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Optimization of the Environmental Conditions for alkaline protease production using *Streptomyces griseus* in Submerged Fermentation Process

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Abstract: The objective of this study is the production of an extra cellular alkaline protease by a new local isolate which was characterized and identified by 16S rRNA as *Streptomyces griseus* sub sp *griseus* and it was submitted in gene bank with accession number AB723782. The culture conditions for higher protease production by *Streptomyces griseus* were optimized with respect to carbon source, nitrogen source and pH. Maximum protease production was obtained in medium no. 5 supplemented with 3% glucose as a carbon source, 0.4 % $NaNO_3$ as a nitrogen source, the optimum pH was 8 at 30°C and the highest production was obtained after 6 days of incubation. Wheat bran proved as the best food waste for protease production (9208.5 U/mg protein) compared to other wastes, and finally the optimum gamma radiation was (1.6 kGy) led to increase the specific activity to 14450.5 U/mg protein. The protease profile of the isolate shows its potential as a good source for industrial application.

Key words: *Streptomyces griseus,* protease, 16S rRNA, production, agro-industrial wastes.

INTRODUCTION

Enzymes are extensively used in food-manufacturing and analysis of chemicals, thus becoming one of the most important branches of different industries (Priolo *et al*., 1991, Pandy *et al*., 2000, and Moharib &Gad, 2010). Proteases are still the most important group of enzymes play an important role in biological systems, such as viral maturation, protein degradation (fungal peptidase) and blood pressure regulation (rennin). Proteases have been detected in some fungi (Dohmae *et al*., 1995, Palmieri *et al.,* 2001 and Moharib, 2007), but only few of them have been isolated and characterized.

Proteases known as proteinases or proteolytic enzymes which occur naturally in all organisms, they act on the peptide bonds formed by specific amino acids to hydrolyze them (Tunga *et al*., 2003; Prathamesh, 2011).

Microbial proteases represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sale in the world and they are the leaders of the industrial enzyme market world wide. Alkaline proteases are of immense interest due to their wide application in detergent, tanning, textile, dairy industry, organic synthesis, peptide synthesis, instant recovery of silver and wastewater treatment (Gupta *et al*., 2002).

Microbial proteases are extracellular and would simplify the downstream processing of the enzyme as compared to those obtained from plant and animal sources (Oztruk *et al*., 2009). Despite the long list of protease producing microorganisms, only a few microorganisms are considered as appropriate producers for commercial exploitation, being non-toxic and non-pathogenic. Actinomycetes are gram positive mycelium–forming soil bacteria that include many species that are able to degrade many macromolecules such as lipids, starch, chitin, xylan, pectin and proteins (El-Shanshoury *et al*., 1995). The ability to produce variety of enzymes can be an alternative phenomenon in these prokaryotes. *Streptomyces* species producing protease include *S. griseus, S. rimosus* and *S. thermovulgaris* (Yeoman & Edwards, 1994; Hadeer, 1999 and Yang & Wang, 1999). The present study was carried out to isolate and screen diverse actinomycetes for protease production. A soil isolate, *S. griseus* was identified as a producer of alkaline protease, also this study describes the optimization of some culture conditions for enzyme production.

MATERIALS AND METHODS

1. Microorganism:

The soil samples were collected from different locations in Egypt, air dried and powdered. The samples were diluted in sterile saline solution (0.89 % w/v). The diluted samples were plated onto solid sterile soy bean plates (pH 7) containing (gl^{-1}) soy bean, 30; dextrin, 15; $CaCO_3$, 10; $MgSO_4$, 1 and agar 20 and were incubated

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at 30 ºC. After 7 days, the isolated actinomycetes colonies were subcultured in fresh plates and then the single uniform colonies were transferred into slants of the same medium and preserved.

2. Primary Screening for Protease Production:

All the isolated actinomycetes were screened for protease production by culturing in nutrient broth medium containing (gl^{-1}) peptone, 10; yeast extract, 5; glucose, 10; and NaCl, 10 for 4 days with agitation at 200 rpm at 30 ºC. The culture filtrate was assayed for protease activity by Tsuchida *et al*. (1986).

