

Physico: Chemical Characterization of Alkaline Protease Produce by *Bacillus Sp*

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Abstract:

In this study, Alkaline protease producing bacterial strains were isolated from various sample using skimmed milk agar. Among the isolates, Strain RE-1 shows highest protease activity which was identified as *Bacillus sp.* by partial 16S rRNA sequencing. The isolate showed maximum alkaline protease production (89.396 U/ml) at pH 8.0 and 30°C within 24 h of incubation. The enzyme exhibited thermal stability in the range of 40 to 70°C. Specific activity of partially purified enzyme is found to be 0.53 U/mg with 37.8% yield.

Keywords: Alkaline protease, Enzyme Assays, 16S rDNA, Industrial effluent.

1. Introduction:

Proteases are enzymes that break down proteins into polypeptides or amino acids. They make up 59% of the global industrial enzyme market. They're widely used in detergents, leather, food, and pharmaceuticals. Proteases are classified as acid, neutral, or alkaline based on their optimal pH. Acid proteases work best at pH 2.0-5.0, mostly from fungi. Neutral proteases, mainly from plants, function around pH 7.0. Alkaline proteases, often from microorganisms like *Bacillus sp.*, thrive at pH 8 and above, crucial in industries like detergents and pharmaceuticals. *Bacillus sp.* is widely distributed and can tolerate extreme conditions, making them valuable for industrial protease production [1]. Isolation and screening of *Bacillus sp.* from diverse environments can lead to the discovery of new proteases with unique characteristics. For instance, the *Bacillus sp.* CEMB 10370 produces a serine protease with optimal activity at pH 11.5 and 50°C.

2. Materials and Methods

2.1 Isolation and screening of alkaline protease producing bacteria:

Different samples namely soils, industry effluents, wastewater were collected from various source in and surrounding Surat City, Gujrat, India. One gram of soil or one milliliter liquid sample was added to a glass tube containing 10 mL sterile distilled water and serial dilution was carried out. 100 µL aliquot from diluted sample was spread on the sterile Skimmed milk agar plate. These plates were incubated at 30°C for 24 h. Colonies forming transparent zones, because of partial hydrolysis of milk casein, were selected. Purified cultures of selected isolates were streaked on Skimmed milk agar slants and stored at 4°C [5].

2.2 Biochemical and molecular characterization of selected isolate:

The isolates obtained from various sources were examined for colony characteristics, cell shape, Gram reaction. The following biochemical tests viz., Indole, Methyl red, Voges-Proskauer, Citrate utilization, Nitrate reduction, H₂S production, Urea hydrolysis, Starch hydrolysis, Sugar fermentation tests, Catalase

and Oxidase were carried out for biochemical characterization. Molecular identification of selected isolate was carried out by extracting genomic DNA. 16S rRNA sequencing reaction of PCR amplicon was carried out with using BDT v3.1 Cycle sequencing kit on ABI 3730*1 genetic analyzer. The partial sequence of 16S rRNA of bacterial isolates were amplified using 27F – 5'AGAGTTTGATCCTGGCTCAG-3' and 1391R – 5'GACGGGCGGTGGWTRCA3' as forward and reverse primer respectively. Identification of isolates were carried out by using BLAST (Basic Local Alignment Search Tool) with the database of NCBI (National Centre for Biotechnology Information) gene bank database based on maximum identity score [3].

2.3 Phylogenetic tree analysis of selected isolate:

The phylogenetic analysis of selected isolate was carried out on the basis of partial 16S rRNA sequence. The nucleotide sequence of related organisms was obtained from the NCBI database and used for alignment and calculating the homology level. Cluster W programme was used to align the sequences. The Phylogenetic tree was constructed by the neighbor-joining method using MEGA 11 (Molecular Evolutionary Genetics Analysis) software. Partial sequence of 16S rRNA of R1 was deposited in NCBI database using BankIt submission tool of Gene bank [2].

