revealed that growth stages of yeast have pronounced influence on transformation rate. Transformation rates in the case of expression (α amylase gene(s)) were ranged from 5.25 (25.722/4.898) to 10.09 (4.398/1.130) fold higher in the exponential phase in comparison with stationary phase. On the other hand, transformation rate was increased gradually with the elapsing of time during the exponential period, where transformation ratio was 2.25 fold at the end of this phase as compared with transformation at the beginning of this phase.

Similar results were obtained in the case of the expression of cellulase gene(s).

Table (1): Growth kinetics of *S. cerevisiae* (5) during 55 hours incubation period at 30°C on (YEP) medium:

Incubation period (h)	Growth phases	Specific growth rate (h^{-1})	Doubling time (h)	Multiplication rate (h^{-1})	Number of generation
Up to 4	Early exponential phase	0.3346	2.07	0.48	1.92
Up to 8	Medium exponential phase	0.2497	2.78	0.36	1.44
Up to 21	Late exponential phase	0.1119	6.19	0.16	1.28
Up to 55	Stationary phase	-	-	-	-

Table (2): Effect of different growth phases on the efficiency of transformation parameters of yeast to accept (α-amylase gene(s)):

Time (h)	Growth phases	Compet-ents No.	Transformants No.	Transform-ation %	Transform-ation rates as compared to zero time
0	Start	930	1	0.108	-
4	Early exp. Phase	325	4	1.231	11.398
8	Med.Exp. phase	290	7	2.414	22.352
21	Late exp. Phase	180	5	2.778	25.722
29	Stationary phase	567	3	0.529	4.898

43	Stationary phase	456	1	0.219	2.028
52	Stationary phase	817	1	0.122	1.130
55	Stationary phase	789	1	0.127	1.176

Table (3): Effect of different growth phases on the efficiency of transformation parameters of yeast to assimilate cellulose:

Time (h)	Growth phases	Compete-nts No.	Transform-ants No.	Transform-ation %	Transformation rates as compared to zero time
0	Start	980	3	0.306	-
4	Early exp. Phase	453	5	1.104	3.608
8	Med.Exp. Phase	320	6	1.875	6.127
21	Late exp. Phase	210	4	1.905	6.225
29	Stationary phase	390	3	0.769	2.513
43	Stationary phase	420	2	0.476	1.555
52	Stationary phase	480	2	0.416	1.359
55	Stationary phase	392	1	0.255	0.833

Table (3) shows that the optimizing growth phases enhanced the transformation process. But this natural transformation system gave slightly different percentages of transformation in compared of expression (α-amylase) gene(s). However, the transformation percentages were relatively higher in the exponential phase (1.04 to 1.905) in comparison with stationary phase. Transformation rates ranged from 2.4 (6.225/2.513) to 4.33 (3.608/0.833) higher in the exponential phase in comparison with stationary phase. This results shows that the transformation rates of cellulase expression gene(s) were lower than the transformation rates of α-amylase expression gene(s). These results might be due to the different genes which are proposed to explain the ability of producing the enzymes by the same yeast transformed isolates.

These results are never can be due to the same structure gene(s) introduced through transformation. More than a gene in a common pathway had been introduced through transformation which opened the blocked pathway in yeast enable it to produce (α-amylase) in a large amount than the cellulase enzyme. Because, some species of yeast can be able to produce α-amylase in a different concentration, while the yeast (*Saccharomyces cerevisae*) can not be able to produce cellulase to metabolise cellulose as a sole source of carbon. Moreover, the different rates of transformation of each enzymes might be due to the different locations of those gene(s) affecting its action.

Second, the introduction of regulatory genes through transformation having different locations in the two different experiments.

On the contrary to the above results, the high concentrations of NaCl decreased the transformation percentage after treatment with the monovalent cation (Li^+).

Data in table (4) revealed that the transformation percentage was increased gradually with the elapsing of time during the exponential period. On the other hand, no transformants can be obtained in the high

concentrations of NaCl on the early exponential phase or stationary phase. The transformation decreased with the high concentration of NaCl. This is true up to a concentration value at which the number of transformants drops

drastically, the stronger the concentration of NaCl the lower the transformation percentage. This may also due to a very low survival rates of cells.

Data also revealed that the exponential phase is the best phase to introduce DNA across the cell membrane. Whereas transformation percentage reached its peak 2.778%, 1.905%, 1.66%, 1.7%, 0.53% and 0.86% in the expression of α-amylase gene(s), cellulase gene(s) and Osmo genes at NaCl 4%, 6%, 8% and 10% respectively at the end of exponential phase (late exponential phase). The greatest transformation rate which recorded at the exponential phase in general and in the end of the exponential phase (late exponential phase) in particular, may be attributed to the thickness of cell wall of yeast cell than other phases. Rose and Harrison (1970) and Ramadan *et al.* (1983) reported that the thickness of cell wall was increasing gradually in the stationary phase of growth. While the lower number of transformants and transformation percentages were observed at zero time and in the stationary phase. The frequency of transformation was dependent on growth phase of yeast cells. Although regulation of the time for the growth rate of the cells was required to give large numbers of transformants and transformation percentages.