3. Identification:

The most enzyme producing strain was identified by 16S rRNA sequencing data collection. A database containing 16S rRNA gene sequences of all validly published filamentous actinomycetes (Euze´by, 2002) 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 Stackebrandt *et al.* (1997) and the NCBI Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/ Browser/wwwtax.cgi).

A. Organisms and Culture Conditions:

The organism was isolated on Czapek solution agar (Atlas, 1993), Middlebrook 7H9 agar (Difco Laboratories) or Streptomyces general defined medium [(800 ml): 0.17 g Na_2HPO_4. $2H_2O$; 0.14 g KH_2PO_4; 0.05 g $MgSO_4.7H_2O$; 0.01 g $FeSO_4.7H_2O$; pH 7.0; autoclaved at 15 p.s.i. (103.5 kPa) for 15 min; after cooling, 100 ml of 100 mM glucose, 50 ml of 50 mM $(NH4)_2SO_4$ and 50 ml of 50 mM L-glutamic acid, sodium salt, were added)]. All media contained cycloheximide at 50 mg m^{-1}. Cultures were incubated at 30 °C for 14–28 h. Colony selection was based on the colour of aerial and substrate mycelium, differences in morphology and rate of growth.

B. DNA Extraction:

Actinomycete strain was grown in 10 ml International *Streptomyces* Project Medium 1 (ISP 1) (Shirling & Gottlieb, 1966) 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 mM Tris-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 (300 ml) 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.

PCR amplification was carried out in 50 ml volumes containing 2 mM $MgCl_2$, 2U Taq polymerase (JMR Holdings, USA), 150 mM of each dNTP, 0.5 mM of each primer and 2 ml template DNA. Primer F1 (59-AGAGTTTGATCITGGCTCAG-39) and primer R5 (59-ACGGITACCTTGTTACGACTT-39) were modified from primers FDI and rP2, respectively (Weisburg *et al*., 1991). Primer F1 binds to base positions 7–26 and primer R5 to base positions 1496–1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877T (rrnD operon; GenBank accession no.M27245). The primers were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96ºC for 2 min), 30 cycles of denaturation (96 °C for 45 s), annealing (56 °C for 30 s) and extension (72 °C for 2 min), and a final extension (72 °C for 5 min). The PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide (10 mg m^{-1}), to ensure that a fragment of the correct size had been amplified.

C. DNA Sequence:

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 GeneDoc (www.NCBI / blast.com), and determine translation encoded regions at (www.expasy.org/cgi-bin/dna_aa). Phylogenetic tree establish my mega program.

4. Inoculum Preparation and Fermentation Conditions:

The inoculum was prepared by growing the most enzyme producing organism on soy bean slants for 9 days; then a spore suspension of optical density 0.6 at 660 nm was made by using sterile saline solution. Two ml of spore suspension was inoculated on 48 ml liquid medium (medium No. 5) which contain (gl^{-1}) 20, sucrose; 0.5, NaCl; 2, KNO_3; 1, K_2HPO_4; 0.5, $MgSO_4$; 3, $CaCO_3$ and 0.01 for each of $FeSO_4$, $ZnSO_4$ and $MnCl_2$ in 250 ml Erlenmyer flasks. Thereafter, the flasks were incubated for 6 days at 30 ºC in a shaking incubator (200 rpm). The culture medium was centrifuged at 6000 rpm for 10 min and the supernatant was assayed for protease activity and protein content.

5. Quantitative Assay of Protease:

protease activity in the culture filtrate of *S. griseus* was assayed by modified method of Tsuchida *et al*.(1986) 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^{-1} w/v of casein in 10 mM Tris-HCl buffer (pH 8)]. The mixture was incubated at 37 ºC for 30 min. Reaction was terminated by the addition of 1ml 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 ºC; 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 Folin Ciocalteau reagent was measured at 660 nm. All assays were done in triplicate. One protease unit is defined as the amount of enzyme that releases 1µg of a tyrosine per ml per minute under the above assay conditions. The specific activity is expressed in the units of enzyme activity per milligram of protein.

