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Production and Optimization of Gelatinase Producing Lentzea sp. Strain Isolated from the Soil Rhizosphere

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Abstract

Proteases are the major enzymes that have been used widely in health, food, and nutritional supplement fields. Rare actinobacteria are considered a new resource for proteolytic enzyme activity. Rare actinobacteria are used to be isolated from marine and have been studied under restricted limits. The strain used in this study was identified as Lentzea sp. which was successfully isolated from the soil at the Faculty of Science, Damietta, Egypt. It was subjected to gelatin liquefaction as primary screening for gelatinolytic activity. Based on this study, the best gelatinolytic (gelatinase) activity was noticed within 8 days at 37oC (17.59 U/ml ±0.06). The maximum activity was obtained at pH 9 (28.33 U/ml ±0.04) and agitation speed of 150 rpm (29.10 U/ml ±0.04). Upon carbon and nitrogen source optimization, the basal medium of combination between gelatin and peptone produced maximal gelatinase activity (29.12 U/ml ±0.06) and it was found that 10% sucrose was the best carbon source that produced maximum gelatinase activity at (30.17 U/ml ±0.05).

Keywords: Lentzea sp., actinomycetes, gelatinase activity, optimization.

Introduction

Enzymes are vulnerable to environmental condition, so it was optimized for their optimal activity conditions **(Konjerimam *et al*., 2020)**. Microbial proteases became one of the major popular industrial enzymes contributing 20% of global enzymes sales with a value of $2.767 million in the pharmaceutical industry field by 2019 **(Razzaq *et al*., 2019).** Extracellular enzymes are usually optimized to media components variation in the C/N ratio (**Gupta *et al*., 2002)**. Protease-producing microorganisms possess some good assets such as high production with high economic impact **(Bhatia *et al*., 2021).** Major proteases are produced from *Bacillus* and *Streptomyces*. However, proteases have been produced by *Bacillus* genus strains are the predominant enzymes used in several fields, alkaline proteases were being produced from other genera. It was reported by **Boughachiche *et al*., (2016)** that *Streptomyces* sp. isolated from the saltpan of Algeria was able to produce alkaline proteases. Enzymes from non-*Streptomyces* sources have not been explored in detail till

recent years and little is known from *Nocardiopsis* sp**. (Kim *et al*., 1993)**. Common gelatinases producing strains are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Serratia marcescens*. Rare actinobacteria, as new sources for gelatinolytic activity giving novel properties of the enzyme, would be a great baseline for different industrial applications. Proteolytic enzymes can be used in medical antitumor and metastasis **(Dudani *et al*., 2018)**, homeostasis, infections, immunity, and disease improvement **(Patel *et al*., 2018)**, waste management (**Naveed *et al*., 2021)**, food industry and supplements **(Dos Santos Aguilar and Sato., 2018)**, peptide synthesis **(Białkowska *et al*., 2017; Zanutto-Elgui *et al*., 2019)**, wool processing **(Su *et al*., 2020)**, pharmaceutical drugs field and basic biological science. Gelatinases proteolytic enzyme application at the high impact of biotechnology is associated closely with discovering novel producing strains that produce novel enzymes or enzymes with novel properties. Such nonpathogenic enzymatic source and production of extracellular enzyme give a positive impact for the enzyme to be used commercially safely "genetically regarded as safe "or Generally Recognized as Safe GRAS with food and drug administration **(Zhang *et al*., 2019).**

Gelatinases are matrix **metalloproteinases** (**MMP-2** and **MMP-9**) which can play a vital role in human life as they cleave extracellular matrix, contributing global role in embryonic development, morphogenesis, reproduction, and tissue remodeling and diseases like arthritis, cardiovascular, neurological diseases and cancer and metastasis by cleaving. MMPs are important in the medical field **Da Silva *et al*., (2018)** and pharmaceutical for drug development. Both bacterial and eukaryotic metalloproteases are involved in the curing of precursors that modulate tumor formation **(Makinen, and Makinen, 1994)**. MMPs have great value in tumor therapy by enhancing tumor inhibitor development **(Tamaki *et al*., 1995)**, and playing a major role in the degradation of connective tissue correlated with tumor metastasis, so it shares in drug improvement. Gelatinases A and Gelatinase B hydrolyze gelatin and also type IV and V collagens and elastin **(Senior *et al*., 1991)**.

