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Penicillium citrinum: A Promising Candidate for Protease Production

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ABSTRACT

Proteases are one of the most important classes of industrial enzymes accounting for 60% of the global industrial enzyme market. Microbes are the most preferred sources of proteases because of their rapid growth, easy genetic manipulation and limited cultivation space. The aim of this study was to isolate and characterize potent fungal candidates for protease production. Protease-producing fungi were isolated from abattoir soil using standard mycological methods while screening for protease production was determined by the presence of halozones on skimmed milk agar medium. Enzyme activity using best isolate was evaluated on time course and at various pH regimes (5.0-8.0) and temperatures (25-45^{o}C). Best isolate had halozone diameter of 69 mm and identified as *Penicillium citrinum* by sequencing its 18S rRNA. Optimum protease activity was recordedon the 4th day for both immobilized and free spores. When pH was optimized, pH 6 (19.25± 0.32 U/mL) and pH 6.5 (14.21 ± 0.65 U/mL) were best for immobilized and free spores respectively whereas 35^{o}C was the best temperature condition for optimum protease activity for both immobilized (22.44 ±0.71 U/mL) and free spores (17.16 ± 0.51 U/mL). We therefore conclude that the abattoir soil environment is a potential reservoir of protease-production fungal determinants while spore immobilization resulted in better protease production.

***Keywords*:**Protease, Fungi, Immobilization, Enzyme Activity, Halozones, *Penicillium*

Introduction

Proteases are enzymes that show the ability to cleave peptide bonds of proteins in a specific way. They consist of one of the three largest cluster of enzymes used in industrial processes, constituting over 65% of the worldwide enzyme market, and stand amongst the most valuable commercial biocatalysts [1,2]. According to the Enzyme Commission (EC), proteases belong to group 3 of the hydrolases and subgroup 4, hydrolysis of peptide bonds, however can still be classified according to the source of isolation (animal, vegetable or microbial), catalytic action (endo or exopeptidase), active site, charge, molecular size and substrate specificity [3]. They are in high demand due to their applications in several sectors of industry and biotechnology such as waste, food, pharmaceutical, leather, detergent and textile industries etc[4,5,6]. Microbial proteases are among the most important hydrolytic enzymes. Proteases from microorganisms have attracted a great deal of attention in the last decade because of their biotechnological potential in various industrial processes such as detergent, textile, leather, diary and pharmaceutical preparations [7].

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Soil is said to be a home for a large proportion of world's diversity. There is an incredible complex between soil organisms and soil function, this soil function takes into consideration the interaction within the soil community. Presence of suitable substrates acts as a converging factor for specific group of organisms in the soil habitat. The abattoir soil environment is predominantly characterized by animal based wastes that are rich in proteins [6]. Microbial diversity in such protein-rich soil environment may be of great importance in sourcing for protein-hydrolyzing microorganisms [6]. Several protein-hydrolyzing fungi which include *Aspergillus flavus*[8], *Aspergillus niger*[9], *Aspergillus fumigatus*, *Penicillium italicum* and many others have been successfully isolated from the soil environment [10,11,12,13].

Fungal enzymes are most widely used enzymes in the industry due to a variety of technological factors, including the possibility of getting enzymes in the fermentation medium at high amounts while providing a clear benefit over bacterial enzymes in respect to simplifying the downstream processing [14]. Specifically, fungal proteases in recent years have garnered the concerns of most scientists because they can grow on cheap substrates and can also elaborate copious amounts of enzymes into the culture medium, and are equally more readily and easier to detach their mycelium by filtration over bacterial proteases.

Nevertheless, some of the outstanding challenges encountered in fungal protease production are reduced yield and difficulties in separating microbial cells from the product (enzyme) during downstream processing.

Owing to this, metabolites production using microbial cell confinements in the medium through immobilization has been encouraged [14]. Cell immobilization offers several benefits when compared with suspended cells. For instance, increased cell to cell communication, increased rate of substrate uptake, high rate of metabolite excretion and cell continuous reuse after recovery are some of the benefits of cell immobilization [14, 15]. Immobilization technique

has proven to enhance enzyme production than the free mobile spores. For example, protease activity of 119.67 U/mL was recorded with immobilized spores of *Bacillus licheniformis* as against 78.3 U/mL with free spores using corn cob as carrier [16]. Similarly, the use of sodium alginate beads as immobilization matrix, gave rise to higher protease production in the entrapped cells than the free cells of *Pseudomonas aeruginosa* [17]. Since it has been reported that the abattoir soil environment is profusely characterized by animal-derived wastes that are rich in proteins [6], the aim of this study was therefore to isolate potent strains of protease-producing fungi from soil and evaluate the efficiency of the same in enzyme production using immobilized calcium-alginate beads.