6. Protein Concentration:

The protein concentration was determined by the method of lowry *et al*. (1951) with bovine serum albumin as a standard.

7. Optimization of Cultural and Environmental Protease Production:

7.1 Production Media:

Six different types of media were used for primary evaluation for medium optimization process. The composition of these media gl^{-1} were as follows: medium 1: 0.7, K_2HPO_4; 0.3, KH_2PO_4; 0.5, $MgSO_4$; 0.01, $FeSO_4$; 0.001, $ZnSO_4$ and 7, baker´s yeast (modified from Reynolds, 1954).

Medium 2: 10, peptone; 5, yeast extract; 10, glucose and 10, NaCl (modified from Rahman *et al*., 2007).

Medium 3: 30, soy bean; 15, dextrin; 10, $CaCO_3$ and 1, $MgSO_4$ and 1, $MgSO_4$. (Gad *et al*., 1971).

Medium 4: 40, starch; 0.9, $(NH_4)_2SO_4$; 0.8, KH_2PO_4; 17.5, soy bean; 3, $CaCO_3$; 0.02, $ZnSO_4$; 0.01, $FeSO_4$ and 0.01, $MnCl_2$.

Medium 5: 20, sucrose; 0.5, NaCl; 2, KNO_3,; 1, K_2HPO_4; 0.5, $MgSO_4$; 3, $CaCO_3$; 0.01 for each of $FeSO_4$, $ZnSO_4$ and $MnCl_2$.

Medium 6: 20, starch; 0.5, NaCl; 2, KNO_3; 1, K_2HPO_4; 0.5, $MgSO_4$; 3, $CaCO_3$ and 0.01 $FeSO_4$.

For all media used, the pH was adjusted to 7 before sterilization. This was carried out in 250 ml conical flasks containing 48 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, USA) at 200 rpm.

7.2. Optimization of the Culture Medium:

The effect of different carbon sources on protease production was studied by substituting of sucrose in the selected medium with different sugars. These were glucose, starch, lactose, xylose, fructose, molass, sorbose and dextine at 2 % (w/v). Also the best concentration of the best carbon source was studied.

To test the effect of different nitrogen source on protease production, in the optimum liquid medium potassium nitrate was substituted by equivalent amount of various nitrogen sources such as casein, soy bean, yeast extract, corn steep liquor, $NaNO_3$, NH_4NO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and urea. The culture media were inoculated and incubated at 30°C for 6 days at 200 rpm . The cell free supernatant was analyzed for protease activity and total protein. Furthermore, different concentrations of the best nitrogen source were tested.

7.3. Effect of Initial PH Values:

To determine the most favorable pH values for protease production, the initial pH value of the incubation medium was adjusted in the range from 4 to 10 before sterilization using 0.1 N NaOH or 0.1 N HCl. The cultures were incubated at 30°C at 200 rpm for 6 days and analyzed for cell growth and protease activity.

7.4. Effect of Different Wastes:

For this propose, different wastes such as rice straw, sugar beet, sweet whey and wheat bran were tested as a sole carbon and nitrogen sources. The inoculated flasks were incubated for 6 days in shaking incubator adjusted at 200 rpm with the same conditions as described before.

8. Gamma Radiation:

The *Streptomyces griseus* strain was cultured in the optimized medium at 30 °C for 18 h in 500 ml conical flasks, the medium was dispensed in 10 ml quantities into test tubes exposed at room temperature, to various doses (0.00, 0.2, 0.4, 0.6, 0.8, 1.00, 1.2, 1.4, 1.6, and 1.8 kGy) of gamma radiation. Duplicate tubes were used for each dose. The obtained irradiated standard cultures were inoculated into the medium and incubated at 30 °C for 18 h, to estimate protease activity.

9. Statistical Analysis:

Results are expressed as the mean ± S.E. of three independent astrocytic culture preparations performed in triplicate. The statistical analyses were carried out using one way anova followed by Dunnett's multiple comparison tests with statistical significance set at $p < 0.05$. All analyses were performed with Graph Pad Prism 4.02 for Windows (Graph Pad Software, San Diego, CA) version (2007).

