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# Induction of low-temperature tolerant yeast mutants by chemical mutagens and evaluation of their performance

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ABSTRACT

Yeasts have been used for thousands of years to make fermented foods and bread. Therefore, it was necessary to improve its fermentation ability at low temperatures and used in making bread process during the winter without the need to provide certain conditions. Therefore, we have inducing yeast mutants that tolerate low temperatures below 4°C. In this study, a total of 64 yeast isolates were isolated from five food samples and two commercial yeast: Mango juice (N=13), Strawberry juice (N=12), sugarcane juice (N=9), Milk (N=8), Cheese (N=9), Compressed yeast (N=6) and powdered yeast (N=7). The results showed that, 14 out of 51 yeast isolates which isolated from food samples are growing rapidly on YPD medium at 10 °C. Three mutants out of eight mutants CH1EMS, CH2EMS and CH3EMS were able to tolerate of low temperature. Fermentations were performed at 4, 6, 8 and 10 °C, using different mutants yeast induced by Ethyl Methane Sulfonate (EMS). In the present work randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) with six primers was applied to determine the genetic similarity between 10 yeast mutants and one yeast wild type. RAPD-PCR analysis of the different yeast isolates gave distinctive band profiles that allowed a clear differentiation of all the considered isolates.

## 1. Introduction

Low temperature is one of the most important environmental stresses that influences the life and distribution of living organisms. In the yeast, reductions in environmental temperature have widespread effects on growth and survival. At low but permissible temperatures (10-18ºC), metabolic activity and growth rates decrease. This applies to the industrial exploitation of yeast, as the fermentation process and some dough fermentation processes take place at about 10-18ºC during the winter. Therefore, it was necessary to obtain genetically improved yeast strains that have the ability to withstand low temperatures and use them in the processes of manufacturing bread. Yeasts are variable temperature organisms, so their lives depend on the ambient temperature [1]. Generally, low temperature increases the production of glycerol, ethyl esters and fatty acids and decreases the formation of acetic acid, acetate esters, and higher alcohols [2]. These compounds are formed through intricate and dynamic metabolic processes during fermentation, which is intimately associated with yeast metabolism [3]. However, researches associated with the influence of low temperature on the metabolite formation of Saccharomyces cerevisiae are mainly based on the lower temperatures (15–20ºC) and higher temperature (25–30ºC) [2,4]. Temperature is one of the important factors affecting the growth of S. cerevisiae, the optimum temperature for growth is around 30°C [5]. At low temperatures (1-10ºC), the cells are viable but they do not grow well [6]. The common uses of S. cerevisiae are for bread rising, brewing beer, and fermenting wine, which depend on the ability of fermentation of S. cerevisiae. According to Babiker [7], the temperature is controlled to grow the yeast at optimum level, since the metabolic pathway of S. cerevisiae produces heat. This production of heat could affect the fermentation process. Since this particular strain of yeast is widely used in bread and wine making industries, the effect of temperature can be very useful in producing better quality goods. The expression of genes of S. cerevisiae can be altered by heat stress, which induces protein folding that ultimately affects the growth [8].

Temperature is one of the main relevant environmental variables that microorganisms have to cope with. It has an influence on the life and distribution of nearly all microorganisms. In addition, temperature is also a key factor in some industrial processes involving microorganisms, such as the yeast species [9]. In the yeast S. cerevisiae, reductions in ambient temperature have several effects on the biochemical and physiological performance of cells: poor protein translation efficiency; slow protein folding; lower membrane fluidity; a reduction in oxygen solubility; changes in lipid composition; changes in nutrient uptake, transport and consumption; an increase in the biosynthesis of some protective compounds; and a reduction in the rate of

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biochemical reactions [10]. The effects of low temperatures on the growth and survival of yeast cells depend on the severity of the stress. At 10–18ºC, metabolic activity and growth rates decrease. When the temperature falls further, near freezing (0–4ºC), yeast cells could trigger series of biochemical reactions to respond and adapt to the stress, protect them against sever cold and freeze injury [11]. However, most studies have focused mainly on the adaption of S. cerevisiae at low but still permissive temperatures (10–18ºC), there have been limited studies on the temperature below 10ºC. The low-temperature fermentation technologies of compelling interest are currently as follows: preparing cooled dough for shaping; preparing cooled shaped dough pieces for proofing [12]. At present, the optimal technological parameters for dough making (temperature and duration of fermentation) have not been determined [13, 14], and their effect on the properties of semi-finished products and the quality of the finished rich bakery products was not assessed [13]. This study aimed to obtain different mutants industrial yeast that has ability to withstand low temperatures and have high fermentation efficiency under low temperature. As well as genetically evaluating of these mutants resulting from different treatments, also determine the genetic similarity between mutants yeast and wild type using the randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR).

## 2. Materials and methods

### 2.1. Chemical and buffers

Ethyl Methane Sulfonate (EMS) (1mg/ml) was procured from Fisher scientific, UK, whereas chemical used in different media were pushed from Hi-media, India. PCR buffer (10x), master mix and RAPD primers were procured from GeneDirex, Inc.

### 2.2. Collection of samples

Five samples from each food source; sugarcane juice, strawberry juice, mango juice, milk and cheese were collected from the local markets in Fayoum governorate, Egypt. Samples were collected in March 2021.

