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Gamma Radiation in Improvement the Production and Anticancer Activity of Novel L-Asparaginase Fungal Producer *Fusarium Incarnatum*

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ABSTRACT

The Fungi have a great ability to produce a wide range of benefit and applicable enzymes. L-asparaginase is one of them, this study concerned about new fungal producer *Fusarium carnatum* and the enhancement of γ-ray in the enzyme production. Experimentally, *F. incarnatum* without exposure to γ-ray recorded 4.33 IU/ml where that exposed to 75Gy produced 10.14 IU/ml with 2.3 fold increase. The enzyme was purified from irradiated *F. incarnatum* using ammonium sulphate precipitation followed by dialysis and gel filtration by Sephadex G-100 leading to 11.58 fold purification and 7.43% yield. As anticancer drug, the enzyme dose at 10, 5 and 2.5 IU is very effective against three human tumor cell lines namely, HepG2 (Human hepatocellular carcinoma cell line), MCF 7 (Breast cancinoma cell line), CACO-2 (colorectal adenocarcinoma cell line) as it reduce the survival of all tested cell lines by 90%. The fungal enzyme showed low IC_{50} (0.87 and 0.94 U/ml) for HepG2 and MCF-7 respectively where it recorded 1.81 U/ml for CACO-2 tumor cell line. In conclusion, the low dose γ-ray irradiated *F. incarnatum* produce significant amount of l-asparaginase in simple and cheap solid state fermentation with promising anticancer activity.

Keywords: L-Asparaginase, *Fusarium incarnatum*, γ-ray irradiation, solid state fermentation, MTT assay.

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INTRODUCTION

L-asparaginase (E.C. 3.5.1.1) belongs to amidase group catalysis the conversion of l-asparagine to l-aspartic acid and ammonia (Ghasemi *et al.,* 2008). It is one of the most important biomedical and biotechnological groups of enzymes as it constitutes nearly 40% of the total worldwide enzyme sales (Warangkar and

Khobragade, 2009). This enzyme is widely used in treatment of certain malignancies such as Hodgkin's disease, leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma. Hence, the exponential increase in the application of enzymes in various fields, demands extension in both qualitative improvement and quantitative enhancement.

The enzyme present in a wide range of organisms including animals, microbes, plants, and in the serum of certain rodents but not in human beings, its production has been reported from *Serratia marcescens* (Rowley and Wriston 1967), *Erwinia carotovora* (North *et al.,* 1969), *Proteus vulgaris* (Tosa *et al.,* 1972), *Saccharomyces cerevisiae, Streptomyces karnatakensis, S. venezuelae.* Recently, Eukaryotic microorganisms like yeast and filamentous fungi *Aspergillus, Penicillium* and *Fusarium* have a potential for the production of l-asparaginase (Pinheiro *et al.,* 2001; Dange and Peshwe, 2015; Vijay and Jaya Raju, 2015). Human beings are more closely related to fungi as compared to bacteria. Hence, chances of immunological reaction against fungal l-asparaginase will be lesser (Shrivastava *et al.,* 2012).

For practical cultivation of l-asparaginase producing fungi, agricultural wastes represent one of the most abundant, cost effective substrates and energy rich sources for cultivation and production of various fungal enzymes. Practically, solid state fermentation (SSF) has emerged as an effective technique to increase the product yield at low capital cost, low energy input, simple fermentation media, and low water use, and offers simple operational control. Other advantages include ease of product extraction (Baysal *et al.,* 2003).

Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme as the quantities produced by wild strains are usually too low (Szengyel *et al.,* 2000; Chand *et al.,* 2005; Li *et al.,* 2009; Pradeep and Narasimha, 2011). The improvements of strain in industry are mostly attributed to the extensive application of mutation and selection (Xu *et al.,* 2011; Vu *et al.,* 2011), such improved strains can reduce the cost of the processes with increased productivity (Karanam and Medicherla, 2008). Irradiation by γ-ray may cause some mutations to the genes of cells through the DNA repair mechanisms within cells. This type of radiation is short wave high energy electromagnetic radiations emitted from certain radioactive isotopes such as Co^{60}. The effect of γ-ray doses on fungal enzyme production was studied by many researchers (Macris, 1983; Shimokawa *et al.,* 2007; Yousef *et al.,* 2010).

