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THE EFFICIENCY OF TRANSFORMED *SACCHAROMYCES CEREVISIAE* STRAINS TO PRODUCE ß-GALACTOSIDASE FROM CHEESE WHEY

[41]

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ABSTRACT

Four different strains of yeast; two conventional of *S.cerevisiae* and the same had been transformed by introduction of shuttle vectors YCplac111 and YCplac33 coding for ampicillin (Amp) and ß-galactosidase(*Lac-z*). The YCplac111 plasmid containing LEU2 gene, while YCplac33 plasmid containing URA3 gene. All strain types were propagated in media based on whey or on lactose; under constant conditions. Three parameters were used for detection of fermentation ability of transformed and non-transformed yeast. They are cell number or density of cells /ml ,change in *p*H and consumption of lactose as a carbon source. When the yeast was propagated on whey based medium, the maximum of cell number reached $5X10^9$ for constructed yeast transformed with the plasmid YCplac111 and $12X10^8$ for constructed yeast transformed with the plasmid YCplac33. Respective cell number; $2X10^9$ and $5X10^8$ were obtained on lactose-based media. The TGT111 constructed strain had higher cell number than original strain (without plasmid), they were increased by $4.9X10^{10}$ % on whey. The TGM33 constructed strain, had higher cell number than original strain, they were increased by $2X10^9$ % on whey. On the other hand, TGT111 constructed strain, was the highest in cell number on whey than lactose medium, they were increased by 250%. While, the TGM33 constructed strain was the highest cell number on whey than lactose medium, they were increased by 240%. No change of initial *p*H in the two non-transformed yeast strains while considerable change in initial *p*H was noticed in transformed strains. The *p*H were 4.4 and 4.6 in whey for TGM33 and TGT111,respectively. But its were 4.6 and 4.5 in a synthetic medium ,respectively. Cellular ß-galactosidase active averaged 6.41 and 6.25 Miller units/mg of the yeast cells of strains TGT111 and TGM33, respectively, propagation in whey-based medium. Respective mean ß-galactosidase activities of 3.64 and 2.38 Miller units/mg of yeast were obtained on lactose-based medium. 73.8 and 70.2% of the lactose present initially in the whey and in the lactose media, respectively were consumed as a sole carbon source by the yeast. In conclusion, whey a cheap byproduct of the cheese industry proved to be a valuable substrate for constructed yeasts. The yeast strain transformed with the plasmids YCplac111 had excellent properties.

Key words: *Saccharomyces cerevisiae*, Lac-Z gene, ß-galactosidase, Cheese whey

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INTRODUCTION

In the production of cheese, milk is inoculated with lactic acid bacteria that are responsible for acid formation prior to enzymatic precipitation of the curd ,and subsequently for flavor formation during the ripening period **(Kosikowsky, 1977)**. The typical flavor is produced in some cheeses after inoculation with mold spores, such as *Penicillium roquefortii* **(Bottazzi, 1983)**. In other mold-ripened cheeses a considerable population of yeasts develops; in particular *Kluyveromyces lactis* , *Saccharomyces cerevisiae,* and *Debaromyces hansensi* **(Becerra *et al* 1997)**. In a Roquefort cheese, the yeast population may reach 10^9 cells /g after 30 days of ripening **(Gripon, 1987)**. As a secondary flora, yeasts play a part in the development of the flavor of such cheeses.

The lactase or ß-galactosidase (ß-Dgalactoside galactohydrolase, EC 3.2.1.23) from *Kluyveromyces lactis* is an enzyme which has attracted our attention since it represents an essential material to convert the waste product cheese whey into a substrate valuable for biotechnology industries **(González Siso, 1996)**.

