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ORIGINAL ARTICLES

Saccharomyces cerevisiae Transformant Genes as a Tool For The Production of Antiaflatoxigenic Agents

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ABSTRACT

Saccharomyces cerevisiae is a safe, non-toxic, non-pathogenic, thermophilic yeast and has become a powerful model system for biological research in a wide range of fields of study. The aim of the current study was to use *S. cerevisiae* for the production of thymol through the transformation of *Thymus vulgaris* genetic material and evaluation of the new microbial products as antiaflatoxigenic and antioxidant activity. The study revealed that 40% of the yeast strains were able to produce thymol in variable amount. Using different types of sugar (fructose, lactose, sucrose, mannose, arabinose, glucose and corn steep) as a source of carbohydrate enhanced thymol bio-production, whereas glucose and corn steep produced thymol more efficiently. The antioxidant properties of thymol were examined by two methods DPPH scavenging activity examination, and β-Carotene/linoleic acid assay and both examinations showed the highest antioxidant properties. Mycelia dry weight (MDW) and aflatoxin (AF) production by *Aspergillus parasiticus* were decreased using different concentration of thymol (75, 100, 125, 150, 175, 200 and 250 ppm) and were dependant on the presence of active components and their concentrations. In conclusion the transformation of plant genes 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 mycotoxin production. Moreover, this is an easy, safe and economic approach.

Key words: Thymus vulgaris; Saccharomyces cerevisiae; thymol; antiaflatoxigenic; antioxidant; gene transformation; biotechnology.

Introduction

Thymus vulgaris L. (thyme) is an ancient herb widely used in folk medicine by the Greeks, the Egyptians and the Romans for the treatment of a variety of diseases. (Rustaiyan *et al*., 2000: Aydin *et al*, 2005). Thyme has been commonly used in foods mainly for the flavour, aroma and preservation. (Croteau 2000) Thymol (Fig 1) is a phenolic compound and is considered as the main constituent of the volatile oil of thyme plant which produces primary and secondary metabolites and encompasses a wide array of functions. (Croteau *et al*, 2000): Hamid *et al*, 2011). Thymol is widely, used as well as different volatile components as active principle and flavouring agents in pharmaceutical, cosmetic and food industries. (de Melo *et al*, 2000).

Several screening studies with essential oils verified the antibacterial and antifungal activity of the essential oil of thyme. It was shown to inhibit a broad spectrum of bacteria; generally Gram-positive bacteria. (Blakeway 1986: Farag 1986: Deans and Ritchie 1987). Furthermore, the essential oil of thyme showed a wide range of antibacterial activity against microorganisms that had developed resistance to antibiotics. (Nelson (1997) In the antimicrobial active constituents of thyme oil, the monoterpenes thymol and carvacrol were found to play an outstanding role. These terpenes bind to the amine and hydroxylamine groups of the proteins of the bacterial membrane altering their permeability and resulting in the death of the bacteria. (Juven *et al*., 1994).

Recently there are many synthetic compounds that are currently in use by industry and in modern agriculture, whereas there is a strong international tendency to replace some of these synthetic chemicals with natural compounds that are often safer and friendly in concern to environmental aspects. Traditional methods for isolating essential oils from plant materials, such as steam distillation and solvent extraction, have some drawbacks due to the heat instability of essential oils and the presence of residual organic solvents in the extract. Thus, the use of supercritical fluids for the extraction of essential oils has received increasing attention as an alternative to these traditional techniques. (Stahl *et al*, (1987).

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

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a considerable role in food production, alcoholic fermentation, and preparation of wine, beer and bread. (Phaff (1990: Legras *et al*., 2007) *S. cerev*isiae has become a powerful model system for biological research in a wide range of fields of study. (Abo-Sereih 2011: Aly *et al*., 2005) 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. (Klich (2007) They are of great concern because of their detrimental effects on human and animal health, including mutagenic, teratogenic, immunosuppressive and potent carcinogenic effects. (Eaton, D.L. and Gallagher 1994: Abdel-Wahab and Aly (2003) 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).

