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MICROBIAL PROTEASE: A POTENT INDUSTRIAL TOOL

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Abstract:-Microbial proteases are among the most important hydrolytic enzymes and have been studies extensively since the advent of enzymology. Proteases not only play important role in cellular metabolic activity but also gained considerable attention in the industrial community.

Bacillus Stearothermophilus is used for the production of enzyme. It was carried out by using soy meal and wheat husk protein source at 33C $\pm^0$2C temperature with 7.2 pH for 5 days of incubation.

The crude enzyme was then checked against different proteinaceous substances, such as blood clot, blood stain feathers, x-ray film, milk coagulation, casein hydrolysis etc. All these substrates get acted upon by proteases enzyme at 370C of temperature within 19 to 94 hrs of incubation.

Enzyme shows, 0.478 mg protein/ml, 226.2U/ml activity and 556.9 U/mg protein in the extracted broth.

Keywords: Microbial proteases, proteolytic, caseinolytic assay, blood clot lysis, stain removal, detergent.

INTRODUCTION:

Proteases are one of the industrially most important enzymes. These proteiolytic (protein digesting) biocatalysts have been in use for many centuries, at first in the dairy industry as milk clotting agents (rennet) for the manufacture of cheese (A.Sumnatha et al.2006)

Hydrolysis of protein resulted into peptides and amino acids. These constitute a very large and complex group of enzymes, which differ in the properties such as substrate specificity, active site and catalytic mechanism, pH, and temperature optima and stability profile. (C.Sandhya et al. 2004).

Microbial proteases have been reviewed several times with emphasis on different aspects of proteases. Proteases are essential constituent of all forms of life on the earth, including prokyarotes, fungi, plants and animals (Feroz Khan 2013).

Microbial proteases are classified into various groups, dependent on whether they are active under acidic , neutral or alkaline conditions and on the characteristics of the active site groups of the enzyme i.e. metallo (E.C. 3.4.24), aspartic (E.C. 3.4.23) cystein or suphydryl (E.C. 3.4.22) or serine type (E.C. 3.4.21) (Kalisz 1988; Rao et al. 1998).

Alkaline proteases account for a major share of the enzyme market all over the world. Alkaline proteases from bacteria find numerous applications in various industrial sectors and different companies worldwide have successfully launched several products based on alkaline proteases (Abhijit Ray 2012).

Extracellular proteases are important for the hydrolysis of proteins in cell free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz 1998).

APPLICATIONS OF PROTEASES:

1.In food industry: Proteases are used as a milk clotting & de-bittering agent for cheese making e.g. protteases from *Mucor miehi* and *Aspergillus niger*. It is also used in degradation of turbidity from fruit juices & alcoholic beverages. Trade name Flavourzyme™ - a fungal enzyme from A. oryzae and Kojizyme™ used for fermentation of soy sauce. In bakery for partial hydrolysis of wheat gluten which increases the quality of loaf formation. In other applications preparation of black pudding, meat tenderization and recovery of protein from fish/ meat etc.

2.Leather and wool industry: Replacement of harsh, slow and waste generating treatment of hydrogen sulphide by easy,

controlled , speed and waste reduction alkaline proteases is one of the great achievement. Soaking, de-haring, bating processes of preparing skins and hides etc. requires proteases treatments. To obtain shrink proof wool proteases is useful. For elastolytic and keratinolytic activity proteases can be used in leather-processing (Feroz Khan 2013).

3.Photographic industry: In the bio-processing of used X-ray or photographic films for silver recovery. These waste films contain 1.5–2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled (R. Gupta 2002).

4.Silk degumming: To make a silk fabric soft and glossy it is necessary to remove the sericin or gum by a treatment is called degumming. In earlier time silk is treated with soap at a temperature of 105ºC. Use of enzyme reduces the temperature requirement & also restricts addition of pollutants to water bodies. (R. Gupta 2002).

5.Medical usage: Anti-inflammatory activity, treatment of blood clots in ischemic stroke & CVD, Wound debridement, Applications of proteases auxiliary to antibiotic therapy e.g. Serratia E-15 protease, Treating cancer with enzymes &detoxification with coffee enemas (under clinical trials) (Abhijit Ray 2012).

6.Detergent industry: The use of enzymes in detergent formulations is now common in developed countries. To remove protein from clothes soiled with blood, milk, sweat, grass etc. proteases is used. In most detergent preparations, only 0.4 - 0.8% crude enzyme by weight is present. Granulated forms & liquid preparations both are available now days (Feroz Khan 2013).

7.Cleaning of environment, Management of industrial & household waste: As a means for enhancing bioremediation. Transformation into less toxic and more biodegradable forms. Enzymes remain effective in a wide range of pH and temperature ranges, particularly if they are immobilized. To reduce pathogen counts, reduce the solids content, and increase de-flocculation in sludge (R. Gupta 2002).