The foreign DNA uptake may be enhanced by Li^+ as it was acting to:

i. protect the exogenous DNA against nuclease degradation; ii. increase the permeability of plasma membrane (Hahermann and Stacey, 1990). Li^+ cation enhanced the absorption of the transferring DNA to plasma membrane by interacting with the negative changes of both the DNA molecules and the membrane surface.

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تأثير مراحل نمو خلايا خميرة السكارواميسيس سرفيسيا على كفاءة تعبير الجينات الغريبة

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تم دراسة النقل المباشر للمادة الوراثية على خلايا الخميرة بإستخدام جينات الأمبليز والسليوليز والملوحة كجينات دالة لقياس كفاءة التحول الوراثى خلال منحنى النمو للخلايا، وقد كان الإطار الأساسى للدراسة هو تحديد مقارنة ملائمة للنتائج المتحصل عليها خلال المراحل المختلفة للنمو. وقد دلت النتائج على أن المرحلة الأخيرة من الطور الأس (اللوغارتيمى) هو أحسن المراحل لإدخال قطعة من جزى الـDNA من خلال أغشية الخلية وقد وصلت نسبة المحولات الوراثية أعلى قيمة لها فى نهاية هذا الطور.

وأوضحت النتائج أيضا أن نسبة المحولات الوراثية فى حالة تغيير الجينات السليوليز كانت أقل من نسبة المحولات الوراثية لتعبير جينات الأميليز. وقد تعزى هذه النتيجة إلى إختلاف الجينات المنظمة لإنتاج كلا الإنزيمين وإحتلالهما لمواقع مختلفة فى نفس خلية الخميرة وعكس ذلك فإن التركيزات المرتفعة من الصوديوم كلوريد قد خفضت تدريجيا نسبة المحولات الوراثية خلال الطور الآس ومن الناحية الأخرى فلم يتم الحصول على أى نسبة من المحولات الوراثية فى المراحل الأولى للطور الآس أو الطور التمهيدى وهذا قد يرجع إلى إنخفاض نسبة الإعاشة. وقد تم تسجيل أكبر نسبة للمحولات الوراثية عامة خلال الطور الآس (خاصة خلال الطور الآس المتأخر) وهذا يعزى إلى سمك الجار الخلوى لخلايا الخميرة الذى يختلف عن أى طور آخر.

ومن هنا يتضح من خلال النتائج أن ليس من الممكن زيادة كفاءة الخلايا لزيادة التعبير عن الجينات الغريبة فقط لكن هناك أيضا وسيلة مباشرة لتطبيق وتنظيم إستخدام إستراتيجية معدل النمو فى تكنيك إستخدام النقل المباشر للمادة الوراثية.

Table (4): Effect of different growth phases on the efficiency of transformation parameters of yeast to express Osmo gene(s):

Time (h)	4% NaCl					6% NaCl			
	Growth rate	Competents No.	Transform. No.	Transform. %	Transf. rates as compared to 0 time	Compet-ents No.	Transform. No.	Transform. %	Transf. rates as compared to 0 time
0	Start	394	2	0.50	-	333	2	0.60	-
4	Early exp. phase	466	4	0.86	1.720	415	3	0.72	1.200
8	Med. exp. phase	297	3	1.01	1.174	757	10	1.32	1.833
21	Late exp. phase	282	3	1.06	1.049	751	13	1.73	1.311
29	Stat. phase	757	3	0.396	0.374	328	2	0.610	3.526
43	Stat. phase	789	2	0.202	0.510	382	2	0.524	0.8590
52	Stat. phase	521	1	0.192	0.950	356	2	0.560	1.068
55	Stat. phase	322	0	0	-	402	1	0.250	0.893

Table (4):

Time (h)	84% NaCl					10% NaCl			
	Growth rate	Compet-ents No.	Transform. No.	Transform. %	Transf. rates as compared to 0 time	Compet-ents No.	Transform No.	Transform. %	Transf. rates as compared to 0 time
0	Start	394	0	0	-	451	0	0	-
4	Early exp. phase	405	0	0	-	369	0	0	-
8	Med. exp. phase	387	2	0.51	-	246	2	0.81	-
21	Late exp. phase	375	2	0.53	1.039	466	4	0.86	1.062
29	Stat. phase	369	0	0	-	303	0	0	-
43	Stat. phase	322	0	0	-	284	0	0	-
52	Stat. phase	422	0	0	-	295	0	0	-
55	Stat. phase	389	0	0	-	264	0	0	-