Therefore, 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 to enhancing the thymol production through the cultivation of the transformant strain on different types of sugar as well as to evaluate its functional properties of the new microbial products as antiaflatoxigenic and antioxidant agent as well as.

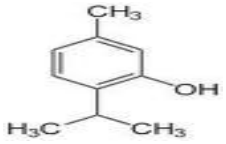


Fig. 1: Chemical structure of standard thymol

Materials and Methods

Strains:

A diploid wild type of *S. cerevisiae* was kindly provided by the Egyptian Sugar and Distillation Company, Hawamdia, Egypt and was grown and maintained on Yeast Extract Peptone Medium (YEP) (1% yeast extract, 2% peptone, 2% D-glucose).

Aspergillus parasiticus NRRL 2999 was obtained from US Department of Agriculture, Agriculture Research Service, National Centre for Agriculture, Peoria, IL, USA and was maintained on potato dextrose (PDA, Difco, Detroit, MI, USA) agar slants.

Media and chemicals:

AFs standards and all chemicals used in the current study were of the highest purity commercially available and were purchased from Sigma Aldrich Chemical Co., P. O. Box 14508, St. Louis, MO 63178, U. S. A. Potato Dextrose Agar (PDA) was purchased from DIFCO Laboratory, Detroit, MI 48232-7058, USA. Yeast Extract was purchased from Fluka Biochemika Company, Switzerland.

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**.**

Thymol Sensitivity:

For growth on media containing thymol, logarithmically growing cells of *S. cerevisiae* were diluted to OD_{600} = 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).

DNA isolation and purification:

The DNA was isolated using the CTAB method and was dissolved in 1 mL of sterile double distilled water (DDW) and purified using a column (Genelute plant genomic DNA extraction kit- G2N 70, Sigma Aldrich). (Vural (2009).

Transformation:

Recipient cells (0.2 mL of 1 x 10^7 cells/mL) were mixed with different concentrations of thymol and 0.3% lithium acetate. After 5 to 10 min, 0.1 mL of the donor DNA in 50 mM $CaCl_2$ was added to the mixture and incubated at 0°C for 15 min. The mixture was diluted 3 to 10 times after incubation and plated and incubated at 30°C for the selection of the transformants. (Diatchenko *et al*, 1996).

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% $NaHCO_3$ 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.

The effect of different sources of carbohydrate:

Seven types of sugar fructose, lactose, sucrose, mannose, arabinose corn steep liquor, and glucose were used in this study. Sugars were added individually to the principal medium to evaluate the effect of these sugars on the ability of transformed yeast to produce thymol.

Detection of thymol on TLC plates:

The produced thymol was identified on silica gel 60 F_{254} TLC plates (E. Merck, Darmstadt), using toluene–ethyl acetate–methanol, 9:1:0.5 v/v/v, as mobile phase according to Verma and Joshi (2006).

Antioxidant activity:

DPPH radical scavenging 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 indicate 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

β. carotene/ linoleic acid assay:

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxide formation from linoleic acid oxidation. (Matthaus 2002).

Antifungal and antiaflatoxigenic activity:

The antifungal activity and antiaflatoxigenic activity of thymol (75, 100, 125, 150, 175, 200 and 250 ppm) was studied. All treatments were incubated at 28 ± 2ºC for 8 days. MDW and AF content were determined according to Aly *et al*., (2011). AFs were analyzed using HPLC.

A Waters (Milford, MA) HPLC equipped with a model 600 pump, and a model 474-fluorescence detector and Millennium 2010 software (Waters) was used to quantify AFs. Separations were carried out at ambient temperature on Phenomenex 4m ODS column, (250 x 4.6 mm). AFs were eluted with acetonitrile / methanol /

water (1:3:6 v/v/v) as the mobile phase at a 1 mL/min flow rate. The detection wavelength for excitation and emission were set at 365 and 450 nm, respectively.

