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Original Article

Protease Inhibitor Expression with Immobilized Cells of Egyptian *Streptomyces lavendulae* on Different Radiated Matrices Using Gamma Radiation

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ABSTRACT

Immobilization technique using different matrix (agar, polyacrylamide and gelatin) to enhancement protease inhibitor activity and specific activity from *Streptomyces lavendulae* (Egyptian isolate from marine) which identified by 16SrRNA with accession number AB795940. The best specific activity of protease inhibitor using numerous factors plus UV radiation was 1055 u/mg however the specific activity without any factor 234 u/mg. Comparing free cells data with immobilization data great different observed, specific activity of protease inhibitor when *Streptomyces lavendulae* immobilized on gelatin after three days less cell leakage 0.7 and specific activity was 660 u/mg. when gelatin matrix exposure different doses of gamma radiation then immobilized bacterial cells specific activity reached to 1900 u/mg at 8 KGy. The gelatin immobilized cells of *Streptomyces lavendulae* can be proposed as an effective biocatalyst for repeated usage for maximum production of protease inhibitor.

Key words: *Streptomyces lavendulae***,** protease inhibitor, identification, immobilization, UV and gamma radiation.

INTRODUCTION

Proteases inhibitor catalyzes the cleavage of peptide bonds in proteins [**1**]. They conduct highly selective and specific modification of proteins i.e. zymogenic form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, processing and transport of secretory proteins across the membrane. They catalyze important proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms. Their involvement in the life cycle of disease-causing organisms has led to become a potential target for developing therapeutic agents against fatal disease such as cancer and AIDS [2]. Protease inhibitors (PIs) are a class of antiviral drugs that are widely used to treat HIV/AIDS and hepatitis caused by hepatitis C virus. Protease inhibitors prevent viral replication by selectively binding to viral proteases (e.g. HIV-1 protease) and blocking proteolytic cleavage of protein precursors that are necessary for the production of infectious viral particles [3, 4]. Protease inhibitors may be classified either by the type of protease they inhibit, or by their mechanism of action [5] introduced a classification of protease inhibitors based on similarities detectable at the level of amino acid sequence. This classification initially identified 48 families of inhibitors that could be grouped into 26 related super family (or clans) by their structure. According to the MEROPS database there are now 85 families of inhibitors. These families are named from I followed by a number, for example, I14 contains hirudin-like inhibitors [5]. Researchers are investigating whether protease inhibitors could possibly be used to treat cancer. For example, nelfinavir and atazanavir are able to kill tumor cells in culture in a Petri dish [6, 4**].** This effect has not yet been examined in humans; but studies in laboratory mice have shown that nelfinavir is able to suppress the growth of tumors in these animals, which represents a promising lead towards testing this drug in humans [4]. Inhibitors of the proteasome, such as Velcade/Bortezomib are now front-line drugs for the treatment of various cancers, notably multiple

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Myeloma.Protease inhibitors are proteins or peptides capable of inhibiting catalytic activities of proteolytic enzymes [7].These inhibitors have been demonstrated in a variety of biological materials and are widely distributed in many plant materials used as food, especially in legumes, potato and cereals, [8]. Bacteria such as *Streptomyces* and *Pseudo alteromonas* were also found to be good producers [9, 10]. Inhibitors of various proteases are also well known in the organs of mammals and their products such as milk and semen. Beside mammals such substances have also been found in nematodes, fish and in the eggs of certain birds [11].

The aim of the present study was to screen some bacterial isolates for their ability to produce protease inhibitor and identification of the most promising isolate by 16SrRNA. Optimization of the cultural and environmental production conditions. In addition to immobilize *Streptomyces* cells on different matrices.

MATERIALS AND METHODS

Primary screening for protease inhibitor:
The following method was developed for screening and isolating protease inhibitors producing microorganisms according to [12]. A double layer of agar in petri dishes (90mm) was used. The bottom layer consisted of skim - milk agar, which contained skim - milk powder 10g, and agar (12g/l) of 0.02 M phosphate buffer pH 7.5. After spreading 0.1 ml of the diluted sample on the surface, the plate was incubated for 2 days at 28±2ºC. After this time, it was cooled at 2 ºC for 1h. Protease from *Streptomyces lavendulae*, was sterilized by filtration and added to agar solution (45 ºC), containing 12 g agar/l of the same phosphate buffer. Thereafter, this agar was poured on top of the bottom layer. The plate was incubated for an additional 24h at 28ºC. The skim milk agar turned transparent during this incubation, except around colonies producing protease inhibitors. These colonies were used for inoculation of 250 ml Erlenmeyer flasks containing 25 ml of nutrient broth. The flasks were incubated at 28±2ºC as shake cultures. After 3 days, the trypsin inhibiting activity in the culture filtrate was estimated.

Estimation of trypsin –inhibiting activity:
Crystallized salt- free trypsin was used for the estimation of inhibitor effects. 0.2 ml filtrate from shake culture and 0.8 ml trypsin solution, containing about 5 µg trypsin was incubated for 30 min at 28°C. Blanks were incubated with 0.8 ml trypsin solution and 0.2 ml distilled water. The remaining free trypsin, which was not inhibited, was estimated as caseolytic activity at pH 9.5 according to method of [13]. Because the correlation between inhibition and concentration of inhibitor is linear only to a certain limit [14]. Culture filtrates diluted to give about 50% inhibition of the trypsin were used. Inhibitor activity was calculated as the amount of trypsin (µg) that was inhibited to 50% by 1µl of culture filtrate.

