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Optimization of L-Asparaginase Production from *Fusarium oxysporum* F-S3 using Irradiated Pomegranate Peel under Solid-State Fermentation

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Abstract

L-Asparaginase is an important enzyme used in the pharmaceutical and food industry and can be produced by wide variety of microorganisms using affordable agro-based materials. The current study aimed to produce L-asparaginase by fungal isolates, which were screened from soil and optimize the production from the most effective fungus. The data showed that among the 7 tested isolates fungus (F-S3) gave the highest L-asparaginase yield, which was completely identified based on the cultural, morphological and molecular properties as *Fusarium oxysporum*F-S3. This strain was applied on the solid-state fermentation using 7 agro-based materials (peels of orange, pea, potato, banana, lemon, pomegranate and tea waste) for L-asparaginase production. Results revealed that the pomegranate peel has promoted maximum enzyme production. Our study revealed that when pomegranate peel was treated with gamma irradiation at 15.0 kGy for decontamination, the undesired microbial growth was totally inactivated and L-asparaginase production was increased 1.45-fold compared to the pomegranate peel sterilized by autoclave. Optimization of the solid-state fermentation parameters using the one factor at a time technique resulted in maximum L-asparaginase production (280.4 U/gds) was observed using the irradiated pomegranate peel that was supplemented with 0.2 % (w/w) glucose, 1.0 % (w/w) ammonium chloride, L-asparagine 0.6% (w/w), 70% (v/w) initial moisture content, pH was adjusted at 7.0 and inoculated with 3% inoculum volume (10^7 spores/ml) for 96 h of incubation at 30°C. Therefore, results concluded that the production of L-asparaginase by the F-S3 strain after optimization of the solid-state fermentation parameters was significantly increased by about 1.74-Fold compared to the production before irradiation and optimization.

Keywords: Pomegranate peel; *Fusarium oxysporum*; physiological parameters;Gamma radiation;L-Asparaginase; Nutritional parameters; Solid state fermentation (SSF).

1. Introduction

L-Asparaginase (L-Asparagine amidohydrolase E.C.3.5.1.1) is an enzyme belongs to the amidase group, which catalyzes the hydrolysis of the amino acid asparagine in aspartic acid and ammonia. It is widely used as a therapeutic agent for the treatment of acute lymphoblastic leukemia (seen mainly in children), Hodgkin's disease, acute myeloid leukemia, chronic lymphocytic leukemia, lymphosarcoma, and melanosarcoma. L-Asparaginase also has important applications in the food industry to reduce the formation of carcinogenic acrylamide in carbohydrate-rich foods[1]**.** The catalytic process is modulated together with two consecutive processes. In the first stage, the nucleophilic fragment of L-asparaginase is driven by a strong base and acts on the amide carbon atom of L-asparagine to generate beta-acyl-enzyme. The second catalyst stage is achieved by inducing nucleophilic fragments to attack the ester carbon produced in the first step in the presence of water molecules [2, 3].

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L-Asparaginase is widely distributed in plants, animals, and microorganisms. However, the production of L-asparaginase from fungi species has attracted much attention for its stability, high productivity, and easy cultural conditions [1]. Conversely, bacterial L-asparaginase can lead to a variety of side effects including skin rashes, fever, hepatic dysfunction, leukopenia, pancreatitis, diabetes, neurological seizures, and abnormal coagulation of plasma, which causes intracranial thrombosis or hemorrhage [4]. Therefore, finding new eukaryotic sources for L-asparaginase production can be considered a vital way to overcome these problems.

The Solid-State Fermentation (SSF) technology for culturing molds is the best alternative to the submerged fermentation technique (SMF), primarily used for industrial applications. Several advantages of SSF over conventional SMF have been reported. These advantages include low energy inputs, simpler downstream processing with lower catabolic repression, high volumetric yield, higher concentration, reasonable stability of the products, and finally, no complex mechanical obligations to reduce energy demand. In addition, SSF is using an economical and simple medium, because it mainly uses inexpensive and abundantly available industrial and agricultural wastes as substrates [5, 6].

Pomegranate peel is one of the important agricultural food wastes with great potential due to its wide availability and low cost. It is an abundant by-product of pomegranate harvesting in many countries, including Egypt. Pomegranate peel powder mainly consists of carbohydrate approximately of 59.98±1.52 g/100g[7], reducing sugars 30.4%, crude fiber 21%, fat 9.4%, and protein 8.7% [8].

Sterilization of agro-industrial materials for solid-state fermentation is a very important process, unsterilized substrate probably contributes to unwanted microbial contamination that has a negative impact on the fermentation process [9]. The agro-industrial materials are often subjected to thermal sterilization such as steaming and autoclaving but, these processes can damage nutrients and produce toxic compounds that suppress microbial growth and have a negative impact on the production of the required metabolites [10]. Gamma irradiation is now globally getting recognition throughout the world as a phytosanitary treatment of agro-industrial materials. It can improve the hygienic quality of various herbal raw materials and reduce the loss caused by microbial contamination. In addition, it is a quick, safe, convenient, and eco-friendly method [11]. Moreover, gamma irradiation as a non-destructive measure could be an alternative to autoclaving and preserving more heat-sensitive components of the substrate [12].

The purpose of the current research was to avoid the potential adverse effects of L-asparaginase, obtained from bacterial origin. Another goal of the present study was to provide an innovative process such as gamma rays to obtain good yields of L-asparaginase, which can be employed for production at an economic and industrial scale. Therefore, the present investigation suggests that agro-based materials, carbon, and nitrogen sources are very vital for producing maximum levels of L-asparaginase enzyme.

2. Materials and methods

2.1. Substrate and chemicals

Seven agro-industrial wastes, including peels of orange, pea, banana, potato, lemon, and pomegranate peels, as well as tea waste, were obtained from a Cairo market. It was washed, sun-dried for 3 days, milled, and sieved to a particle size of 3 mm before usage. All chemicals used in the present study were of analytical grade and were obtained from Sigma–Aldrich (Sigma-Aldrich, Egypt).

2.2. Isolation and selection (qualitative and quantitativeassessment) of L-asparaginase producing fungal isolates.

Ten grammes of rhizosphere of the broad bean (*Vicia faba*) plant were obtained from the Faculty of Agriculture, Ain Shams University, Qalyubia Governorate, Egypt and suspended in 90 milliliters of sterilized distilled water, which was then agitated for 15 min and left stable for 5 min. The serial dilutions technique [13]was used to isolate fungi on modified CzapekDox's (MCD) agar medium [14]which includes the following ingredients (g/L): glucose, 2.0; L-asparagine, 10.0; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; traces of $CuSO_4$, $ZnSO_4$.$7H_2O$ and $FeSO_4$.$7H_2O$, agar, 18 and pH 6.0. This medium was supplemented with 0.3 ml of phenol red dye (2.5% *w*/*v*) prepared in ethanol at pH 6.5 with incorporating L-asparagine into the medium for evaluation of L-asparaginase activity. The plates

were incubated at 28 °C for 2-5 days. The pink zone appeared after 72 h of growth [14].