### 2.2. Isolation of yeasts from collected samples

Ten g of each solid sample was suspended in 90 ml of sterile water and shaken at 200 rpm for 30 min under room temperature environment. The suspensions were then serially diluted ($10^{-1}$–$10^{-10}$) and plated on Yeast Peptone Dextrose (YPD) agar plates and cultivated at 28 ºC for 48-72 h. The culture plates were examined daily and colonies showing different morphologies were randomly peaked and streaked on YPD agar and further stored at 4°C for further experiments [15].

### 2.3. Phenotypic characterization of low-temperature tolerance for yeast isolates

The low temperature tolerance of yeast isolates was characterized by growth on plates using the methods reported by Yang [16] with some modifications. Yeast isolates were inoculated in 5 mL YPD medium and grown at 28◦C for 24 h. Then, 200 µL pre-culture were inoculated in 5 mL YPD and cultured at 28◦C, afterward, 5 µL of each serial dilution of the cell cultures was streaked onto agar plates containing YEPD agar for checking the effect of low temperature on the growth. The plates were incubated at 28, 25, 23, 20, 17, 15, 13 and 10 ºC for 48 hrs. After 48 hrs plates were observed for colony formation and the extent of growth was recorded.

### 2.4. Morphological and biochemical characteristics of low temperature tolerance yeast isolates

Morphological and biochemical studies of low-temperature for yeast isolates were carried out as given by Martini and Martini [17] and species was identified as given by Barnett [18], the yeast isolates were tested for colony morphology, textures (mucoid, fluid or viscous, butyrous), elevation (flat or raised), color (yellow, orange and red), surface (glistening or dull, smooth, rough and sectored), margin (entire, undulating, lobed and filaments) and spore staining. Starch hydrolysis, nitrate reduction, ammonia from urea, fermentation of carbohydrates.

### 2.5. Induction of yeast cells for ascospores formation

Yeast sporulation was performed according to Parts [19]. The cultures were grown overnight and plated on acetate agar medium contained, 2.5% yeast extract, 1% dextrose, 10% potassium acetate and 3% agar, pH 6.5 and the plates were incubated at 28ºC for 6 days. When the sporulation efficiency has reached >90%, cells were collected and resuspended in sterile water and treated with an equal amount of ether to kill unsporulated cells. After 6 days cells were examined microscopically under 40 x and 100 x objectives for formation of ascospores. The percentage of cells containing ascospores was determined for each select isolates.

### 2.6. Mechanical disruption of ascus and isolation of haploid spore

Glass bead vortexing probably the most widely used method is disruption of yeast cells by agitation with glass beads (0.4 – 0.5 mm). The simplest method for agitating in glass beads is with the use of a vortex mixer. Several cycles of agitation (60 sec) must be interspersed with cycles of cooling on ice to avoid overheating of the cell suspension. The resultant suspension is shaken with sterile paraffin oil. The latter then is separated by centrifugation. The spores, because of their lipophilic properties, are taken up in the oil, while undamaged cells and cell debris remain in the aqueous phase. The oil suspension is streaked on a suitable agar medium in order to obtain single-spore colonies (usually haploids). Single colonies which obtained in complete medium transferred in sporulation medium to exanimate their own ploidy genome (diploid or haploid). Single colonies were re-plated and tested for mating type. The haploid colonies streaked in slant of complete medium and stored in refrigerator at 4 ºC for further tests.

2.7. Induction of yeast mutants by Ethyl Methano Sulphate (EMS)

Chemical mutagenesis was carried out according to the method of Morikawa [20]. Appropriate dilutions 50, 100,150 µ/ml were prepared from EMS solution (1mg/ml) in 0.2M sodium phosphate buffer, pH 7 and then 30 ml of yeast haploid isolate was treated with each concentration of EMS and shaked at 30 ºC for zero, 30, 60, 90 or 120 min. At intervals, 0.1 ml of the suspension was withdrawn and added to 9.9 ml of 3% sodium thiosulfate solution to stop the effect of EMS. The treated isolates were washed three times in centrifuge at 5000 rpm for 10 min with %3 sodium thiosulfate solution. 1ml of treated and untreated strains suspensions were spread separately on the surface of YPD medium in petri plates and incubated at 28 ºC for 28 – 72 hr. After incubation, colony developed from single colony was transferred on fresh YPD medium and incubated at 4 ºC for further studies.

2.8. Construction of diploid mutant yeast from haploid mutant yeast isolates

2.8.1. Diplodization

The diploid yeast isolates were constructed from different haploid mutant's isolates according to the method of Anthony and Louis [21]. An inoculum of approximately $10^6$ of the opposite mating types (α and a - mutant isolates) were mixed and incubated in 10 ml of conjugation medium using a shaking incubator at 30 ºC for 24 hr. The magnesium salt is necessary in conjugation medium in order to keep the cell agglutinated.

2.8.2. Isolation of diploids yeast mutants isolates

A 0.1 ml from each cross between α and a - mutant strain was plated on complete medium and incubated at 30 ºC for 3 days. Colonies appeared on complete medium should be tested for ploidy. The single colonies were suspended in sterile distilled water and 0.1 ml cell suspension was plated on presporulation medium at 30 ºC for 24 hour. The single colonies appeared were transferred to sporulation medium and incubated at 30 ºC for 15 days. Sporulation indicates that they are diploids and diploid colonies were grown on YEP medium and kept for further investigation.