Identification by 16S rRNA:
The most protease inhibitor producing strain was identified by 16S rRNA sequencing data collection. A database containing 16S rRNAgene sequences of all validly published filamentous actinomycetes [15] was compiled from Gen Bank (http://www.ncbi.nlm.nih.gov). All sequences used were longer than 1400 bp. The sequences were grouped by genus according to [16]. Actinomycete strain was grown in 10 ml International *Streptomyces* Project Medium 1 (ISP 1) [17] with agitation at 30 ˚C for 18–24 h and examined by gram stain. Cells (4 ml) were harvested by centrifugation (7500 g for 2 min), washed once with 500 ml of 10 mMTris-HCl/1 mM EDTA (TE) buffer (pH 7.7) and resuspended in 500 ml TE buffer (pH 7.7). The samples were heated in boiling water for 10 min, allowed to cool for 5 min and centrifuged (7500 g for 3 min). The supernatant was transferred to a clean tube and stored at 4 ˚C. If melanin or other pigments were produced during growth, cultures were grown in Middlebrook 7H9 broth, as these pigments interfered with the PCR. Full length 16S rRNA (1500 bp) were amplified from isolates (*streptomyces*) by PCR using (universal forward primer P1 and universal reverse primer) Forward: (5'-AGAGTTTGATCCTGGCTCAG-3'), Reverse: (5'-CGGTTACCTTGTTACGACTT-3') under the (optimum conditions denaturation 94ºC ,1 min, annealing 45ºC, 30 sec and extension 72ºC, 2 min, 35 cycles). Amplified 16S rRNA was purified from 0.8% melting point agarose gel. Bands obtained from PCR product were eluted and purified by (Qiagen elution kit) PCR instructions, DNA band desired was excised from ethidium bromide stained agarose gel with a razor blade, transferred to Ependorf tube. DNA was sequenced directly using specific primer with concentration 20 pmol in Promega company lab. The sequence alignment was prepared with DNA STAR software program. Nucleotides sequences of the products were edited using Bioedit version 5.0.6 [18] and phylogentic tree by mega 4 program. DNA sequenced at sequencer lab in labs of Promega Company in USA. The sequence alignment was prepared with DNASTAR software programs (DNASTAR. INC., Madison, Wis.), and manually

edited with Gene Doc (www.NCBI / blast.com), and determine translation encoded regions at (www.expasy.org/cgi-bin/dna_aa).Phylogenic tree establish my mega program.

Growth media and fermentation conditions: Four different types of media were used through this study. Medium 1 [19]: basal components of fermentation medium as follows (g/l):1.0, KH_2PO_4; 3.4, K_2HPO_4; 0.01, $FeSO_4.7H_2O$; 0.3, $MgSO_4.7H_2O$; 0.1, $ZnCl_2$; 0.01 $CuSO_4.7H_2O$; 0.003, $MnCl_2.4H_2O$; 0.01, CaCl2; 0.03, NaCl. Medium 2(g/l):18, glucose; 10, K_2HPO_4; 1.0, KCl; 0.5, $NaNO_3$; 3.0, $MgSO_4.7H_2O$ and 0.01, $FeSO_4.7H_2O$. Medium 3(g/l): 10, peptone; 5, yeast extract; 10, glucose and 10, NaCl. Medium 4(g/l): 30, soy bean; 15, dextrin; 10, $CaCO_3$ and 1.0, $MgSO_4$, the last three media according to,Mostafaet al.,[20].For all media used, the pH was adjusted to 7.0 before sterilization. This was carried out in 250 ml conical flasks containing 50 ml of different media and inoculated with 2 ml of spore suspension and incubated for 7 days at 30 ºC in a shaking incubator (New Brunswick Scientific Co., NJ, and USA) at 150 rpm.

Quantitative assay of protease:

Protease activity was assayed by a modified method of [13] with some modification by using casein as the substrate. Hundred µl of enzyme solution was added to 900 µl of substrate solution (2 mg/ml (w/v) casein in 10 mMTris-HCl buffer, pH 8.0). Themixture was incubated at 37 °C for 30 min. Reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 12,000 rpm for 10 min at 4 °C; the acid soluble product in the supernatant was neutralized with 5 ml of 0.5 M Na_2CO_3 solution. The color developed after adding 0.5 ml of 3-fold diluted FolinCiocalteau reagent and was measured at 750 nm. All assays were done in triplicate. One protease unit is defined as the amount of enzyme that releases 1 µg of a tyrosine / ml / minute under the above assay conditions. The specific activity is expressed in the units of enzyme activity per milligram of protein.

Protein concentration:

Protein concentration was determined by the method of [21] with bovine serum albumin as standard.

Ultraviolet (UV) rays irradiation of samples: *Streptomyces lavendulae* cells were grown at (pH 7, 37°C and shaking 150 rpm), harvest and washed three times with physiological solution (NaCl 0.9 %), the pellet was resuspended in deionized water. *Streptomyces lavendulae* suspensions in saline solution or in deionized water were exposed to short-wavelength UV-B rays (254 nm) in sterile Pyrex glass Petri dishes. The time of exposure was 10, 20 and 30 min. Lamp irradiance was quantified with a UV detector (IL1400A; International Light, Newburyport, Mass.) by placing the detector at the same height as the sample surface 100000 cells/ ml.