These colonies were pre-selected and then pure cultivated using the streak plate technique [15]. The pure cultivated fungal colonies were grown on potato dextrose agar (PDA) medium slants, stored at 4 °C and sub-cultured regularly for further investigation.

Quantitative assay of L-asparaginase activity from producing isolates. It was performed in 250 ml plugged Erlenmeyer flasks containing 100 ml of MCD broth medium (which it was the same MCD agar medium without agar, as described above), and inoculated with 2% v/v individually tested isolates. These flasks were incubated at 28 °C and 120 rpm of shaking speed for 3 days. L-Asparaginase activity in the culture filtrates was determined by the method of **Imada et al.** [16]. The hydrolysis rate of L-asparagine was determined by quantification of free ammonia using Nessler's reagent. A blend of 0.5 ml of 0.04 M L-asparagine, 0.5 mL of enzyme extract, 0.5 mL of 0.1 M Tris-HCl buffer (pH 7.5) and 0.5 mL of deionized water was incubated at 37 °C for 30 min, then 0.5 ml of 1.5 M trichloroacetic acid (TCA) was added to stop the reaction. A blank sample was prepared by the addition of TCA before adding the enzyme. The ammonia liberated in the supernatant was determined spectrophotometrically (LW Scientific, Model V-200-RS) by adding 0.2 ml of Nessler's reagent intotubes containing 0.1 ml of enzyme mixture and 3.7 ml of deionized water and incubated at room temperature for 10 min. The enzyme activity was expressed in U/ml or U/gds of the dried substrate. The absorbance was measured at 450 nm and L-asparaginase activity was expressed as the amount of ammonia liberated per ml per minute under the standard assay conditions.

2.3. Identification of the most active L-asparaginase producing fungal isolate.

Identification of the selected fungal isolate was carried out by morphological and microscopic observations using the procedures described by **Leslie and Summerell**[17] The phenotypic identification was confirmed by applying genotypic identification using 18S rRNA sequencing. Primers for the 18S rRNA were used to amplify a DNA sequence (primers (*ITS-1*) F (5′-TCCGTAGGTGAACCTGCGG-3′) and *(ITS-4)R (5′*TCCTCCGCTTATTGATATGC-3′) with Product Size 600 bp. The PCR amplification was performed in a total volume of 50 ul, containing 1X reaction buffer, 1.5 mM $MgCl_2$, 1U *Taq* DNA polymerase (Promega), 2.5 mM dNTPs, 30 poml of each primer and 30 ng genomic DNA. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfil 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 30 sec., an annealing step at 45ºC for 30 sec. and an elongation step at 72ºC for 1 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.The amplification products were detected by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. A 100bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000). Amplified products for all PCR were purified using EZ-10 spin column PCR products purification PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes was added of binding buffer 1 after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 minutes after that centrifuge, 750 ul of wash solution was added to the column and centrifuge at 10.000 rpm for two minutes, repeated washing, 10.000 rpm was spine for an additional minute to remove any residual wash solution. The column was transferred into a clean 1.5 ml microfuge tube and adds 50 ul of elution buffer, incubated at room temperature for 2 minutes and when store purified DNA at -20 °C. The sequencing of the product PCR was carried through in an automatic sequencer ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle Sequencing Kits following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using Rbcl Forward primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Microgen Company).

***In silico* analysis of internal transcribed spacer (ITS) region.** The sequences were analyzed using Sequences Nucleotide Basic Local Alignment Search Tools (BLASTn) program on National Center for

Biotechnology Information Website (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Inoculum preparation

Fifty milliliters of potato dextrose broth (PDB) medium in plugged Erlenmeyer flask (100 ml) were inoculated with *Fusarium oxysporum* F-S3 spores obtained by scratching from a grown slant. The culture was incubated at 28 °C for 7 days under shaking cultivation conditions at 120 rpm using a shaker (WiseCube®, Fuzzy control system). A hemocytometer slide was used to count the number of fungus spores, as described by **Gautam and Bhadauria**[18].

2.5. Cultivation using solid-state fermentation

A total of seven agro-based materials were used, (tea waste, peels of orange, pea, banana, potato, lemon, and pomegranate) and screened for L-asparaginase production. Five grams of each substrate were placed separately in a 100 mL Erlenmeyer flask and moisturized with 60% of deionized water. The flasks were sterilized by autoclaving at 121 °C for 20 min. After cooling, the flasks were inoculated with 2 % v/w of spore suspension (10^7 spores/ml). The contents of the flasks were thoroughly mixed and incubated at 28 °C for 3 days. After incubation, the crude enzyme was extracted using the technique described by **Ahmed and Abou-Taleb**[19], which involves filling flasks with 0.1M phosphate buffer at pH 7.0 (50 ml) and shaking for 1 hour at 200 rpm. After that, the solution was filtered through a Whatman No. 1 filter paper. The slurry was centrifuged at 8,000 rpm for 10 min at 4°C in a cooling centrifuge (Thermo fisher scientific, Model Megafuge 8R, Max speed 10,000).The supernatant was collected as a crude enzyme and enzyme activity was determined as reported earlier.

2.6. Sterilizationof pomegranate peels by gamma irradiation

To compare the sterilization efficiency of gamma irradiation and autoclaving technique of pomegranate peel as a substrate for solid-state fermentation by *F. oxysporum* F-S3strain. Pomegranate peels were preserved in polyethylene bags (5g for each bag). Bags were split into four groups, the first group was left without irradiation as a control, while the second to four groups were exposed to different doses of gamma irradiation at levels of 5, 10 and 15 kGy (kilogray - Radiation Absorbed Dose Unit), respectively. The irradiated pomegranate peel was then mixed with the pre-sterilized deionized water to reach a moisture content of 60% in a 250 ml Erlenmeyer flask. For substrate autoclaving, 5.0 g of pomegranate peel was mixed with deionized water to reach 60% of moisture content in a 250 ml Erlenmeyer flask and autoclaved at 121°C for 20 min. According to both treatments, the sterilization efficiency was determined by counting the remaining microorganisms using the plate count agar technique [20]. Among the irradiation doses in the series, 15 kGy was the most effective dose in sterilizing the substrate. The Irradiation treatment was performed at the National Center for Radiation Research and Technology, NCRRT (Nasr City, Cairo, Egypt) at a dose rate of 3.49269 kGy/h using the-Indian Gamma Chamber 4000 A‖ with a 60 Co source.

2.7.Optimization studies for L-asparaginase production

The irradiated pomegranate peel at a dose of 15 kGy was used as a substrate for L-asparaginase production under SSF. The optimization studies were performed by one factor at a time (OFAT) approach by varying only a single factor at a time and keeping the remaining factors constant [1].

