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Molecular identification of protease producer ORSK-4 strain and determination of optimum enzyme production conditions

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Abstract: A microorganism resistant to high temperatures and producing alkaline proteases was isolated from soil samples from a protein-rich region (Kırıkkale/Yahşihan). This isolate, with high protein production, was identified as ORSK-4 by determining its morphological and biochemical properties using the 16s rRNA molecular approach and the Amplified ribosomal DNA restriction analysis (ARDRA) technique employed in strain differentiation. The optimum enzyme production conditions of the strain ORSK-4 were found to be the enzyme media, 3 days of incubation, 27.0 °C, and pH 7.0. Different components were utilized to determine the effect of changing the medium content on enzyme activity. Under the optimal production conditions determined in this way, the enzyme activity of ORSK-4 was found to be higher than that of some ATCC reference *Bacillus* species. To purify the extracellular protease of ORSK-4, precipitation with ammonium sulfate (30% and 80%), dialysis, and DEAE ion exchange chromatography were performed. SDS-PAGE analysis determined the molecular weight of the purified enzyme as approximately 30 kDa. Although the enzyme showed activity at various pH ranges, it showed its maximum activity when increased up to pH 9.0. In conclusion, the stability of the obtained alkaline protease enzyme under different conditions shows that it can be used in industrial and environmental applications.

Keywords: *Bacillus*, enzyme characterization, molecular identification, protease, purification,

1. Introduction

Proteases (proteinases or peptidases) catalyze the hydrolysis of peptide bonds. They represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sales worldwide (Rao et al 1998). Proteases are widely used in detergents, the leather industry, medical diagnostics, silver X-ray films, and stickiness removal in silk, food, and feed industries. Therefore, many companies have started producing proteases commercially (Solanki et al. 2021).

The primary consideration when isolating enzymes on an industrial scale for commercial purposes is to reduce the cost of production concerning the value of the end product (Adrio and Demain 2014). From this perspective, bacterial proteases are one of the best options. Proteases, mostly produced extracellularly and efficiently by bacteria, seem more suitable for industrial applications because they are thermostable and active in a wide pH range (Adrio and Demain 2014).

Although most alkaliphilic microorganisms, which are more preferred in protease production, produce alkaline proteases, the interest is limited to the microorganisms that produce large amounts of enzymes, and the microorganisms used to obtain proteases are toxic and non-pathogenic (Adrio and Demain 2014). These organisms with optimal growth conditions are essential for increasing enzyme production. The culture conditions that promote the production of these proteases have been found to differ significantly from those that promote cell growth (Mason and Joyce 2011). Technical media containing high concentrations (100-150 g dry weight/liter) of complex carbohydrates, proteins, and other media components have produced industrial alkaline proteases (Asha and Palaniswamy 2018). The fermentation medium and production conditions should be optimized to develop an economically viable technology, and the yields of alkaline proteases should be improved (Sharma et al. 2017).

Alkaline proteases are indispensable enzymes hence ubiquitous in nature. They are isolated from various sources by surface plating on an alkaline medium and subsequent screening for the desired characteristics (Sharma et al. 2017).

From the very beginning, these enzymes have been produced by using *Bacillus* species, starting with *Bacillus amyloliquefaciens* and *Bacillus licheniformis* (Zhang et al. 2022). Each organism or strain has specific conditions for maximum enzyme production (Sharma et al. 2017). Therefore, various aspects must be optimized for the strain to reach its maximum enzyme production capacity. In addition, identifying the strain is important for optimizing the production conditions. In this respect, a simple, fast and cost-effective microbial method is preferred for strain identification (for example, ARDRA).

This study isolated and identified microorganisms by taking samples from alkaline, protein-rich soil (Kırıkkale/Yahşihan). It aims to extract and purify the protease enzyme obtained by determining the optimum culture and enzyme production conditions for the microorganisms.

2. Materials and Method

Study area: Collected from the Yahşihan district of the province of Kırıkkale, microorganisms used in this study were isolated from alkaline, protein-rich soil (39º52'36.6"N and 33º26'25.5"E). Two soil samples were collected in total, one from the soil surface and one from the soil surface at a depth of 2 cm. Studies on the protease enzyme produced by the isolated microorganism were carried out after it was determined to be the best-suited protease-producing microorganism from the soil sample. The circumstances under which the acquired protease enzyme had the highest suitable activity were identified. Thus, the purified enzyme's applicability to the industry was evaluated.

2.1. Isolation, identification of isolates and biochemical tests

A location with a high concentration of proteins was identified to help identify the protease-producing bacterium, and soil samples from this stratum were collected and placed in sterile tubes. 1 g of dirt was serially diluted with 0.9% sterile saline. Singh et al. medium was modified to create the dilutions, and 0.1 ml of the material was added to this media by using the smear technique (Singh et al. 2001). The best isolates with various colony architectures and forming the biggest zone in proteolytic activity on skim-milk agar were chosen, purified, and stored at +4 °C for later use in Petri dishes left to incubate for 24 hours at 37°C. The macromorphological (gram staining, spore staining) and biochemical (IMVeC, starch hydrolysis, gelatin hydrolysis, catalase test) characteristics of the strain were determined.

2.2. Molecular identification of isolated microorganisms by 16S rRNA analysis and distinguishing microorganisms from one another with the ARDRA method

The strain was then identified by using molecular techniques (DNA isolation, 16S rRNA analysis, PCR, sequence analysis, ARDRA). Genomic DNA isolation of microorganisms was carried out by modifying the method of Cutting and Horn (Bron et al. 1990). After determining the nucleoid sequence of the 16S rRNA gene, it was amplified by Polymer Chain Reaction (PCR) with universal primers 27F(5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3'). Since PCR can be affected by many factors, the PCR products used to extract the nucleotide sequencing of 16S rRNA gene must be optimally synthesized. 5 different concentrations of $MgCl_2$ between 1 mM-2.33 mM and coupling temperatures between 50-55 °C were tested for PCR optimization. Sequence analysis of 16S rRNA gene amplified by PCR was carried out by MedSanTek company contracted under a service procurement. The obtained sequencing results were matched with the data on the 16S rRNA database of the National Center for Biotechnology Information (NCBI). The Molecular Evolutionary Genetics Analysis 7 (MEGA7) program was used to determine the kinship relations with the results obtained. First, the data on the database and the nucleotide sequences of our samples were synchronized, the distance matrix was drawn by providing statistical reliability with these data, and the degree of closeness was determined by the neighbor-joining method. Although 16S rRNA analysis successfully determines the genera of microorganisms, differentiation at the species level is very difficult, especially for the genus *Bacillus*. ARDRA, a cutting method of 16S rRNA gene with restriction enzymes, was utilized to recognize the genus *Bacillus*, which is found very similar to the other microorganisms in 16S sequence analysis, and the differences between microorganisms were investigated. Three restriction enzymes (HaeIII (BsuRI), TaqI, and AluI) were used in ARDRA (Kurabachew et al. 2003).

2.3. Protease production medium and culture conditions of the isolate

A nutrient broth (N.B) medium was used to determine the optimum growth temperature and pH of the isolated isolate strain. The medium pH was adjusted to 5.0, 7.0, 9.0, and 12.0, and sowing was carried out. The cultured media were incubated at 17 °C, 27 °C, 37 °C, and 50 °C and the growth was checked on the first three days. After incubation, the growth density of the microorganism was measured in a microplate reader (Biotek, PowerWave XS2) set to 600 nm. For protease production, Gessesse et al. (1997) suggested that organic (peptone, yeast extract, casein, meat extract, gelatin, skim milk, urea, tryptone, soy flour) and inorganic components (NaCl, $MgSO_4$, $CaCl_2$, and K_2HPO_4, $((NH_4)_2SO_4)$, $C_2H_7NO_2$, $NaNO_3$, Ca $(NO_3)_2$) and their effects on enzyme activity were determined by changing their amounts (negative, 2.5-fold)

(Gessesse and Gashe 1997). Accordingly, the optimum growth media for the protease activity of the isolate was determined.

2.4. Enzyme production

At each step of the enzyme purification process, the protein concentration was measured by a modified Bradford method. The measurement of the protease activity was determined by modifying the method employed by Takami (Takami et al. 1989). This method used the supernatant part of the samples, whose incubation was completed in the appropriate medium, as the enzyme source. Through three repetitions, 0.5 mL of 50 mM Glycine-NaOH buffer containing 0.6% casein (pH 9.0) as a substrate were taken into 1.5 mL centrifuge tubes, and 0.1 mL of the previously prepared medium supernatant (enzyme source) was added. It was incubated at 37 °C for 20 minutes. Then, 0.5 mL of TCA solution was added, the tubes were turned upside down to stop the reaction and kept at room temperature for 30 minutes. Then, the tubes were centrifuged at 12000 rpm for 7 minutes to remove the excess substrate. After centrifugation, three repetitions were completed in 96 well plates; 25 µL of supernatant was taken, 125 µL of 0.5 M Na_2CO_3 and 25 µL of 1 N Folin-ciocalteu's phenol reagent was added, and it was kept in the dark at room temperature for 30 minutes. After 30 minutes, well plates were read in a microplate reader set to 660 nm, and the results were recorded by averaging the repetitions. Enzyme units (U/mL) were then determined by the amount of tyrosine released from the tyrosine standard curve (Kazan et al. 2005). The enzyme units (U/mL) were divided by the total protein content of the sample to determine the specific activity (mg/mL). While the specific activity was calculated as enzyme activity per mg of protein and expressed as units (U/mL), the sample activity was expressed as units (U/mL).