Lentzea is a Gram-positive mesophilic and aerobic genus belonging to the family Pseudomonadaceae of suborder Pseudonocardia belonging to the order Actinomycetales. *Lentzea* genus was named by **Yassin *et al*., (1995)**. The name Lent'ze.a. N.L. fem. n. *Lentzea*, was called after Friedrich A. Lentze, a German microbiologist who devoted a considerable part of his life to studying pathogenic actinomycetes. Consequently, was presented by **Fang *et al*., (2017)**

This work aimed to characterize and optimize extracellular gelatinase production by rare actinobacteria (non-Streptomyces).

Materials and Methods

Isolation and identification of Actinomycetes

Lentzea strain was isolated from the rhizosphere soil at the Science faculty, Damietta. The soil sample was kept in plastic bags and was transferred to the lab in an icebox to maintain the temperature at 4ºC.The sample was undergone serial dilution up to 10^{-5} then 0.1 ml aliquots of each dilution were cultured in starch nitrate agar medium **(Hayakawa and Nonomura., 1987).** The Petri dishes were incubated at 30ºC for 7 days. The strain was purified by further streaking on sterilized starch nitrate agar plates. The isolate was kept on a slant culture in screw cab tubes at 4ºC for use, and also was preserved in 30% glycerol at -80ºC freezer for further research.

Gelatin liquefication

The strains were stabbed in gelatin media (20% v/v) deep tube and incubated at 30ºC, the presence of liquefaction even after refrigeration indicated hydrolysis of gelatin. The result was recorded for 21 days by placing them in the refrigerator at 4ºC till the solidification occur if gelatin was hydrolyzed the medium was still liquid after settling in the refrigerator, but if it was not hydrolyzed the medium re-solidify in the refrigerator **(Prihanto and Nursyam, 2018)**.

Gelatinase assay

The selective strain was taken for gelatinase quantitative assay according to a modification of **Tran and Nagano method (2002)**. The reaction mixture contains 2.05 ml substrate

gelatin (0.2%) in water and is adjusted at pH 7.8. The reaction was initiated by adding (0.2ml) of extracellular supernatant (crude enzyme), Then was incubated at 30°C for 1hr. The stopping of the reaction occurred by boiling for 10 min at constant temperature in a water bath, then the reaction mixture was completed to a volume of 4.2 ml using distilled water. The released free α -amino acid was measured by Ninhydrin method (**Rosen, 1957)** and enzyme activity was expressed in terms of L-leucine**.** One unit of gelatinase enzyme was expressed as the amount of enzyme that release 1µmol of L-leucine per min per ml of culture media under specified condition **(Hamza *et al*., 2006).**

Submerged fermentation for gelatinolytic production

The culture basal medium containing peptone 5gm/L, and gelatin 20% (w/v) was used. Gelatin was dissolved by good homogenizing using a glass rod for stirring during heating at 40°C to achieve homogenization of the medium mixture and pH was adjusted using 1M HCl and 1M NaOH. The medium was dispensed into falcon tubes, then autoclaved at 121°C for 15 min after cooling of media the culture was inoculated with the selected strain of constant growth and the uninoculated tube was served as a negative control for enzyme assay. The tubes were incubated in an incubator shaker and the temperature was adjusted according to the parameter tested. Each parameter was optimized during the experiment and was fixed during the subsequent steps of achieving the others.

Physicochemical optimization for maximal gelatinolytic production

The incubation period was carried out during 10 days with 2 days intervals and temperature effects on the enzyme activity have been studied at 25°C, 30°C, 35°C, 37°C, 40°C, 45°C, and 50°C during this period. The effect of pH on the gelatinolytic activity was established as initial pH at a range of (6 – 13) with 1 interval, adjusting the pH of the medium have been done using 1M HCl and 1M NaOH, and pH after incubation also was measured. The effect of agitation occurred by incubation of the media after inoculation at 37°C at an incubator shaker at 50, 100, 150,200, and 250 rpm.

The different nitrogen sources (gelatin, KNO_3, proline, hydroxyproline, threonine, methionine, valine, serine, phenylalanine, cysteine, peptone, and a mix of gelatin & peptone) have been studied at the same molecular weight of nitrogen atoms equivalent to the nitrogen content of 5 g/l peptone.

The effect of various carbon sources (maltose, arabinose, glucose, galactose, raffinose, fructose, sucrose, carboxymethylcellulose (CMC), starch, mannitol, and lactose) has been studied at the same molecular weight of carbon atoms equivalent to the carbon content of 1 g/l glucose, then studying of sucrose concentration effect on the enzyme activity was carried out at a range of 1% - 10% with 1 interval and then from 10% - 60% with 10 intervals.