The effect of various physiological and nutritional parameters such as incubation period (ranged from 3 to 5 days), initial moisture content of irradiated substrate (ranged from 40% to 90%), initial pH (ranged from 5.0 to 9.0), incubation temperature (25, 30, 35, 40 and 45 °C) on L-asparaginase production have been studied. The influence of supplementation of different carbon sources (glucose, fructose, mannitol, citric acid, lactose, sucrose, starch soluble and carboxymethylcellulose) at 0.1% (*w*/*w*) and nitrogen sources (peptone, tryptone, yeast extract, casein, ammonium chloride, ammonium nitrate, ammonium sulfate, potassium nitrate, sodium nitrate and urea) at 0.1% (*w*/*w*) on L-asparaginase production have been studied. Effect of different inoculum sizes (ranged from 1.0 to 5.0 % v/w) of seven-days-old culture containing 10^7 spore's suspension/ml and the effect of L-asparagine concentration (ranged from 0.2% to 1.0%) on the yield of L-asparaginase was also investigated. For

each experimental, all other parameters were kept at their optimum level.

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2.8. Statistical analysis

All experiments were carried out in triplicate, with an average of three repetitions being reported. The data were evaluated statistically using IBM ® SPSS® Statistics software version 19 and Duncan's Multiple Range Test was applied at the 0.05 level [21].

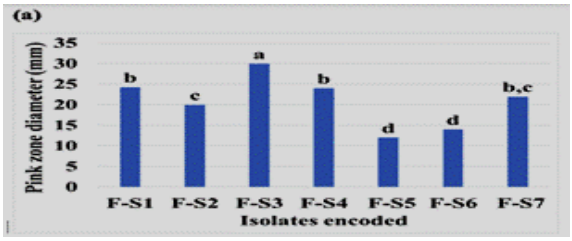
Results

Isolation and selection of L-asparaginase producing fungal isolate

A total of 7fungal isolates were isolated from rhizosphere of the bean plant. All isolates were screened as qualitative assessment for L-asparaginase production on MCD agar medium supplemented with 0.3 ml phenol red dye (2.5%) and L-asparagine using plate assay technique. All isolates exhibited a pink zone appeared around colonies growth after 72 h of incubation periods with zone diameter ranged from 12 to 30 mm, which are said to be the L-asparaginase producers. Fungal isolate encoded F-S3 appeared the largest diameter zone (30 mm) as shown in **Fig. (1a).**

Quantitative selection of L-asparaginase producing isolates on agar medium was done in MCD broth medium are shown in **Fig. (1b).** Results showed that the enzyme activity produced by these isolates was ranged from 13 to 122.6 U/ml. As well as an increase in pH due to ammonia accumulation in the medium ranged from 8 to 10.5. The most significant enzyme activity and pH value were recorded by isolate encoded F-S3 being at 122.6 U/ml and pH 10.5, respectively.

Furthermore, isolate encoded F-S3 was selected as the most active isolate for L-asparaginase production and was used for further studying.



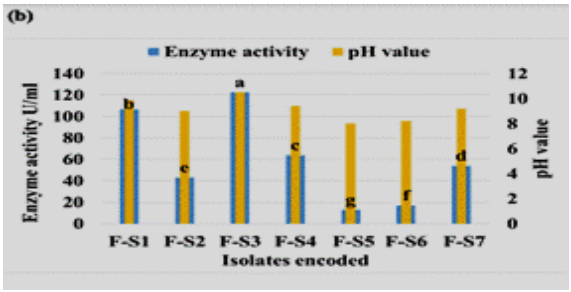


Fig. (1): Qualitative and quantitative estimation of L-asparaginase produced by various isolates isolated from rhizosphere bean plant on MCD agar and broth media, respectively which expressed as pink zone diameter (mm) on agar medium (a) and enzymeactivity and pH value in broth medium (b) after 72h incubation.

[a,b]-Means with small letters above column followed by different letters are significantly different at $p< 0.05$ level.

Identification of the most efficient isolate

The fungal isolate encoded (F-S3) was identified based on the cultural and morphological characteristics according to the key of **Campbell et al.** [22]as presented in **Fig.(2 a&b).** It showed pale violet colony color on PDA agar medium **Fig. (2a).**The microscopic appearance showed the formation of macroconidium with medium length with 3-5 septate, slightly curved and thin walled. Microconidium with short monophialides in kidney shape with 2-septate and chlamydospore arranged in a single form with rough walled and located at the surface hyphae **Fig. (2b).** Therefore, this isolate was classified as *Fusarium* sp.Phenotypic identification was confirmed with molecular identification using 18S rRNA. The results indicated the total genomic DNA was isolated, purified and used as a template for PCR reaction and DNA products were amplified by 600 base pairs **Fig. (1C-i).**The sequence data showed that the fungal isolate had the highest sequence similarity of 99.4 %with the species of *Fusarium oxysporum* Fo-Sh01 with Quary cover 96%. Various sequences taken from the GenBank database were used to build the phylogenetic tree **Fig. (1C-ii).** The phylogenetic tree indicated that each isolate was clustered together as one, with more similar strains found in GenBank. The sequence was subsequently deposited in GenBank (National Center for Biotechnology Information) withaccession number OK356604.

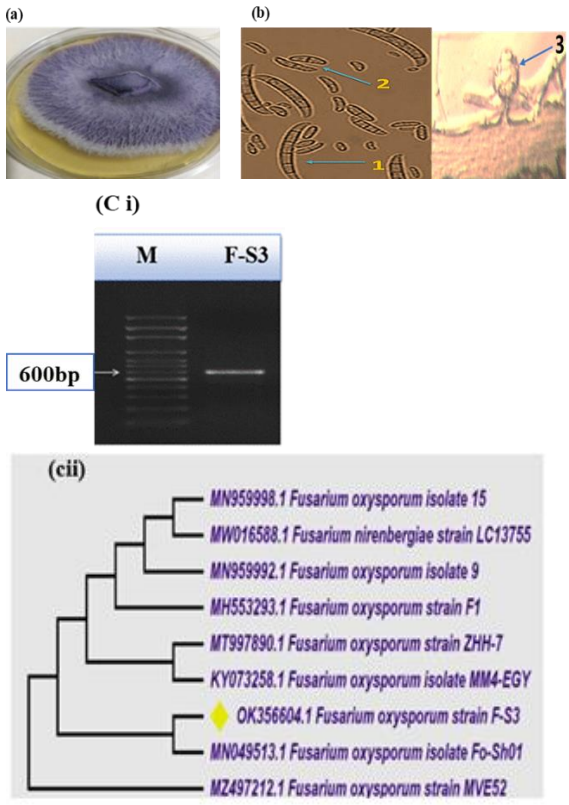


Fig. (2): Identification of F-S3 isolate based on (a) cultural properties on PDA medium (b) morphological properties under light microscopic photos at magnification power (600X) (1): Macroconidium, (2): Microconidium and (3): Chlamydospore. (c) genotypic identification based on 18S rRNA sequences; i-agarose gel electrophoresis shows PCR product of 18S rRNA sequence from F-S3 isolate (Lane M represents DNA base pair Marker). ii-phylogenetic tree showing the position of F-S3 isolate and closely related strains, generated by the Neighbor-Joining method using MEGA 11.0 software (the selected isolate with rhombus symbol).