Almost all yeast strains including *S.cerevisiae* are constitutive to metabolize glucose; fructose and mannose in descending order **(Stewart *et al* 1979)** .In the case of *S. cerevisiae*, a number of transports by systems for sugars have been described: a constitutive system common for glucose; fructose and mannose; inducible system for galactose, α-methyl-D-glucoside, maltose and maltotriose, respectively. **(Stewart *et al* 1979)**. Sucrose is hydrolyzed outside the cell membrane by the cell membrane by the extracellular enzyme invertase (ß-O-fructofuranoside fructohydrolase. E.C. 3.2.1.26) to fructose and glucose. Lactose; the milk sugar is a disaccharide, consisting of galactose and glucose units. Whey, the by-product from the cheese industry is quite a rich source of lactose. There are two species of yeasts; kluyveromyces *fragilis* and kluyveromyces *lactis,* that can utilize lactose as the energy source; with an uptake mechanism quite similar to that of maltose **(Stewart *et al* 1979)**. The lactose is transported across the cell membrane by means of a lactose permease system. Once inside the cell, lactose is hydrolyzed by ß-galactosidase (ß-galactoside galactohydrolase, E.C.3.2.1.23) into constituent monosaccharides galactose and glucose. Both monosugars enter next the common glycolytic pathway of the cell **(Stewart *et al* 1979)**. Baker's yeast, like most other galactose-utilizing microorganisms require three important enzyme systems to convert galactose into glucose-1-phosphate.Those enzymes referred to as Leloir pathway enzymes include galactokinase; galactose-1-phosphate uridyltransferase and uridine diphosphogalactose-4-epimerase **(Rao *et al* 1988)**. The respective encoding genes for the three enzymes are GAL 1, GAL 7, and GAL 10. The plasmid transformed into the commercial baker's yeast strain had been reported to be of poor efficiency **(Van *et al* 1998)**. Instability problem could be overcome by the development of a new vector containing the LEU 2 gene to complement the yeast LEU 2 mutation **(Adam *et al* 1999)**. The vectors had been reported to integrate at the site of homology resulting in the direct duplication of the homologous sequence; which stabilizes the transformed gene. Traditionally, the manufacturing of Baker's yeast was

dependent completely on molasses as the substrate. Due to constant increase in the prices of molasses concomitant with a decline in the quality, there is a world-wide trend to grow baker's yeast on whey.

Such strategy requires the introduction of new strains of baker's yeast capable to utilize lactose efficiency and with good gas characteristics (**Reed and Nagodawithana, 1991**). The uptake and utilization of lactose are under the control of the polymeric gene system as those of maltose. The lactose and maltose are transported first into the cell before being hydrolyzed within the cell matrix; unlike sucrose and melibiose, which are hydrolyzed extracellularly (**Stewart, 1981**).The structural gene for ß-galactosidase (LAC 4) (**Sheetz and Dickson, 1981**), and for lactose permease (LAC 12) (**Sreekrishna and Dickson, 1985**) had been identified.

The mechanism of the regulatory system for the induction of ß-galactosidase enzyme in *kluyveromyces lactis* had been elucidated and the induction of the LAC 4 gene by gene lactose is regulated at the transcriptional level (**Lacy and Dickson, 1981**).

In Egypt new constructive yeast strains (TGM33 and TGM111) were produced which utilize lactose as the source of energy (**Sharaf El-Deen and Khalil, 2003**). The whey in Egypt representing a big problem for environmental pollution . The dairy industries elimination it in waste water .So the aim of the present work is to study the efficiency of constitutive yeast strains for the propagation in whey-based media .It is essential material to convert the waste product whey into a substrate valuable for biotechnology industries.

MATERIAL AND METHODS

Materials

I. Strains

The yeast strains *(Saccharomyces cerevisiae)* used in the present study are listed in Table (1).Detailed description of the processes of their construction were described earlier (**Sharaf El-Deen and Khalil, 2003**).

Table 1. Constructive yeast strains

Strains	Description
GM3	a-gal_{10},trp_1,ura_4,ade_7,leu_2,lys_2,lys_1,$ilvs_1$,aro_1D,Can_1,Suc,mal, Cupr.
GT160-34B	a-ade_1,leu_2,his_6,met_{14},lys_9
TGM33	GM3 Transformants carried YCplac33
TGT111	GT160-34B Transformants carried YCplac111

II. Media

Four different media were used; Yeast peptone dextrose medium, for growth; Edinburgh minimal medium, for required testing; and Yeast peptone lactose (**Sherman, 1991**) and Sweet Whey obtained from a cheese manufacturer were prepared as reported **Foda *et al* (1988)**, for propagation. Media are listed in Table (2).

