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Production, partial purification, characterization, and application as laundry detergent additive of an alkalophilic protease from *Bacillus* sp. strain i51

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Abstract: A collection of 88 strains was subjected to the screening of proteolytic activity in solid medium. Thirty-six strains showed a halo of inhibition which correspond to the protease activity. The strain i51, isolated from wastewater at the EJM Company, which exhibited the largest halo of inhibition, was retained for further studies and assigned as *Bacillus* sp. based on physiological and biochemical properties and 16S rRNA gene sequencing. The study of the effect of carbon, nitrogen, and energy sources on the activity of the studied protease showed that the best activity of 4500 U/ml was obtained by combining the powdered milk and yeast extract. The characterization of the physico-chemical properties showed that the partial protease has an optimum activity at 50 °C and pH 8. The gene encoding for the studied protease was amplified by PCR and showed a size of 1.5 kb. The preliminary purification and SDS-PAGE analysis revealed that the studied protease possess a molecular mass of about 32 kDa.

Keywords: *Bacillus* sp.; Alkaline protease; Detergence formulations.

1. Introduction

Proteases represents one of the major groups of industrial enzymes and a number of detergent stable proteases have been isolated and characterized because of its widespread use in detergents. It is worthwhile to screen microbes from new habitats for proteases with novel

properties to meet the needs of rapidly growing detergent industry. High-alkaline serine proteases have been successfully applied as protein degrading components of detergent formulations and are subject to extensive protein engineering efforts to improve their stability and performance. Protein engineering has been extremely used to study the structure-function relationship in proteases and led to deeper understanding of the factors influencing the cleaning performance of detergent proteases. The aim of this work is to produce an alkaline protease having characteristics allowing its potential incorporation and application in the detergence industry.

2. Results and Discussion

Eighty-eight strains were isolated from wastewater of the "EJM" detergence company. They were subjected to a screening on solid nutrient agar milk media. This study showed that 36 strains possess a halo of inhibition. Among these, the one exhibiting the largest halo of inhibition was retained for subsequent studies **(Figure 1.)**. The taxonomic identification of this strain showed a PCR product of 1.5 kb corresponding to the size of the gene coding for the ARNr 16S **(Figure 2.)**. The sequencing of this gene showed that the studied strain was identified as *Bacillus* sp. The study of the effect of the cultivation period on the protease activity from *Bacillus sp.* i51 exhibited that the maximum protease activity was reached after 48 h of incubation **(Figure 3.)**. The same result was obtained in previous works for proteases from *Bacillus* sp. MIG [1] and *Bacillus subtilis*. PE-11 [2]. However, higher periods of incubation of 72 and 96 were needed to obtain a maximum proteolytic activity [3,4]. The study of the combination of different carbon and nitrogen sources on the protease activity from *Bacillus* sp. i51 revealed that the highest protease activity of 4500 U/ml was obtained using the powdered milk as a source of carbon and yeast extract as a source of nitrogen **(Figure 4.)**. Other combinations of carbon and nitrogen sources were used for the production of protease activity like starch, yeast extract and casamino acids [5] or peptone, yeast extract and casein [1]. The study of the different pH of culture on the protease activity from *Bacillus* sp. i51 showed that the most important protease activity was obtained at a pH of culture of 8 **(Figure 5.)** as it was reported for the proteases from *Bacillus* sp. Po2 [6]. The preliminary physico-chemical characterization of the protease from *Bacillus sp.* i51 revealed that the optimal pH is 8 **(Figure 6.)** and the optimal temperature is 50 °C **(Figure 7.)**. Proteases from several *Bacillus* strains have been described to have an optimal proteolytic activity at 50 °C [7] and pH 8 [4,8]. The protease from *Bacillus sp.* i51 was purified to homogeneity and exhibited a size of nearly 32 kDa and comparable to those from *Bacillus cereus* VITSN04 [8] and *Bacillus pumilus* UN-31-C-42 [9] **(Figure 8.)**. The amplification of the mature protease gene from *Bacillus* sp. i51 revealed that it has a size of about 1.5 kb **(Figure 9.)**.

Figure 1. Casein hydrolysis seen as zone of clearance around colonies on nutrient agar milk media

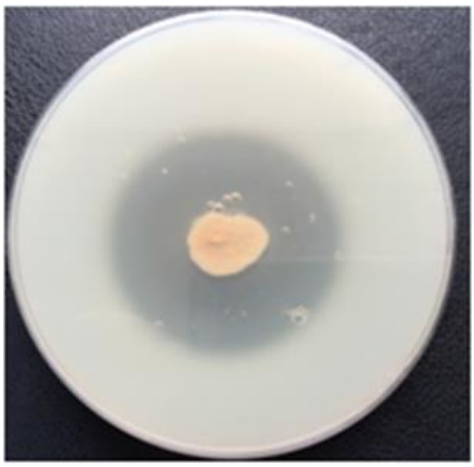


Figure 2. PCR amplification of the gene encoding the ARNr 16S.

