Effect of Irradiation Treatment on the Microbial Production of α-Terpineol

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Abstract: Recently, several techniques have arisen to counteract the low yields of α-terpineol during the biotransformation process. The aim of this research is to study the role of gamma radiation in the increasing of α-terpineol yield. A native *Penicillium italicum* AUMC13045 that have ability to metabolize the orange oil was isolated, identified and irradiated. Irradiated fungal strains gave α-terpineol yield higher than no irradiated ones. The highest α-terpineol yield 91.79% obtained when the irradiated native *Penicillium italicum* AUMC13045 used for the bioconversion process.

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1. Introduction

Over the past few decades, an increasing trend on the way to effective utilization of natural resources has been observed around the world. Limonene molecule is very similar to the ones from very impacting aroma compounds, like carvone, α-terpineol, eugenol and thymol, *etc*. Due to the increasing amounts of limonene available from orange juice processing, the searches increased on the conversion of the inexpensive hydrocarbon R-(+)-limonene intooxyfunctionalized compounds. R-(+)-limonene is one of the most abundant monocyclic terpenes in nature and represents more than 90% of orange peel oil, while being an inexpensive citrus by-product available in bulk amounts (Pérez-Mosqueda *et al.*, 2015).

Biotransformation process of readily monoterpene precursors, for example R-(+)-limonene, into their more important oxygenated derivatives is recognized as being of great economic potential to the food and perfume industries as well as, limonene is an attractive starting substrate for conversion due to its abundance and little cost(de Carvalho and da Fonseca, 2006).

The first data on fungal bioconversion of limonene back to the late sixties. The double bond of limonene (1,2 position) was, first found to be attacked by *Cladosporium* sp. T7, yielding trans-limonene-1,2-diol, and small amounts of the cis-diol (Mukherjee *et al*., 1973).

Low yields can be generally regarded as the major problem on the biotransformation of limonene (de Carvalho and da Fonseca, 2006). In order to counteract the low yields, several techniques have arisen. Some of them are: suspended or immobilized cells, induction of enzymes through adaptation of pre-cultures with substrates and/or products, two phase reaction systems, and other techniques (Marostica- Jr and Pastore, 2007).

The most interesting end products resulting from the bioconversion of limonene was α-terpineol where, fungi which isolated from rotting orange rind were found to be able to convert limonene to α-terpineol. Terpineols are unsaturated monocyclic terpene alcohols, α- and β-terpineol occur in optically active forms and as racemates. These terpenoids are naturally found in low quantities in many volatile oils and used extensively in cosmetics and flavouring of foods and beverages. α-terpineol is an important commercial product, has anticancer activity and can play a role in antioxidant and antibacterial processes (Paduch *et al*., 2007).

Gamma-irradiation affects the activity of some fungal species during fermentation processes. Where, low dose of ionizing radiation on some fungal strains is responsible for accelerated enzymatic activity (Chakravarty and Sen, 2001), using gamma radiation increases production of enzymesin filamentous fungi, and irradiation at dose 500 Gy stimulates *Penicillium* sp. to produce phenoloxidases enzymes Baptista *et al.* (2015). Gherbawy (1998) showed that the lowest dose of gamma irradiation (1 millicurie / 10 min) enhanced three isolates of *Aspergillus niger* to produce more biomass, polygalactronase, pectinmethylglacturonase, cellulase and protease. While, the higher doses (1 millicurie for 20 and 30 min) were inhibitory to the growth of *A. niger*.

Haggag and Mohamed (2002) irradiated *Trichoderma harzianum, Trichoderma viride* and *Trichoderma koningii*a t dose 0.5 kGy resulted in producing highly active exo-enzymes caused the highest percentage of pathogen growth reduction.

Afify *et al.* (2013) indicated that the biomass of *Trichoderma spp.* was increased and reached its maximum at 250 Gy and as a general trend, the gamma radiation over than 0.25 kGy reduce the growth of *Trichoderma spp.*

The role of gamma radiation in the biotransformation of orange essential oil processes has never been reported. So, the objective of this research is to study the microbial transformation of orange peel essential oil using irradiated fungal species.

2. Materials and Methods

Materials

Orange fruits *(citrus sinensis)* at mature stage harvested in season 2016-2017 were obtained from local market, Sharqia Governorate, Egypt.