RESULTS AND DISCUSSION

Screening of Actinomycetes for Protease Production:

It is important to identify hyper-producing microbial strains in order to meet the ever-growing industrial demand of protease. Research on terrestrial actinomycetes brought out a large number of actinomycetes strains with diverse potential including enzyme production. Therefore, the present study was aimed to isolate actinomycetes from three different soil samples collected from different areas in Egypt and screen them for protease activity. Among the ten isolates, one isolate coded as S13 was selected for further studies as it produced maximum proteolytic activity of 241.6 U/ml after 4 days of incubation on medium no. 2 compared to the rest of isolates (data not shown).

Identification of S. no. 13:

The partial 16S rRNA sequence followed by construction of phylogenetic tree by neighbor joining method have revealed that the isolate S13 was *S. griseus*. The 16S rRNA sequences for *Streptomyces griseus subsp. griseus* and the relevant sequences were downloaded and phylogenetic analysis has been carried out. Isolate was submission in gene bank with accession number AB723782 similarity 95% with same species found in genebank. Fig. (1) show phylogenic tree of *Streptomyces griseus subsp. griseus*

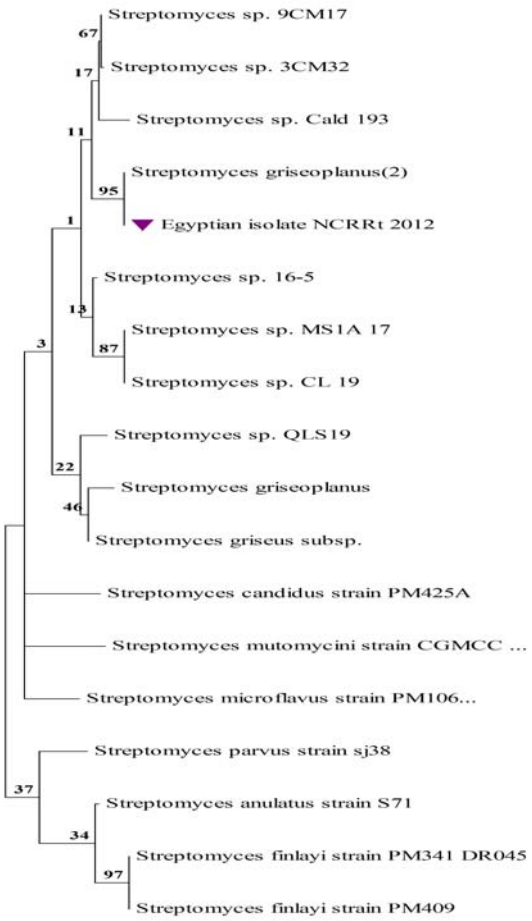

Fig. 1: Distance neighbor-joining phylogenetic tree of Egyptian isolate (*Streptomyces griseus subsp. Griseus* spp) NCRRT 2012 Consensus bootstrap.

PCR-based methods have provided a rapid and accurate way to identify bacteria (Gurtler *et al*., 1991; Kohler *et al*., 1991; Beyazova and Lechevalier, 1993; Telenti *et al*., 1993; Soini *et al*., 1994; Mehling *et al*., 1995; Steingrube *et al*., 1995; Wilson *et al*., 1998 and Laurent *et al*., 1999). In particular, amplified rDNA restriction analysis (ARDRA) has proved to be very useful (Harvey *et al*., 2001 and Alves *et al*., 2002). ARDRA has been shown to be useful in differentiating between bacterial species within a genus, for example, Clostridium (Gurtler *et al*., 1991), and in differentiating bacterial strains within a species, for example, *Lactococcus* (Kohler *et al*., 1991). 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 *Tsukamurella* (Wilson *et al*., 1998; Harvey *et al*., 2001 and Laurent *et al*., 1999). 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 Eucarya (Olsen *et al*., 1992; Olsen & Woese, 1993 and Thompson *et al*., 1994). Second, it revolutionists the classification of microorganisms, and makes the classification of non-cultivable microorganisms possible (Relman *et al*., 1990 and 1992). 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 the 16S 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 (Woo *et al*., 2000). 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 (Woo *et al*., 2001). 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 (Woo *et al*., 2001). The phylogenetic tree generated from partial 16s rRNA gene sequences, including the sequences of (*Streptomyces griseus subsp. Griseus* 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.

