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Research Article

Statistical optimization of culture medium for neutral protease production by *Streptomyces* sp. using sardine viscera

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Abstract

Proteases are the most important industrial enzymes and widespread in nature. In the present work, sardine waste (viscera) was used to optimize the protease production by newly isolated *Streptomyces* sp. strain. A comparative study of proteolytic activity was first carried out on a synthetic medium, sardine waste broth (in submerged fermentation), and powder of the same waste (in solid-state fermentation). The enzyme production media were optimized according to statistical methods, while using two plans of experiences. The first corresponded to the Plackett-Burman matrices and the second was the Box-Wilson central composite design. The protease characteristics study showed an optimum temperature of 40°C and an optimum pH of 7, which is a typical characteristic of neutral proteases. The results of protease production optimization showed maximum activity on the following fermentation medium: sardine viscera broth (25%), NaCl (7.99 g/L), and gelatin (9.82 g/L). All these results confirmed the high biotechnological potential of this strain for neutral protease production on sardine viscera, which provides an interesting and promising strategy for large-scale enzyme production on fish waste.

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Introduction

World fish production from aquaculture and fisheries was estimated at 185.4 million tons in 2023, a modest increase of 0.6 percent compared to the preceding year (FAO, 2023). Overall, although there has been an increase in fish utilization, the potential for waste generation remains high due to its perishable nature and the presence of inedible fractions (Thirukumaran *et al*., 2022).

The fishery industry generates large amounts of waste (20-75% w/w) of the total caught fish weight (Cardeira *et al*., 2022). Every year, fishing discharges more than 20 million tons of waste. Generally, this waste is dumped, burnt, discarded or abandoned, leading to a whole range of environmental, biodiversity, and health problems (Racioppo *et al.*, 2021). The common sardine (*Sardina pilchardus*) belongs to the Actinopterygians class, Clupeiformes order, and Clupeidae family. It is found in the North-East Atlantic, from Norway to Scotland to Senegal, and the Mediterranean Sea. Sardine has a high nutritional value and their co-products possess quite similar composition (Bahammou *et al.*, 2019).

Among the natural sources of proteolytic enzymes (plants, animals, and microbes) microorganisms represent excellent sources of these enzymes; in fact, microbial proteases are of prime interest due to their rapid cell production rate and ease of maintenance of microbial cultures (Swarna and Gnanadoss, 2021; Liya *et al.*, 2023). Proteolytic enzymes are mainly applied in major industrial sectors such as the textile, pharmaceutical, food, leather, cosmetics, and detergent industries (Razzaq *et al*., 2019). The ability of microorganisms to grow at high temperatures, alkaline pH, and high salinity makes them an attractive target for enzyme production with several industrial applications (Mechri *et al.*, 2022).

The producers of proteases were found among *Aspergillus*, *Bacillus*, and *Streptomyces* genera (Blieva *et al*., 2021). The genus *Streptomyces* is one of the richest sources of secondary metabolite biosynthetic gene clusters BGCs (Chung *et al*., 2021). This active genus is a representative group of Actinobacteria and one of the largest taxa in bacteria, including approximately 700 species with validly published names (Komaki, 2023). The aim of this study was to use sardine waste (viscera) as a base medium for the production of proteolytic enzymes by a *Streptomyces* sp. strain in two fermentation modes: solid state fermentation (SSF) on waste powder and submerged fermentation (SmF) on waste broth, then to optimize the production medium on the basis of this waste by applying statistical plans.

Materials and methods

The producing micro-organism

The micro-organism studied was a strain belonging to the Actinobacteria. It was isolated from decomposing olive pomace using the decimal dilution method on ISP_2 medium (glucose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L, agar 20 g/L). The strain was sub-cultured on the same medium until complete purification.

Phylogenetic strain identification

The Actinobacteria strain studied, coded PO5, and it was phylogenetically characterized by 16S rRNA gene sequence

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analysis. DNA extraction was carried out by CTAB/NaCl method (Wilson, 1987); PCR was achieved with the universal bacterial primers SD-Bact-0008-a-S-20 (5'-AGA GTT TGA TCC TGG CTCAG-'3) and S-D-Bact-1495-a-A-20 (5'-CTA CGG CTA CCTTGT TAC GA-'3). PCR final volume was 25 µl containing: 1µL DNA, 0.5 µM of primers, 1.25 mM $MgCl_2$, 1.25µM dNTP, 2.5 µL PCR buffer, 0.22 UI Taq polymerase.

The amplification cycle was: 1X 95°C (5min), 2 X 94°C (45s), 35 cycles X [50°C (1 min); 72°C (1 min)] and 1 X 72°C (7 min). Sequence analysis was performed by Big Day terminator cycle sequencing kit V3.1, using capillary electrophoresis ABI Prism 3130TM DNA sequencer (Applied Biosystems, Hitachi, Japan); and the sequence similarities were analyzed with NCBI Blast (https://blast.ncbi.nlm.nih.gov/). The bootstrap consensus tree was constructed with 1000 replications by the method of Saitou and Nei (1987) using MEGA version 11.