Table 2. Presents the composition of the media used for the growth and the propagation of the yeast

Media	Ingredients	*p*H	Lactose%
YPD	2% peptone, 1% yeast extract, 2% glucose	5.5	0.0
YPL	2% peptone, 1% yeast extract, 2% lactose	5.5	2.0
EMM2	0.3% potassium hydrogen phthalate, 0.22%$NaHPO_4$, 0.5%NH_4Cl, 2% (w/v) glucose, 2 ml salts solution, 0.1ml vitamins solution, 0.01 ml minerals solution, with 2% agar for solid phase	5.5	0.0
Cheese Whey	Powder from Misr Co. of Dairy	4.8	4.6

III. Reagents

- Ortho-nitrophenyl ß-D-galactopyranoside crystals (ONPG); $C_{12}H_{15}NO_8$; Mf: 301.3 (SIGMA). Phosphate buffer (Z-buffer), *p*H 6.5 ;0.06M Na_2HPO_4, 0.04M NaH_2PO_4, 0.01M KCl, 0.001 M $MgSO_4$, add 0.03M 2-mercaptoethanol to Z-buffer immediately used. Monitoring the cellular ß-galactosidase activity **(Guarente, 1983)**
- Trichloroacetic acid (TCA); 30 g/l aqueous solution of ;*0*-toluidine reagent (0.15 g of thiourea dissolved in 94 ml of glacial acetic acid, 6 ml of distilled 0-toluidine is added and mixed with shaking. The solution thus prepared is stored in a brown bottle. Glucose standard solution freshly prepared; 2.78mM. (**Stroev and Makarova, 1989).**

Methods

Enumeration of the yeast cells

100 µl of the cell suspension was applied on a haemacytometer. The number of cells lying was counted at least 80 small square. Examined microscopically at 30x magnification. After correcting for the dilution factor, the cell numbers was number multiplied by 10000 to get the cell enumeration /mm^3.

Assay for cellular ß-galactosidase-activity

Preparation of the cells for the enzymatic assay, by grinding cells with glass bead (0.55mm) to rupture the cell wall. The following Table (3) presents the sequences of the addition of reagents to assay the activity of yeast ß-galactosidase.

Table 3. The sequences of the addition of reagents for enzyme assay

1	Grow up selectively a 2ml yeast culture and record the OD_{595},which should be mid-log (0.1-0.5)
2	Washing the yeast cells with the KH_2PO_4, *p*H5 buffer
3	Spin down 1ml and wash with 1ml cold Z buffer
4	Resuspend in 1ml cold Z buffer, and add 1-2 drops of 0.1% SDS from a Pasteur pipette and 1-2 drops of chloroform and vigorously to permeabilise the cells
5	Equilibrate for 5 min at 30°C
6	Add 200 µONPG to each tube, record the time of addition and allow the reaction to run until the solution has turned yellow
7	Stop the reaction with 0.5ml of 1M Na_2CO_3, record the time; calculate the elapsed time in min.
8	Spin out the cell debris and read the OD_{420} of the supernatant
9	Calculate "Miller units"

Miller units following formula:

$$Units= 1000XOD_{420}/ VxTxOD_{595}$$

Where 1000; the molar extinction coefficient of *0*-nitrophenol (ONP), V; volume cells assayed (1ml), T; time of reaction (min). One enzyme unit (EU) is defined as the quantity of enzyme that catalyzes the release of 1 µmol of ONP from orthonitrophenyle-ß-D-galactopyranoside (ONPG) / minute under assay conditions.

Analysis of lactose

The colorimetric acid phenol reaction **(Stroev and Makarova, 1989)** was used for the analysis of the glucose; the hydrolytic product of glucose. Standard glucose solutions (5.55 mmol/l) were run in parallel for calculating the concentration of glucose. The results were expressed as glucose equivalent by multiplying in the factor 342/360.

Statistical analysis

The results were expressed as the mean and standard error. Student's t-test was used to estimate statistical differences between mean values. The significant level was set at P< 0.05.

RESULTS AND DISCUSSION

Its will known that the growth of microorganisms lead to increasing of the cell number as well as , consumption of nutrients specially carbon source i.e., lactose and change of initial *p*H . The results of the previous parameters are shown in Table (4).This table represents number of the cells as direct response for growth, the lactose consumption and change in initial *p*H as indirect response for growth.