2.9. Estimating the fermentative ability of diploid yeast mutants' at low temperature

Diploid mutant yeast isolates were cultivated on YPD liquid medium for 48 – 72 h and then the cell pellets were collected by centrifugation. Fermentation was started by addition of sugar solutions to cell pellets and resuspended prior to insert durham tubes to collect any gas produced during fermentation. The fermentation abilities were inspected at frequent intervals for accumulated gas in the Durham tubes, as well as changes in indicator (phenol red) color were investigated according to Kurtzman [22].

2.10. Genetic similarity between mutant and wild type of yeast isolates

2.10.1. Genomic DNA isolation from yeast isolates

Genomic DNA was extracted from wild type and mutant yeast isolates according the method described by Tillett and Neilan [23]. The quantified DNA were electrophoresed on a 1% agarose gel in 1x TAE buffer containing 0.5 µg/mL of ethidium bromide and visualized under UV light and stored at -20°C until further processing of PCR amplification of RAPD-PCR analysis.

2.10.2**.** Random Amplified polymorphic DNA-PCR (RAPD-PCR)

A total number of six primers (OPA-09, OPA-10, OPC-06, OPC-07, OPC-08 and OPC-09)) were used in present study to identify the one wild type and ten mutants of yeast isolates. The sequence of the six primers was presented in Table (1). The PCR reaction mixture (25µl) contained 12.5ml Master Mix (onePCRTM), 3 µl of Primer, 2 µl of template DNA preparation and 7.5 µl of H2O2. PCR conditions were as follows; one cycle of initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation for 1 min at 94 °C, annealing primer for1 min at 37 °C and extension for 1 min at 72 °C, and one cycle for final extension step at 72 °C for 10 min using thermal cycler 2720 (Applied Biosystems, USA). PCR products were separated in 2% agarose gels. Standard molecular size markers (100- bp ladder and 1-kb ladder) were used to estimate the sizes of PCR generated fragments. Bands were detected and molecular fingerprints were analyzed using the gel documentation. RAPD-PCR fingerprinting patterns were carried out with Computer assisted analysis using RAPD software package, version 1.4. Similarity of the band profiles was based on Excoffier matrix [24]. The correlation coefficient was used to compare the number of the DNA patterns obtained. The clustering of the strains was determined by the UN weighted Pair Group Method using Arithmetic Average (UPGMA).

**Table 1:** Nucleotide sequence of random primers used for RAPD analysis.

| Number | Primer code | Primer sequences (5` – 3`) |
|---|---|---|
| 1 | A-09 | -5` GGGTAACGCC 3`- |
| 2 | A-10 | -5` GTGATCGCAG 3`- |
| 3 | C-06 | -5`GAACGGACTC 3`- |
| 4 | C-07 | -5` GTCCCGACGA 3`- |
| 5 | C-08 | -5` TGGACCAATG 3`- |
| 6 | C-09 | -5` CTCACCGTCC 3`- |

## 3. Results and discussion

### 3.1. *Isolation and morphological identification of yeast isolates*

The yeast isolates were characterized by distinctive morphological characters that includes rapid, colony appearance and pigment. A total of 64 isolates of yeast were isolated from the collected samples. 6 isolates from compressed yeast, 7 isolates from powdered yeast, 13 from Mango juice, 12 from Strawberry juice, 9 from sugarcane juice, 8 from Milk and 9 from Cheese.

### 3.2. *Phenotypic characterization of low-temperature tolerance for yeast isolates*

The effect of temperature on growth of yeast isolates is shown in Table (2). The results showed that, all the 24 yeast isolates are growing rapidly on YPD medium at 28, 25, 23, 20, 17 and 15 ºC. At low temperature up to 13 ºC, 17 yeast isolates, St1, M1, M2, St2, CH1, ML1, St5, ML2, CH2, S1, MD3, ML4, CH3, S2, S3, M3 and M4 have shown good growth. Whereas 7 isolate, St1, M1, M2, CH1, ML1, St5 and ML2 have shown good growth up to 10 ºC. The results in Table (2) showed that, the isolates of yeast which isolated from samples are growing rapidly on YPD medium at low temperature up to 10 ºC. On the other hand the commercial compact yeast which obtained from local markets and used as control in our study were failed to grow at low temperature under 15 ºC. It was also noted that all isolates failed to grow at 8 ºC (Table 2). According to the results obtained in Table (2), 8 yeast isolates (CH 1, CH 2, CH 3, ML 2, M 2, M 3, St 2 and MD 4) out of 13 yeast isolates that had good growing at low temperature 10 ºC were selected for further study (Table 3).

