Application of Yeast as a Bioreactor for the Production of Microbial Flavors

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ABSTRACT

Thymol (5-methyl-2-isopropylphenol) is a phenolic compound that is used to inhibit oral bacteria. Because little is known regarding the effects of this compound on ruminal microorganisms and it is a halal aroma, the objective of this study was to use yeast as a bioreactor to produce thymol as a halal flavor. Isolation and molecular identification were done by using 5.8S ITS. The results indicated that the strain which we isolated is *Saccharomyces cerevisiae*. This strain is very important in halal functional food, it produces zinc and selenium. The transformation of *Thymus vulgaris* L.DNA to *S. cerevisiae* is considered a major technique for the production of active components that can be used as food preservatives against fungal growth and their mycotoxin occurred in food production so that the aim of the current study was to use *S. cerevisiae* for the production of thymol through the transformation of *Saccharomyces cerevisiae* with genetic material of *Thymus vulgaris* and evaluation of the new transformant able to produce antioxidant products.

1. Introduction

Thyme (*Thymus vulgaris* L.) is known to produce a variety of phenolic monoterpenes. Thyme has been commonly used in foods mainly for the flavor, aroma and preservation (Croteau et al., 2000) agents in pharmaceutical, cosmetic and food industries (de Melo et al., 2000). Thymol is widely used as well as different volatile components as active principle and flavouring agents in pharmaceutical, cosmetic and food industries. *Saccharomyces cerevisiae* is safe, non-toxic, non-pathogenic, thermophilic yeast has become a powerful model system for biological research in a wide range of study. Transformation is a technique by which exogenous DNA is introduced into a cell and is an indispensable method in cell manipulations (Pham et al., 2011). Using biotransformation technologies, could lead to the manufacture of a wide range of natural, novel active ingredients such as flavours, fragrances, antioxidants and antimicrobial agents. (Shimoni et al., 2000) The yeast S. cerevisiae known as bakers or brewer yeast has played a considerable role in food production, alcoholic fermentation, and preparation of wine, beer and bread. (Legras et al., 2007) S. cerevisiae has become a powerful model system for biological research in a wide range of fields of study. Abo-Sereih (2011) produced eugenol (medicinal constituent) using the protocol of transformation which included isolation of the DNA from clove and then insertion into S. cerevisiae.

Aflatoxins (AFs) are one of the most potent mycotoxins produced as secondary metabolites by the toxigenic strains of *Aspergillus flavus and A. parasiticus*, and represent an economic problem and public health hazard. They are of great concern because of their detrimental effects on human and animal health, including mutagenic, teratogenic, immunosuppressive and potent carcinogenic effects. (Aly 2005) The International Agency for Research on Cancer classified aflatoxin B_1 (AFB_1) as group 1A carcinogens, (IARC 1993) contributing to the high prevalence of cancer in regions such as Central Africa and China (WHO 2002). Therefore, to reduce and/or prevent human and animal exposure to AFs, a practical and effective method of decontamination and/or detoxification is urgently required. Different strategies have been used for AF regulation such as natural compounds (essential oils). This work was undertaken to study the possibility of producing thymol which is the major constituent of thyme essential oil, via genetic transformation from thyme plant to the yeast *S. cerevisiae*, and evaluate its functional properties of the new microbial products as antiaflatoxigenic and antioxidant agent as well as.

2. Materials and methods

Isolation and purification of yeast isolates: Samples from different sources such as rotten fruits and vegetable, commercial yeasts, milk, wine, yogurt, preserved food, garden soil from different areas of Lahore and Faisalabad , were collected and stored at 4ºC in airtight bags. Serial dilutions up to 10-3 of each 1% sample were prepared and 100 µl from each dilution was spread on YEPD (Kurtzmann & Fell, 1998). The plates were incubated for overnight at 30oC. Colonies appeared on plates after incubation were selected and streaked on specific medium Eosin-Methylene Blue Differential (EMBD) medium contained 0.3%Peptone, 1% Glucose, 0.05% Chloramphenicol, 0.0065% Methylene blue, 0.01% Triphylterazolium, 0.04% Eosin, 0.3% Malt extract and 2% Agar (Deák, 2007). Different colored colonies appeared on EMBD plates were selected and streaked on new YEPD plates and incubated at the same condition as before to get pure single colonies. Purified cultures were preserved as 30% glycerol stocks at -80ºC for further use.