Statistical analysis

The analysis of statistics was carried out by expressing the standard deviation for each experimental triplicate result. In this study, Results were shown as the mean value of three independent determinations. Bars correspond to standard deviation. Data were analyzed to determine significance using one-way ANOVA for all experiments and two ways ANOVA for incubation period and temperature effect on enzyme activity with a significance of 0.05 (ANOVA software version 25).

Results and Discussion

The isolated strain used in this study was identified as *Lentzea* sp. This strain is belonging to *Lentzea* genus according to (**Yassin *et al*., 1995),** and it was found to have the ability of gelatin liquefication.

Effect of incubation period and temperature on gelatinase activity:

The gelatinase enzyme of *Lentzea sp*. showed maximal enzyme activity on the 8th day (Figure 1). This was nearly similar to the result performed by **Ramesh *et al*., (2009)** on *Streptomyces fungicidicus* which possesses maximal proteolytic activity on the 6th day. On the other hand, the enzyme production was maximum after 4 days by *Saccharomonospora viridis* SJ-21 **(Younis *et al*., 2009).** The study by **El-Hadedy *et al*., (2014)** revealed that the maximal alkaline protease activity by *Streptomyces griseoflavus* was at the first three days of growth, while **Hosseini *et al*., (2016)** showed maximal proteolytic activity after 4

days.

Results revealed that 37ºC was the optimal temperature for gelatinolytic enzyme activity, it was recorded at 17.6 U/ml of enzyme activity Figure 1. The activity was increasing gradually during the 2nd, and 4th 6thdays till the 8th day was the maximal activity at 37ºC, then a slight decrease was observed. This agreed with the findings in the study of *Streptomyces pseudogrisiolus* NRC-15 by **Mostafa *et al*., (2012); Ash *et al*., (2018)** also reported that the optimum temperature of *Bacillus laterosporus* protease activity was the best at the temperature of 37ºC. Related studies also reported that the optimum temperature of protease activity by *Bacillus subtilis* possessed maximum activity when incubated at 37ºC (**Ashwini *et al*., 2014)**.

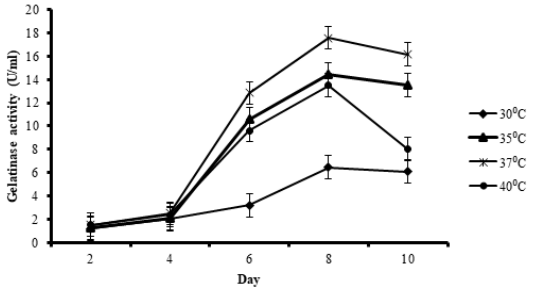

Fig.1. Effect of incubation period and temperature on gelatinase activity by *Lentzea* sp. Data are expressed as mean (n = 3) ±SD.

Effect of pH on gelatinase activity

The effect of initial pH of the culturing medium on the proteolytic gelatinase hydrolysis was studied at the optimum temperature 37ºC and different pH values. In Figure 2 the results showed that maximal activity was at optimum pH 9. This was agreed with **Maruthiah *et al*., (2013)** who recorded the highest activity was in the range pH (8-9). Furthermore, it was reported that the activity of different proteases from *Bacillus pumilus*, *Fervidobacterium islandicum*, *Alcaligenes faecalis*, and *Vibrio fluvialis* was maximal at pH 9 as studied by **Wang *et al*., (2007)**. **Haddar *et al*., (2009)** showed that the highest activity was at pH 8.5 for *Bacillus mojavensis* A21. The pH parameter was a critical physico-chemical factor affecting enzyme productivity. **Naveed *et al*., (2021)** reported that the pH range of (8-12) was the optimum value for alkaline proteases.

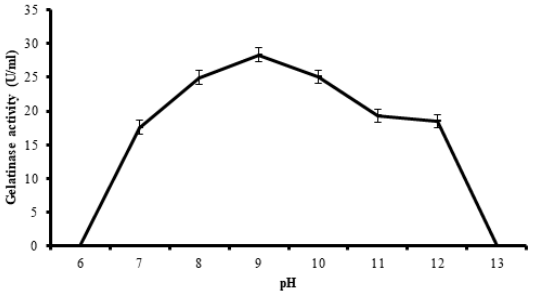

Fig.2. Effect of pH on gelatinase activity by *Lentzea* sp. Data are expressed as mean (n = 3) ±SD.