Primary screening of proteolytic activity on solid media

The proteolytic activity of strain P05 was detected on a solid medium (milk agar medium) with two concentrations (30% and 20%) of skimmed milk at pH 7, using the Williams and Cross (1971) technique. After incubation at 30°C for 5 days, the presence of light areas around the colonies indicated the presence of proteolytic activity.

Fermentations

Inoculum preparation

The inoculum was prepared by scraping the surface of the studied strain solid cultures on ISP_2 medium in order to obtain spores and mycelial fragments suspensions in physiological water.

Fermentation media

Synthetic medium

Fermentation was carried out on a synthetic medium (Peptone 5g/L, yeast extract 3g/L, gelatin 4g/L) in SmF inoculated with 2% of the prepared inoculum.

Sardines waste media

Sardine viscera were boiled with water for 20min, centrifuged at 1132g for 10min and the supernatant was used as broth after sterilization. The centrifugation pellet was dried at 80°C for 48h and made into a powder. Finally, two types of fermentation were prepared from these wastes, one based on broth in SmF and the other based on powder prepared in SSF.

SmF with Sardines waste broth

Sardine viscera broth was prepared at different concentrations (25%, 50%, and 75%) in 200 mL Erlenmeyer flasks, at a rate of 50 mL.

SSF with Sardines waste powder

Sardine viscera powder was introduced into 200 mL Erlenmeyer flasks containing 50 mL saline solution (K_2HPO_4: 0.05%, NaCl: 0.5%, $MgSO_47H_2O$: 0.25%, NH_4NO_3: 0.005%), in order to prepare three concentrations 1%, 2.5% and 5%. After incubation for 4days at 30°C, the cultures were centrifuged at 1132g for 10 min. The

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supernatant representing the crude enzyme extract was recovered and frozen for subsequent analytical studies.

Proteolytic activity assay

Proteolytic activity was measured using the method described by Tsuchida *et al.* (1986).

Enzymatic reaction

1mL of 2% (w/v) casein in phosphate buffer (pH7) was mixed with 1mL of culture supernatant. After incubation for 30 min at 40°C, the enzymatic reaction was stopped by adding 2mL of trichloroacetic acid (0.4M). The mixture was then centrifuged at 1132g for 10 min at 4°C.

Assay protocol

1mL of the supernatant was mixed with 5mL of Na_2CO_3 (0.4M) and 1mL of 10% Folin-Ciacolteu reagent. After incubation for 20 min at 40°C in the obscurity, the absorbance of the mixture was measured at 750 nm. Protease unit (U) is equivalent to 1 µg of tyrosine released per mL per hour under essay conditions.

Application of statistical designs for the optimization of protease production media based on sardine waste broth

Selection of factors with a positive effect on protease production

This first optimization step was carried out on sardine viscera broth using the Plackett and Burman design (PBD) (Plackett and Burman, 1946), which consisted of a square matrix where the factors were taken at two levels: (-1) for the lower level and (+1) for the upper level. They were constructed by circular permutation of a baseline row. The factors in the last row take always the (-1) level.

A matrix of 12 combinations was adapted in order to select the factors influencing the production of proteases, allowing the study of 11 factors: 8 real and 3 errors (Table 1). The real values of the coded levels (-1 and +1) are presented in Table 2.

Table 1: Plackett and Burman matrix of 12 combinations.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
2	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
3	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
4	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
5	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
6	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
7	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
8	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
9	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1
10	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
11	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

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Two series of media were also prepared, in order to study the effect of agitation on protease production. The first set was incubated in a stirred water bath, and the second was incubated without agitation, both at a temperature of 30°C for 4 days.

Optimum level determination using the central composite design

The factors selected in the PBD were included in the central composite design (CCD) in order to search for their optimal values **(**Box and Wilson, 1951).

Table 2: Real and coded values of the various factors used in the Plackett and Burman design.

Factors	Low level (-1)*	High level (+1)*
X_1: Glucose	0	3
X_2: Fructose	0	3
X_3: Mannose	0	3
X_4: Error	-	-
X_5: Casein	0	8
X_6: Gelatine	0	8
X_7: Yeast extract	0	8
X_8: Error	-	-
X_9: $CaCO_3$	0	1
X_{10}: NaCl	0	4
X_{11}: Error	-	-

* Concentrations in (g/L).

In this design, each variable was studied at 5 levels: (-1) lower level, (+1) higher level, (0) central point, and (-α, +α) two levels determined based on the number of factors being studied. The two selected factors were studied in CCD of 11 combinations (Table 3):

- a full factorial design (2^2) with two levels of factors (-1, +1)
- a 2×2 design formed either with two levels (-α) and (+α) where α = 1.414
- 3 repetitions of central point (0)

Table 3: Central composite design for the study of 2 selected factors.