2.1. Morphological characteristics of isolated yeast

The purified yeast strains were morphologically identified by wet mount method at 40X and 100X magnification of compound microscope and budding yeasts of different shapes and sizes were observed.

2.2. DNA isolation from different yeast strains

DNA from yeast strains was isolated by modified CTAB method (Doyle, 1990; Shahzadi et al., 2010). Overnight grown yeast cultures in YEPD broth were centrifuged at maximum speed. About 10mg of yeast cells for each strain were taken and pre warmed 200 µl of solution I at 65°C containing 1.4M NaCl, 2% CTAB, 20mM EDTA (pH 8.0), 0.2% β-mercaptoethanol and 100mM TrisHCl (pH 8.0) was introduced, mixed well and incubated at 65°C for 15-20 minutes in water bath. After incubation, all tubes were cooled for 3-5 minutes and same volume of solution II (Chloroform: Isoamyl alcohol, 24:1) was added, mixed thoroughly and centrifuged at 14,000 rpm for 10 minutes at room temperature. Aqueous phase (upper) were taken from each eppendorf separately and 3M Na acetate (1/10) was introduced in each eppendorf along with equal volume of cold iso-propanol or double volume of cold absolute ethanol, mixed it gently and placed on ice for 10 minutes. All tubes after incubation were centrifuged at 12000 rpm at 4°C for 15 minutes and supernatant was disposed off. Five hundred microlitre of chilled 70% ethanol (solution III) was added directly for washing pellet and then centrifuged at 14000 at 4°C for 2 minutes. The pellet was air dried after discarding supernatant from each tube. The pellet was resuspended in 50µl double deionized water or TE-buffer to store at – 20^{O}C. The yield of DNA was quantified by Spectrophotometer (Sambrook et al., 2004).

2.3. Amplification of 5.8S-ITS region by polymerase chain reaction

Amplification of 5.8S-ITS region of rRNA gene was done by using ITS1 (F) 5'TCCGTAGGTGAACCTGCGG3' and ITS4 (R) 5'TCCGTAGGTGAACCTGCGG3' primers (White et al., 1990) in thermocycler (Bioerxp cycler). The reaction mixture contained 100ng DNA, 5µl of 10pmol each oligonucleotide primer, 3µl of 25mM MgCl2, 3µl of 250mM dNTPs mixture and Taq DNA polymerase (5units) in a total volume of 50 µl. PCR conditions were as follow: 3 min. at 94Co followed by 35 cycles (45 sec. at 94oC, 45 sec. at 55oC (annealing temperature), 1 min. at 72oCo and final extension for 7 min. at 72oC. The amplified product was checked by running on 0.8% agarose gel and visualized by using UV illuminator and photographed (El-Sharoud et al., 2009).

2.4. Electrophoresis

Gel electrophoresis was run for about an hour. Band sizes were estimated by comparison against 100 bp DNA ladder. The amplicons were viewed, photographed and documented using the gel documentation system. The amplicon/band of interest (600 bp) was cut out and stored in Eppendorf tube at 4°C until ready to use.

2.5. DNA sequencing and phylogenetic analysis

For the direct confirmation of specificity of the PCR products, the DNA fragments generated by each of the primer pairs were sequenced. Purified 5.8S ITS products were loaded into sample loading plate and directly sequenced using theedited and assembled using MEGA6 and then subjected to GenBank BLASTN (Yeast-id database of the National Center for Biotechnology Information (NCBI) (blast.ncbi.nim.nih.gov/Blast. cgi)) to identify them by sequence homology with described yeast taxa.

2.6. Plant material

T. vulgaris L. flowers and leaves were bought from local markets in Cairo, Egypt. The plant was identified by the Department of Botany, National Research Center and the voucher was kept in the herbarium of National Research Centre.