Effect of agitation:

Agitation rate is considered an important factor affecting protease enzyme productivity from different strains. At low agitation speeds our selected strain produced low gelatinolytic enzyme activity due to poor nutrient uptake and poor medium aeration which may lead to weak strain growth. The agitation speed at 150 rpm was the best for maximal gelatinolytic enzyme activity because this speed caused sufficient nutrient uptake **(Beg *et al*., 2003)** and good media aeration which makes good oxygen dissolving **(Kumar and Takagi., 1999)**. At high agitation speed, up to 200 rpm leads enzyme activity decreases gradually because high speed can cause strain cell rupture raising cell permeability due to corrosion of cell surface resulting from collision **(Darah and Ibrahim, 1996)**. A high agitation rate might lead also to protein and enzyme denaturation **(Burkert *et al*., 2005)**. Our result coincided with **Norazizah *et al*., (2005)** as the best activity of protease was at 150 rpm Figure 3.

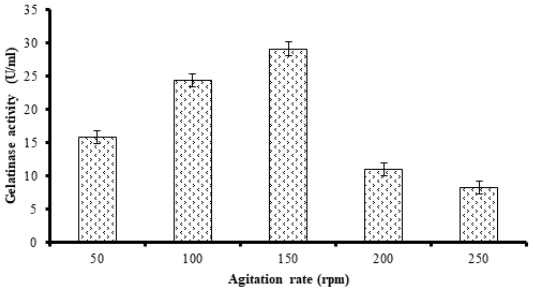

Fig.3. Effect of agitation (rpm) on gelatinase enzyme activity by *Lentzea* sp. Data are expressed as mean (n = 3) ±SD.

Effect of nitrogen source on gelatinase activity

The study demonstrated that varying nitrogen sources influence the enzyme activity rate. Results showed that some nitrogen sources stop the gelatinase activity completely (KNO_3,

hydroxyproline, threonine, methionine, serine, and cysteine), while others (proline, valine, phenylalanine, peptone, and gelatin) increase the activity. It was noticed that gelatin itself as a sole nitrogen source enhanced the hydrolytic activity of the enzyme (17.68 U/ml) followed by peptone (17.4 U/ml) then phenylalanine (16.7 U/ml) as indicated in Figure 4. The combination of the best two nitrogen sources (gelatin and peptone) enhanced the gelatinase activity (29.1 U/ml) which could be a recommended formula for the highest activity.

Balan *et al*. (2012) stated that the best nitrogen source for maximum gelatinase activity was gelatin. On the other hand, it was reported that peptone was the best nitrogen source for proteases production from *Bacillus* sp. (**Gaur *et al*., 2014)**. Our results indicated that KNO_3 possessed no gelatinase activity effect nearly as recorded by **Mostafa *et al*., (2012)** who noticed that KNO_3 was associated with the lowest enzyme activity yield.

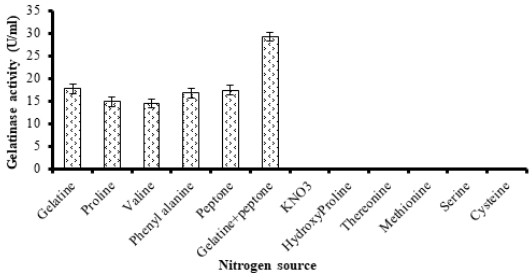

Fig.4. Effect of nitrogen sources on gelatinase enzyme activity by *Lentzea* sp. Data are expressed as mean (n = 3) ±SD.

Effect carbon source on gelatinase activity

The study investigated the effect of additional carbon sources on gelatinolytic activity. The results revealed an obvious decrease with all used carbon sources (Figure 5) and a slight increase when nearly 0.75% sucrose was added (29.87 U/ml) as shown in Figure 5. Contrary, **Mostafa *et al*., (2012)** showed that glucose was the best carbon source for stimulating protease activity.

In this study, the organic carbon source carboxymethylcellulose CMC and starch suppressed the gelatinase activity. This disagreed with what was revealed by **Umayaparvathi *et al*., (2013)** that starch was the best carbon source for protease enzyme activity from *Bacillus* sp., *Bacillus pumilus*, *Bacillus* sp. RGR-14 and *Bacillus cereus* SU12, respectively.

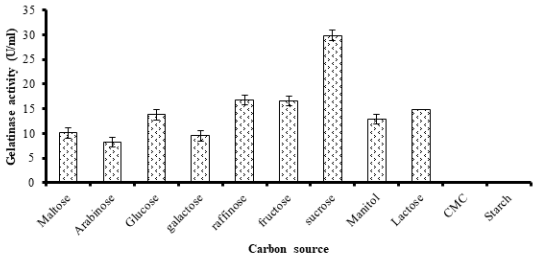

Fig.5. Effect of additional carbon sources on gelatinase activity by *Lentzea* sp. Data are expressed as mean (n = 3) ±SD.

