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GENETIC MODIFICATION OF SOME *Saccharomyces cerevisiae* STRAINS TO INCREASE UNSATURATED FATTY ACIDS PRODUCTION

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ABSTRACT

Fatty acids are main important for building prostaglandins, which are necessary for several body processes, including dilation of blood vessels, cholesterol metabolization and other critical bio-chemical functions and physiological reaction. The total lipids determination were detected in supernatant of each hydrolysis cells and cultures. Important variation were recorded between the extracellular and intracellular total lipids for all yeast strains. All strains were grown at the same condition but with Yeast peptone Glucose (YPG) medium and 3 % glucose. For selecting the highest total lipid producing strains, results showed that the strains GT160.34B and STX23-5B were the highest for lipid of extracellular (7.5 and 10.2 g/l), respectively. While the intracellular lipid of the tested strains GT160-34B, GM3 and C321RE221R were the higher where reached to (14 g/l). The tested yeast strains showed considerable range in the iodine values that indicated varying amounts of unsaturated fatty acids. The two hybrids and protoplasts strains; F (GT160-34B**×** GM3), H_1(GT160-34B**×** STX23-5B) and H_2(GT160-34B**×**STX23-5B) were higher producers of the extracellular, intracellular fatty acid and total lipid concentration, respectively. . The conclusion of these data, it's provided that the improved strains by (protoplast fusion and hypradzition), F(GT160-34B**×**GM3), H_1(GT160-34B**×**STX23-5B) and H_2(GT160-34B**×**STX23-5B) were better strains contained fatty acids comparison with the organal strains.

Keywords*: Saccharomyces cerevisiae* hybrid, protoplast fusion, fatty acids.

INTRODUCTION

Fatty acids that are required by the body, but cannot be made in sufficient quantity by the body from other substrates, therefore must be obtained from food and are called essential fatty acids. In the body, essential fatty acids are primarily used to produce hormone-like substances that regulate a wide range of functions, including blood pressure, blood clotting, blood lipid levels, the immune response, and the inflammation response to injury infection (Bethesda, 2005; Herbaut , 2006).

Fatty acids play an important role in the life and death of cardiac cells because they are essential fuels for mechanical and electrical activities of the heart(Honoré, *et al* 1994; Reiffel and McDonald 2006; Landmark and Alm 2006).

Microorganisms are being considered oleaginous if they can accumulate lipid to more than 20% of their cell dry weight (Ratledge 1991). The oil is accumulated intracellular in the form of oil droplets, consisting mainly (more than 80%) of triglycerides. Lipid accumulation specifically