Chemicals

Authentic D-limonene and α-terpineol were purchased from Fluka™ (~ 99%), Sigma-Aldrich, Switzerland. Diethyl ether and Pentane (Alkan C_5) were purchased from SDFCL™, Mumbai, India. All other chemicals and solvents were of the best grade available.

Microbial stains and media

Penicillium digitatum AUMC525 strain was obtained from center of fungi, Asuit University. Identified native *Penicillium italicum* AUMC13045 was isolated from orange. Potato Dextrose Agar (PDA): 2% glucose, 0.4% potato extract, 1.5% agar. Malt Extract Agar (MEA): 2%malt extract, 0.1%peptone, 2%glucose, 1.5%agar. Malt Yeast Broth (MYB): 2% malt extract, 1%glucose, 1%peptone, 0.3%yeast extract.

Extraction of orange peel oil

The essential oil of orange was extracted by applying water/steam distillation method using a Clevenger-type apparatus. Orange fruits were peeled off carefully, to avoid any damage of oil glands. The separated part of orange (flavedo) was employed in round flask, add deionized water in another connected flask and then boil. The apparatus of water/steam distillation is set-up. The boiling time was 120 – 160 min., the extracted volatile oils was collected, dried over anhydrous sodium sulphate, put in opaque glass bottles and kept at –20 ^{o}C until needed.

Isolation of fungi converted orange oil

Isolation of different fungus species

Various types of spoiled oranges were selected, with purplish to dark brown rot, blue rot, green rot as well as those with black rot from local fruits markets in Zagazig, Sharkia, Egypt. Each spoiled orange was collected into sterile polythene bag; these samples were kept unwashed and transported to lab for the experiments. Isolation of fungi from each of the orange samples was carried out using the method of (Baiyewu *et al.*, 2007). Segment of tissue from the margin of the decayed fruit were cut with a sterile scalped and placed on the previously prepared potato dextrose agar (PDA) in petri dishes and incubated at 28±1°C for 5 days. The detected fungi were carefully isolated into pure cultures of PDA in plates and slants. The plates and slants were incubated for seven days at 28°C.

Identification of the selected fungal species

The fungal colonies arising on the plates were purified. The purified cultures were prepared into mounts in microscopic slides by placing portion of mycelial growth carefully picked with the aid of sterile inoculating needle in a drop of lactophenol cotton blue. The microscopic slide was covered with a cover slip and was examined under the microscope, first with (X10) and then with (X40) objective lens for morphological examination with descriptions by (Samson and Reenen-Hoekstra, 1988; Fawole and Oso, 1998 and Oyeleke and Manga, 2008). The fungal isolates were identified by comparing their morphology and characteristics with descriptions given by (Samson and Reenen-Hoekstra, 1988).

Selection of the most fungi utilized orange peels oil

The ability of fungal isolates which isolated from moldy oranges for using orange peels oil as carbon source and converted it were studied in this experiment. MEA media prepared with different concentration of carbon source; group A (2% glucose, 0% orange oil), group B (1.5% glucose, 0.5% orange oil), group C (1% glucose, 1% orange oil), group D (0.5% glucose, 1.5% orange oil) and group E (0% glucose, 2% orange oil). In all cases, the media from each group were inoculated with 1 ml of spore suspension (1 x 10^7spores/m) of each tested isolates. After inoculation, petri dishes were incubated at 25±2°C for 7 days, fungal growth was determined to select the best fungi have the ability to utilize orange oil.

Identification of native *Penicillium* sp.

Native *Penicillium* sp. isolate selected for biotransformation in further experiments was identified by Mycological Center, faculty of science, Assiut University (AUMC). Identification was done morphologically based on macro- and microscopic characteristics and descriptions given by Pitt (1979) and Domsch *et al.* (2007). It is close to *Penicillium italicum* Wehmer. Thus, the fungal strain of *Penicillium* sp. was identified as *Penicillium italicum* AUMC13045 (Figure 1).

Irradiation treatments

The most potent orange oil transformer fungi were applied in this study to investigate the role of gamma radiation in the biotransformation process. Fungal strains were stopper carefully on growing slant of PDA medium to be ready for irradiation by gamma ray. The applied irradiation doses were 0.4, 0.5, 0.6,

0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 kGy. The source used for the irradiation process was cobalt-60 Gamma cells 220, located at the National Center for Radiation Research and Technology, Nasr City, Cairo, Egypt. This source provided an average exposure rate of 2.85 kGy / 1 hour at the time of the experiments. After irradiation, survival percentages of nonirradiated fungal strains (control) and irradiated ones were determined.

