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RESEARCH ARTICLE

STUDY OF EFFECT OF TEMPERATURE ON ENZYME ACTIVITY OF PROTEASE OF DOON VALLEY

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ABSTRACT

Proteases are typical enzymes that are being investigated for their potential usage in many areas of application, such as detergent, brewing meat, photography, leather and dairy. A large number of proteases have been purified and characterized from bacteria and few from fungi. Bacteria were isolated from the soil sample of Lachiwala, Doon Valley. Out of 64 bacterial pure cultures, 10 best protease producing microorganisms were isolated through activity zone methods. Protease activity was checked spectrophotometrically using azocasein dye as a substrate. 1 bacteria was selected from them. The thermos table enzyme showed activity up to at 90C.

INTRODUCTION

Proteases (E.C.3.4) are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases are hydrolytic enzymes that hydrolyze protein by adding water across peptide bonds and break them in smaller peptides in organic solvents (Al-Shehri, 2004). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes (Rao *et al.,* 1998) with 2/3 of this amount, being of microbial origin (Cowan, 1994). Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as homeostasis and inflammation (Rao *et al.,* 1998). Proteases play an important role in a wide rang of industrial process viz., baking, brewing, detergents, leather processing, pharmaceuticals, meat tenderization, cosmetics, peptide synthesis, waste treatment and medical diagnosis (Gupta *et al.,* 2002).

MATERIALS AND METHODS

Study area

The vegetation at Lachiwala is a mix of moist deciduous and Sal trees. It contains the marshes and grassland along with the Song River. The point coordinates of Lachiwala is E 78^0 06' 38.6" and N 30^0 13' 37.2" with attitude of 1683 feet above the sea level. The site of Lachiwala with species of Sal, Syzygium, rich in plant litter and content of organic carbon (oc) is law. The soil surface is compact. The texture of soil is sandy loam. Moisture on the surface is moderate while at sub-surface it is moist (Kumar and Bhalla, 1994).

Sampling

Sediment samples were collected by sterile spatula or by a corer from 6 different locations of Lachiwala area of Doon Valley. For this survey the sampling was done in 3 times a year-in the month of January (S_A), May (S_B) and September (S_C). The collected samples were brought to laboratory under refrigeration and were preserved at -20˚c.

Sample Preparation and spreading

Samples (S_A,S_B,S_C) were taken out of -20^0C freezer and kept at 4^0C for 1 h to 2 h. Then they were kept at RT for 12h to avoid the heat shock. The samples were prepared by serial dilution and spread plate methods.200uL of each dilution was spread into the nutrient agar plates (Beef extract 3.0g., Peptone 5.0g.,Nacl 5.0g ,Glucose10.0g.,Yeast extract 5.0g., Agar agar 20.0g., SDW 1000ml.,pH 7.2-7.4) (Sevinc and Demirkan, 2011).

Primary stock cultures preparation

After 7 days all the plates were taken out and they were then picked and streaked again in the nutrient agar plates with sterile tooth picks. The plates were kept at 30^0C in the incubator for 24 h. The bacterial cultures were then inoculated into nutrient broth (beef extract 3.0g, peptone 5.0 g, NaCl 5.0 g, glucose 10 g, yeast extract 5 g, SDW q.s. 1000 ml, pH 7.2 – 7.4) and

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placed in the orbital shaker at 30^0c and 120 r.p.m for 24 h. The cultures were restreaked onto the nutrient agar plates and the process was repeated until pure bacterial cultures were obtained.

Preservation of pure cultures

The selected microorganisms were streaked onto solid nutrient agar medium (1 organism in 1 plates) in order to make final and pure working cultures and at the same time glycerol stock were prepared for the final selected bacteria for long term preservation and preserved in-70^0c freezer. The entire procedure was repeated until a certain number of pure bacterial cultures (64) were found to carry on the further experiments.

Screening of microorganisms for proteolytic activity

The selection of most potent isolates was performed by two steps:

Activity zone technique according to Sonia Sethi *et al.* (2012), (Palsaniya *et al.,* 2012) and assay of enzymes according to Kole *et al.* (1988).

Activity zone technique

Determination of the most potent isolates was performed by gelatin clear zone technique.64 bacterial colonies(from each sample s_a,s_b,s_c) were screened for proteolytic activity in Petri dishes with production medium PM1 which contains: Beef extract 3.0 g, Peptone 5.0 g, NaCl 5.0 g, Glucose 10 g, Yeast extract 5.0 g, Gelatin 4.0g, Casein 10.0 g, Agar agar 20.0 g, SDW 1000 ml, pH 7.2 – 7.4. The plates were incubated at room temperature for 24 hour. At the end of incubation period, the remainder supernatant was removed and plates were flooded with 1% tannic acid (Kobayashi *et al.,* 1996).