Statistical analysis:

Statistical analysis was performed using SPSS statistical program for windows (Version 16) (SPSS Inc., Chicago, IL, USA). All data were statistically analyzed using analysis of variance. Results were considered significant at $P < 0.05$.

Results

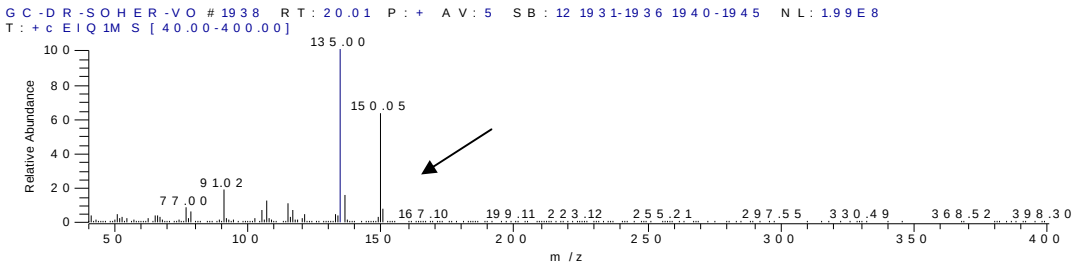
Production of thymol using transformant yeast strains:

Twenty-five isolates were chosen and examined for their ability to produce thymol as a biomarker of transformation process. Cell Free Supernatant (CFS) of transformed yeast strains were harvested after 48h by direct solvent extraction and subjected to GC/MS analysis. Table (1) revealed that 40% of transformed yeast samples efficiently produced thymol through the biotransformation process. Other volatile components were identified with which thymol was abundant in the volatile CFS extract of the transformed *S. cerevisiae* (Fig. 2a, b and c). On the other hand, CFS of transformed yeast samples 2 and 4 were more efficient and produced higher amounts of thymol in comparison with other samples (Table 1). It was also noticed in Fig (2c), the presence of a new compound $C_{12}H_{17}NO_2$, which may affect the flavour as well as the antimicrobial and antioxidant activity of the thymol produced. Thus, indicating the necessity to analyze all CFS of transformant yeast to select the more suitable and favourable transformant strain for the production of thymol.

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

Trans. No.	Efficiency	Trans. No.	Efficiency	Trans. No.	Efficiency	Trans. No.	Efficiency	Trans. No.	Efficiency
1	-	6	-	11	-	16	-	21	-
2	++++	7	++	12	+++	17	+++	22	++
3	-	8	-	13	-	18	-	23	-
4	++++	9	+++	14	++	19	+++	24	+++
5	-	10	-	15	-	20	-	25	-

- not detected ++ Good +++ very Good ++++ Excellent * visually on TLC

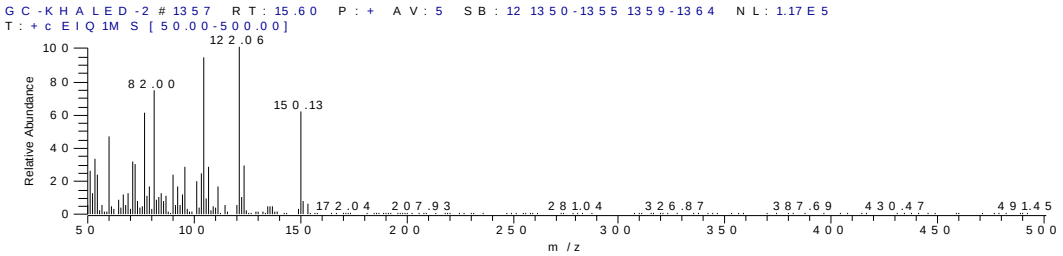


Name	Molecular Formula	Molecular Weight	SI	RSI
Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	$C_{10}H_{14}O$	150	916	917
Phenol, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{14}O$	150	918	918
Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	$C_{10}H_{14}O$	150	929	931

Fig. 2a: GC/MS chromatogram of standard thymol.