L-asparaginase production under SSF using agro-industrial wastes by *F. oxysporum* F3 strain

Furthermore, SSF was carried out using various agro-industrial materials (orange, pea, potato, banana, lemon, pomegranate peels and tea waste) as a substrate (carbon sources) with an initial moisture content of 60% for L-asparaginase production by *F. oxysporum* F-S3 at 28 ◦C for 3 days. In this experiment results in **Fig. (3)** revealed that the autoclaved pomegranate peel was found to be the most suitable substrate for L-asparaginase synthesis by giving the maximum enzyme yield at 160.3 U/gds followed by potato peel (59.2 U/gds), orange peel (35.6 U/gds), and tea waste (32.2 U/ gds), respectively. The minimal yield of L-asparaginase was obtained from pea and banana peel (24.3 U/gds and 15.4 U/gds), respectively**.** Therefore, the further studies were carried out with pomegranate peel as a substrate.

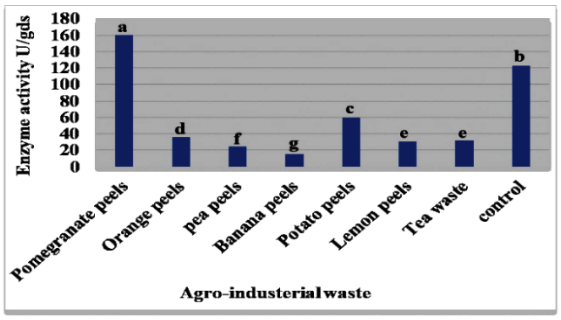


Fig. (3): Effect of different agro-industrial residues on L-asparaginase production by *F. oxysporum* F-S3 at 28◦C for 3 days under solid-state fermentation.
*The control sample served as L-asparaginase activityin SMF
[a,b]-Means with small letters above column followed by different letters are significantly different at $p< 0.05$ level.

Effect of irradiation on L-asparaginase production by *F. oxysporum* F-S3 strain

The current work was designed to sterilize pomegranate peel as the best substrate for production of L-asparaginase under solid-state fermentation by *F. oxysporum* F-S3 with gamma irradiation at different doses of (0, 5, 10 and 15 kGy)**.** Data showed in **Fig. (4 a)** revealed that non-irradiated pomegranate peels have 2350 microbial colony forming units (CFU / gram of dry substrate), whereas, the irradiation treatment of the pomegranate peel inactivate undesired microbial growth, with a dose-dependent effect on CFU/ gram of substrate. When the irradiation dose increased to 15 kGy, no microbial growth was detected. Moreover, the sterilization of pomegranate peel at 15 kGy was compared with autoclaving for enzyme production by the selected strain. Data illustrated in **Fig. (4 b)** concluded that irradiated pomegranate peel at a dose of 15 kGy gave a higher yield of enzyme (230 U/gds) more than those obtained by autoclaved substrate (160.3 Ugds). Moreover, autoclaved pomegranate peel gave dark brown color while irradiated pomegranate peel retained

their color **Fig. (5).** Therefore, the irradiated pomegranate peel at 15 kGy was used for the further studies.

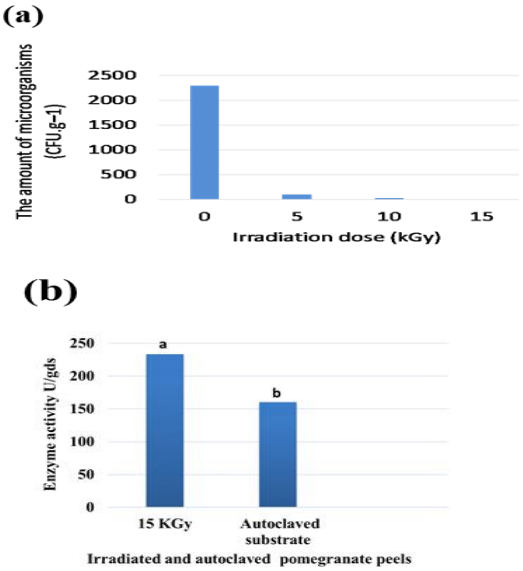


Fig.(4): Effect of gamma ray doses on sterilization of pomegranate peel substrate (a) and it compared withautoclaved pomegranate peel (b) for L-asparaginaseproduction under solid-state fermentation.

[a,b]-Means with small letters above column followed by different letters are significantly different at $p< 0.05$ level.

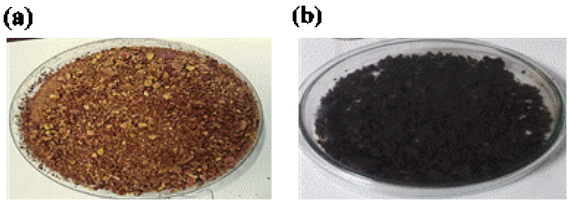


Fig. (5): Effect of gamma irradiation at dose level 15 kGy (a) and autoclaving (b) on colour of pomegranate peel.

Optimization of cultural parameters for L-asparaginase production by *F. oxysporum* F-S3 strain under SSF

In the solid fermentation process, the fermentation period is very important to study the maximum L-asparaginase production**.** To estimate fermentationtime, SSF was performed with a variety of fermentation period schedules ranging from 24 to 144 h. For this, the irradiated pomegranate peels (5 g) were inoculated with 2% of spore suspension of *F. oxysporum* F-S3 containing 10^7 spores/ml with an initial moisture content of 60 % (v/w) and incubated at 28 °C. Samples were taken every 24 h and analysed for the enzymatic activity. The formation of L-asparaginase gradually increased with increasing the fermentation time to reach a significant peak (234 U/gas) after 96 h and then reduced to 210 and 198 U/gds after 120 and 144 h of fermentation time, respectively as shown in **Fig. (6 a).** The correlation coefficient was found to be highly correlated ((r= 0.76).

The optimal level of initial moisture in SSF had a direct impact on the enzyme activity. Using pomegranate peel, SSF gained various moisture levels varying from 40 to 90% (v/w). The results are shown in **Fig. (6 b)** achieving a significant enzyme yield of 239 U/gds after 96 h of fermentation when the initial moisture content was 70%, followed by 232 U/gds at 80%, 225.0 U/gds at 90%, and 184.3 U/gds at 50% while the lowest L-asparaginase formation was 167.7 U/gds at 40% moisture content, respectively. The correlation coefficient was found to be highly correlated (r= 0.8).