Optimization of Protease Production:

Effect of Different Media and Incubation Period:

Enzyme formation is largely dependent on the condition of growth of the culture and composition of nutrient medium (Fujiwara and Masui, 1993). To find the best medium for production of protease from *S. griseus,* six tested media differing in their carbon and nitrogen sources were studied at 30 ºC, 200 rpm for different incubation periods (3 to 7 days) as shown in Table (1). Results showed that all media used gave protease productivity by different degrees, this indicate that the organism can produce protease on different substrates, where medium no. 5 was the most effective one for enhancing the yield (644.3 U/ml) after 6 days of incubation at pH 7. While, medium no. 6 was the lowest for protease production, this may be due to induction of enzyme secretion by sucrose and potassium nitrated in medium no. 5 compared to starch in medium no. 6, furthermore, the low concentration of starch (2%) in medium no. 6 gave a low activity of no. 4 which contain 4% starch. Chaloupaka and Cesko-slovenska (1956) mentioned that the presence of more quantity of sugar in the medium may enhance the protease production. On the other hand, baker´s yeast and soy bean in media no. 1 and 3 promote the production compared to medium no.2 which contains peptone that may reduce the productivity. Lazim *et al*. (2009) reported that malt extract, peptone, ammonium nitrate, sodium nitrate and casein greatly reduced enzyme productivity by *S. sp* CN902.

Protease activity produced by *S. griseus* was detected in the culture supernatant from day 3 up to 7 days and the highest level (644.3 U/ml) was reached its maximum value after 6 days of incubation by using medium no. 5 and the other media except medium no. 4 reached its maximum at 5 days, then the production was declined beyond this period (Table 1). This result was in accordance with Yang and Wang (1999) who mentioned that the optimum protease production from *S. rimosus* was recorded at 166 h. Ramesh *et al.* (2009) observed that growth of *S. fungicidicus* MML1614 continued to increase from day 3 till day 8, but the increase in protease production

was only up to 6 days and after wise it was declined. On the other hand, Singh and Thumar (2007) found that *S. clauvligerus* gave high protease production at 110 h (130 U/ml).

Table 1: Effect of different media on the production of alkaline protease from *Streptomyces griseus.*

Medium No.	Enzyme activity (U/ml)				
	Incubation period (days)				
	3	4	5	6	7
1	194.1±1.15	314.3±1.20	414.7±0.88	463.0±0.58	365.9±1.73
2	131.0±1.53	241.0±.0.58	355.7±1.45	371.7±0.33	321.4±0.88
3	217.0±2.88	384.7±1.76	437.3±0.82	460.1±1.23	351.0±0.57
4	194.3±2.19	335.3±1.20	502.6±0.88	385.0±1.73	373.7±1.20
5	275.6±0.58	394.7±1.33	533.7±1.21	644.3±1.86	450.7±0.66
6	206.0±1.15	236.7±1.20	299.0±0.55	365.7±1.86	363.3±0.33

T test using Prism program version 2007, $P < 0.05$
Note: mean ± SE (stander error)

Carbon Source and Protease Production:

