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RESEARCH ARTICLE

EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF PROTEASE ENZYME FROM ISOLATED BACTERIUM *BACILLUS SUBTILIS* AND OPTIMIZATION OF FEW CULTURE CONDITIONS

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Abstract

The aim of this study is to optimize the environmental conditions during fermentation, developing an industrial media formulation and evaluating the characteristic properties of the crude alkaline protease enzyme produced from *Bacillus* sp. isolated from natural habitat. Bacteria of genus *Bacillus* are active producers of extracellular proteases. *Bacillus subtilis was* allowed to grow in broth culture for purpose of inducing protease enzyme. Proteases are enzymes that hydrolyse proteins via the addition of water across peptide bonds and catalyses peptide synthesis in organic solvents and in solvents with low water content. The purification was carried by applying successively dialysis, DEAE-cellulose ion exchange chromatography to the supernatant. In this study the optimum temperature (40ºC), optimum pH (7.0), optimum incubation period (42 h), salt concentration (0.05gm) and sugar source (sucrose) were determined. Molecular weight of the obtained enzyme was investigated by SDS-PAGE. Molecular weight of the protease was determined, and it was found that the weight of enzyme was 63 kDa. Data emphasized the possibility of extraction and partial purification of protease enzyme from *Bacillus subtilis* for industrial scale applications.

Introduction

Biotechnology can be simply defined as a technique which comprises the "controlled and deliberate application of simple biological agents in technically useful operations either of productive manufacture or as service operation. Recombinant DNA technology has opened new horizons in the study of gene function and the regulation of gene action. Thus, bacteria and yeast can be created to metabolize specific products or to produce new products.

B. subtilis is one of the most widely used bacteria for the production of Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids. Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzymes that hydrolyse proteins via the addition of water across peptide bonds and catalyse peptide synthesis in organic solvents and in solvents with low water content (Sookkheo et al., 2000; Beg et al., 2003).

Proteases represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market accounting for about 60% of total sale of enzymes (Beg et al., 2003). Microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals.Proteases the most important group of enzymes produced commercially are used in detergent, protein, brewing, meat, photographic, silver recovery, leather, waste treatment and dairy industries (Gupta et al., 2002a ;Ito et al., 199; Anwar et al., 1998 ; Agrawal et al., 2004 ;Gupta et al., 2002b). Bacillus strains are specific producers of

extracellular proteases (Singh,et al.,2001) and can be cultivated under extreme temperature and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (Han et al.,1997).

In enzyme technology which is a sub-field of biotechnology ,new processes have been and are being developed to manufacture both bulk and high added value products utilizing enzymes as biocatalysts The microorganisms from diverse and exotic environments are an important source of enzymes, whose specific properties are expected to result in novel process applications (Kumar and Takagi, 1999). With time, research, and improved protein engineering methods, many enzymes have been genetically modified to be more effective at the desired temperatures and pH.

The focus of this study was to produce and purify protease from *B.subtilis* isolates in the laboratory. Also the optimization of some culture conditions of the bacteria under study was done.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PROCESSING

Soil Samples were aseptically collected from Simhachalam hills of Visakhapatnam. The soil sample was processed with phosphate buffer to decrease the microbial count and serially diluted.10^{-3}, 10^{-4}, 10^{-5}, dilutions were inoculated in Nutrient agar plates by spread plating method and incubated at 37^0C for 24-48 hrs. Identification of bacteria by bacteriological analysis-Gram staining, Biochemical test or phenotypic test.

OPTIMIZATION OF CULTURE CONDITION

EFFECT OF TEMPERATURE

Temperature has profound effect on protease production The culture medium were incubated at different concentration for optimum enzyme production. For this reason, equal quantity of inoculums was added in each conical flask containing 60mL of selected suitable medium. All the other physical parameters are maintained at optimum level only varying the temperatures. The flasks were incubated at temperatures ranging from 20-50ºC.

EFFECT OF INCUBATION PERIOD

The effect of incubation periods on the growth and protease activity was studied. For this 60mL of selected medium was taken in each separated test tubes. All the tube were autoclaved at 121 ºC and 15 lb pressure for 20mins.After cooling the flask were inoculated with equal quantity of inoculums .The flasks were incubated at 48 ºC .At 24,48,72 and 96 hr of incubation ,the culture filtrates were collected.

EFFECT OF MEDIUM pH

To observe the effect of medium pH on enzyme production, 60 mL of selected medium of different pH (such as 5.0, 6.0, 7.0, 8.0, and 9.0 respectively) was taken in each test tubes. All the tubes were autoclaved at 121 ºC and 15 lb pressure for 20mins. After cooling the flasks were inoculated with equal quantity of inoculums and incubated at 48 ºC.