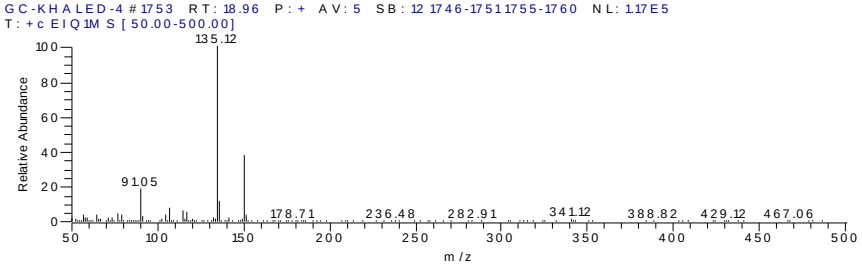
The effect of different sources of carbohydrate:

To enhance the production of thymol, (Transformant strain sample 2), seven types of sugar were used individually as a source of carbohydrate to evaluate the effect of these sugars on the ability of transformed yeast strain to produced thymol. Data in Table (2) revealed that thymol was more efficiently produced in the presence of glucose and corn steep liquor in the media.



Name	Molecular Formula	Molecular Weight	SI	RSI	Probability
Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	$C_{10}H_{14}O$	150	739	816	23.33
Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	$C_{10}H_{14}O$	150	739	816	23.33
3,4-dimethylbicyclo[3.3.0]oct-3-en-2-one	$C_{10}H_{14}O$	150	739	816	23.33

Fig. 2b: GC/MS chromatogram of thymol produced by transformed *S. cerevisiae* sample 2.



Name	Molecular Formula	Molecular weight	SI	RSI	Probability
Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	$C_{10}H_{14}O$	150	848	886	27.83
Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	$C_{10}H_{14}O$	150	849	921	27.83
Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate (CAS)	$C_{12}H_{17}NO_2$	207	850	950	28.95

Fig. 2c: GC/MS chromatogram of thymol produced by transformed *S. cerevisiae* sample 4.

Table 2: Effect of different sources of carbohydrate on thymol production*.

Carbohydrate Source	Efficiency
Glucose	++++
Mannose	+
Arabinose	++
Lactose	-
Fructose	++
Sucrose	-
Corn steep	++++

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

Antioxidant activity:

DPPH radical scavenging activity:

Free radical scavenging activity of thymol produced by transformant yeast strains measured by DPPH is presented in Fig (3). These results showed that thymol was able to reduce the stable free radical DPPH with an IC50 of less than 200 ppm.

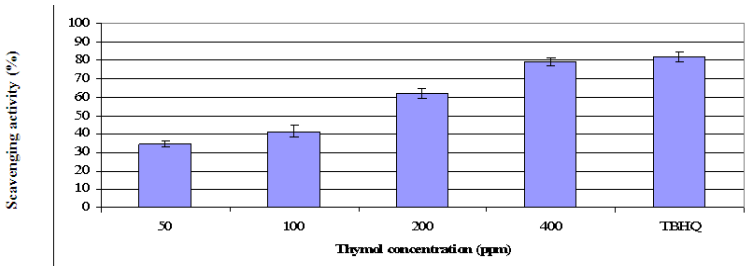


Fig. 3: Free radical-scavenging capacities (%) of thymol measured in DPPH assay. Bars indicate means ± SD obtained from three independent measurements. Results revealed significant differences ($P < 0.05$).

β. carotene/ linoleic acid assay:

The antioxidant activity of thymol was evaluated using β-carotene bleaching by linoleic acid assay (Fig 4). 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.

Antifungal and antiaflatoxigenic activity:

Data in Fig (5) 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 AFB_1, AFB_2, AFG_1 and AFG_2 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 AFG_1 was more sensitive to thymol showing the highest percentage of inhibition.

