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MICROBIAL PRODUCTION OF PROTEASE AND ITS MEDIUM OPTIMIZATION FOR FOOD INDUSTRY APPLICATION

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Abstract: Proteases are one of the most extensively used industrial enzymes because they catalyze the breakdown of peptide bonds in proteins. Proteases are protein groups that belong to the subcategory hydrolases of the larger class enzymes. These hydrolytic enzymes are used in the food industry to improve nutritional content, digestibility, palatability, and flavor, reduce allergenic chemicals, and treat household and industrial waste. Proteases are an important class of enzymes, accounting for more than 65% of the overall industrial enzyme market. Proteases are utilized in a variety of industrial applications, including food, protein recovery, and meat tenderization Proteases are found in a wide variety of sources, including plants, animals, and microorganisms, however, microbial sources are favored for protease production due to technological and economic advantages. Members of the genus *Bacillus* were discovered to be the most prevalent and prolific source of protease among all microbes evaluated for usage in diverse industrial applications. Among several bacterial species, *Bacillus* is the most active in creating proteases. *Bacillus subtilis* is commonly used for protease enzyme isolation. The synthesis of protease by a bacterial organism isolated from soil was studied. Various biochemical tests were performed on the organisms, which led to their identification as *Bacillus subtilis*-generating protease enzymes. These bacteria were tested in a casein agar medium with casein as a substrate. The bacterial strain with the highest clear zone production was chosen for further optimization tests. Nutritional elements such as carbon and nitrogen sources, as well as physical parameters such as incubation time, pH, and inoculum size, were optimized for maximal protease output. The Plackett-Burman design was used to screen the cultivation factors. The current work aimed to establish the culture conditions that allowed protease production by the strain *Bacillus subtilis* as a locally isolated strain utilizing low-cost ingredients, and high yield as well as to investigate some features of the enzyme generated.

Keywords: Protease, *Bacillus subtilis*, Plackett-Burman design, Food application.

Introduction: Enzymes are well-known biocatalysts that are commercially used and execute a wide range of chemical reactions[1]. In order to have a high product yield and selectivity, various critical conditions for enzyme manufacturing must be met. There are three of them: (1) microorganisms, (2) medium composition, and (3) physicochemical factors (pH, temperature, and oxygen transfer). It is critical to select the appropriate microbe in order to obtain the desired product. There are several

microbial products utilized in industries today, and the globe is rapidly evolving to fulfill the needs of the food processing, pharmaceutical, and textile industries. Though enzymes from many sources were previously utilized for this purpose, microbial enzymes are now thought to be the most beneficial for a variety of reasons[2]. The majority of the 4000 enzymes known to be used in these sectors are of bacterial or fungal origin. This is due to the enormous diversity and ease with which bacteria produce enzymes. Microbes are a desirable source of protease enzymes due to their rapid proliferation and low space requirements for cultivation. 75% of the world's industrial enzyme sales are hydrolytic enzymes, with two-thirds being proteolytic enzymes[3]. Because proteases are very selective throughout the hydrolysis process, they are widely used in various industries, including food, laundry detergent, leather, and pharmaceuticals. Proteases are a type of enzyme that is widely employed in the industry[4]. Their catalytic role is to hydrolyze protein peptide bonds and break them into free amino acids. Proteases are found in all species and play a role in various physiological responses ranging from simple protein digestion to highly regulated cascades such as the blood clotting cascade. Because of their wide metabolic diversity and vulnerability to genetic manipulation, microorganisms are a valuable source of enzymes. Proteases are often categorized as endo or exoenzymes depending on where they operate on protein substrates. Depending on their catalytic mechanism, they can also be classed as serine, aspartic, cysteine, or metalloproteases[5]. According to Jellouli *et al.* (2009), microorganisms, particularly *Bacillus spp.*, create the majority of the commercially available proteases. Studies have shown that dietary components, such as sources of carbon and nitrogen, can affect the development of the protease enzyme by bacteria[6]. In addition to nutritional parameters, physical factors including inoculum concentration, temperature, pH, and the incubation period can all have a big impact on protease synthesis Since they display qualities like broad substrate specificity, substantial activity, stability, ease of downstream purification, quick fermentation times, and low cost, *Bacillus sp*. are among the best protease producers researched to date[7]. The bacterial proteases are best suited for a larger range of industrial applications due to their characteristics. The *Bacillus species B. sterothermophilus, B. cereus, B. megaterium, B. mojavensis*, and *B. subtilis* are among those that produce proteases[8]. *B. subtilis* has been demonstrated to be very responsive to genetic manipulation and has been widely used as a model organism for lab investigations[9]. Cellular morphology, growth conditions, endospore staining, gram staining, capsule staining, and biochemical tests were used to identify the isolated bacteria. The following ingredients were utilized in the media for the best protease enzyme production: Dextrose (1% w/v), casein (1%), yeast extract (0.5%), KH_2PO_4(0.2%), $MgSO_4.7H_2O$ (0.2%) and pH 8.0. The optimization of the basic media used for the generation of protease included consideration of several sources of carbon and nitrogen as well as physical factors like pH, temperature, salinity, inoculum size, and incubation time. The current study's objectives were to characterize and purify *B. subtilis* protease and maximize the conditions for extracellular protease synthesis. Application of proteases**:** In the production of industrial goods like food[10], pharmaceuticals, detergents, leather, dairy, brewing, managing industrial and household waste, photographic, silk degumming, proteases in peptide mapping and sequencing, baking process, meat tenderization, animal feeds, and other goods in industries, microbes are important sources that have included protease[11].

