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Isolation, purification and mass production of protease from *Bacillus subtilis*

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Abstract
Isolation and identification of protease producing strains of bacteria were carried out from four different soil samples collected from various places in Tiruchirappalli. The organisms were tested by various biochemical tests, which led to their identification as *Bacillus subtilis* producing protease enzyme. These *Bacillus subtilis* could grow up to 60ºC and in pH range of 6-9 with optimal growth temperature and pH of 55ºC and 9.0 respectively. It was also optimized for carbon test and nitrogen test with optimal growth in dextrose and peptone respectively. Enzyme production was carried out in 1 litre of optimized media in the fermenter at 55ºC for 48 hours at pH 9.0. Harvested protease product was purified by salt precipitation method. Finally the enzyme protease was purified by column chromatography. The protein was characterized using SDS-PAGE. This result showed that *Bacillus subtilis* is a good producer of extra cellular protease. The enzyme produced by *B. subtilis* is pH stable and thermo stable, which can be utilized in local detergent and leather industry.

Keywords: protease, *Bacillus subtilis*, staining, peptone, casein agar, Inoculum, column chromatography

Introduction
Proteases are found in several microorganisms such as protozoa, bacteria, yeast and fungi. Protease constitutes 60-65% of the global industrial market most of which are alkaline proteases [1, 2]. Among the various proteases, Bacterial proteases are more significant compared with animal and fungal proteases [3]. The inability of the plant and animal proteases to meet the current world demands (due to extensive use in food, pharmaceutical and detergent industry) has led to an increased interest in microbial proteases [4].
And among Bacteria *Bacillus* species are the specific producers of extracellular proteases. Bacteria are most important alkaline protease producers with the genus *Bacillus* being the most prominent source [5], because of their ability to produce large amount of protease having significant proteolytic activity and stability at high pH and temperature.
The microbial proteases are usually produced by either free or immobilized cells. Proteases production by micro-organisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, incubation time, agitation and inoculums density [6]. *Bacillus subtilis* is one of the most widely used bacteria for the production of specific chemicals and industrial enzymes and also a major source of amylase and protease enzymes. It is also used in baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, cheese making, certain medical treatments of inflammation and virulent wounds and in unhairing of sheepskins. It has also wide application in Bioremediation process [7, 8].
In this study an attempt was made for, the isolation and selection of *Bacillus* strain that is a potent producer of extracellular alkaline protease, and the optimization of culture conditions required for enzyme production.

Materials and Methods
Isolation, Identification and screening of Protease Producing Bacteria
Soil samples were collected from various places in Tiruchirappalli. The samples were labeled after collection. These were spread onto isolation media and incubated at 35°C for 48 hours after serial dilution of 10^{-4} to 10^{-9} times. The isolated bacteria were examined for cellular morphology, growth condition, gram staining, endospore staining, capsule staining and biochemical tests as per Bergey's Manual of determinative Bacteriology [9]. The sample culture was spread on casein agar medium containing casein 2.0%; peptone, 0.5% and agar 1.5% and then incubated at 37°C for 48h. The clear zone of casein hydrolysis was an indication of protease secretion [10]. The isolates were selected on the basis of larger zone on casein agar medium and the best one was selected for further study.

Effect of Various Temperatures, pH and Incubation Periods on Alkaline Protease Production
Incubation temperature was shown to influence protease production. To study the effect of incubation temperature for maximum protease production, the flasks with the production medium were inoculated and incubated at various temperatures such as 37, 40, 45, 50, 55 and 60^{0}C for 48 h. The initial pH of the medium was shown to influence protease production. The effect of initial pH on alkaline protease production was studied. The production medium was adjusted at various levels of pH by sodium hydroxide solution (6.0, 7.0, 8.0, 9.0, 10.0, and 11.0). General procedure was followed for protease production.The duration of incubation plays an important role in the production of a microbial metabolite. To study the optimal incubation period for maximum protease production, the flasks with the production medium (pH 9.0) were inoculated and incubated at 55^{0}C. Samples were withdrawn

periodically at every 12 h up to 96 h and assayed for protease activity.

Effect of level of inoculum on alkaline protease production

The effect of level of inoculum was studied for optimal alkaline protease production. Experiments were carried out using 1-6% inoculum volume each, containing OD of 1.0. The flasks with the production medium (pH 9.0) were inoculated as above and incubated at 55^0C for 48 h.