Immobilization

Agar: The 47 µl of cell suspension (equivalent to 0.03 g cell dry weight) was added to molten agar (2%) maintained at 45˚C and poured into sterile flat bottom Petri plates and allowed to solidify. The solidified agar blocks were cut into equal size cubes and added to sterile 0.1 M phosphate buffer (pH 7.0) and kept at 4°C for 1 h. After curing, phosphate buffer was decanted and cubes were washed with sterile distilled water 3-4 times and were stored at 4˚C.

Gelatin: The 47 µl of cell suspension was added to gelatin (2%) and poured into sterile flat bottom Petri plates and allowed to solidify. The gel was over layered with 5 ml of 5% glutaraldehyde for hardening. The blocks were cut into equal size cubes and washed thoroughly with sterile distilled
water for complete removal of excess glutaraldehyde. Cubes were preserved at 4°C.

Polyacrylamide:

Acrylamide (9.0 g) and bisacrylamide (0.24 g) were mixed and 0.45 g ammonium persulphate was added. After this 3 ml TEMED was mixed and 47 µl of cell suspension was added to polyacrylamide solution and poured into flat bottom petriplate for solidification. After polymerization, polyacrylamide gel was cut into equal size cubes. The cubes were transferred to 0.2 M phosphate buffer (pH 7.0) and kept at 4°C for curing for 1 h. The cubes were washed with sterile distilled water and stored at 4˚C until use. All operations were carried out aseptically under a laminar flow hood.

Protease inhibitor production by immobilization Batch process:

The batch experiment was performed in 250 ml Erlenmeyer flask containing 50 ml of medium. The immobilized beads of different matrices were transferred to 50 ml of production medium [g/l: glucose, 5.0; peptone, 7.5 and salt solution, 5% ($MgSO_4.7H_2O$, 5.0; KH_2PO_4, 5.0 and $FeSO_4.7H_2O$, 0.1) pH 9.0]. The flasks were incubated at 37˚C for 48 h on shaker incubator at 200 rpm. Similarly, one flask containing production medium was inoculated with 47 µl of cell suspension (equivalent to 0.03 g cell dry

weight) and was incubated at 30˚C for 48 h. This was treated as free cell system. Samples were withdrawn at regular interval of 6 h from each flask and protease inhibitor activity was calculated by using casein as a substrate as mentioned above and cell leakage of each sample was determined.

Repeated batch process:
The repeated batch culture was established in 250 ml Erlenmeyer flask containing 50 ml medium. The immobilized beads of each matrix were filtered, washed with 30 ml saline solution and transferred to 50 ml fresh production medium. Samples were withdrawn at regular interval of 6 from each flask and protease inhibitor wascalculated. The process was repeated until the beads get disintegrated.

Cell leakage:
Cell leakage from the gel matrix was determined as cell weight by measuring the optical density at 700 nm. From the standard graph of biomass, the corresponding absorbance was converted to µg of cell leakage from each matrix.

Gamma Radiation:
The *Streptomyces lavendulae* strain was cultured in the optimized medium at 30 ℃ for 24h in 500 ml conical flasks, the medium was dispensed in 10 ml quantities into test tubes exposed to various doses (2, 4, 6, 8, 10 and 14 kGy) of gamma radiation at room temperature. Duplicate tubes were used for each dose. The obtained irradiated standard cultures were inoculated into the medium and incubated at 30 °C for 24 h to estimate protease inhibitor.

Statistical analysis: Results are expressed as the mean ± S.E. of three independent astrocytic culture preparations performed in triplicate. Statistical analysis was done using prism software programmed one-way ANOVA.

RESULTS AND DISCUSSIONS

Protease inhibitors may be classified either by the type of protease inhibited, or by their mechanism of action. In [5] introduced a classification of protease inhibitors based on similarities detectable at the level of amino acid sequence.

Selected bacteria producing protease inhibitor: From the results in fig. (1), among 7 bacterial isolates which were isolated from soil (3 isolates) and marine water (4 isolates), two strains (isolates no.5 and 6)produced good amounts of protease inhibitor as trypsin – inhibiting capacity (234 and 92 units/mg protein respectively).The other five isolates produced negligible amounts of protease inhibitor. Thus, organism no. 5 with the highest trypsin –inhibiting capacity was selected for the present study (**Fig 1**).

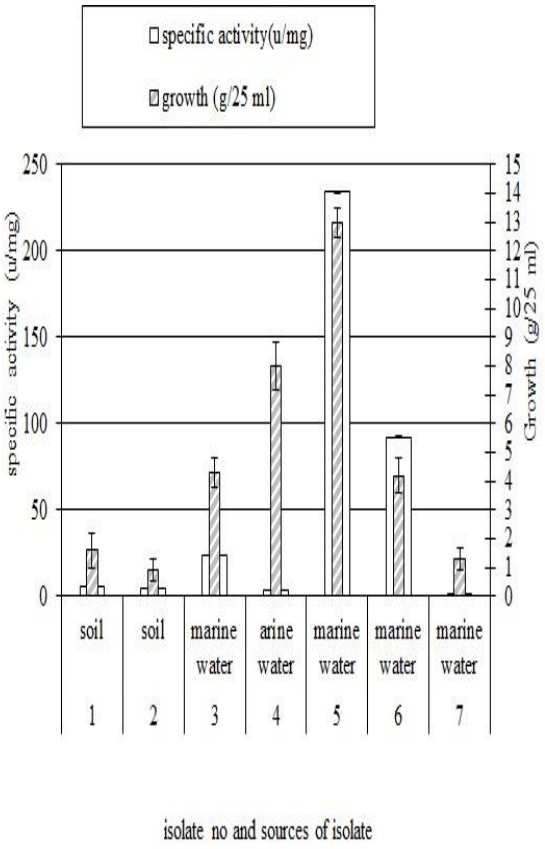

Fig.1. Screening of different Streptomyces isolates for protease inhibitor production

