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Genetically Construct *Saccharomyces cerevisiae* Strains Harboing α-Amylase Gene From Amylolytic Yeasts

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A HIGHLY active α-amylase gene secreted by five raw starch-degrading yeasts has been transferred to four *S. cerevisiae* strains using $CaCl_2$ and spheroplast transformation (PEG methods) . Lower percentage of transformation was obtained in response to direct transformation using $CaCl_2$, while transformation using PEG method showed higher percentages. In addition, PEG method yielded the maximum transformation percentage 4.0 %, whereas, that in $CaCl_2$ method was reached to 2.7 %. The higher number of transformation experiments using $CaCl_2$ was more than that produced by PEG which may due to lower regeneration of yeast spheroplasts on spheroplast transformation plates. Transformants were differed in their ability to grow on soluble starch due to the genetic background of various isolates contributing to the differential levels of α-amylase secretion which infuence their growth rate and genetic stability of expression α-amylase gene. All transformants were able to secrete extracellular α-amylase and expression of *α-Amy* gene was differed significantly in their activity to utilize soluble starch. Furthermore, transformants containing the *AMY* gene showed significant differences in halo size, which may be due to the different levels of expression of α-amylase gene. Recombinant isolates exerted significant differences in hydrolysis percentage of raw potato starch. Positive relation was achieved between ethanol production from raw potato starch and glucose values and the expression of α-amylase genes. Also, differences were obtained between amylolytic yeast strains and their transformants based on molecular weight of protein bands, the similarity degree and genetic distance.

Keywords : α-Amylase gene, Ethanol yielding, Genetic distance, Protein bands, Starch degradation, Transformation.

The yeast *Saccharomyces cerevisiae* has been used extensively for the production of many heterologous proteins, since it is safe eukaryotic microorganism with well established fermentation technology for large-scale production (Romanos *et al.*, 1992). Large amounts of yeast cells can easily be grown at lower cost than any other eukaryotic expression system. In addition, as the yeast *Saccharomyces cerevisiae* can not produce the starch degrading

enzymes, genetically engineered strains of *Saccharomyces cerevisiae* secreting heterologus raw-starch-degrading enzymes were developed by Janse and Pretorius (1995) . Genetically engineering strain of *S. cerevisiae* was useful in production of single-cell protein, maltose syrup, baking and brewing industry (Steyn and Pretorius, 1990).

Raw starches as unexpensive substrates, found in large amounts in agriculture residues , could be utilized for alcoholic fermentation and yeast biomass on an industrial scale by construction of genetically modified *S. cerevisiae* strains (synthesis and secretion of two enzymes α-amylase and glucoamylase). The use of starch as a renewable biological resource has both social and economic advantages.

Various S. *cerevisiae* transformants were employed for ethanol production which is considered one of the important role in the economy of number of developing countries, *e.g.* Brazil and India (Hacking, 1987 and Rosillo-Calle *et al.* 1992). Also , it makes basal industry for various organic compounds including acetic acid, vinegar, acetaldehyde, perfumes, medicine and laboratory uses. The trends to use ethanol as a fuel to replace gasoline to overcome environmental pollution is one of the aims for its production.

Therefore, the aim of the present study was screening yeast strains-degrading starch and selecting the efficient ones that completely degrading starch very well. The efficient strains (*Saccharomycopsis* sp.; *Lipomyces* sp. and *Schwanniomyces* sp.) were used for isolating and transferring their α-amylase gene to *Saccharomyces cerevisiae* and evaluate their transformants for the expression of α-amylase gene as, which can be used in ethanol production from raw potato starch. Genetically modified strains were also biochemically evaluated for secreting α-amylase- which can degrad starch completely, in order to recycl starch resulting from some industries in a trial to reduce its polluting effect in the environment.

Material and Methods

I- Material

1-Microbial strains

The sources and relevant genotypes of yeast strains used in this investigation are listed in Table 1. In addition, transformant isolates developed and used in this work are listed in Table 2.

2- Media

(a) Yeast medium broth (YB): It contained (g / l), yeast extract, 3 ; malt extract, 3 ; peptone, 5 and glucose , 10 and distilled water 1000 ml according to Agricultural Research Service Culture Collection, USA.

(b) Yeast peptone dextrose (YPD) : It contained (g %) 1 yeast extract, 2 peptone, 2 glucose (dextrose) and 2 agar. The medium was used for general growth (Sherman *et al.*, 1986).

(c) Synthetic complete medium (SC): Transformants of *S. cerevisiae* were cultured and selected on synthetic complete (SC) medium containing 0.67 % yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 0.5 % $(NH_4)_2SO_4$. No growth factors were added and 0.4 % soluble starch was added as a selectable marker for starch utilization and as a carbon source instead of 2 % glucose (Eksteen *et al.*, 2003 a).

(d) Maintenance the amylolytic yeast strains (MAYM): The solid medium used for maintenance of yeast strains able to secrete α-amylase enzyme has the following composition (g/L): peptone, 1.25; yeast extract, 1.25; starch, 10 and agar, 20 (Gogoi *et al.*, 1987).

(e) Amylase production medium (AM): The amylase production medium has the following composition (g /L); starch, 5.0; yeast extract, 5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1 (Gogoi *et al.*, 1987).

(f) The fermentation medium: It was prepared using potato starch extract (about 2% starch), 0.67 % YNB without amino acids. The pH was adjusted to 5.5 after autoclaving at 121 °C for 15 min (Eksteen *et al.*, 2003 b).

TABLE 1. Yeast strains used in this study.

Yeast strains	Genotypes	Source / Reference	Designation
Yeast strains secretable α-amylase:			
Saccharomycopsis fibuligera NRRL Y-2388	Wild type	USDA	SF
Saccharomycopsis capsularis NRRL Y-17639	"	USDA	SCA
Lipomyces kononenkoae NRRL Y-11553	"	USDA	LK
Lipomyces starkeyi NRRL Y-11557	"	USDA	LS
Schwanniomyces occidentalis NRRL Y-10	"	USDA	SO
Saccharomyces cerevisiae strains:			
S. cerevisiae NRRL Y-2043	Wild type	USDA	SC_1
S. cerevisiae M1	"	France	SC_2
S. cerevisiae M2	"	Turkey	SC_3
S. cerevisiae M3	"	China	SC_4

NRRL = National Regional Research Laboratory. USDA = United States Department of Agriculture.

M1 = Commercial strain isolated from France beaker yeast powder.
M2 = Commercial strain isolated from Turkey beaker yeast powder.
M3 = Commercial strain isolated from China beaker yeast powder.

TABLE 2. Designation of transformed *S. cerevisiae* strains with α- amylase gene(s) from amylolytic yeast strains using $CaCl_2$ and PEG methods.