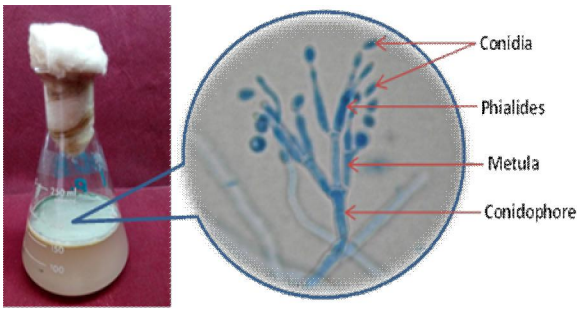

Figure 1 Identification of *Penicilliumitalicum* AUMC13045 as described by (Pitt, 1979 and Domsch *et al*., 2007)

Influence of Gamma rays on the bioconversion process:

MYB medium is used in comparing the bioconversion process of orange peel oil between irradiated fungal strains and nonirradiated ones. Liquid cultures of tested fungal strains run with shaking in the bioconversion experiments according to Marostica- Jr and Pastore (2007) with some modifications concerning; substrate amount and sampling time. The mold was cultivated in 250 ml flask, filled with 150 ml of sterile liquid media. The culture media was inoculated with 1ml of fungi spore suspension and pre-incubated at 27°C for 72 h with agitation of 150 rpm, using an orbital shaker.

After pre-incubation, biotransformation was initiated with the addition of 50 µl of orange peels oil each 12 h (200 µl, total addition) under sterile condition. Chemical blanks were performed in all biotransformation experiments (media containing only orange peels oil without mycelium) to ensure the absence of chemical transformation reactions. After 48 hfrom the first addition of orange peels oil, 5 ml samples were taken for extraction and analysis using GC analysis.

Extraction of samples and Analysis by GC:

At the end of each bioconversion experiment, 5 ml fungal cell were extracted (Marostica- Jr and Pastore, 2007) by diethyl ether and pentane (1:1, v/v) and identified by gas chromatography GC**.** GC analysis was performed using Perkin Elmer Auto system XL equipped with flame ionization detector (FID). A fused silica capillary column DB – 5 (60 m × 0.32 mm, i.d.) was used. Helium was used as the carrier gas, at flow rate 1.1 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The injector and detector temperatures were 240 and 250°C, respectively. The retention indices (Kovats index) of the separated volatile compounds were calculated with hydrocarbons (C8 – C22, Aldrich Chemical Co.) as references.

The reduction in the limonene area (at each sampling time) between blank and sample experiments was used in the calculation of limonene bioconversion (%) according to Demyttenaere *et al.* (2001) as follows:

$$Limonene\ bioconversion(\%) = \frac{(area\ of\ limonene\ in\ a\ blank\ -\ area\ of\ limonene\ in\ a\ sample)}{area\ of\ limonene\ in\ a\ blank} \times 100$$

Where, the α-terpineol production was followed using GC analysis in all culture extracts to calculate the percentage of limonene bioconversion (The major monoterpenes components of orange peel essential oil were d-limonene representing 82.87%).

3. Results

Isolation and identification of different fungi from orange

The fungi responsible for the spoilage of orange were isolated and identified; in order to select the most fungal strain had the ability to metabolize orange peel oil. Fungal species such as *Penicilliumsp.*, *Aspergillus sp., Fusarium sp., Rhizopus sp.,* and *Alternaria sp.* were isolated from decayed orange (Table1). These results in the line with Matny (2015). In addition,(Barth *et al.*, 2009)who isolated about seven different fungal genera from different fruits including sweet orange fruits.