Identification of bacterial isolate by 16S rRNA: The partial 16S rDNA sequence followed by construction of phylogenetic tree by neighbor joining method has revealed that the isolate no.5 was *S. lavendulae* (fig.2).The 16S rDNA sequences for *Streptomyces lavendulae* and the relevant sequences were downloaded and phylogenetic analysis has been carried out. Isolate was submission in gene bank with accession number AB795940.1similarity 95% with same species found in genebank (**Fig 2)**. In laboratories in developed countries, a partial 16S rDNA sequence of a new *streptomyces* isolate can be obtained quickly and at low cost to give an unambiguous identification of the genus to which the isolate belongs. This is certainly not the case in developing countries, such as South Africa, where high sequencing costs and possible restricted access to sequencing facilities preclude the use of 16S rDNA sequencing as a routine genus-identification tool. PCR-based methods have provided a rapid and accurate

way to identify bacteria [22, 23, 24,25,26,27,28,29,30 and 31]. In particular, amplified rDNA restriction analysis (ARDRA) has proved to be very useful [32, 33]. ARDRA has been shown to be useful in differentiating between bacterial species within a genus, for example, *Clostridium* [22], and in differentiating bacterial strains within a species, for example, *Lactococcus* [23].

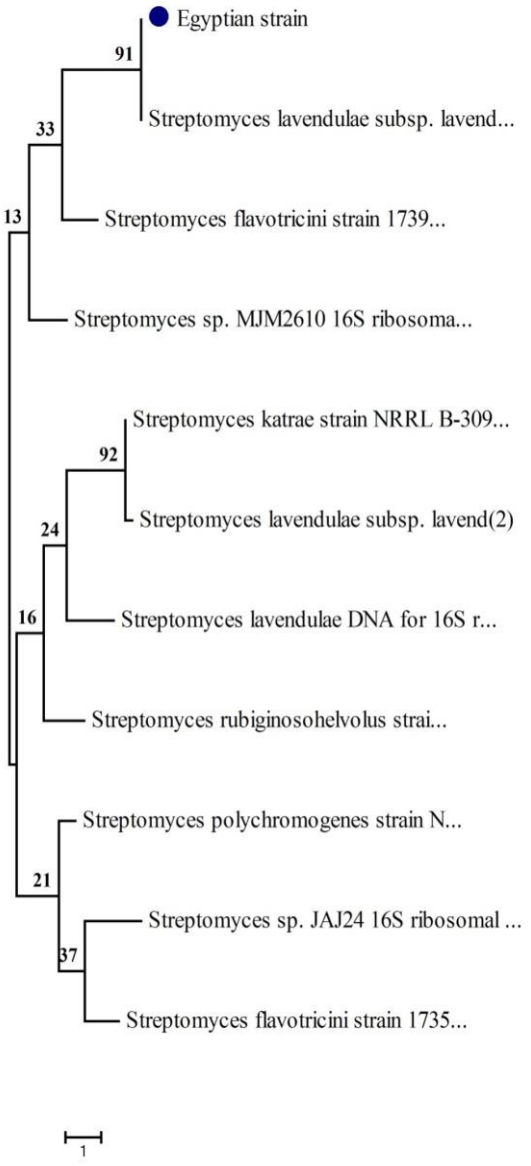

Fig.2. Distance neighbor-joining phylogenetic tree of Egyptian isolate (*Streptomyces lavendulae*).

It has also been shown to be useful in identifying several medically important species of aerobic actinomycetes belonging to the genera *Actinomadura*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomyces* and [29, 30 and 32]. Small rRNA gene sequencing, particularly 16S rRNA sequencing in bacteria, has led to advances on multiple fronts in microbiology. First, the construction of a universal phylogenetic tree classifies organisms into three domains of life, bacteria, Archaea, and [32, 33, 34, 35, 36]. Second, it revolutionists the classification of microorganisms, and makes the classification of non-cultivable microorganisms possible [37, 38]. Third, it helps to elucidate the relation of unknown bacterial species to known ones. 16S rRNA gene sequencing will continue to be the gold standard for the identification of bacteria, and the automation of the technique could enable it to be used routinely in clinical microbiology laboratories, as a replacement of the traditional phenotypic tests. Modern technologies have made it possible to construct a high density of oligonucleotide arrays on a chip with oligonucleotides representing the16S rRNA gene sequence of various bacteria. Such a design will facilitate automation of the annealing and detection of the PCR products of 16S rRNA gene amplification, and hence routine identification of most clinical isolates will be possible [39]. The use of 16S rRNA gene sequencing has several advantages. First, the turnaround time is short, because amplification of the 16S rRNA gene takes only four to six hours, and the annealing and detection of PCR products takes only another few hours, theoretically the identification can be completed within one day. Second, it can be used for slow growing bacteria, unlike most commercially available kits that are based on phenotypic tests that require the detection of growth of the organism in the presence of certain specific substrates, and hence the slow growing bacteria are usually “unidentified” when the growth control shows a negative result [40]. Third, the problem of “unidentifiable strains” will be overcome and there would be minimal misidentification – the identification of a clinical strain is clearly defined by the number of base differences between it and the existing species. Fourth, oligonucleotides representing all bacterial species, including those rarely encountered clinically, can be included in the array, making it easy to identify the rare species. Lastly, such a technique will be applicable not only to pyogenic bacteria, but also to other organisms such as mycobacteria, [40]. The phylogenetic tree generated from partial 16s rRNA gene sequences, including the sequences of *Streptomyces lavendulae* and other sequences from the database of gene bank showed that the isolate formed one clusters. Our data were in agreement with the division of the 16s rRNA topology

into major clusters as described. Data obtained in this study illustrate primer design will be useful to identify many bacterial genera.