The effect of replacement of sucrose (2%) in the basal medium (no. 5) by various carbon sources on protease synthesis is shown in Fig. 2. All carbon sources slightly increased the production except starch, xylose and sorbose which had the lowest activity (611.0, 622.9 and 325.4 U/ml, respectively). On the other hand, glucose gave the highest activity (1452.0 U/ml) and specific activity reached 9680.0 U/mg protein compared to sucrose in the control (643.6 U/ml and 2145.3 U/mg protein). These findings were in agreement with Ramesh *et al.* (2009) who found that replacement of glucose instead of sucrose and reducing the quantity of peptone in the modified medium remarkably enhanced the productivity on 6th day. Also, El-Shafei *et al*. (2010) observed that glucose (1.25%) was finally the best carbon source for protease production by *S. albidoflavus*. Mostafa *et al*. (2012) observed that the maximum protease yield was obtained from glucose. On the other hand, addition of glucose and other carbon sources lead to the reduction in alkaline protease production by *S*. sp CN902 (Lazim *et al.*2009). Since glucose (2%) was the most favorable carbon source for the enzyme production, it was necessary to study the effect of its concentration. Different concentrations of glucose ranged from 1 to 6 % were tested (Fig. 3). The best concentration of glucose was 3 g% with activity reached 1652.4 U/ml and specific activity reached 11016.0 U/mg protein.

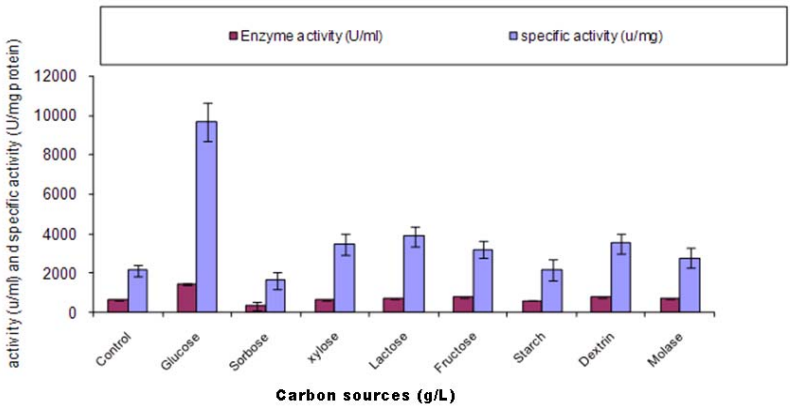

Fig 2: Effect of different carbon sources on activity and specific activity of protease produced from *Streptomyces griseus.*

Effect of Various Nitrogen Sources:

This was done by eliminating KNO_3 (0.2%) from the culture medium and using (on equivalent N-basis) sodium nitrate, ammonium sulphate, ammonium nitrate, ammonium hydrogen phosphate, casein, corn steep liquor, urea, Yeast extract and soy bean as nitrogen sources. The results (Table 2) showed that the Yeast extract, soy bean and ammonium sulphate increased protease activity to 2919.2, 2518.0 and 2006.3 U/ml, respectively versus 1167.3 and 323.8 U/ml for casein and corn steep liquor. On the other hand, sodium nitrate increased the enzyme specific activity to 11067.5 U/mg protein compared to the control, so sodium nitrate and potassium nitrate (control) are the best nitrogen sources according to the specific activity. Also, Johnvesly and Naik (2001) noticed that 1% (w/v) of sodium nitrate and potassium nitrate seems to be good nitrogen sources for protease production. These results in contrast with Mostafa *et al.* (2012) who found that the lowest protease yield was recorded using potassium nitrate in the production medium. Furthermore, Lazim *et al*. (2009) and Mostafa *et al*.

(2012) found that yeast extract and ammonium sulphate increased protease production. While, malt extract, peptone and casein reduced enzyme secretion. Moreover, the results obtained by Bajaj and Sharma (2011) indicated that mustared cake and soy bean meal induced significant levels of protease production but urea and gelatin were relatively ineffective as nitrogen sources.

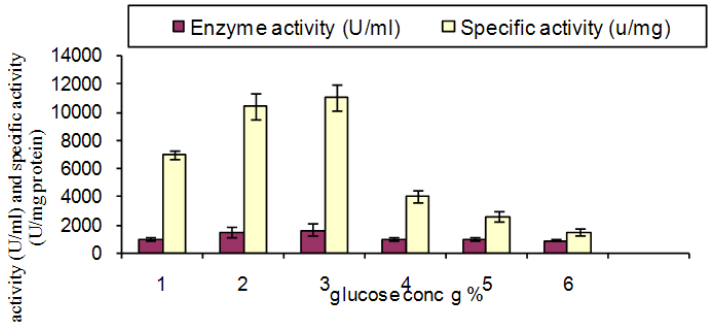

Fig. 3: Effect of different glucose concentrations on activity and specific activity of protease produced by *Streptomyces griseus.*

Table 2: Effect of different nitrogen sources on activity and specific activity of protease produced by *Streptomyces griseus*.