2.5. Determination of the molecular size of protease enzyme by enzyme purification and SDS-PAGE

As to ammonium sulfate precipitating, 30% and 80% ammonium sulfate precipitation was performed. 50 mM (pH 7.0) phosphate buffer was added to the precipitated sample and dissolved in the lowest volume. The pellets dissolved in phosphate buffer with 80% precipitation were added to the dialysis membranes, and dialysis was performed against the same buffer for 12 hours at +4 °C. 5 mL of DEAE-Sepharose ion exchange column material (DCL6B100 SIGMA) was taken, and 50 mL of 50 mM (pH 7.0) phosphate buffer was added to it. The column material was mixed thoroughly and became ready for use. The sample from the dialysis was loaded on the column, and by changing the salt density (10, 100 mM, 1 M), a 1.5 mL (approximately 32) fraction was obtained at a flow rate of 1 mL/min. SDS-PAGE electrophoresis was performed to determine the molecular size of the purified protease enzyme.

2.6. Enzyme characterization

2.6.1. Determination of the optimum operating temperature and the pH value of the enzyme

The effects of temperature (17-80 °C) and pH (7.0-12.0) on enzyme activity were analyzed at varying intervals.

2.6.2. Effects of metal ions, surfactants, inhibitors, detergents, organic solvents, oxidants, and different substrates on enzyme activity

Enzyme activity was measured by incubating all reaction mixtures in 50 mM Glycine-NaOH buffer having a pH of 9 and containing 0.1 mL enzyme source (media supernatant) and 0.5 mL 0.6% casein, for 20 min at 37 °C. +1 valent (LiCl, NaCl, KCl), +2 valent ($Co(NO_3)_2$, $ZnSO_4$, $Fe(NH_4)_2(SO_4)_2$, $Sr(NO_3)_2$, $MnSO_4$, $Pb(NO_3)$ at the concentrations of 2 mM and 10 mM, $NiSO_4$, $SrCl_2$, $CuCl_2$, $MgSO_4$, $ZnNO_3$, $CaCl_2$, $MgCl_2$) and +3 valence ($Cr(NO_3)_3$, $AlCl_3$, $FeCI_3$) metal salts were used. Serine protease inhibitor PMSF, Metalloprotease inhibitor EDTA, $CuSO_4$, and DPPH with final concentrations of 2 mM and 10 mM were used. 10% (v/v) and 25% (v/v) volumes of toluene, heptane, methanol, acetone, DMSO, ethanol, 2-propanol, hexane, acetonitrile, and benzene were used. 1% (v/v) and 5% (v/v) volumes of tween-80, Triton X-100, SDS and H_2O_2 were used. Gelatin, bovine serum albumin (BSA), ovalbumin, and soy flour were used as substrates.

2.7. Statistical analysis

All experiments were repeated three times. The results were expressed by adding standard deviation (SD) error bars on the graph. Differences between tested groups were determined by the Tukey test at $p<0.05$ after a one-way analysis of variance (ANOVA). Analyzes were performed using the R v.4.0.3 program.

3. Results

3.1. Isolation, prescreening of strains and biochemical properties

One of the six isolates (ORSK-4) that formed the largest zone in proteolytic activity in the preliminary screening was selected to be used in subsequent studies.

The microbiological and biochemical properties of the isolate are given in Table 1. Various biochemical tests are vital for the types of bacteria to be identified and characterized. For this purpose, biochemical properties of isolate ORSK-4 were found as (gram (+), spore (+), gelatin hydrolysis (+), catalase (+), starch hydrolysis (-), indol (-), methyl red (-), Voges-Proskauer (+), citrate (-)).

Table 1. Biochemical test results of the isolated microorganism

	Gram	**Spore**	**Gelatin hyd.**	**Starch hyd.**	**Catalase**
ORSK-4	+	+	+	-	+
	I. test	**M.R. test**	***V.P.* test**	**S. test**	
	-	-	+	-	

(+) indicates positive result and (-) denotes negative result.

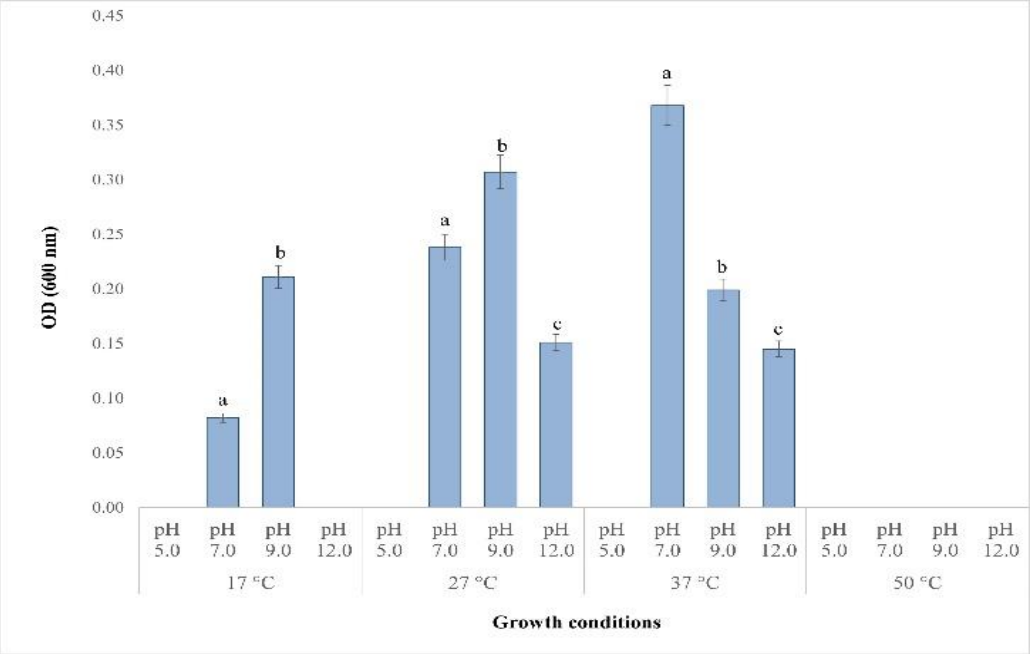

Fig. 1. Growth density of isolate ORSK-4 under different growing conditions. Different letters (a, b, c) on the graph indicate the significant differences for different pH values at the same temperature ($p < 0.05$).

3.2. Determination of isolate's optimal growth temperature and pH value

The optimum growth medium of isolate ORSK-4 was found to be 37 °C and pH 7.0. Growth densities of isolate ORSK-4 under different growth conditions are given in Figure 1.

3.3. *Molecular identification of isolated microorganisms by 16S rRNA analysis and distinguishing microorganisms from each other with the ARDRA method*

The images of the isolated chromosomal DNAs carried out in 1% agarose gel electrophoresis at 80 Volts for an hour are shown in Figure 2.

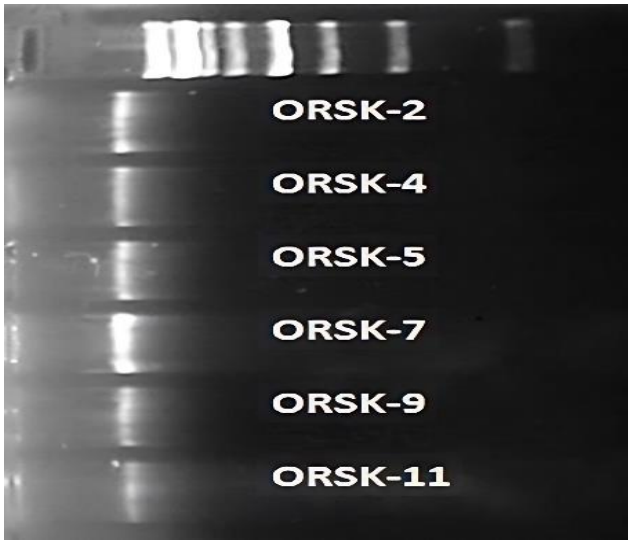

Fig. 2. Agarose gel electrophoresis of isolate ORSK-4

The optimum bonding temperature of isolate ORSK-4 was determined to be 50 °C (b), and the optimal final $MgCl_2$ concentration at 50 °C was 1.67 mM (a). Optimization results of the isolate's binding temperature and final $MgCl_2$ concentrations are given in Figure 3.