The effect of various factors on the production of protease inhibitor

Effect of different media: The ability of *S.lavendulae* to produce active extracellular alkaline protease inhibitor was investigated by culturing the organism on four different media varied in their composition after 4 days of incubation periods under shaking conditions (150 rpm) (Fig. 3). The results indicated that the enzyme production varied greatly with the culture media used. It was found that medium number 2 was the most effective medium for the production of extracellular alkaline protease inhibitor (350 units/mg protein) after 4days of incubation at 28±2°C. This media contained glucose as carbon source, sodium nitrate as nitrogen source and some minerals. Many authors were used different media for production of protease inhibitor from *Streptomyces* spp., most of these media contained glucose as carbon source and organic nitrogen source (peptone or poly peptone) in contrast of our medium which contained inorganic nitrogen source [40,41,42,43,44,19,10] (**Fig 3).**

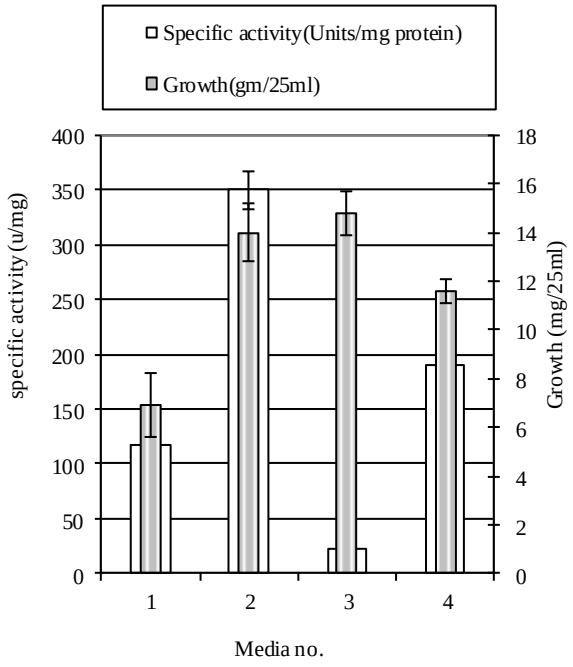

Fig.3. Effect growth media on protease inhibitor production

Effect of incubation period: The time course of the production of protease inhibitor during cultivation was examined. Maximum production of growth and protease inhibitor was reached after 5 days of incubation at 28 °C with shaking (357 units/mg proteins) results in fig. (4).These results are in agreement with that obtained by [12]; they found that maximum production of protease inhibitor was obtained when *Streptomyces violascens* incubated for 5 days at 15-20 °C. The same result was obtained by [10]; when different strains of *Streptomyces* were used to produce protease inhibitor. In contrast, our results

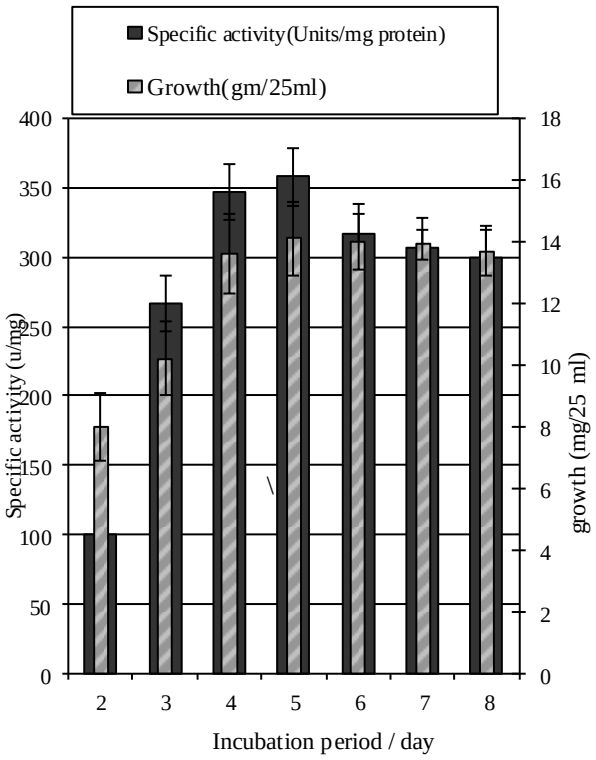

Fig.4. Effect different incubation periods on protease inhibitor formation.

incompatible with that obtained by [42]; they found that the maximum production of protease inhibitor was obtained after 72h of incubation with *Streptomyces griseoincarnatus* strain KTo-250, and [44]; Who found that 28h was optimum for production of protease inhibitor by *Streptomyces* sp (**Fig 4).**

Effect of carbon sources on protease inhibitor production: Efficiency of protease inhibitor production was checked under different supplementation of the culture medium with different carbon sources. Results represented in Fig. 5, showed that *S. lavendulae* utilized all tested sugars as C-

sources with one exception in case of raffinose.The protease inhibitor activity produced in good level when sorbose, glucose, starch and galactose were used as carbon sources(603,366,366 and 313 units/mg protein respectively).Moreover cellobiose , glycerol, maltose and fructose gave less amounts of protease inhibitor productivity (225,188,114 and 26 units/mg protein respectively).The present results can be assumed that protease inhibitor are produced constitutively. Many authors investigated the effect of carbon sources on the production of protease inhibitor in different *Streptomyces* species. Glucose was found to be the ideal carbon source at concentration of 0.05-3%, [19, and 43].While starch at concentration of 1-2% was used to produce protease inhibitor abundantly, [45, 46] (**Fig 5**).