Materials and Methods

Collection of samples

Soil samples were collected from two different locations in abattoir soil at Nsukka metropolis, Nigeria at a depth of 10-15 cm with a sterile hand trowel and put in sealed plastic bags in June, 2022. The soil samples were immediately taken to the laboratory for further analysis

Isolation and Purification of Fungi

A 10g quantity of the experimental soil was dissolved in 90 mL of sterile distilled water and further diluted serially up to 10^{-9}. Thereafter, the spread plate method was adopted by pipetting 0.1 mL aliquot of the sample suspension and inoculating onto freshly prepared potato dextrose agar (PDA) plates aseptically [16]. Inoculated plates were subsequently incubated at 30^{o}C for four days. Pure fungal isolates were obtained by repeated subculturing on the same medium [18].

Screening for Protease Production

Protease production screening was performed on skim milk solid medium. The composition of skim milk agar medium (g/L) is as follows: skim milk powder, 28; casein enzymatic hydrolysate, 5; yeast extract, 2.5; dextrose,1; agar, 15 and pH was adjusted to 7.2. The isolates were individually spot inoculated onto fresh skim milk agar medium and incubated at room temperature for 6-8 days. Zones of hydrolysis that emerged around fungal colonies after incubation were measured to the nearest millimeter using a metre rule.

Identification of fungal isolates

Preliminary identification of the isolates was performed macroscopically. Fungal morphology, colour and texture, elevation, margin and optical characteristics were determined and their probable identities determined with soil fungi manual [19]. The best isolate in terms of optimum enzyme production was picked and identified on molecular basis by sequencing its 18S rRNA. Applied Bio Systems' Genetic Analyzer 3130xl sequencer was used to sequence the amplified fragments, and the sequencing kit utilized was the Big Dye terminator v3.1 cycle sequencing kit. The phylogenetic analysis was conducted using MEGA 6 and Bio-Edit software. Amplification was carried out using the polymerase chain reaction.

Alginate beads immobilization

Separate solutions of calcium chloride (2%) and sodium alginate (2%) were produced and autoclaved for 15 minutes at 121 degrees Celsius. Later, 5 mL quantity of the standard spore suspension (1.0×10^6 spores/mL) of best isolate (S4) were introduced to the cool solution of sodium alginate and gently shaken to mix [20,21,15]. Using a 5 mL automated pipette, the mixture was then poured dropwise into the calcium chloride solution while the beaker was gently swirled at 70 rpm with a magnetic stirrer (Magnetic Stirrer OP-912/3, Radelkis, Hungary). Alginate gel beads were created when the alginate droplets came into contact with the calcium chloride solution. Each bead contained an average spore concentration of 3.8×10^5 spores/bead and bead diameter was 3mm. The resulting beads were incubated in calcium chloride solution for twenty-four (24) hours to enhance bead cross linking and stability. Two hundred beads were inoculated to the sterile 150 mL protease-specific fermentation broth

Protease Activity Assay

Determination of the protease activity was done by a modified procedure of Potumarthi*et al.*[22] making use of casein as the substrate. A 0.5 ml aliquot of enzyme solution was introduced into 4.5 mL of the substrate solution (1%v/v, casein with 50 mMTris-HCL buffer, pH 8) and incubated for 30 min at 30 °C. The reaction was stopped by introducing 5 mL of 5% TCA (Trichloro acetic acid) mixture (5%TCA, 9%Na-acatate, 9% acetic acid) followed by 30 min holding at room temperature and centrifugation at 8000 rpm for 20 min. The precipitates were eliminated by filtration through Whatman No. 1 filter paper, and the filtrate's absorbance was determined at 660 nm. The amount of enzyme liberating 1 µg of tyrosine per minute under assay conditons defines the protease activity. Tyrosine solutions (0–50 mg) were used as the reference standard for measuring enzyme units. [10,16].

Effects of Different Environmental Parameters on Protease Activity

Two hundred calcium alginate beads containing 3.8×10^5immobilised spores/bead and bead diameter of 3mm were inoculated in sterile 150 mL protease-specific fermentation broth. While the control (containing 5.0×10^5 spores/mL of best fungal isolate free spores) was also inoculated in sterile 150 mL of protease-specific fermentation broth. The effects of time cause, temperature, and initial pH were investigated for protease activity. Time course was studied using 5.0×10^5 spores/mL under room temperature and initial pH of 6.5 for free spores (control) while 200 beads containing about 3.8×10^5immobilised spores/bead were used for the optimization processes. The incubation temperatures were varied between 25°C and 45°C, while the initial pH was also varied between 5.0 and 7.5 using 1M NaOH or HCl. For both immobilized and free spores (control) experiments, cultivation time was 7 days.