Run	Factors	
	X_{10}	X_6
1	-1	-1
2	+1	-1
3	-1	+1
4	+1	+1
5	-1.414	0
6	0	-1.414
7	+1.414	0
8	0	+1.414
9	0	0
10	0	0
11	0	0

The coded and real values of the 2 studied factors are given in Table 4.

Table 4: Real and coded values of the 2 selected factors.

Factors	Levels				
	-α	-1	0	+1	+α
X_{10}: NaCl	1.172	2	4	6	6.828
X_6: Gelatine	3.172	4	6	8	8.828

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The analysis of variance allows to estimate the significance of the obtained results by CCD. If the test is significant, the effect of factors (X_6 and X_{10}) on protease production is expressed as a quadratic equation, whose resolution gives the optimal coded levels:

$$Y = \beta_0 + \beta_1 X_6 + \beta_2 X_{10} + \beta_{11} {X_6}^2 + \beta_{22} {X_{10}}^2 + \beta_{12} X_6 X_{10}$$

Study of the protease properties

Effect of temperature on proteolytic activity

The Effect of temperature on the proteolytic activity was studied by incubating the reaction mixture at temperatures of 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C.

Effect of pH on proteolytic activity

The effect of pH on the proteolytic enzyme's activity was studied by carrying out the enzymatic reaction in buffer solutions at a pH range of 5 to 12.

- Citric acid-Na_2HPO_4 (pH 5 and 6);
- Phosphate (pH 7 and 8);
- Glycine - NaOH (pH 9 and 10);
- Phosphate-NaOH (pH 11 and 12).

Results

Phylogenetic strain identification

The studied strain (PO5), isolated from olive waste, produced on ISP_2 medium: white substrate mycelium, pink aerial mycelium, and diffusible violet pigment (Fig. 1). Alignment of the partial 16S rDNA sequence obtained from the strain (accession number OR_793850) showed that it was related to species of the genus *Streptomyces*. The bootstrap consensus tree of the strain with related species showed that *Streptomyces tritolerans* DAS165, was the closest species to our studied strain occupying the same phylogenetic line (Fig. 2), with a high degree of similarity (95.90%), a bootstrap percentage of 70%, and a genetic distance of 0.0198.

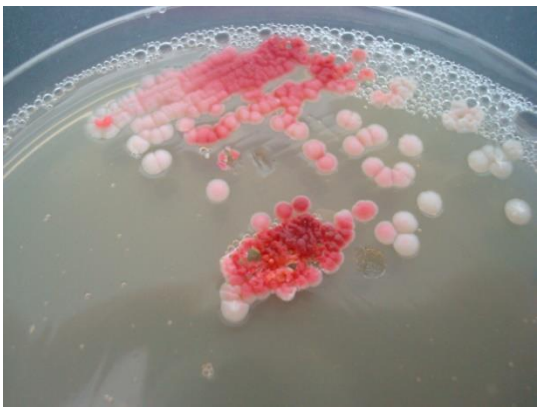

Figure 1: Macroscopic aspect of PO5 strain on ISP_2 medium.

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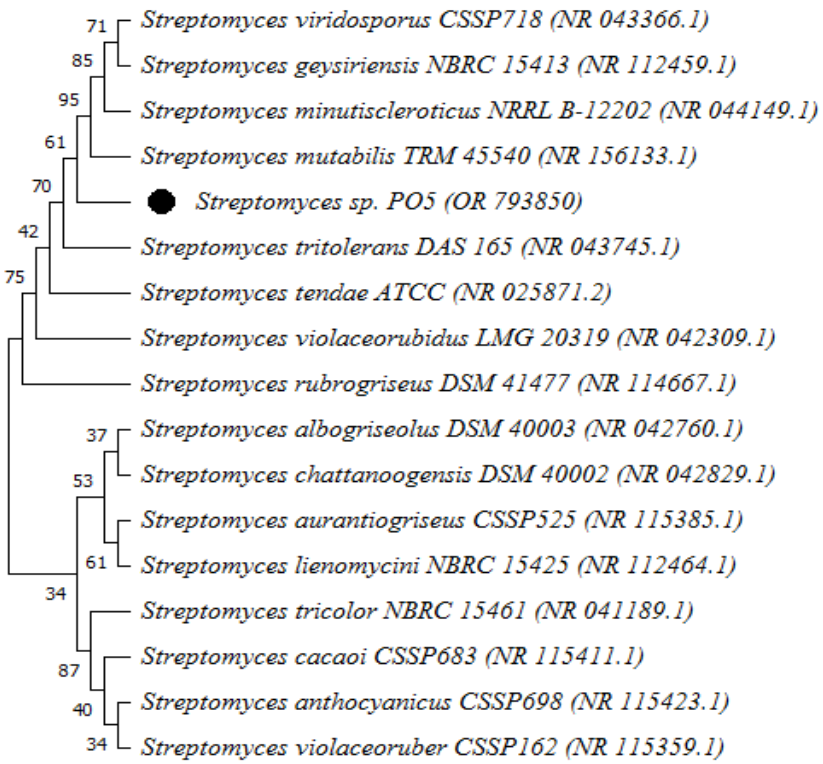

Figure 2: Neighbor-Joining phylogenetic tree based on partial 16S rRNA gene sequences showing PO5 strain and the nearest related taxa (bootstrap support are based on 1000 replicates).