Table 2: Ability of yeast isolates to tolerate different temperature

| Isolates | source | Temperature ( ºC) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 28 °C | 25 °C | 23 °C | 20 °C | 17 °C | 15 °C | 13 °C | 10 °C | 8 °C |
| St 1 | Strawberry juice | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | - |
| St 2 | Strawberry juice | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | - |
| St 3 | Strawberry juice | +++ | +++ | +++ | +++ | +++ | + | - | - | - |
| St 4 | Strawberry juice | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | - |
| M 1 | Mango juice | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | |
| M 2 | Mango juice | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | - |
| M 3 | Mango juice | +++ | +++ | +++ | +++ | +++ | - | - | - | - |
| M 4 | Mango juice | +++ | +++ | +++ | +++ | ++ | - | - | - | - |
| M 6 | Mango juice | +++ | +++ | +++ | +++ | ++ | - | - | - | - |
| CH 1 | Cheese | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | - |
| CH 2 | Cheese | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | - |
| CH 3 | Cheese | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | - |
| ML 1 | Milk | +++ | +++ | +++ | +++ | +++ | ++ | + | + | - |
| ML 2 | Milk | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | - |
| ML 3 | Milk | +++ | +++ | +++ | +++ | +++ | + | - | - | - |
| ML 4 | Milk | +++ | +++ | +++ | +++ | +++ | + | - | - | - |
| ML 5 | Milk | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | - |
| S 1 | sugarcane juice | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | - |
| S 2 | sugarcane juice | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | - |
| S 3 | sugarcane juice | +++ | +++ | +++ | +++ | +++ | + | + | + | - |
| MD 1 | Compressed yeast | +++ | +++ | +++ | ++ | + | - | - | - | - |
| MD 2 | Compressed yeast | +++ | +++ | +++ | ++ | + | - | - | - | - |
| MD 3 | Compressed yeast | +++ | +++ | +++ | + | + | - | - | - | - |
| PW 1 | Powdered yeast | +++ | +++ | +++ | + | + | - | - | - | - |

(+++) High growth (++) Moderate growth (+) Low growth (-) No growth

**Table 3:** The yeast isolates showed good tolerance for growth at low temperature (10 ºC).

| No. | Isolates | Source |
|---|---|---|
| 1 | St 1 | Strawberry juice |
| 2 | M 1 | Mango juice |
| 3 | M 2 | |
| 4 | CH 1 | Cheese |
| 5 | CH 2 | |
| 6 | CH 3 | |
| 7 | ML 2 | Milk |
| 8 | S2 | Sugarcane juice |

Charoenchai [25] conducted an experiment of the effect of temperature on the cell biomass of S. cerevisiae. They found, the cell biomass increased within the optimal temperature range. Psychrophilic microorganisms have a maximum temperature for growth of 20 °C or below and are restricted to permanently cold habitats, whereas psychrotrophic microorganisms have maximum temperatures for growth of more than 20 °C. Growth at low temperatures is often associated with thermolability [26]. Such microorganisms can have slower metabolic rates and higher catalytic efficiencies than mesophiles, making them considerably interesting for biotechnological applications [27]. Some cryotolerant strains with good adaptation to low temperature belonging to Saccharomyces species (Saccharomyces uvarum, Saccharomyces kudriavzevii, and Saccharomyces eubayanus) can be used in industrial fermentation processes [28].

### 3.3. *Morphological and biochemical characteristics of low-temperature tolerance yeast isolates*

The eight selected yeast isolates were further used for identification studies. Colonies formed by yeast isolates were round, smooth and cream, white to whitish cream colored. Individual cells were oval, elongate, ovoid to spherical when young and hexagonal when aged. Cells showed oval, globose, spherical and ellipsoidal budding. Also all 8 yeast isolates did not show, true mycelium (Figuer 1). Glucose, Fructose and sucrose were fermented and assimilated by all isolates except ST2. All the 8 yeast isolates were fermented soluble starch (Table 4). Moneke [29] isolated six morphologically different yeast strains from orchard soil of Nigeria, which were identified as Saccharomyces yeasts by adopting similar methodology. Similar isolation and identification of yeasts was carried out by Brooks [30] in which eight yeasts were isolated from ripe banana peels and five were subjected to identification studies.

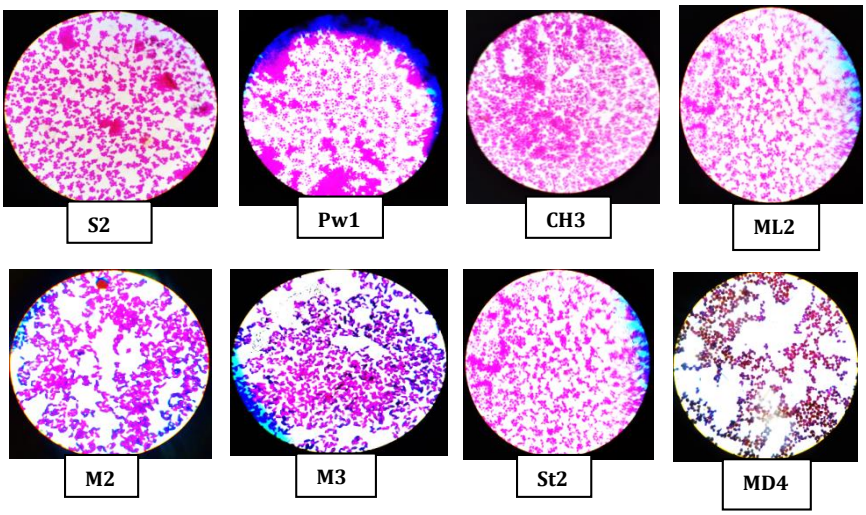


**Fig.1.**The image represents the microscopical observation of yeast isolates**.**

**Table 4:** Biochemical characteristics of low-temperature tolerance yeast isolates

| Characteristics | Yeast isolates | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | St 1 | M 1 | M 2 | CH 1 | CH2 | CH 3 | ML 2 | S2 |
| Glucose | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Fructose | - | w | w | +++ | + | +++ | + | +++ |
| Sucrose | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ |
| Starch Soluble | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Magnesium nitrate | +++ | ++ | + | ++ | +++ | +++ | +++ | ++ |