The target of this study is improvement of l-asparaginase produced by *Fusarium incarnatum* via γ-ray in cost effective SSF of wheat bran and in vitro application of the produced enzyme as anticancer.

MATERIAL AND METHODS

Fungal isolate

F. incarnatum used in this study was previously isolated and identified in the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University, Cairo, Egypt.

Preparation of sample for γ-irradiation

Conidial suspension was prepared from freshly raised seven-day old culture of *F. incarnatum* on Czapek-Dox agar slants by suspending in 10 ml of 0.85% sterile saline solution.

Mutagenesis with γ-ray

The Spore suspension in triplicates was irradiated by different doses 75, 125, 250, 500, 1000 Gy using Co^{60} gamma source according to Iftikhar *et al.,*. (2010) by the Indian γ cell of Co^{60} located at the National center of radiation research and technology (NCRRT), Nasr City, Cairo, Egypt.

Qualitative plate assay for l-asparaginase activity

For rapid qualitative technique for l-asparaginase activity, 100 µl of spore suspension was poured into the agar well of diameter 8 mm prepared in plates containing modified Czapek-Dox agar medium containing l-asparagine as the sole source of carbon with 3 ml/l of 2.5% of phenol red indicator dye (Gulati *et al.,* 1997)

The inoculated plates with spore suspension of different γ-ray doses were incubated for 3 to 5 days at 28 ±2ºC. Positive doses were detected based on the appearance of a pink zone around the well in yellow colour medium indicating l-asparaginase activity due to the change in colour of phenol red indicator as NH_3 is liberated.

L-asparaginase production by *F. incarnatum* on SSF of Wheat Bran

The fermentation medium had the following composition: 10 g wheat bran (WB) moistened with 10 ml of deionized water and placed in 250 ml Erlenmeyer flasks. The medium was sterilized by autoclaving for 15 min., inoculated with spore suspension of different γ-ray doses under sterile conditions and incubated under static conditions at 28±2°C for 4 days.

Extraction of l-asparaginase from SSF medium

The extracellular crude enzyme was eluted at the end of the fermentation period by the addition of 50 ml of a 0.01 M phosphate buffer pH 6.2 to the fermented medium, shaking for 30 min. followed by centrifugation at 8000 rpm for 20 min. The cell-free filtrate was used as crude enzyme solution to estimate l-asparaginase activity.

Quantitative assay of l-asparaginase activity

L-Asparaginase enzyme assay was performed by a colorimetric method by quantifying ammonia formation in a spectrophotometric analysis using Nessler's Reagent (Gurunathan and Sahadevan, 2011). For routine assay 0.1 ml of diluted enzyme solution was added to 0.4 ml of 0.025 M l-asparagine solution in 0.1 M Tris-HCl buffer (pH 8.0). Incubation was conducted for 30 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1N H2SO4. The precipitated protein was removed by centrifugation and 0.2 ml of supernatant was diluted with 3.8 ml of distilled water and 0.5 ml of Nessler`s reagent was added, and the absorbance was measured at 400 nm within 1 to 3 min. Enzyme and substrate blanks were included in all assays, and a standard curve was prepared with ammonium chloride (Roberts, 1976). The enzyme activity was expressed as unit IU/ml. One unit of l-asparaginase activity is defined as that amount of enzyme that liberates one micromole (µmol) of ammonia per min under the standard assay conditions. The specific activity is defined as the units of l-asparaginase per milligram protein.

Cell viability test

L-asparaginase was tested for cytotoxic effect against three tumor cell lines namely, HepG2 (Human hepatocellular carcinoma cell line), CACO-2 (colorectal adenocarcinoma cell line) and MCF 7 (Breast cancinoma cell line). When the cells reached confluence (75–90%) (Usually after 24 h), the cell suspension was prepared in complete growth medium (RPMI 1640).