Inoculum volume is also an important factor in the production of L-asparaginase. To determine the effect of inoculum volume on the L-asparaginase synthesis, different inoculum volumes of 1% to 5%from seven-days old culture were added to different flasks. Fermentation was carried out for 4 days and the results are illustrated in **Fig. (6 C).** The maximum significant L-asparaginase production (244 U/gds) was achieved when an inoculum volume of 3 % while the lowest enzyme production (152 U/gds) was observed when an inoculum volume of 1 %. The correlation coefficient was found to be highly correlated (r= 0.75).

The incubation temperature affects microbial growth, metabolic activity, and enzyme synthesis of microorganisms. In the present study *F. oxysporum* F-S3 was inoculated at various temperatures degrees (25, 30, 35, 40 and 45 °C) into irradiated pomegranate peel medium under SSF. The data shown in **Fig. (6 d)** revealed that, the maximum yield of extracellular L-asparaginase was 245 U/gds at 30 °C and then decreased to reach 230.3, 225 and 188 U/gds at 35, 40 and 45 °C, respectively. The correlation coefficient was found to be highly correlated (r= -0.84).

In this experiment, the pH level of the moistening solution used was set from 5 to 9. As shown in **Fig. (6 e),** the maximum yield of L-asparaginase was recorded at pH 7.0 (250.0 U/gds) but with an acidity

equal to pH 5.0 or an alkalinity equivalent to pH 9.0, it decreased to 223.6 and 133.6 U/gds, respectively. The correlation coefficient was found to be highly correlated (r= -0.72).

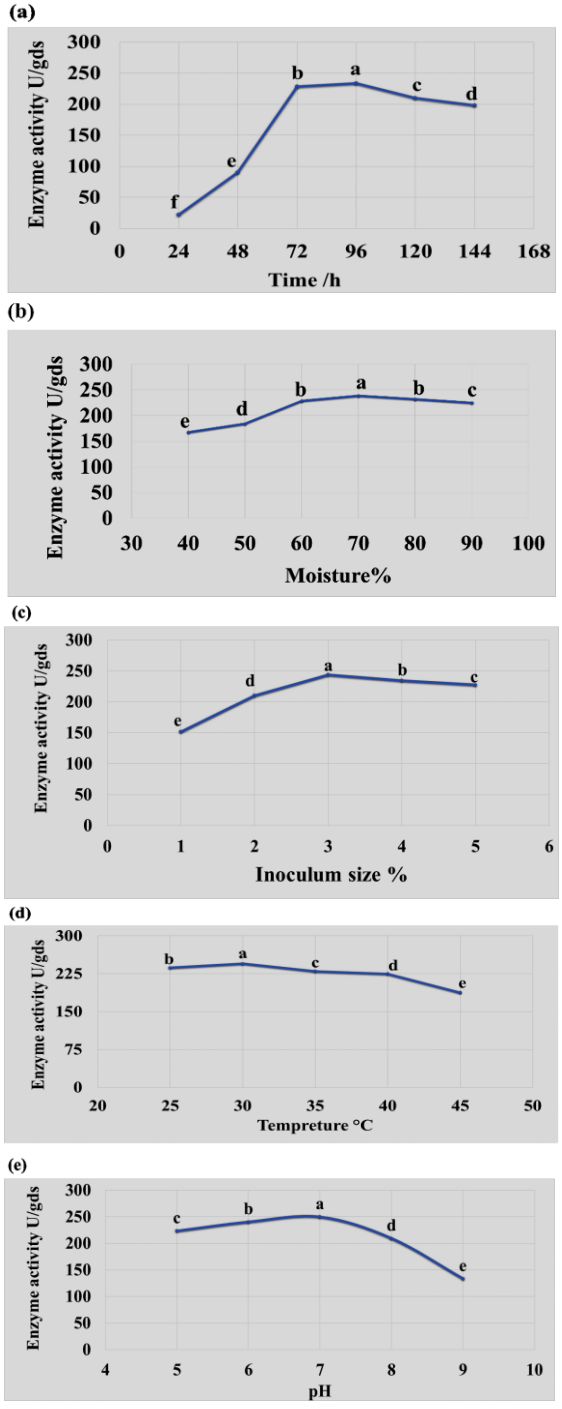


Fig. (6):L-asparaginase production by *F. oxysporum* F3 strain under SSF affected by some cultural parameters of fermentation time (a), moisture content (b), inoculum size (c), incubation temperature (d) and pH (e).

[a,b]-Means with small letters above point followed by different letters are significantly different at $p< 0.05$ level.

Optimization of nutritional parameters for L-asparaginase production by *F. oxysporum* F-S3 strain under SSF

In order to find out the optimum carbon source, different carbon sources such as glucose, fructose, mannitol, citric acid, lactose, sucrose, starch soluble and carboxymethylcellulose, at 0.1% (w/w), were incorporated with irradiated pomegranate peel medium and incubated at 30 ºC for 96 hours with moisture level of 70%, at pH 7 and inoculum size of 3%. From results presented in **Fig. (7 a),** it was observed that glucose as a carbon source had a remarkable impact on L-asparaginase production and enhanced enzyme activity up to (256 U/gds), followed by sucrose (230.3 U/gds). While the supplementation of irradiated pomegranate peel with lactose and citric acid showed a marginal decrease in the production of L-asparaginase (223 U/gds) and (201 U/gds), respectively. On the other hand, the addition of carboxy methyl cellulose and mannitol decreased the L-asparaginase production to 134 and 103 U/gds, respectively, while starch soluble gave the lowest L-asparaginase production at 60.3 U/gds.

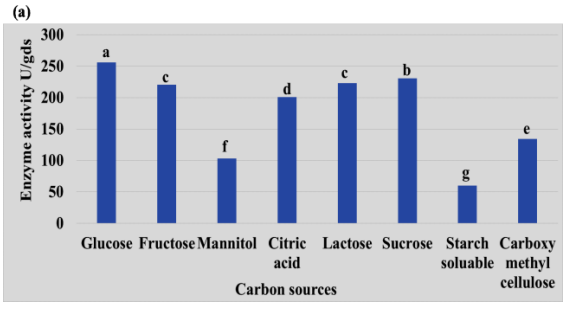
To determine the effect of different concentrations of glucose which exhibited superiority among other tested carbon sources for L-asparaginase production, SSF was carried out by irradiated pomegranate peel that was supplemented with five concentrations of glucose varying from 0.2 to 1.0 % (w/w). From the data obtained in **Fig. (7 b)**, it could be observed that the maximum significant production of L-asparaginase (261 U/gds) was obtained with the optimum glucose concentration of 0.2 % (w/w). Further increase in glucose concentrations resulted in the significant a decrease of L-asparaginase production.