Statistical Analysis

All data was reported as mean ± standard deviation of triplicate experiments. SPSS was used for the analysis

Results and Discussion

Colonial Characteristics of the Fungal Isolates

The colonial morphology of the fungal isolates is presented in Table 1. The isolates varied in morphological forms, most of them were filamentous and round while others were round with raised margin, concentric and wrinkled. Observations on margin formation indicated that 40% of the isolates were irregular while 33.3% had entire margins including 8 isolates representing 26.7 % that were wooly (Table 1).

Protease production

Zones of hydrolysis that emerged around fungal colonies upon qualitative screening for protease production is presented in Table 2. The diameters ranged from 0 mm to 69 mm. Observation showed that 50% (15 isolates) had diameters above 40 mm, 9 isolates (30 %) recorded zone diameter less than 40 mm, whereas 6 isolates (20%) had 0 mm zone diameter. For clearer appreciation of the result, a pictorial presentation of the control experiment (plate showing no zone of hydrolysis (Figure 1) and plate showing the widest halo zone (69 mm) is presented in Figure 2.

In the present study, qualitative screening for protease production revealed protease-production potentials at varying degrees except isolates with white colored colonies that had negative results. In other words, they may not have the capability to utilize skim milk as a source of carbon owing to the absence of visible zone of hydrolysis on skim milk (Figure 1). Isolate S4 was dark green in colour and was designated the best candidate when different filamentous fungi isolated from abattoir soil were screened for protease-production potentials. It was observed that all green and dark-green coloured colonies isolated had excellent potentials for protease production, including our best performed isolate (Isolate S4) with dark green-coloured colonies. At this level, S4 is arguably a strong protease producer and evidently so (Table 2 and Figure 2). This shows that the abattoir soil environment is a reservoir of fungi with diverse morphological characteristics though they may possess similar physiological characteristics. Similar observation had been widely

documented [23,2,12]. Consequently, S4 was therefore employed in the protease activity assay.

Protease activity

Industrial organisms perform optimally at various environmental conditions. Therefore, it is a common biotechnological practice to optimize environmental parameters such as pH, temperature and time to achieve maximum result. Owing to the low yield and difficulties in downstream processes especially in separating enzymes from cells, microbial immobilization was necessary. In this study therefore, the effects of cell immobilization was investigated. Other parameters studied were effects of incubation time course, initial pH and incubation temperatures on protease activity by S4.

Effects of time course

The time course for protease activity in both free and immobilized fungal spores within a seven-day period is presented in Table 3. Protease activity rose from 3.70 ± 0.66 (first day) to a peak on the 4th day (15.94 ± 0.69 U/mL) and then declined to 6.86 ± 0.93 U/mL on the last day (7th day) in the immobilized spores. However, suspended spores had a similar trend by recording 2.40 ± 0.98 U/mL on the day one and optimum protease activity on the same 4th day (11.38 ± 0.91 U/mL) before declining subsequently to 6.29 ± 0.99 U/mL on the last day (Table 3). It was observed that protease activity on time course increased appreciably from the first day of the incubation period and peaked on the fourth day after which there was a gradual decline till the seventh day of the experimental period. This trend was notably consistent in both the immobilized spores and free spores giving indication that the peak cultivation time for either of the two systems was on the fourth day. Comparative studies recorded different incubation time optima for protease activity. Maagsoodi[18] reported a similar trend in which protease activity rose from 91.67U/mL to 119.67 U/mL within a 20-50 (48 hr optimum) time regime using immobilized spores of *Bacillus licheniformis*. Javed *et al*[24] reported 48 hours for maximum protease production (23.57±1.19 PU/mL) by *Bacillus subtilis* M-9 strain. However, engineered strains were found to take shorter time for maximum protease activity. Twenty-four hours was the time optima for a mutant strain of the same *Bacillus subtilis* to produce the highest concentration of protease (81.21 ± 1.85PU/mL) [24]. Notably, in the present work, higher protease activity was achieved with the immobilized spores (Table 3). This phenomenon may be attributed to the fact that immobilization improves the metabolic activities of microbial cells[14]. In addition, entrapped cells witness increased substrate uptake and higher product formation. Perhaps the persistent characteristics of higher protease enzyme activities recorded using immobilized microorganisms as against suspended microorganisms in this study may be due to reduction in cell to cell distance and higher communications among cells [14,15,21]. Arguably, the gradual decline after the fourth day may be due to enzyme inhibition. Accumulation of protease enzyme in the culture medium might have engendered negative feedback mechanism (enzyme inhibition), weakening the protease-producing fungal spores. In this study therefore, four (4) days is the optimum length of time recorded for protease activity by S4 (Table 3).