2.7. Thymol Sensitivity

For growth on media containing thymol, logarithmically growing cells of *S. cerevisiae were diluted to OD600* = 0.3 and 10-fold serial dilutions were spotted onto synthetic media containing the indicated thymol. The thymol was added when the media was at approximately 50°C, followed by immediate pouring of the agar into plastic plates. Plates were incubated at 30°C for 2 to 3 days and photographed (Stepanov et al., 2008).

2.8. Isolation and identification of thymol using GC/MS

To extract extracellular volatile compounds (thymol), transformed S. cerevisiae were grown in YEP broth for 48 h. Cells were removed from the suspension culture by centrifugation for 10 min. A 200 mL portion of cell free supernatant (CFS), was adjusted at pH 7.8 with 2% NaHCO3 solution and extracted three times with diethyl ether (20 mL). The thymol was obtained by evaporation of the solvent in a water bath at 40°C. (Abraham and Berger 1994) Thymol was analyzed using GC/MS technique. The mass spectrometer was an Agilent 6890 N GC/5973MSD-SCAN (Agilent Technologies, Palo Alto, CA, USA). The split ratio was 10:1 onto a 30 mm×0.25mm HP-5 (cross-linked phenyl–methyl siloxane) column with 0.25 mm film thickness to a mass spectrometer and sniff port. Injector and detector temperatures were set at 220 °C.

2.9. The antioxidant activity

The antioxidant activity of the samples was measured by the DPPH radical scavenging assay. A known antioxidant, TBHQ was used to validate the assay (Miliauskas et al., 2004). Decreasing the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. This

activity was given as percent DPPH radical scavenging, which was calculated with the equation: *% DPPH radical scavenging = (control absorbance- sample absorbance) /control absorbance X 100*

2.10. Determination of the accumulated selenium and zinc in yeast cells

The methods of Pankiewicz U., Jamroz J., Schodzinski A.(2006) and De Nicola et al.(2009) were used to determine selenium and zinc in accumulated yeasts with slightly modification. A loop of yeast culture S. cerevisiae was used to inoculate 100 ml Erlenmeyer flask containing 25 ml of sterilized medium containing (g/l-1) peptone 1.0, yeast extract 0.5, glucose 3.0 the flask was incubated at 30oC in 90 rpm shaking over night, After this period 1 ml of culture inoculate 5 flasks of 100 ml capacity each containing above medium and add 50-300 mg/l-1 sodium selenite or 25-100 mg/l-1 zinc sulfate (pH 5.5) which sterilized by membrane filter. The yeast cultures were inoculated from different treatment. All treatment performed in 90 rpm shaking at 37oC for 72 hrs incubation. Atomic absorption spectroscopy (AAS) at (NRC) was used to determine the concentration of selenium Pankiewicz et al. (2006) and zinc in yeast after collection by centrifuge 5000 rpm /5 min and several washings with deionized water to remove culture medium which detected]. The weighted samples were digested in concentrated pure salfonic acid (95%) and per chloric acid (70%) in ratio 2:1. Samples digestion was carried out with acids at hot plate. Samples then converted to soluble matter in deionized water to appropriate concentration level.

2.11. Transformation

Transformation of *S. cervisia* whole cells; an overnight yeast culture was diluted 1:100 in 50 ml YPAD agar medium (for 10 transformations). The culture was incubated at 37ºC to an OD600. The cells were centrifuged at 1000 × g for 5 minutes and the supernatant was discarded. The cells were resuspended in 10 ml of 0.1 M LiAc and the suspension was centrifuged at 1000 × g for 5 minutes then the supernatant was discarded. The cells were resuspended in 10 ml of 0.1 M LiAc and incubated for 1 hour at 30ºC. The suspension was centrifuged at 1000 × g for 5 minutes washed with 0.5 ml of 0.1 M LiAc, aliquotoe 50 µl of the cells were added into micro centrifuge tubes containing 0.1 ml of fragmented genetic material of *Thymus vulgaris*. The mixture was diluted 3 to 10 times after incubation, plated and incubated at 30 ^{O}C for the selection of transformant.