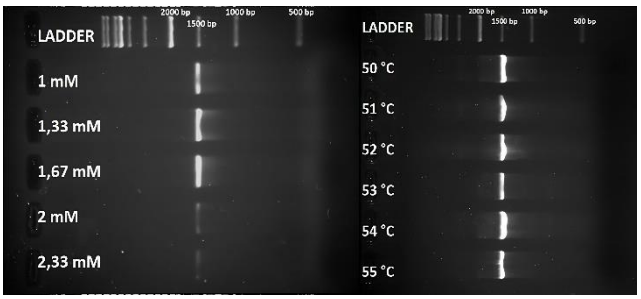

Fig. 3. Optimization results of the ORSK-4 binding temperature and final $MgCl_2$ concentrations

The nucleotide sequences of 14 different microorganism species obtained from NCBI 16S rRNA database together with the 16S rRNA sequence analysis result of the isolate ORSK-4 were aligned with each other in MEGA7 within the content of this study. According to the alignment results, the distance matrix was drawn by providing statistical reliability (bootstrap analysis with 1000 repetitions) (Figure 4). Evolutionary closeness levels were determined by drawing pedigrees with the neighbor-joining method using the distance matrix results (Figure 5). According to the aligned distance matrix results, the isolate ORSK-4 was found to be the closest relative of *Bacillus cereus* (accession number: NR_074540.1)

The 16S rRNA gene was PCR-amplified with primers 27F and 1492 R. It was revealed in the previous studies that this area includes approximately 1500 base pairs (Yu et al. 2012).

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ORSK-4														
NR_074540.1 *Bacillus cereus*	0.0411													
NR_114581.1 *Bacillus thuringiensis*	0.0418	0.0006												
NR_121761.1 *Bacillus toyonensis*	0.0418	0.0006	0											
NR_117414.1 *Bacillus marcorestinctum*	0.0432	0.0018	0.0012	0.0012										
NR_113991.1 *Bacillus pseudomycoides*	0.044	0.0036	0.0042	0.0042	0.0055									
NR_024697.1 *Bacillus weihenstephanensis*	0.0453	0.0036	0.003	0.003	0.003	0.0073								
NR_113990.1 *Bacillus mycoides*	0.0453	0.0036	0.003	0.003	0.003	0.0073	0							
NR_116644.1 *Bacillus gaemokensis*	0.0482	0.0073	0.0067	0.0067	0.0067	0.0061	0.0073	0.0073						
NR_125530.1 *Bacillus manliponensis*	0.0531	0.0116	0.0122	0.0122	0.0135	0.0116	0.0153	0.0153	0.0166					
NR_074914.1 *Bacillus cytotoxicus*	0.0546	0.0129	0.0135	0.0135	0.0147	0.0104	0.0166	0.0166	0.0154	0.0135				
NR_133704.1 *Bacillus tianshenii*	0.066	0.0238	0.0244	0.0244	0.0245	0.0238	0.0264	0.0264	0.0265	0.027	0.027			
NR_041942.1 *Bacillus acidicola*	0.0674	0.0251	0.0257	0.0257	0.027	0.0225	0.029	0.029	0.0243	0.0303	0.0219	0.0324		
NR_025373.1 *Bacillus shackletonii*	0.068	0.0257	0.0263	0.0263	0.0276	0.0231	0.0296	0.0296	0.0249	0.0309	0.0218	0.0338	0.0042	
NR_118437.1 *Bacillus marisflavi*	0.0683	0.0258	0.0264	0.0264	0.0277	0.0231	0.0297	0.0297	0.025	0.0323	0.027	0.0278	0.0141	0.016

Fig 4. Distance matrix of ORSK-4 isolate

ARDRA *is* a commonly used method for the classification of the species (Panigrahi et al. 2019). This method was utilized to determine ORSK-4's similarities with and differences from *B.cereus* ATCC 10876 and *B. subtilis* ATCC 6633. 16S rRNA gene region of isolate ORSK-4 generated 3 bands (680, 240, and 180 base pairs) after cut by Alu I restriction enzyme, 4 bands (590, 480, 310, and 120 base pairs) after cut by Hae III restriction enzyme, and 3 bands (800, 560 and 140 base pairs) after cut by Taq I restriction enzyme (Figure 6a).

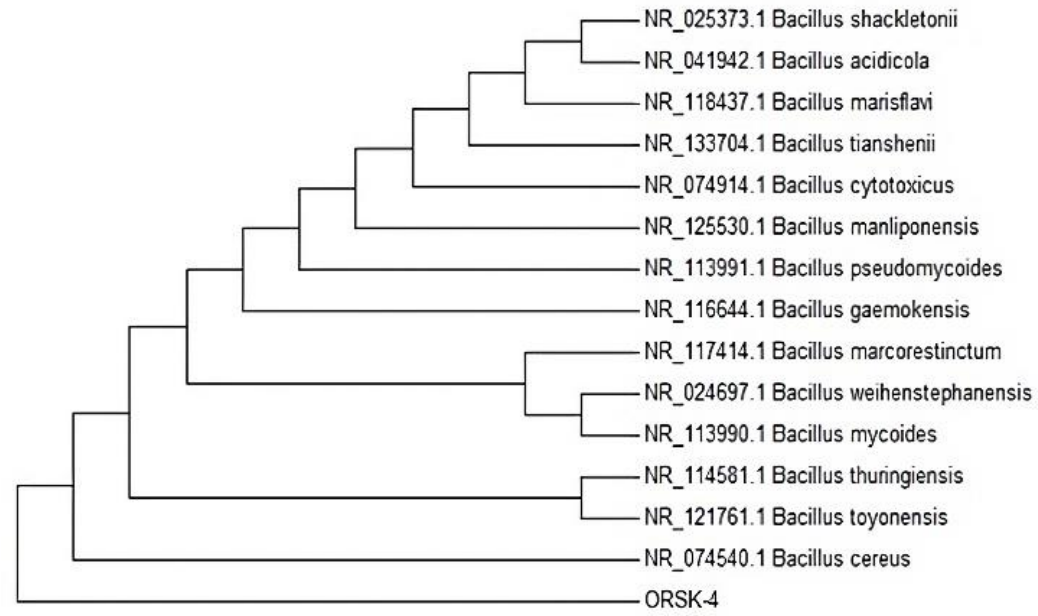

Fig. 5. Distance matrix results used to determine the pedigree of the isolate ORSK-4