Effects of pH

The pH is another important kinetic parameter for enzyme production and activity as microbial activities are affected by variations in pH, hence the introduction of buffers in culture media. pH changes observed during microbial growth also affects stability of products in the growth medium.

Protease activity in the immobilized and free spores at different pH regimes is presented in Table 4. Between pH 5.0 and 7.5, protease activity rose from 13.75 ± 0.95 U/mL at pH of 5.0 to an optima at pH of 6.0 (19.25 ± 0.32 U/mL) before finally dropping to 9.12 ± 0.81 U/mL at the pH of 7.5 in the immobilized spores. At pH 5, protease activity of 8.88 ± 1.12 U/mL was recorded in the suspended spores and this value increased as the pH was increased to record 14.21 ± 0.65 U/mL at pH 6.5 before declining to 9.65 ± 1.08 U/mL when the pH was further increased to pH 7. The optimum pH recorded for enzyme activity was pH 6.0 (fairly acidic). While a comparable study reported 6.3-6.5 for *Bacillus Sp*. MIG [25], others reported neutral initial pH in separate studies involving different organisms and culture conditions [26,27,28].

Table 1: Cultural and Morphological Characteristics of isolated Fungi

Sample No	Form	Elevation	Margin	Surface texture	Color
S1	Filamentous	Raised	Wooly	Fluffy	Brown
S2	Concenric	Umbonate	Entire	Powdery	Green
S3	Filamentous	Raised	Wooly	Fluffy	Brown
S4	Round with raised margin	Flat	Entire	Powdery	Dark green
S5	Filamentous	Flat	Wooly	Fluffy	Green
S6	Filamentous	Umbonate	Irregular	Smooth	Creamy
S7	Filamentous	Raised	Wooly	Fluffy	Creamy
S8	Wrinkled	Raised	Irregular	rough	Black
S9	Round with raised margin	Flat	Entire	Smooth	Brown
S10	Concentric	Raised	Irregular	Smooth	Creamy
S11	Filamentous	Flat	Wooly	Fluffy	Brown
S12	Wrinkled	Umbonate	Irregular	Rough	Creamy
S13	Round with raised margin	Raised	Irregular	Rough	White
S14	Concentric	Raised	Wooly	Fluffy	Brown
S15	Round	Umbonate	Entire	Smooth	Creamy
S16	Wrinkled	Flat	Entire	Rough	Brown
S17	Round	Umbonate	Entire	Rough	Creamy
S18	Round	Raised	Irregular	Smooth	Creamy
S19	Concentric	Umbonate	Irregular	Rough	Brown
S20	Round with raised margin	Raised	Wooly	Fluffy	Creamy

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S21	Filamentous	Umbonate	Irregular	Smooth	White
S22	Round	Umbonate	Entire	Fluffy	Brown
S23	Round	Flat	Wooly	Fluffy	White
S24	Filamentous	Flat	Irregular	Smooth	White
S25	Round with raised margin	Flat	Entire	Powdery	Dark green
S26	Filamentous	Flat	Irregular	Fluffy	White
S27	Round	Umbonate	Entire	Smooth	White
S28	Round	Flat	Irregular	Fluffy	Brown
S29	Round	Raised	Irregular	Smooth	Creamy
S30	Round with raised margin	Flat	Entire	Smooth	Brown

Table 2: Diameter of Zones of hydrolysis around isolates

Sample No	Mean values (mm)
S1	45 ± 3.2
S2	66 ± 3.0
S3	63 ± 4.5
S4	69 ± 4.1
S5	61 ± 3.9
S6	24 ± 4.2
S7	32 ± 3.7
S8	41 ± 3.5
S9	55 ± 4.2
S10	33 ± 3.8
S11	48 ± 4.3
S12	29 ± 3.3
S13	00 ± 00
S14	53 ± 2.8
S15	31 ± 3.9
S16	59 ± 3.2
S17	29 ± 3.3
S18	20 ± 4.6
S19	49 ± 4.1
S20	19 ± 3.8
S21	00 ± 00
S22	53 ± 4.7
S23	00 ± 00
S24	00 ± 00
S25	62 ± 3.5
S26	00 ± 00
S27	00 ± 00
S28	48 ± 2.9
S29	19 ± 4.2
S30	50 ± 3.5