2.4 Fermentative production of alkaline protease:

Inoculum preparation:

The Inoculum was prepared by suspending pure colonies of 24 hours old culture of the selected isolate into sterile 10ml distilled water tube. Optical density of the suspension was set to 0.1 at 600 nm [8].

Production of alkaline protease:

The liquid 100ml production medium in a 250 ml Erlenmeyer's flask was inoculated with 1% Inoculum and incubated at 30°C in incubator at 24h. After incubation cells were separated by centrifugation at 10,000 rpm for 10 min at 4°C and supernatant was used to estimate protease activity [8].

2.5 Alkaline protease enzyme assay:

Protease activity was measured following the procedure by modified method of Keay and Wildi. The enzyme solution was added to 0.6% (w/v) casein solution (dissolved in 50 mM citrate buffer with pH of 7.6) and incubated at 30°C for 30 min. The reaction was terminated with the addition of 1 ml of 10% (w/v) Trichloroacetic acid and allowed to stand at 30°C for 30 min and centrifuged at 10,000 rpm for 10 min. Aliquots of 0.5 ml supernatant were mixed with 2.5 ml of 0.5 M Na₂CO₃ and 0.75 ml of Folin – Ciocalteu's reagent: water (1:2 v/v) and incubated at room temperature for 30 min in dark condition. The optical density of the solutions was determined at 610 nm and compared against a tyrosine standard curve. Zero-time blanks and controls without enzyme were also carried out for the study. One unit of enzyme activity was defined as the amount of the enzyme that liberates 1µg of tyrosine per min under the standard assay conditions [4].

2.6 Physio-chemical characterization of crude extracellular alkaline protease:

Determination of optimum pH:

The optimum pH for enzyme activity was determined by performing protease activity assay using buffer with different pH (6,7,8,9,10). For this purpose, 0.6% casein (substrate) used in the assay was dissolved in the following buffers: 0.1 M citrate buffer (pH 5–6), 0.1 M phosphate buffer (pH 7–8), 0.1 M Tris buffer

(pH 9) and 0.05 M borax buffer (pH 10). The enzyme and substrate mixtures were incubated at room temperature. After 30min, the reaction was terminated by adding 1 ml of 10% TCA. Then the protease activity was measured as described earlier [5] [7].

Determination of optimum temperature:

Protease activity was determined by incubating reaction mixtures containing crude enzyme at different temperatures (20, 30, 40, 50, and 60°C). Substrate solution was pre-warmed at the required temperatures before the addition of enzyme. After incubation, the enzyme activity was assayed [5] [7].

Determination of thermal stability:

Prior to conducting protease assay, 2X diluted crude enzyme was incubated at different temperature (30, 40, 50, 60 and 70°C) for 30min. after incubation at different temperature, enzyme was shifted immediately to ice and used for the determination residual protease activity [5] [7].

2.7 Partial purification of alkaline protease:

Ammonium Sulphate Precipitation:

The enzyme was partially purified by ammonium Sulphate fractionation. Selected isolates were grown as describe earlier under flask condition. After 24h, the cells were separated by centrifugation at 10,000rpm for 15minutes and supernatant was used as crude enzyme for precipitation using various percentage of ammonium sulphate saturation for precipitation. Activity was measured using above assay [1].

Dialysis:

About 10ml of the enzyme precipitate with ammonium Sulphate was taken out in a dialysis bag for dialysis. The bag was then dipped in 100 ml of 0.025M phosphate buffer and was kept for 24 hrs. for stirring on a magnetic stirrer. Purity of the enzyme was checked by calculating specific activity and fold purification [6].

3. Results and Discussion

3.1 Isolation and screening of alkaline protease producing bacteria:

Sample (soils, industry effluents, waste water etc.) collection was carried out from different sites in and around Surat, Gujrat, India for primary screening. 28 Isolates were screen by performing serial dilution technique on Skim Milk Medium from total 11 Samples on the basis of colonies showing clear zone of partial hydrolysis of milk Casein, All Isolates were maintained on minimal salt agar slants at 4°C for further study.