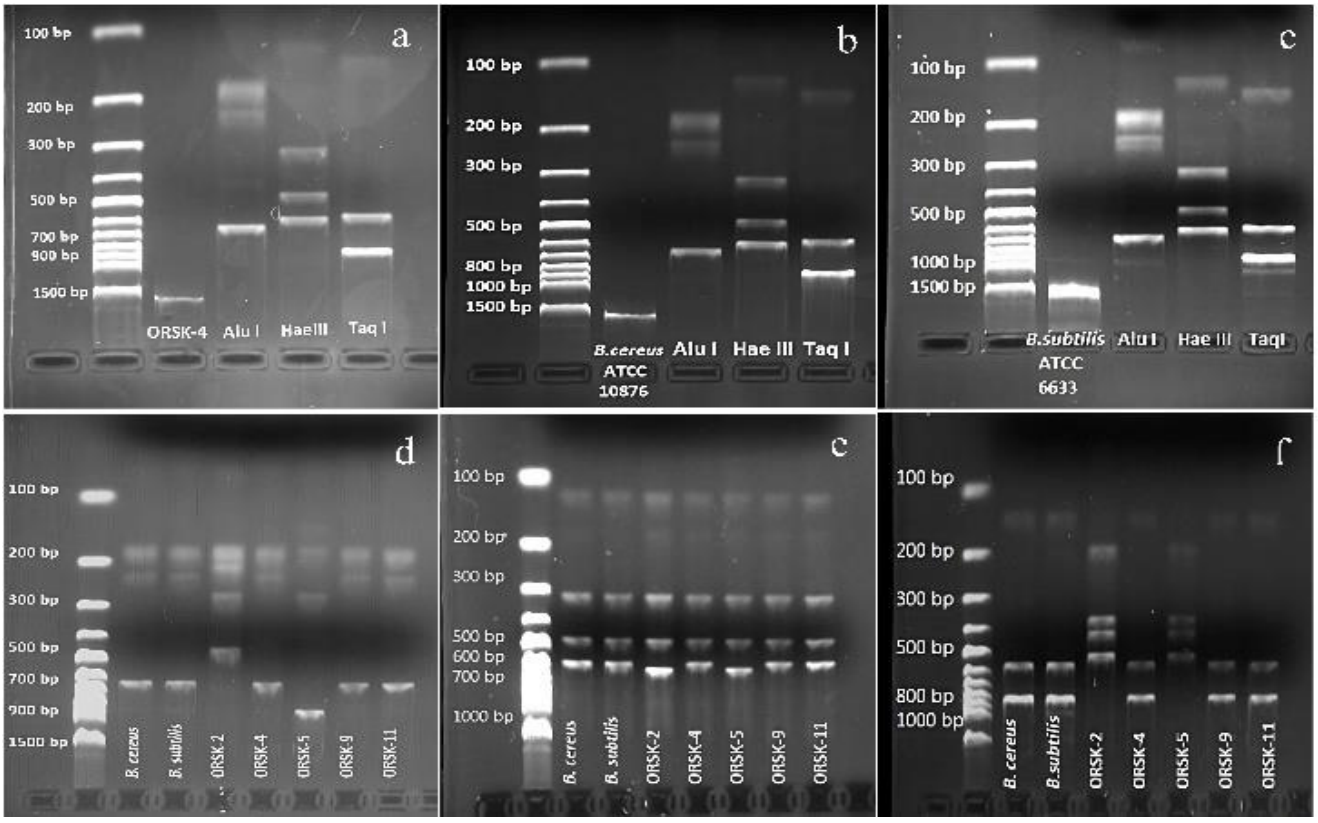

Fig. 6. a: ARDRA profile of ORSK-4 isolate cut by Alu I, Hae III and Taq I enzymes, b: ARDRA profile of *B. cereus* ATCC 10876 strain cut byAlu I, Hae III and Taq I enzymes cut, c: ARDRA profile of B. cereus ATCC 6633 strain cut by Alu I, Hae III and Taq I enzymes cut, d: ARDRA Profile of Alu I cleavage enzyme in reference strains and all isolates, e: ARDRA Profile of Hae III cleavage enzyme in reference strains and all isolates, f: ARDRA Profile of Taq I cleavage enzyme in reference strains and all isolates

16S rRNA gene region of *B. cereus* ATCC 10876 generated 3 bands (680, 240, and 180 base pairs) after cut by Alu I restriction enzyme, 4 bands (590, 480, 310, and 120 base pairs) after cut by Hae III restriction enzyme, and 3 bands (800, 560 and 140 base pairs) after cut by Taq I restriction enzyme (Figure 6b). 16S rRNA gene region of *B. subtilis* ATCC 6633 generated 3 bands (680, 240, and 180 base pairs) after cut by Alu I restriction enzyme, 4 bands (590, 480, 310, and 120 base pairs) after cut by Hae III restriction enzyme, and 3 bands (800, 560 and 140 base pairs) after cut by Taq I restriction enzyme (Figure 6c).

As a result of the cleavage of 16S rRNA gene regions of reference strains and isolate with Alu I restriction enzyme, isolate ORSK-4 showed the same cut profile as reference strains (Figure 6d). As a result of the cleavage of 16S rRNA gene regions of reference strains and isolate with Hae III restriction enzyme, that the isolate ORSK-4 showed the same cut profile as reference strains (Figure 6e). As a result of the cleavage of 16S rRNA gene regions of reference strains and the isolate (with Taq I restriction enzyme), ORSK-4 showed the same cut profile as reference strains (Figure 6f).

3.4. Optimum enzyme production conditions for strain ORSK-4

Different conditions were tested for the optimum protease activity of isolate ORSK-4 (Figure 7). Optimum protease enzyme activities of the isolate ORSK-4 on enzyme medium and N.B medium were measured, and the highest enzyme activity was found to be the enzyme medium at 27 °C and pH 7.0, and after 3 days of incubation.

It was seen that the partially purified protease enzyme obtained from ORSK-4 complies with the conditions characterized above. The effect of organic enzyme medium content changes on ORSK-4 protease enzyme activity is given in Figure 8A. As a result of the modification of organic compounds, the compound increasing the protease enzyme activity the most was the amount of peptone increased five times when compared to the control. The absence of the casein on the medium significantly reduced the protease enzyme activity. The effect of inorganic enzyme medium content changes on ORSK-4 protease enzyme activity is given in Figure 8B. As a result of the replacement of inorganic compounds, the compound increasing the protease enzyme activity the most was found to be the amount of K_2HPO_4, which was increased by 25.81% when compared to the control. The absence of $CaCl_2$ in the medium reduces protease activity by 39.78% compared to the control.

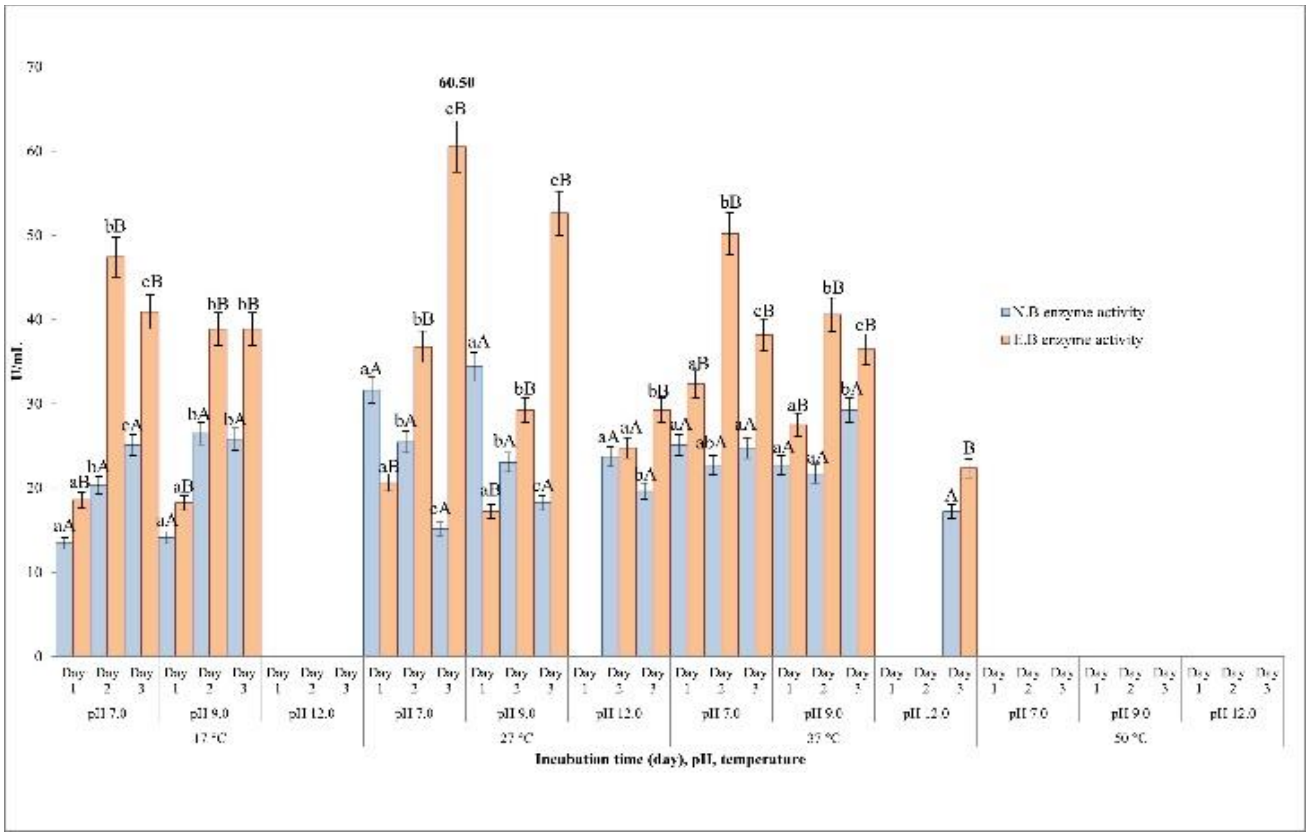

Fig. 7. Optimal protease activity of isolate ORSK-4 under different conditions. While different lower-case letters (a, b) on the graph show significant differences between different incubation times at the same temperatures, same pH ($p < 0.05$) and same medium, capital letters (A, B) indicate significant differences between different incubation times, same temperature and pH ($p < 0.05$) on different media.

Types of carbon and nitrogen sources affect the production of extracellular enzymes (Reddy and Kanwal 2022). The effect of the medium using only nitrogen source as an organic compound on ORSK-4 protease enzyme activity is given in Figure 9A. The use of 12 g/L casein in the medium prepared as the sole nitrogen source increased the protease enzyme activity by 71.91% when compared to the control. When only 5 g/L of urea was present in the medium, the protease enzyme activity decreased by 89.89% when compared to the control group.

The effect of different inorganic nitrogen sources on ORSK-4 protease enzyme activity is given in Figure 9B. Although there were no significant changes compared to the control when different inorganic nitrogen sources were added, a 12.50% increase in protease activity was observed with the addition of 0.1 g/L sodium nitrate.