3. Results and discussion

3.1. Isolation and purification of yeast strains

Purified yeast strains on YEPD were initially morphologically identified as budding yeast of different shape and sizes under microscope. It was identified based on the colony morphology and microscopic observation. The two isolates exhibited smooth surfaces with circular margins. The color of the colonies was white creamy. The cells were found to be of various shapes such as round and oval (Fig 1). The isolates were named isolate1 and isolate2

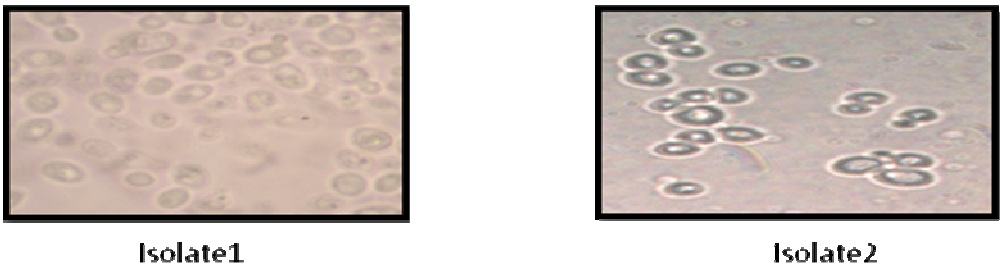

Fig 1. Purified colonies of yeast strains under light microscope

3.2. PCR amplification of 5.8s ribosomal gene

Amplification of 5.8S ITS gene was done by PCR. PCR amplified products were resolved on 1% Agarose gel. The sizes of amplified products of yeast strains about 620 bp were observed by comparing with 100 bp DNA ladder (Fig. 2) The partial sequence of 26S ribosomal gene obtained from yeast strains were aligned with the available 5.8S ribosomal gene sequences in Gen Bank data base. The 5.8S ribosomal gene partial sequence of yeast isolates are presents in Figures (3). As a result, a phylogenetic tree was mapped using the neighbor joining method, and is shown in figure (4) Phylogenetic analysis using the 5.8S ribosomal gene sequences indicated that isolate 1 and isolate 2 were belonged to the genus Saccaromyces and according to blast results, they were identified as *saccaromyces cerivisia* strains. These results are in agreement with Keita et al. (2016).

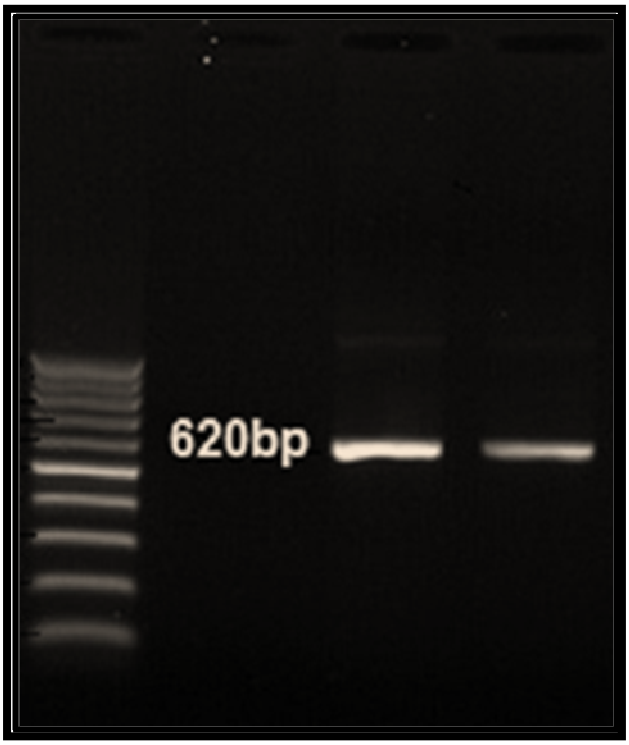

Fig 2. PCR amplified of 5.8S ITS. M= 100 bp ladder, Lane 1 and 2 yeast isolate.