Nitrogen sources	Activity (U/ml)	Specific activity (U/mg)
Control	1648.0±2.2	11010.6±7.7
Sodium nitrate	1770.8±3.3	11067.5±10.2
Ammonium sulfate	2006.3±3.6	5279.7±11.3
Ammonium nitrate	1987.2±3.9	4516.4±11.8
Ammonium hydrogen phosphate	1692.8±1.1	7053.3±3.6
Casein	1167.3±1.6	3647.8±4.2
Corn steep liquor	323.8±1.1	431.7±3.5
Urea	1476.9±2.5	3886.6±7.9
Yeast extract	2919.2±2.8	4169.9±8.8
Soy bean	2518.0±2..0	2595.9±6.3

T test using Prism program version 2007, $P < 0.05$
Note: mean ± SE (stander error)

Furthermore, $NaNO_3$ was used at series of different concentrations (0.05-0.50 g %) in the production medium for examining its effect on the protease activity. It was observed that the optimum concentration was 0.30 g % for the activity but 0.40 g % gave the highest specific activity (13143.4 U/mg protein) (Table 3).

Table 3: Effect of different concentration of sodium nitrate on activity and specific activity of protease produced by *Streptomyces griseus.*

Nitrogen concentration (g %)	Enzyme activity (U/ml)	Specific activity (U/mg protein)
0.05	1678.6±3.3	8909.6±10.6
0.1	1722.2±5.2	10802.9±12.1
0.2	1765.9±4.3	11076.9±12..2
0.3	1984.3±6.6	12447.1±15.6
0.4	1809.6±5.0	13143.4±12.5
0.5	1717.5±4.2	12564.5±11.0

T test using Prism program version 2007, $P < 0.05$
Note: mean ± SE (stander error)

Effect of Different PH Values on Protease Production:

Production of protease was observed at various pH values ranging from 4 to 10 (Fig. 4). Protease activity and specific activity gradually increased by increasing the pH and reaching its optimum level at pH 8 (1879.2 U/ml and 13505.2 U/mg protein, respectively), thereafter enzyme production decreased reaching 1803.6 U/ml with specific activity 9552.6 U/mg protein at pH 10. Generally the enzymes posses many ionizable groups so that pH changes may alter the conformation of the enzyme (Chectham, 1995). Furthermore, Li *et al.* (2005) mentioned that alkaliphilis *S*. sp grows at an optimum pH 8-9 with scant growth at pH 7. Moreover, Lazim *et al.* (2009) found that protease activity gradually increased until reach the optimum level at pH 9 (182.5 Ug^{-1}) using *S*.spCN902.

Effect of Different Agro-Industrial by Products on Protease Production:

Among various cheap sources, agro- industrial by-products such as rice straw, sugar beet, and wheat bran and other cheaper components like sweet whey have focused considerable attention, as they support both cell mass and enzyme production (Kanekar *et al*., 2002). The crude sources, employed in the present study,

supported protease synthesis with a moderate value compared with the control which contain glucose and sodium nitrate as a carbon and nitrogen sources, respectively. Wheat bran when used as the sole carbon and nitrogen source (according to the carbon and nitrogen ratio), induced protease production compared to the other wastes (Fig. 5). These findings were in agreement with an alkaline protease from *Bacillus* JB 99 (Johnvesly and Naik, 2001), the ability of the organism in this study to grow and produce enzyme with amino acids as well as cheaper agro-industrial by-products assumes significance. The production of the enzyme with these sources would be economically attractive repositions.