Donor	Recipient	Trans formation methods	Designation
Saccharomycopisis fibuligera NRRL-Y 2388	*S. cerevisiae* M1	$CaCl_2$	T_1
	S. cerevisiae M2	$CaCl_2$	T_2
	S. cerevisiae M3	$CaCl_2$	T_3
	S. cerevisiae M2	PEG	T_4
Saccharomycopisis capsularis NRRL-Y 17639	*S. cerevisiae* NRRL-Y 1204	$CaCl_2$	T_5
	S. cerevisiae M1	$CaCl_2$	T_6
	S. cerevisiae M2	$CaCl_2$	T_7
	S. cerevisiae M3	$CaCl_2$	T_8
	S. cerevisiae M1	PEG	T_9
	S. cerevisiae M3	PEG	T_{10}
Lipomyces kononenkoae NRRL-Y 11553	*S. cerevisiae* NRRL-Y 1204	$CaCl_2$	T_{11}
	S. cerevisiae M1	$CaCl_2$	T_{12}
	S. cerevisiae M2	$CaCl_2$	T_{13}
	S. cerevisiae M2	PEG	T_{14}
Lipomyces starkeyi NRRL-Y 11557	*S. cerevisiae* NRRL-Y 1204	$CaCl_2$	T_{15}
	S. cerevisiae M1	$CaCl_2$	T_{16}
	S. cerevisiae M2	$CaCl_2$	T_{17}
	S. cerevisiae M1	PEG	T_{18}
	S. cerevisiae M2	PEG	T_{19}
Schwanniomyces occidentalis NRRL-Y 10	*S. cerevisiae* NRRL-Y 1204	$CaCl_2$	T_{20}
	S. cerevisiae M1	$CaCl_2$	T_{21}
	S. cerevisiae M2	$CaCl_2$	T_{22}
	S. cerevisiae M3	$CaCl_2$	T_{23}
	S. cerevisiae NRRL-Y 1204	PEG	T_{24}
	S. cerevisiae M1	PEG	T_{25}
	S. cerevisiae M2	PEG	T_{26}
	S. cerevisiae M3	PEG	T_{27}

Transformants selected for protein pattern using SDS - PAGE depending on starch degradation presented in Table 5 .

II- Methods

1- DNA isolation

Five milliliter of a 48 hr-old yeast strains secreting α- amylase enzyme culture (donor) were inoculated into 50 ml of YM broth medium and incubated at 28 °C for 48 hr. The cells were harvested and washed once with 10 ml of distilled water and once with 10 ml sodium acetate buffer pH 5.5. DNA isolation was carried out according to Maniatis *et al.* (1982).

2- Transformation procedures

Yeast cells were transformed using direct transformation ($CaCl_2$ method) with some modifications and Spheroplast transformation (PEG method) according to Maniatis *et al.* (1982) and Glover and Hames (1995).

3-Detection of the activity of yeast transformants secreted α-amylase

Amylase hydrolyzing activity was determined by assaying halo zone-forming around the colonies indicated starch-degrading activity intensified by iodine staining according to Rothstein *et al.* (1985) and quantification in cell free supernatant fluid which was used as the crude enzyme source.

4- Genetic stability of different recombinant yeasts

The genetic stability of different recombinant yeasts was determined using the following formula according to Rothstein *et al.* (1985):

$$\text{Genetic stability (\%)} = \frac{\text{Number of colonies on YPD}}{\text{Number of colonies on YNBS}} \times 100$$

5-Alcoholic fermentation

Recombinant *Saccharomyces cerevisiae* strains were grown on fermentation medium at 30 °C for 168 hr with agitation by orbital shaker. Samples were taken every 24 hr for determination of : ethanol production according to Harwitz, (1980) with some modifications, utilization starch determined by iodine colored method according to De Mot *et al.* (1984), biomass dry weight (Birol *et al.* 1998) and reducing sugars using Nelson (1944)-Somogyi (1952) methods. The amount of glucose was estimated from standerd curve of glucose.

6- α-amylase assay and glucoamylase activity

The extracellular activity of α-amylase samples taken at different phases of growth was assayed as described by Hemandez and Pirst (1975) and glucoamylase determined according to Ribeiro dos Santos (1988).

7- SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970). Extracted proteins of yeast cells were resolving in electrophoresis run buffer using a small vertical electrophoresis unit with 4 % acrylamide stacking gel and 12 % acrylamide resolving gel. A constant voltage of 100 V at 4 °C was maintained in each gel over nigh. After electrophoresis, the gel was stained for protein with Coomassie brilliant blue R-250.

Jaccard's similarity coefficient may be used which only takes positive matches into account (both bands are presented) according to Patwary *et al.* (1993).

$$J_{ij} = C_{ij} / (n_i + n_j - C_{ij})$$

Here, C_{ij} is the number of positive matches between two individuals, while n_i and n_j is the total number of bands in individuals i and j, respectively.

8- Statistical analysis

All experiments in this study were conduced in a completely randomized design with three replications. This design, the simplest possible, is set up by assigning treatments at random to a previously determined set of variation in this units. There are only two sources of variation in this design, among experimental units within a treatment, which call experimental error and that among treatment means.

Results and Discussion

1- Yeast transformation

The results presented in Table 3 and Fig. 1 showed the lower number of transformants and lower percentage of transformation obtained in response to the direct transformation using $CaCl_2$ or PEG. This may due to strain poor transformant recovery and high frequency of diplodization. The results also indicated that there are three of transformation experiments did not give any transformant colonies using $CaCl_2$ method, while there are ten experiments of transformation did not gave any transformant colonies using PEG which may due to lower regeneration of yeast spheroplasts on spheroplast transformation plates (Traver *et al.*, 1989). Transformation of PEG method showed higher transformant percentages than that of $CaCl_2$ method. This indicated that PEG method was efficient than $CaCl_2$ method for inserted DNA through the cell wall of the yeast cells. The PEG method yielded the maximum transformation percentage 4.0%, whereas, that in $CaCl_2$ method was reached to 2.7 % . Similar results were obtained by Hafez (2000), who reported that the efficiency of genetic transformation in *S. cerevisiae* using PEG gave higher transformants and transformation percentages more than that of $CaCl_2$. The results revealed that all experiments of *Schwanniomyces occidentalis* NRRL Y-(10 transformation) used a donor strain induced transformant colonies using $CaCl_2$ and PEG. This was due to easily entrance of DNA across cell walls of all four industrial strains of *S. cerevisiae* and mitotic stability of the transformed DNA in recombinant isolates. Furthermore, in transformation the combined effect of alkali ion for generation of membrane distortions, allow the uptake of donors DNA. The effect of PEG method can probably be attributed to changes in membrane charges caused by interactions among negatively charged PEG, monovalent cations, and the yeast cell surface (Hafez , 2000).

TABLE 3. Transformation of yeast strains using $CaCl_2$ and PEG methods.