Materials and Methods

Microorganism and culture conditions: For the purpose of isolating bacteria, soil samples were obtained from the V.S.B Engineering College campus in Karur. The samples were serially diluted up to a 10^{-7} dilution, spread out on nutrient agar plates, and incubated for 24 hours at 37 °C. On the basis of visual and biochemical traits, including endospore staining, the colonies were recognized as *B. subtilis*[12]. On nutrient agar plates, colonies were subcultured and maintained to generate a pure culture, which was then transferred to a medium (pH 8.0) containing 1% Dextrose, 1% Casein, 0.55% Yeast Extract, 0.2% KH_2PO_4, 0.2% $MgSO_4 7H_2O$, pH 8 and incubated at 37 °C with constant shaking.

Characterization of Bacteria: Cellular morphology, growth conditions, endospore staining, gram staining, capsule staining, and biochemical tests were used to identify the isolated bacteria[13].

Protease Assay: The culture broth was centrifuged and the supernatant was used for further analysis[14]. Tubes were filled with casein (5 ml) and enzyme (1 ml) and kept at room temperature for ten minutes. Add 5 ml of 110 mmol of trichloroacetic acid, and then incubate for 30 minutes at 37 °C. Add 5 ml of sodium carbonate and 1 ml of Folin's reagent, and incubate for 30 minutes at room temperature. Read the spectrophotometer's OD reading at 660 nm.

Effect of pH on protease production: The culture broth was centrifuged and the supernatant was used for further analysis. The prepared buffer and casein (0.2 g) were combined & enzyme(1ml) was added to four beakers, and they were left at room temperature for ten minutes[15]. Add Trichloroacetic acid (5ml) 110mmol and incubate at 37°C for 30 minutes. Add Sodium carbonate (5ml) & Folin's reagent(1ml) & incubated at room temperature for 30 mins. Read the OD value at 660nm in the spectrophotometer.

Effect of various temperatures on protease production: The culture broth was centrifuged and the supernatant was used for further analysis. The prepared buffer and casein (0.2 g) were combined & enzyme(1ml) was added to four beakers, and they were left at various temperatures like 23,33,43,53°C for ten minutes[16]. Add Trichloroacetic acid (5ml) 110mmol and incubate each sample at various temperatures like 23,33,43,53°C for 30 minutes. Add Sodium carbonate (5ml) & Folin's reagent(1ml) & incubated at room temperature for 30 mins. Read the OD value at 660nm in the spectrophotometer.

Substrate concentration: Casein (0.2 g) with different concentrations was combined & enzyme(1ml) was added to four beakers, and they were left at room temperature for ten minutes. Add Trichloroacetic acid (5ml) 110mmol and incubate each sample at room temperature for 30 minutes. Add Sodium carbonate (5ml) & Folin's reagent(1ml) & incubated at room temperature for 30 mins. Read the OD value at 660nm in the spectrophotometer. Substrate concentration will be identified using Michaelis–Menten kinetics[17].

Media Optimization: The following ingredients were utilized in the media for the best protease enzyme production[18]: Dextrose (1% w/v), casein (1%), yeast extract (0.5%), KH_2PO_4(0.2%), $MgSO_4.7H_2O$ (0.2%) and pH 8.0. The optimization of the basic media used for the generation of protease included consideration of several sources of carbon and nitrogen as well as physical factors like pH,

temperature, salinity, inoculum size, and incubation time.