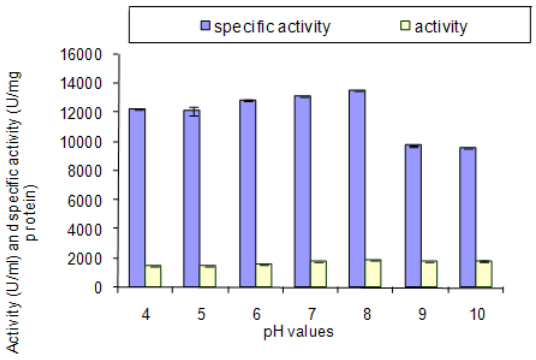

Fig. 4: Effect of different pH values on activity and specific activity of protease produced by *Streptomyces griseus*

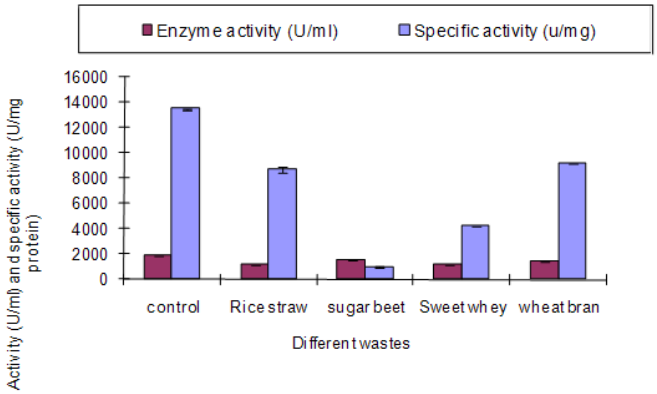

Fig. 5: Effect of different wastes on activity and specific activity of protease produced by *Streptomyces griseus.*

Effect of Different Doses of Gamma Radiation on Protease Production:

An experiment was conducted to study the effect of gamma irradiation on protease production. Results given in Table 4 using different doses of gamma radiation (0.0, 0.2, 0.4, 0.6, 0.8, 1.00, 1.2, 1.4, 1.6 and 1.8 kGy) illustrated that values of protease specific activity gradually increased, using 0.2 until 1.6 KGy and decreased thereafter at 1.8 KGy. The highest protease activity was at 1.4 kGy (2200.0 U/ml) and the specific activity was 14450.5 U/mg protein at 1.6 KGy. The lowest protease activity was 1892.1 U/ml and specific activity was 13338.3 U/mg protein with 0.2 KGy. Data also indicated that increasing doses of gamma radiation showed rapid decreasing in growth after 1.6 kGy dose level.

Table 4: Effect of gamma radiation on activity and specific activity of protease produced by *Streptomyces griseus*.

Different doses of gamma radiation (kGy)	Enzyme activity (U/ml)	Specific ctivity (U/mg)
control	1880.2±6.5	13515.9±12.0
0.2	1892.1±8.5	13338.3±11.5
0.4	1949.1±8.8	13410.3±12.0
0.6	1963.9±10.6	13456.6±14.6
0.8	2079.1±13.2	13489.6±18.8
1	2116.6±12.0	13603.5±15.2
1.2	2158.4±13.6	13810.1±18.7
1.4	2200.0±14.5	14155.6±20.1
1.6	2139.3±13.7	14450.5±20.0
1.8	1943.8±11.9	13885.2±16.1

T test using Prism program version 2007, $P < 0.05$
Note: mean ± SE (stander error)

An increase in protease activity was shown because radiation made the activation of histone-specific proteases depended on the duration of postradiation period. Also, Simultaneously, nuclear proteases tightly bound to histones and specifically cleaving histones were observed to be activated by apoptogenic factors of the mitochondrial intermembrane fraction. Probably, the apoptogenic action of gamma-radiation involves not only a direct DNA damage that induces activation of DNA-dependent proteases but also an indirect component destructive alterations in mitochondria leading to the exit of apoptogenic factors from the intermembrane space (Gaziev et al., 2002). Ten species of bacteria which survived low-dose gamma radiation were isolated from fresh Gulf oysters and identified. Irradiation at 0.2–0.3 (mico rad) reduced proteolytic activity of the bacteria considerably. Reduction of proteolysis was significantly correlated with a decrease in bacterial numbers produced by irradiation (Liuzzo *et al*., 2006).

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