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ISOLATION AND SCREENING OF THERMOPHILIC BACTERIA PRODUCING THERMOSTABLE PROTEASE ENZYME FROM SOIL

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ABSTRACT

Isolation and screening of thermophillic Bacteria producing thermostable protease enzyme from soil .A most important factor influencing the effect of temperature on growth and the temperature sensitivity of enzyme catalysed reactions ,like that of any reactions will roughly double for every 10°C rising temperature .these are bacteria that live in salt water solution, in boiling hot springs ,in acid water at <2.0,around the case of atomic reaction ,in the fuel tanks of airplanes, at the bottom of the ocean >110^{0}C to view a video of life around volcanie pents and hundreds of feet below the surface of the ground. Protilytic enzymes are involved in agreat valacity of phyliological process and their action action can be divided into different categories like limited proteolysis, unlimited proteolysis, protease, proteinase or peptide endopeptide & endopeptides are included in proteinases, and other mechanistic classes include the science proteinases cystenine proteinases aspartic proteinases, metallo proteinase. Different methods like sexeining for the thermophillic bacteria isolation of pure cultures, protease activity, morphology tests (like grams staining, biochemical tests oxide test, urase test) are included culture medias are identified through macconkeyagar, blood agar, stimmed milk broth, protein precipition method. SDS-page etc. composition of media consists of various chemical medias and different reagents. By the screening of bacteria more than 50 thermophilic bacteria colonies were grown the surface of agar medium pink and rod shaped bacteria was examined under microscope.

KEYWORDS: Temperature, Bacteria, Enzymes, proteinases, Thermophillic Bacteria.

INTRODUCTION

Environmental temperatures profoundly affect microorganisms like all other organisms. Indeed, microorganisms are particularly susceptible because they are usually unicellular. Their temperature various with that of the external environment for these reasons, microbial cell temperature directly reflect that of the cell surroundings. A most important factor influencing the effect of temperature on growth and the temperature sensitivity of enzyme-catalyzed reactions, like that of any chemical reactions will roughly double for every 10°C rising temperature. Because the rate of each reaction increases, metabolism as a whole is more active at high temperatures and the microorganisms can grow faster. High temperatures damaged macroorganisams by denaturing enzymes transport carries, and other proteins. Microorganisms, membranes are also disrupted by heat. The lipid bilayer simply melts and disintegrates. Thus although functional enzyme operate more rapidly at high temperatures. The macroorganisams may be damaged to such an extent that grow is inhibited because the damaged cannot be adequately repaired.[1]

The traditional temperatures very greatly between microorganism's options normally range from 0^{o}C to as high as 75^{o}C; where as microbial growth occurs at temperatures extendly from -20^{o}C to over 100^{o}C. The growth temperatures range for particular microorganisms usually about 30^{o}C. Some species (eg.*Neisseria gonorrhoeae)* have a small range and are called stenothermal others, like *Emterpcpccis faecalis*, will grow over a wide range of temperatures and are eurythermal. Until recently the highest reported temperatures for bacteria growth was 105^{o}C. It seemed that the upper temperature limit for life was about 100^{o}C.[2]

Normally the Bacteria live in the most diverse range of environments of any forms of life. There are bacteria that live in salt water below freezing in melted ice pockets in the Antarctic, in saturated salt solutions, in boiling hot springs, in acid waters at pH<2.0, around the core of atomic, reactors, in the fuel tanks of airplanes, at the bottom of the ocean at >110^{o}C to view a video of life around volcanic vents and hundreds of feet below the surface of the ground. A basic principle of microbial growth is that very microbe has a preferred environment and it is a challenge to the investigator to tease out the unique set of conditions, called the optimal growth condition that suits each microbe best. Thermophilic submarine microoraganisms are found as normal inhabitants of continental and volcanic areas, geothermally heated.[3]