occurs under conditions where there is an excess of carbon source and a limiting amount of one of the other nutrients, preferably nitrogen (Granger *et al.* 1993). Among the group of oleaginous yeasts, *Cryptococcus curvatus* ATCC 20509 has attracted considerable attention because it can accumulate large amounts of oil, 60% of cell dry weight (Ratledge 1991), during growth on cheap carbon sources like whey permeate (Ykema 1989) and other carbohydrate- rich agricultural or food processing wastes (Vega *et al.* 1988; Bednarski *et al.* 1986). The yeast oil as produced by *C*. *curvatus* resembles plant seed oils like palm oil (Davies 1988). Production of yeast oil will always be more expensive than the production of vegetable oils. Therefore, single-cell oil fermentations can only be economically feasible if a particular oil can be produced with a high added value. In the past 15 years attention has been focused on the production of yeast oil with a fatty acid composition resembling cocoa butter. Several approaches have been followed. Moreton (1985) used the ∆-9 desaturase inhibitor sterculia oil, while Davies *et al* (1990) tried to increase the degree of saturation by lowering the dissolved oxygen concentration. A genetic approach was followed by Ykema *et al* (1989), who isolated ∆-9 desaturase mutants defective in the conversion of stearoyl-CoA to oleoyl- CoA. Revertants of this mutant produced lipids with a fatty acid composition comparable to cocoa butter (Ykema *et al*. 1990). Important parameters determining the price of yeast oil are substrate costs, production rate and final product concentration. *C*. *curvatus* can be cultivated on many different feedstocks like sugars, fatty acids and oils. Although glycerol is stoichiometrically not one the most favourable substrates, [Ratledge (1988) reported a maximal theoretical yield of 0.3 g lipid/g glycerol] it could become an interesting feedstock when biodiesel is produced and applied on a large commercial scale. With the production of 10 kg biodiesel from rapeseed oil, 1 kg glycerol becomes available. Different cultivation modes, including batch, fed-batch and continuous fermentations, have been used to maximize the lipid production rate (g lipid l~1 h~1) in *C*. *curvatus*. Evans and Ratledge (1983) have investigated the use of different carbon sources in continuous cultures resulting in a maximum lipid production rate of 0.27 g l~1h~1. Hassan *et al* (1993) further optimized growth in a continuous culture on glucose and reached, at an optimal dilution rate of 0.123 h~1, a lipid production rate of 0.42 (g/l/h). Ykema *et al.* (1988) examined lipid production in various culture modes with whey permeate. They reported the highest lipid production rates (0.995 g l~1 h~1) with a partial recycling method. In this study we describe growth and lipid accumulation of *C*. *curvatus* in a culture with glucose as carbon and energy source.

The mating reaction in *Saccharomycopsis lipolytica* is shown to be bipolar through the analysis of whole meiotic tetrads. Genetic evidence is provided that alkane metabolism group proceeds through a fatty acid or a fatty acid intermediate since several mutants unable to grow on n-tetradecane were also unable to utilize palmitic acid (Bassel and Mortimer; 1973).

Protoplast fusion is versatile technique for inducing genetic recombination in a variety of prokaryotic and eukarotic microorganisms. Protoplast is induced to fuse and form transient hybrids. During this hybrid

state, the genomes may re-assort and genetic recombination can occur (Martins, 2004).

This work aimed to genetic improvement by different methods (hybridization, protoplast fusion) of some *Saccharomyces cerevisiae* strains to increase their production of unsaturated fatty acids.

MATERIALS AND METHODS

Strains:

Saccharomyces yeast strains that used through out this work are described in (Table 1).

Table (1): collected *Saccharomyces cerevisiae* strains.

Strain	Genotype	Source
GT 160-34 B (a)	a, MATa, ade1, leu2, his 6, met 14, lys 9.	YGSC-Barkly-California USA
STX23-5B (α)	α, ade4, trp1	Microbial Genetic Dept., NRC
GM3 (a)	MAT a, ura 1 P+ W-	YGSC-Barkly-California USA
C321 RE221R(a)	a, gal10, trp1, ura3, ura4, met8, ade5,7, leu2, lys1, ilv1, aro1D, can1, Suc mal Cup^r	Microbial Genetic Dept ., NRC

Media:

(1) Yeast Peptone Glucose (YPG): 1% yeast extract, 2 % peptone, and 2 % glucose, (Sherman and Stewart 1982).

(2) Minimal medium yeast nitrogen base without amino acids (MM-AA) 0.67 % glucose 2% agar 2% .

(3) Minimal medium 0.3 % KH_2PO_4 , 0.23 % Na_2HPO_4 , 0.5% NH_4Cl , 0.2 % Glucose , 2 % Agar + 2ml salts solution / 100 ml , 0.1 ml vitamin solution /100ml, 0.01ml minerals solution /100ml.

(4) Medium to increase lipid, content yeast extracts 0.5 % peptone 1.5 % glucose 3 %

(5) **Medium for lipid production**: it contained 30 g of glucose, 0.8 g of K2HPO4 ,0.2 g of KH_2PO_4 ,0.2 g of yeast extract, 0.2 g of $MgSO_4.7H_2O$, 0.05 g of $CaCl_2.2H_2O$ and 0.025 g of $FeSO_4.7H_2O$ (Ray *et al* 1984).