Proteolytic activity investigation

The results of this test gave very similar hydrolysis zones (5 to 6 mm) for the two skimmed milk concentrations tested (20% and 30%).

Comparison of proteolytic activities in the different fermentation media tested

The results of this study showed that protease production was very high in the liquid medium prepared from sardine viscera broth (in SmF) compared with the synthetic medium and the viscera powder medium (in SSF) (Fig. 3).

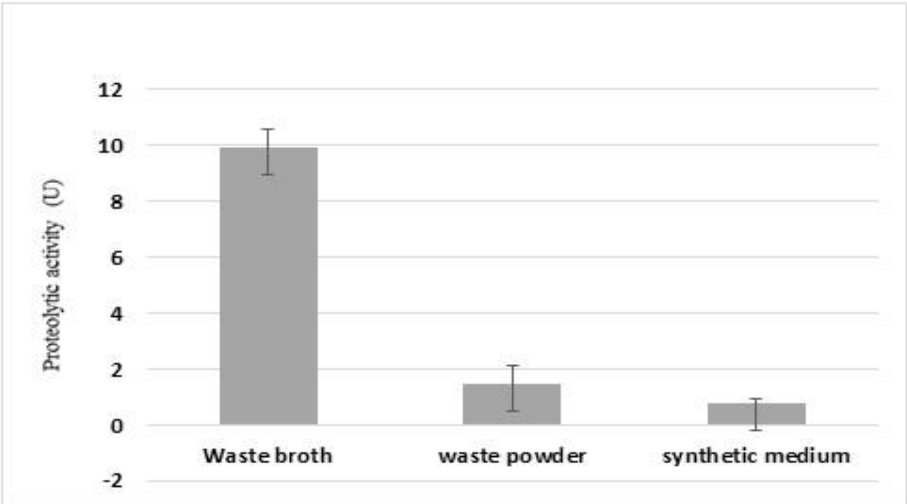

Figure 3: Proteolytic activity on the fermentation media tested.

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Study of proteolytic activity in SSF at different concentrations of sardine waste powder

The proteolytic activity assay, determined after solid fermentation of different concentrations of sardine waste powder, showed better activity on the medium with the lowest concentration of powder (Fig. 4).

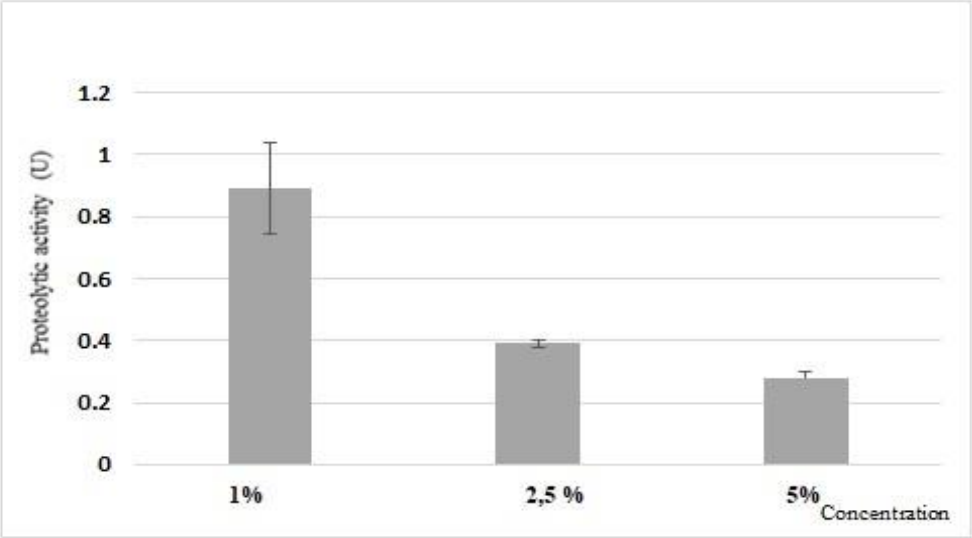

Figure 4: Proteolytic activity on the solid state fermentation at different concentrations of sardine waste powder.

Study of the proteolytic activity on SmF at different concentrations of sardine waste broth

The results of this test, presented in Figure 5, showed a maximum proteolytic activity at the lowest broth concentration, which decreased with increasing broth concentration. The large difference in proteolytic activity produced in the two types of fermentation led us to proceed with the optimization of the production media based on sardine waste broth in SmF.

