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Optimization of the Growth Conditions on Protease Production by *Laceyella Sacchari*, and *Thermus Aquaticus* Isolated from Maha Oya Geothermal Springs in Sri Lanka.

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Abstract

Thermophilic bacteria have a greater advantage in biotechnological application due to their production of enzymes such as proteases with high thermostability enzymes. The aim of the present study was the optimization of the growth conditions on protease production by *Laceyella sacchari*, and *Thermus aquaticus* isolated from Maha Oya Geothermal springs in Sri Lanka.

The selected thermophilic bacteria belonging to the genera of *Laceyella* and *Thermus* which were previously being isolated and identified were used in the present investigation to study their protease production. Among the two isolates, *Laceyella sacchari* (DMBUK 14901) reached a maximum protein concentration with a level of 490 µg/ml and showed maximum protease activity of 1.7831 units/ml at 55°C, at pH 7. According to the final result observation of temperature and pH on protease activity discovered that the extreme protease activity was detected at 60°C over a pH range of 6. The isolates of *Laceyella* and *Thermus* consumed several carbon and nitrogen sources for the synthesis of proteases. Both *Laceyella* and *Thermus* showed maximum protease activity in the presence of sucrose as a carbon sources. Furthermore, isolates of *Laceyella* and *Thermus* showed a maximum protease activity when the gelatin was used as the nitrogen source.

Keywords

Thermophilic bacteria; Protease; Temperature; pH; Carbon source; Nitrogen source

Introduction

Laceyella sacchari belongs to the genus of *Laceyella*, isolated from a bagasse in Thailand [1,2]. *Laceyella sacchari* is a Gram-positive, aerobic, non - acid fast, endospore forming thermophilic bacteria [3].

Genus *Thermus* is Gram-negative, rod shaped, anaerobic and immotile bacteria [4]. *Thermus* have been found in hydrothermal systems like shallow or deep - sea marine, low saline solfataric springs. In 1969, a new species of thermophilic bacterium were reported by Thomas D. Brock and Hudson Freeze, they named it as *Thermus aquaticus* [5]. First detected in the lower geyser basin of Yellowstone National Park [6].

Thermus is able to produce yellow, pink, red colour pigments when it is exposed to sunlight *Thermus aquaticus* is a useful microorganism in biotechnology because it produces a Taq polymerase enzyme, the enzyme used in polymerase chain reaction (PCR), [7] which supports to DNA fingerprinting, enzyme production and medical diagnoses. Taq polymerase will not denature at high temperatures.

A high temperature should be a temperature above the thermophile boundary for growth (>55°C). Most, but not all proteins from thermophiles are thermostable. Extracellular enzymes generally show high thermostability, as they cannot be stabilised by cell-specific factors like compatible solutes [8].

Proteases are widely distributed in nature and perform significant biological processes. Present sources of proteases are animals, plants, bacteria, archaea and viruses. Proteases are involved in performing completely different catalytic activities at temperature range 5°C -100°C and Ph range 0-14. Proteases are involved in protein processes apoptosis, regulation of protein function, digestion, viral pathogenesis, photosynthesis and other vital processes. More than 70% of the world-wide industries use proteases in their physiological and biotechnological applications [9].

This project aims to report optimization of the growth conditions on protease production by *Laceyella sacchari* and *Thermus aquaticus* [10] isolated from hot springs in Maha Oya. *Laceyella sacchari* and *Thermus aquaticus* were studied to investigate their optimum growth parameters of temperature and pH. Furthermore, using these two isolates investigated the temperature, pH, carbon sources and nitrogen sources effects on activity of protease production.

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Materials and Methods

Chemicals

All the chemicals, media, and medium components used in this study were of analytical grade, obtained from Sigma Chemicals (USA), HiMedia Laboratories, (India).

Isolated Bacterial Cultures

Two bacterial cultures *Laceyella sacchari* (DMBUK 14901) and *Thermus aquaticus* (DMBUK 2901) previously been isolated, identified and deposited at the Departmental Culture Collection, Department of Microbiology, University of Kelaniya, Sri Lanka were used in the present study.