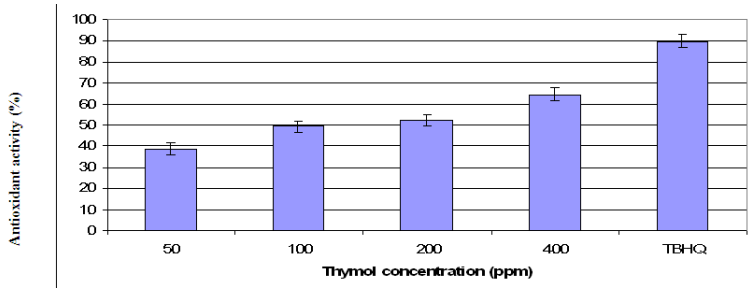


Fig. (4): Antioxidant activities of thymol measured by β- Carotene /linoleic acid assay. Bars indicate means±SD obtained from three independent measurements. Results revealed significant differences ($P < 0.05$).

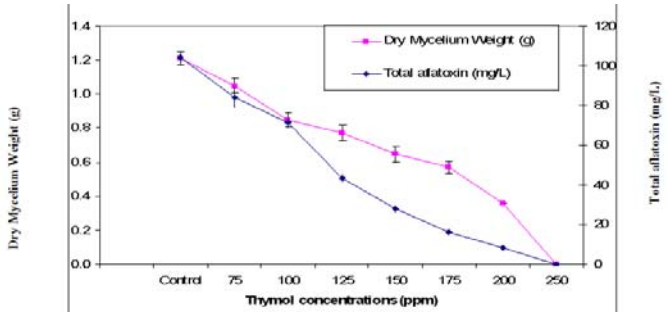


Fig. 5: Antifungal and antiaflatoxigenic activity of thymol. Bars indicate means ± SD obtained from three independent measurements. Results revealed significant differences ($P < 0.05$).

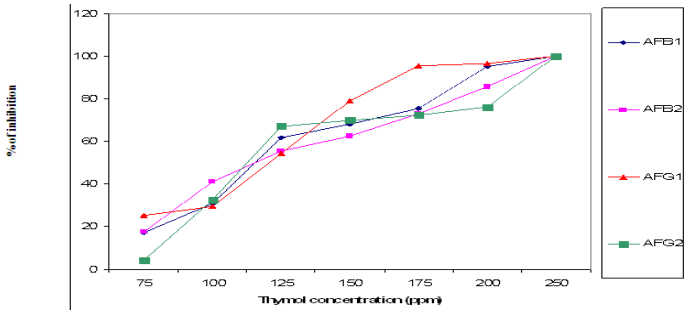


Fig. 6: Percentage of inhibition of AFs (AFB_1, AFB_2, AFG_1 and AFG_2) using different concentration of thymol. Results are man ± SD obtained from three independent measurements.

Discussion:

Transformation is an important technique in which exogenous DNA is introduced into a cell, resulting in genetic modification. In this study, we investigated the possibility of producing thymol via genetic transformation from thyme plant to the yeast *S. cerevisiae*. In this respect, Akada *et al*., (2002) found that however, transformation efficiencies obtained with drug resistance markers are usually lower than those obtained with auxotrophic markers. Moreover, background colonies that are resistant to the drugs often arise in addition to the true transformants, because many drug resistance markers are derived from bacterial antibiotic resistance genes.

Our results revealed that forty percent (40%) of transformed yeast samples were efficient in producing thymol through the biotransformation process indicating the genetic ability of the yeast transformants to produce these volatile compounds. On the other hand, 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.

Thymol was identified using GC/MS from different cultures of transformant yeast in different quantities (Fig 2a, b and c) compared to thymol standard. Our results suggested that DNA transformation of thyme (donor) to the yeast cells (host) could enhance the development of flavouring and or medicinal compounds.

Moreover, different constituents identified with thymol which have the same molecular weight as showed in Fig (2) may be explained by the presence of some leakage through the pathway of thymol biosynthesis, which may be due to the introduction of regulatory gene (s) through transformation in different locations in the different yeast colonies. (Awad *et al*., 1993).