(6) **Salts solution**; 52 g /L MgCl2.6H2O, 0.74 g/L CaCl2.2H2O, 50 g /L, 2g/L Na2SO4.

(7) **Vitamin solution;** 1g/L Pantothenic acid , 10 g/L Nicotinic acid , 10 g/L Inisitol , 10 mg/L Biotin .

(8) **Minerals solution;** 5 g/L Boric acid , 4 g/L $MnSO_4$, 4 g/L $ZnSO_4$.7H2O , 2 g/L $FeCl_2.6H_2O$, 0.4 g/L molybidic acid , 1 g/L KI , 0.4 g/L $CuSO_4.5H_2O$, 10 g/L Citric acid.

Methods:

Hybridization: Three strains with two mating types were used in this work;

1- STX23-5B (α) 2- GM3 (a) 3- GT160-34B (a)

Diploid assays*:* Mating assays were essentially as described by Sprague (1991). Approximately $3x10^3$ cells from exponentially growing cultures of strain. The cells were collected on a nitrocellulose filter disk (25-mm diam.,

0.45 µm pore size). The filters were on the surface of a plate (cell side up) and incubated for 4.5 h at 30ºC. Cells were resuspended in sterile water, and various dilutions were plated on synthetic complete medium (SC plates) to determine the total number of colony-forming units and on synthetic minimal medium (S minimal plates) to select for diploid.

Protoplast fusion

Yeast strains used with protoplast system (the same mating type)

Used 2 strains 1 - GM3 (a) 2- GT160-34B (a)

Selection markers: amino acids requirement as biological protoplasts markers were formed with a lytic enzyme from *Trichoderma harzianum* (Novozym^{TM} 234 SIGMA 2mg/cm^3 0.6 M KCl, incubation up to 1h at 30°C). 0.6 M KCl was used as osmotic stabilizer. The rate of protoplasts formation [%] and regeneration of the protoplasts [%] were determined. The fusion of protoplasts of distillery and selected requirement of yeasts was induced using a solution of polyethylene glycol (PEG 6000) in 10mM $CaCl_2$ (60 min., 30°C). The frequency of fusion was calculated according to Sakai *et al.* (1986). The obtained hybrids were transferred to YM slants and kept at 4°C. After 4, 8, 12 and 24 weeks of storing, the stability of hybrids was examined by controlling their growth in selection medium.

Total lipid determination: The method is based on the ability of unsaturated lipid metabolites to produce, by reaction with phosphorvanillin reagent, a colored compound whose intensity coloration is proportional to the total lipid concentration in each exogenous and endogenous of yeast strains grown different media (James, *et al* 1990).

Vanillin-phosphate reagent, 4 parts (by volume) of concentrated orthophosphoric acid are mixed with 1 part of 0.6% aqueous vanillin solution. The reagent is kept in a brown bottle at room temperature. The total lipid concentration in samples was calculated as g/liter, estimated by analytical curve according to James *et al* (1990).

Lipids extraction and fatty acid identification: Lipids were extracted from washed yeast cells with 10 vol., each of chloroform-methanol-water mixture, (4:8:3 v/v). And chloroform –methanol-mixture, (1:1 v/v). Combined extracts were evaporated to dryness. Absolute methanol-diethyl ether (3:1 v/v) was added to the lipid extract following by saponification with1 ml of 5 N Na OH for 5 hr. Fatty acids were then extracted in n-hexane after adding water and lowering the pH to 1.0 (Purschmann,1982).