Laceyella sacchari (DMBUK 14901) and *Thermus aquaticus* (DMBUK 2901) freeze-dried cultures were reviving on culture media so, those cultures were re-suspended the content of ampule was poured into nutrient agar plates. The plates were incubated at 55°C for 48 hours. The pure cultures were stored as slant cultures in the refrigerator at 4°C.

Quantitative analysis of the growth of thermophilic bacteria

Serial dilution technique and colony count method were used in the quantitative analysis of bacteria at various temperatures (30°C -70°C) and pH (6, 7, 8) [11].

Screening for proteolytic activity

The ability of the bacterial isolates to hydrolyze casein by extracellular caseinases was studied using Casein Hydrolysis Method [12].

Preparation of thermophilic bacterial crude enzyme

The isolated thermophilic bacteria were inoculated in protease specific medium broth [13]. The medium was dispensed in 5 ml amounts in test tubes and tubes were sterilized by autoclaving at 121°C for 15-20 minutes. The sterilized protease specific medium broth was cooled and inoculated with 5% inoculum of 48 hours old cultures of the isolate. The cultures were maintained at 55°C at 140 rpm for 48 hours in shaking incubator. After the fermentation period, each fermentation broth was centrifuged at 10,000 rpm at 4°C for 15 minutes. The clear supernatant was used as the crude enzyme preparation.

Approximation of Protein Concentration

The concentration of protein in the crude cell- free extract was estimated by Lowry's method [14].

Assay of protease enzyme activity

Protease assay was performed by a modified method of Anson [15] with casein as the 125 substrate.

Protease assay

Test tubes were taken and labeled them all and 5 ml of 0.65% casein solution was added, equilibrated in a water bath at 37°C for 15 minutes, and 1 ml enzyme solution was added. The test samples were mixed by swirling and incubated at 37°C for exactly 10 minutes. The protease activity and production of tyrosine started during this incubation time. After incubation, 5 ml of the Trichloroacetic acid reagent (TCA) was added to each test tube to stop the reaction. Then 1ml amount of enzyme solution was added to each tube. After all, tubes were mixed by swirling and incubated at 37°C for 30 minutes. Test samples were filtered through Whatman number 50 filter paper to remove any insoluble matter from the samples. After pipetting out 2 ml of test filtrate to the new test tube and 5 ml of sodium carbonate reagent was added, followed by 1 ml of Folin's reagent immediately afterward, mixed by swirling and incubated at 37°C for 30 minutes then kept the samples to cool to room temperature. Samples were filtered through Whatman filter

papers and immediately prior to record the reading using the spectrophotometer, Thermo scientific Multiskan GO at 660 nm.

Effect of Temperature on Protease Invention

Protease specific medium broth (pH 7) was inoculated with 5% inoculum of an actively growing respective thermophilic bacterial cultures and incubated at different temperatures, 40°C, 55°C, 60°C in an orbital shaker at 120 rpm for 48 hours. After incubation period broth samples were centrifuged at 10,000 rpm at 4°C for 15 minutes. Finally, the protease activity was assayed using supernatant by following the procedure indicated in a protease assay for test reagents.

Effects of pH on Protease Invention

The effect of pH on protease production by the bacterial isolates was determined by growing the thermophilic bacteria in protease specific medium broth at varying pH values, pH 6, pH 7, and pH 8. The growth medium was inoculated with 5% inoculum of isolated microorganisms. The inoculated culture flasks of varying pH values were incubated at 55°C for 48 hours. After incubation, broths were centrifuged at 10,000 rpm at 4°C for 15 minutes. The supernatant was used to measure protease production through assay of protease.

Effect of Carbon Sources on Protease Invention

The sterilized protease specific medium broth (pH 7) was prepared with the various carbon sources. These carbon sources were used to replace the carbon source (glucose) available in the protease specific medium broth. The culture tubes were inoculated with 5% inoculum on different carbon sources and incubated at 55°C for 48 hours on a rotary shaker at 120 rpm. The resulting culture broth was centrifuged at 10,000 rpm at 4°C for 15 minutes. Finally, the protease activity measured by the assay of protease as per the procedure given in section of protease assay for test reagents.