Sea-sediments and hydrothermal vents and thus are considered their particular areas. Cellular components of thermophilic organisms (enzymes, proteins and nucleic acids) are also

thermostable. Apart from high temperature, they are also known to withstand denaturants of extremely acidic and alkaline conditions. Thermostable enzymes are highly specific and thus have considerable potential for many applications. The use of such enzymes in maximizing reactions accomplished in the food and paper industry, detergents, drugs, toxic wastes removal and drilling for oil is being studied extensively. The enzymes can be produced from thermopiles through either optimized fermentation of the microorganisms of fast-growing mesophiles by recombinant DNA technology.[4]

Proteolytic enzyme s are involved in a great variety of physiological processes and their action can be divided into two different categories:

1. Limited proteolysis, in which a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formerly inactive protein i.e., conversion of prohormones to hormones.

2. Unlimited protelysis, in which proteins are degraded into their amino acid constituents. The protiens to be degraded are usually first conjugated to multiple molecule of the polypeptide ubiquitin. This modification marks them for rapid hydrolysis by the proteasome in the presense of ATP. Another pathway consists in the compartmentation of proteases e.g. in lysosomes. Protiens transferred into this compartment undergo a rapid degradation.

Protease, Proteinase or peptidase

The international union of Biochemistry and molecular biology (1984) has recommended wing the term peptidase for the subset of peptide bond hydrolases. The widely used term protease is synonyms with peptidases comprise two groups of enzymes: the endopeptidases and the exopeptidases, which cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C- terminus respectively. The term proteinase is also used as a synonym word for endopeptidase and four mechanistic classes of proteinases are recognized by the IUBMB as detailed below. The modern scheme of nomenclature is that Proteinases:

1. Endopeptidase.
2. Exopeptidase.

Proteinases are classified according to their catalytic mechanisms. The international Union of Biochemistry and molecular Biology have recognized four mechanistic classes:

a.The serine proteinases

b.The cystenine proteinases

c.The aspartic proteinases

d.The metallo proteinases

This classification by catalytic types has been suggested to be extended by a classification by families based on the evolutionary relationship of proteases.[5]

MATERIALS AND METHODS

Media

Nutrient agar medium, skimmed milk agar medium.

Methods

Screening for thermophillic bacteria[6]

First autoclaved the petriplates, pipettes and glass wavers and also what are the requirements required in the process. Prepare nutrient agar 100ml and add the 0.15 grams agar autoclaved at 121 ^{0}C for 15 min. After completion of the autoclaving remove the nutrient agar mediums cool it. And pour in to the required Petri plates. In between take the 1 gram of soil sample and dissolve 99ml of the distilled water. Keep the cotton plugged cover with paper. This soil sample containing tube keptto the shaking water the 50^{o}c for 1 hrs. Ever 5 min can rotate the soil sample containing tube. This diluted sample to 100 times represent 10^{-2} dilution. 1 ml of homogenized sample (10^{-2}) was taken then the pipette in to test tube containing 9 ml of the dilytes using sterilized pipette. The represents 10^{-3} dilution. This procedure was repeated using sterilized pipette for each dilution up to 10^{-9} dilution. From 10^{-7}, 10^{-8}, 10^{-9} 1 ml was pipette out in to a sterilized petriplate in triplactes. Petriplates was labeled properly. Care was taken to mix the sample solution of each dilution prior to pip petting out.

About 15-20 ml of molten and cooled nutrient agar medium was taken and poured in to the petriplates under strict aseptic condition. Percolates before the solidification the medium were rotated in clockwise and anti lock wise direction for through for mixing of inoculcums. After the solidification the plates were inverted and incubate at 50^{0}c in an incubator for 24 hrs. after incubation the thermophillic bacteria colonies were identified.

Isolation of Pure Cultures

Prepare the nutrient agar medium 100ml in that add the more amount of agar and sterilized at 121^{o}c for 15 min.

After completion of the sterilization medium was plottede in sterilized petriplates, allow the solidification. Taks thew single colony of the thermophillic bactgeria colony streak in to the nutrient agar plate. The plates were kept for the incubation at 50°C for 24 hrs.