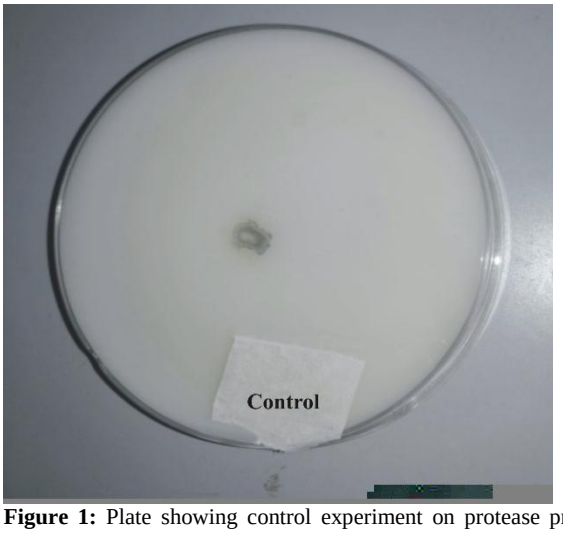

Figure 1: Plate showing control experiment on protease production screening

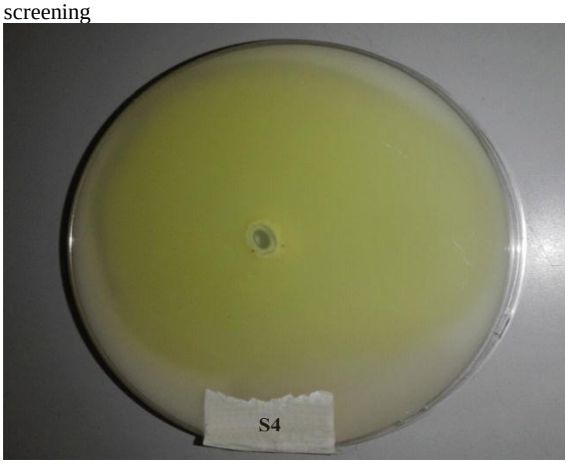

Figure 2: Plate showing zone of hydrolysis around *Penicillium citrinum*

Day	Protease activity using Immobilized spores (U/mL)	Proteaseactivity using free spores (U/mL)
1	3.70 ± 0.66	2.40 ± 0.98
2	5.10 ± 0.32	3.98 ± 0.69
3	11.80 ± 0.77	7.27 ± 0.49
4	15.94 ± 0.69	11.38 ± 0.91
5	14.55 ± 0.91	10.59 ± 1.39
6	10.90 ± 1.12	8.12 ± 1.03
7	6.86 ± 0.93	6.29 ± 0.99

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Table 4: Protease activity using entrapped and free fungal spores at different pH

pH	Protease activity using Immobilized spores (U/mL)	Protease activity using free spores (U/mL)
5.0	13.75 ± 0.95	8.88 ± 1.12
5.5	16.30 ± 0.59	11.50 ± 0.54
6.0	19.25 ± 0.32	12.50 ± 0.92
6.5	16.10 ± 0.84	14.21 ± 0.65
7.0	10.32 ± 1.21	13.92 ± 0.73
7.5	9.12 ± 0.81	9.65 ± 1.08

Table 5: Protease activity using entrapped and free fungal spores at different temperature

Temperature (^oC)	Protease activity using Immobilized spores (U/mL)	Protease activity using free spores (U/mL)
25	13.99 ±0.85	9.54 ± 1.09
30	18.31 ±0.39	15.12 ± 0.43
35	22.44 ±0.71	17.16 ± 0.51
40	13.01 ±1.03	9.31 ± 0.75
45	5.43 ±0.95	3.64 ± 1.77

Enzyme activities have been found to vary with pH among different fungi under different conditions. Maximum protease activity of 78.3 U/mL was reported at pH 7.6 and decreased at pH>9 for immobilized *Bacillus licheniformis* spores [14]. However, Agrawal et al [29] reported the highest protease activity of 8907±407 U/g at pH 9 with *Penicillium* Sp. Arguably, it might be laborious to find exactly the same pH requirement for even closely-related organisms in literature. This is because pH is affected by the type of organism and the source of isolation [12]. Other culture conditions also interplay with pH. However, there could be a significant difference between the effect of pH on protease production and protease activity [30].