Donor	Recipient	No. of competent colonies		No. of transformed colonies		Transformation (%)	
		I	II	I	II	I	II
Saccharomycopsis fibuligera NRRL Y-2338	*S. cerevisiae* NRRL Y-2043	6500	546	0.0	0.0	0.0	0.0
	S. cerevisiae M1	6830	602	102	0.0	1.5	0.0
	S. cerevisiae M2	7170	733	120	29	1.7	4.0
	S. cerevisiae M3	6750	621	22	0.0	0.33	0.0
Saccharomycopsis capsularis NRRL Y-17639	*S. cerevisiae* NRRL Y-2043	6320	564	27	0.0	0.43	0.0
	S. cerevisiae M1	6620	583	168	22	2.5	3.8
	S. cerevisiae M2	6870	672	15	0.0	0.22	0.0
	S. cerevisiae M3	6600	636	35	5	0.53	0.79
Lipomyces kononenkoae NRRL Y-11553	*S. cerevisiae* NRRL Y-2043	6750	437	34	0.0	0.50	0.0
	S. cerevisiae M1	6980	506	20	0.0	0.29	0.0
	S. cerevisiae M2	7220	588	120	13	1.7	2.2
	S. cerevisiae M3	7280	645	0.0	0.0	0.0	0.0
Lipomyces starkeyi NRRL Y-11557	*S. cerevisiae* NRRL Y-2043	6480	378	30	0.0	0.46	0.0
	S. cerevisiae M1	7050	412	60	7	0.85	1.7
	S. cerevisiae M2	7150	396	88	12	1.2	3.0
	S. cerevisiae M3	6720	334	0.0	0.0	0.0	0.0
Schwanniomyces occidentalis NRRL Y-10	*S. cerevisiae* NRRL Y-2043	6650	582	173	16	2.6	2.8
	S. cerevisiae M1	7120	568	156	11	2.2	1.9
	S. cerevisiae M2	6950	676	187	24	2.7	3.6
	S. cerevisiae M3	7250	606	175	10	2.4	1.7

I= $CaCl_2$ method. II= PEG method.

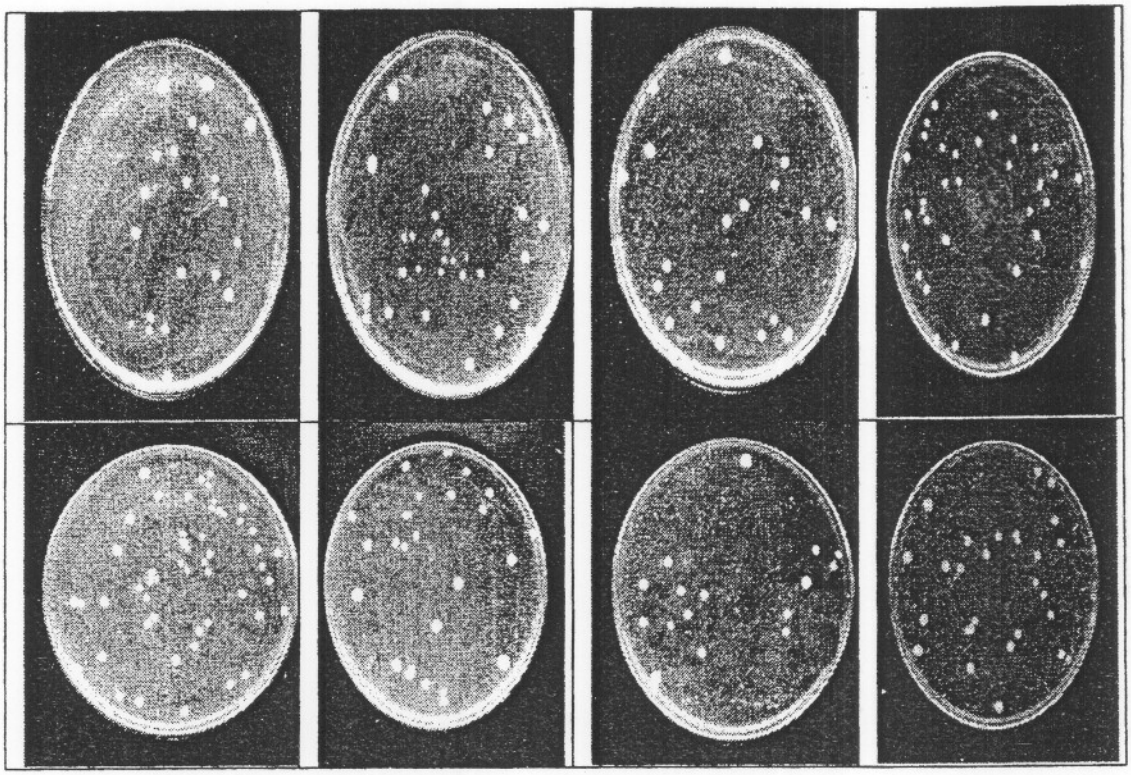

Fig. 1. Photographs of some transformed *S. cerevisiae* colonies with α- amylase gene (s) from amylolytic yeast strains on solid SC medium by using $CaCl_2$ (above) and PEG (below) methods.

2- Secretion of α-amylase and starch utilization in transformant yeasts

The results presented in Table 4 showed that *S. cerevisiae* transformants acquired the ability to secrete active α-amylase for 48 hr old period and grow well on starch medium using starch as a sole carbon source. These transformants displayed significant differences in terms of the level of secretors expression of α-amylase-encoding gene (*α-amy*), as well as, their ability to produce and secrete the α-amy-encoded, raw starch-degrading α-amylase and to ferment starch. The results revealed that transformants are differed in their ability to grow on soluble starch medium. This may due to the genetic background of various yeast strains which affect levels of growth and secretion of α-amylase. These results are in agreement with those obtained by Moses *et al.* (2002), who found that specific genetic background of the recombinant yeast strain, play a significant role in heterologous protein production. The results also show that, these transformants exerted significant differences in biomass production (fresh weight). This indicated that the production of biomass is under the control of many genes with different expression and reflects the complicated nature of the gene expression and its inheritance. The results obtained herein are in harmony with those found by Levine and Cooney (1973), who found that the maximal dry weight of the yeast on methanol was 0.36 g/g methanol, whearas the yield on oxygen was 0.37g per g of O_2 . In a significant breakthrough, Birol *et al.* (1998) reported the utilization of 100 g of starch per liter with production of 44 g of ethanol per liter and 8 g of cells per liter.

TABLE 4. Secretion of α-amylase and starch utilization by different *S. cerevisiae* transformants.