Ptotease activity

Prepare the prtein containing skim milk agar medium 100ml and autoclaved at 15lb for `5min. The medium was poured into the sterilized petriplates and allowed to solidify. After solidification the thermophillic bacteria were inoculated in to the skimmed milk agar plates and incubatede for 24hrs at 50°c.[7]

Morphology Test[8]

Gram Staining

Pink and Rod shaped bacteria was examined under the microscope.

Identification using culture media

Nutreint agar

Peptic digest of animal tissue	0.5
Beef Extract	0.3
NaCl	0.5
Agar	1.5
pH	7.0
Dist. Water	100ml

Blood agar medium

To the Nutrient agar medium 7% sheep blood is added after sterilization.

MacConkey agar

Peptone:	2.0
Lactose:	1.0
Sodium truant chlorate:	0.2
Sodium Chloride:	0.5

Agar:	1.5
Natural red:	0.03
D. Water:	100
pH:	7.4

Note: All the numbers enlisted are in Grams/100ml.

Protein precipitation method

After the enzyme release in the broth take the 1ml of enzyme containing broth centrifuge it 10,000rpm at 4^{o}C for 20 min. the extra cellular protease enzyme in the supernatant liquid was properly purified by 80% ammonium sulfate. The enzymatically active fraction were finally purified the protease enzyme. After the purification, apply the SDS-PAGE.

SDS-PAGE

Wash thoroughly the notched & rectangular glass plates and dry. Insert them into a fresh & clean polythene bag. Notched plate should be facing top. Insert a pair of 1.0mm thick spacers in between the notched & rectangular plates. Move the spacers to the each side. Clamp this arrangement on to the gel-casting stand with the dummy plates otherwise leave it. Prepare the following separate gel mix in a 25-ml conical flask.

Acryl amide monomer-2.3501
4Xseparate gel buffer-1.750ml
10%SDS-0.070ml
10%APS-0.035ul
d. water-2.800ml. (Final volume-7.0)

Degas the solution for 5min and add TEMED 3 UL, rotate the flask gently and pour the mix in the polythene bag. Immediately add 100ul of n-butanol over the get mix and leave the arrangement undisturbed for 20min. appearance of the interface between the bottom separating gel and top UN polymerized solution indicates the completion of polymerization. Remove the top Butanol & UN polymerization layer and wash. Prepare the following stock gel mix:

Acryl amide monomer-0.266ml
4X stacking gel buffer-0.500ml
10%SDS-0.20ml

10%APS-0.010ml

d. water-1.204ml & TEMED 1-2ul (final volume-2.0ml).

Mix gently pours on the separating gel and inserts the comb to 1 cm depth. Leave the arrangements for 20min. seal the bag kept UN disturbed at room temperature. Take samples in different concentration in 20ul volume and add 20ul of 2X - sample buffer. Keep samples in boiling water bath for 3 min. Cut open the polythene bag and take the gel. Remove the comb slowly. If there is buffer in wells remove the filter paper. Clamp the glass sandwich on the tank, notched glass facing the notch of the tank. Apply the sample (six different samples and a standard MW marker) to the wells and slowly layer the tank buffer on the each sample. Pour tank buffer 100ml each to bottom as well as the top buffer reservoir slowly. Connect the tank to the power pack, switch on. Chang the constant mA mode and run at 10mA constantly till the sample reaches the top of the separating gel. Chang to constant Volt mode and run at 75V till the BPB tracking dye reaches the bottom of the gel. After the run, take the gel plate, remove the slide spacers, and lift the notched plate using a scalpel. Take the gel-developing tray and pour about 50ml stain. Slowly tilt the rectangular glass plates and drop the gel in to the stain. Gently agitate the gel in stain for 30min. Decant the stain and add 50ml of destain 1-10min. Decant the destain 1 and 2; agitate till clear background is obtained. Take the gel document system and analyze the images. Determine the M.W of UN known peptides comparing the standard M.W markers.