Plackett-Burman design: R.L. Plackett and J.P. Burman published "The Design of Optimal Multifactorial Experiments" in Biometrika (vol. 33) in 1946. This study described how to build very inexpensive designs with run numbers that are multiples of four (rather than powers of two). When only the major effects are of interest, Plackett-Burman designs are particularly efficient screening designs[19]. These designs have run numbers that are multiples of four. Plackett-Burman (PB) designs are employed for screening tests because the main effects are severely confounded by two-factor interactions in a PB design. For an experiment with up to 11 factors, the PB design with 12 runs, for example, might be utilized. However, these designs are very useful for detecting large main effects economically, assuming all interactions are negligible in comparison to the few important main effects. Response Surface Methodology is frequently used to increase product yield, decrease development time, and lower overall process costs.

Statistical Experiment Design: Response surface designs for central composites are those that can match a complete quadratic model. Imagine a central composite design where there are a number of variables that can have low or high values. For ease, let's assume that each factor has a range of -1 to +1. One central composite design consists of center points at the origin, star points along the axes at or outside the cube, and cube points at the corners of a unit cube that is the product of the intervals [-1, 1]. The whole experimental design is shown in table, together with the achieved values for the response (protease activity) and the real values of the independent variable.

Statistical analysis: The same software was used to gather the data, and regression analysis was done on it. The derived Second-order polynomial model was then fitted to the data[20]. As a result, we were able to develop an empirical model that connected the measured response to the experiment's independent factors. The proposed model equation predicts the response as a function of the various values of independent variables; the significance of each coefficient of the resulting model was assessed using the p-value. ANOVA was used to perform the analysis of variance. The proportion of variance, which is represented by the coefficient of multiple determinations R^2, was used to assess the suitability of the polynomial model. The fitted polynomial equation was shown as surface plots to show the link between the response and experimental levels of each factor and to determine the concentration of each factor for maximum protease synthesis. The interactions between the three variables were examined using Stat-Ease Inc.'s Software Design Expert Version 8.0.6, which is based in Minneapolis, USA. The same programme was used to create surface plots. Experimental testing was done to verify the model using combinations of several optimized parameters that produced the most protease.

RESULT AND DISCUSSIONS

The soil samples were collected from the campus of V.S.B Engineering college, karur and used for the isolation of bacteria. Serial dilution was carried out and after being serially diluted between 10^{-5} to 10^{-7}

times, these were spread onto media and incubated at 32°C for 48 hours. The morphological character of *Bacillus subtilis* was identified by gram staining. Shape - rod , Color – Purple, Gram staining - positive

Figure.1: Soil collection

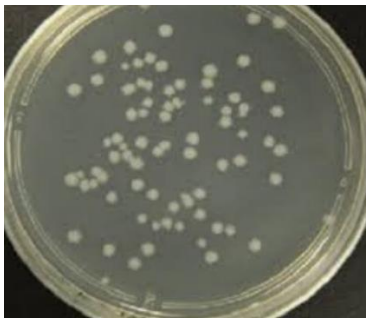

Figure.2: Isolation of Bacteria

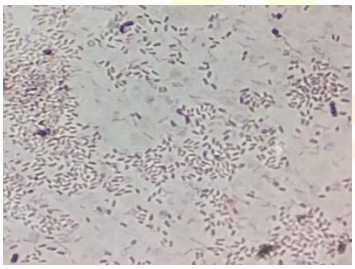

Figure.3: Gram staining

Table.1: Biochemical test

Biochemical test	Results
Catalase test	Positive
Citrate utilization test	Positive
Indole test	Negative

Protease assay: The modified Folin's method was used to conduct the alkaline protease assay. In a buffer solution of 50 mM glycine and 10.0 mM sodium hydroxide, 0.5% casein was dissolved. The reaction mixture was then incubated at 80 °C for 30 minutes. The addition of 10% TCA then halted the reaction. OD was measured at 660 nm and the amount of released tyrosine was calculated using the Folin's reagent. The amount of protease needed to release 1 mg of tyrosine under test conditions in 1 minute was the unit of protease.

Effect of pH on protease production: The pH of the fermentation medium has a significant impact on the enzyme's productivity in culture. As a result, the impact of pH (6.0 to 8.0) on the generation of protease by *Bacillus subtilis* was investigated (Table.2). Protease synthesis gradually increased from pH 6.0 to 7.2, with pH 6.6 showing the highest levels of enzyme production (Figure.4).