No of trans-formant	*Final pH	O.D at 600 nm Absor-bance	Fresh weight g/100ml culture	Culture filtrate						
				EA u/ml	Protein mg/ml	Specific activity u/mg	Residual starch		Starch utilization	
							mg/l	%	mg/l	%
Trans. 1	9.00	1.867	1.30	485	5.11	95	0.0	0.0	5000	100
Trans. 2	8.47	1.896	2.07	432	4.74	91	46	0.92	4954	99.08
Trans. 3	8.60	1.932	1.41	451	4.90	92	0.0	0.0	5000	100
Trans. 4	9.15	1.795	1.70	397	4.30	92	0.0	0.0	5000	100
Trans. 5	8.91	1.920	1.38	406	4.50	90	0.0	0.0	5000	100
Trans. 6	8.74	1.945	1.18	387	3.90	99	0.0	0.0	5000	100
Trans. 7	8.81	1.985	1.29	433	4.90	88	0.0	0.0	5000	100
Trans. 8	7.60	1.496	0.59	379	3.50	108	141	2.82	4859	97.18
Trans. 9	9.06	1.789	1.80	395	3.80	104	0.0	0.0	5000	100
Trans. 10	7.83	1.905	1.36	428	4.60	93	29	0.58	4971	99.42
Trans. 11	8.53	1.832	1.73	418	4.40	95	0.0	0.0	5000	100
Trans. 12	9.78	1.258	1.01	266	2.08	86	1388	27.76	3612	72.24
Trans. 13	9.72	1.119	1.00	251	2.09	81	1658	33.16	3342	66.84
Trans. 14	8.92	1.756	1.84	417	3.90	107	0.0	0.0	5000	100
Trans. 15	9.62	1.285	1.03	336	3.40	99	953	19.06	4047	80.94
Trans. 16	8.78	1.845	1.74	432	3.80	114	0.0	0.0	5000	100
Trans. 17	9.06	1.742	1.60	455	4.10	111	0.0	0.0	5000	100
Trans. 18	9.28	1.378	1.34	290	2.70	107	536	10.72	4464	89.28
Trans. 19	9.17	1.802	1.37	419	4.00	105	0.0	0.0	5000	100
Trans. 20	9.10	1.1726	1.30	468	4.40	106	0.0	0.0	5000	100
Trans. 21	9.17	1.702	1.24	430	4.39	98	0.0	0.0	5000	100
Trans. 22	7.73	1.701	1.00	232	1.82	82	1835	36.7	3165	63.3
Trans. 23	9.18	1.745	1.08	420	3.90	108	0.0	0.0	5000	100
Trans. 24	9.41	1.663	1.29	387	3.80	102	278	5.56	4722	94.44
Trans. 25	9,22	1.809	1.08	460	3.92	117	0.00	0.0	5000	100
Trans. 26	9.30	1.805	1.52	415	3.60	115	0.00	0.0	5000	100
Trans. 27	9.16	1.786	1.25	438	4.30	102	0.00	0.0	5000	100
F. test		*	**	**	*	*				
L.S.D	0.01	0.08	0.20	50	0.5	6				
	0.05	0.11	0.5	70	0.8	10				

Note: EA= Enzyme Activity. Initial pH was 5.5.

The results obtained in this study indicated that *S. cerevisiae* transformants were able to secrete extracellular α-amylase and the expression of the *α-Amy* gene was observed in all of the transformants (Table 4) . Transformants revealed significant differences in the activity of α-amylase activity due to the different levels of expression leading to differences in starch utilization. Some transformants (No. 12, 13, 22) appears to be much slower in the utilization and degradation of starch than others. Others (No. 15, 18, 24) were relatively the fastest in starch utilization. This was due to integration of the α-amylase gene in these recombinant isolates.

These results agree with those of Kato *et al.* (2001), who found that recombinant strains of *S.cerevisiae* harboring α-amylase gene were differed in the degradation ability of soluble starch. Recombinant isolates harboring more than one copy of α-amylase gene exhibited larger starch-digestion zones on YPS medium and had increased levels of starch hydrolysis if compared with strains harboring a single copy of α-amylase gene.

3- Halo zone- assay for α-amylase detection

The results presented in Table 5 and Fig. 2 revealed that spotting of the transformants exreted significant differences in halo size, due to the different levels in the expression of α-amylase gene. Some transformants produced a large halo size, whereas, some others produced a smaller halo size. These results agree with those reported by Kato *et al.* (2001), who found that *Pichia pastoris* transformants obtained by insertion of mouse salivary α-amylase gene revealed difference in clear halo size. They suggested that the large halos might have been caused by multicopy integration of the α-amylase expression cassette in *Pichia* chromosome.

TABLE 5. Halo zone-forming assay for α-amylase activity of different *S. cerevisiae* transformants grown on agar plates containing soluble starch.

No. of trans.	Halo zone Diameter (cm)	No. of trans.	Halo zone diameter (cm)	No. of trans.	Halo zone diameter (cm)
Trans. 1	6.9	Trans. 10	7.2	Trans. 19	6.8
Trans. 2	6.4	Trans. 11	6.7	Trans. 20	7.1
Trans. 3	6.6	Trans. 12	3.3	Trans. 21	6.7
Trans. 4	6.2	Trans. 13	3.4	Trans. 22	3.5
Trans. 5	6.8	Trans. 14	6.8	Trans. 23	6.5
Trans. 6	6.7	Trans. 15	4.6	Trans. 24	5.6
Trans. 7	6.8	Trans. 16	7.5	Trans. 25	7.1
Trans. 8	5.9	Trans. 17	7.0	Trans. 26	6.9
Trans. 9	6.8	Trans. 18	5.2	Trans. 27	6.5
F. test	*		*		*
L.S. 0.05	0.4		0.4		0.4
D 0.01	0.9		0.9		0.9

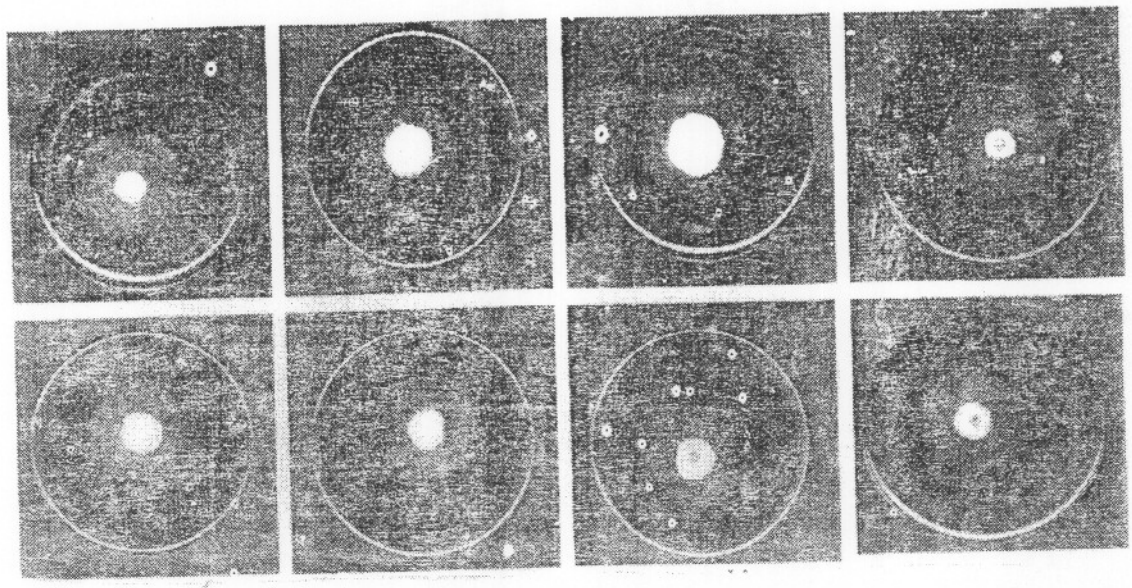

Fig. 2. Halo zone-forming assay for α-amylase activity secreted by some *S. cerevisiae* transformants on SC medium after staining by iodine-vapour.