3.2 Results of biochemical and molecular characterization of isolates:

The results of biochemical characteristics of the selected isolates (RE-1) on the basis of enzyme activity are listed in Table 1. Isolate RE-1 is found gram positive rod shape bacteria.

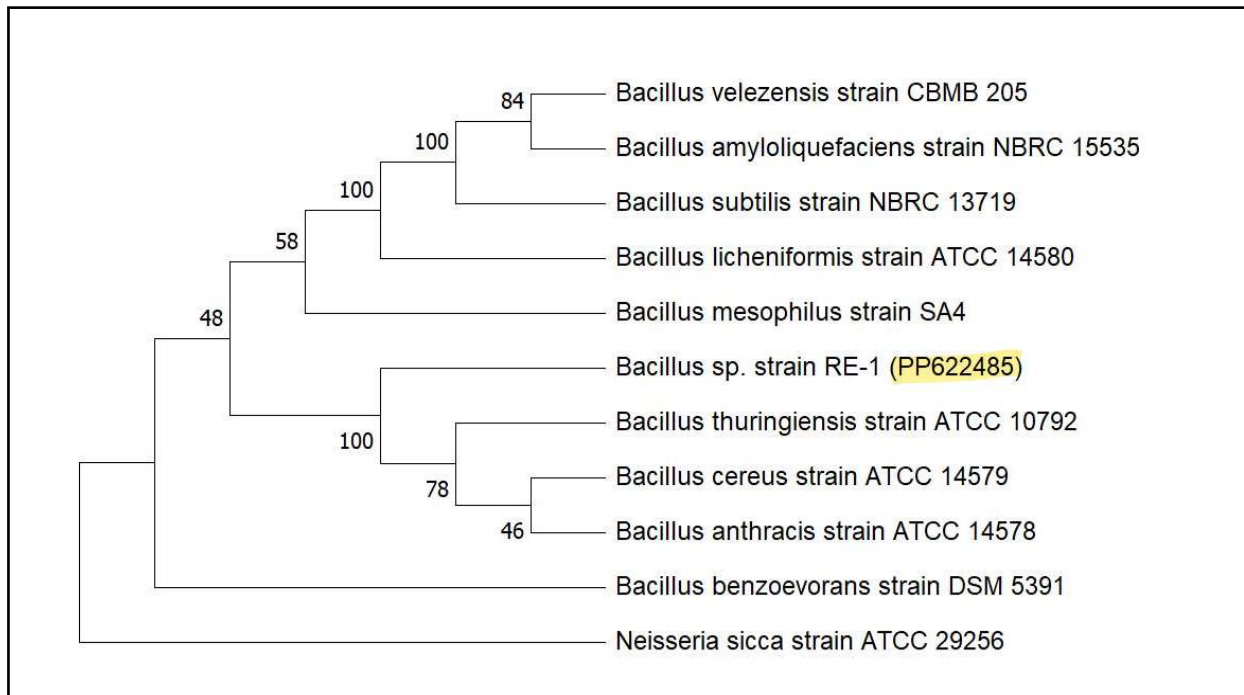
Table: 1 Biochemical Characterization of RE-1

Name of the Biochemical test	Result	Name of the Biochemical test	Result
Indole test	-	Nutrient Glucose broth	+
MR test	+	Nutrient Sucrose broth	+
VP test	-	Nutrient Lactose broth	-
Citrate utilization test	+	Nutrient Mannitol broth	+
Starch hydrolysis test	+	Nutrient Maltose broth	

Nitrate reduction test	-	TSI agar slant	
Urea hydrolysis test	-	Slant	Al
Catalase test	+	Butt	A
Oxidase test	+	CO ₂ Production	-
+ = Positive test, - = Negative test, A = Acid, Al = Alkaline,			

Isolate RE-1 was further identified by performing the 16S rRNA sequencing by using Sanger dideoxy sequencing method. The partial sequence of 16S rRNA of RE-1 is 710 nucleotide long. RE-1 was isolated from the pharmaceutical industry wastewater and identified as *Bacillus sp.* using BLAST tool (Basic Local Alignment Search tool) of NCBI (National Center for Biotechnology Information). This partial sequence of *Bacillus sp.* RE -1 has been submitted to NCBI having accession number PP622485. The phylogenetic tree was constructed for isolate RE-1 using MEGA software (version 11). Evolutionary tree analysis of isolate RE-1 along with set of organisms or group of organisms (taxa) was shown in Figure 1.