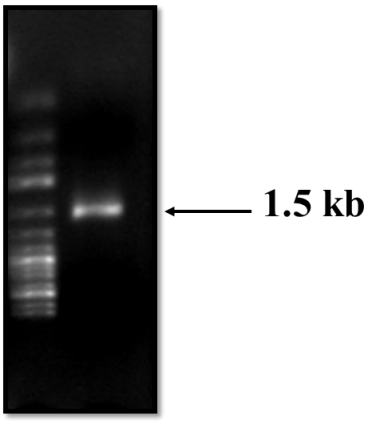


Figure 3. Effect of the cultivation period on the protease activity from *Bacillus* sp. i51

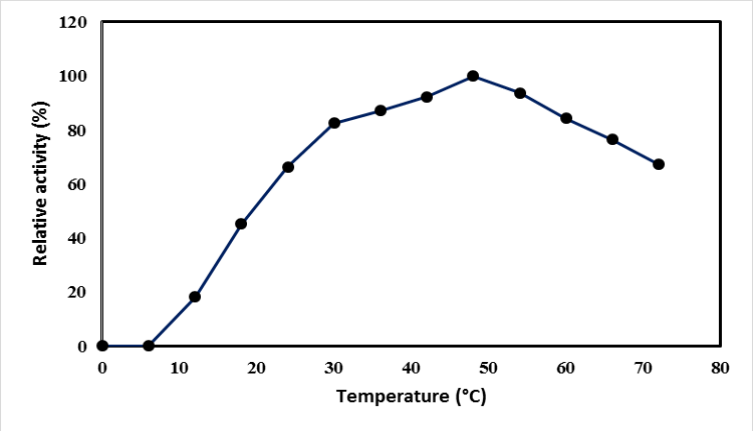


Figure 4. Effect of the combination of different carbon and nitrogen sources on the protease activity from *Bacillus* sp. i51

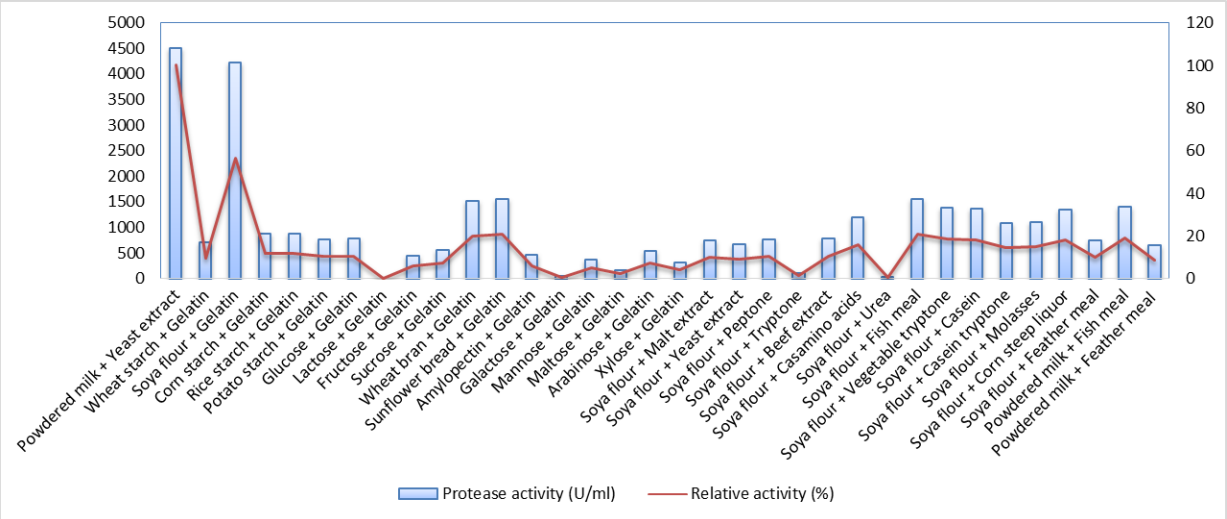


Figure 5. Effect of pH of the culture on the protease activity from *Bacillus* sp. i51