GAGAAGGGGTTCTGGAACGTGGCTCTTCGGAGTGTTATAGCCAGGGCCAGATGCTGCGTGCGGGGA
CCGAGGACTGCGGCCGTGTAGGTCACGGATGCGAAACATGGCACGTGAAATTGTTGAAAGGATATC
AATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTC
AGATTTGAAATCGTGCTTTGCGGCACGAGTTGTAGATTGCAGGTTGGAGTCTGTGTGGAAGGCGGTG
TCCAAGTCCCTTGGAACAGGGCGCCCAGGAGGGTGAGAGCCCCGTGGGATGCCGGCGGAAGCAGTG
AGGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCCAAGCGGGTGGTAAATTCCATCTAAGG
CTAAATACTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAA
GAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGCGCCCGACATGGGGATTGCGGA
TGCTGCGTGCGGGGACCGAGGACTGCGGCCGTGTA

Fig 3. The nucleotide sequence of 5.8S ITS 620 bp

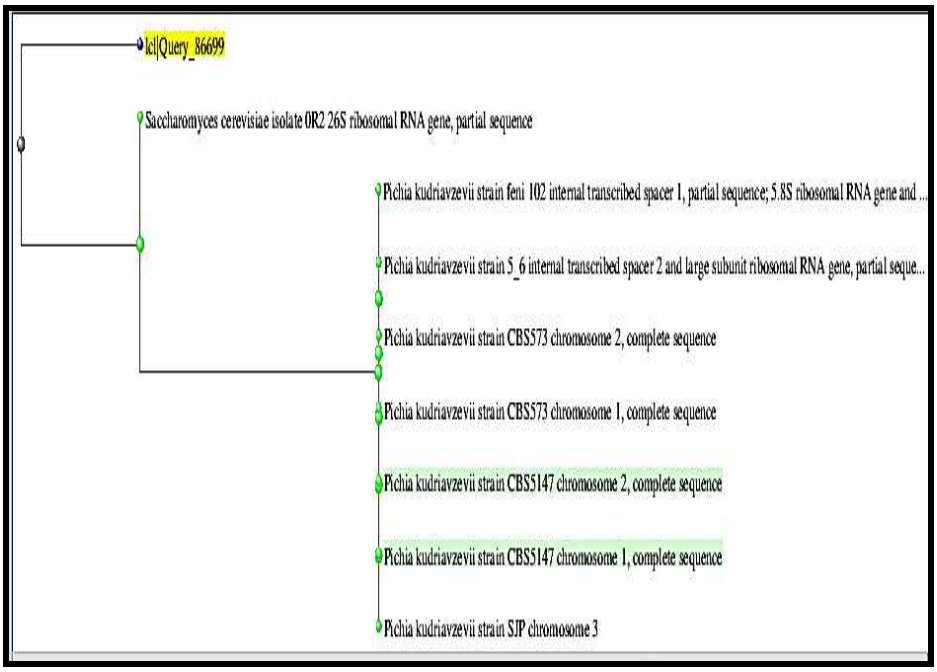

Fig 4. Phylogenetic tree of *saccaromyces cervisia* (query_86699) based on 5.8S ITS *ribosomal* gene sequences using neighbor joining method.

3.3. β. carotene/ linoleic acid assay

The antioxidant activity of thymol was evaluated using β-carotene bleaching by linoleic acid assay (Fig 5). Thymol was efficient in inhibiting the oxidation of linoleic acid, which is an important issue in food preservation. It was noticed that the antioxidant activity increased by increasing thymol concentration. The IC50 was found to be around 100 ppm. Our results demonstrated that thymol showed high antioxidant activity due to the phenolic structures of the thymol constituent, which is the main component of the essential oil of thyme. These results are in agreement with previous studies (Nguyen et al., 2000) who revealed that thymol is known to inhibit lipid peroxidation. The antioxidant activity may be due to different mechanisms, such as prevention of chain initiation, decomposition of peroxides, and prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts (Gulcin et al., 2003). These mechanisms explain the different data obtained in our investigation, thus, it is important to evaluate the effectiveness of antioxidants by several analytical methods and different substrate.