Effects of temperature

It has been reported that incubation temperature plays a critical role in several microbial processes [12]. Even minor deviations from optimum temperatures can affect microbial processes. In this study, incubation temperatures were varied from 25-45^{o}C.

The protease activity of the spores at different temperature regimes are presented in Table 5. Between 25 and 45^{o}C, protease activity rose from 13.99 ±0.85 U/mL to a peak (22.44 ±0.71 U/mL) at the temperature of 35^{o}C before declining finally to 5.43 ±0.95 U/mL at 45^{o}C. Similarly, protease activity of 9.54 ± 1.09 U/mL recorded at 25^{o}C was increased to a peak (17.16 ± 0.51 U/mL) when the temperature was increased to 35^{o}C before dropping to 3.64 ± 1.77 U/mL on further increase in temperature to 45^{o}C while using suspended spores. However, the same trend was recorded when suspended spores were used for protease activity.

Optimum temperature of 35^{o}C was recorded for protease activity (Table 5). However, Chandrasekaran[12] in a similar study recorded a slightly lower incubation temperature of 30^{o}C with a Paddy Indian soil. Beyond 35^{o}C, protease activity consistently declined in this study. High temperatures are found to denature proteins, leaving dysfunctional polypeptides. Arguably, denaturation of the protease elaborated by S4 in the current study could have led to the downward trend observed in protease activity. Ganesh-Kumar *et al*[30] also recorded optimum temperature of 35^{o}C with a mesophilic fungus. In a similar vein, *Morimura et* al[31] reported consistent decline in protease activity at higher incubation temperatures.

Molecular analysis

PCR amplification of theinternal transcribed spacer (ITS) region of the isolates revealed that it was positive, with an amplicon size of 0.6kb (Figure 3). The conserved ITS region is peculiar to fungal isolates, hence isolate 4 and 9 are fungi isolates.

Molecular identification of sample isolates 4 by sequencing of its PCR amplicons, revealed that it shares similarities with a submitted GenBank reference sequence, with the BLAST search showing it has a 98.92% pair wise identity with *Penicillium citrinum* strain P18 with NCBI accession number OM397070.1 (Supplementary data).

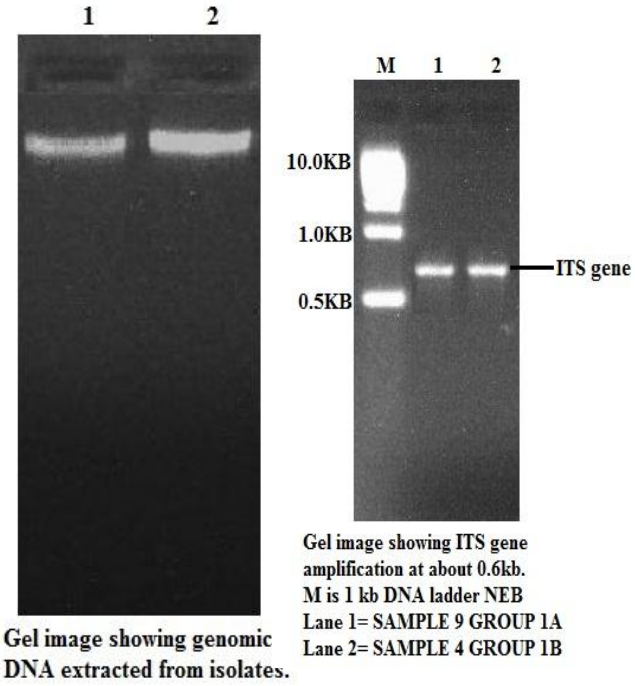

Figure 3: Gel image of isolates
Gel image of genomic DNA extracted (b) PCR amplification of ITS gene.