The isolates were evaluated for their proteolytic activity by measuring a clear zone of gelatin hydrolysis formed on milky agar. A total of 10 isolates were selected. The activity was then detected by the appearance of clear-cut clearing zones around the bacterial growth indicated the ability of the isolate to hydrolyze gelatin due to the biosynthesis of the protease enzyme. The resulting diameters of the clearing zones were recorded.

Enzyme assay

Protease activity in microbial culture is estimated by different ways. Estimation of Protease activity by Spectrophotometer is the cheap and best method. Spectrophotometric assay of protease could be done in many ways. But azoprotein substrates would be the best choice for specific as accurate enzymatic reaction (Ghosh, 2004). Azocasein is a chemically modified protein containing sulphanilamide group covalently linked to peptide bond of casein. During incubation for 30 min. proteases hydrolyse peptide bond, liberating shorter peptide and amino acid from the chain, which is orange in colour. The intensity of colour, is measured spectrometrically to determine protease activity. One unit of enzyme activity was defined as the amount of enzyme required to solubilize 1µg. of substrate/min under assay condition.

Culture preparation

Microorganisms were collected form respective working culture plates and inoculated into the production medium. The fermentation was carried out at 30^0C with orbital shaking at 120 rpm for 48 h in 100 ml. Erlenmeyer flasks containing 20 ml Lauria- Bertani (LB) medium .It contains: Yeast extract 5.0 g, Peptone 10.0 g, NaCl 10.0 g, SDW q.s. 1000 ml, pH 7.2 – 7.4.

Reagents and Instruments

1.0.1 M Tris- HCl (pH 8.5)
Preparation: 0.1 M of Tris- HCl solution was prepared in water and the desired pH was adjusted as required with 0.1 M Hydrochloric acid (HCl).

2. Azocasein (2500 $\mu g.ml^{-1}$ solution): Substrate
Preparation: In accurately weighed azocasein half if the required amount of water was mixed in a container and it was dissolved by very slow and gentle shaking. After a part of azocasein was dissolved it was kept in refrigerator at 4^0 C for30min. Next the solution was taken out and rest half of water was mixed and remaining undissolved azocasein was dissolved with slow shaking of the container and kept at 4^0 C for few min before use. It should be freshly prepared before use and should not be shaken vigorously during preparation, which might lead to degradation of azocasein.

3. 10% tri- chloro acetic acid (TCA) solution
Preparation: 10.0 g of TCA was dissolved in distilled water and kept at 4^0 C before use.

4. Spectrophotometer

5. Centrifuge

Procedure

Reaction mixture was prepared with 100 µL culture supernatant (1.5ml culture centrifuged at 10×10^3 r.c.f. for 5 min at 4°C to separate supernatant and cells),125 µL azocasein solution, 50 µL 0.1 M Tris-HCl and 225 µL distilled water. The entire mixture was incubated for 30 min at 37°C and then the assay was terminated by 500 µL ice cold 10% TCA solution (18). TCA was precipitated by centrifugation at 10×10^3 r.c.f. for 5 min at 4°C. Finally the O.D. was measured with required dilution at λ_{440}.

Control: Media filtrate without the substrate was taken as blank. Volume was made up by adding 0.1 M Tris-HCl buffer only.

Precaution: Azocasein being a very delicate and light substrate should be shaken gently.

Study of enzyme activity -Effect of temperature

Any enzyme has an optimum temperature requirement for its activity. Temperature is also an important parameter for enzyme activity. Enzymatic activities were checked by altering the temperature e.g. 25^0c, 37^0c, 40^0c, 50^0c, 60^0c, 70^0c, 80^0c, 90^0c, and 100^0c. The activity found in various incubation temperature were plotted in order to get the temperature profile.