Fermentation experiments were carried out using cultures of different age (12, 24, 36, 48 and 60 h old culture as inoculums).

The flasks with the production medium were inoculated using cultures of different age at 5% level and incubated at 55^0C for 24 h.

Effect of Nitrogen Source on Protease Biosynthesis

Effect of peptone concentration (0.25 - 1.5%) on protease production and growth of microorganism was studied with a range of 0.25 - 1.5%. Various nitrogen sources (casein soluble, casein hydrolyzed, tryptone, corn steep liquor, yeast extract, urea (NH2– CO–NH2), sodium nitrate ($NaNO_3$), ammonium nitrate, potassium nitrate (KNO_3), ammonium chloride and ammonium sulphate) were utilized.

Mass Production of Alkaline Protease through Fermentation

The culture media used for mass production of protease contains Dextrose 1% (w/v), peptone 0.5%, KH_2PO_4 0.2%, $MgSO_4$. $7H_2O$ 0.2%, Casein 1% and pH 8.0. It was maintained at 37°C for 48hrs in a shaking incubator. After inoculation, fermentation was carried out at 37°C at 200 rpm for 48hrs. At the end of each fermentation period, the whole culture broth was centrifuged at 10,000 rpm for 15minute, to remove the cellular debris and the clear supernatant was used as enzyme preparation [11]. The purification of the enzyme was done by ammonium sulphate precipitation method.

Purification

The enzyme was purified from the culture by ammonium sulfate precipitation method and the precipitate 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 on a magnetic stirrer. Further purification of dialyzed protein was done by column chromatography using Diethyl amino ethyl (DEAE) column. The protein concentration was measured using BSA [12] as a standard, Protease activity was determined by a modified procedure (Tsuchida *et al*, 1986).

Results

Identification of *B. subtilis*

Five bacterial isolates were obtained from soil samples of which S5 strain was identified as *Bacillus subtilis*, morphologically and biochemically. The colonies were subjected to grams staining; capsule staining by negative staining method and endospore staining. The colonies, which were positive for Grams staining, capsule and endospore staining were considered for further studies (Table 1, 2 & 3).

Table 1: Tabulation for results of Colony Characteristics.

Strain No.	Colony Surface	Colony Colour	Visual Characteristics	Shape of the Colony	Height of the Colony
S1	Smooth	Brown	Opaque	Irregular	Raised
S2	Smooth	Off white	Translucent	Irregular	Flat
S3	Smooth	Off white	Opaque	Circular	Raised
S4	Smooth	Off white	Opaque	Circular	Raised
S5	Smooth	Off white	Opaque	Irregular	Flat

Table 2: Tabulation for results of staining techniques.

Strain No.	Gram Staining	Morphology (*Bacillus*/ Cocci)	Endospore Staining	Capsule Staining
S1	Negative	Rod	Positive	Positive
S2	Positive	Rod	Positive	Positive
S3	Positive	Rod	Positive	Positive
S4	Positive	Both Rod & Cocci	Positive	Positive
S5	Positive	Cocci	Positive	Positive

Table 3: Tabulation for Results of Various Biochemical Tests.

Sl. No.	Samples	Indole	MR	VP	Amylase	Nitrate	Oxidase	Catalase	Urease	Gelatin	Caesin	TSI
1	S1	- ve	- ve	+ ve	+ ve	- ve	+ ve	- ve	- ve	- ve	+ ve	+ ve
2	S2	- ve	- ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	- ve	+ ve	- ve
3	S3	- ve	+ ve	- ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve
4	S4	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve	- ve	+ ve	+ ve
5	S5	- ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve

S 1-S5 designates the different kinds of bacterial colonies isolated after serial dilution. (Presence +, Absence -)

Screening for Protease Production

The selected colonies were streaked on skim milk agar plates. The plates were subjected to incubation for a period of 48 hours at 55°C. The plates which showed clear zone around the streaked area of test organism were selected as protease producing strain. The organism named S5 showed the inhibition zone and was subjected to various biochemical tests (Table 3). S5 showed the following results for the biochemical tests. It was positive for Methyl red test, starch hydrolysis, citrate utilization test, oxidase test, TSI, gelatin hydrolysis, urease test and nitrate reduction test, and was negative for Voges Proskauer test, indole test and catalase test. After biochemical tests the test organism was confirmed to belong to the *Bacillus* species producing Protease.