TLC Chromatography for identification of fatty acids: TLC is used to separate and determined the concentrations of different types of lipid groups in yeasts, e.g., triacylglycerols, diacylglycerols, monoacylglycerols, cholesterol. A TLC plate is coated with a suitable absorbing (Silica gel) material and placed into an appropriate solvent. A small amount of the lipid samples to be analyzed is spotted onto the TLC plate. With time the solvent moves up the due to capillary forces and separates different lipid fractions on the basis of their affinity for the absorbing material. At the end of the separating the spots visualized by UV. Comparing the distance that the spots move with standards of known composition it is possible to identify the lipids present. Spots scraped and dissolved in the suitable solvents used. Fatty acids were fractionated by thin-layer chromatography (TLC) on 0.25 nm.

layers of silica gel GF 254 Merck,using hexane-ethyl acetate (65:35, v/v) as solvent 10 µl from each samples was pointed on a distance 0.5 cm of silica gel sheet. ,beside linolenic acid as standard fatty acid request detecting 10,20, 30, 40 ,and 50 µl from 55 µl liolenic acid was solvated in 100 µl n-hexane. After solvent running (about 4 hrs) the spots visualized by UV. , and the parallel spots for linolenic were scraped from the TLC plates, dissolved in methanol and analyzed by UV. Spectroscopy (535 nm).Linolenic as the important fatty acid in this study (sigma chemical co.) was used as internal standard, (Purschmann, 1982).

Iodine value (wijs method): The iodine value is a measured of unsaturation and expressed as the number of iodine absorbed, under the prescribed conditions. The reaction with iodine was long used for analyzing the number of double bonds that is, the degree of saturation. Iodine solutions have a violet color. In the reaction with a double bond, one iodine molecule will lose its color. In iodine number determinations (titration) the quantity of iodine of is determined which will just be a decolored by the fat or oil. This provides a direct measure of the number of double bonds present in the sample. One of the most commonly used methods for determining the iodine value of lipids is "Wijs Method" .The lipid to be analyzed is weighed and dissolved in suitable organic solvent, you which a known excess of iodine chloride is added. Some of the I Cl reacts with the double bonds in the unsaturated lipids, while the rest remains. The amount of I Cl that has reacted in determined by measuring the amount of I Cl remaining after the reaction has gave to completion (I Cl reacted = I Cl excess - I Cl remaining) .The amount of I Cl remaining is determined by adding excess potassium iodide to the solution, and then titrating with a sodium thiosulfate solution in the presence of starch to determine the concentration of iodine released .Thus the concentration of C=C in the sample can be calculated by measuring the amount of sodium thiosulfate needed to complete the titration .The higher the degree of unsaturation, the more iodine absorbed, and the higher the iodine value (IV). Laboratory manual in biochemistry (Book).

RESULTS AND DISCUSSION

The total lipid of extracellular and intracellular yeast strains.

Yeast extract was suitable nitrogen source for lipid activation and yeast cells propagation. However, the increase of glucose to up 3% with yeast extract increased lipid production.

The total lipids determination was detected in supernatant of each hydrolysis cells and culture. The extracellular and intracellular total lipid was described in Fig (1) and Table (2). In extra YPG was higher strains STX23-5B, GT160-34B, C321 RE221R and GM3, respectively, in the production of high-lipid College while in the Intra strains GT160-34B,GM3 and C321 RE221R in the production of high-lipid College. For selecting the highest total lipid production strains Fig.(1)showed that the strains STX23-5B and GT160-34B were the highest for lipid of extracellular, (10.2 and 7.5 g/l) on YPG medium, respectively. While the intracellular lipid of the strains GT160-34B, GM3andC321RE221R were the highest,14g/l onYPG medium.Wild *et al* (2010)

reported the use of soluble potato starch as a carbon source resulted in higher cell yield as well as higher fraction of lipids in the cells. Growth on starch increased the fraction of C18:1 fatty acid in the cells, a feature that will be desirable for improved cloud point in biodiesels produced from these lipids.

Table (2): The total lipid (gram/liter) of extracellular and intracellular of *Saccharomyces cerevisiae* strains.