EFFECT OF DIFFERENT CONCENTRATION OF SALTS

The culture medium were incubated at different concentration of salts for optimum enzyme production .For this reason ,equal quantity of inoculums was added in each conical flask containing 60mL of selected suitable medium with selected pH. All the tube were autoclaved at 121 ºC and 15 lb pressure for 20mins.After cooling the flask were inoculated with equal quantity of inoculums .The flasks were incubated at 48 ºC .

EFFECT OF DIFFERENT SUGAR SOURCE

The culture medium were incubated at different sugar source for optimum enzyme production .For this reason ,equal quantity of inoculums was added in each conical flask containing 60mL of selected suitable medium with selected pH and different sugar source are added in different test tube containing media. All the tube were autoclaved at 121 ºC and 15 lb pressure for 20mins. After cooling the flask were inoculated with equal quantity of inoculums. The flasks were incubated at 48 ºC.

RESULTS AND DISCUSSION

The present work has been taken up with a view of exploring the possibilities to optimize the environmental conditions during fermentation, developing an industrial media formulation and evaluating the

characteristic properties of the crude alkaline protease enzyme produced from *Bacillus* sp. isolated from natural habitat.

BACTERIAL IDENTIFICATION

STAINING REACTION

The significant isolated colony was stained with Gram's stain and examined under oil immersion microscope. The bacteria were identified to be Gram's positive Bacilli as the colour of the cell appeared to be violet in colour.

BIOCHEMICAL CHARACTERIZATION

CATALASE TEST

This test is performed to detect the presence of enzyme catalase which converts the hydrogen peroxide to water and oxygen. The bacteria were found to be catalase positive as the culture produced effervesces.

ENDOSPORE STAINING

Schaeffer-Fulton method is used for staining endospores. The endospores have retained the malachite green , appearing green colour .Hence there is presence of endospores.

STARCH HYDROLYSIS TEST

The bacteria were producing starch hydrolyzing enzyme Protease which was detected by addition of Iodine solution to the culture plates.

MANNITOL FERMENTATION

The bacterial isolates were found to be positive for mannitol fermentation.

PROTEIN ESTIMATION

The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution. The amount of protein in the crude extract is estimated to be 0.44µg/ml

DETERMINATION OF PROTEOLYTIC ACTIVITY

Alkaline protease activity was determined by applying a modified form of the method given by Takami et al .(Table 1)

PURIFICATION OF ENZYMES

ION-EXCHANGE CHROMATOGRAPHY

More than one fractions were found to be showing peaks near 260-280 nm. This indicated presence of proteins in all these fractions. Presence of enzyme was confirmed testing the enzyme activity. NaCl precipitation spectrophotometry readings.

ENZYME ACTIVITY OF ELUTED PROTEIN SAMPLES

0.2 – 2 TEST : 0.449
CONTROL: 0.269
Inference: Enzyme present

0.2 – 3 TEST : 0.426
CONTROL: 0.337
Inference: Enzyme present

0.2 – 4 TEST : 0.348
CONTROL: 0.223
Inference: Enzyme present (very less)

0.3 – 2 TEST : 0.174
CONTROL: 0.173
Inference: Enzyme absent

RESULT:

According to the absorbance the graph (Figure 1) shows the presence of protein in the given sample

SDS PAGE

Molecular weight

Phosphorylase	94kd
Bovine serum albumin	66kd
Ova albumin	43kd
Carbonic anhydride	29kd

Lysozyme 14.3kd
Relative mobility(Rm) :
Rm = Distance travelled by protein/Distance travelled by dye (bromo phenol blue).