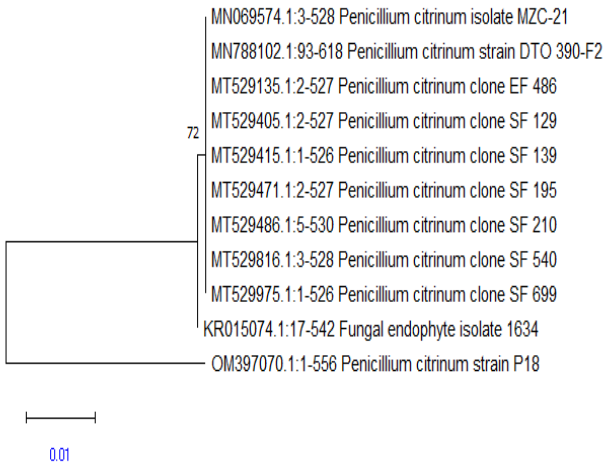

Figure 4: Phylogenetic tree of isolate S4

Phylogenetic analysis

Figure 4 shows the phylogenetic tree of the sample isolate 4. Eleven (11) of the best hits with different similarity was used for the analysis, with the bootstrap value of 1000 replication used the test of phylogeny. The evolutionary distances were calculated using the Maximum Composite Likelihood technique and are in base substitutions per location. The fungal isolates' phylogenetic tree showed a clustering pattern based on its close ancestorial similarity, with the 3 clades belonging to the *Penicillium* genus. The alignment matrix had 11 nucleotide sequences with 555 locations in the final dataset, according to a phylogenetic tree based on the ITS gene.

All isolates *Penicillium* species were clustered with ex-type strains from Genbank with cluster identity of above 70%, with the isolate *P. citrinum*strainP18 being the outgroup.

In the present study, it was noted that more protease activity was observed in the immobilized cells than their free counterparts and this trend was consistent under different culture conditions (pH, time course and temperature). Generally, as mentioned earlier, different organisms with different physiology and metabolism; would always respond differently to various cultural and/or environmental conditions. This fact, to a large extent, might have contributed to the wide discrepancy in protease activity results consistently reported in literature. The homology percentage gene sequence of S4 isolate was similar with *Penicillium citrinum* when the ITS sequence region of the 18S rDNA was identified.Therefore, S4 isolate was identified as *Penicillium citrinum* OM397070.1. *Penicillium citrinum*OM397070.1 however proved to be a very good protease producer in this study.

Conclusion

In this work, *Penicillium citrinum*was found to hydrolyze an appreciable quantity of the milk protein more than other fungi isolated. Optimum pH, temperature and incubation time for protease activity were 6.0, 35^{o}C and 4 days respectively. Immobilized spores were found to perform better than the free fungal spores. Therefore, *Penicillium citrinum*, a filamentous soil funguswas found to be a very good candidate for protease production.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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References

1. Adrio JL, Demain AL. Microbial enzymes: tools for biotechnological processes. Biomolecules. 2014 Jan 16;4(1):117-139.
2. Sharma KM, Kumar R, Panwar S, Kumar A. Microbial alkaline proteases: Optimization of production parameters and their properties.J. Genet. Eng. Biotechnol. 2017 Jun 1;15(1):115-126.
3. Sumantha A, Larroche C, Pandey A. Microbiology and industrial biotechnology of food-grade proteases: a perspective. Food Technol. Biotechnol. 2006 Mar;44(2):211.
4. Hassan MA, Haroun BM, Amara AA, Serour EA. Production and characterization of keratinolytic protease from new wool-degrading Bacillus species isolated from Egyptian ecosystem. Biomed Res. Int. 2013 Jan 1;2013.
5. Tork SE, Shahein YE, El-Hakim AE, Abdel-Aty AM, Aly MM. Purification and partial characterization of serine-metallokeratinase from a newly isolated Bacillus pumilus NRC21. Int.J. Biol. Macromol. 2016 May 1;86:189-96.
6. Nwokoro O EkwemOH, Oyeagu U. Extracellular Alkaline Protease production By *Bacillus licheniformis* in Submerged Fermentation.Ann., Food Sci. Technol..2017. Vol 18, Issue 3, 467-477
7. Saran S, Isar J, Saxena RK. A modified method for the detection of microbial proteases on agar plates using tannic acid. J. Bioch.Bioph Meth. 2007 Jun 10;70(4):697-9.
8. Nwokeoma CI, Arotupin DJ, Olaniyi OO, Adetuyi FC. Enhanced production, purification and characterization of pectinase from *Aspergillus flavus*. Trop J Nat Prod Res. 2021, 5 (10):1876-1882
9. Ametefe GD, Lemo AO, Orji FA, Kolawole LA, Iweala EE, Chinedu SN. Comparison of Optimal Fungal Pectinase Activities Using the Box-Behnken Design. Trop J Nat Prod Res. 2021, 5(9):1656-1654
10. .ElHadj-Ali N, Agrebi R, Ghorbel-Frikha B, Sellami-Kamoun A, Kanoun S, Nasri M. Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated Bacillus licheniformis NH1.Enzyme Microb Technol. 2007 Mar 5;40(4):515-23.
11. Josephine FS, Ramya VS, Devi N, Ganapa SB, Siddalingeshwara KG, Venugopal N, Vishwanatha T. Isolation, production and characterization of protease from Bacillus sp. isolated from soil sample. J Microbiol Biotech Res. 2012;2(1):163-8.
12. Chandrasekaran S, Kumaresan SS, Manavalan M. Production and optimization of protease by filamentous fungus isolated from paddy soil in Thiruvarur District Tamilnadu. J. App Biol. Biotechnol. 2015 Dec 19;3(6):066-9.
13. Paula MD, Monaliza, DA.B, Perola OM. A biotechnology, perspective of fungal protease. Braz. J. Microbiol., 2015 46(2):337-346.
14. Joo DS, Cho MG, Lee JS, Park JH, Kwak JK, Han YH, Bucholz R. New strategy for the cultivation of microalgae using microencapsulation. J. Microencapsul. 2001 Jan 1;18(5):567-76.
15. Oyeagu U, Nwuche CO, Ogbonna CN, Ogbonna J C.Amylase Production by *Aspergillusniger* immobilized in Microporous Calcium Alginate Gel Beads. Rom Biotechnol Lett. 2021, 26(4): 2825-2833.
16. Maghsoodi V, Kazemi A, Nahid P, Yaghmaei S, Sabzevari MA. Alkaline protease production by immobilized cells using B. licheniformis. Sci. Iran. 2013 Jun 1;20(3):607-10.
17. Al-Meani S. production of protease using immobilization cells technique. Biochem. Cell. Arch. 2020, 20 (1):0000-000
18. Sinha P, Singh RK, Srivastva R, Sharma R, Tiwari SP. Characterization and optimization of alkaline protease enzyme produced by soil borne bacteria. Trends Life Sci. 2013;2(2):38-46.
19. Nagamani A, Kunwar IK, Manoharachary C. Handbook of Soil Fun. Handbook of Soil Fun. 2006.
20. Ogbonna JC, Amano Y, Nakamura K. Elucidation of optimum conditions for immobilization of viable cells by using calcium alginate. J. Biosci. Bioeng, 1989 Jan 1;67(2):92-6.
21. Oyeagu U, Nwuche CO, Ogbonna CN, Ogbonna JC. Addition of fillers to sodium alginate solution improves stability and immobilization capacity of the resulting calcium alginate beads. Iran. J. Biotechnol.. 2018 Apr;16(1):66-72
22. Potumarthi R, Ch S, Jetty A. Alkaline protease production by submerged fermentation in stirred tank reactor using Bacillus licheniformis NCIM-2042: effect of aeration and agitation regimes. Biochem. Eng. J. 2007 May 1;34(2):185-92.