The effect of different carbon sources on ORSK-4 protease enzyme activity is given in Figure 10. The effects of carbon sources on enzyme activity were investigated by using equal amounts of maltose, fructose, sucrose and starch instead of carbon sources on the medium. Adding glucose and fructose to the medium significantly decreased ORSK-4 protease enzyme activity while adding starch, lactose, sucrose, and galactose to the medium increased the protease enzyme activity of isolate ORSK-4.

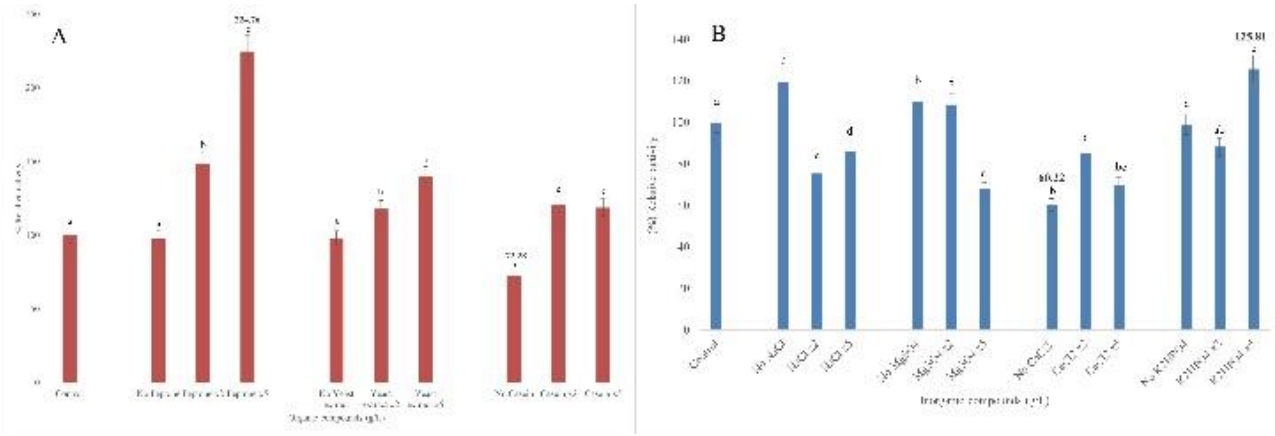

Fig. 8. A: Effect of content change in organic enzyme medium on the protease enzyme production of ORSK-4. Different letters (a, b, c) on the graph indicate significant differences between the control and the different organic compounds on the medium ($p<0.05$), B: Effect of inorganic enzyme medium content changes on the protease enzyme activity of ORSK-4. Different letters (a, b, c) on the graph indicate significant differences between the control and the different inorganic compounds on the medium ($p<0.05$).

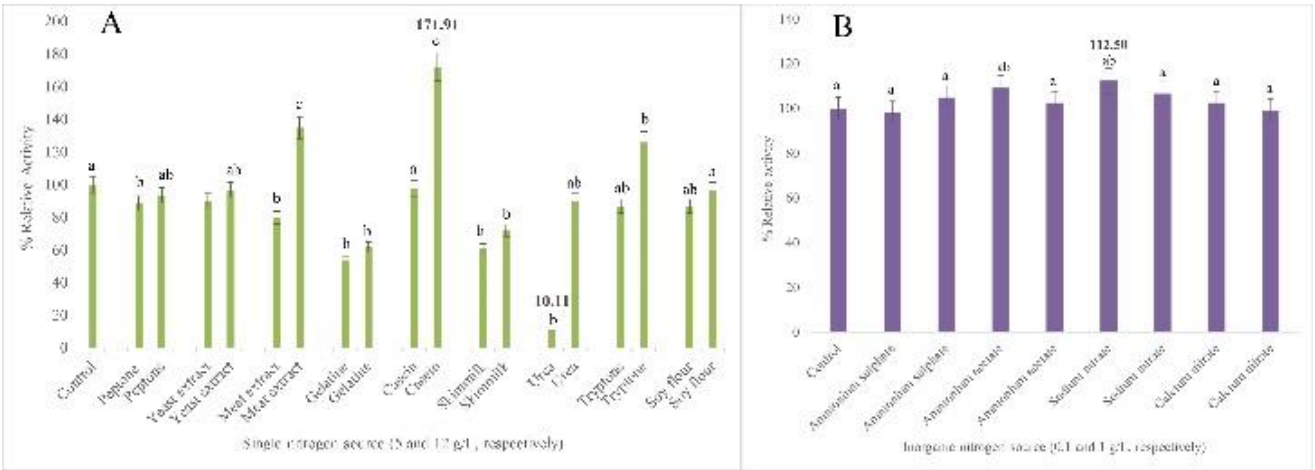

Fig. 9. A: The effect of the medium using only nitrogen source as organic compound on the protease enzyme activity of ORSK-4. Different letters (a, b, c) on the graph indicate significant differences between the control and the different nitrogen sources on the medium ($p<0.05$), B: Effect of different inorganic nitrogen sources on the protease enzyme activity of ORSK-4. Different letters (a, b) on the graph indicate significant differences between the control and the different inorganic nitrogen sources on the medium ($p<0.05$).

ORSK-4 protease enzyme activity on the optimized modified and meat-casein medium is given in Figure 10. The protease enzyme activity was measured approximately three folds higher in the optimized modified medium than in the normal enzyme medium (Figure 11A). The protease enzyme activity of ORSK-4 was measured approximately two folds higher in the meat-casein medium compared to the normal medium (Figure 11B).

3.5. Comparison of protease activity of some Bacillus genus microorganisms and isolated ORSK-4

A comparison of the protease activity of ORSK-4 isolated with *Bacillus cereus ATCC 10876* (Figure 12A) and *Bacillus subtilis ATCC 6633* (Figure 12B) strains is given in Figure 11. The enzyme activity of isolate ORSK-4 showed higher activity than the compared ATCC strains.

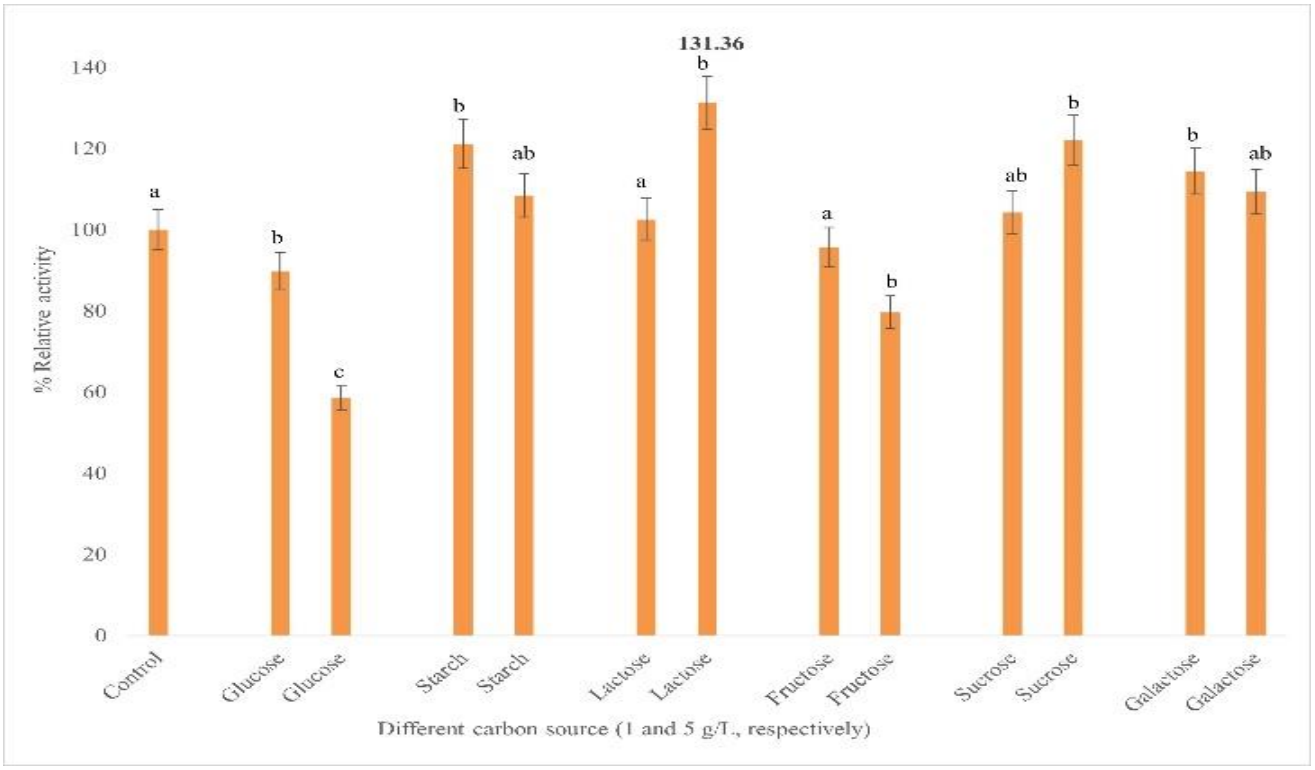

Fig. 10. Effect of different carbon sources on the protease enzyme activity of ORSK-4. Different letters (a, b, c) on the graph indicate significant differences between the control and the different carbon sources on the medium ($p<0.05$).