4- Genetic stability of different recombinant yeasts harboring α-amylase gene

Genetic stability of recombinant isolates as shown in Table 6 was expressed as the percentage of colonies that possessed amylolytic activity in the total population versus growth generation without selection pressure by growing in the YPD medium. Generally, transformant *S. cerevisiae* strains carrying α-amylase gene exhibited significant difference in the degree of genetic stability. Data clearly show that some transformants appeared higher genetic stability after many generations of growth under non-selective conditions (YPD medium), while others showed drastically lower stability. As, the transformant cells were grown on YPD medium containing glucose, there was no selective pressure to retain α-amylase gene residing in the cell. However, since starch was the primary carbon source, there was some selective pressure to retain the ability to produce α-amylase. These results agree with that obtained by Kim *et al.* (1988), who found that transformant *S. cerevisiae* containing the genes encoding mouse salivary α-amylase were differed in their genetic stability after many generations of growth under non-selective conditions (YPD medium). This may be due to various degrees of mitotic instability and multicopy integration of the α-amylase gene (Kato *et al.* , 2001) .

5-Evaluation of transformants in the utilization of potato starchy extract in small-scale fermentations

1-Growth characterization

The ability of different transformants to utilize potato starch extract as the only carbon source in small–scale fermentations over a period of 168 hr was studied (Table 7). Recombinant *S. cerevisiae* strains showed significant differences in the percentages of hydrolysis of raw potato starch. Most of the constructed strains hydrolyzed raw potato starch more rapidly during the first 24 hr of fermentation. After 72 hr, some transformants hydrolyzed raw potato starch faster than others, and completely exhausted available potato starch in the medium reached about 100%. Some others consumed the raw potato starch completely after 96 hr. However , some recombinants were lower in raw potato starch degradation .

TABLE 6. Genetic stability of different recombinant yeasts harboring α-amylase gene .

No. of trans.	Genetic stability (%)	No. of trans.	Genetic stability (%)	No. of trans.	Genetic stability (%)
Trans. 1	100	Trans. 8	85.1	Trans. 19	95.8
Trans. 2	94.3	Trans. 9	100	Trans. 20	100
Trans. 3	100	Trans. 8	85.1	Trans. 21	91
Trans. 4	100	Trans. 9	100	Trans. 22	18.6
Trans. 5	100	Trans. 8	85.1	Trans. 23	100
Trans. 6	100	Trans. 15	69.4	Trans. 24	86.7
Trans. 7	91.7	Trans. 16	100	Trans. 25	100
Trans. 8	85.1	Trans. 17	100	Trans. 26	100
Trans. 9	100	Trans. 18	72.3	Trans. 27	96.5
F. test	**		**		**
L.S. D 0.05	4.2		4.2		4.2
L.S. D 0.01	6.1		6.1		6.1

These results agreed with those of Janse and Pretorius (1995), who found that transformant *S. cerevisiae* strains contained the genes encoding a bacterial α-amylase (*AMY1*) were differed in their ability to hydrolyae and assimilate of soluble starch. This may be due to integration of the different amylolytic genes into transformant *S.cerevisiae* strains. On the other hand, transformants containing two copies of α-amylase gene produced larger starch-digestion zones on YPS medium and possessed higher levels of starch hydrolysis , compared to transformants containing a single copy. Moses *et al.* (2002) found that utilization of soluble starch by different transformants of *S. cerevisiae* harboring α-amylase gene was fastered . They secreted amylolytic enzymes continuously during the growth of yeast cells, where sugar uptake and carbohydrate content of the culture broth at a given time will reflect the activities of the amylolytic enzymes present. Also , Abouzied and Reddy (1986) found that incomplete utilization of potato starch by *Aspergillus* species may due to lake of enough oxygen or to feedback inhibition of amylase activity by glucose released from starch hydrolysis.

The obtained data revealed that there is a positive relation between the percentage of hydrolysis raw potato starch and biomass yield (Table 7) . The data clearly demonstrated that some transformants were consumed completely starch more rapidly, because of their increased growth rates and biomass yielding . In contrast, some others consumed raw potato starch more slowly than others, these achieved lower growth rates and slower biomass yielding . These results agree with those of Moraes *et al.* (1995), who found that lower growth rates and biomass yields of transformant *S. cerevisiae* strains grown in corn starch may not be due to defect in sugar uptake system but in the activity of the amylolytic domain of fusion protein and anchored this enzymes on starch granules.

TABLE 7. Utilization of potato raw starch and biomasses dry weight of transformants at batch fermentation .

Trans.	Fermentation period (h)													
	24		48		72		96		120		144		168	
	Yield		Yield		Yield		Yield		Yield		Yield		Yield	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
SF	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_1	1.1	1.3	1.1	1.4	1.0	1.4	1.1	1.3	1.1	1.3	0.0	1.2	0.0	1.2
T_2	0.8	0.7	0.9	1.0	0.8	1.1	0.9	1.1	1.1	1.1	0.0	1.0	0.0	1.0
T_3	0.9	0.9	0.8	0.9	0.9	1.3	0.9	1.2	1.1	1.2	0.0	1.2	0.0	1.2
T_4	1.6	1.9	1.3	1.4	1.2	1.8	1.3	1.7	0.0	1.7	0.0	1.5	0.0	1.5
SCA	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_5	0.6	0.6	0.9	0.7	0.9	1.2	1.0	1.3	1.0	1.3	0.0	1.2	0.0	1.1
T_6	1.4	2.0	1.2	1.1	1.2	1.7	1.3	1.8	0.0	1.7	0.0	1.5	0.0	1.4
T_7	0.9	1.6	0.9	1.2	1.1	1.4	1.1	1.6	1.1	1.5	0.0	1.4	0.0	1.2
T_8	0.7	1.0	0.8	1.0	1.0	1.2	1.0	1.2	1.0	1.2	0.0	1.3	0.0	1.2
T_9	1.6	6.0	1.5	2.2	1.3	2.5	1.3	2.4	0.0	2.1	0.0	1.9	0.0	1.7
T_{10}	1.2	2.0	1.3	1.1	1.3	1.5	1.3	1.8	0.0	1.8	0.0	1.6	0.0	1.4
LK	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_{11}	2.0	4.0	2.1	4.0	2.0	4.6	1.8	4.2	1.5	1.7	0.0	1.3	0.0	1.4
T_{12}	1.8	2.0	1.5	3.2	1.3	2.6	1.4	2.4	1.1	1.1	1.0	0.8	0.9	0.9
T_{13}	0.8	0.5	1.3	1.0	1.2	1.3	1.1	1.7	1.0	0.8	0.8	0.7	0.8	0.7
T_{14}	2.4	6.0	2.3	4.8	2.2	5.4	2.0	4.8	0.0	2.0	0.0	1.6	0.0	1.5
LS	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_{15}	3.6	3.0	1.6	1.7	1.3	1.1	0.9	0.8	0.9	0.9	0.8	0.9	0.0	1.1
T_{16}	7.1	15.0	3.2	9.7	2.7	6.4	1.6	2.5	0.0	1.7	0.0	1.7	0.0	1.7
T_{17}	9.5	20.0	3.9	15.0	3.1	7.9	1.6	2.7	0.0	1.9	0.0	1.7	0.0	1.7
T_{18}	2.6	1.0	1.5	1.3	1.3	1.0	0.8	0.6	0.7	0.5	0.7	0.4	0.0	0.8
T_{19}	8.6	14.0	3.8	10.0	2.9	5.6	1.6	2.1	0.0	1.7	0.0	1.5	0.0	1.5
SO	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_{20}	1.2	2.1	1.1	1.2	1.3	1.5	1.4	1.7	0.0	1.5	0.0	1.5	0.0	1.4
T_{21}	1.0	1.6	1.1	1.4	1.2	1.6	1.4	1.7	0.0	1.4	0.0	1.4	0.0	1.3
T_{22}	1.6	0.0	0.3	0.1	0.4	0.1	0.5	0.2	0.4	0.3	0.0	0.4	0.0	0.5
T_{23}	1.3	2.9	1.2	2.1	1.4	1.8	1.4	1.8	0.0	1.6	0.0	1.5	0.0	1.5
T_{24}	0.9	1.6	0.9	1.1	1.0	0.8	1.0	0.9	0.8	0.9	0.0	0.9	0.0	1.0
T_{25}	1.5	4.6	1.3	2.6	1.3	1.9	1.4	1.9	0.0	1.7	0.0	1.7	0.0	1.6
T_{26}	1.2	2.0	1.0	1.3	1.1	1.4	1.2	1.4	1.0	1.3	0.0	1.4	0.0	1.3
T_{27}	1.2	2.1	1.2	1.5	1.2	1.6	1.4	1.6	0.0	1.5	0.0	1.5	0.0	1.4