One liter of RPMI 1640 contains: Glucose (2 g), pH indicator (phenol red, 5 mg), Salts (6 g sodium chloride, 2 g sodium bicarbonate, 1.512 g disodium phosphate, 400 mg potassium chloride, 100 mg magnesium sulfate, and 100 mg calcium nitrate), Amino acids (300 mg glutamine; 200 mg arginine; 50 mg each asparagine, cystine, leucine, and isoleucine; 40 mg lysine hydrochloride; 30 mg serine; 20 mg each aspartic acid, glutamic acid, hydroxyproline, proline, threonine, tyrosine, and valine; 15 mg each histidine, methionine, and phenylalanine; 10 mg glycine; 5 mg tryptophan; and 1 mg reduced glutathione) and

Vitamins (35 mg i-inositol; 3 mg choline chloride; 1 mg each para-aminobenzoic acid, folic acid, nicotinamide, pyridoxine hydrochloride, and thiamine hydrochloride; 0.25 mg calcium pantothenate; 0.2 mg each biotin and riboflavin; and 0.005 mg cyanocobalamin) (Moore *et al.,* 1967; Atlas and Snyder, 2006**)**. The medium was supplemented with 50 mg/ml gentamycin. The aliquots of 100 µl of 105 cell suspension were added to each well on a 96-well tissue culture plate. The blank wells contained complete RPMI medium in place of cell suspension. The cells were incubated for 24 h at 37°C in a humidified incubator with 5% CO2.

After the formation of a complete monolayer cell sheet in each well of the plate, the samples were added ranging from15.63 µg/ml to 500 µg/ml. Serial two-fold dilutions of the samples was added into a 96-well tissue culture plate using a multichannel pipette (eppendorff, Germany). After treatment (24 h), culture supernatant was replaced by fresh medium. Then, the cells in each well were incubated at 37°C with 100 ml of MTT solution(5 mg/ml) which consisits of (3-(4,5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide, a tetrazole) for 4 h.

After the end of incubation, the MTT solution was removed, then 100 ml of DMSO was added to each well. The absorbance was detected at 570 nm using a microplate ELISA reader (SunRise TECAN, USA).The absorbance of untreated cells was considered as 100%. The results were determined by three independent experiments.

The percentage cell viability was calculated according to the following equation: the percentage of cell viability = [1 − (ODt/ODc)] × 100%, where ODt is the mean optical density of wells treated with the tested compound and ODc is the mean optical density of untreated cells. The absorbance is proportional to the number of surviving cells in the culture plate. The tested sample was also compared using the IC50 value, i.e., the concentration of an individual compound leading to 50% cell death that was estimated from graphical plots of viable cells vs compound concentrations (Cheng *et al.,* 2003).

Purification of l-asparaginase of *F. incarnatum*

The purification process of l-asparaginase from the crude extract obtained from potent strain was carried out at 4°C in a cold room

Ammonium sulfate precipitation

A four-day old culture filtrate (200 ml) of *F. incarnatum* was collected after centrifugation at 8000 rpm for 10 min at 4°C. The crude enzyme was subjected to ammonium sulfate precipitation and the protein precipitate at 80% salt saturation was allowed to stand overnight. The precipitate was collected by centrifugation at 10,000 rpm for 15 min and resuspended in 0.01M Tris-HCl buffer (pH-7.2) and dialyzed overnight against the same buffer.

Gel filtration chromatography

The lyophilized samples were loaded onto Sephadex G-100 (1.5×15cm) column pre-equilibrated with 100 mM phosphate buffer pH 7.0 and eluted with Tris-HCl buffer (0.01M; pH 7.2). Fractions were collected (1ml/tube) and the absorbance of all the fractions was recorded at 280 nm and active fractions were pooled and concentrated. The purified l-asparaginase was stored at 4°C and used for further characterization.

Estimation of protein

The concentration of protein was estimated by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976).