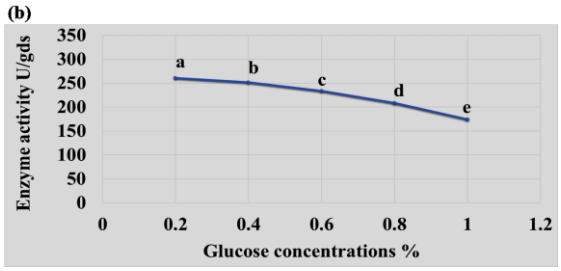
A nitrogen source is the limiting factor and that plays a key role in the L-asparaginase production. The organic (urea, peptone, yeast extract, casein and tryptone) and inorganic nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate) were incorporated to pomegranate peel medium at 0.1% (w/w) showed a various effect on the production of L-asparaginase by *F. oxysporum* F-S3 under SSF, as shown in **Fig. (7 C).** Among the different nitrogen sources tested,

ammonium chloride as an inorganic nitrogen source in the medium improved L-asparaginase production with a significant yield of 266.0 U/gds, followed by ammonium sulphate (238.3 U/gds) and ammonium nitrate was achieved (235 U/gds). While potassium nitrate, sodium nitrate, casein and urea gave the lowest enzyme production which achieved 176.3, 170, 166 and 156 U/gds, respectively.

To evaluate the impact of ammonium chloride levels as the best inorganic nitrogen source on L-asparaginase production in the fermentation medium. L-asparaginase production varied according to different concentrations of ammonium chloride from 0.25 to 1.25% (w/w). The results in **Fig.(7 d)** revealed that the maximum yield of L-asparaginase was 272.0 U/gds with ammonium chloride concentration of 1% (w/w). Further increase in ammonium chloride concentration resulted in a significant decrease of L-asparaginase production.

The production of L-asparaginase stimulated by the addition of L-asparagine. Therefore, individual addition of various concentrations of L-asparagine (0.2, 0.4, 0.6, 0.8 and 1.0%) was added to irradiated pomegranate peel medium. The best concentration of L-asparagine was 0.6% that gave the maximum production of enzyme (280.4 U/gds). Moreover, it has been noted that any increase of L-asparagine resulted inasignificant reduction in L-asparaginase synthesis, the results are depicted in the **Fig. (7 e)**.





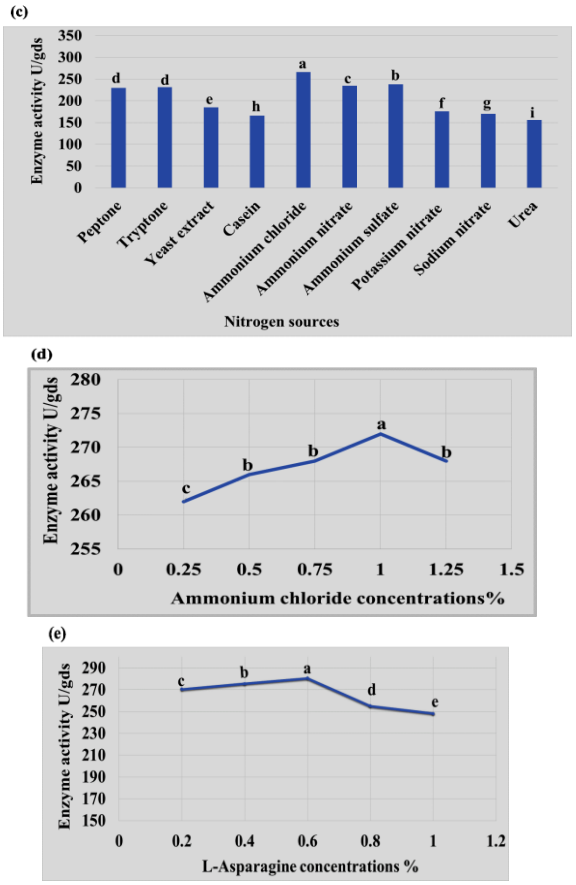


Fig. (7):L-asparaginase production by *F. oxysporum* F-S3 strain under SSF affected by some nutritional parameters of carbon sources (a), glucose concentrations (b), nitrogen sources (c), ammonium chloride concentrations (d) and L-asparagine concentrations (e).

[a,b]-Means with small letters above point followed by different letters are significantly different at $p< 0.05$ level.

Discussion

Recently, the eukaryotic fungal genera such as Aspergillus, Penicillium, and Fusarium have been explored as L-asparaginase producers [2, 23]. Interestedly, the fungal L-asparaginase is more favorable than prokaryotic L-asparaginase, such as bacteria and actinomycetes, which usually accompanies with some problems during patient treatment such as hypersensitivity and immune inactivation [24, 25]. The toxic side effects of some currently used clinical formulations of bacterial origin have necessitated the search for alternative sources of L-asparaginase. The eukaryotic source such as filamentous fungi possess antitumor

properties with non-toxic effects. On the other side, it has revealed

better compatibility with the human body, therefore extensively explored for L-asparaginase [26, 27].

Several publications conducted to L-asparaginase production by submerged fermentation (SMF), solid state fermentation (SSF), and recombinant DNA technology [28]. At both industrial and laboratory scales the production of L-asparaginase can be done by submerged fermentation (SMF) or by solid-state fermentation (SSF). Comparison of both methods revealed that SSF is preferable over SMF because it is inexpensive, environment-friendly and provides a high yield of L-asparaginase enzyme [29]. Furthermore, another notable advance that the contamination is considerably reduced in SSF attributable to the presence of low moisture content Moreover, during this process, the specific activity of the enzyme is often very high. Therefore, L-asparaginase production in SSF was higher than in SMF [30, 6, 31] **.**

In the present study, pomegranate peel was found to be one of the best substrates for production of L-asparaginase. Therefore, further studies were carried out using pomegranate peel for SSF because the production cost of any bioprocess mainly depends on the cost and availability of the substrate utilized. Interestingly, the biochemical composition of pomegranate peel makes it a notable substrate for production of L-asparaginase. The nutritional profile of the pomegranate peel shows high levels of fiber, organic acids, polyphenols, minerals, vitamins, and proteins, making the fruit peel attractive and profitable substrate [32, 33, 34]. Asimilar conclusion from **Nair et al.** [35]**,** who noted that pomegranate peel had proven to be the best substrate to support maximum production of L-asparaginase (253U/gds) by *Aspergillus terreus* MTCC 1782.According to the reported data, agro-industrial materials used in SSF possess excellent buffering capacity [36]. In specific, **Isaac and Abu-Tahon**[37]**]**found that wheat bran supported maximum L-asparaginase production of 187.9 U/gds followed by rice bran at 105 U/gds and corn cob at 97.1 U/gds by *F. solani*AUMC 8615. In addition,**Pallem**[38]who noted that the maximum L-asparaginase yield (21.54 U/gds) was achieved by *F. oxysporum* NCIM 1008 when wheat straw was used as a substrate under SSF.