MATERIALS AND METHODS:

Microorganism used: Previously isolated and identified *B. stearothermophillus* was taken which was maintained on nutrient agar slant and its 5% of nutrient broth inoculum used for enzyme production.

Production medium: Soy meal Wheat husk Medium composed of following g/l: Soy meal-50.0, Wheat husk-10.0, K_2HPO_4-1.0, NaCl-5.0, Glucose-10.0, pH-7.0-7.2. This broth sterilized by autoclaving at 121C for 20 min, cooled and inoculated with 5% inoculum of B.stearothermophilus.

Enzyme production: It was carried out by adding 5% inoculum of *B. stearothermophillus* to soy meal wheat husk medium and the flask kept for 5 days of incubation at 37 C temperature.

Extraction and partial purification: Broth was centrifuged at 10,000 rpm for 15 min to separate all microbial growth and nutrient particles. The supernatant collected and labeled as crude enzyme. Partial purification was carried out by precipitation of enzyme at 70% of saturation with ammonium sulphate salt followed by dialysis against 0.2 M Tris-HCl buffer of pH 8.0. The dialyzed enzyme stored in small vials at 0C.

Total protein determination: Protein content was determined by Lowry method using BSA as a standard.

Caseinolytic Activity: For this 5.0 ml of 0.65% w/v casein solution prepared in phosphate buffer of pH 7.0 taken to this 1.0 ml of enzyme added and incubated at 37C for 10 min. the reaction was terminated by adding 5.0 ml of 110mM trichloro acetic acid. After well mixing again kept for 30min at 370C and filtered through 0.45 micron filter or centrifuged at 4000 rpm for 10 min. To the 2.0 ml of filtrate or centrifugate 5.0 ml of 500mM sodium carbonate solution added and 1.0 ml of 1:4 diluted folin-ciocalteu's phenol reagent was added immediately afterwards. Mixed well and incubated at 37C for 30 min. finally absorbance was measured at 660 nm and caseinolytic activity calculated. One caseinolytic enzyme unit is that amount of enzyme which liberates 1µmole of tyrosine in one min under the assay conditions (folin and Ciocalteau 1929).

APPLICATION OF ENZYME ON DIFFERENT SUBSTRATES:

Blood clot lysis: 1.0 ml of neutralized enzyme added to blood clot and incubated at 37C temperature up to the complete dissolution of clot.

Feather degradation: 1% hen feather treated with crude enzyme for up to 4 days at 37C temperature.

Recovery of silver particles: X-ray sheet strips treated with enzyme for 72 h at 37C temperature along with control.

Casein hydrolysis: 1.5% casein and 1.0% sterile agarose mixture poured in the sterile petri plates. After solidification wells were punched at the centre. About 30 micro liter of enzyme added to the well and incubated at 37C° temperature and 55C^{0} temperature for overnight.

Milk coagulation: 5.0 ml milk inoculated with 0.1 ml of enzyme and kept for overnight incubation at 37C^{0} temperature.

Blood stain removal: Blood stained cloth patches taken for this test. One of the stained cloth patch and one unstained cloth patch kept as control. The four stained cloth patches separately treated with- Water, Detergent, Enzyme and Enzyme + Detergent. The treatment was continued till any of cloth patch shows complete removal of stain.

Egg protein degradation: Coagulated egg white mixed with 5.0 ml of enzyme and incubated at 37C^{0} temperature and observed for egg protein degradation.

RESULTS:

1.Enzyme production reached maximum within 5 days of incubation. Protein content of enzyme was determined by Lowry method which was found 0.478 mg/ml. Caseinolytic activity was 266.2U/ml of enzyme and 5556.90 U/mg protein was the specific activity (Table-01).
2.Blood clot gets completely lysed within 19 h of incubation at 37C^{0} temperature (Fig-01).
3.Complete removal of feather hairs within 4 days of incubation was obtained (Fig-02).
4.White precipitate with chlorine water gives confirmation for release of silver particles hence the degradation of gelatin layer (Fig-03).
5.Zone of casein hydrolysis was clearly observed when the incubated plates flooded with 10% tri chloro acetic acid. The enzyme was active at 37C^{0} temperature as well as at 55C^{0} temperature (Fig-04).
6.Milk protein casein gets accumulated as a clot in the enzyme treated milk solution within overnight incubation (Fig-05).
7.Blood stain removal was successfully carried out within 15 min with enzyme and enzyme+ detergent combination when compared with controls (Fig-06).
8.Coagulated egg protein gets particulates and dissolved after 72 h of incubation at 37C^{0}tempoerature (Fig-07).

Table and Figures:

Enzyme source	Protein concentration in mg/ml	Caseinolytic activity U/ml	Specific activity U/mg
B. stearothermopjilus	0.478	226.2	556.9

Table-01: Caseinolytic activity of *B. stearothermophilus*

Figure-01: Complete blood clot lysis within 19 h of incubation at 37C^{0} temperature.

Figure-02: Complete removal of feather hairs within 4 days of incubation at 37℃ temperature.