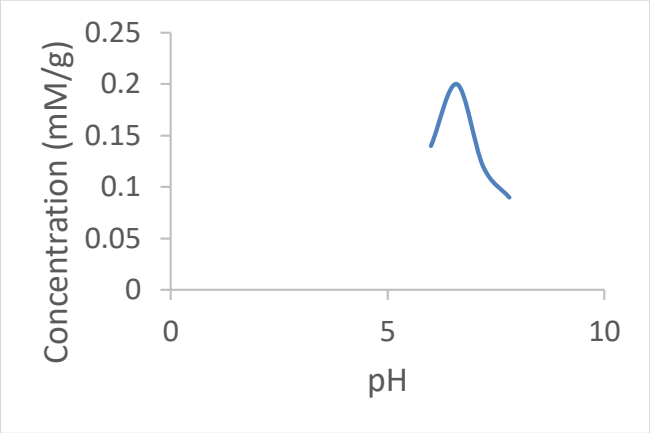

Figure.4: Effect of pH on protease activity

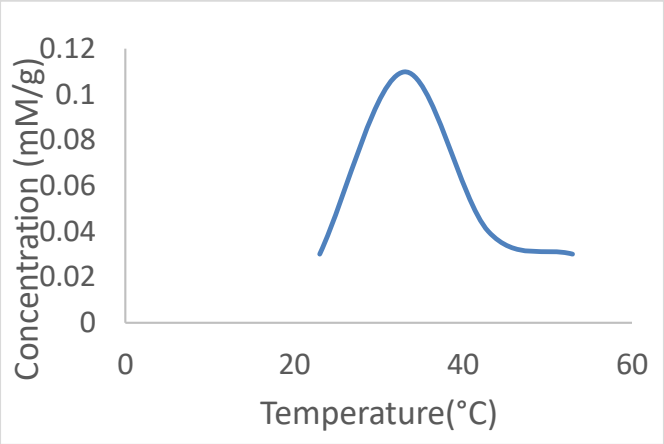

Figure.5: Effect of various temperature on enzyme activity

Effect of various temperatures on protease production: The medium was maintained at various temperatures to help with Protease formation (Table 3). For the generation of protease, 33 °C was determined to have the highest protease activity (Fig.5). The organism's preferred temperature depends on the type of organism. Numerous studies demonstrated that bacillus strains prefer lower and moderate temperatures (23–53 °C) for the production of protease [23, 25, 28, 33]. Aeration, pH, and medium composition are three critical factors that have an impact on enzyme synthesis.

Substrate concentration: Utilizing various substrates, including Casein, kcl, and Yeast extract, the impact of various substrates on protease activity was evaluated. Casein demonstrated the highest level of protease activity, whereas yeast extract and kcl showed lower levels than casein of activity for each of the other substrates.

Table.4: Substrate concentration

Substrate concentration		OD	Concentration (p)	r=p/t	1/r	1/s	s/r	r/s
0.2	2	0.15	0.14	0.014	71.42	0.5	142.85	0.007
0.4	4	0.28	0.26	0.026	38.46	0.25	153.84	0.0065
0.6	6	0.31	0.29	0.029	34.48	0.166	206.89	0.0048
0.8	8	0.26	0.24	0.024	41.66	0.125	333.33	0.003
1	10	0.19	0.18	0.018	55.55	0.1	555.55	0.0014