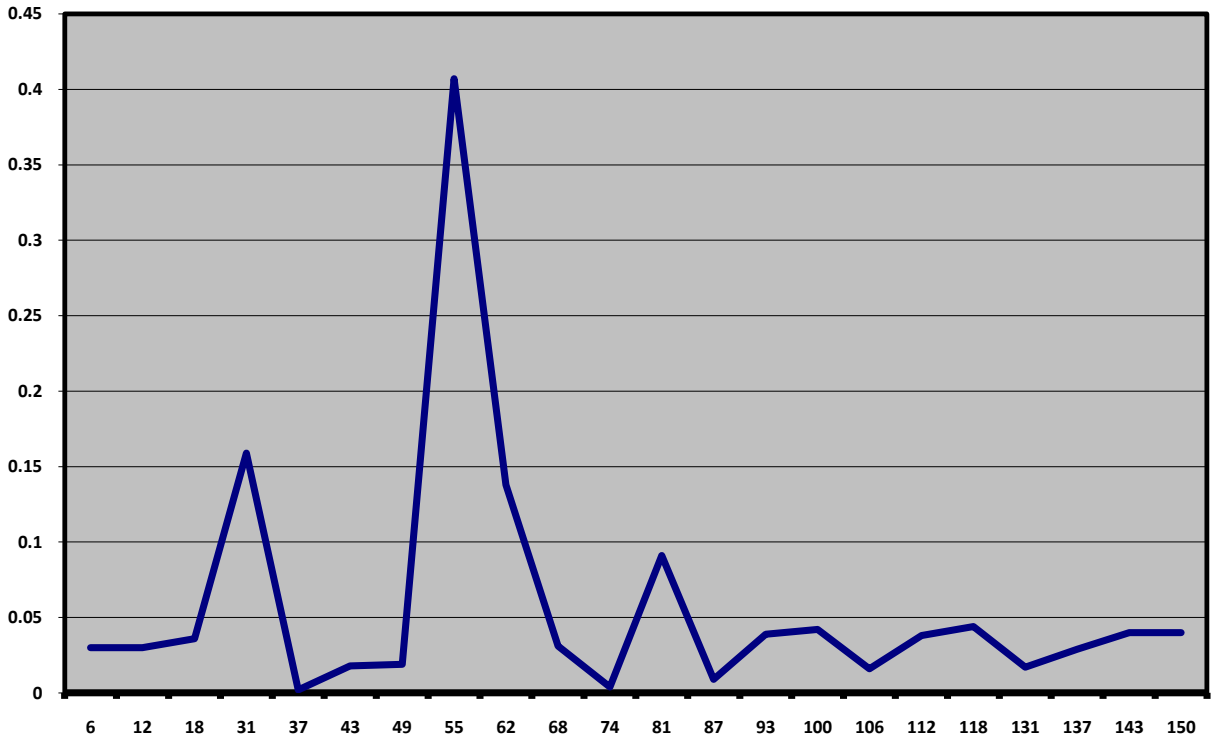

Figure 1 : OD at 280 nm for eluted volume

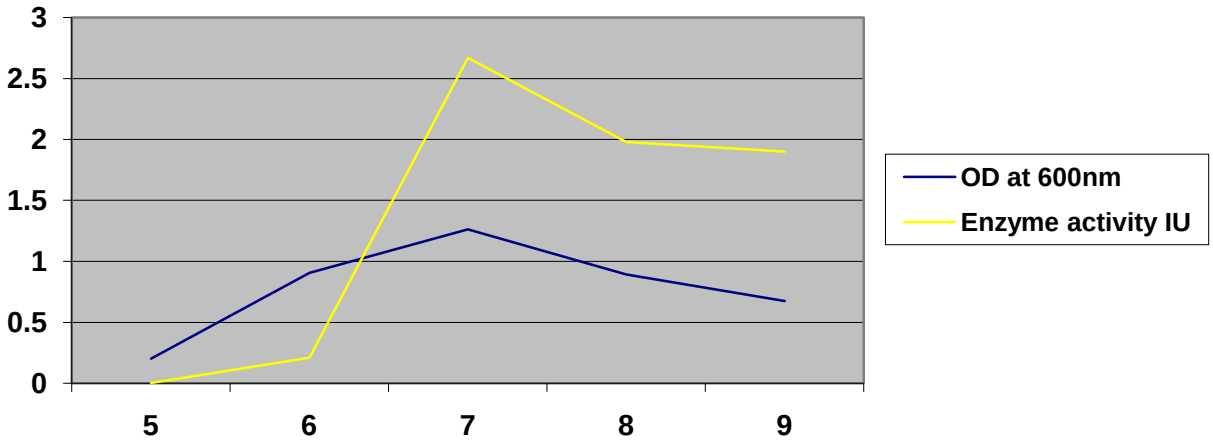

Figure 2: Effects of the medium pH on the production of protease .

Table 2 EFFECT OF TEMPERATURE ON *BACILLUS SUBTILIS* FOR THE PRODUCTION OF PROTEASE.

SALT CONCENTRATION	GROWTH
0 (Control)	heavy growth ++++
0.05%	heavy growth ++++
1.0%	less growth +++
2.0%	Very less growth ++
3.0%	no growth --
4.0%	no growth --

Table 3 EFFECTS OF INCUBATION PERIOD ON THE BACTERIAL ISOLATE BACILLUS SUBTILIS FOR THE PRODUCTION OF PROTEASE.