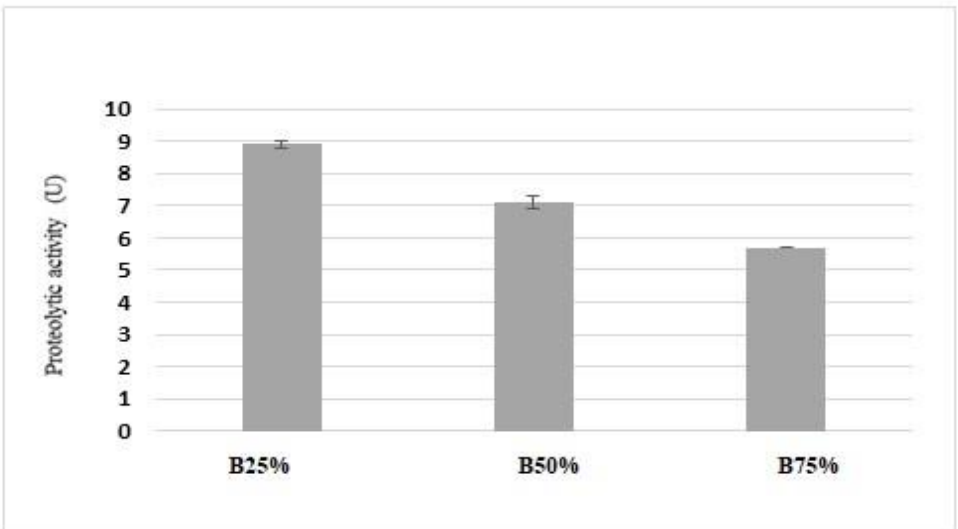

Figure 5: Proteolytic activity on submerged fermentation at different broth concentrations.

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Optimization of SmF protease production media based on sardine waste

Selection of factors influencing proteolytic activity

The results of the proteolytic activities in different media according to the PBD matrix are presented in Figure 6. It turns out that under agitation, trial 1 was the best combination of factors for protease production, while it was trial 10 without agitation. These findings are reflected in the statistical analysis and modeling of the results using Minitab 18 software to evaluate the effect of each factor and its level of significance on proteolytic activity (Table 5). This analysis allowed us to select a single significant factor, X_{10} (NaCl), with a Student's value of 5.97.

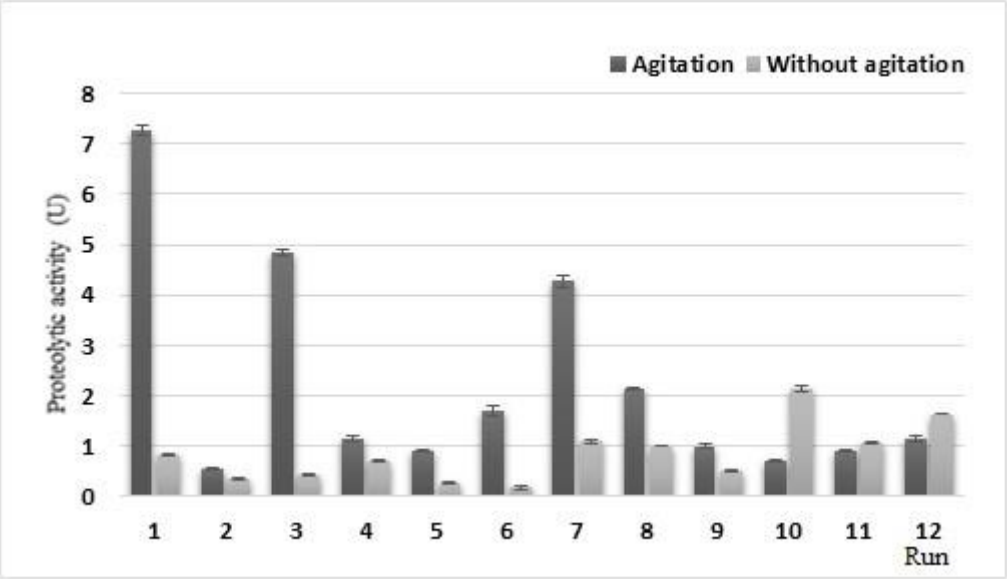

Figure 6: Proteolytic activities of the different Plackett and Burman design assays.

Table 5: Results of the statistical treatment of the Plackett and Burman design.

Source	Effect	Coefficient	F value	*p*-value
X_1	0.337	0.337	0.09	0.781
X_2	2.585	2.585	0.71	0.461
X_3	2.990	2.990	0.82	0.432
X_5	0.027	0.027	0.01	0.937
X_6	2.832	2.832	0.78	0.443
X_7	4.260	4.260	1.17	0.359
X_9	3.597	3.597	0.99	0.394
X_{10}	21.735	21.735	5.97	0.092
Error	10.923	3.641		
Total	49.287			

t : Student test, p : probability

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Regression equation:

$$\text{Activity} = 2.22 - 0.70\,X_1 + 0.46\,X_2 + 0.50\,X_3 + 0.05\,X_5 + 0.49\,X_6 + 0.60\,X_7 + 0.55\,X_9 + 1.35\,X_{10}$$