Selection of fungi utilized orange peels oil

The metabolism of limonene by microorganisms was different, some microorganisms resistant against limonene and have the ability to metabolize it. Otherwise, there are some microorganisms which sensitive against limonene and limonene have toxic effect on it. In this experiment, fungal isolates isolated from orange peels were cultivated in MEA medium containing different concentration of orange peel oil as carbon source. The results are tabulated in Table 2 where, a native *Penicilliumsp.* was grown in MEA

medium with different concentrations of orange essential oil represented the highest growth in all groups comparing with other fungal isolates. The previous results were in the line with Marostica- Jr and Pastore, (2007) who found that different species of *Penicillium, Aspergilllus and Fusarium* had the ability to grow on different media supplemented with limonene and the fungal able to convert limonene to α-terpineol. Thus, *Penicillium sp.* was the best selection for biotransformation in further experiments.

Survival percentages % of gamma irradiated *Penicilliumdigitatum* AUMC525and *Penicilliumitalicum* AUMC13045 at different doses

Microorganisms differ greatly in their sensitivities to ionizing radiation. The radiation sensitivity differs with species and even with strains. Fungal strains growing on slants of PDA agar medium were gamma irradiated at doses of 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 kGy. Figure 2 showed the survival percentages of the *Penicilliumdigitatum* AUMC525 and identified a native *Penicilliumitalicum* AUMC13045 irradiated at different doses of gamma rays. The correlation between the decreasing survival percentages and the increasing gamma doses was recorded. The survival percentages at (0.4 and 0.5 Gy) showed no difference compared to the control. On the other hand, a dramatic decrease was recorded starting from 5 and 7 kGy until complete killing which observed at doses of 6 and 8 kGy for the *Penicilliumdigitatum* AUMC525 and identified a native *Penicilliumitalicum* AUMC13045, respectively. The previous data were in the same trend with Dadachova and Casadevall, 2008, who found that irradiation doses up to 4 kGy were not fungicidal for different fungal strains like; *Aspergillus* (13 species), *Penicillium* (5 species) and *Cladosporium* (4 species).

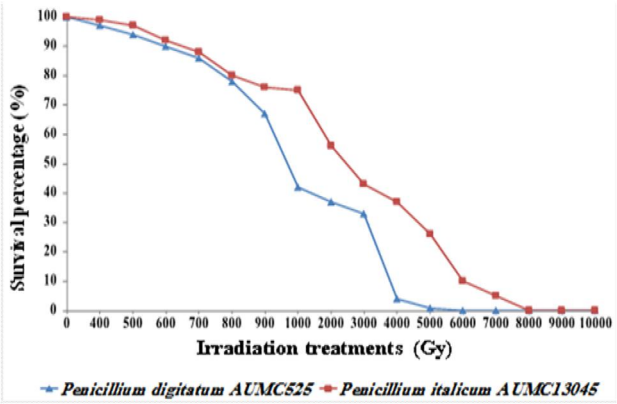

Figure 2 Survival percentage (%) of gamma irradiated *Penicilliumdigitatum* AUMC525 and native *Penicilliumitalicum* AUMC13045 at different doses

Table1 Identification of fungal isolates from spoiled oranges

Spoilage	Morphology description	Microscopic Examination	Organism	References
Green rots	Woolly in texture, colonies initially white and become green while reverse white to yellowish	Septate hyaline hyphae simple or branched conidiophores, phialides grouped in brush-like clusters at the end of conidiophores	*Penicillium sp.*	Samson and Pitt, 2000
Dark brown discoloration, sunken spots fruits become spongy	Yellow to white hyphae, turning black with conidial formation. The reverse is pale yellow	Septate hyphae, unbranched, smooth, long, hyaline conidiophores arise from foot cell and terminating in a globose vesicle. conidia arranged as catenulate	*Aspergillus sp.*	Samson and Pitt, 2000
Whitish-pink mycelial growth	Mycelium sparse or abundant ranging in color from white to pale violet or violet white	Mycelium producing terminal or intercalary chlamydospores, usually singly, in pairs, in clusters or in short chain. Conidia straight to slightly curved usually 3 to 5 septate	*Fusarium sp.*	Leslie and Summerell, 2006
Watery, soft rot, wrinkled appearance with depression	Colonies are cotton-candy like in texture. Colony color is witish becoming gray to yellowish, reverse white to pale	Sporangiophores non septate, simple or branched arising from stolon opposite rhizoids in groups of 3 or more. Sporangiospores are ovoid in shape	*Rhizopus sp.*	Oyeleke and Manga, 2008
Brown or black, sunken spots	Woolly and fuzzy in texture, grayish in color while reverse side is brown to black	Septate hyphae, conidiophores septate, simple or branched. Conidia, septate, muriform, oval, chains or single	*Alternaria sp.*	Fawole and Oso, 1998