3.4. Antifungal and antiaflatoxigenic activity

Data in Fig (6) showed that the extracted thymol caused a reduction in MDW and total aflatoxin production. Results also revealed that there is a parallel relationship between the fungal growth and the total amount of AFs which was affected in a descending order as the thymol concentration in the medium increased. Fig (6) revealed that the inhibition of the four types of AF recorded 75.55%, 72.94%, 95.58% and 72.29% for AFB1, AFB2, AFG1 and AFG2 respectively in the presence of 175 ppm thymol. In the presence of 250 ppm thymol AFs were completely inhibited (100%). In this respect, it could be noticed that AFG1 was more sensitive to thymol showing the highest percentage of inhibition. In our investigation, results revealed that the addition of different concentrations of thymol extracted from transformant S. cerevisiae decreased fungal growth and aflatoxin production. This was in agreement with previous studies (Aly, 2011) who studied the antifungal activity of thyme oil, towards some food spoilage fungi, especially Aspergillus. It demonstrated that thyme oil (T. vulgaris) inhibited both mycelia growth and aflatoxin synthesis of *A. parasiticus*. Zambonelli et al. (1996) revealed that the antifungal activity of thyme oils is also attributed to thymol and carvacrol, which cause degeneration of the fungal hyphae that seems to empty their cytoplasmic content. Antimicrobial activity of thymol was evaluated for the control of

10 pathogenic microorganisms. Results revealed that thymol possessed antimicrobial properties and is a potential source of antimicrobial ingredients for food industry (Kumar et al., 2008).

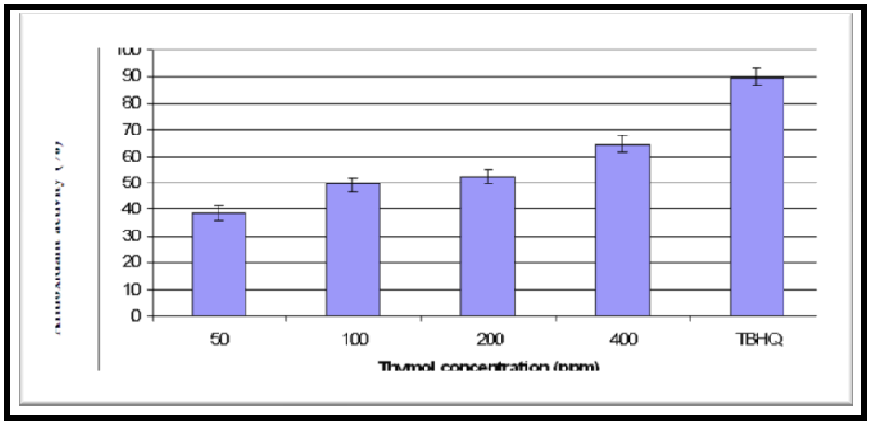

Fig 5. Antioxidant activities of thymol measured by β- Carotene /linoleic acid assay. Bars indicate means ± SD obtained from three independent measurements. Results revealed significant differences ($P < 0.05$).