Effect of Nitrogen Sources Protease Invention

The sterilized protease specific medium broth (pH 7) was prepared with different nitrogen sources (sodium nitrate, urea and gelatin). These nitrogen sources were used instead of the nitrogen source (peptone) contained in the original protease medium broth. The prepared broth cultures were inoculated with 5% inoculum of the respective thermophilic bacterial culture and incubated at 55°C for 48 hours on a rotary shaker at 120 rpm. After incubation period broth samples were centrifuged at 10,000 rpm at 4°C for 15 minutes. The protease activity measured by assay of protease as per the procedure previously described in the text.

Calculating Enzyme Activity

The absorbance of the samples was measured by a spectrophotometer at the wavelength of 660 nm. Absorbance values for the standards, standard blank and different test samples were recorded. After collecting all of the data, a standard curve was created.

Unit definition

Hydrolyzed casein one unit produces colour that, equivalent to 1.0 μ mole (181 μ g) of tyrosine per minute at pH 7.5 at 37°C (coloured by Folin and Ciocalteu's Phenol Reagent) [16].

Statistical Examination

The protease assay data analysis was carried out using minitab 17 statistical software, and one way Analysis of Variance (ANOVA) method was used. Randomized Complete Block Design was used to determine the effect of various treatments on protease production and Turkey's pairwise comparison was used to determine the significant difference among the groups of treatments.

Results and Discussion

Reviving the thermophilic bacterial cultures

Freeze- dried cultures are revived on nutrient agar medium at 55°C for 48 hours given in the following table 1.

Effect of temperature for thermophilic bacterial cultures

Serial dilution technique and colony count method were used to estimate the number of colonies (30 300) presented in the test sample. Table 2 shows the Log CFU/ml (colony forming units per ml) present in 30°C – 80°C of *Laceyella sacchari* and *Thermus aquaticus* [11].

According to the results (Table 2) and comparison with literature [11]. According to the results (Table 2) and comparison with literature [11] all isolates showed very good to excellent growth between 40°C - 60°C but *Laceyella sacchari* growth were inhibited at 70°C. At temperature 80°C both *Laceyella* and *Thermus* growth were inhibited.

These isolates can be withstand at high temperature (above 40°C), due to their physiological adaptations and it's not effect on *Laceyella* and *Thermus* metabolic reactions. Temperature effects on organisms in two ways. One is when the temperature rises, chemical and enzymatic reactions proceed at a faster rate and the growth rate increases. Another way is when the temperature rises, proteins are invisibly damaged. As a molecular adaptation thermophiles are much more heat stable because of their intracellular enzymes and some of factors such as salts, high protein concentration, coenzymes, substrates, activators and general stabilizers [17].

Effect of pH for thermophilic bacterial cultures

Serial dilution technique and colony count method were used to estimate the number of colonies (30 300) presented in the test sample. Table 3 shows the Log CFU/ml (colony forming units per ml) present in pH 6, 7 and 8.

Laceyella and *Thermus* were showing maximum growth at pH 8. Previous references mentioned pH 7.4 and pH 7.6 values observed from Maha Oya hot water springs [18,11]. Each organism has a particular pH range, which is possible to grow. Most neutral environments have pH values between 5 - 9 and most microorganisms have pH optimal in this range. High [H⁺] ions are required for thermophiles to stabilize the cytoplasmic membrane, but it dissolves when the pH value is raised to neutrality [19].

Proteolytic activity

Isolates were screened for the protease activity on the casein agar plates. The results obtained are presented in the Table 4. Selected isolates were used to further experimental studies.

Casein hydrolysis method was used to study the protease activity from isolates [20]. The proteolytic activity was observed from using casein agar and expressed as diameter of clear zone in mm. According to the results (Table 3) *Laceyella* and *Thermus* were taken as the positive result and they were selected for further experimental studies. Similar literature [11] was used to confirm the results.