(I) = Utilization starch (g/L). (II) = Biomass dry weight (g/L).
Note = Concentration of potato starch at initial incubation was 20 g/l.

2- Ethanol production

The results in Table 8 revealed that the ability of transformants to produce ethanol from starch (potato starch extract) in batch fermentations were differed. The concentration of raw potato starch decreased drastically during fermentation as the ethanol concentration increased. Some transformants produced high concentration of ethanol yield . This might be due to the ability both of α-amylase and glucoamylase activities to degrade the starch and release higher amount of glucose which could be incorporated into biomass production and ethanol yield. In contrast, some

transformants produced lower ethanol which may be due to the lower amount of glucose librated from starch degradation, reflected the lower activities both of α-amylase and glucoamylase enzymes. These results agree with those reported by Eksteen *et al.* (2003 b), who found that significant differences between recombinants of *S.cerevisiae* which containing α-amylase and glucoamylase genes to produce ethanol from soluble starch in batch fermentations. It appeared that ethanol production was depended on the hydrolysis of soluble starch by amylolytic enzymes and release glucose which converted to ethanol by constructed yeast strains.

TABLE 8. Yield of ethanol produced by transformants of *S. cerevisiae* grown on potato raw starch at batch fermentation.

Trans.	Fermentation period (h)													
	24 Yield		48 Yield		72 Yield		96 Yield		120 Yield		144 Yield		168 Yield	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
SF	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_1	1.1	0.0	1.2	1.5	1.1	1.3	1.1	0.9	1.2	1.5	1.2	0.0	1.1	0.0
T_2	0.9	0.0	0.8	1.3	0.7	1.0	0.8	1.6	0.9	4.3	0.9	0.0	0.9	0.0
T_3	1.1	0.0	1.0	1.3	1.0	0.8	1.0	1.7	1.1	3.5	1.1	0.0	1.1	0.0
T_4	1.4	0.0	1.4	1.8	1.3	2.0	1.3	0.7	1.4	0.0	1.4	0.0	1.4	0.0
SCA	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_5	0.5	0.0	0.6	1.3	0.7	1.6	0.8	1.8	0.9	1.5	1.0	0.8	1.0	0.0
T_6	1.1	0.0	1.3	1.8	1.3	2.4	1.2	1.3	1.3	1.1	1.3	0.0	1.3	0.0
T_7	0.8	0.0	1.1	1.7	1.1	1.6	1.2	0.9	1.3	0.8	1.3	0.0	1.5	0.0
T_8	0.7	0.0	0.9	1.1	0.9	1.4	1.0	1.8	1.2	1.6	1.2	1.4	1.1	0.0
T_9	1.3	0.0	1.5	2.3	1.6	2.6	1.5	1.0	1.7	0.4	1.7	0.0	1.8	0.0
T_{10}	1.0	0.0	1.2	1.2	1.2	1.7	1.3	0.6	1.3	0.6	1.3	0.0	1.4	0.0
LK	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_{11}	2.5	0.0	1.8	3.2	1.8	4.1	1.8	1.5	1.7	0.3	1.5	0.0	1.6	0.0
T_{12}	1.6	0.0	1.3	1.6	1.2	1.9	1.0	2.8	1.0	0.9	0.8	0.6	1.0	0.6
T_{13}	0.8	0.0	0.6	0.6	0.9	1.3	0.8	2.0	0.8	1.1	0.7	0.9	0.7	0.8
T_{14}	2.7	0.0	2.1	3.8	2.3	4.0	2.1	1.3	1.9	0.4	1.7	0.0	1.8	0.0
LS	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_{15}	1.4	0.0	1.2	3.0	1.2	2.4	1.2	1.1	1.0	2.5	1.1	3.5	1.2	0.0
T_{16}	2.7	0.0	2.8	10.0	2.7	7.0	2.5	7.1	2.0	0.0	1.9	0.0	2.0	0.0
T_{17}	2.8	0.0	2.3	11.0	2.4	7.6	2.4	1.6	1.9	0.7	1.9	0.0	2.1	0.0
T_{18}	0.7	0.0	0.7	1.5	0.7	1.4	1.0	0.7	0.8	1.8	0.7	4.5	0.8	0.0
T_{19}	2.7	0.0	2.4	7.0	2.4	6.4	2.3	1.1	2.1	0.6	2.9	0.0	1.8	0.0
SO	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T_{20}	1.1	1.3	1.3	1.9	1.3	2.1	1.3	1.0	1.4	0.6	1.5	0.0	1.6	0.0
T_{21}	0.7	0.8	0.9	1.4	1.3	1.9	1.3	1.2	1.5	0.9	1.6	0.0	1.4	0.0
T_{22}	0.0	0.0	0.0	0.1	0.2	0.1	0.3	0.5	0.3	1.2	0.3	9.5	0.4	0.0
T_{23}	1.1	1.5	1.4	1.9	1.6	2.2	1.6	1.5	1.8	1.1	1.7	5.0	1.6	0.0
T_{24}	0.7	0.0	0.9	0.7	0.9	1.5	0.9	1.3	1.1	1.0	1.0	1.5	0.9	0.0
T_{25}	1.0	1.9	1.3	2.5	1.4	1.5	1.4	1.2	1.6	0.0	1.7	0.0	1.7	0.0
T_{26}	0.9	0.0	1.1	0.9	1.2	1.4	1.3	1.2	1.4	0.9	1.4	0.0	1.4	0.0
T_{27}	1.0	0.0	1.3	1.1	1.5	1.7	1.4	1.1	1.5	0.7	1.5	0.0	1.5	0.0

(I) = Ethanol production (%). (II) = Total reducing sugar (g/L).
Note = Concentration of potato starch at initial incubation was 20 g/l.