The present work was designed to compare gamma irradiation with autoclaving for sterilization of pomegranate peel as the best substrate for production of L-asparaginase under solid-state fermentation by *F. oxysporum* F-S3. When the irradiation dose increased to 15 kGy, no microbial contaminations were detectedand generated higher L-asparaginase production with 1.43-Fold than the autoclaved substrate. Moreover, irradiated substrate retained its color, but autoclaved substrate generated black color, this could be attributed to the degradation of sugars by autoclaving and formation of 5-hydroxymethylfurfural (5-HMF). In the same line **Ni et al.** [12]reported that autoclaving of citrus peels produced a dark brown color, more reducing sugar and 5-hydroxymethylfurfural (5-HMF) could be reduce microbial growth, reduce fungal protein excretion and enzyme production, while irradiated substrate at dose 6 kGy sufficiently inhibited possible microbial contaminations and produced higher enzyme production than autoclaved substrate. It is known that a high content of glucose and sucrose inhibits or decreases the synthesis of enzymes [39]**.** Whereas polysaccharides degraded less by irradiation than autoclaving [40]. This difference explains why irradiated pomegranate peel enhanced L-asparaginase production. In addition, irradiation preserves more thermosensitive components in the substrate.

The experimental activity of L-asparaginase was analyzed to study the independent effects of incubation time, moisture content, inoculum volume, pH, temperature, carbon source, nitrogen source and L-asparagine concentrations on L-asparaginase production by *F. oxysporum* F-S3 using irradiated pomegranate peel as a substrate under solid-state fermentation. Normally, microbial enzyme synthesis was dependent on the time of fermentation. After 96 h, the enzyme yield started to decrease gradually because the microorganism might be reached a level of growth, from which it could no longer balance its steady growth with the available nutrient sources [38]. A similar result was reported by **Pallem**[38]showed that the maximum L-asparaginase activity (7.04 U/gds) was observed after 96 h by *F. oxysporum*NCIM 1008. In addition, the results obtained were also consistent in agreement with**Soniyamby et al.** [30]recorded that a maximum yield of L-asparaginase was 25.2 U/gds after 96 h of incubation by *Aspergillus oryzae* using pineapple

peel. The decrease in enzyme yield with prolonged incubation may also be due to inhibition and denaturation of the enzyme [41].

In the current study L-asparaginase production increased with the increase in moisture content up to 70%. In the same line, **Meghavarnam and Janakiraman**[1]reported that the maximum yield of L-asparaginase (7.33 U/gds) was recorded at a moisture content of 70% by *F. culmorum* (ASP-87) using raw materials of agricultural origin. **Pallem et al.** [36]also, pointed out that the optimum moisture level for L-asparaginase production by *F. oxysporum* was obtained at 60% using wheat bran as a substrate. It was observed that reducing water content decreased the yield of L-asparaginase, probably due to lower nutrient solubility in the solid substrate, reduced substrate swelling, and decrease the water retention by the substrate. On the other hand, when the moisture level is increased, oxygen transport becomes insufficient, reduction in gas volume, decrease gaseous exchange and change in the decomposition of the lignin and increases the risk of bacterial contamination [36, 4 2] .The present result is in agreement with the findings of **Mishra**[26]who reported that the maximum L-asparaginase yield was 38.7 U/gds at 70% moisture content using bran of Glycine max as a substrate. In fermentation process, a reduction in enzyme production at high initial moisture content may be attributed to a reduction in substrate porosity, changes in structure of substrate particles, and reduced fungal growth [43] . All these conditions affected the fungal growth and ultimately the enzyme production.

In the present investigation, L-asparaginase production increased readily with the increase in the inoculum level and maximum enzyme activity was obtained at 3% (v/w) whereas the least activity was recorded at 1% (v/w). **Pallem et al.** [36**]**stated that the maximum L-asparaginase production of 4.81 IU with the inoculum volume of 1.5 ml of 7 days old *F. oxysporum*. Whereas it was observed that 5% inoculum volume of *Aspergillus niger* gave the maximum activity of L-asparaginase (5.40 U/ml), further increase in inoculum size caused overcrowding of spores that decreased the enzyme activity [44] .**El-Sayed** [45]and **Mao et al.** [46]found that 2.0% inoculum concentration supports maximum L-glutaminase production by *Trichoderma koningii* and *Bacillus amyloliquefaciens*, respectively. Also, **Soren et al.**[47]extracted that the best inoculum volume was 2.0% for L-glutaminase production by *Fusarium nelsonii* KPJ-2. On the other hand, the minimum amount of L-glutaminase production was observed at 5% of inoculum level. A low inoculum volume may give insufficient biomass resulting in low product formation, whereas a higher inoculum volume may produce too much biomass and may deplete the substrate nutrients or accumulation of self-inhibitors that leads to inhibition of product formation [48].

The microbial enzyme production is highly influenced by incubation temperature as it is a critical environmental factor for L-asparaginase production by tested fungal strain. Temperature influences the rate of the chemical reaction thus affecting the rate of enzymatic activity [49]. In the current study, the maximum activity obtained was (245 U/gds) at 30 °C and then decreased to 230.3, 225 and 188 U/gds at 35, 40 and 45 °C respectively, which may be due to conformation incorrect structure of enzyme molecules due to denaturation of the mesophilic enzymes at higher temperatures [49] .Based on our results *F. culmorum, F. solani* AUMC 8615, *F. brachygibbosum, Penicillium* sp., *A. terreus*, *Mucor hiemalis* and *Emericellanidulans* showed the maximum L- asparaginase activity at 30 °C [50, 37, 49, 51, 52]. In contrast, the maximum production of L-asparaginase from *A. fumigatus* and *A. terreus* CCT 7693 was observed at 40 and 34.6 °C, respectively [53 ,54].

The pH of the fermentation medium is essential for the transportation of various nutrients across the cell membrane and for increasing the production of L-asparaginase. In general, the enzyme activity can be either inhibited or enhanced depending on the change in the pH [55, 56]. Different organisms have different pH optima and any modification in their pH optima could result in a decrease in their enzyme activity. In the present study experiments were carried out in order to maintain the favorable conditions and to find the optimum pH to obtain maximum L-asparaginase production. This was performed by carrying out the fermentation process by using various pH from 5.0-9.0. The maximum enzyme activity was observed at pH 7.0 with an activity of (250.0 U/gds). Our result in the same way with **El-Gendy et al.**[52]found that the highest yield of L-asparaginase (19.25 U/ml) was achieved at pH 7.0. While **Pallem**[38]reported that maximum L-asparaginase productivity was noticed with pH 6.0.

Furthermore, the highest L-asparaginase activity was observed at pH 5.8 by *A. terreus* and at pH 9.0 from *A. niger*[27 ,57]. It was observed that the peak of L-asparaginase production was reached at pH 6 (0.096 U/mL) from *A. caespitosus*[58]. In general, fungal strains are noted for their best performance in the range of 3.5-6.0 and the low pH prevents contamination by other microorganisms. Further increases in pH can reduce enzyme activity due to denaturation or ineffectiveness of the microbial strain at extremely high basic and acidic pH values [38].