Quantification of Protein Content

The protein level of the crude enzyme of each isolate was estimated by Lowry's method. Using absorbance values results a standard curve plotted (Figure 1) and the protein concentration of each thermophilic bacterium was deduced using the standard curve.

The standard curve and the protein concentration present in each isolate are presented in the

Figure 1 and the protein concentration of isolates are given in the Table 4.

Tyrosine Standard Curve

The tyrosine standard curve was prepared by taking a different concentration of L-tyrosine standard solution. The tyrosine standard curve is used for the calculation of the amount of tyrosine released by the enzyme and thereby the activities of the enzyme can be calculated.

Protease activity for thermophilic bacterial cultures

Protease activities of the *Laceyella* and *Thermus* were determined at 55°C and pH 7 as controlled samples. The protease activities of isolates were deduced using the tyrosine standard curve.

Protease activities of thermophilic bacterial isolates are shown in the Figure 2.

The protease activity was determined by using the tyrosine standard curve. From the standard curve the activity of protease samples can be determined in terms of units, which is the amount in micromoles of tyrosine equivalents released from casein per minute [15]. As shown in Figure 2 the protease activity was highest in *Laceyella sacchari* (DMBUK 14901), its protease activity was 1.7831 units/ml and 1.606 units/ml was shown the lowest protease activity by *Thermus aquaticus* (DMBUK 2901).

According to previous results (Table 2 and Table 3) the crude enzyme samples were tested for the effect of temperature and pH on protease production as previous references mentioned [20,21].

Effect of the temperature on protease production

The crude enzyme samples were tested for the effect of temperature on protease production. Protease activity at temperatures 40°C, 55°C and 60°C are presented in the Figure 3.

The temperature was a critical parameter affecting the bacterial growth and protease enzyme production. Therefore, *Laceyella* and *Thermus* were grown at temperature 40°C, 55°C and 60°C but the highest protease production (Figure 3) was shown by *Laceyella sacchari* from overall temperature values. The ANOVA statistical methods used to compare the effect of temperature on protease production and according to results at 60°C both *Laceyella* and *Thermus* were shown their optimum growth. Previous study of thermophilic protease producing *Geobacillus* isolates were grown optimally at a temperature between 60°C-62°C [22].

Effects of pH on protease production

The crude enzyme samples were tested for the effect of pH on protease production. Protease activity in pH 6, pH 7 and pH 8 are given in the Figure 4.

The pH is an important parameter for the thermophilic bacterial growth because metabolic reactions are active in the bacterial cell and it leads to the production of protease [23] with respect to pH, it was evident that the thermophilic bacterial isolates were able to grow and produce protease over a selected pH values of 6, 7 and 8. According to Figure 4, *Laceyella* and *Thermus* were shows pH 6 was

Sample numbers	Freeze- dried thermophilic bacteria species
DMBUK 14901	<i>Laceyella sacchari</i>
DMBUK 2901	<i>Thermus aquaticus</i>

Table 1: Revived thermophilic bacterial cultures

Sample numbers	30°C (Log CFU/ml)	40°C (Log CFU/ml)	50°C (Log CFU/ml)	55°C (Log CFU/ml)	60°C (Log CFU/ml)	70°C (Log CFU/ml)	80°C (Log CFU/ml)
DMBUK 14901	7.056905	8.214844	5.033424	6.745855	6.766413	-	-
DMBUK 2901	6.190332	7.264818	5.380211	7.858537	8.825426	5.843855	-