Effect of Incubation Temperature

To study the effect of various temperatures on the growth and alkaline protease production, different temperature ranges (37^0C, 40^0C, 45^0C, 50^0C, 55^0C and 60^0C) were used. The fermentations and assays were carried out in triplicate. The results indicated that the organisms grew over a wide range of temperatures (37^0C to 60^0C). At 37, 40, and 45^0C, the protease production was 109, 148 and 204 U/ml respectively. The maximum alkaline protease production (222 U/ml) was observed at 55^0C at 48 h (Fig 1). Increase in incubation temperature to 60^0C decreased the yield to 186

U/ml. Hence the optimum incubation temperature for protease production by this organism is 55^0C.

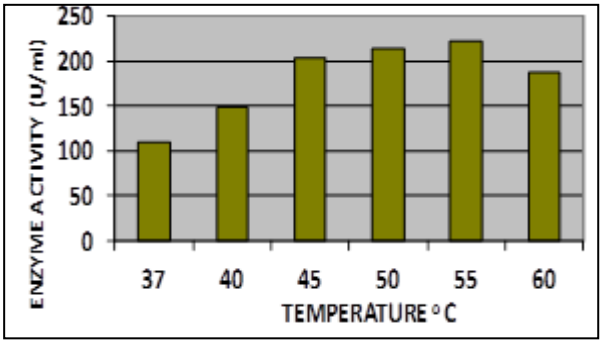

Fig 1: Effect of temperature on Enzyme production

Effect of Initial pH

The effect of initial medium pH on protease yield was studied. Different initial pH values (6.0-11.0) were used to study their effect on the protease production. The fermentations and assays were carried out in triplicate as per the general procedure and the results are shown in Fig 2. The organism produced reasonable amounts of protease in acidic and highly alkaline conditions with highest yield (266.2 U/ml) at pH 9.0 where it had maximum growth. So the optimum pH for protease production was found to be 9.0. It is clear from Fig 2 that the organism grew well at a wide range of pH 7.0- 11.0.

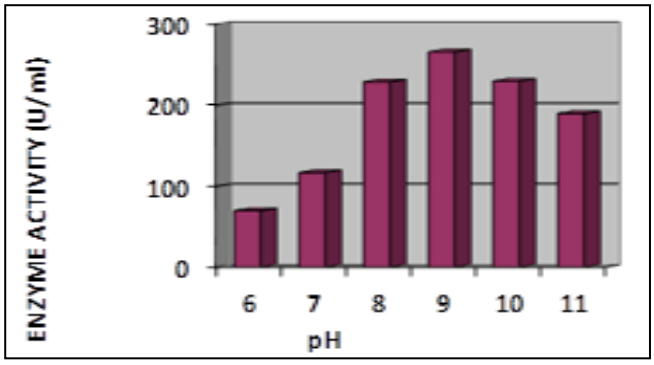

Fig 2: Effect of Initial pH on Enzyme production

Effect of Level of Inoculum and its Incubation Period

Initial microbial load to a medium does affect the growth and in turn metabolite production. To study the effect of inoculum level the experiments were conducted using 1,2,3,4, 5 and 6% of inoculum volume (Fig 4). The results indicate that protease production was increased with increase in level of inoculum upto 5% level (230 U/ml) and further increase in inoculum level did not increase the protease production.

To analyse the optimal incubation time for maximum protease production, the fermentation samples were withdrawn periodically at every 12 h up to 60 h and assayed. The results are shown in Fig 3. The results indicate that the organisms grew well in the medium and maximum protease production (224 U/ml) was achieved at 48 h. After that the protease production decreased gradually with increased incubation periods.