Determination of molecular weight of purified protein by SDS-PAGE

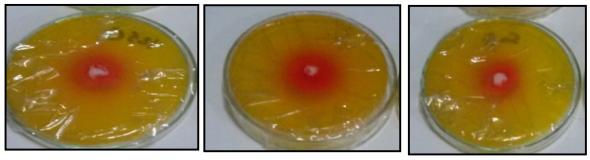
The molecular weight of purified l-asparaginase was determined by (SDS-PAGE) the method of Laemmli (1970). SDS-PAGE was performed using a 12% polyacrylamide gel. The proteins were

stained with coomassie brilliant blue R-250. The molecular weight of purified enzyme was determined using standard molecular weight markers (Bio-Rad).

RESULTS

Screening of wild and γ-irradiated *F. incarnatum* for l-asparaginase production using rapid plate assay method

The wild type of *F. incarnatum*, when exposed to different γ-ray doses ranging from 75 to 1000 Gy gave five irradiated samples with different abilities to produce l-asparaginase. As showen in fig 1, the change of the indicator color from yellow to pink resulted from the increase in pH due to ammonia release so the pink color around fungal colony confirmed the l-asparaginase production. The unirradiated *F. incarnatum* showed pink zone of 1.5 cm where 75Gy γ-ray exposed showed the maximum diameter of pink zone diameter (3.0 cm) followed by 125Gy (2.5 cm), 250Gy (2.0 cm). Minimum activity was observed at 500Gy (1.4 cm) and 1000Gy (0.9 cm).



Control 75 Gy 1000 Gy

Fig 1: Rapid plate assay for the screening of l-Asparaginase production at different doses of γ-ray.

Determination of *F. incarnatum* l-asparaginase activity

The quantitative assay of l-asparaginase production from γ-ray irradiated *F. incarnatum* revealed that the low doses of a γ-ray (75, 125 and 250 Gy) significantly increased the enzyme activity of *F. incarnatum* as compared with un-irradiated**.** On the contrary, *F. incarnatum* irradiated with 500 and 1000 Gy doses resulted in inhibition of the enzyme production compared with unirradiated one. Digitally, the data in fig (2) revealed that the enzyme activity of *F. incarnatum* (control) without exposure to γ-ray recorded 4.33 IU/ml where that exposed to 75Gy produced 10.14 IU/ml with 2.3 fold increase. At 125Gy the enzyme activity slightly decreased to 9.38, where that irradiated by 1000 Gy produce only 3.94 IU/ml.

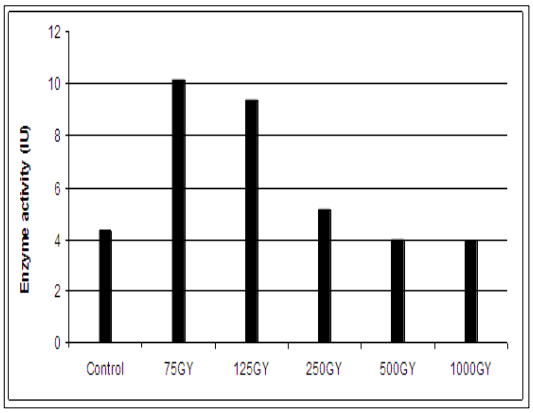


Fig. 2: Effect of different doses of γ radiation on of l-Asparaginase production using *F. incarnatum*.

Partial purification of l-Asparaginase

L-asparaginase enzyme was purified from irradiated *F. incarnatum* at 75 Gy using ammonium sulphate precipitation followed by dialysis and gel filtration by Sephadex G-100 (table 1). Asparaginase activity of culture supernatant was recorded (10.14 U/ml) and after concentrated by ammonium sulphate precipitation (80% w/v saturation) it was recorded 18.63 U/ml with specific activity 2.44 U/mg protein/ml with 5.77-fold increase in specific activity compared to the unconcentrated supernatant. After fractionation with Sephadex G-100, the specific activity of l-asparaginase was increased from 2.44 to 11.46 IU/mg with 11.58 fold purification and 7.43% yield.