(+++) High growth (++) Moderate growth (+) Low growth (---) No growth. (W) Weak response

### 3.4. *Induction of yeast cells for ascospores formation*

Yeast isolates were tested for their ability to sporulate on sporulation medium. Growth scoring and microscopial examinations were carried out daily for a period of 10 days. Growth was obtained at 28°C. The yeast isolates was formed asccus on sporulation medium (Figure 2). This result indicates that all isolates was diploid (2n). The plates were incubated for 48 – 72 hr at 28°C for growth. After incubation, colony developed from single colony was transferred on fresh YPD medium and incubated at 4°C. Diploid cells of S. cerevisiae modify their growth in response to nutrient availability. In the presence of nutrients they grow in budding form. The presence of a poor nitrogen source such as proline will trigger the onset of mitotic growth in a pseudohyphal form [31]. The complete absence of nitrogen, and the presence of a non-fermentable carbon source such as acetate, causes the cells to exit the mitotic cycle, undergo meiosis, and sporulate [32]. When triggered to enter the sporulation program, cells exit the mitotic cycle from the G1 phase. This is followed by premeiotic DNA synthesis and entry into the meiotic divisions. As with mitosis in yeast, meiosis is “closed”; that is, it takes place without breakdown of the nuclear envelope. The spindle microtubules are nucleated from the nuclear face of spindle pole bodies (SPBs) that span the nuclear envelope [33].

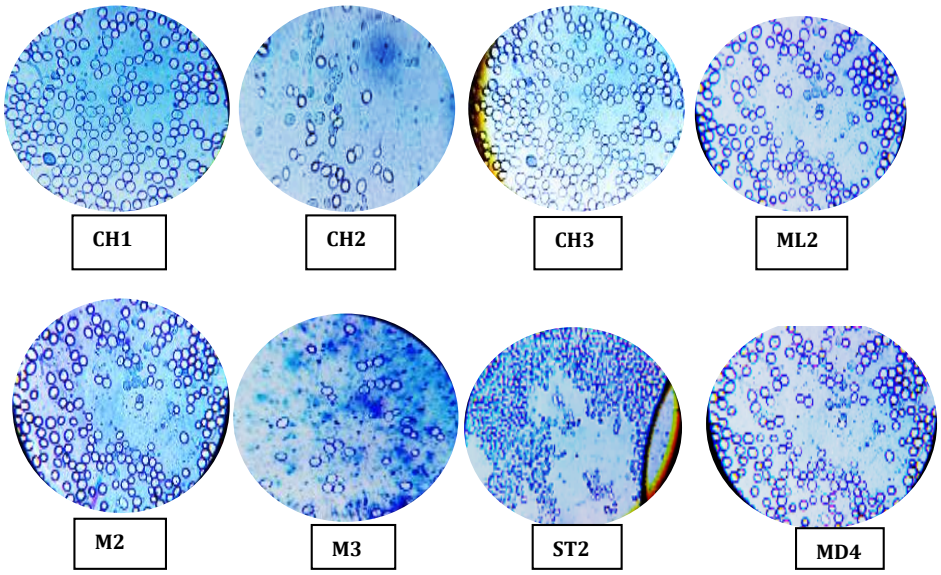


**Fig. 2.** The image represents the sporulation of yeast isolates

3.5. *Induction of yeast mutants by Ethyl Methano Sulphate (EMS)*

In mutagenesis induced using EMS, the viability of yeast cells was determined by total count method. Cell viability decreased with increasing exposure time of EMS. The viabilities at 0, 30, 60, 90 and 120 min. of EMS treatment were 100%. 85, 76, 48 and 0 %, respectively. This decreased to 0% after 120-min exposure. On the basis of viability variation at different time points, we assumed that EMS affected yeast cells actively, and nearly 48% of yeast cells survived and EMS mutagenized the cells after 90 min. Then, we used this yeast suspension to isolate tolerant mutagenized cells because their cells viability was suitable for tolerant mutant screening. The results showed that, mutants were induced by EMS. Among many mutagenized cells of yeast, only three mutants (2 mutants obtained from CH1 and CH2 yeast haploid isolate were induced by EMS for 30 min and 90 min respectivelly, and one mutant obtained from CH3 yeast haploid isolate) showed the ability to grow on medium incubated at low temperature 4°C (Table 5). Although the growth of wild-type cells was inhibited in the low temperature 4°C, mutant cells did not grow in medium incubated under 4°C. After incubation, colony developed from single colony was transferred on fresh YPD medium and incubated at 4°C.