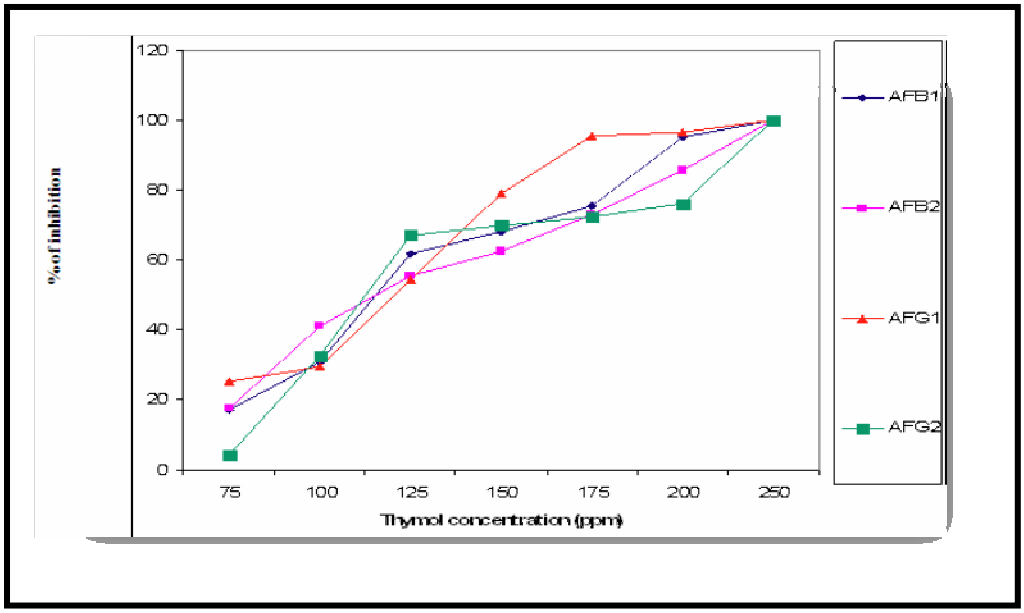

Fig 6. Percentage of inhibition of AFs (AFB1, AFB2, AFG1 and AFG2) using different concentration of thymol. Results are man ± SD obtained from three independent measurements.

3.5. Yeast transformation

Saccharomyces cerevisia is safe, nontoxic, nonpathogenic and thermophilic yeast. It is considered a rich source of B vitamins and chromium as well as treatment alone reduced bacterial translocation so that useful as biotherpeutic agent. Thymol is a is widely, used as well as different volatile components as active principle and flavoring agents in pharmaceutical, cosmetic and food industries. The present investigation aims to transform the probiotic agent *Saccharomyces cerevisia* whole cells. 40% of transformant cells showed high efficacy for thymol production (Table 1) as biomarker for positive transformation. yeat samples. the negative results may be due to the limited number of competent cells in the population that can take up DNA very efficiently, (Ramboseck and Leach 1987). or to the capacity of the cell wall to absorb DNA which is at least one of the determinants of transformation efficiency and frequency. (Pham et al., 2011). Recently, Kawai et al. (2010) presented a model for the mechanism of transformation of intact *S. cerevisiae* cells. In this model, they proposed that (1) DNA initially attaches to the cell wall, (2) passes through the cell

wall and (3) enters into the cells via endocytotic membrane invagination. They added that PEG is essential for the attachment of DNA and possibly acts on the membrane to increase the transformation frequency. Moreover LiAc and heat shock help the DNA to pass through the cell wall.

Table 1. Efficiency of S. cerevisiae transformants to produce thymol*

Trans. No.	Efficiency	Trans. No	Efficiency
1	-	6	-
2	+++++	7	++
3	-	8	-
4	++++	9	++++
5	-	10	-

Not detected ++ Good +++ very Good ++++ Excellent * visually on TLC

3.6. Selenium accumulation

The results of this experiment on the (Figure 7) indicated that the increase of yeast biomass *S. cerevisiae* and their transformant when increase the sodium selenite in culture medium it was shown that higher amount of sodium selenite in the culture medium have a strong inhibitory effect on the growth of wild type strain of *S. cerevisiae* (0.63-0.17mg/l) comparison with their transformant, the range become (0.15-0.17mg/l) The increase of yeast biomass *S. cerivisia* and transformant. These results were close to the results of Saito and Takahashi (2000). They reported that the activity of selenium dependent glutathione peroxidase (which is a selenocysteine containing protein found in all microorganisms) and glutathione reductase and retained within the cell as red metabolically selenium into organic compounds. Izquierdo et al. (2010) reported that unlike in higher organisms, selenium is not essential for growth in S. cerevisiae. In this species, it causes toxic effects at high concentrations. In the present study, we show that when supplied as selenite to yeast cultures growing under fermentative metabolism, its effects can be dissected into two death phases. From the time of initial treatment, it causes loss of membrane integrity and genotoxicity. Many transformant identified affected either mitochondrial or vacuolar function and these groups showed similar effects on the accumulation of many different elements. Finally, ionome profile data allowed to correctly predicting a function for a previously uncharacterized gene, *YDR065W*. They had shown that this gene is required for vacuolar acidification Clark et al. (2005) indicated that the results referring to the power of bionomics to identify new aspects of mineral homeostasis and how these data can be used to develop hypotheses regarding the functions of previously uncharacterized genes.