Antitumor activity of purified *F. incarnatum* l-asparaginase

The *in vitro* cytotoxic effect of purified l-asparaginase from *F. incarnatum* irradiated with 75 Gy dose of γ-ray on the growth of three human tumor cell lines namely Hep G2 (Human hepatocellular carcinoma cell line), MCF 7 (Breast carcinoma cell line), CACO-2 (colorectal adenocarcinoma cell line) were studied. Incubation of carcinoma cells for 24 hours in tissue culture medium with different dilutions of l-asparaginase from

0.3 to 10 IU revealed that the inhibition of three human tumor cell lines is dose dependent (Fig. 4). The enzyme dose at 10, 5 and 2.5 IU dose is very effective as it causes about 90% reduction in survival of all tested cell lines moreover, 1.2 U is also effective in Hep G2, MCF 7. The half maximal inhibitory concentration (IC_{50}) of the fungal enzyme as figured in fig 5 are 0.87 and 0.94 IU for Hep-G2 and MCF-7 respectively where it recorded 1.81 IU for CACO2 tumor cell line.

Table 1: The purification summary of l-asparaginase from 75 Gy γ-ray irradiated *F. incarnatum*

Steps	Total volume (ml)	Enzyme Activity (U /ml)	Total activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude	200	4.33	866	9.43	0.46	0	100
Ammonium sulfate precepitation	50	9.56	478	6.87	1.39	3.02	55.20
Dialysis	20	20.43	408.6	3.43	5.96	12.96	47.18
Sephadex G-100	5	27.85	139.25	2.59	10.75	23.37	16.08

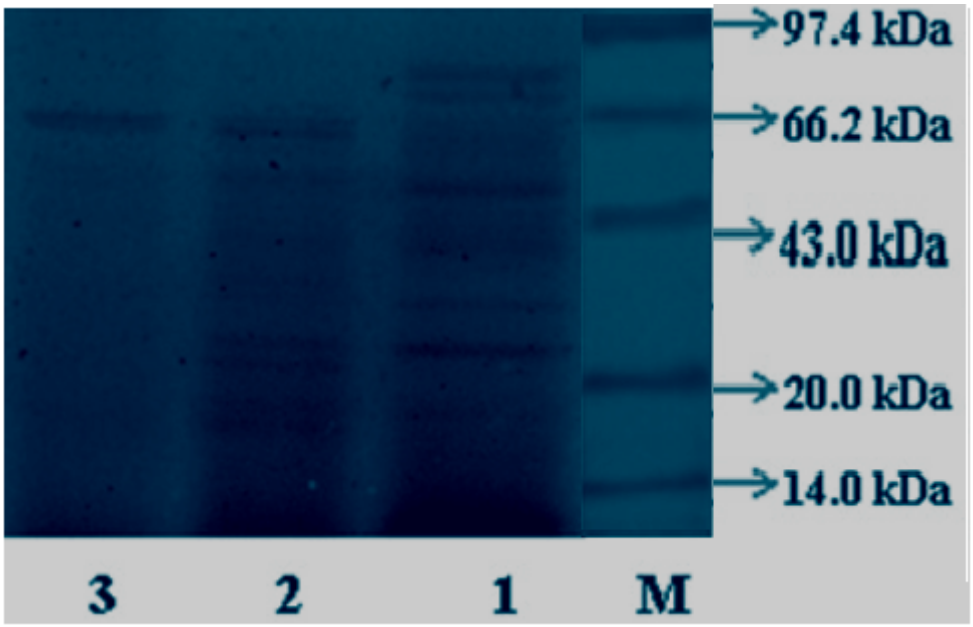


Fig 3: SDS-PAGE of partially purified L-asparaginase from γ-ray (75 Gy) irradiated *F. incarnatum*. M-molecular markers, 1- Crude preparation, 2-Ammonium sulfate precipitated protein, 3- Purified protein.