Figure 1: Phylogenetic tree of isolate RE-1



3.3 Alkaline Protease enzyme assay:

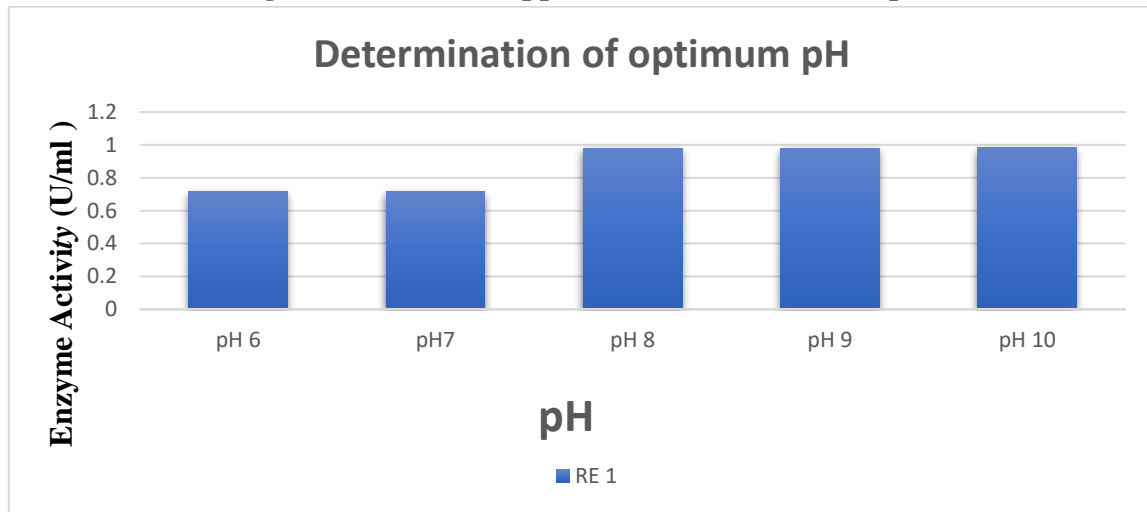
Enzyme activity was carried out by modified method of Keay and Wildi method using Tyrosine as a standard Casein as a substrate. shows the alkaline protease activity of all 28 isolates. Among the isolates RE-1 gave highest enzyme activity 89.396 U/ml respectively. So, these isolates were selected for further study.

3.4 Physio-chemical characterization of crude extracellular alkaline protease:

Determination of optimum pH:

RE-1 shows good activity between pH 8 to 10. This suggests that enzyme from isolates having good alkalophilic activity. (Figure 2)

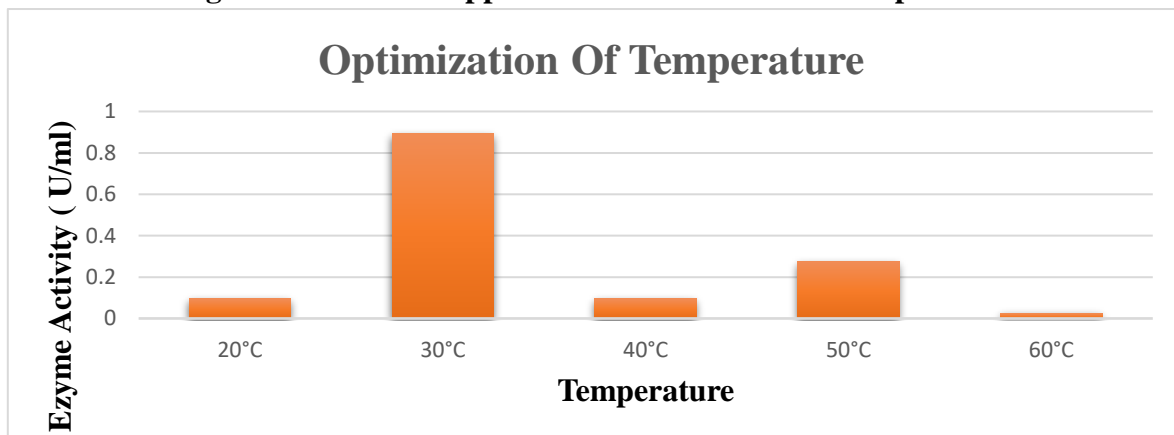
Figure 2: Effect of supplementation of different pH