EMS, an alkylating agent, is commonly used as a chemical mutagen for DNA lesions and induces base changes or nucleotide substitution, which consequently alter codon sequences, leading to either nonsynonymous or synonymous effects also and induces a biased spectrum of G/C-to-A/T transitions and these transitions occur due to the alkylation at the O6 or N7 position of guanine, which leads to the replacement of cytosine with thymine base pairing [34]. EMS is a mutagenic agent that induces point mutations in a DNA molecule by A-T transition to G-C. In the presence of EMS, native sequences of affected genes are changed and their related products are modified structurally, causing inactivation of functional proteins [35]. Wahlbom [36] reported that EMS is a suitable mutagen for related purposes. In addition, French [37] reported that EMS is a powerful chemical mutagen and its effect on cells is related to its concentration in a medium. Our findings about mutagenesis induced by EMS were in good agreement with the above reports and confirmed the usefulness of this potent mutagenic agent for inducing mutagenesis in yeast. The reason for the ability of a few yeast mutant cells to grow at low temperature may be the presence of an additional tolerance mechanism(s) or mutations in their nonvital genes [38].Therefore, the majority of EMS-mutagenized cells were dead under this condition.

Table 5: Growth of yeast mutants induced by different concentrations of EMS (min) and incubated at 4°C.

| | Growth of yeast mutants at 4°C | | | | |
|---|---|---|---|---|---|
| Yeast mutants | 0 min | 30 min | 60 min | 90 min | 120 min |
| MD 4 | - | - | - | - | - |
| St 2 | - | - | - | - | - |
| M 2 | - | - | - | - | - |
| CH 1 | - | + | - | + | - |
| CH 3 | - | - | - | - | - |
| ML 1 | - | - | - | - | - |
| M 3 | - | - | - | - | - |
| CH 2 | - | + | - | - | - |

(+) growth (-) No growth

3.6. *Construction of diploid mutant yeast from haploid mutant yeast isolates*

Our strategy for construction of homozygous diploid cells is shown in Table (6), all the cross between haploid mutant isolates MD4, St2, M1, CH1, ML2, CH2, M2 and CH3 were done to detect the mating type for each haploid mutant yeast isolate. The results showed the 5 isolates (MD4, St2, M2, CH1 and CH 3) had ɑ- mating type, whereas the three isolates (ML2, M3 and CH2) had a- mating type.

The yeast sexual cell types are designated a and ɑ, which are conferred by the MATa and MATTɑ- alleles of the Mating-Typ Locus (MAT), respectively [39]. In general, homozygous diploid mutant strains are constructed by crossing strains of the opposite mating-type, which need to be constructed individually. When the two haploids have different prototrophic or antibiotic resistance markers, the diploids can be easily selected on plates lacking both nutrients or containing both antibiotics because auxotrophy or antibiotic sensitivity are complemented by each genotype. The HO endonuclease, which mediates mating-type switch, can be used to obtain diploids via mating of MATa and MATɑ cells within colonies [40]. Alternatively, zygotes (dumbbell-shaped cells) can be isolated by micromanipulation during conjugation of two cells. However, these methods are unsuitable for large-scale analysis. Thus, there has been no easy way to construct and select diploid strains from single haploids at high throughput so far.

**Table 6:** Determination of yeast mating type by different crossing between ɑ- and a- haploid isolate

| Yeast haploid Isolates | SU 4 | St 2 | M 1 | CH 1 | ML 2 | CH 2 | M 2 | CH 3 |
|---|---|---|---|---|---|---|---|---|
| SU 4 | - | - | + | - | + | + | - | - |
| St 2 | - | - | + | - | + | + | - | - |
| M 1 | + | + | - | + | - | - | + | + |
| CH 1 | - | - | + | - | + | + | - | - |
| ML 2 | + | + | - | + | - | - | + | + |
| CH 2 | + | + | - | + | - | - | + | + |
| M 2 | - | - | + | - | + | + | - | - |
| CH 3 | - | - | + | - | + | + | - | - |

3.7. *Estimating the fermentative ability of diploid yeast mutant isolates under low temperatures*

To analyze the effect of low-temperature fermentation of dough on properties of dough, its acidity and moisture content, shape stability of dough pieces, and the duration of their proofing were assessed. The acidity of the dough pieces increased during fermentation under low temperature conditions, which was related to the formation and accumulation of a number of organic acids (lactic, acetic, succinic, malic, etc.). The moisture content in all dough pieces was similar in value to that in the control. It was found that the shape stability of dough pieces during fermentation decreased within the whole temperature range. Proofing of dough pieces was required only after 2-hour fermentation at 4 °C; the rest of the samples were not proved due to spreading of dough, and dough pieces were immediately sent for baking. The optimal time of dough fermentation at 4 °C was found to range within 2–3 h. After baking, we assessed physical and chemical properties (acidity, moisture content, porosity, shape stability, aromatic content and sugar mass fraction), and organoleptic quality indicators of the bakery products. The minimum shape stability was found for bakery products made from dough fermented for 4°C. The organoleptic evaluation of bakery products showed that products made from dough fermented for 4°C was round in shape; they showed a smooth brown surface without cracks and breaks, and had a good elastic crumb with a flavor peculiar to bakery products (Figure 3).