Strains	Extra YPG	Intra YPG
GT160-34B	7.5	14
STX23-5B	10.2	5
GM3	4	14
C321 RE221R	6	14

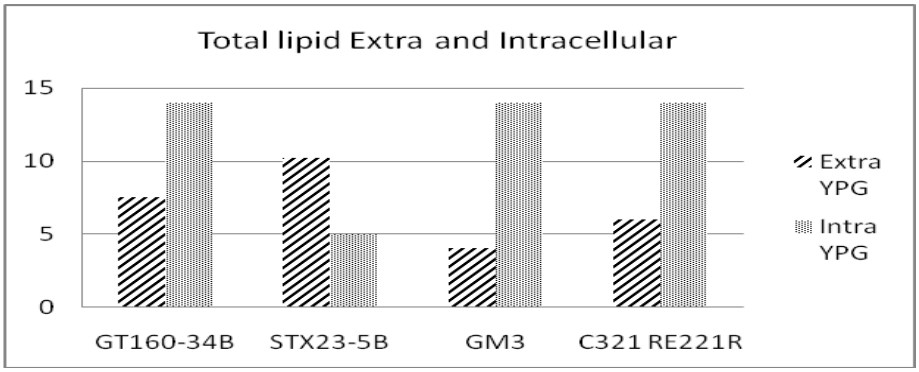


Figure (1): The total lipid of extracellular and intracellular of *Saccharomyces cerevisiae* strains.

Lipids extraction and fatty acid identification:

Yeast strains (4 strains) were compared with linolenic, standard concentrated, 55µl diluted in 100 µl n-hexane ,and detected the absorbency value to each, 10, 20, 30, 40, and 50µl. as shown in Table (3).

Table (3). The standard absorbency values of linolenic acid

Sample,conc. µl/100µl solvent	Conc. ,mg /l
10	31.79
20	44.84
30	63.25
40	89.21
50	192.8

However, the samples of yeast strains at the same conditions were detected the linolenic and /or others fatty acids, as showed in Fig. (2), Table (4). The total lipids and fatty acids were higher in strains GM3, GT160-34B, STX23-5B and C321RE221R respectively. On the other hand there is a relationship between increased the total lipids and fatty acids in the collection of strains. Glucose is the major source of acetyl Co-A for fatty acid synthesis-Acetyl Co-A is a central intermediate in lipid metabolism. The putative fatty acid transporter (FAT) family includes hundreds of sequenced homologous which include fatty acyl Co-A ligases (fatty acyl Co-A synthases), carnitine Co-A lingases, and putative transporters (Hirsch and Lodish 1998). The fluorescence intensity corresponding to the intracellular lipid amount was determined at the peak of the corrected spectrum (Kimura *et al*. 2004).

Table (4): The linolenic (mg/L) and/or fatty acid in *Saccharomyces cerevisiae* strains grown on YPG.

Strains	fatty YPG	absorbency v. YPG
GT160-34B	32	68.79
STX23-5B	30	63.25
GM3	34	70.17
C321RE221R	15	39.19

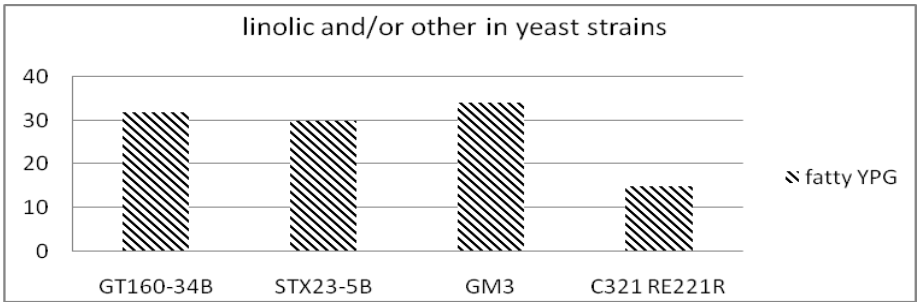
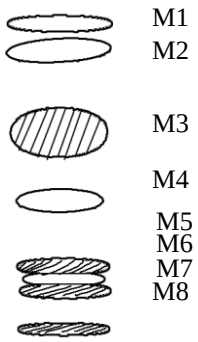


Figure (2): The linolenic and/or fatty acid in *Saccharomyces cerevisiae* strains grown on YPG.