Table2 Fungi growth on Malt Extract Agar (MEA) media containing different concentration of carbon source

	Group A	Group B	Group C	Group D	Group E
Penicillium sp.	+++	+++	+++	+++	++
Aspergillus sp.	+++	+++	+++	++	±
Fusarium sp.	+++	++	++	++	+
Rhizopus sp.	+++	+	+	±	±
Alternaria sp.	+++	++	+	±	±

Group A = 0% oil + 2% glucose, Group B = 0.5% oil + 1.5% glucose, Group C = 1% oil + 1% glucose, Group D = 1.5% oil + 0.5% glucose, Group E = 2% oil + 0% glucose, +++ = Full growth, ++ = heavy growth, + = moderate growth and ± = slightly growth

Biotransformation by irradiated *Penicillium digitatum* AUMC525

To investigate the role of gamma radiation in the biotransformation process, irradiated *Penicillium digitatum* AUMC 525 was applied in this study, irradiated at different gamma ray doses (0.4, 0.6, 0.8 and 1.0kGy). The results displayed in Table3found that the highest α-terpineol yield 81.19%was obtained using irradiated *Penicillium digitatum* AUMC525at 0.4kGy, compared to non-irradiated (53.83%). On the contrary, α-terpineol was not produced at 1.0 kGy.

Table3 Metabolite production during the orange peel oil biotransformation, using irradiated *Penicillium digitatum* AUMC525

Extract content (%)	**Irradiation Treatment (kGy)**				
	0	**0.4**	**0.6**	**0.8**	**1.0**
Unconverted Limonene	22.76	17.12	37.94	47.23	48.01
Produced *α*-terpineol	44.61	67.29	26.04	10.87	0.0
Other products	32.63	15.59	36.02	41.9	51.99
Biotransformation (%)					
Total Biotransformation	77.24	82.88	62.06	52.77	51.99
α-terpineol Biotransformation	53.22	64.81	22.01	2.92	1.31
Yield of *α*-terpineol	53.83	81.19	31.42	13.11	0.0

Biotransformation by irradiated native *Penicillium italicum AUMC13045*

In this experiment, identified *Penicilliumitalicum* AUMC13045 was subjected to gamma irradiation at different doses (0.4, 0.6, 0.8 and 1.0 kGy). Then the results tabulated in Table 4 where, 0.4 kGy represented the best dose that induce identified *Penicillium* sp. to produce α-terpineol from the biotransformation process of limonene. The yield of α-terpineol was 91.79% compared to non-irradiated 70.01%. As a point of view, this might be low doses of gamma radiation were used to enhance the α-terpineol production.

Table4 Metabolite production during the orange peel oil biotransformation, using irradiated a native *Penicilliumitalicum* AUMC13045

Extract content (%)	**Irradiation Treatment (kGy)**				
	0	**0.4**	**0.6**	**0.8**	**1.0**
Unconverted Limonene	14.27	9.08	38.84	20.02	40.05
Produced *α*-terpineol	58.02	76.07	25.46	32.11	29.15
Other products	27.71	14.85	35.7	47.87	30.8
Biotransformation (%)					
Total biotransformation	85.73	90.9	61.16	79.98	59.95
α-terpineol Biotransformation	70.67	81.33	20.16	58.85	17.68
Yield of *α*-terpineol	70.01	91.79	30.72	38.75	35.18

4. Discussion

Microorganisms and their enzymes have proven to be versatile biocatalysts (Jones *et al.*, 1993) and are extensively used for biotransformations of various terpenoids. A large variety of enzymes occur in several microorganisms which are effective in

biotransformations of various terpenoids and can be used *invivo*. In addition, whole cells are generally less expensive compared to purified enzymes, and, in some cases, enzymes are more stable within the cell, thus extending the life of the biocatalyst. Moreover, when comparing biotransformations with isolated enzymes and whole microbial cells, microorganisms generally produce compounds with lower enantioselectivity because a microbial cell may have multiple enzymes that are capable of transforming a substrate (Nakamura, 1998).