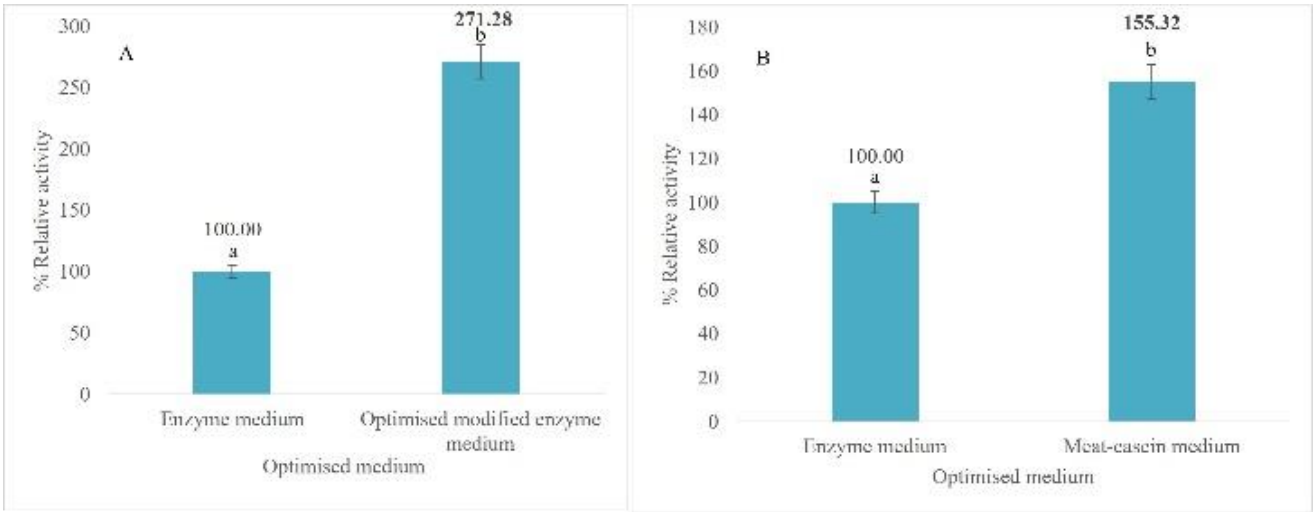

Fig. 11. ORSK-4 protease enzyme activity in optimized modified meat-casein medium. Different letters (a, b) on the graph indicate significant differences between enzyme medium and optimized modified media ($p<0.05$).

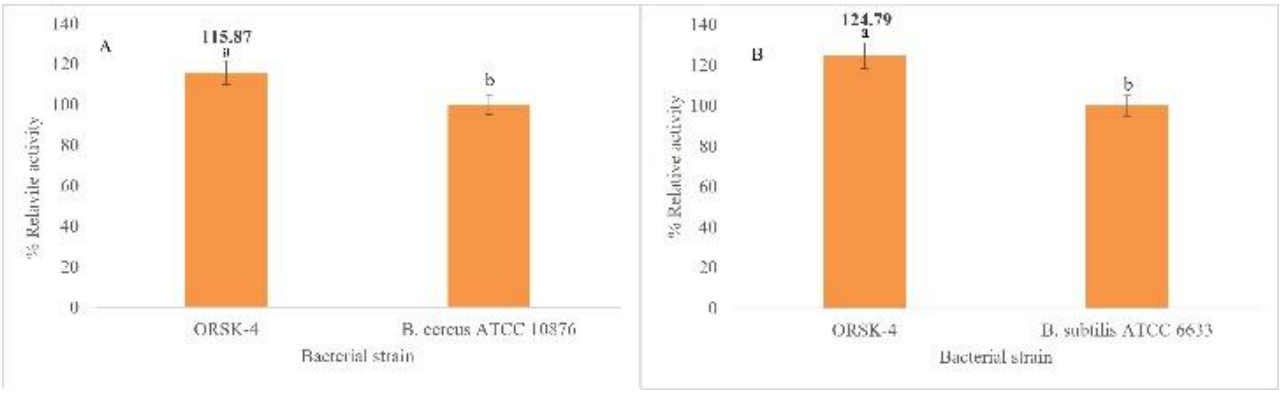

Fig. 12. A: Comparison of protease activity of strain ORSK-4 with *B. cereus* ATCC 10876. Different letters (a, b) in the graph indicate significant differences between ORSK-4 and *B. cereus* ATCC 10876 and *B. subtilis* ATCC 6633 protease activities ($p<0.05$), B: Comparison of protease activity of strain ORSK-4 with *B. subtilis* ATCC 6633.

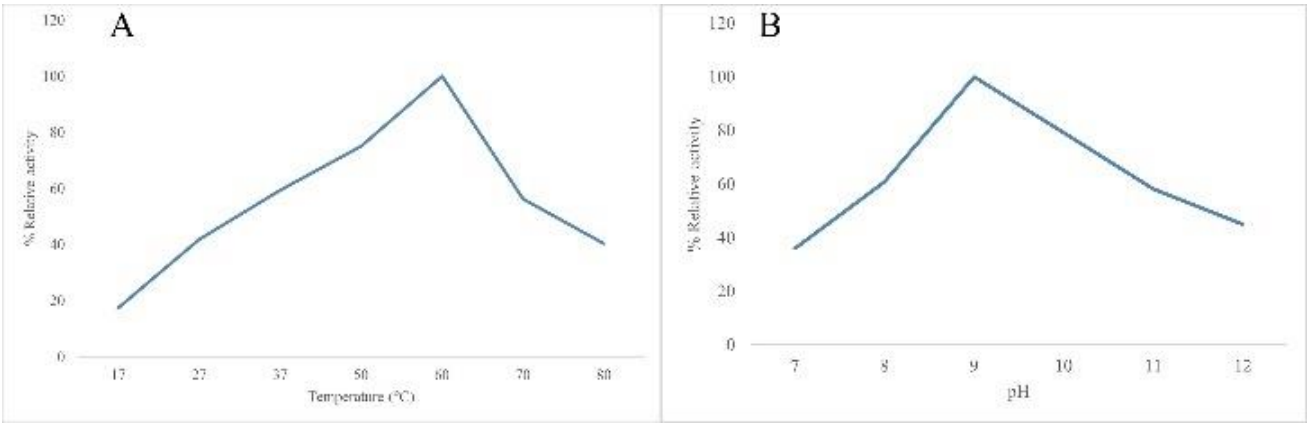

Fig. 13. A: Determination of the optimum working temperature of the protease enzyme, B: Determination of the optimum working pH of the protease enzyme.

3.6. Stability of protease enzyme obtained from isolated ORSK-4

The determination of the optimum operating temperature of the enzyme is given in Figure 13A. It was observed that the protease enzyme obtained from the isolated ORSK-4 increased until reaching 60 °C but decreased at 70 °C and 80 °C. While the ability to isolate ORSK-4 to produce protease was evaluated between 17 °C and 80 °C, and maximum enzyme production was observed at 60 °C, a decline in the production of enzyme was detected at temperatures above 60 °C, which is also an indicator for ORSK-4 isolate's heat-proof nature.

The determination of the optimum working pH of the enzyme is shown in Figure 13B. It was observed that the activity of protease enzyme obtained from isolated ORSK-4 increased up to pH 9.0 and then gradually decreased until reaching pH 12.0. This pH value demonstrates that the obtained enzyme is alkaliphilic.

It is known that pH and temperature are of great importance during the process of enzyme production by microorganisms. The optimum pH value for protease enzyme production was reported as 10.0 in a similar study. When the pH value was increased, a decrease in enzyme activity was observed (Verma and Pandey 2019). The effect of +1 and +3 valence metal ions on the enzyme activity is given in Figure 14A. 2 mM LiCl increased the protease activity by 33.79% compared to the control, while 10 mM $AlCl_3$ decreased the activity by 35.13%. It was observed that $Mn(SO_4)_2$, one of the bivalent metal ions, increased the enzyme activity by 64.37% compared to the control, while $Fe(NO_3)_2$ metal ions inhibited the activity by 81.61%. In one of their studies, Ullah et al. (2022) evaluated the enzyme activities based on the control group after the incubation with metal ions and found that Mg^{+2} and Ca^{+2} ions had a considerable stimulating effect on protease activities (Ullah et al. 2022). The effect of bivalence metal ions on the enzyme activity is given in Figure 14B.