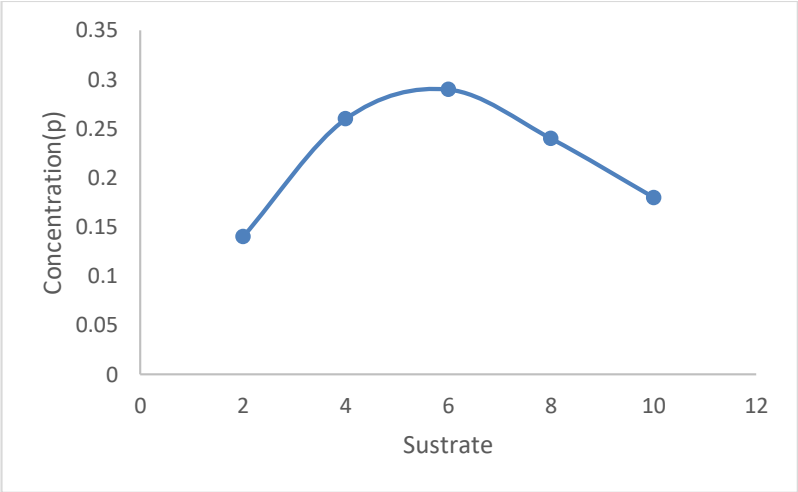

Figure.6: Substrate concentration

Table.5: Plackett-Burmen method

TRIAL	VARIABLES							Yield (Mm/g) Protease
	Dextrose	Peptone	KH_2PO_4	$MgSO_4$	Casein	Yeast extract	KCL	
1	H	H	H	L	H	L	L	0.25
2	L	H	H	H	L	H	L	0.40
3	L	L	H	H	H	L	H	0.47
4	H	L	L	H	H	H	L	0.65
5	L	H	L	L	H	H	H	0.68
6	H	L	H	L	L	H	H	0.55
7	H	H	L	H	L	L	H	0.28
8	L	L	L	L	L	L	L	0.13

Media Optimization: A Plackett-Burman design was used to examine the effects of a total of seven variables on protease production (Table 5). It displays the design matrix chosen for the screening of relevant variables for protease production together with the appropriate responses. The model's suitability was assessed, and using the ANOVA, the variables showing statistically significant impacts were identified (Table 8).

Table.6: Result for placket burmen table

	Dextrose	**Peptone**	**KH_2PO_4**	**$MgSO_4$**	**Casein**	**Yeast extract**	**KCL**
∑(H)	1.73	1.61	1.67	1.8	2.05	2.28	1.98
∑(L)	1.68	1.8	1.74	1.61	1.36	1.13	1.25
∑(H)-∑(L)	0.05	0.19	0.07	0.19	0.69	1.15	0.73
Effect	0.0125	0.0475	0.0175	0.0475	0.1725	0.2875	0.1825
Mean Square	0.0003125	0.0045125	0.0006125	0.0045125	0.0595125	0.1653125	0.0666125
F-Test	0.002694	0.038913	0.005281	0.038913	0.51320	1.42557	0.57443

Table.7: Box-Behnken design

Yeast Extract	KCL	Casein	Protease Activity	
			predicted	Actual
-1	1	0	0.4537	0.3175
1	0	1	0.3175	0.4266
0	0	0	0.4266	0.2562
0	-1	1	0.2562	0.4937
0	1	-1	0.4937	0.4512
1	1	0	0.4512	0.5787
-1	-1	0	0.5787	0.7887
0	-1	-1	0.7887	0.6825
-1	0	-1	0.6825	0.6312
0	1	1	0.6312	0.4266
0	0	0	0.4266	0.335
1	0	-1	0.335	0.4266
0	0	0	0.4266	0.2462
1	-1	0	0.2462	0.305
-1	0	1	0.305	0.24

Response Surface Plots: The optimum point on the response form a three-dimensional surface plot is located on the highest point. It was clear that the provision of the concentration of Casein, KCl, and Yeast extract higher or lower as well as changes in pH and volume of inoculum will affect the increase in protease activity.

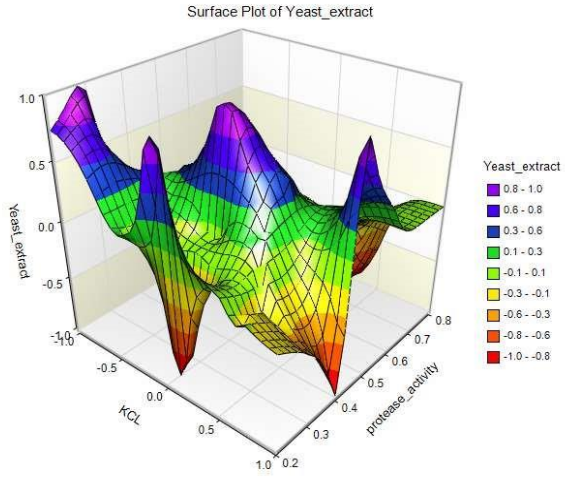

Figure.6: 3D Surface plot of Yeast Extract

Figure.6 shows that protease activity increase with the increase in reaction yeast extract and kcl.