6- Protein analysis using gel electrophoresis technique

The results summarized in Table 9 compared between amylolytic yeast strains and their transformants based on molecular weight of protein bands. The data revealed the polymorphic bands are common between amylolytic yeast strains and their transformants. There are one common band (94.5, 79.6 kD) appeared in each protein banding pattern of *Saccharomycopsis fibuligera* NRRL Y-2388 and their transformants. Also, there are four bands (74.4; 68.7; 55.5 and 43.9 kD) named a partial polymorphic bands. There are two common bands (94.6 and 74.3) appeared in each protein banding pattern of *Saccharomycopsis capsularis* NRRL Y-17963 with their transformants. Aso , there are fife partial polymorphic bands of molecular weight 79.4, 70.4, 65.5, 60.6 and 56.8 kDa. There are fife partial polymorphic bands of molecular weight 83.6, 81.2, 78.5, 68.2 and 44.7 kDa in *Lipomyces kononenkoae* NRRL Y-11553. But, there is one common band (10.6 kDa) found in each protein pattern of *Lipomyces starkeyi* NRRL Y-11557 and four partial polymorphic bands of molecular weight 68.7, 43.4, 37.8 and 22.7 kDa with their transformants. In addition, there are one common band appeared in each protein banding pattern of *Schwanniomyces occidentalis* NRRL Y-10 and two partial polymorphic bands of molecular weight (32.0, 21.7 kDa) with their transformants. These results are in agreement with those of Ibrahim *et al.* (2000), who found that three common band appeared in protein banding pattern of *S. fibuligera* and their modifying strains. Also, they found two common protein bands appeared in each protein banding pattern of *Saccharomycopsi* recipient . However, the two modified strains showed greater similarity with the recipient being 55.6 and 51.6 % respectively.

TABLE 9. Molecular weight of protein bands in amylolytic strains and their transformants harboring α-amylase gene .

Marker	SF	T_1	T_3	T_4	SCA	T_4	T_5	T_7	T_9	LK	T_{11}	T_{12}
92.5	94.5	94.3	94.5	94.3	94.6	96.4	97.3	97.1	96.2	97.7	98.0	84.3
78.0	90.5	90.3	81.3	91.1	91.9	82.7	82.9	83.2	83.3	83.6	84.5	78.7
62.5	81.2	88.1	79.4	79.4	80.5	80.3	79.6	79.6	79.9	81.2	80.8	67.8
45.5	79.6	84.6	74.3	74.2	74.7	74.7	75.2	77.4	75.8	78.5	78.6	41.0
36.0	74.4	81.0	68.2	70.4	65.2	71.9	71.2	73.3	71.8	75.6	69.5	28.2
28.0	68.7	79.7	54.5	65.5	60.6	67.5	65.5	67.1	66.3	68.2	61.7	19.3
21.2	55.5	69.4	44.9	56.8	56.5	61.1	57.9	60.3	59.6	62.4	57.4	15.7
16.7	43.9	57.4		44.7		46.2	53.4	53.5	55.5	57.2	43.4	11.2
		42.2					44.1	45.7	44.4	44.7		

T_{14}	LS	T_{16}	T_{17}	T_{19}	SO	T_{20}	T_{21}	T_{23}	T_{25}
88.2	90.4	89.8	84.1	79.8	79.1	76.4	84.8	84.3	78.8
80.6	68.7	80.0	76.1	68.7	42.1	51.6	79.0	79.4	65.3
71.2	62.8	66.7	65.7	37.0	32.0	22.5	42.4	68.7	44.0
45.1	43.4	60.1	40.9	31.0	24.7	18.1	34.0	33.8	31.1
36.2	37.8	44.1	32.4	25.4	21.7	14.5	29.1	31.8	21.4
22.9	22.7	15.5	20.5	19.1	17.5	10.5	21.1	27.0	15.2
15.1	16.2	10.6	18.0	13.6	10.9		14.6	14.7	11.0
11.0	10.3		15.7	10.7			10.6	10.9	
			11.3						

The results presented in Table 10 and Fig. 3 clarified the similarity and genetic distance resulted from genetic transformation processes. The similarity between *S. fibuligera* NRRL Y-2388 and their derivative transformants was 54.5, 66.7 and 60.0. On the other hand, the genetic distance ; 45.5, 33.3 and 40.0. While the similarity degree between *S. capsularis* NRRL Y-17963 and their derivative transformants was 36.4, 33.3, 23.1, and 45.5 and the genetic distance was 63.6, 66.7, 76.9, and 54.5. The similarity degree between *L. kononenkoae* NRRL Y-11553 and transformants derivative was 88.9, 21.4 and 13.3 and the genetic distance was 11.1, 78.6 and 86.7. The similarity degree between *L. starkeyi* NRRL Y-11557 and transformants derivative was 36.4, 13.3, and 23.1, although the genetic distance was 63.6, 86.7 and 76.9. The similarity degree between *Schw. occidentalis* NRRL Y-10 and transformants derivative was 30.0, 36.4, 25.0 and 40.0 and the genetic distance was 70.0, 63.6, 75.0 and 60.0. These results are in harmony with those of Ibrahim *et al.* (2000) .

TABLE 10. The similarity and genetic distance between amylolytic yeast strains and their transformants based on protein patterns.

Yeast strains	Total no. of protein bands	Genetic distance (%)	Similarity (%)	Yeast strains	Total no. of protein bands	Genetic distance (%)	Similarity (%)
SF	8	0.0	100	T_{12}	8	78.6	21.4
T_1	9	45.5	54.5	T_{14}	8	87.7	13.3
T_3	7	33.3	66.7	LS	8	0.0	100
T_4	8	40.0	60.0	T_{16}	7	63.6	36.4
SCA	7	0.0	100	T_{17}	9	86.7	13.3
T_5	8	63.6	36.4	T_{19}	8	76.9	23.1
T_6	9	66.7	33.3	SO	7	0.0	100
T_7	9	76.9	23.1	T_{20}	6	70.0	30.0
T_9	9	54.5	45.5	T_{21}	8	63.6	36.4
LK	9	100	100	T_{22}	8	75.0	25.0
T_{11}	8	11.1	88.9	T_{23}	7	60.0	40.0

Note Similarity was calculated between the donor strains against transformant isolates.

This result achieved different degrees of expression. Some transformants showed the amplification in protein expression as shown in lans T_1, T_{11}, LS, T_{16}, T_{17}, T_{21} and T_{25}. This may due to over expression of protein amylase enzyme under the effect of starch as a sole source of carbon for yeast transformants.