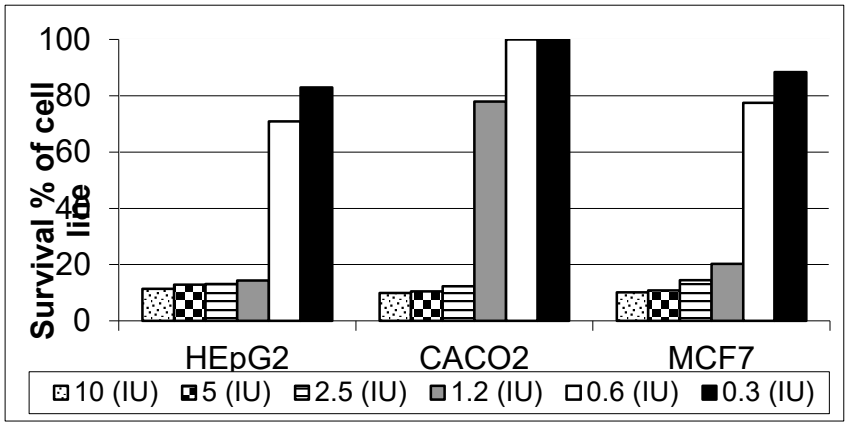


Fig 4: Antitumor activity of the partially purified l-asparaginase from 75 Gy gamma ray irradiated *F. incarnatum* after 24 hours treatment against three tumor cell lines using MTT assay (mean ± s.d., n = 3)

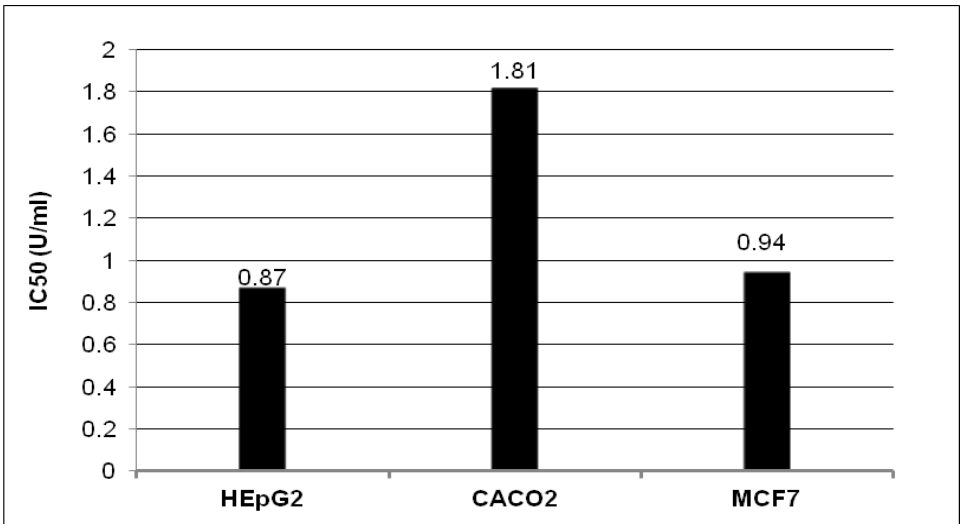


Fig 5: IC50 of the partially purified l-asparaginase from 75 Gy gamma ray irradiated F. incarnatum

DISCUSSION

L-asparaginase is one of very important applicable enzymes in medicine. Fungi consider as a good source for this enzyme with less adverse effects and lesser chances of immunological reaction (Shrivastava *et al.,* 2012). Gupta *et al.,* (2009) recorded that *Aspergillus*, *Penicillium*, *Fusarium* and *Paecilomyces* were found to be good source of l-asparaginase enzyme. Many Fusarium species were studided for its l-asparaginase productivity but to the best of our knowledge this is the first report about *F. incarnatum* as l-asparaginase producer.