RESULTS

Results of effect of temperature on enzyme activity by LW-1, LW-2 and LW-3

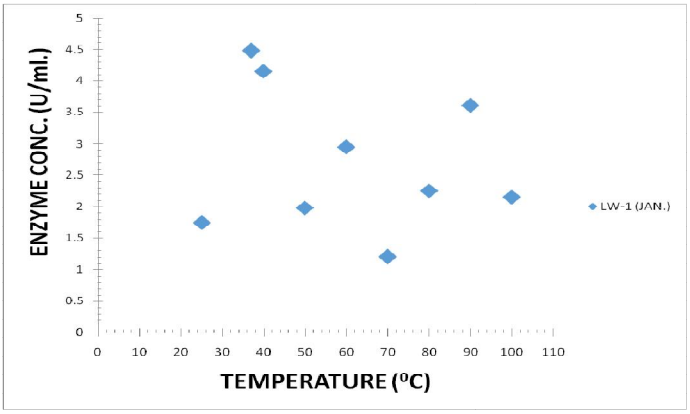

Fig.1. Effect of temperature on enzyme activity by microorganism LW-1

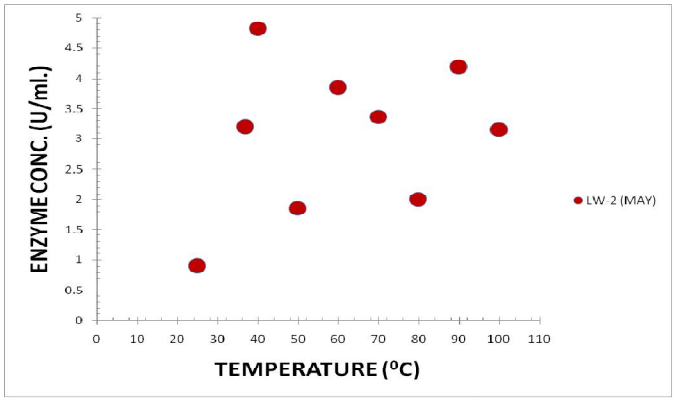

Fig. 2. Effect of temperature on enzyme activity by microorganism LW-2

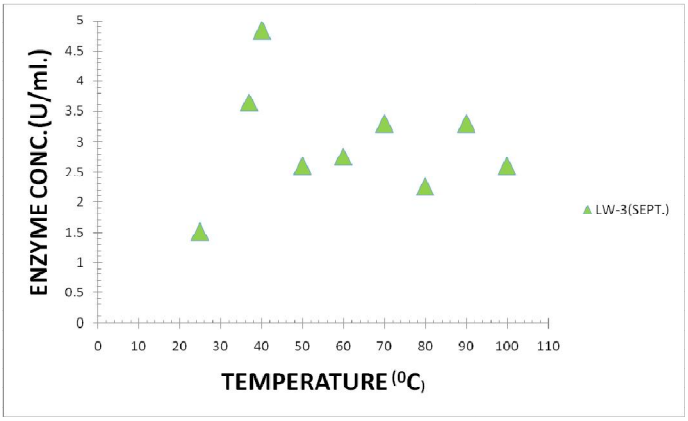

Fig. 3. Effect of temperature on enzyme activity by microorganism LW-3

According to Fig.1 microorganism LW-1 in fermentation broth gave a temperature optimum at 37^0c (4.48 U/ml) and as the temperature increased the enzyme stability started decreasing. Again from 70^0c the enzyme was found to be stable (1.2 U/ml). It gave 3 peaks, one at 37^0c (4.48 U/ml) and others at 60^0c (2.95 U/ml) and 90^0c (3.61 U/ml).

Fig.2 depicts that enzyme from LW-2, when fermented gave maximum stability at 40^0c (4.82 U/ml). LW-2 enzyme started losing its stability with the increase in temperature but again showed thermostability at 60^0C (3.85 U/ml) and 90^0C (4.18 U/ml).

While in Enzyme from LW-3 (Fig.3) the temperature optima was also found to be at 40^0C (4.85 U/ml). LW-3 enzyme started losing its stability with the increase in temperature but again showed thermostability at 70^0C and at 90^0C (3.3 U/ml).

DISSCUSION

In the current work, the maximum biosynthesis protease enzyme(s) was recorded within incubation temperature of 37^0C for LW-1, 40^0c for LW-2 and LW-3 (4.48, 4.82 and 4.85U./ml. respectively). The present result is in complete accordance with the finding of other investigators viz. 37^0c (Thankamani *et al.,* 2011; Kumar *et al.,* 2002; Maghsoodi *et al.,* 2013) and 40 ^{0}C (Sierecka, 1998; Miyaji *et al.,* 2005).

On the other hand, other optimum incubation temperatures for protease production by *Bacillus* species at 35^0C (3), 50^oC (Sumantha *et al.,* 2006; Kim *et al.,* 2001; Ali, 1991) and 60^oC (Kumar and Bhalla, 1994; Maghsoodi *et al.,* 2013) were reported.

Conclusion

The enzyme was stable up to 90^0C indicating that it was thermostable enzyme. The results of temperature stability of the investigated neutral protease suggesting that it may have potential application in detergent, pharmaceutical formulations, leather, laundry, food (Rao *et al.,* 1998) and waste processing industries (Hazem *et al.,* 2012).

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