Table 2: The Log CFU/ml of the isolates at temperature ranges 30°C to 80°C

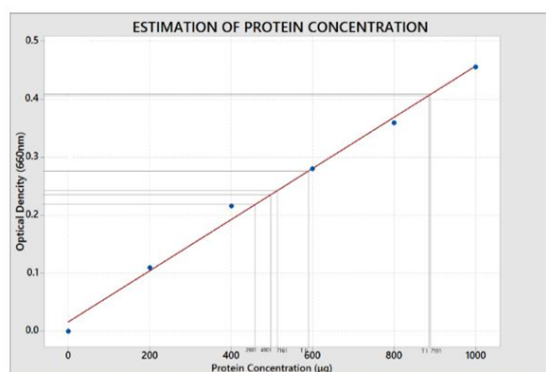
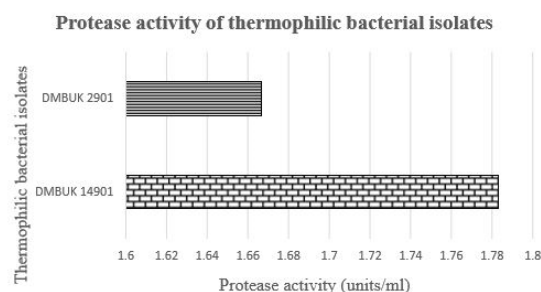
Sample numbers	pH6 (Log CFU/ml)	pH7 (Log CFU/ml)	pH8 (Log CFU/ml)
DMBUK 14901	7.100371	6.745855	9.269513
DMBUK 2901	7.68842	7.858537	7.884795

Table 3: The Log CFU/ml of the isolates at pH 6, 7 and 8

Isolates	Zone of Inhibition (mm)
DMBUK 14901	19
DMBUK 2901	23

Table 4: Inhibition zone (mm) of isolates

Sample numbers	Optical density at 660nm	Isolates protein concentration (µg/ml)
DMBUK 14901	0.2356	490
DMBUK 2901	0.219	450

Table 5: The protein concentration of isolates**Figure 1:** The protein concentration standard curve - Lowry's method**Figure 2:** Protease activity of thermophilic bacterial isolates

effect to protease production, thus ANOVA was applied to compare the effect of pH on the protease production the conclusion was that the effects of selected pH levels on protease production was high at the pH 6, when compare with pH 7 and pH 8. Previous studies showed the optimum pH of the enzyme was found to be 8. After incubation of crude enzyme solution for 24h at pH 5.5, 8 and 9 for protease producing *thermophilic Bacillus sp* [24].

Effect of carbon source on protease production

The different carbon sources fructose, mannitol and sucrose on the cells growth and protease production was investigated by replacing the carbon sources in the protease specific medium.

Protease activity in the presence of fructose, mannitol and sucrose respectively, are given in the Figure 5.

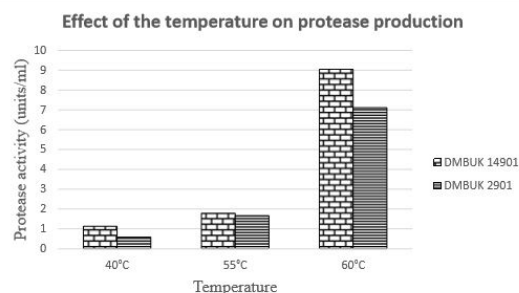
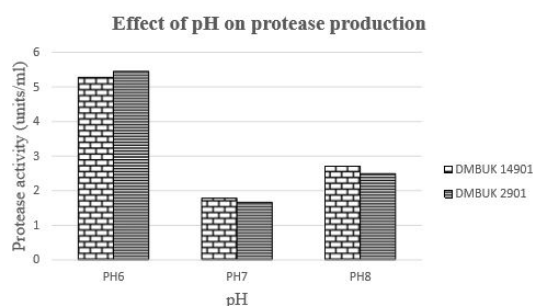
The results indicated that different carbon sources have a different impact on the production of protease from isolates. All tested carbon sources supported the growth of isolates. According to Figure 5, *Laceyella* and *Thermus* were shown maximum protease production with sucrose. The effects of carbon sources on protease production was analysed using ANOVA statistical methods and belong to that results *Laceyella* has shown maximum protease production with fructose, mannitol and sucrose as selected carbon sources. *Thermus* was showing low production of protease compare to *Laceyella* but among these three carbon sources, sucrose and fructose affect was high with *Thermus*. These results are in accordance with some previous studies which showed the addition of more carbon sources in the peptone medium failed to improve the protease production [25].

Effect of nitrogen source on protease production

The selected nitrogen sources as sodium nitrate, urea and gelatin on the thermophilic bacterial growth and protease production was evaluated replacing the nitrogen sources present in the protease specific medium. Protease activity in sodium nitrate, urea and gelatin are given in the Figure 6.