INCUBATION PERIOD	COLOUR	GROWTH	BIOMASS CHARACTERISTIC absorbance at 600 nm
24	Golden yellow	Suspended growth ++	Turbid 0.830
48	Golden yellow	Suspended growth +++	Turbid 1.262* protease activity
72	Golden brown	Heavy growth ++++	Turbid with sedimentation 1.720
96	Golden brown	Heavy growth ++++	Sedimentary growth 1.947

Table 4 GROWTH OF *BACILLUS SUBTILIS* AT DIFFERENT SALT CONCENTRATION %

Temp °C	Growth
20	Less growth, turbidity
25	Moderate growth, turbidity
30	Good growth, turbidity
35	Heavy growth, turbidity
40	Heavy growth, turbidity + precipitation
45	Growth, precipitation
50	Growth, precipitation

RESULT

Band of protein size is 63K.D molecular weight, confirmed that the purified protein is the α- protease.

YIELD OF ENZYME (%)

Yield of enzyme is calculated by the following formula :

$$\text{Yield} = \frac{\text{Amount of purified enzyme protein}}{\text{Amount of total protein in crude}} \times 100$$

The amount of crude enzyme extracted is 442 µg /ml.

And the amount of total purified protein is 158 µg /ml.

So the yield of enzyme obtained is 36%.

OPTIMIZATION OF CULTURE CONDITIONS

EFFECT OF TEMPERATURE ON THE PRODUCTION OF PROTEASE BY THE BACTERIAL ISOLATE *BACILLUS SUBTILIS*

Temperature has profound effect on protease production. Experiments were done on protease enzyme production by varying the temperature from 20 to 50 ºC with 5 ºC increment and the results were shown in the table below. From the data it is evident that the optimum temperature for the production of protease was at 40 ºC(Table2). However, the temperature below or above 40 ºC caused a sharp decrease in protease yield as compared to the optimal temperature.

EFFECTS OF THE MEDIUM pH ON THE PRODUCTION OF PROTEASE BY THE BACTERIAL ISOLATE *BACILLUS SUBTILIS*

To find out the optimum pH, the production medium was adjusted at different pH values i,e varying pH between 5 to9 of different buffers and results indicated that the best pH for production of protease was at phosphate buffer pH 7.0 .(Figure 2)

EFFECTS OF INCUBATION PERIOD ON THE PRODUCTION OF PROTEASE BY THE BACTERIAL ISOLATE *BACILLUS SUBTILIS*

Experiments were conducted on protease enzyme production by varying the incubation period from 24 to 96 hours. For this 60 mL of selected medium was taken in each separated test tubes. All the tube were autoclaved at 121 ºC and 15 lb pressure for 20 mins. After cooling the flask were inoculated with equal quantity of inoculums .The flasks were incubated at 48ºC . At 24, 48,72 and 96 hr of incubation ,the culture filtrates were collected. Results indicated that, as time increase the enzyme activity increase. The optimum incubation period for protease activity was 48 hrs (Table3)

EFFECT OF DIFFERENT CONCENTRATION OF SALTS ON THE PRODUCTION OF PROTEASE BY THE BACTERIAL ISOLATE *BACILLUS SUBTILIS.*

Protease enzyme production was tested for different concentration of salts (NaCl). It is evident from the results that media supplemented with 0.05gm of NaCl concentration has showed heavy growth(Table 4), hence it can be considered as the optimum salt concentration.

EFFECT OF DIFFERENT SUGAR SOURCE ON THE PRODUCTION OF PROTEASE BY THE BACTERIAL ISOLATE *BACILLUS SUBTILIS.*

Protease enzyme production was tested for different sugar sources. Various sugars like glucose, lactose, maltose, starch, sucrose, citric acid, xylose were incorporated separately into production medium. It is evident from the results that sucrose showed heavy spread growth whereas glucose showed very scanty sedimentary growth. Lactose, Starch, Xylose and Maltose showed flat dry growth. Hence it can be considered as the condition which enables good yield of protease enzyme is media supplemented with sucrose.

CONCLUSION

The present work has been taken up with a view of exploring the possibilities to optimize the environmental conditions during fermentation, developing an industrial media formulation and evaluating the characteristic properties of the crude alkaline protease enzyme produced from *Bacillus* sp. isolated from natural habitat. Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Agrawal et al., 2004). Although there are many microbial sources available for producing proteases, there is always a demand for the cheapest possible method for getting this enzyme for the economic gain. Bacteria isolated from local environment can be very cheaper source of enzyme as they are easily isolated and maintained. Also these bacteria are more resistant to stress conditions which make them economical for industries. Optimization of the culture condition will help us to standardize the production parameters. The bacteria were tested for their ability to withstand the adverse conditions like temperature (Johnvesly and Naik, 2001), extreme salt concentration and pH, sugar concentration which often arise in the fermentors during

the enzyme production processes. This vast diversity of proteases application, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Rao et al., 1998).

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