23. Maitig, AM. Alhoot MAM. Tiwari K. Isolation and Screening of Extracellular Protease Enzyme from Fungal Isolates of Soil.J Pure Appl Microbiol, 2018, 12(4), 2059-2067
24. Javed S, Meraj M, Bukhari SA, Irfan R, Mahmood S. Hyper-production of alkaline protease by mutagenic treatment of Bacillus subtilis M-9 using agroindustrial wastes in submerged fermentation. J. Microb. Biochem. Technol. 2013;5(3):74-80.
25. Abusham RA, Rahman RN, Salleh AB, Basri M. Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant Bacillus subtilis strain Rand. Microb. Cell Fact. 2009 Dec;8(1):1-9.
26. Ikram-Ul-haq HM, Umber H. Production of protease by Penicillium chrysogenum through optimization of environmental conditions. J. Agric. Soc. Sci 2006;2(1):23-25.
27. Maal KB, Emtiazi G, Nahvi I. Production of alkaline protease by Bacillus cereus and Bacillus polymixa in new industrial culture mediums and its immobilization. Afr J Microbiol Res. 2009 Sep 1;3(9):491-497.
28. Agrawal D, Patidar P, Banerjee T, Patil S. Production of alkaline protease by Penicillium sp. under SSF conditions and its application to soy protein hydrolysis. Process Biochem. 2004 Apr 30;39(8):977-81.
29. Braaksma M, Smilde AK, van der Werf MJ, Punt PJ. The effect of environmental conditions on extracellular protease activity in controlled fermentations of Aspergillus niger. Microbiol. 2009 Oct;155(10):3430-9.
30. Ganesh-Kumar AG, Nagesh N, Prabhakar TG, Sekaran G. Purification of extracellular acid protease and analysis of fermentation metabolites by Synergistes sp. utilizing proteinaceous solid waste from tanneries. Bioresour Technol. 2008 May 1;99(7):2364-72.
31. Morimura SK, Sonada Y. Production of protease using waste water from the manufacture of Shochu. J.Ferment.Bioeng. 199477: 183-187.