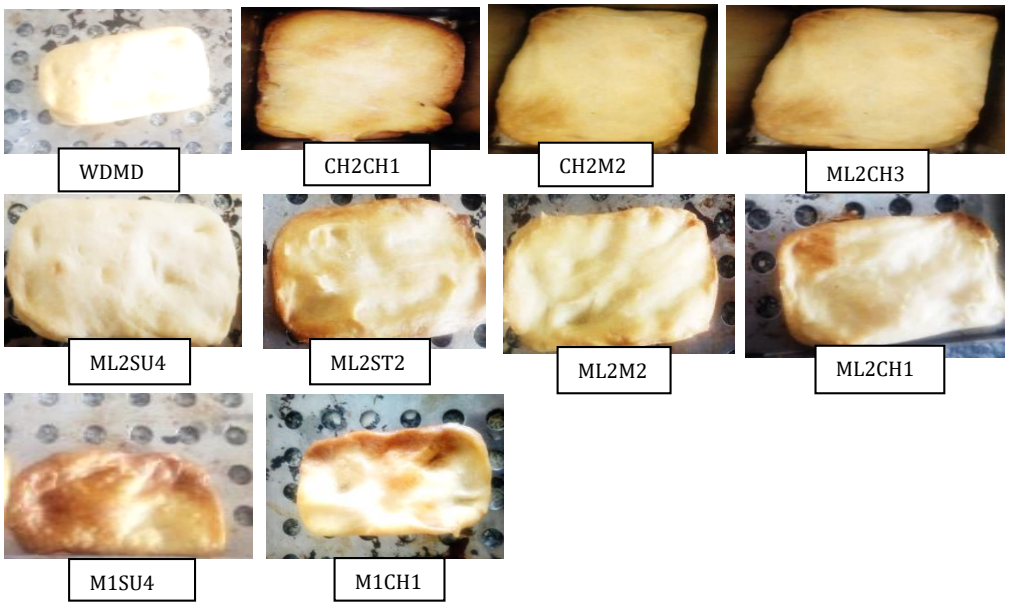


**Fig.3.** Samples of bread prepared by straight-dough method fermentation at 4°C for 2hours

The long, low-temperature fermentation technology implies the time of dough fermentation increased by 10–36 h at low temperatures. Semi-

finished products slowly rise to the required degree of ripeness within this time due to the decreased temperature varying from 28–30°C to 0–5°C. The low-temperature fermentation technologies of compelling interest are currently as follows: preparing cooled dough for shaping; preparing cooled shaped dough pieces for proofing [12]. At present, the optimal technological parameters for dough making (temperature and duration of fermentation) have not been determined [8, 9], and their effect on the properties of semi-finished products and the quality of the finished rich bakery products was not assessed [13].

3.8. *Genetic similarity between mutant and wild type of yeast isolates by RAPD-PCR*

In the present study six random primers were used to identify the selective 10 mutants yeast isolates and one wild type yeast isolate. These primers generated reproducible and easily securable RAPD profiles (Figure 4). The number of scorable bands for corresponding primers ranged from 9 to 16 with an average of 13.5 bands. A total of 81 bands were scored against 11 isolates of the yeast (Table 7). The number of amplified DNA fragments was scored for each primer and Primer OPA09 and OPC06 amplified the highest numbers of amplicons fourteen polymorphic and two monomorphic, fourteen polymorphic and zero monomorphic, respectively. The number of polymorphic amplicons per primer ranged from 71.4% by primer OPC09 to 100% by primers OPA10 and OPC06. The applicability of the method for determining genome similarities among baker yeast isolates was investigated by performing cluster analysis on the RAPD data. Cluster analysis of RAPD showed distinct genetic heterogeneity among the selective ten mutants and the wild type yeast isolates. The dendrogram was constructed considering all bands generated by six primers and suggested two primary genetic clusters by six primers and suggested two primary genetic clusters; the first cluster consisted of two sub-clusters that include yeast isolates S1,S2,S5,S11,S7,S10,S9,S6 and S8. The second cluster includes two yeast isolates (S3and S4). The isolates (S7 and S10) were closely related in one linage (Figure 5). RAPD analysis is considered an important molecular biology technique, which is used for the identification of indigenous yeast strains. In comparison to other molecular typing methods, RAPD is faster, less labor- intensive only a small amount of template DNA is required for amplification reaction [41]. Rieseberg [42] stated that primers with arbitrary sequences give different banding patterns with the same DNA even by applying on the same individual genotypes of species which may be obtained due to the recombination generating species. [43]. stated that the combination of different typing techniques was useful when discriminating similar organisms. So, the introduction of a second typing technique can be more advantageous than increasing the number of characters obtained with a single method. Pina [44] used the combination of PCR fingerprinting and RAPD assays to discriminate fifty-eight yeast isolates from carbonated orange juice factory that showed to be very useful in tracking the route of contamination in a carbonated juice production chain.