There is variation in total lipid either extracellular or intracellular for all yeast strains. All strains were grown at the same condition but with YPG medium difference carbon source and 3% glucose as carbon source. The fatty acid; omega-3 concentration of yeast strains were compared with linolenic standard 55µl /100µl n.hexan, after scraped the spots visualized on TLC plates Table Fig (3). The fatty acid Linolenic acid and/or other in intracellular was the highest, on YPG medium GT160-34B and GM3 respectively. In spites GM3 and GT160-34B strains have highest fatty acid and iodine value detected is contained more unsaturated fatty acid or more carbon double bond.

Table (5): Iodine value of *Saccharomyces cerevisiae* strains.

Strains	IV
GT 160-34B	380
STX23-5B	300
GM3	375
C321 RE221R	300



M1: hydrocarbons
M2: cholesterol esters
M3: triacylglyceroals
M4: free fatty acids
M5: 1,3diacylglyceroals
M6: cholesterols
M7: 1, 2 diacylglyceroals
M8: monoacylglycerols

Fig. (3): View of hydrocarbons separated by TLC (Thin layer chromatography) for identification fatty acids.

Hybrdization :-

Two hybrid strains resulted from the hybrid between H_1 (GT160-34B×STX23-5B) and H_2 (GM3×STX23-5B) and examined for total lipids, fatty acid and iodine value , H_1produce (68.18, 32, 380) and H_2 (63.25, 30, 350) respectevly. (Table 7)

The mating reaction in Saccharomycopsis lipolytica is shown to be bipolar through the analysis of whole meiotic tetrads. Genetic evidence is provided that alkane metabolism proceeds through a fatty acid or a fatty acid intermediate since several mutants unable to grow on n-tetradecane were also unable to utilize palmitic acid (Bassel and Mortimer 1973).

Verwoert *et al* (1989). Reported that level of saturated fatty acids in lipids of hybrid strains is higher than in wild-type *A. curvatum* and in some hybrids even approaches cocoa butter. Intraspecific spheroplast fusion seems a promising approach for the production of cocoa butter equivalents.

Two-hybrid yeast approach to detect direct protein interactions between fatty acid synthase components. Enoyl-acyl carrier protein (ACP) reductase was found to interact with stearoyl-ACP desaturase and acyl-ACP thioesterase, but none of these proteins interacted with ACP in the yeast nucleus (Honeyman and Fawcett, 2000).

Protoplast fusion:-

For determining the efficiency of protoplast induction and number of regenerated protoplasts for each parental strain, two equal samples of protoplast suspension, were used. The first sample was sprerated onto YPG medium after osmotic shock to allow the osmotically stable, non- protoplasted cells which were not transformed to protoplast form to grow. The ratio of

protoplasting was calculated after subtracting the number of colonies appeared on YPG medium after osmotic shock from the total number of cells before protoplasting treatments. The second sample was added to the top layer medium then overplayed on the regeneration medium and grown cells were also counted. The regeneration ratio was calculated after subtracting the number of remainder cells after protoplasting treatment from the number of the colonies appeared on the regeneration medium.These results are illustrated in Table (6).

Table (6): Protoplast and regeneration ratio of two parent *Saccharomyces cerevisiae* strains.