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. (Daphevicius *et al*., 1998) These results are in agreement with previous studies (Aeschback *et al*., 1994; Yanishlieva *et al*., 1999; 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 substrates.

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 (Conner and Beuchat, 1984; Farag *et al*, 1989). who studied the antifungal activity of thyme oil, towards some food spoilage fungi, especially *Aspergillus*. In the same trend Elaraki and Beraoud (1994). demonstrated that thyme oil (*T. vulgaris*) inhibited both mycelial 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 microorganism, and results revealed that thymol possessed antimicrobial properties and is a potential source of antimicrobial ingredients for food industry. (Rota *et al*., 2008; Kumar *et al*., 2008; Solomakos *et al*., 2008).

The mechanism of the inhibitory effect of the active constituents may be related to their hydrophobicity, which enables them to partition the lipids of the cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. (Knobloch *et al*., 1986; Denyer and Hugo, 1991 Sikkema *et al*., 1994). Rastogi and Mehrotra (2002). reported that extensive leakage from bacterial cells or the existence of the critical molecules and ions will lead to death.

Conclusion:

Our results concluded that the new strain of *S. cerevisiae* produced thymol in measurable quantities, which gave promising results in the production of these compounds in cost effective amounts suitable for production of pharmaceutical formula. These results open up new opportunities for the set up of drug delivery systems based on engineered yeasts secreting compounds directly in the digestive tract. The main potential medical applications include the development of oral vaccines, the correction of metabolic disorders and the in situ production of biological mediators.

References

Abdel-Wahab, M.A. and S.E. Aly, 2003. Antioxidants and radical scavenging properties of vegetable extracts in rats fed aflatoxin contaminated diet. J. Agric. Food Chem., 51(8): 2409-2414.

Abo-Sereih, N.A., H.A. Mohamed, A.A. Haggran and R.C.S. Salim, 2011. Genetic and biochemical characterization of some food borne properties of Saccharomyces cerevisiae modified strains. Int. J. Academic Res., 3(1): 533-540.

Abraham, W.R. and R.G. Berger, 1994. Higher fungi for generating aroma components through novel biotechnologies. J. Agric. Food Chem., 42: 2344-2348.

Aeschback, R., J. Löliger, B.C. Scott, A. Murcia, J. Butler, B. Halliwell and O.I. Aruoma, 1994. Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food Chem. Toxicol., 32(1): 31-36.

Akada, R., Y. Shimizu, Y. Matsushita, M. Kawahata, H. Hoshida and Y. Nishizawa, 2002. Use of a *YAP1* over expression cassette conferring specific resistance to cerulenin and cycloheximide as an efficient selectable marker in the yeast *Saccharomyces cerevisiae*. Yeast, 19: 17-28

Aly, S.E., N. Abo-Sereih and K.E. El-Massry, 2005. Production of flavouring compounds through genetically engineered Saccharomyces cerevisiae and their evaluation as antioxidant and antimicrobial. Arab University J. Agric. Sci., 11(1): 205-219.

Aly, S.E., A.A. El-Shaffy, M.E. Selim, K.F. El-Massry and B.A. Sabry, 2011. Chemical profile, antioxidant, antifungal and antiaflatoxigenic activity of parsley and ginger volatile and Non-volatile extracts. J. Biologically Active Products Nat., 1(1): 81-96.

Awad, N.E., F.M. Hafez and A.M.M. Ali, 1993. The production of *Nigella Sativa* L. oil constituents through genetically engineered yeast strain. Assiut J. Agric. Sci., 24(4): 225-240.

Aydin, S., A. Basaran and N. Basaran, 2005. The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C. Mutat. Res., 581: 43-53.

Blakeway, J., 1986. The antimicrobial properties of essential oils. Soap Perfume Cosmetic, 59: 201-203.