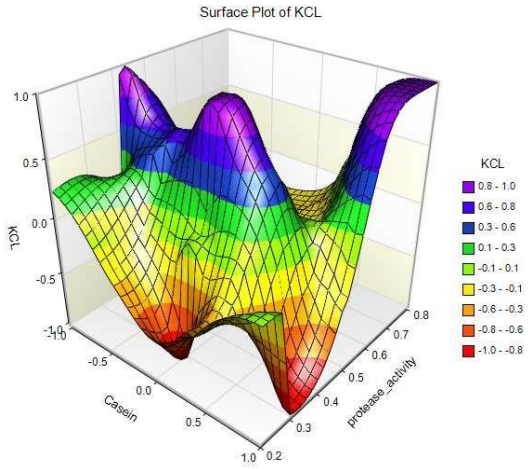

Figure.7: 3D Surface plot of KCL

Figure.7 shows that protease activity increase with the increase in reaction kcl and Casein.

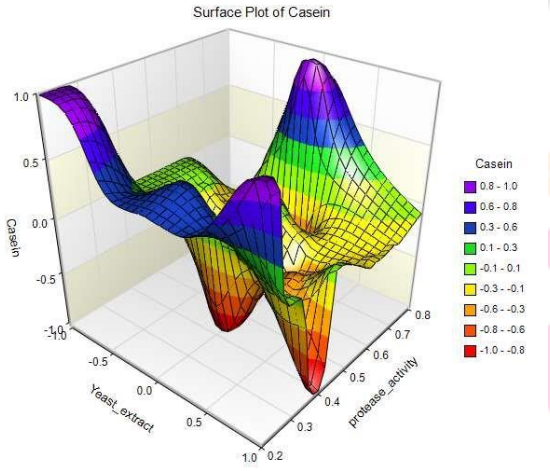

Figure.8: 3D Surface plot of Casein

Figure.8 shows that protease activity increase with the increase in reaction Casein and Yeast Extract

The response surfaces were chosen among the possible combinations to visualize the simultaneous effects of Yeast extract, kcl and Casein on protease production patterns

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The experimental design and results are shown in Table 7. The quadratic model calculated (using NCSS) for maximum protease yield after eliminating the statistically insignificant terms was:

$y=0.4266667-0.08375*A+0.02*B-0.09875*C-0.06333333*A^2+0.06916667*B^2+0.04666667*C^2+0.0825*A*B+0.09*A*C+0.1675*B*C.$

Analysis of Variance Table: The ANOVA (obtained by StatistXL) reproduced in Table 8 showed that the model was significant. The F-test was greater than the table value of 2.82 and the p-value of 0.63115 clearly reveals that this regression was statistically significant at the 95 % confidence level. Further, the R^2 of the regression obtained was 0.488 which means that the model can explain 48.8% of the variation in the response.

Table.8: Analysis of variance table

Model Term	DF	Sum of Squares	Mean Square	F-Ratio	P-Value
Between(protease activity)	13	7.5	0.5769231	1.1538	0.63115
Within(error)	1	0.5	0.5		
Adjusted Total	14	8			
Total	15				

Other tests revealed that the maximum levels of protease production occurred at an incubation temperature of 37 C, pH 9.0, and glucose as the carbon and sodium nitrate as the nitrogen sources, respectively. However, in the present experiment, the organism produced the highest levels of protease at pH 6.6 and 33 °C, with dextrose and peptone serving as the carbon and nitrogen sources, respectively. Temperature and pH have a major impact on the protease yields because of the different components of the medium and their combined effects on the metabolism of the bacterial species. In the current experiment, a significant protease output was found when diverse nitrogen and carbon sources were used at 33°C. Similar outcomes in *Bacillus subtilis* with pH ranging from 1 to 5 were also reported by Arunkumar in 2012. Additionally, Sidra Hashmi et al. (2002) used *Bacillus subtilis* S1 with various carbon & nitrogen sources.

Conclusion: Proteases are essential biological enzymes that play a role in cell division, managing protein turnover, and blood clotting. The detergent, leather, pharmaceutical, silver recovery, and food

sectors all depend on proteases. 60% of all industrial enzyme sales, which are currently expected to be worth over $3 billion globally, are made up of proteases. For the isolation process, soil samples were collected from the V.S.B. college in the Karur area. The natural isolates were identified and given morphological and biochemical descriptions. The isolated species showed a zone of hydrolysis on the casein agar medium, which suggested that an enzyme was being synthesized. Casein served as the substrate and tyrosine as the standard plot for calculating protease activity. Alkaline protease production was enhanced for nutritional circumstances like carbon and nitrogen sources. pH, temperature, aeration, and agitation of the culture were optimized for the best enzyme production. It was found that 33°C and pH 6.6 were appropriate. All of the optimized settings resulted in the highest enzyme production.

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