Carbon is the main structural and functional component of microbial cells and plays an important role in the nutrition of fungi. A source of carbon is required for all biosynthesisprocesses leading to reproduction, product formation and cell maintenance. Microbial production of major metabolites is strongly influenced by their growth, which is determined by the availability of nutrients in the substrate. Many species of fungi can thrive on different types of aliphatic hydrocarbons. Therefore, fungi can use a variety of carbon compounds, but most fungi prefer simple sugars [59].In **Fig. (6 a),** it was observed that glucose as a carbon source has a remarkable impact on L-asparaginase production and enhanced enzyme activity, this may be attributed to the positive influence of glucose as a co-metabolic agent for enhanced L-asparaginase biosynthesis have been reported by **Hosamani and Kaliwal**[60]. In current work, the highest amount of L-asparaginase yield was noticed by irradiated pomegranate peel medium supplemented with glucose, an increase in the production of L-asparaginase with glucose could be due to the fact that it is a simple and easily soluble sugar, which is easily metabolized by fungi [1]. It was also reported that glucose supported L-asparaginase production in other fungi such as *A. terreus* MTCC1782 and *Trichoderma viride* F2 [61 , 62]. Otherwise, glucose was also reported to be a repressor of L-asparaginase in *Enterobacter aerogenes*[63].

In the present work, among different glucose concentrations ranging from 0.2 to 1%, the glucose concentration of 0.2% showed the highest L-asparaginase activity (261 U/gds) after 4 days of incubation at 30 ^{o}C.Further increase in glucose concentration resulted in the decrease of L-asparaginase production it might be due to the inhibitory effect of glucose at higher concentrations. In accordance with this result, **El-Hefnawy et al.** [64]reported that a different concentration of glucose gave a significant increase in the enzyme activity with increase in the glucose concentration up to 0.5% and later decreased with an increase in the fermentation period by *F. solani*. The similar glucose effect was observed for the L-asparaginase produced by *A. terreus*MTCC 1782 [61]and *Bipolaris*sp. BR438 [65]. The data showed that the release of extracellular L- asparaginase by *F. oxysporum* F-S3was not only affected by the kind of carbon substrates supplied but also was sensitive to the level of specific carbon source used in the fermentation medium. It was recorded that the yield of L-asparaginase production depend on the microorganisms, as well as the nature of carbon source and its level [66].

Effect of adding different sources of inorganic and organic nitrogen on L-asparaginase production were evaluated. L-asparaginase production increases with incorporation of nitrogen sources, suggesting that it is regulated by nitrogen; therefore, it requires supplementary nitrogen sources in addition to the carbon source to enhance L-asparaginase production. Many microorganisms consume both organic and inorganic sources of nitrogen to synthesis components of the cell wall, proteins, nucleic acids and amino acids [67]. Fungi have previously shown high specificity for different nitrogen sources, thereby influencing microbial metabolism [68]. In the current investigation, among the different nitrogen sources tested, ammonium chloride as an inorganic nitrogen source in the irradiated pomegranate peel medium improved L-asparaginase production with a yield of 266.0 U/gds, followed by ammonium sulfate (238.3 U/gds). These results are in agreement with **Meghavarnam and Janakiraman**[50]found that ammonium chloride enhanced the production of L-asparaginase by 12-fold in *Fusarium culmorum* but 4-fold in *Fusarium brachygibbosum*, while yeast extract and casein repressed the production of L-asparaginase in both the *Fusarium* sp. **El-Hefnawy et al.**[64]reported that ammonium sulfate and yeast extract at (0.5%) were found to be the best nitrogen source for *Fusarium solani* and *Penicillium oxalicum*, respectively. Moreover, **Yadav and Sarkar**[69]demonstrated no substantial variance between the type of nitrogen sources and L-asparaginase yield obtained from *F. oxysporum* but ammonium sulphate

and sodium nitrate were the best inducers for its production by *F. oxysporum, F. semitectum, F. equiseti*and *Penicillium* sp. [60, 50].

The influence of various ammonium chloride concentrations as the best inorganic nitrogen source which gave the highest L-asparaginase activity have been studied. The maximum L-asparaginase production (272.0 U/gds) was obtained with ammonium chloride concentration of 1% (w/w). Further increase in ammonium chloride concentration resulted in a significant reduction of enzyme production.Similar results were reported by **Baskar and Renganathan**[70**]**which extracted thatammonium chloride was found to be the best nitrogen source for L-asparaginase production using *A. terrus* MTCC 1782. Another investigation by **Varalakshmi and Raju**[42**]**reported the highest L-asparaginase yield was (267.8 U/gds) with ammonium sulphate concentration of 2% (w/v), whereas any increase in ammonium sulfate concentrations resulted in a decrease in L-asparaginase synthesis may be due to the inhibitory effect of ammonium sulfate at higher concentrations. From current data, it can be concluded that inorganic nitrogen source in irradiated pomegranate peel medium showed significant induction in the activity of L-asparaginase, while organic sources showed significant decrease in the enzyme activity by *F. oxysporum* F-S3.

The present data revealed that L-asparaginase activity of *F. oxysporum* F-S3 enhanced with the incorporation of L-asparagine into irradiated pomegranate peel medium and reached a significant optimum value (280.4 U/gds) at 0.6%. With a further increase in L-asparagine concentration, there was a significant decrease in the enzyme yield, which may be attributed to the negative effects of L-asparagine at higher concentrations on L-asparaginase gene expression or down regulation of nitrogenous compounds availability [66 ,38]. Similar observation by **Pallem**[38**]**whostated that the maximum L-asparaginase activity (21.54 U/gds) was noticed with 0.75 % L-asparagine dose.

Conclusion

The findings of this study provide important evidence illustrating the effect of irradiated pomegranate peel on L-asparaginase production. Interestingly, gamma irradiation as a sterilization physical method for sterilization of pomegranate peel gave harboring effect on L-asparaginase production than autoclaving with increasing in enzyme activity 1.43-fold more than the autoclaved substrate. The observations made in the current study are very promising regarding the maximum value of L-asparaginase activity obtained after optimization of fermentation parameters such as incubation time, initial moisture, inoculum size, pH, incubation temperature, carbon source such as glucose, inorganic nitrogen source such as ammonium chloride and L-asparagine concentration as an inducer for the maximum L-asparaginase production using *Fusarium oxysporum* F-S3 under solid fermentation (SSF) and the enzyme activity increased 1.74 fold in optimized media than non-optimized. This clearly demonstrates that the discovery of soil fungi to produce L-asparaginase as a therapeutic enzyme using cheaper and irradiated substrates in SSF.

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