Effect of sucrose concentration:

The best carbon source (sucrose) showed maximal gelatinolytic activity at concentration of 10% (30.17) U/ml as shown in Figure 6, which was coincided with the results revealed that sucrose have moderate protease activity **(El Hadedy *et al*., 2014).** On the other hand, it was proved that sucrose was the best carbon source for the alkaline protease production by *Streptomyces avermitilis.* **(Kumar, and Takagi, 1999)**. While, disagreed with results showed that glucose was the best carbon source with good impact for further industrial study (**Mostafa *et al*., 2012; El-Shafei *et al*., 2010).** It was reported by **El-Shafei *et al*., (2010)** that 1.25% was the finally concentration for the best carbon source protease activity from *S. Albidoflavus* due to glucose accumulation produced from lactose hydrolysis leading to enzyme activity catabolic repression **(Mehta *et al*., 2006),** so it can be deduced why 10% was the finally concentration for the sucrose to enhance the enzyme activity due to sucrose hydrolysis to its constituents glucose and fructose leading to the enzyme being catabolically repressed by glucose **(Mehta *et al*., 2006).**

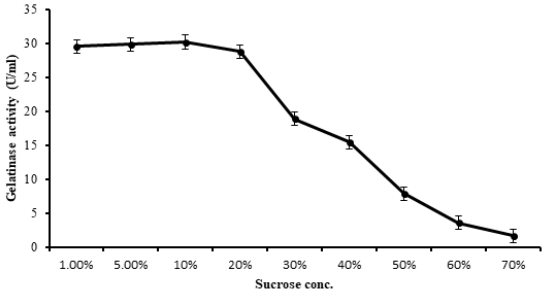

Fig.6. Effect of sucrose concentration on gelatinase activity by *Lentzea* sp. Data are expressed as mean (n = 3) ±SD.

Conclusion

In this study, gelatinase enzyme producer *Lentzea* sp. was isolated from soil (rhizosphere of palm tree) at the Faculty of Science, Damietta, Egypt. Lentzea sp. strain as rare actinobacteria is considered one of the new species producing gelatinase. The research explored the potentiality of the selected strain for the production of extracellular gelatinase enzyme by submerged fermentation of basal medium with a combination of gelatin and peptone as nitrogen sources. The maximal gelatinase activity was recorded within 8 days at 37°C and pH 9 with agitation at 150 rpm. Further study should be performed to establish its usage either in the industrial or medical fields including its purification and characterization.

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الملخص العربي

عنوان البحث: إنتاج وتحسين انزيم الجيلاتينيز بواسطة السلالة المعزولة Lentzea sp. من التربه الملاصقه للنبات

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انزيمات البروتييز تعد من الإنزيمات الرئيسية التي تم استخدامها على نطاق واسع في مجالات الصحة والغذاء والمكملات الغذائية. تعتبر بكتيريا الأكتينوبكتيريا النادرة موردا جديدا لنشاط الإنزيم المحلل للبروتين. تعزل الأكتينوبكتيريا النادرة عادة من مياه البحار وقد تمت دراستها على نطاق محدود. فى هذه الدراسة تم تحديد السلالة المستخدمة باسم Lentzea sp. والتي تم عزلها من التربة في كلية العلوم، دمياط، مصر. تم استهداف قدرة هذه العزله على إسالة الجيلاتين كفرز أولي لنشاط تحلل الجيلاتين. وقد دلت النتائج على أن الظروف المثلى لانتاج اقصى نشاط لانزيم الجيلاتينيز من العزله كان في غضون ٨ أيام عند ٣٧ م° بنشاط (١٧,٥٩ وحدة / مل ± ٠,٠٦)، ورقم هيدروجينى ٩ بنشاط (٢٨,٣٣ وحدة / مل ±٠,٠٤) وعند اهتزاز بعدد لفات ١٥٠ لفة / الدقيقة بنشاط (٢٩,١٠ وحدة / مل ±٠,٠٤). وعند استخدام المصادر الكربونية والنيتروجينية المختلفة أظهرت النتائج أن أقصى نشاط للانزيم كان بواسطة مكونات الوسط الغذائى الاساسي الذى استخدم لزرع العزله لانتاج الانزيم وذلك بالدمج بين مصدرى النتيروجين الجيلاتين والببتون فاعطى نشاط (٢٩,١٢ وحدة / مل ±٠,٠٦) ووجد أنه عند استخدام المصدر الكربونى السكروز بتركيز ١٠% كانت قيمة النشاط الانزيمى عند (٣٠,١٧ وحدة / مل ±٠,٠٥).