Limonene Hydratase enzymeis involved in the bioconversion of the monoterpene limonene. One part of this pathway involves the conversion of limonene to α-terpineol, which is the corresponding hydration product. Limonene hydratase are often found in fungi (especially in those that grow on rotting citrus peel) such as *Fusarium oxysporum* (Marostica- Jr and Pastore, 2007and Molina*et al*., 2015), *Aspergillus niger* (ATCC 16404, ATCC 9642 and ATCC 1004 strains) (Rottava*et al*., 2010) and *Penicillium digitatum* (Badee*et al*., 2011).

The bioconversion of limonene by micro-organisms is often catalyzed by catabolic enzymes, with the main purpose of making limonene available as an energy source. Although there are some examples of micro-organisms that carry out such oxidations with rather high specificity, many other micro-organisms oxidize limonene with a low specificity, leading to a large number of unwanted side products (Chang and Oriel, 1994). Furthermore, several studies indicated that initial enzymes involved in limonene catabolic pathways are not always completely regiospecific. Possibly this is due to the absence of a sufficiently strong selective pressure; complete regiospecificity of catabolic oxygenases is only essential if the regioisomers cannot be assimilated (and would thus be lost as a source of carbon and energy, after the investment of NADH). However, many strains capable of growth on D-limonene can utilize a wide range of hydroxylated derivatives (van der Werf and de Bont, 1998).

In spite most terpenes have antimicrobial properties, dilution with the organic solvent likely helps in reducing their toxicity towards the microorganisms being used for the biotransformation (de Carvalho and da Fonseca, 2002a).

Various investigations directed to the reduction of fungi in food products by gamma irradiation. The inactivation doses for fungi, particularly in dry spices, are reported generally in the limit of 6 kGy Dadachova and Casadevall, 2008 where, radioresistance of different fungal strains accounted as lethal doses (LD_{10}) were; 5 kGy for *Alternaria* sp. and *Cladosporium*sp. and ≈ 4.3 kGy for *Cryptococcus* sp. This radioresistance of fungi is not widely appreciated and should be taken into consideration when gamma radiation is used for sterilization of food or medical supplies.

One of suggested explanation of the fungal radioresistant, cultures in agar provide a heavier and more diversified bioburden, along with nutrient conditions that favor the function of repair systems. In addition, organic compounds in the medium compete with the fungus for the hydrolytic products of irradiation (Silverman, 1983).

On the contrary, gamma radiation nowat low dosesis widely used for improving the microbial activities such as increasing the production of hydrolytic enzymes for industrial applications, hyphal growth and spores germination (Tugay*et al*., 2006). This observation called "radiostimulation" and also named "radioadaptive response". However, the presence of adaptive properties in fungi exposed in the long term to elevated radiation levels are need further investigations.

For instance, utilization of gamma rays for enhancing the cellulase enzyme from *Trichoderma reesei* through a successive mutagenesis steps was applied (Shahbazi *et al.*, 2014). They obtained number of mutant strains showing rapid growth rate and enhanced cellulase production compared to the parent strain. Huma *et al.* (2012) reported the hyper production of α-amylase by gamma radiation treatment from *Phialocephala humicola*. This enzyme has diverse industrial applications. In this regard, enhancement of polygalctouronase enzyme (PGase) from *Penicillium citrinum* was achieved by optimizing of all conditions in solid state fermentation (SSF) associated with applying gamma rays at dose of 0.7 kGy as the best dose (El-Batal *et al.*, 2013).

Mutagenesis by gamma rays radiation is well used for improving microbial strains for enhancing production and synthesis of many products. Enhanced production of glucose oxidase was performed by mutagenesis of *Aspergillus niger*by gamma irradiation. It was found that a dose of 800 Gy was optimum for derivation of positive mutant strains and the selected mutants (*A. niger* G-80A, *A. niger* G-80-B and *A.niger* G-80-C) showed a higher glucose oxidase activity compared to parental strain as reported by (Anjumzia *et al.*, 2012).

Conclusion

The study concluded that the best fungal strain (*Penicillium* sp.), had the ability to grow in MEA medium fortified with orange essential oil, as a carbon source, comparing with other isolated fungal strains. Gamma irradiation experiments indicated that 0.4kGy was the best irradiation dose that induce identified *Penicillium italicam* AUMC13045 to convert d-

limonene into α-terpineol (81.33%, conversion) yielding 91.79% of converted pure α-terpineol.

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