Conner, D.E. and Beuchat, R.L. (1984). Effects of essential oils from plants on growth of food spoilage yeasts. J. Food Sci., 49: 429-434.

Croteau, R., 2000. Function of secondary metabolites in plants. Volume 40, Pp. 115-117.

Croteau, R., T.M. Kutchan and N.G. Lewis, 2000. Biochemistry and Molecular Biology of plants. American Society of plant physiologist, Rockville, MD, pp: 1250 -1318.

Daphevicius, A., R. Venskutonis, T. Van Beek and J.P.H. Linssen, 1998. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. J. Sci. Food Agric., 77:140-146.

de Melo, M.L.S., N. Narain and P.S. Bora, 2000. Food Chem., 68: 411-414.

Deans, G.G. and G. Ritchie, 1987. Antibacterial properties of plant essential oils. Int. J. Food Microbiol., 5: 165-180.

Denyer, S.P. and W.B. Hugo, 1991. Biocide-induced damage to the bacterial cytoplasmic membrane. In Mechanisms of Action of Chemical Biocides. Denyer SP, Hugo WB. (eds), The Society for Applied Bacteriology, Technical Series No 27. Oxford Blackwell Scientific Publication, Oxford. Pp.171-188.

Diatchenko, I., Y. Lau and A. Campball, 1996. A method for generating differentially regulated or tissue specific DNA probes and libraries. Proc. Nat. academic Sci., 93: 6025-6030.

Eaton, D.L. and E.P. Gallagher, 1994. Mechanisms of aflatoxin carcinogenesis. Ann. Rev. Pharmacol. Toxicol., 34: 135-172.

Elaraki, A. And L. Beraoud, 1994. Inhibition of growth and aflatoxin production in *Aspergillus parasiticus* by essential oils of selected plant materials. J. Food Protect., 61: 616-619.

Farag, R.S., Z.Y. Daw and S.H. Abo-Raya, 1989. Influence of some spice essential oils on *Aspergillus parasiticus* growth and production of aflatoxins in a synthetic medium. J. Food Protect., 54: 74-76.

Farag, R.S., H. Salem, A.Z.M.A. Badei and D.E. Hassanein, 1986. Biochemical studies on the essential oils of some medicinal plants. Fette Seifen Anstrichm, 88: 69-72.

Gulcin, I., M.E. Buyukokuroglu, M. Oktay and O.I. Kufrevioglu, 2003. Antioxidant and analgesic activities of turpentine of *Pinus nigra* Arn. subsp. *pallsiana* (Lamb.) Holmboe. J. Ethno-pharmacol., 86: 51-58.

Hamid, A.A., O.O. Aiyelaagbe and L.A. Usman, 2011. Essential oils: it medicinal and pharmacological uses. Int. J. Current Res., l3 (2): 86-98.

International Agency for Research on Cancer (IARC), 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. In: IARC monographs on the evaluation of carcinogenic risks to humans. IARC, Lyon, France. Vol. 56, Pp. 245-395

International Agency for Research on Cancer (IARC) (WHO), 2002. Mycotoxins. In: IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans: Some natural occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC, Lyon, France

Juven, B.J., J. Kanner, F. Schued and H. Weisslowicz, 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J. Appl. Bacteriol., 76: 626-630.

Kawai, S., H. Wataru and M. Kousaku, 2010. Transformation of *Saccharomyces cerevisiae* and other fungi, Methods and possible underlying mechanism Bioen. Bugs, 1(6): 395-403.

Klich, M.A., 2007. *Aspergillus flavus*: the major producer of aflatoxin. Mol. Plant Pathol., 8:713-722.

Knobloch, K., H. Weigand, N. Weis, H.M. Schwarm and H. Vigenschow, 1986. Action of terpenoids on energy metabolism. In Progress in Essential Oil Research: 16th International Symposium on Essential Oils. Brunke, E.J. De Gruyter (eds), Berlin. pp: 429-445.