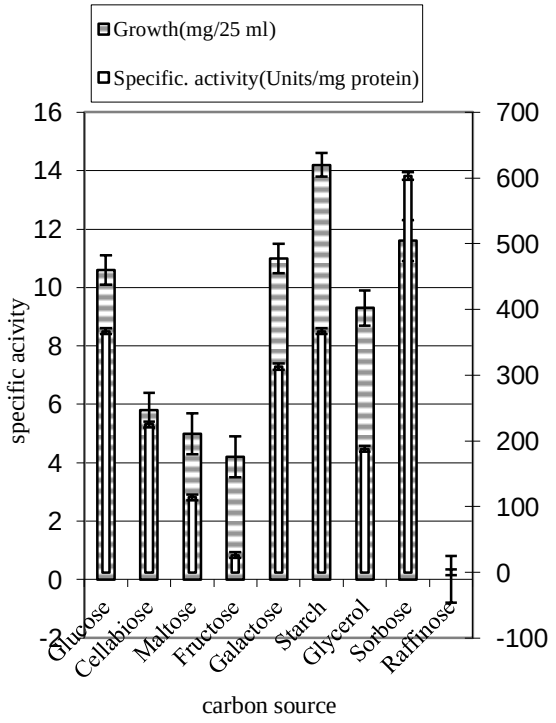

Fig.5. Effect of different carbon sources on growth and protease inihbitor production

Furthermore, the results indicated that the protease inhibitor production was increased as the increasing sorbose concentration up to 3% (708 units/mg protein) .Further increases in the initial concentration of sorbose gradual decreased the production (fig.6). The use of sorbose as carbon source for protease inhibitor production not recommended by any authors but [43] was used sorbitol with many carbon sources to study their effect on protease inhibitor production. Sorbose turns onto sorbitol during fructose and mannose metabolism in bacteria and enters in the phosphoenol pyruvate dependent phosphotransferase system the major mechanism used by bacteria for uptake of carbohydrates particularly hexoses and hexitols, [46, 47]. For this explanation, *Streptomyces lavendulae* may be prefer sorbose for its metabolism to produce protease inhibitor **(Fig 6).**

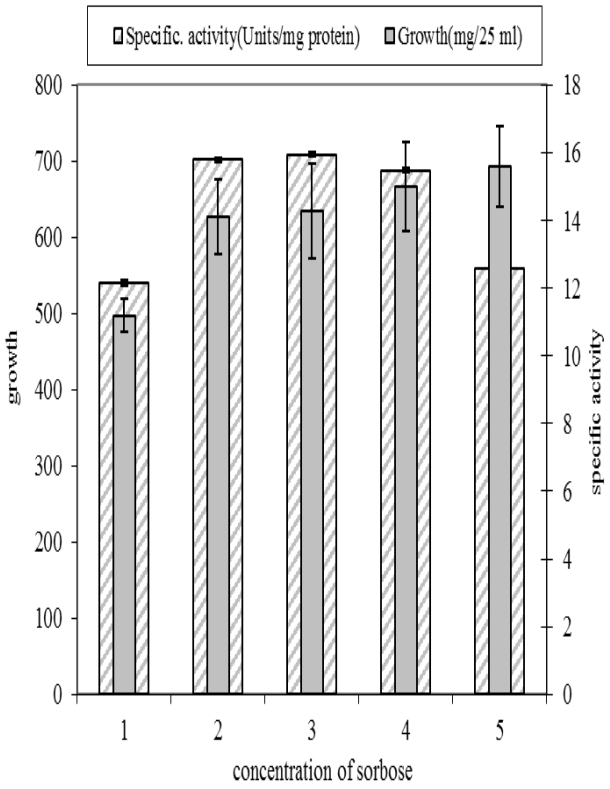

Fig.6. Effect of differenet concentration of sorbose on protease inhibitor production

Effect of nitrogen sources on protease inhibitor production: Protease inhibitor production depends heavily on the availability of nitrogen sources so the nitrogen sources of the fermentation medium under study were investigated as shown in Figure 7.Our results revealed that the maximum production of protease specific activity was obtained from the culture when yeast extract was used (703 U/mg protein) followed by peptone (650 units/mg protein) compared to the control. When inorganic nitrogen sources were used, the culture containing sodium nitrate (control) followed by potassium nitrate produced (603 and 500U/mg protein, respectively). Organic nitrogen sources such as yeast extract and peptone being rich in amino acids and short peptides

and yeast extract is an inexpensive organic source of amino acids, proteins, vitamins for cell growth and synthesis of enzymes such as amylase and protease [48].These data are agreement with that obtained by [43], they found that a few complex organic nitrogen sources like poly peptone followed by bacto-casitone were most suitable for protease inhibitor production by marine bacteria. [44, 19, 45, 10] were used poly peptone for protease inhibitor production by different *Streptomyces* strains. From the above mentioned results, organic nitrogen sources were favorable for the production of protease inhibitor (**Fig 7).**