The effect of inhibitors on enzyme activity is given in Figure 15A. In the presence of 10 mM EDTA, the enzyme's activity loss of up to 72.28% indicates that the protease is a metalloprotease. However, an inhibition rate of 33.66% in the presence of PMSF also indicates the presence of serine protease. While isolate ORSK-4 secretes a large amount of metalloprotease out of the cell, it also synthesizes serine protease.

The effects of surfactants, detergents and oxidants on enzyme activity are given in Figure 16A. When compared to the control group, it was observed that while 1% hydrogen peroxide increased the protease enzyme activity by 250%, 1% and 5% of Triton-X100 inhibited protease enzyme by 100%.

3.7. Partial purification of the protease enzyme obtained from ORSK-4 and the protein amounts

Available protease produced by ORSK-4 was partially purified with a combination of several steps (ammonium sulfate precipitation, dialysis, DEAE). Enzyme activities and protein amounts before and after ammonium sulfate precipitation are given in Table 3.

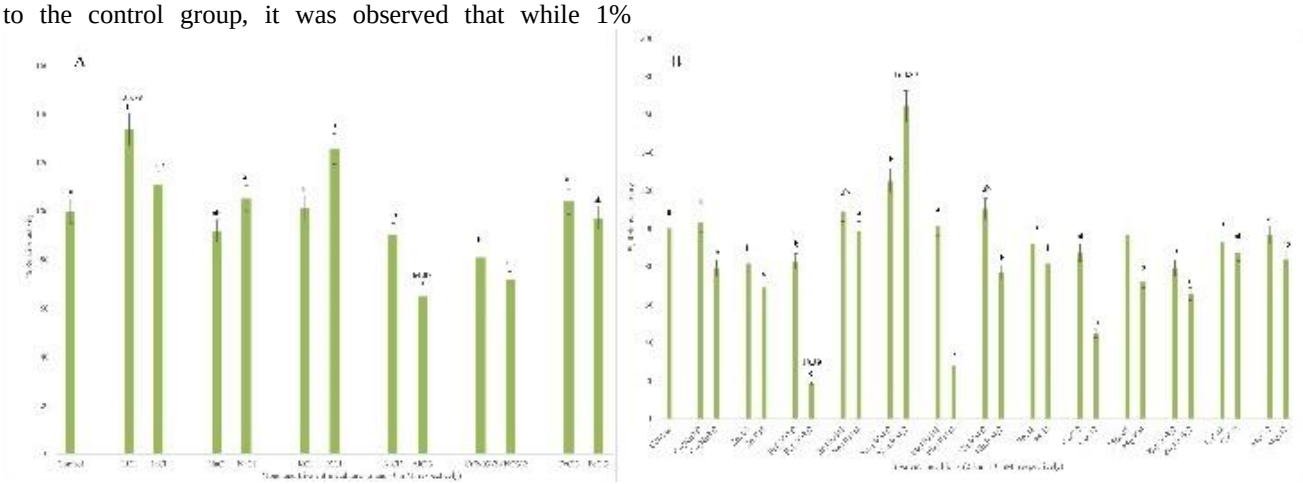

Fig. 14. A: Effects of +1 and +3 valence metal ions on enzyme activity. Different letters (a, b, c) on the graph indicate significant differences between the control and the medium containing +1 and +3 charged metal ions on the media ($p<0.05$), B: Effects of +2 valence metal ions on protease enzyme. Different letters (a, b, c) on the graph indicate significant differences between the control and the media containing +2 charged metal ions on the medium ($p<0.05$).

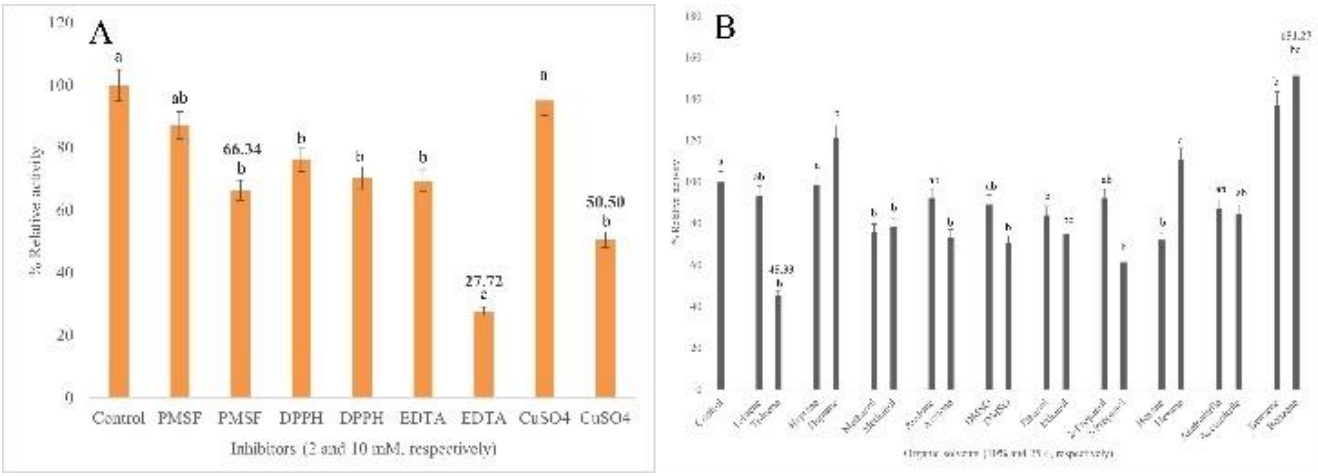

Fig. 15. A: Effects of inhibitors on protease enzyme activity. Different letters (a, b, c) on the graph indicate significant differences between control and media containing protease inhibitor ($p<0.05$), B: Effects of organic solvents on protease enzyme activity. Different letters (a, b, c) on the graph indicate significant differences between the control and the media containing organic solvents ($p<0.05$).

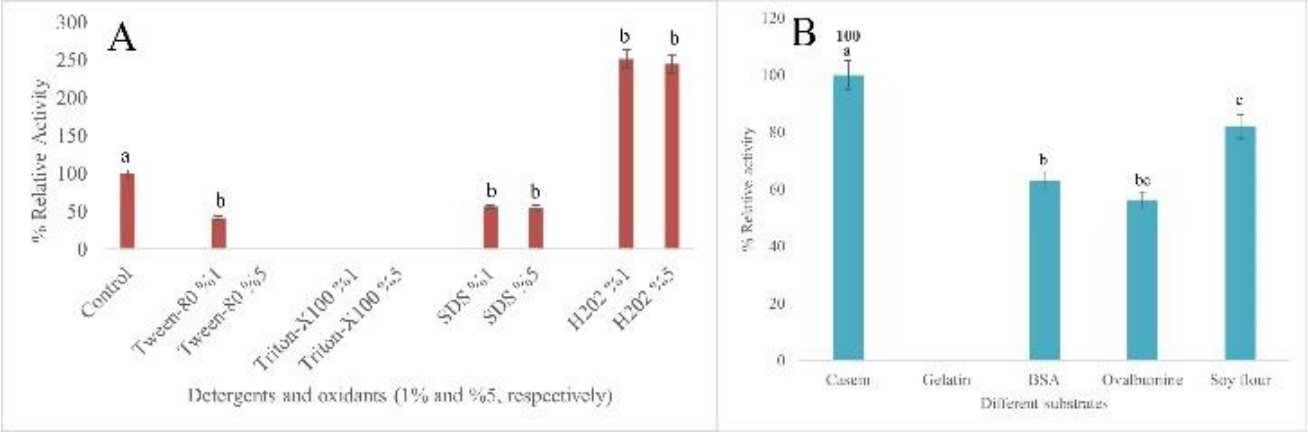

Fig. 16. A: Effects of surfactants, detergents and oxidants on enzyme activity. Different letters (a, b, c) on the graph indicate significant differences between the control and media containing surfactants, detergents and oxidants ($p<0.05$), B: Effects of different substrates on enzyme activity. Different letters (a, b, c) on the graph indicate significant differences between control and media containing different substrates ($p<0.05$).