Kumar, A., R. Shukla, P. Sing, S.C. Prasad and N.K. Dubey, 2008. Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. Innovative Food Sci. Emerg. Technol., 9(4): 575-580.

Legras, J.L., D. Merdinoglu, J.M. Cornuet and F. Karst, 2007. Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. Mol. Ecol., 16(10): 2091-2102.

Matthaus, B., 2002. Antioxidant activity of extracts obtained from residues of different oil seeds. J. Agric. Food Chem., 50: 3444-3452.

Miliauskas, G., P.R. Venskutonis and T.A. Beek, 2004. Screening of radical scavenging activity of some medical and aromatic plant extracts. Food Chem., 85: 231-237.

Nelson, R.R., 1997. *In vitro* activities of five plant essential oils against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. J. Antimicrob. Chemother., 40: 305-306.

Nguyen, D.V., M. Takascova, T. Jakubik and M.N. Dang, 2000. Antioxidative effect of thyme in rape-seed oil. Biologia, Bratislava, 55(3): 277-281.

Phaff, H.J., 1990. Isolation of yeast from natural sources. In: Isolation of biotechnological organisms from nature. Labedo DP (ed), Mc Grew Hill, New York. pp: 53-79.

Pham, T.A., S. Kawai, E. Kono and K. Murata, 2011. The role of cell wall revealed by the visualization of *Saccharomyces cerevisiae* transformation. Curr. Microbiol., 62: 956-961.

Ramboseck, J.A. and J. Leach, 1987. Recombinant DNA in filamentous fungi: progress and prospects. Crit. Rev. Biotechnol., 6: 357-393.

Rastogi, R.P. and B.N. Mehrotra, 2002. Glossary of Indian Medicinal Plants. National Institute of science communication. New Delhi, India.

Rota, M.C., A. Herrera, R.M. Martinez, J.A. Sotomayor and M.J. Jordan, 2008. Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. Food Control, 19 (7): 681-687.

Rustaiyan, A., S.H. Masoudi, A. Monfared, M. Kamalinejad, T. Lajevardi and S. Sedaghat, 2000. Volatile constituents of three Thymus species grown wild in Iran. Planta Med., 66: 197

Shimoni, Y., T. Kurihara, M. Ravazzola, M. Amherdt, L. Orci and R. Schekman, 2000. Lst1p and Sec24p cooperate in sorting of the plasma membrane ATPase into COPII vesicles in *Saccharomyces cerevisiae*. J. Cell Biol., 151: 973-984.

Sikkema, J., J.A.M. De Bont and B. Poolman, 1994. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem., 269: 8022-8028.

Solomakos, N., A. Govaris, P. Koidis and N. Botsoglou, 2008. The antimicrobial effect of thyme essential oil, nisin and their combination against *Escherichia coli* O157:H7 in minced beef during refrigerated storage. Meat Sci., 80 (2): 159-166.

Stahl, E., K.W. Quirin and D. Gerard, 1987. Dense Gases for Extraction and Refining, Springer Verlag, Berlin.

Stepanov, A., K.C. Nitiss, G. Neale and J.L. Nitiss, 2008. Enhancing drug accumulation in Saccharomyces cerevisiae by repression of pleiotropic drug resistance genes with chimeric transcription repressors. Mol. Pharmacol., 74: 423-431.

Verma, J.K. and A.V. Joshi, 2006. Rapid HPTLC method for identification and quantification of curcumin, piperine and thymol in an ayurvedic formulation. J. Planer Chromatogra., 19(111): 398-400.

Vural, H.C., 2009. Genomic DNA isolation from aromatic and medicinal plants growing in Turkey. Scientific Res. Essay, 4(2): 059-064.

Yanishlieva, N.V., E.M. Marinova, M.H. Gordon and V.G. Raneva, 1999. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem., 64(1): 59-66.

Zambonelli, A., A.Z. Daulerio, A. Bianchi and A. Albasini, 1996. Effects of essential oils on phytopathogenic fungi *in vitro*. J. Phytopathol., 144: 491-494.