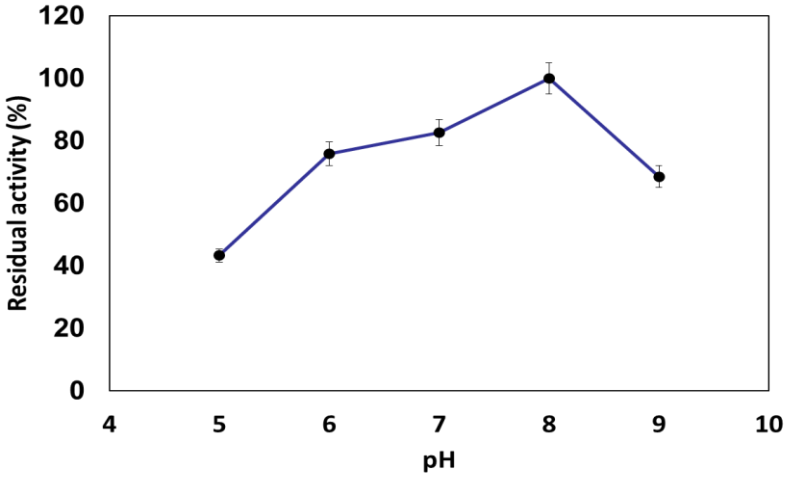


Figure 6. Effect of pH on the protease activity from *Bacillus* sp. i51

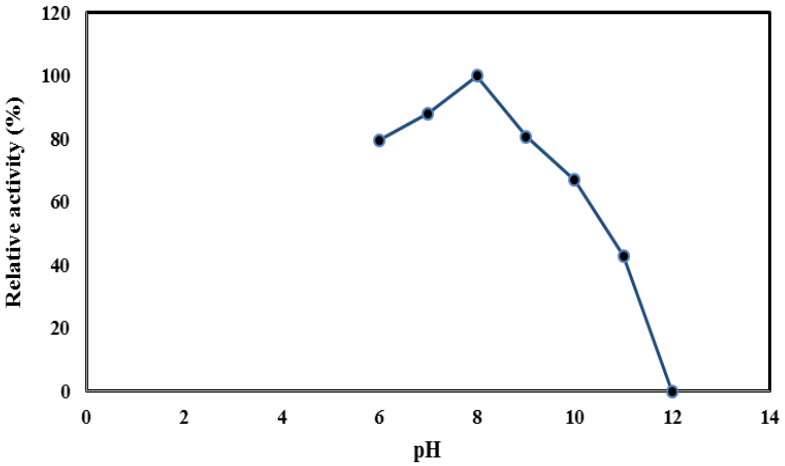


Figure 7. Effect of temperature on the protease activity from *Bacillus* sp. i51

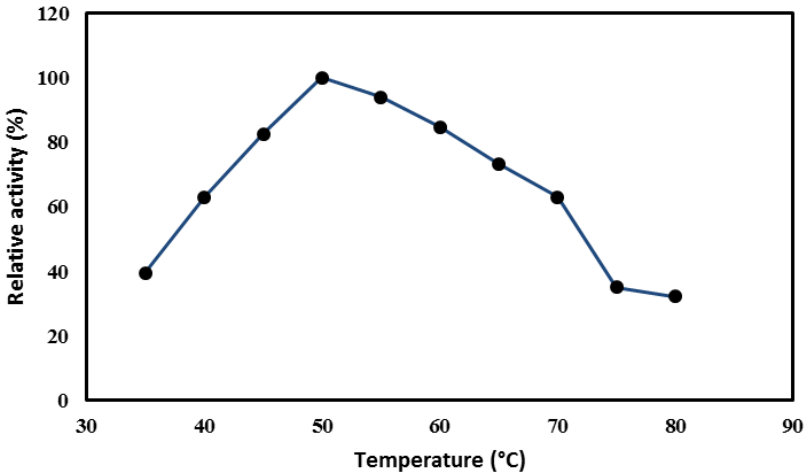


Figure 8. SDS-PAGE analysis of the protease from *Bacillus* sp. i51

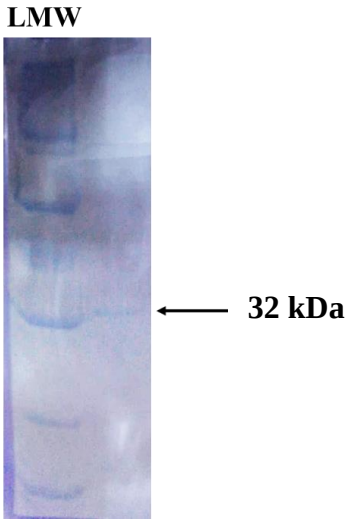
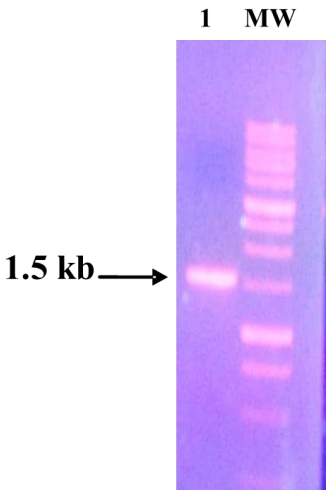