Silver staining[9]

Procedure: Place the gel in a clean tray and add 50ml of TCA-30min Gentle shaking. Remove TCA and add 50ml of cross-linking solution-30 min gentle shaking. Wash in 100ml distilled water 10min X thrice. Incubate the get in 50ml of DTT-20min. remove DTT and add 50ml of silver nitrate solution 10min slow shaking. Wash in DD water -2min. remove water and add the Developing solution 50ml-10min, slow shaking. Change the developing solution twice every 10 min. stop the developing by adding distilled water or destain 1 solution protein bands appear as brown spots.

Gel Drying & Preservation

After destining the gel thoroughly in Distains 2. place the gel in a clean try. Add 50ml of methanol\ethanol and gently shake for 10 min. Decant the alcohol and add fresh Methanol\alcohol and leave for 15 min.

Take the gel and dry under fan. Place it on a polythene bag and press in between glass plates to make the surface flat. Dry under air current and seal in a polythene bag.

SDS-PAGE

Acryl amide monomer, Acryl amide, Methylene bis Acrylamide, D, water, 4X separating gel buffer. Tris, D. water.

10% SDS: SDS, d. water

10% APS: APS, D, water, 2X sample treatment buffer 4X stacking get buffer, 10%SDS, Glycerol, Bromophinol blue, DTT, D. Water

Tank buffer: Tris, Glycine, SDS, Coomassie brilliant blue stain

Coomassie brilliant blue: Ethanol, Acetic acid, Distilled water., Destain 1:50% ethanol, 7% acetic acid, Distilled water.

RESULTS

Screening for the bacteria

After completion of the incubation, more than 50 thermophilic bacteria colonies were grown on the surface of the agar medium.

Protease activity

Thermophilic bacteria were releasing the enzyme in the skim milk agar plate. So the enzyme was formed clear zone around the bacterial colony. The protease enzyme digest the protein into polypeptide and small peptide so that the zones were formed around the bacteria colony/

Identification using culture media

S.NO	MEDIA	RESULTS
1.	Mecconkey agar	Non-lactose fermentative colonies
2.	Blood agar	Non-hemolytic bacteria colony

Biochemical tests

S.NO	Tests	Results
1	Urease	Positive
2	Catalase	Positive
3	Oxidase	Negative

SDS-PAGE

The sample protein along with the marker are loaded into the well and running the electrophoresis. After completion of get running stained with silver staining and observed on

the U.V Trans-illuminator. Several proteins bands pattern were observed and the molecular weights of the protein are compared with that of markers to characterized the protein.

Fig1:-Sub culture of thermophilic bacteria

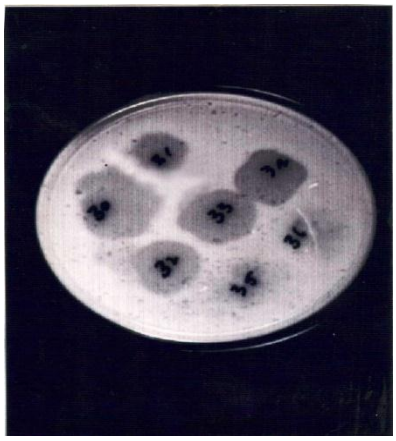

Fig 2:-Protease activity of screened of thermophilic bacteria

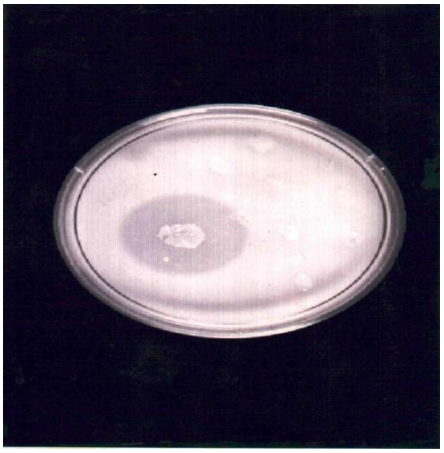

Fig 3:-Protease producing bacterial pure culture.