Table 3. Summary of the purification step for the proteolytic enzyme from *B. cereus* ORSK-4

Purification steps	T.P. (mg)	T.A. (U)	S.A. (U/mg)	Recovery (%)	Purification
Crude extract	28.8	363.5	12.61	100	1
30% ASP	17.9	289.9	16.23	79.74	1.29
80% ASP	10.4	239.7	23.14	65.93	1.83
Dialysis	4.22	180.1	42.71	49.54	3.39
DEAE IEC	1.97	125.3	63.48	34.48	5.03

ASP (Ammonium sulphate precipitation), IEC (Ion exchange chromatography), T.P. (Total protein), T.A. (Total activity), S.A. (Specific activity)

The specific activity of the crude homogenate (12.61 U/mg) increased up to 16.23 U/mg and 23.14 U/mg respectively after 30% and 80% ammonium sulfate precipitation; up to 42.71 U/mg after dialysis; and up to 63.48 U /mg after ion exchange chromatography. It resulted in a yield of approximately 34.48% and a purification fold of 5.03. Verma et al. (2019) first applied 80% ammonium sulfate precipitation and then DEAE ion exchange chromatography for the partial purification of the protease enzyme, and it was found that the enzyme recovery was 77% while the purification layer was 1.66. Furthermore, enzyme purity increased by 1.75 times with 45% yield as a result of enzyme DEAE (Verma and Pandey 2019). After chromatography, SDS-PAGE was performed in the 2nd and 24th fractions. A band was observed in the 2nd fraction and the 24th fraction. The molecular weight of the protease in the second fraction was determined to be 20 kDa, and the protease in the 24th fraction was found to be 30 kDa. The SDS-PAGE execution image is given in Figure 17.

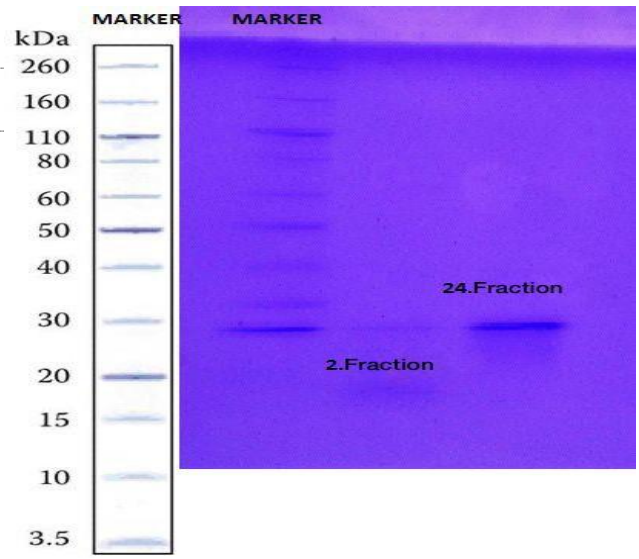

Fig. 17. SDS-PAGE execution

4. Discussion

Within the scope of this study, the protease-producing microorganisms in the soil sample taken from a high-protein region were purified, and the most suitable breeding and protease enzyme production conditions for these microorganisms were determined. Similar to the study carried out by Agasthya et al. (2013), the bacteria isolated from the soil and used in this study were grown on a skim-milk medium, and protease producers were selected based on their zone size and colony morphology differences (Agasthya et al. 2013). Some studies found that most of the bacteria isolated from the soil were *Bacillus* species (Liu et al. 2021; Lopez et al. 2023; Luang-In et al. 2019; Syed et al. 2007).

The maximum activity of the protease enzyme that is commercially significant was reported to be between pH 8-11 intervals. It is also known that many major proteases used in the detergent industry are between pH 7.0-11.0 interval (Hashmi et al. 2022). Therefore, the activity of the ORSK-4 protease enzyme was screened to determine whether it is suitable for both commerce and the detergent industry, and it was found that it maintained the activity within this pH interval. The fact that obtained protease showed its optimum activity at 37°C means *Bacillus cereus* is a mesophilic bacterial strain. On the other hand, a further increase in the temperature resulting in a decline in protease activity suggests that bacteria stopped producing enzyme because of molecular degradation. In a similar study carried out on the protease enzyme produced by *Bacillus* sp., It was found that the optimum temperature for the production of protease enzyme is 37°C (Abusham et al. 2009).

Because substances constituting the growth medium of the bacteria play a key role in biosynthesis and energy production, they are also of great importance for the production of alkaline proteases (Abu-Khudir et al. 2019). It was reported that culture conditions are likely to be a determinant of extracellular protease production by microorganisms. Thus, it became crucial to measure the activity of proteases under different conditions (Suberu et al. 2019). In the study conducted by Jadhav et al. (2020), it was found that the optimum incubation temperature for protease enzyme production of the genus *Bacillus* isolated from the soil was 30°C (Jadhav et al. 2020). Another study conducted by Varol et al. (2023) reported that the pH value in which the bacteria produce the enzyme at the maximum level was 7.0 and the optimum incubation period was 72 hours (Varol et al. 2023).

The pH of the culture medium plays a vital role in the transportation of the components enabling cell growth and product production through the membrane (Suberu et al. 2019). Proteases obtained from *Bacillus* sp. were characterized by their molecular weight of 27-71 kDa, optimum pH of 6-10, temperature of 37- 60 °C and stability values in a wide pH and temperature range (Karray et al. 2021). It was seen that the partially purified protease enzyme obtained from ORSK-4 complies with the conditions characterized above.

In the study conducted by Karray et al. (2021), nitrogen sources in the growth medium were changed to measure the changes in the enzyme activity obtained from *Bacillus stearothermophilus*, and the highest activity was observed in the yeast extract (Karray et al. 2021). Similarly, in another study carried out by Suberu et al. (2019), the effects of nitrogen source change on the activity of protease obtained from *Bacillus* were examined, and it was revealed that the best source of nitrogen for *cereus* ABBA1 and *B. subtilis* RD7 strains was beef extract that was followed by peptone and yeast extract (Suberu et al. 2019).

In a study done by Verma et al. (2019), the effect of different carbon sources such as galactose, lactose, maltose, sucrose and starch on the enzyme activity was investigated, instead of the original carbon source (Verma and Pandey 2019). As a result, a maximum level of enzyme production was found in lactose. Ahmetoğlu et al. (2015) reported that the use of lactose as a carbon source to produce protease gave the best results (Ahmetoglu et al. 2015). In another study carried out by Abu-Khudir et al. (2019), the effects of different nitrogen sources on the production of protease were tested on a medium containing yeast extract, ammonium sulphate, peptone, ammonium chloride, beef extract or urea, and the maximum level of enzyme production was obtained by adding yeast extract (229.03 U/ml) when compared to the control (189.04 U/ml) (Abu-Khudir et al. 2019).

Varol et al. (2023) examined the impacts of inhibitors on protease activity and observed that PMSF (a serine protease inhibitor) and EDTA (a metalloprotease inhibitor) administrations brought about a strong inhibition (Varol et al. 2023). Similarly, the relative activities of PMSF, CuSO4, EDTA at different concentrations resulted in a strong inhibition on the enzyme activity of 66%, 50% and 27% respectively, in our work. The effect of organic solvents on enzyme activity is given in Figure 14B. Heptane, hexane and benzene increased the activity of the protease enzyme, while other solvents decreased the activity of the protease enzyme. When compared to the control group, it was seen that 25% benzene increased the activity by 51,27%, while 25% toluene decreased it by 54.67%.

In one of their studies Hashmi et al. (2022) tried SDS, Triton X-100 and Tween 80 detergents on the protease enzyme obtained from *B. subtilis* S1 and *B. amyloliquefaciens* KSM12 strains, and obtained the lowest inhibition as % relative activity in SDS for *B. subtilis* S1 enzyme (50%) (Hashmi et al. 2022). As to this study, the relative activity obtained because of 1% and 5% SDS application was reported as 14% and 13% respectively. The effect of different substrates on enzyme activity is given in Figure 15B. While the highest protease enzyme activity was observed in the buffer containing casein, no protease activity was observed in the gelatin buffer.

Ullah et al. (2022) applied SDS-PAGE analysis to determine the molecular weight of the protease enzyme obtained from *Bacillus cereus* in their study, and reported that they obtained an enzyme of 35 kDa (Ullah et al. 2022). Besides, in another study performed by Hashmi et al. (2022), the molecular weight of partially purified protease enzyme (produced by *B. subtilis* and *B. amyloliquefaciens*) was found to be 17 and 65 kDa respectively by SDS-PAGE (Hashmi et al. 2022).

Optimizing protease production conditions requires a thorough understanding of microbial physiology, enzyme kinetics and fermentation processes. High yields of active proteases for various industrial applications can be achieved by carefully selecting microbial strains, designing appropriate growth media, controlling pH and temperature, optimizing oxygen transfer and adjusting fermentation parameters. Continued research and innovation in bioprocess engineering can enhance our ability to optimize

protease production and meet the growing demand for enzyme-based technologies.

5. Conclusion

In this study, the isolation of microorganisms producing protease enzyme, selection of appropriate strain for enzyme production, molecular identification of the strain, optimum enzyme production conditions, factors affecting enzyme production and enzyme stability, and enzyme molecular weight were identified by SDS-PAGE. Microorganisms isolated from alkaline protein-rich soil in the Yahşihan district of Kırıkkale province were used. The strain (ORSK-4) was active and stable even in the presence of various metal ions, inhibitors, surfactants, inhibitory agents, and organic solvents. These features supported that it could be a potential alternative in industrial applications.

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