3.7. Zinc accumulation

Zinc sulfate concentration were used (0.0, 25, 50, 75 and 100 mg/l-1) the absorption of wild type strain of S. cerevisiae was (0.0 - 0.3) comparison with their transformant especially ScM2, ScM3 and ScM4, that were (0.0 – 0.7), (0.0 – 0.08) and (0.0 – 0.4) respectively (Figure 8). The increase of yeast biomass of parent yeast strain and their transformant when increase the zinc sulfate in culture medium it was shown that variable increasing in the amount of zinc in the mass cells of the tested transformant of S. cerevisiae (0.63-0.17mg/l) comparison with their parent yeast strain. Because many scientists noted that zinc is the element that is involved in the regulation of metabolic activity of yeast and/or other processes such as flocculation and cell division. The requirements for specific ions may be subject to changes in successive fermentations performed by the same biomass. Zinc levels in the biomass did not exceed 0.6 mg/g yeast dry weight Aleksander et al. (2009) and De Nicola et al. (2009) noted that accumulation of zinc by a distiller's yeast strain of S. cerevisiae was studied during fermentation. Zinc uptake by yeast cells was very rapid in malt wort, as zinc (0.32µg/mL). *S. cerevisiae* have shown numerous beneficial effects on human health. Among these nutritional importance is bioavailability of divalent minerals such as zinc.

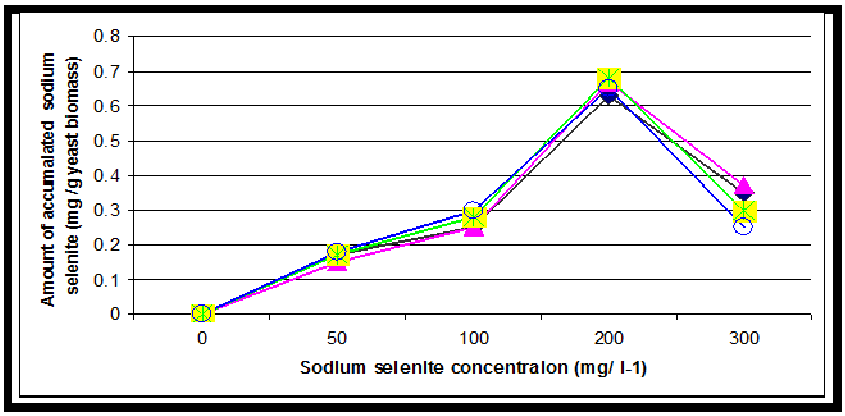

Fig 7. Organically selenium bound in different yeast transformed biomass *S. cerevisiae* after growing on cultures containing different sodium selenite concentrations (0, 50, 100, 200, 300 mg/l-1)

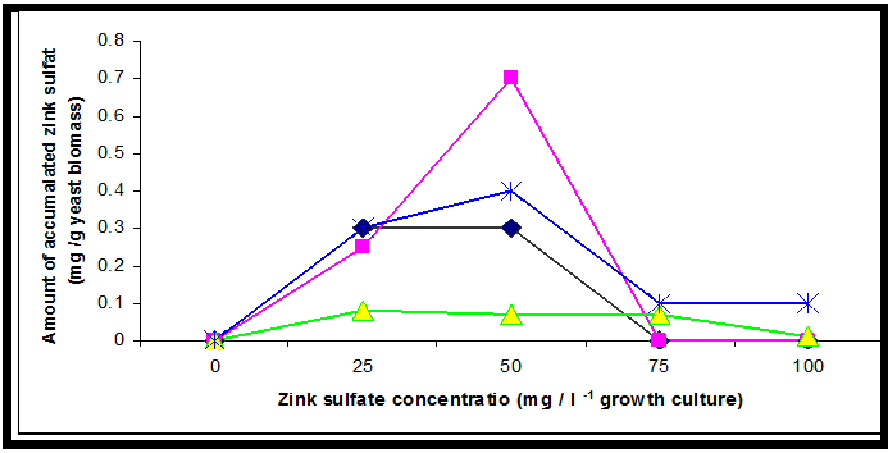

Fig 8. Organically zinc bound in yeast biomass S. cerevisiae after growing on cultures containing different zink sulfate concentrations (0, 25, 50, 75, 100 mg/l^{-1}).

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