Determination of optimum concentrations of the best factors

Figure 7 shows the proteolytic activities of the studied strain, recorded during the various trials in the CCD. The statistical analysis presented in Table 6 confirmed the importance of factor X_{10} (NaCl) as a factor favoring protease production (with a Student's value of 1.86) with a highly significant effect of interactions between the factors (NaCl and gelatin), this analysis also enabled the following equation to be determined:

$$\text{Activity} = 0.084 - 0.036\,X_{10} - 0.019\,X_6 + 0.021\,X_{10}^2 + 0.070\,X_6^2 + 0.040\,X_{10}X_6$$

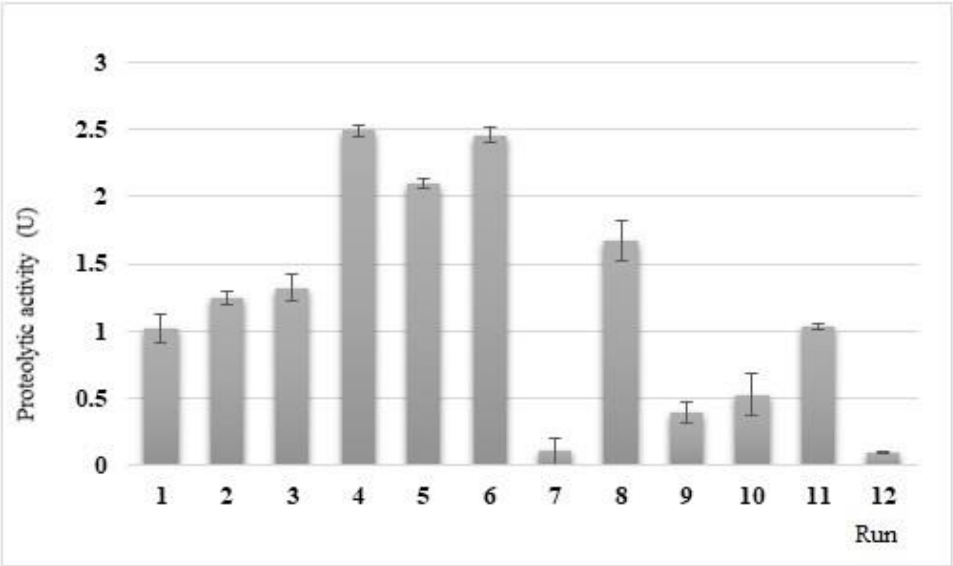

Figure 7: Proteolytic activities of the different tests of the central composite design.

Table 6: Results of statistical processing of the central composite design (R^2 = 74.46%).

Source	Effect	Coefficient	F value	*p* value
Model	0.099	0.020	2.92	0.133
Linear	0.013	0.007	0.99	0.436
X_{10}	0.013	0.013	1.86	0.230
X_6	0.001	0.001	0.11	0.756
Square	0.049	0.025	3.63	0.106
$X_{10}X_{10}$	0.010	0.010	1.44	0.284
X_6X_6	0.045	0.045	6.62	0.050
2 factors interaction	0.018	0.018	2.70	0.162
$X_{10}X_6$	0.018	0.018	2.70	0.162
Error	0.034	0.007		
Inadequate fit	0.028	0.014	7.78	0.065
Pure error	0.005	0.002		
Total	0.132			

t : Student test; p : Probability

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The optima of the factors used were obtained by calculating the partial derivatives of this equation, which makes it possible to determine the coded coordinates of the extremum, which are then converted into real values reflecting the optimal values of the factors selected (Table 7).

Table 7: Optimal coded and real values of the two selected factors.

Variable	Optimal coded value	Optimal real value
X_{10}	1.999	7.99
X_6	1.914	9.82

As a result, the composition of the fermentation medium giving the best proteolytic activity of strain PO5 on sardine waste is as follows:
- 25% sardine viscera-based broth
- 7.99 g/L NaCl
- Gelatin at 9.82 g/L

Study of the protease properties

Effect of temperature on proteolytic activity

Figure 8 presents the effect of temperature on proteolytic activity, showing an increase in the proteolytic activity of the studied strain in a range of temperatures from 30°C to 50°C. Above this temperature, a gradual decrease in proteolytic activity was observed, reaching almost zero at 80°C. The optimum temperature for the protease produced is therefore 50°C.

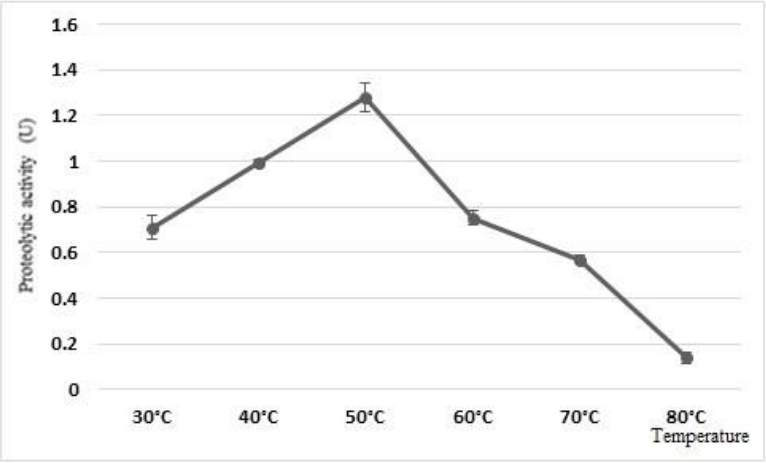

Figure 8: Effect of temperature on the proteolytic activity of strain PO5.