In conclusion, *S. cerevisiae* transformants were able to secrete extracellular α-amylase with different levels of expression leading to differences in starch utilization. Some transformants showed much slower starch utilization, while others were relatively fastest in starch utilization. This leading to recycl starch resulting from some industries in a trial to reduce its polluting effect in the environment , especially , the yeast *Saccharomyces cerevisiae* is safe eukaryotic microorganism with well established fermentation technology for large-scale production.

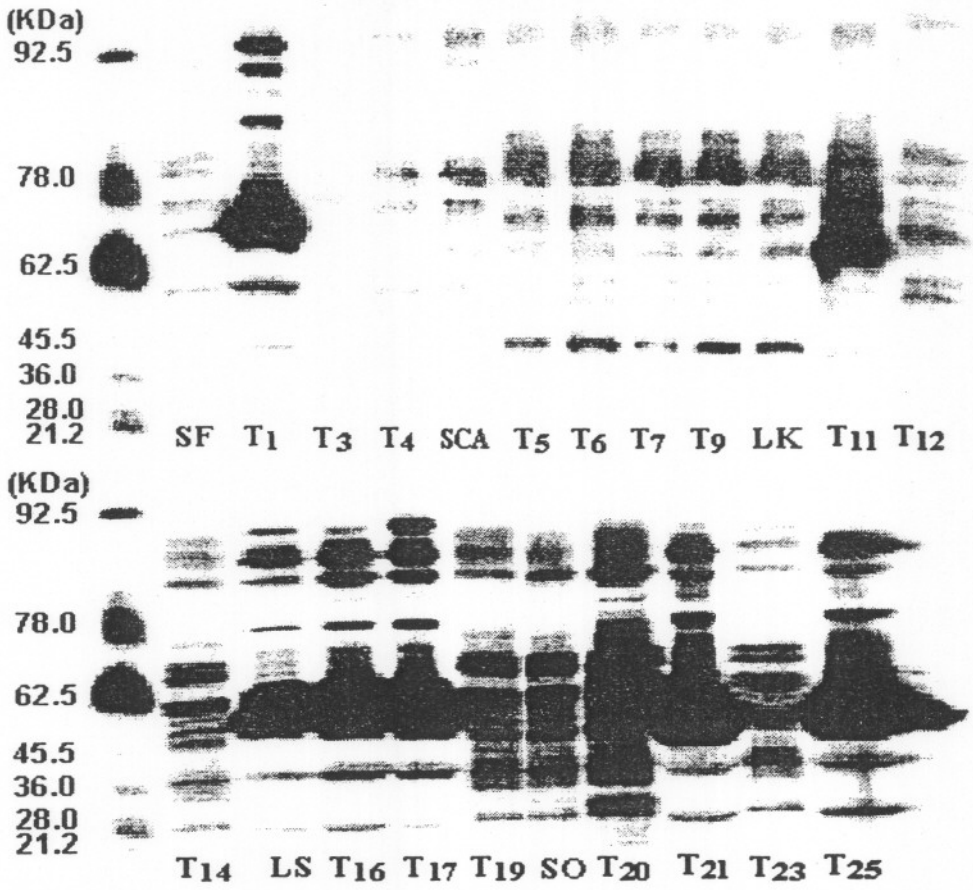

Fig. 3. SDS-PAGE of amylolytic yeast strains and their transformants based on protein patterns, SF: *Saccharomycopsis fibuligera*; T_1, T_3, T_4 transformants, SCA: *Saccharomycopsis capsularis*; T_5, T_6, T_7, T_9 transformants and LK: *Lipomyces kononenkoae*; T_{11}, T_{12}, T_{14} transformants; LS: *Lipomyces starkeyi*; T_{16}, T_{17}, T_{19} transformants SO: *Schwanniomyces occidentalis*; T_{20}, T_{21}, T_{23}, T_{25} are transformants.

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التعديل الوراثى لسلالات من خميرة الخباز بجين الألفا أميليز

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تم فى هذا البحث إجراء عملية التحول الوراثى لأربعة سلالات من خميرة الخبازعن طريق نقل جين الألفا أميليز من خمسة سلالات من الخمائر ذات كفاءه عالية لإفراز إنزيم الألفا أميليز و القادرة على تكسير النشا وتم ذلك باستخدام طريقتى كلوريد الكالسيوم و البولى إثيلين جليكول . أظهرت النتائج إنخفاض عدد المتحولات الوراثية كدليل على إنخفاض نسبة التحول الوراثى باستخدام طريقة كلوريد الكالسيوم إلا أن نسبة التحول الوراثى لطريقة البولى إثيلين جليكول كانت أعلى حيث بلغت حوالى ٤٪ بالمقارنة بكلوريد الكالسيوم التى بلغت حوالى٢,٧ ٪. بينما تميزت طريقة التحول الوراثي بإستخدام كلوريد الكالسيوم عن طريقة التحول الوراثي بإستخدام البولى ايثيلين جليكول فى عدد التحولات الوراثية الناتجة. وبالإضافة إلى ذلك كانت كل تجارب التحول الوراثى للسلالة المستخدمة كمصدر لجين الأميليز ناجحة عند استخدام كل من كلوريد الكالسيوم و البولى ايثيلين جليكول مما يرجع الى سهولة إنتقال جين الأميليز عبر جدر الخلية وأيضا ثبات الإنقسام الميتوزى لقطعة DNA الحاملة لجين الأميليز . أظهرت عزلات الخميرة المحولة وراثيا تباينا واضحا فى مقدرتها على النمو على بيئة النشا مما يرجع الى إختلاف التركيب الوراثى لها كما أظهرت أيضا إختلافات معنوية فى وزن الخلايا الغض و فى مقدرتها على الثبات الوراثى لجين الأميليز . أظهرت كل عزلات خميرة الخباز المحولة وراثيا تباينا فى قدرتها على التعبير الجينى لإنزيم الألفا أميليز عند نموها على بيئة النشا ويرجع ذلك إلى عدد نسخ جين الأميلز المندمجة مع DNA للسلالات المحولة وراثيا. كما أظهرت إختلافات معنوية فى حجم halo zone عند نموها على بيئة أجار النشا الصلبة مما يدل على إختلاف مستويات التعبير الجينى لإنزيم الألفا أميليز كما أنها قادرة على النمو على البيئات المحتوية على النشا سواء كانت صلبة أم سائلة. أظهرت المتحولات الوراثية إختلافات معنوية فى نسبة تكسير مخلف البطاطس النشوى الخام . توجد علاقة موجبة بين إنتاج الإيثانول من مخلف البطاطس النشوى ومعدل النمو والتعبيرالجينى لكل من الألفا أميليز والجلوكو أميليز وكمية الجلوكوز الناتجة. أظهرت سلالات الخمائر المحولة وراثيا إختلافات معنوية فى الوزن الجزيئي للبروتين و فى درجة التشابه و المسافات الوراثية بينها وبين السلالات الأبوية .