Propagation media, whey and YPL medium were different of yeast yield as a cell number which rely cells viable. Four

Table 4. Represents the change in the composition of the whey-based and the lactose-containing media as a function of fermentation with four yeast strains

Yeast	Parameter	Whey-based media		Lactose-based media	
GM3	pH	5.5±	5.5 ±	5.5 ±	5.5 ±
	Cell No.	0.5 x 10^3	0.6 x 10^2	0.5 x 10^3	0.5 x 10^2
GT 160	pH	5.5 ±	5.5 ±	5.5 ±	5.5 ±
	Cell No.	0.5 x 10^3	0.1 x 10^2	0.5 x 10^3	0.1 x 10^2
TGM33	pH	5.5 ±	4.4 ±	5.5 ±	4.6 ±
	Cell No.	0.5 x 10^3	12 x 10^8	0.5 x 10^3	5 x 10^8
TGT111	pH	5.5 ±	4.6 ±	5.5 ±	4.5 ±
	Cell No.	0.5 x 10^3	5 x 10^9	0.5 x 10^3	2 x 10^9

strains of yeast *S.cerevisiae*; original and transformants grew at 30°C, pH 5.5 and initial cell number were approximately 0.5 x 10^3. Its were clear at Fig. (1) which shows the variation in ß-galactosidase-activity based media and Fig. (2) which shows the variation in cell number based media.

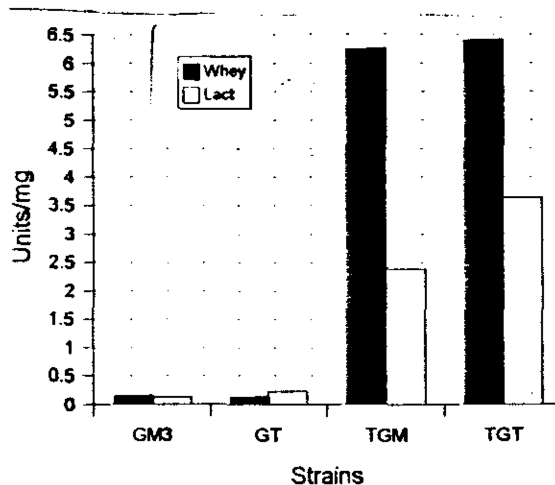

Fig. 1. Comparison between original yeast strains and their transformants on two propagation media (whey and YPL) by β- galactosidase

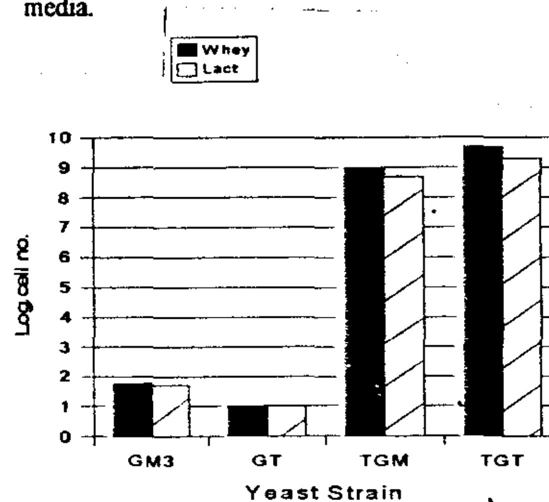

Fig. 2. The variable of log number of original strains and their transformants on the same two propagation media

These figures showed that each two transformants were highly cell number than the two origin strains without plasmid. By means, Fig. (1) shows the transformants were more active in whey than YPL medium. The two transformants yeast strains (TGM33 and TGT111) were transformed by plasmids harboring LEU2 gene that has the ability to complement mutant site in the same gene of the strain, that fail utilize lactose from growth media. No growth of original strains on both whey and YPL media were obtained. While, the two transformants are grown well on both media. On the other hand, whey medium was better than YPL medium from one side. From the other side, the transformants were grown differently. The cell density / ml of transformant TGM33 was 12×10^8 and $5X10^8$ on whey and YPD media, respectively. While transformant TGT111 was $5X10^9$ and 2×10^9 on whey and YPD media, respectively.

The few transformants were highly unstable under nonselective conditions. The problem of instability was greatly minimized by the development of a vector containing the LEU2 gene **(Reed and Nagodawithana 1991)**. ß-galactosidase activity. Fig. (3) shows the transformant; TGT111 was highly producing of enzyme in whey comparing YPL medium. The original strains (untransformants) were containing fall value of this enzyme. The transformant; TGT111 with YCplac111 plasmid was higher efficacy than transformant; TGM33 with YCplac33 plasmid. TGT111 was produced about 6.41 units / mg but TGM33 was produced 6.25 units / mg of ß-galactosidase. The increasing of ß-galactosidase productivity produced by TGT111 by transformed by YCplac111 could be due to the synthesis of *leu* (leucine) by this plasmid. By other words the frequency of *leu* might be high in ß-galactosidase poly peptide chain. This result in according with **Becerra *et al* (1997)**.