Effect of pH on proteolytic activity

Figure 9 shows that the PO5 strain exhibited low proteolytic activity at acid pH and then increased up to pH 7, where it exhibited maximum activity. Above this value, the activity gradually decreased at basic pH levels but remained minimal at extremely basic pH levels. The enzyme produced therefore had a pH optimum of 7, making it a neutral protease.

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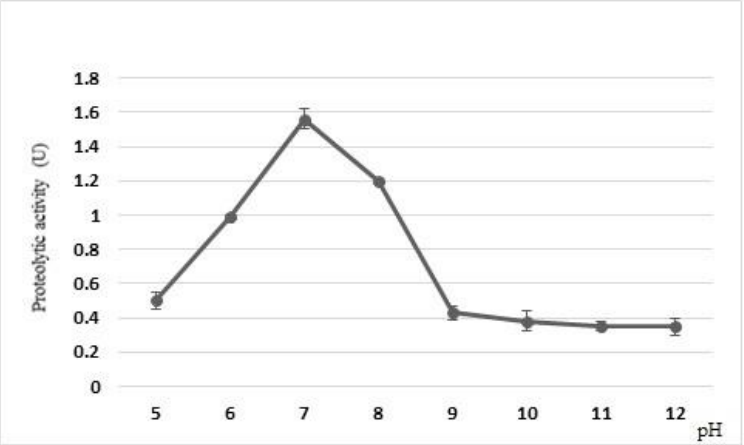

Figure 9: Effect of pH on the proteolytic activity of strain PO5.

Discussion

According to Komaki (2023), the identification of metabolite-producing strains is of great importance for the study of the producer micro-organisms bioactivity. The phylogenetic study showed that the protease producing strain shared the highest similarity with *Streptomyces tritolerans* DAS165 (95.90%), with bootstrap percentage of 70%, and a genetic distance of 0.0198. It may belong to the same species or a different species. *Streptomyces tritolerans* DAS165, was first isolated by Syed *et al*. (2007) from a dry land soil sample in the Gulbarga region, Karnataka province, India. The isolate produced yellow substrate mycelia and grey aerial mycelia on most tested media. However, the strain isolated by Mathew *et al.* (2020), with a similarity percentage of 99.6% with *Streptomyces tritolerans* DAS 165 (NR 043745) presented the same macroscopic appearance (dark yellow substrate mycelium and grey aerial mycelium).

Nofiani *et al.* (2020) also isolated two strains (RC2 and RS2) from sponges and corals collected in the Indonesian water, which showed a high degree of similarity to *Streptomyces tritolerans* DAS165, these two strains formed a grey aerial mycelium on the culture media studied.

In the three research studies cited above, the *Streptomyces tritolerans* strains isolated had a grey aerial mycelium, unlike our strain, which had a completely different microscopic appearance with a white substrate mycelium and a pink aerial mycelium.

These findings, in addition to the small genetic distance (0.0198), suggest that it may belong to different species. Thus, DNA-DNA hybridization and average nucleotide identity (ANI) require further analysis to confirm whether our strain belongs to a new species (He *et al*., 2023).

The second part of this work involved the primary screening of proteolytic activity on solid media. *Streptomyces* sp. showed good activity on all milk concentrations tested. Several studies have demonstrated the richness of Algeria's natural environment by isolating autochthonous *Streptomyces* sp. strains with interesting proteolytic activities (Boulkour, 2016; Boughachiche *et al*., 2021). The comparison test of

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proteolytic activities in the different media showed better activity in SmF based on sardine viscera broth, compared with the synthetic medium and the viscera powder medium in SSF. The difference between the two media prepared from waste was probably due to the nature of the substrates and the fermentation method used in both cases. In fact, the waste broth gave better results than the synthetic medium, both conducted in SmF. This confirms the richness of the waste broth in nutrients. According to a study by Melgosa *et al.* (2020), sardine waste contains 52.2% protein, 25.6% lipids, and 2.9% total sugars, which promote microbial growth and bioactivity.

Several studies have shown the importance of fish waste in the production of proteases by *Streptomyces* sp. such as those by Tran *et al.* (2022) in a study carried out on *Streptomyces speibonae* TKU048, which revealed an interesting potential in the valorization of tuna protein waste for the production of proteases and antioxidants.

Furthermore, the nature of the fermentation, whether solid or submerged, had a direct influence on microbial growth and enzyme production. According to Nighojkar *et al.* (2019), fungi are better adapted to SSF, whereas liquid fermentation promotes bacterial growth and activity. In fact, in SSF, the fungus grows on and inside the inert support independently of the medium; whereas in SmF, the formation of high-density spherical pellets, leads to the best productivity and specific yield of the product (Greco-Duarte *et al*., 2023).