Figure 6: Protease activity of thermophilic bacterial isolates in the presence of sodium nitrate, urea and gelatin.

The maximum protease production analysed using ANOVA method and the selected nitrogen sources on protease production were different therefore the effect of selected nitrogen sources on protease production was different. *Laceyella* and *Thermus*, both thermophilic bacteria were produced maximum protease production with Gelatin. Sodium nitrate and urea were give low protease activity of *Laceyella* and that production value is too low than *Thermus*.

**Figure 3:** Protease activity of thermophilic bacterial isolates at 40 °C, 55°C and 60°C**Figure 4:** Protease activity of thermophilic bacterial isolates at pH 6, pH 7 and pH 8

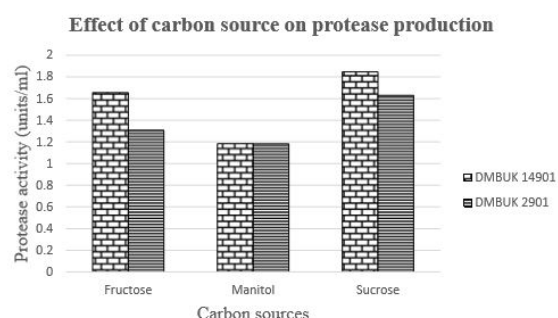


Figure 5: Protease activity of thermophilic bacterial isolates in the presence of fructose, mannitol and sucrose

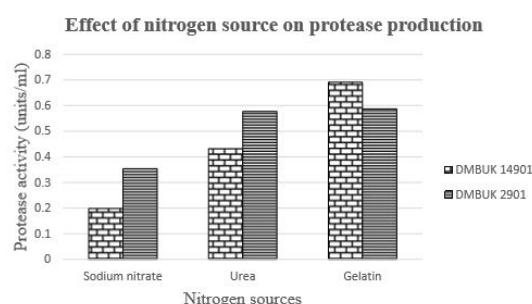


Figure 6: Protease activity of thermophilic bacterial isolates in the presence of sodium nitrate, urea and gelatin

Previous study [25] showed sodium nitrate, ammonium salts and amino acids as sole nitrogen sources interfered with protease formation and protease production was enhanced in the presence of Sodium nitrate.

Conclusion

The present study was based on optimization of protease production by thermophilic bacteria belonging to the genera *Thermus* and *Laceyella* from Maha Oya hot springs in Sri Lanka. According to the results obtained it can be concluded that the thermophilic bacterial isolates were able to grow at temperatures between 30°C -70°C and pH range 6-8.

Among the two isolates maximum growth was shown at temperature 60°C - *Thermus aquaticus* (DMBUK 2901) and *Laceyella sacchari* (DMBUK 14901) was shown 40°C. Both *Thermus* and *Laceyella* growth was inhibited at 80°C. pH 8 was the maximum growth point for both *Thermus* and *Laceyella*.

Laceyella sacchari (DMBUK 14901) and *Thermus aquaticus* (DMBUK 2901) were shown maximum protease production at temperature - 60°C and pH 6, also minimum protease production shown at 40°C and pH 7 according to statistical analysis. Based on the results temperature and pH can be effected on protease production. So, it can be concluded that the maximum protease activity observed at 60°C and pH 6.

Effect of carbon source on protease activity of bacteria showed same results for *Laceyella sacchari* (DMBUK 14901) and *Thermus aquaticus* (DMBUK 2901). Therefore finally observed according to statistical analysis sucrose was given maximum protease activity and mannitol was given minimum protease activity. The statistical data analysis was shown minimum protease activity with sodium nitrate and maximum protease activity with gelatin for both *Laceyella sacchari* (DMBUK 14901) and *Thermus aquaticus* (DMBUK 2901). Therefore finally observed *Laceyella sacchari* (DMBUK 14901) and *Thermus aquaticus* (DMBUK 2901) protease production can be effected from temperature, pH, carbon sources and nitrogen sources.

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