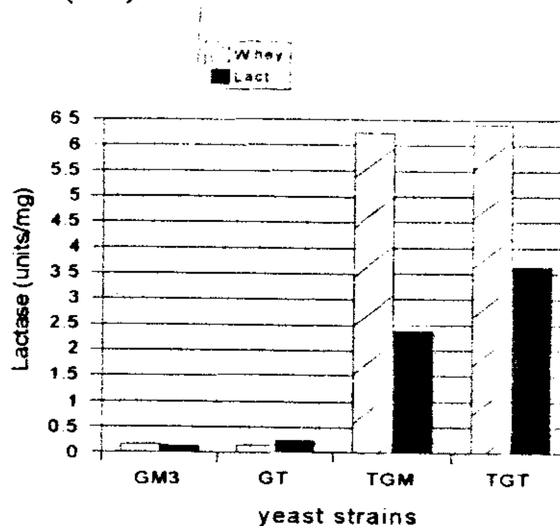

Fig. 3. ß-galactosidase activity of origin strains and their transformants on Whey and Lactose media.

Yeast cells growing on solid media organize themselves into multicellular structures. Colonies, exhibiting patterns specific for particular yeast strains. Identifying genes involved in regulation of the colony formation, enabling the extensive screening of *S.cerevisiae* genes, the expression of which is changed during colony development. **(Minarikova *et al* 2001)**. Noteworthy, the viability of yeast strains were not regarding of cell number, the transformants; TGT and TGM were highly cell number in whey than YPL media, Fig. (2). They contained the high units of ß-galactosidase compared with the origin strains. In is worthily to note

that the two transformants are equal in lactose consumption 73.8% in whey and 70.2% in YPL media. However, they are different in ß-galactosidase production. The transformed strain by plasmid that carry *leu* is higher in production than the other. They could be reflected to synthesis of excess *leu* more than that found in media.

Orderly progression through the eukaryotic cell cycle is controlled by the regulated association of specific cyclins with a CDK (Cyclin-dependent Kinase) In the budding yeast,*S.cerevisiae*,cdc28 is the major CDK and is largely responsible for controlling cell cycle progression (reviewed in **Nasmyth, 1996**). G1 cyclins Clns 1, 2 and 3 are active during G1 up until S phase while B- type cyclins Clbs 1-6 controlled DNA synthesis (**Schwob *et al* 1994**) and mitosis (**Surana *et al* 1991**). The specific association of the appropriate cyclin with Cdc28 is achieved by cell cycle-controlled degradation of the cyclin at key stages of the cycle (reviewed in (**King *et al* 1996 and Deshaires, 1997**).

The mitotic B-type cyclin clb2p is active from late S phase until the end of mitosis when it is rapidly degraded by ubiquitin-mediated proteolysis (**Surana *et al* 1991); Irniger *et al* 1995; Arnon, 1997 and Irniger & Nasmyth, 1997**) Exit from mitosis and entry into the G1 phase of the next cell cycle requires the inactivation of the Clb2 protein; overproduction of Clb2p which has been stabilized by removal of its destruction box causes cells to arrest in telophase with divided chromatin and an elongated spindle (**Surana *et al* 1993**) .

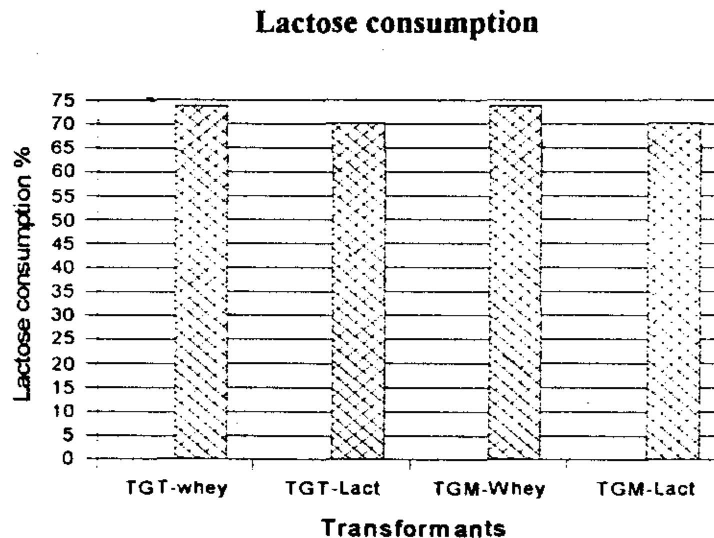

Fig. 4. Lactose consumption in whey and Lactose medium.