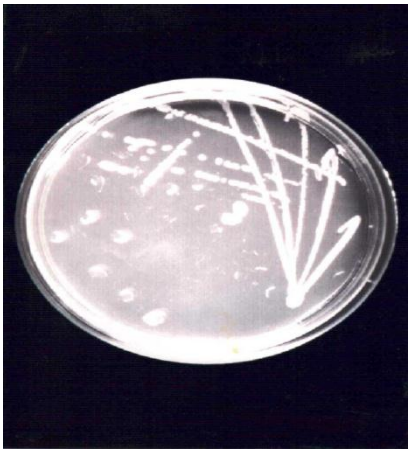

Fig 4:-Single pure culture activity.

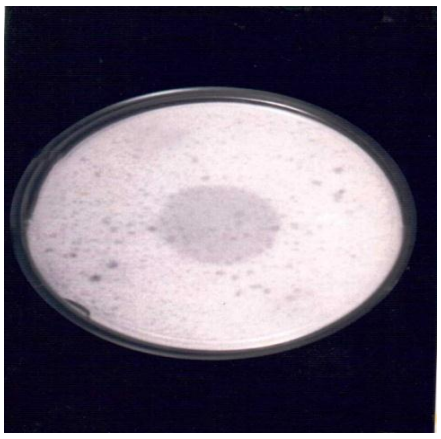

Fig 5:-Protease producing bacterial pure culture.

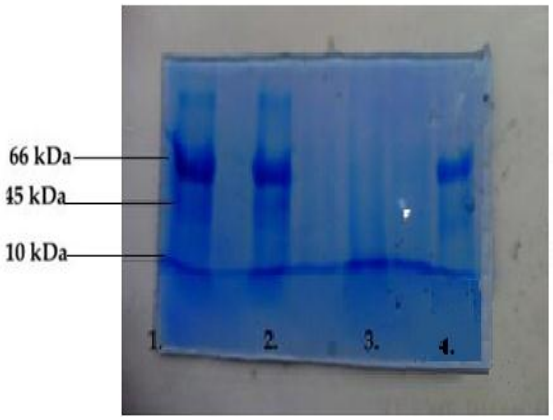

Fig 6:-SDS-PAGE.

DISCUSSION

This is the first report regarding a thermostable protease isolated a strain, tentatively designated *B. thermodenitrificans* TS-3, producing thermostable preotease. This enzyme isolate is most active at around 70^{o}C (at pH6.0). this is the highest temperature for optimal activity among known.[10] The ABNs of *B. substilis* and *Aspergillus niger*.[11] are reported to be highly active at 50 and 60^{0}c, respectively. ABN-TS is highly thermostable, and it has a half-life of 4 h at 75^{o}C, whereas the ABN (PPase-C) from the mesophilic bacterium *B. substilis* IFO3134 is inactivated completely by incubation at 65^{o}C within 30 min.

The present study isolation and indentification of Thermophilic bacteria producing Thermostable protease enzyme from soil in the screening method, the bacterial colonies were appeared more than 50 from these bacterial colonies sub cultured and tested each colony activity in the skimmed milk agar medium plates. Few more colonies were showed more protease activity by degrading the proteins into polypeptides. Based their zone formation the protein were precipitated and applied the SDS-PAGE. This protease enzyme more stable at high temperature.

SUMMARY

Thermophilic bacteria were isolated from soil the sample. Soil sample was serially diluted and taken this dilution sample was incubated first in the nutrient agar medium, and incubated at 18 hrs\ 50^{o}C. The bacterial colonies were sub culture when optical appeared. From this bacterial colonies screened for the protease activity. Few more colonies was showed highly protease activity. After that skin milk broth was prepared and inoculated with pure culture and incubated water bath at 50^{o}C for 24 hrs. After that the protease enzyme was crudly extracted by salt precipitation method. This curde protease activity containing protein was bioassyed in skimmed milk agar plate and it was shown positive result indicates for protease enzyme. The bacteria morphology and biochemical studies identification that gram staining, Catalase, oxidase.

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