Proteolytic activity study in SSF at different concentrations of sardine waste powder revealed better activity on the medium with the lowest concentration of powder. The low proteolytic activity values observed in the three concentrations can be explained by the fact that in the SSF process, the micro-organisms grow on the surface of moist solid substrates, providing nutrients in the absence of free water flow (Colla *et al.*, 2023), but also serves as an anchoring element, which reduces surface availability, which plays an essential role in nutrient transfer and metabolite production. In addition, the small particles of the substrate caused it to agglomerate, which can interfere with aeration and thus hinder growth (Sharma *et al.*, 2017).

Higher values of proteolytic activity were also recorded after liquid fermentation (SmF) based on sardine waste broth compared with those obtained after SSF fermentation of the same waste powder. The study of the proteolytic activity on SmF at different concentrations of sardine waste broth showed that the lowest broth concentration gave the maximum proteolytic activity, which declined with increasing broth concentration. This can be explained by the inhibitory effect of amino acids on protease production. This is similar to the study conducted by Sharma and Singh (2016) which showed that the protease production by Actinobacteria *Nocardiopsis dassonvillei* decreased with the increasing number of amino acids (proline, glutamine, valine, and isoleucine) in the medium. Glutamic acid and threonine individually repressed the synthesis of the protease in minimal medium. In studies by Zhang *et al*. (2023) and Melgosa *et al.*

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(2020), nitrogen metabolism (especially proteolytic activity) is inhibited by elevated nitrogen in *Stropharia rugosoannulata*. Consequently, diluting the samples reduces their concentration and thus enhances protease production.

The optimization of protease production media based on sardine waste broth carried out by factorial statistical plans (PBD) allowed the selection of one factor having a significant effect on the enzyme production "NaCl". The importance of NaCl as a constituent of culture media for protease production by *Streptomyces* sp. has been discussed in several research studies, such as those by Manivasagan *et al*. (2013). In this study, NaCl was identified (with other ingredients) as the most significant variable, influencing protease production in poultry waste, by *Streptomyces* sp. MAB18 strain isolated from Cuddalore coast, India.

Among non-significant effect factors, another one was selected for the second step of the optimization (determination of the optima). Thus, gelatin was chosen for its protein nature and its higher Student's value than casein.

Among the different media tested in PBD, medium 1 proved to be the best production medium, followed by medium 3. Both media contained fructose as a carbon source. The importance of fructose in protease production media has been noted in several studies such as those by Parthasarathy and Gnanadoss (2018), who found that the optimized values showed that fructose at 2.0 g/L enhances the yield of protease up to 120.08±2.2 U/mL. The study conducted by Boughachiche *et al.* (2021) showed that the best protease producing medium from autochthonous *Streptomyces* sp. based on wheat bran was supplemented with 1% fructose. However, fructose can have a repressive effect on protease production, as found by Hurtado-Cantillo *et al.* (2019) where the presence of fructose repressed the proteolytic activity displayed by soil *Streptomyces* sp. AGS-10.

Carrying out the various Plackett and Burman (1946) trials with and without agitation confirmed the importance of agitation as a factor in improving protease production for most of the trials. Our results are in agreement with Lich *et al.* (2022) study, showing that protease production increases with increasing shaking speed, leading to an adequate supply of dissolved oxygen in the medium. Agitation ensures also the homogeneity of the medium, improves mass and heat transfer, and distributes the liquid phase to compensate for the loss caused by evaporation.

The study of the protease characteristics showed an optimum pH of 7 and an optimum temperature of 40°C. Our results are close to those of Ghorbel *et al.* (2014), who characterized a protease produced by *Streptomyces flavogriseus* HS1, which had an optimum activity at 50°C. Al-Dhabi *et al*. (2020) also isolated an alkaline protease from *Streptomyces* sp. Al-Dhabi-82 with an optimal activity temperature of 40°C. Çorbacı and Özcan (2021) also studied a protease produced from *Streptomyces* sp. strain isolated from a Black Sea marine sediment, whose maximum activity is reached at 37°C, which is close to our results. Several research studies have highlighted the production of neutral proteases from the *Streptomyces* genus, such as those by Ghorbel *et al.* (2014) on *Streptomyces flavogriseus* HS1.

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As a conclusion of this study, a culture medium composition was formulated and optimized for maximum protease production. The constituents and their respective quantities were outlined as follows:

- broth (prepared from sardine viscera) at 25%
- NaCl at 7.99 g/L
- Gelatine at 9.82 g/L

The use of sardine waste as a medium for microbial protease production should, therefore, provide an interesting and promising strategy for large-scale enzyme production.

Finally, these results call for further studies and open up new prospects, such as the development of a protocol for the enzyme's recovery and purification, not forgetting the study of the enzyme's areas of application and its mechanism of action.

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Conflict of interest

The authors declare that there is no conflict of interest.

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