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مجلة حوليات العلوم الزراعية، كلية الزراعة ، جامعة عين شمس ، القاهرة ، م(٤٩)، ع (٢)، ٥٩٧-٦٠٨، ٢٠٠٤

كفاءة سلالات خميرة محولة وراثيا لإنتاج إنزيم اللاكتيز من شرش الجبن

[٤١]

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استخدمت أربعة سلالات من خميرة الخباز – اثنان منها محولات وراثية حاوية بلازميدات YCplac111 ، وأخري YCplac33 وكلاهما حاملات لجين (Lac-Z) المسئول عن إنتاج إنزيم β-galactosidase. وكان البلازميد الأول يحوي الأليل البري لجين الحمض الأميني الليوسين (LEU) بينما البلازميد الثاني كان يحوي الأليل البري للقاعدة النيتروجينية اليوراسيل (URA). وهذه السلالات تم إكثارها في كل من بيئة شرش الجبن والبيئة المصنعة الحاوية علي اللاكتوز ، تحت ظروف نمو واحدة.

وكانت هناك ثلاثة دلالات لتحديد قدرة تخمر هذه السلالات:

عدد الخلايا أو كثافة الخلايا لكل مللي لتر؛ معدل التغير في درجة الحموضة؛ وقيمة استهلاك اللاكتوز كمصدر كربوني في البيئة. فقد كان عدد الخلايا للسلالات المحولة وراثيا: $5X10^9$ للسلالات الحاوي للبلازميد YCplac111 بينما الحاوية علي البلازميد YCplac33 كانت $12X10^8$ وذلك علي بيئة الشرش. وكذلك $2X10^9$ و $5X10^8$ علي بيئة اللاكتوز، علي الترتيب . وكانت نسب الزيادة في كثافة النمو % $4.9X10^{10}$ للسلالة TGT111 عن مثيلاتها بدون البلازميد عل شرش الجبن . بينما كانت الزيادة بنسبة % $2X10^9$ للسلالة TGM33 عل نفس البيئة. وعلي الجانب الآخر، فإن السلالة TGT111 كانت الأعلى في عدد الخلايا علي بيئة الشرش عنها في بيئة اللاكتوز بزيادة قدرها 250% ، والسلالة المحولة TGM33 كانت الأعلى في عدد الخلايا علي بيئة الشرش عنها علي بيئة اللاكتوز بنسبة 240%.

وبحساب التغيير في الرقم الهيدروجيني (pH) لم تكن هناك فروق واضحة عند نهاية التجربة عنها في البداية ، لكل من السلالتين الغير محولة وراثيا ، بينما كان التغير واضح في كل من السلالتين المحولتين TGM33 كانت 4.4 والسلالة TGT111 كانت 4.6 وذلك علي بيئة

الشرش. وعلي بيئة اللاكتــوز كانــت 4.6 لسلالةTGM33 و 4.5 لسلالة TGT'111.

وفي النشاط الإنزيمي كان 6.41 وحــدة ميللر لكل مجــم خلايــا لســلالة TGT111 و 6.25 وحدة ميللر لكل مجم خلايا لســلالة TGM33 وذلك علي بيئة الشــرش . بينمــا كان النشاط الإنزيمــي 3.64 و 2.38 علــي الترتيب ولكن علي بيئة اللاكتوز.

وبحساب قيمة استهلاك سكر اللاكتــوز في بيئة النمو كانت نسبته 73.8% مــــن بيئــــة الشــرش و 70.2% مــن بيئــة اللاكتــوز.

والخلاصة: أن شرش الجبن هــو أحــد مخلفات صناعة الجبن – رخــيص الــثمن، يمكن أن يحول إلي مادة لها قيمــة بإكثــار خميرة الخباز المحولة وراثيا والتي تحــوي الجين المسئول عن إنتاج إنــزيم اللاكتيــز (Lac-Z) وقــد كانــت الســلالة الحاويــة للبلازميــد YCplac111 ذو خصـــائص مميــزة.

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