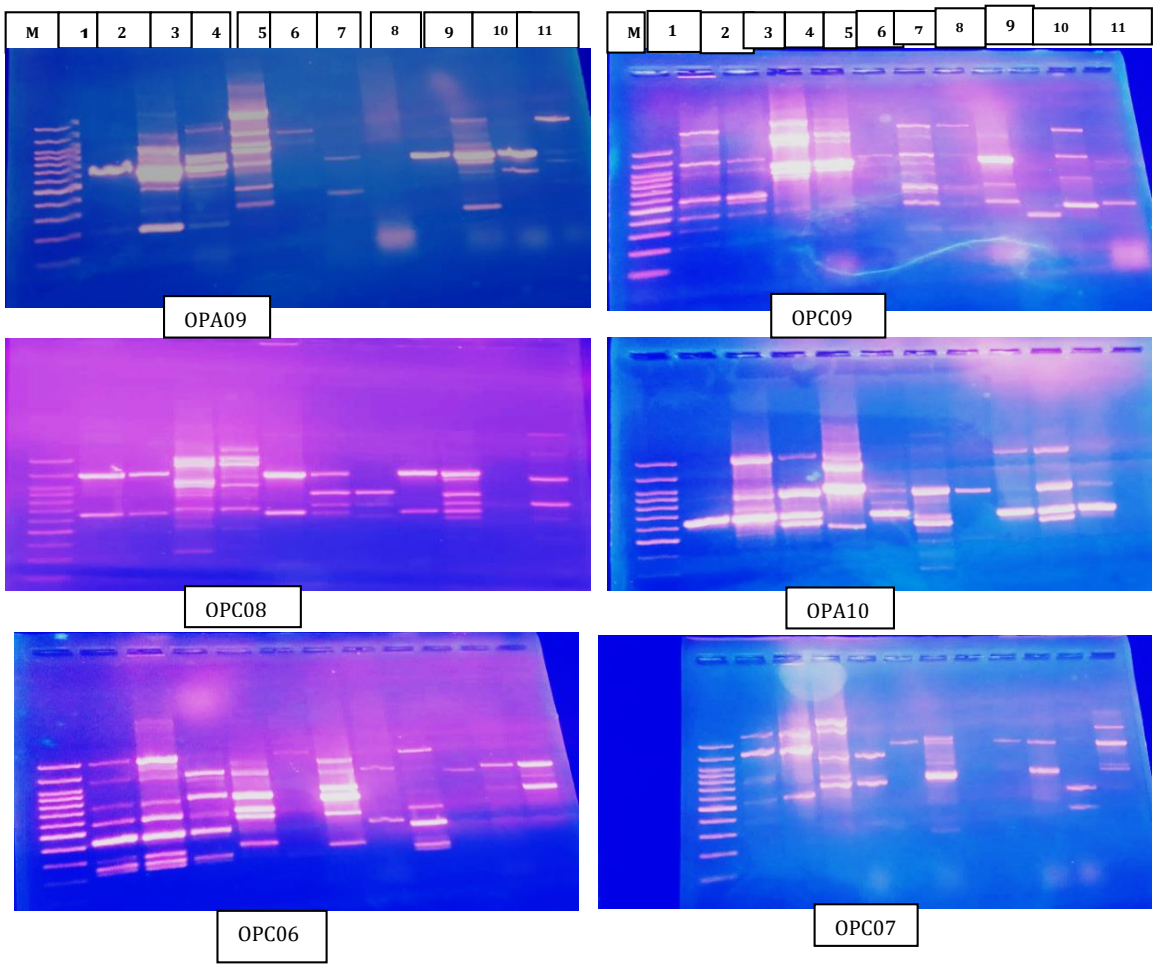


**Fig. 4.** Photograph of RAPD profiles of the ten different yeast mutants isolates amplified and wild type with RAPD primer, OPA-09, OPC-09, OPC-08, OPA-10, OPC-06 and OPC-07, M; (100bp ladder DNA marker); Lane 2: wild type isolate, Lanes from 2 to 11: represent yeast mutants.

Table 7: Random primers showing polymorphism among 10 mutants and one wild type yeast isolates.

| Primer | Total band obtained | Polymorphic band | Monomorphic band | %Polymorphic |
|---|---|---|---|---|
| OPA09 | 16 | 14 | 2 | 87.5 |
| OPA10 | 13 | 13 | 0 | 100 |
| OPC06 | 14 | 14 | 0 | 100 |
| OPC07 | 9 | 8 | 1 | 88.8 |
| OPC08 | 15 | 13 | 2 | 86.6 |
| OPC09 | 14 | 10 | 4 | 71.4 |
| Total | 81 | 72 | 9 | 543.3 |
| Average | 13.5 | 12 | 1.5 | 89.05 |

## 4.Conclusions

In the present study a total of 64 yeast isolates were isolated from food samples. The results showed that, three mutants were obtained using chemical mutagen agent able to tolerate of low temperature (4°C). These yeast mutants were showing fermentation efficiency at low temperature. These effects have a great technological and economic impact because the yeast gassing rate is critical in baking. Consequently, the improvement of the low temperature tolerance in baker's yeast is of significant commercial importance.

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## Author Contributions

All authors contributed to this work. G. Nabil collected the samples and isolated the yeast isolates and completed the experimental measurements. Both G. Hassan and A. Yassein shared writing and followed the performance of the experiments, completed the paper writing, analyzing the data, and validation. E. Eissa, Co-supervisor of the master's thesis from which this research is extracted, G. Hassan followed the revision and submission of the manuscript for publication.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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