Figure 9. PCR amplification of the mature protease gene from *Bacillus* sp. i51



3. Materials and Methods

Isolation, screening and selection of microbial strain

1 g of wastewater sample from the "EJM" detergence company was dissolved in 10 ml of physiological water and then serially diluted, spread on LB broth petri dishes and incubated at 37 °C for 24 h to 48 h. The obtained colonies were screened for proteolytic activity on solid nutrient agar milk media (GNL). The inoculated GNL plates were incubated at 37 °C for 24 h and colonies were observed for the clear zone around the colony. Colonies with a maximum zone of clearance in their vicinity were picked up and subcultured for further studies.

Bacterial strain identification

The taxonomic identification of the retained strain was performed by the amplification and the sequencing of the gene encoding the ARNr16S.

Production of the protease

A culture of 100 ml containing the powdered milk and yeast extract with an initial OD_{600} of 0.1 and pH 8 was incubated at 37 °C for 48 h on a rotary shaker (200 rpm). After completion of 48 h, the cultivated media was centrifuged at 9000 rpm for 20 min and the clear supernatant was assayed for the proteolytic activity.

Estimation of proteolytic activity

The proteolytic activity was measured in the supernatant according to the protocol of Kembhavi *el al*., (1993) [10]. 0.5 ml of casein (10 g/l) was mixed with 0.5 ml of the enzymatic preparation previously diluted in Tris-HCl buffer pH 8. The enzymatic reaction was then incubated at 50 °C for 15 min. The reaction was stopped by the addition of 0.5 ml of TCA at 200 g/l. The mixture was incubated at room temperature for 15 min and then centrifuged at 15000 rpm for 15 min. The absorbance was evaluated at 280 nm.

Preliminary characterization of the protease

The influence of temperature on the proteolytic activity was performed by incubating the enzymatic reactions at different temperatures ranging from 30 to 80 °C while maintaining the pH at 8. The proteolytic activity was assayed as described above.

The effect of pH on the protease activity was investigated by varying the pH of the enzymatic reaction at 50 °C for 15 min (Tris-HCl buffer pH 6, 7 and 8; Glycine-NaOH buffer pH 9 and 10; Na_2HPO_4 buffer pH 11 and 12 at a concentration of 100 mM).

Purification of alkaline protease

A culture of 500 ml at pH 8 and incubated for 48h at 37 °C with a rotary shaker (200 rpm) and performed in the medium cited above was centrifuged at 9000 rpm for 20 min. The obtained supernatant was subjected to an ammonium sulfate fractional precipitation. The first precipitation was performed until 30% of saturation under a gentle agitation at 4 °C. After centrifugation for 20 min at 13500 rpm, the obtained supernatant has been added with ammonium sulfate until 65% of saturation under a gentle agitation at 4 °C. Thereafter, the mixture was centrifuged for 20 min at 13500 rpm and the obtained pellet was dissolved in a minimum buffer volume. The partially purified enzyme was then subjected to High Resolution size exclusion chromatography on Sephacryl S-200 column equilibrated with 50 mM Tris-HCl buffer pH 8. A 0.5 ml/min flow rate was applied to the column and 5 ml fractions were collected. The collected fractions were assayed for alkaline protease activity and protein content.

Estimation of protein content

The protein content of the samples were estimated according to the method of Bradford (1976) [11]. The absorbance was measured at 595 nm and BSA was used as a standard protein.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE analysis was performed according to Laemmli (1970) [12], for the determination of protein profile, molecular weights and purity of the samples. Briefly, the SDS-PAGE gel slabs were prepared with upper 5% stacking gel and lower 10% resolving gel in gel casting stands. The crude and purified samples were diluted in a native sample buffer solution and applied to the wells, resolved by applying a constant current across the gel. After the run, the resolved bands were visualized by Coomassie brilliant blue R-250 staining method. The molecular weights were estimated by comparing with standard broad range protein marker.

4. Conclusions

In this study, the obtained results are encouraging to continue in this path to demonstrate the ability of the protease produced from *Bacillus* sp. i51 to be applied in the detergence industry.

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Author Contributions

Conceived and designed the experiments: WB, BJ.
Performed the experiments: WB, SM.
Analyzed the data: WB, HR, NZJ, SB, BJ.
Contributed reagents/materials/analysis tools: NJ.
Wrote the text of the paper: WB, BJ.
Critical revision of manuscript: NZJ, SB, BJ.
Proofreading and polishing the language of the present paper: SB, BJ.

Conflicts of Interest

"The authors declare no conflict of interest".

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