Strain / Parameter	GT 160-34 B	GM3
Total count /ml	280	300
No. of non-protoplasted cells /ml	80	70
No. of protoplasts /ml	200	230
Protoplasting %	71.4%	76.7%
No. of colonies on regeneration medium /ml	230	240
Regenerated protoplasts /ml	150	170
Regeneration %	75%	73.9%

Data in Table (6) showed the total protoplast and regeneration ratio of two parent strains. Total lipids, fatty acids and iodine value concentration of protoplasts fusion were described in Table (7) the F (GT160-34B,GM3), were the highest of total lipids, fatty acids and iodine value concentration respectively of protoplasts on YPG medium. Protoplast is induced to fusent and form transient hybrids. During this hybrid state, the genomes may-re-assort and genetic recombination can occur (Martins *et al.*, 2004). So far, an increasing number of recombinant strains have been formed.

Table (7): Extra and intra total lipids, fatty acids and iodine value concentration (g/Liter) of two selected hybrids and their protoplast fusion.

Lipid component		Strains		
		H1	H2	F
Total lipids	Extra	9	7	10
	Intra	13	10	18
IV		380	350	440
Fatty YGP		32	30	52
Absorbency YPG		68.18	63.25	198.97

H_1(GT160-34B×STX23-5B)
H_2(GM3×STX23-5B)
F(GT160-34B×GM3)

The conclusion of these data, it's proved that F(GT160-34B×GM3), H_1 (GT160-34B×STX23-5B) and H_2(GM3×STX23-5B) were the better strains contained fatty acid respectively.

Killer character has been successfully transferred into industrial yeast *S. cerevisiae* HAU-1 by protoplast fusion. Examination of 4 fusants showed

that all the desirable fermentation properties of distillery strain *S. cerevisiae* HAU-1 are substantially retained, sustaining the hypothesis that nuclear DNA exchanges if any, had been minimal. Further, it is realized that there is a need to develop a distillery strain of *S. cerevisiae* with a broad spectrum of killer activity, which should be practically active against all types of wild yeasts, *Saccharomyces* as well as non-Saccharomyces spp. present in molasses and other substrates (Bajaj1, B.K; and Sharma, S; 2010).

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التحوير الوراثي لبعض سلالات خميرة الخباز لزيادة إنتاجها من الأحماض الدهنية الغير مشبعة

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الأحماض الدهنية مهمة لبناء البروستاجلاندين ، وهي ضرورية للعديد من العمليات الحيويه مثل التمثيل الغذائي بالجسم ، بما في ذلك تمدد الأوعية الدموية وتقليل نسبة الكولسترول وغيرها من الوظائف الحيوية والكيميائية الهامة. استعمل في البحث اربعة سلالات من خميرة الخباز مختلفه في التركيب الوراثي وتم تنميتها علي بيئة YPG مع جلوكوز 3% . وتم تقدير الليبيدات الكليه في السلالات تحت الدراسه خارج الخلايا وداخلها فكانت أعلي السلالات في الليبيدات الكليه خارج الخلايا هي STX23-5B, GT160-34B (10.2 و 7.5 جرام لكل لتر)علي التوالي اما الليبيدات داخل الخلايا فكانت السلالاتGT160-34B,GM3,C321RE221R (14 جرام لكل لتر)علي التوالي. وأظهرت نتائج أختبار سلالات الخميره أرتفاع في الرقم اليودي والذي يكون مؤشر لارتفاع تركيز الأحماض الدهنيه في الخلايا. وتم عمل هجينين بين ثلاث سلالات مختلفتين في التركيب الوراثي هي

H_1(GT160-3B× STX23-5B) , H_2(GM3× STX23-5B)

وعمل دمج بروتوبلاستي سلالاتين هي (GT160-34B×GM3)F

وكان من اهم نتائج التحسين الوراثي للسلالات باستخدام اساليب التهجين والدمج البروتوبلاستي للخلايا هو ارتفاع نسبة الليبيدات الكليه والأحماض الدهنية في الخلايا وارتفاع الرقم اليودي مقارنة مع السلالات الأبويه . هذه الزياده في نسبة الليبيدات والأحمض الدهنيه تكون نتيجة الخلط للماده الوراثية بين السلالات الأبويه الداخله في عملية التهجين والدمج البروتوبلاستي .

قام بتحكيم البحث

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