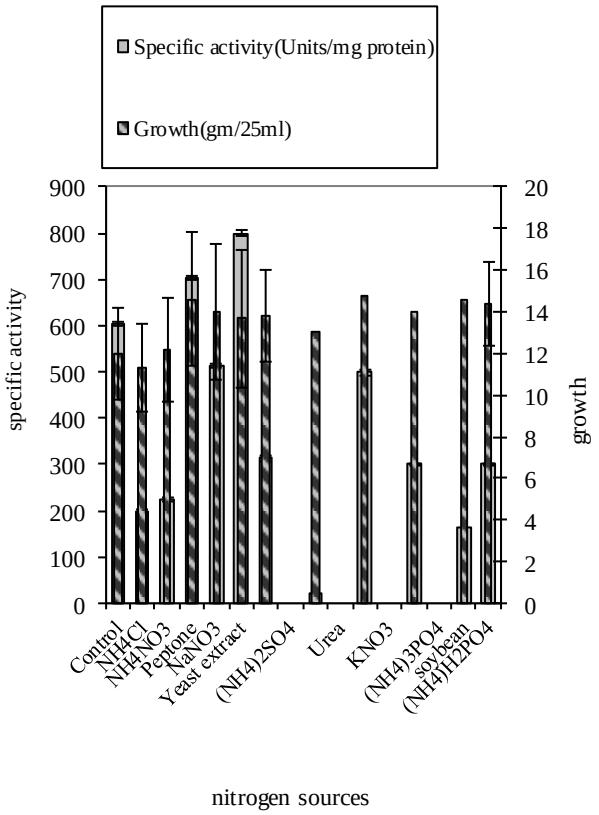

Fig.7..Effect of nitrogen sources on growth and protease ihibitor production

UV radiation: UV radiation is composed of UVA (315 to 400 nm) and UVB (290 to 320 nm). It has a significant contribution to the biological effects of UV radiation since it is absorbed by cellular DNA. Sunlight, due to its content of UV rays, has an important role in the sterilization that occurs under natural conditions. Due to these properties, UV radiation has found applications in control of bacteria in indoor air, water supplies and many heat sensitive food products [49, 50].

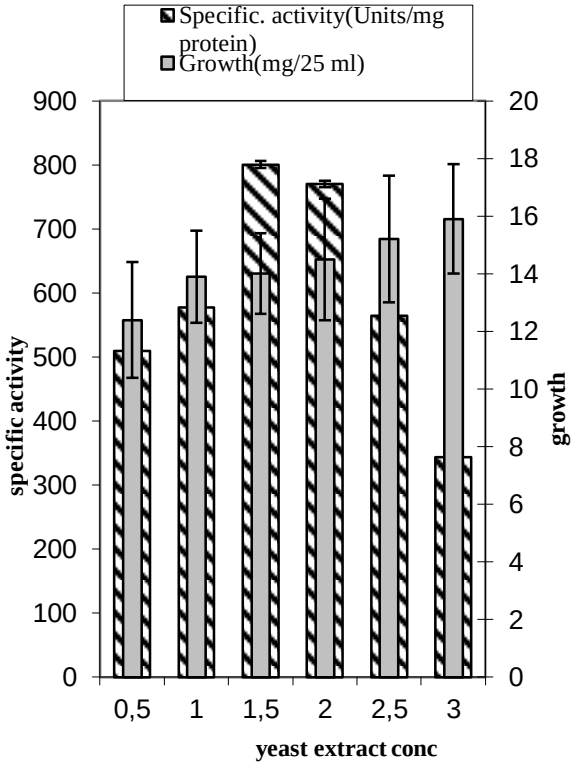

Fig.8. Effect of yeast extract conc on protease inhibitor production

Therefore, yeast extract was used as the most suitable nitrogen source for maximal protease inhibitor production and different concentrations were used. The results in (fig. 8) indicated that yeast extract at concentration of 1.5 % was the most effective concentration for protease inhibitor activity production, when increasing the concentration above 1.5%, gradually decreased in the production was observed (**Fig 8).**

The aim of the present study is directed towards the evaluation of the activity of protease inhibitor from *Streptomyces lavendulae* by physical UV exposure. From the results shown in **table (1**), it seems that 15 min exposure, the activity increased up to optimum value (1055.9 units/mg protein). However before exposure to UV higher protease inhibitor activity was (800.6 units/mg protein) with yeast extract at 1.5%.

Table (1): Effect of different exposure time of UV source on production of protease inhibitor from *Streptomyces lavendulae*.

UV irradiation (min)	Specific activity (units/mg protein) ±SE
10	966.1±13.5
15	1055.9±18.5
20	1010.7±27

Assement of protease inhibitor activity by immobilized strain *Streptomyces lavendulae* on different matrix: Among the immobilization methods for microbial cells, entrapment is the most suitable and common method of practice. Immobilization by entrapment is known to be a simple and gentle procedure and keeps the cells from unfavorable conditions (pH, temperature, etc.) in the surrounding media [51, 52]. Whole cell immobilization technique is generally being used for higher productivity by protecting the cells from shear forces, in addition to this the product and cell separation is easy so that the cells can be reused several times [53].

Low level of protease inhibitor activity was observed with polyacrylamide and free cells. Probably polyacrylamide monomers were toxic for the cells. A low level to moderate titer of protease inhibitor was obtained with agar, and also it was proved to be unstable resulting in a considerable amount of cell leakage. Different matrices like agar, gelatin, and polyacrylamide were used for immobilization of a definite amount of logarithm phase of *Streptomyces lavendulae* cells. A free cell system containing the equivalent cell weight was also run. Results in tables (**3 and 4**) represent the comparative protease inhibitor production by different matrices over a time period of 48 h of incubation and cell leakage, respectively.

Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Last but not least, immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed [54]. Comparison of Alkaline Protease Production by Immobilized Cells in Various Matrices by Entrapment Technique prove, protease inhibitor titer with immobilized cells in gelatin matrix was found to higher followed by agar (**Table 2**).

Table (2): Protease inhibitor activity of *Streptomyces lavendulae* immobilized in different matrices with different times

Matrix Type	Repeat Cycle and Enzyme activity (U/mg)						
	6 h	**12 h**	**18 h**	**24 h**	**30 h**	**36 h**	**42 h**
Gelatin	512±16.5	**560**±8	**660±9**	520.2±12.5	500.3±4.5	460±22	420±18
Polyacrylamide	155±13.2	**151**±4.7	149±10	130.2±14.7	129±8	111±11	100±12
Agar	160.2±18.2	**140**±15	139.9±14.7	0	0	0	0
Free cell	140.0±20.	**150**±11	**200.1**±12.5	**234**±19.8	**136.1**±14	**130**±14	**120.1**±17

The results are in line with the finding by [58] who has reported 21.7 U/mg specific activity of protease by *Bacillus mycoidus*on polyacrylamide matrix at 2% acrylamide concentration.

Agar entrapped cells showed least enzyme production (160.2U/mg) compare to free cells it was only 7.8% increased with maximum cell leakage (0.74 mg/ml). The less protease production was achieved by *Streptomyces avermectinus*at 3% concentration [5]. The gelatin entrapped cells started the specific activity of enzyme at 6 h and gradually it increased to maximum titer 660 U/mg at 18 hrs, among tested different matrices at 24 h of incubation and after this enzyme production was decreased. matrix over the period of incubation was less (0.06 mg/ml) compared to other matrices. Gelatin has used as immobilization matrix for the production of Penicilin G acylase [55], Glucoamylase, invertase and cellobiase [**56**] and for ß- amylase [57]. Compared to free cell system where at 24 h of incubation the maximum specific activity of enzyme titer was 234 U/mg, gelatin entrapped cells showed 51 % increased enzyme production. Cell leakage from the [59] Also reported the least enzyme production with agar by *Pseudomonas diminuta*among tested Chitosen, Gelatin and agar matrices.

Table (3): Comparison of Alkaline Protease Production with Cells Immobilized in Various Matrices by Repeated Batch Cultures

Matrix Type	Fermentation Period for Each Batch (hours)	No. of Batches
Gelatin	**18**	**7**
Polyacrylamide	**6**	**4**
Agar	**6**	**6**
Free cell	24	0

Increasing activity with radiation due to the receptors activation on surface of bacterial cells this do the adhesion on *streptomyces* cells with polymer long time with repeation batch culture cell, however leakage of cell the very low with gelatin so the activity of protease high. At 14 kGy the activity start in decreasing due to denaturation of protease enzyme.

After the immobilized biocatalyst had been in use for about 7 days, it still possessed significant protease inhibitor production. It has been shown that immobilized cells were able to produce protease inhibitor consistently and that they might be used for continuous protease inhibitor production.

The reusability of *Streptomyces lavendulae*was evaluated by transferring the immobilized cells to fresh medium at every 48 h of incubation. Results revel that the gelatin entrapped cells can be reused for maximum times (7 cycles) with total protease titer higher than agar entrapped cells can be reused for only 3 cycles. Bajpai andMargaritis60] reported 10 batch cycles of inulinase production with gelatin entrapped beads by *Kluyveromycesmarxianus* [53, 61, 62].

[63] has reported 5 repeat cycles with Table 10 represents the cell leakage from different matrices during repeated batch process. Maximum cell leakage was observed in agar entrapped cells and minimum with gelatin entrapped cells. With gamma radiation illustrated in table 5 different doses treatment of gelatin carrier for immobilization the bacterial cells of *Streptomyces lavendulae*, higher activity 1900 u/mg with 8 kGy**.**

Table (4): Cell leakage study by immobilized *Streptomyces lavendulae* in different matrices during repeat batch process.

Matrix Type	Repeat Cycle and Cell Leakage (mg/ml) per each batch						
	1 cycle	**2 cycle**	**3 cycle**	**4 cycle**	**5 cycle**	**6 cycle**	**7 cycle**
Gelatin	**0.09**	**0.5**	0.7	0.7	0.8	0.9	0.9
Polyacrylamide	**0.4**	**0.8**	0.9	2.1	0	0	0
Agar	**0.6**	**1.8**	2.4	2	2.2	1.9	0

Table (5): Effect of different doses of gamma on matrices gelatin immobilized with Streptomyces *lavendulae*

Gamma radiation doses	Specific activity (u/mg)±SE
control	**660±9**
2	**1056±11**
4	**1110±24**
6	**1200±27**
8	**1900±24**
10	**1840±26**
14	**1700±21**

CONCLUSION

The results show novelty work using gelatin as matrix immobilized *Streptomyces lavendulae* as enhancement tool for protease inhibitor production and activity, Specific advantages of this technique such as long life-term stability, reusability, and possibility of regeneration to be adaptable also to scale-up the obtained data. In addition, the experiments with repeated batches of gelatin immobilized bacterial growth by introducing fresh nutrients every batch lead to a specific volumetric productivity that is 1.8 times (180%) higher than that obtained with free cells. data promising to apply this technique in large scale as pharmaceutical application with fermenter.

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