Determination of optimum Temperature:

Protease from RE-1 gave highest activity at 30°C temperature. This suggests that enzyme from isolates having good alkalophilic activity (Figure 3)

Figure 3: Effect of supplementation of different Temperature



Determination of Thermal Stability:

Protease from RE-1 shows good thermal stability between 40-70°C. RE-1 shows good thermal stability at 40°C temperature. (Figure 4)

Figure 4: Effect of supplementation of different thermal stability

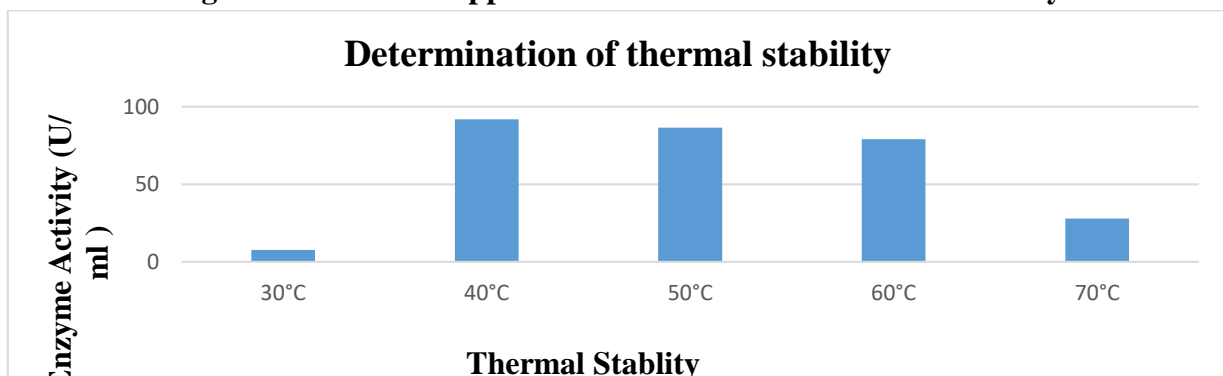


Figure (): Effect of supplementation of different thermal stability

3.5 Partial purification of alkaline protease:

Crude enzyme from *Bacillus sp.* RE-1 was partially purified by ammonium sulphate precipitation followed by dialysis to remove traces of salt. The results of specific activity and fold purification are shown in table 2.

Table : 2 Partial Purification of alkaline proteases enzyme (RE-1)

Enzyme Preparation	Volume (ml)	Protein (mg/ml)	Enzyme Units (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Yield	Fold Purification
Crude	100	23.53	63.05	630.5	0.53	100%	1
Ammonium sulphate Precipitation	10	76.16	45.18	451.84	0.59	71.66%	1.10
Dialysis	2	88.94	47.67	238.35	0.53	37.80%	1.13

Conclusion: Among the 28 isolates, RE-1 was isolated from the pharmaceutical industry effluent and identified as *Bacillus sp.* having highest enzyme activity 89.396 U/ml. Study on physio-chemical parameters of protease produce by RE-1 suggest that it has optimum activity in the alkaline range at 30°C. This isolate may be useful in several industrial applications owing to its thermo-tolerant and alkali-tolerant characteristics.

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