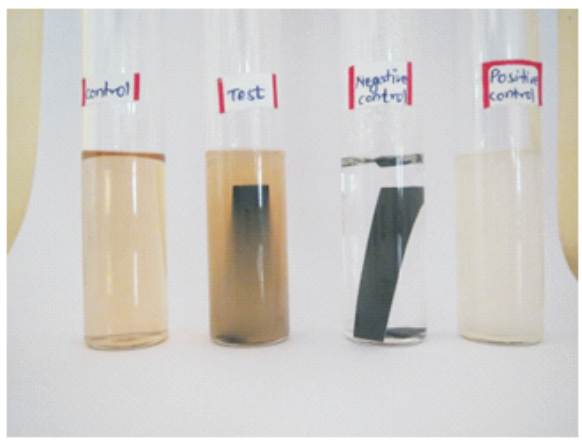

Figure-03: White precipitate with chlorine water confirms release of silver particles

Figure-04: Zone of casein hydrolysis at 37℃ & 55℃ temperature.

Figure-05: Milk protein casein gets accumulated as a clot within overnight incubation.

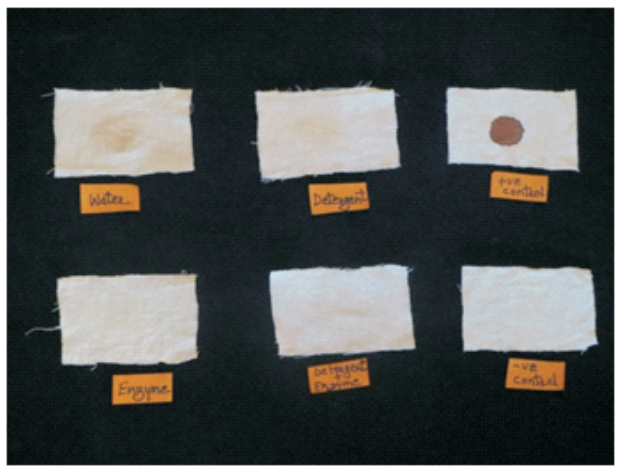

Figure-06: Blood stain removal within 15 min with enzyme and enzyme+ detergent combination when compared with controls.

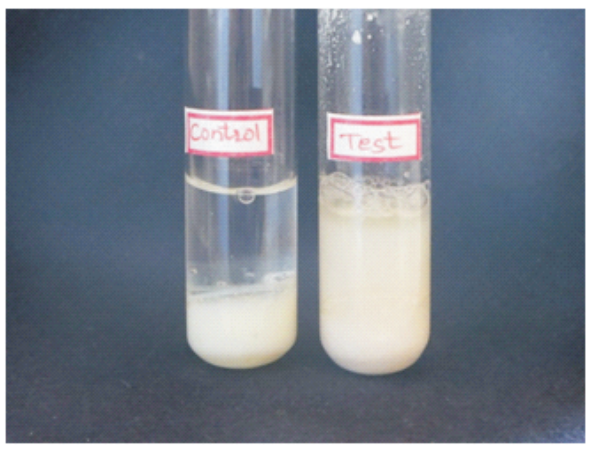

Figure-07: Coagulated egg protein gets dissolved after 72 h of incubation at 37°Ctempoerature.

DISCUSSION AND CONCLUSION:

Protease enzyme has wider application range in number on industrial areas.

Food and feed industry are of the growing industries in developing countries. Processing and its preservation require proteases along with other conditions.

Leather industry and photographic industry generates about 80-90% of wastes pollutants which load in the total environmental pollution. Therefore use of enzymatic processing accomplished by proteolytic enzyme is of great commercial importance.

Wool and silk industries also influenced in the terms of reducing the processing cost as well as ease of work.

In detergent industry about 25-30% total scale is single market for enzymes. Details of the enzymes used and the ways in which they used have rarely been published.

Medicinal use is one of the new research areas where we can develop a enzyme based therapy against from inflammatory reactions to cancerous tissues. Blood clot dissolution is one of the boon for all cardiovascular and stroke patients. But a major need in this field is successful live animal model trails, which is being expensive adds the cost of the therapy.

Another important aspect in which this protease enzyme can be used is environmental cleaning. Number of household garbage and food and fodder industries having proteinaceous wastes generated which finally added to environment and increases the load of protein matter to the pollutants. For this purpose in the waste management this protease enzyme play key role along with other hydrolytic enzymes.

Several aspects of proteases have stimulated research on the study of biochemical regulatory and molecular aspects of proteolytic enzyme system (Rao et al. 1998).

Looking into the commercial success of this enzyme researches have now started aiming at the discovery and engineering of novel enzymes that are more robust with respect ot their pH and temperature kinetics, using techniques of protein engineering and the identification of active site residues through chemical modifications, x-ray crystallographic data and SDM (Gupta et al.2002).

Hence, though microbial proteases already play an important role in several industries, their potential is much greater and their applications in future processes are likely to increase in coming era.

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