For l-asparaginase production, SSF as a fermentation technique was selected for its simplicity, cheapness, less energy consumption, high product formation rate and less catabolite repression. Moreover they mimic the conditions under which the microbe grows natured. For practical application of SSF, wheat bran have been used based on its cheap price, contains various soluble sugars, low lignin content and more amount of protein as compared to other substrates additionally, it remains loose even under moist conditions providing a large surface area (Lequart *et al.,* 1999; Issac and abu-tohan 2016).

Nowadays, ionizing radiation can be used to improve fungal strain in many biotechnological applications (Fadel and Batal, 2000; Geweely and Nawar 2006). In the present study the l-asparaginase production of *F. incarnatum* increased from 4.33 IU to 10.14 IU (2.3 fold increase) when exposed to 75Gy of γ-ray this activity is more than *F. oxysporum* (4.81 IU) (Chanakya *et al.,* 2011) and also more than the maximum yield of l-asparaginase (8.51 IU) of *F. equiseti* using solid state fermentation (Hosamani and Kaliwal, 2011). The fold increase in l-asparginase production is also more than that recorded by Heba *et al.,* (2016) for γ-irradiated *P. cyclopium* at 4 KGy dose level.

This enhancement by γ-ray may be either due to an increase in gene copy number or gene expression or both (Rajoka *et al.,* 1998). The ray act as effective mutagenic agent for fungi and sets off a chain of reactions giving rise to chemical and then to metabolic or physiological changes (Ismail *et al.,* 2010, Mutwakil, 2011). In the other hand, exposure to higher doses of γ-ray inhibits microbial growth and has been extensively used in food or pharmaceutical sterilization and food preservations (Dusan, 2004).

The purified l-asparginase from 75 Gy γ-ray irradiated *F. incrnatum* showed 23.37 purification fold and 16.08% yield which is more than that recorded for *F. culmorum* ASP-87 (Meghavarnam and Janakiraman 2015) and for *Mucor hiemalis* (Monica, *et al.,* 2013) And more than l-asparaginase from marine-derived *Aspergillus niger* AKV-MKBU (Vala *et al.,* 2018) where it less than l-asparaginase from *Cladosporium sp* (Kumar and Manonmani, 2013) and *Penicillium*

brevicompactum NRC 829 (Elshafei *et al.,* 2012).

Molecular weight of l-asparaginase from different microbial sources exhibited varied molecular weights ranged from 40 - 99 kDa (Lincoln *et al.,* 2015; Kalyanasundaram *et al.,* 2015). The present fungus showed 63 KD where l-asparaginase enzyme of *A. niger* had a molecular mass of 39 kDa (Dharmsthiti and

Luchai(2009) Warangkar and Khobragade 2010), The purified l-asparaginase of *F. solani* appeared as two protein bands in SDS-PAGE with apparent molecular weight of 70 and 80 kDa from (Issac and abu-tohan 2016).

The purified enzyme of 75Gy γ-ray irradiated *F. incarnatum* showed 90% inhibition to the three cell line by 2.5 U/ml which is less than 7.9 U/ml used in inhibition of UACC-62 melanoma (Dias *et al.,* 2016) and similar to result reported by Fernanda *et al.,*. (2016) of purified l-asparaginase from *A. oryzae* CCT 3940 which completely inhibited the cell proliferation of UACC-62 (melanoma), 786-0 (kidney), NCI-H40 (lung, non-small cell type), PC-3 (prostate), NCI-ADR/RES (ovary with the multidrug resistance phenotype) and K562 (leukemia) cell lines with TGI values ranging of 3.20–10.00 IU/ml. IC50 of 75 Gy γ-ray irradiated *F. incarnatum* is less than 1 IU/ml against Hep-G2 and MCF-7 which is less than that reported for commercial l-asparaginase from *Erwinia sp.* and *E. coli* (Mahajan, 2014, abakumova 2012) and near to that recorded from marine derived *Aspergillus niger* AKV-MKBU (Vala *et al.,* 2018).

In conclusion, *F. incarnatum* can be considered a new member of l-asparaginase producing family. The fungal enzyme production was highly enhanced by simple treatment with low dose of γ-ray and can be used successfully as antitumor agent.

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