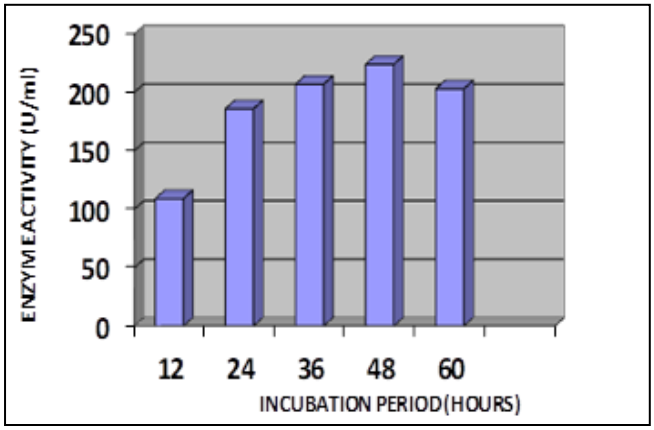

Fig 3: Effect of incubation period on Enzyme Activity

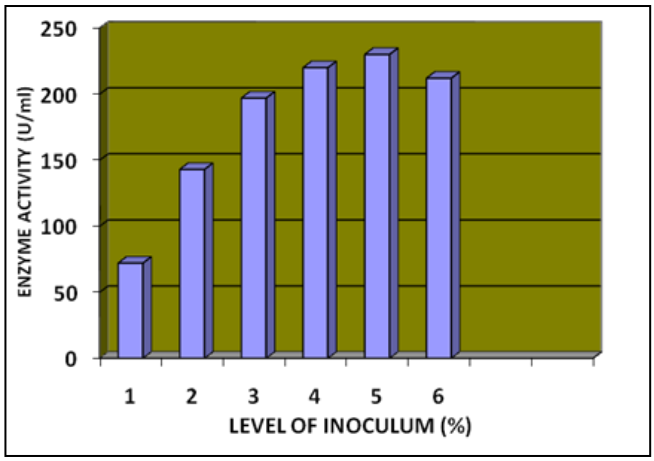

Fig 4: Effect of level of Inoculum on Enzyme activity

Effect of Inhibitors on Protease Production

Fermentation experiments were carried out using cultures of different age (12, 24, 36, 48, and 60 h). The results indicate that culture of 24 h age had maximum protease producing ability (230 U/ml) (Fig 5).

The effect of various inhibitors on protease production was also studied.

It is evident from the Fig 6 that all the inhibitors used inhibit protease production at 5mM level like EDTA and 2, 4-DNP, inhibited strongly. INH had almost no effect and other substances showed moderate inhibitory effect. (Fig.6)

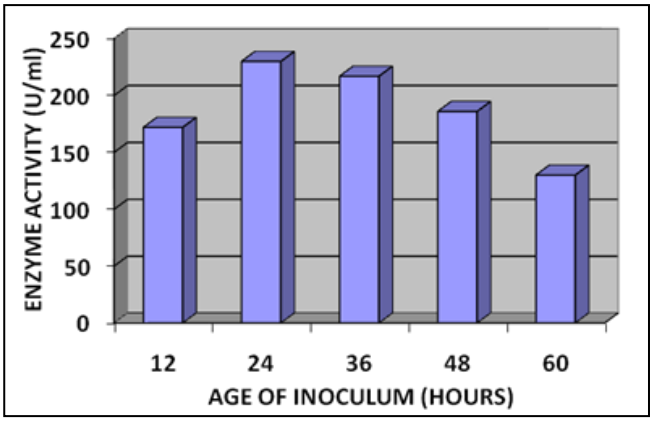

Fig 5: Effect of age of inoculum on Enzyme activity

Effect of nitrogen and other surface-active agents on protease production

The effect of various surfactants on protease production was studied. All the surfactants inhibited protease production at 0.04% concentration. Cetrimide exhibited the highest inhibitory activity followed by Tween-80, SLS and Tween-20 (Fig 7).

B. subtilis have high proteolytic activity, with a clear zone of 22 mm with gelatin matrix. Protease production began 6 h after cell entrapment of gelatin and increased gradually to a maximum level of 10.8 U/mL[12]. *B. subtilis* grew in the pH range 6–9, with optimum protease secretion at pH 7.4 and galactose and peptone as the carbon and nitrogen source, at specific incubation times and temperature. It was observed previously that use of casein as the substrate under the standard assay conditions gave the highest activity at pH 8.0 [13, 20]. Incubation temperature of 37 °C, pH 9.0 and glucose and sodium nitrate as the carbon and nitrogen source, resulted in the highest production of protease [14, 15, 21]. Protease yields vary in their concentration with temperature and Ph [17, 18], which finally influence their anabolism and catabolism of the bacterial species [16, 19]. In the present experiment, maximum protease production was achieved with 0.75% peptone and 2.0% sodium chloride with alternate nitrogen and carbon sources were used.

Conflict of Interest

The authors declared that they have no conflict of interest

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