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Effect of growth media pH and incubation temperatures on the production of proteinases from bacillus *sp.* 26

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Bacillus sps-26 isolated and characterized from dairy effluents was challenged for its ability to produce alkaline proteinase using different temperature and pH as bioparameters with a chemically defined medium containing 5% casein. The maximum enzyme production was achieved at 30.C at pH 10, which was assayed using Genov method. Although moderate enzyme level was obtained at 10.C, it can pave the way for its affordable use in detergent industries.

Key words : *Bacillus sp*-26, Alkaline proteinase, Bioparameters, Tseushida method, Lowry method.

INTRODUCTION

A variety of enzyme products has been developed for use in biological or enzyme detergent to enhance the removal of organic material from textile fibre. The most widely used of these proteinase when function and mode of action is to remove proteins stains such as grease, egg and human sweat by proteolytic degradation to polypeptides that are more soluble and amino acids.

Proteinases are the most important industrial enzymes for about 60% of the total enzyme market and have a number of practical applications in industries such as detergent, tanning, dairy, baking, brewing, leather and textile etc. (Ward *et al.*, 1985). Fungal proteinase is being used for centuries in the Orient for the production of soy sauce. The process involved growing proteolytic strains of A.oryzae on the soybean to produce a heavily sporulated koji (Nahara *et al.*, 1982).

Proteinases are mainly classified into four groups based on their mode of action: Aspartic-proteinase, Serine-proteinase, Metalo-proteinase, Cysteine- proteinase. Alkaline proteinase falls onto either serine-proteinase or metalo-proteinase.Alkaline proteinase are produced by a wide variety of microorganisms such as bacteria, fungi, yeast and actinomycetes.

Bacterial species in particular; *B.subtilis, B.lentus, B.licheniformis* and *B.brevis* are reported to be prolific producers of alkaline proteinase, which constitute a major source of enzymes used in detergents. Different authors reported production, proliferation and characterization of chemo stable alkaline proteinase from *Bacillus* spp. (Takami, 1989; Ferrero *et al.*, 1995; Adinarayana and Elliah, 2002; Prakasham *et al.*, 2005; Enshasy, 2008).

In search of novel extracellular alkaline proteinase active at low and moderate temperatures, isolation and screening of cold active alkaline proteinase have been carried out at RRL, Trivandrum.Extensive screening procedures performed a different stages resulted in the isolation of more than 50 bacterial isolates, which could tolerate alkaline pH. Further screening of those organisms in salt high patent media resulted in the isolation of potent bacterial cultures (identified as *Bacillus* sps-26) that was capable of producing alkaline proteinase in the early growth hours (Sandhia and Prema, 1998).

The influence of various bioparameters on metabolites has been well established. By manipulating the cultural and nutritional parameters of an organism, the enzyme production could be over expressed (Gupta *et al.*, 2002). Following this context, investigation has been carried out to study the effect of growth media, temperature and pH in the production of alkaline proteinase by *Bacillus* sp-26.

MATERIALS AND METHODS

Organism and its maintenance:

Bacillus sps-26 strain used in this study was earlier isolated in RRL, Trivandrum from soil samples. It was maintained on nutrient protein agar medium (pH 10) stored at 4^0C and was periodically subcultured.

Medium used for growth:

The composition of the growth medium (g/100ml) consist of Casein-0.15, Yeast extract-1.0, $MgSO_4$-0.005, KH_2PO_4-0.005, $FeSO_4$-0.001, pH-10.

Inoculum preparation:

Introducing a loop full of culture to the sterilized growth media, incubating at room temperature for 24 hrs and with 5% of the 18hr grown culture serve as inoculum.

Production of enzyme:

Sterilized medium (100ml) taken in 250ml Erlenmeyer flasks inoculated with 5% pre-inoculum were kept in an environmental shaker (New Brunswick) at required different growth temperatures at 150rpm. Samples were collected at frequent intervals and culture free supernatant was obtained by centrifuging the culture broth at cold centrifuge, at temperature 4^0C, with 5000 rpm for 5 minutes. The culture supernatant was used as the enzyme source.

Proteinase assay:

Proteinase activity was assayed according to Genov *et al.*, (1982) using 2% casein as the substrate. 0.5 ml of the buffered substrate (carbonate-bicarbonate buffer, pH-10) was digested with 0.5ml of suitable diluted enzyme for 10 min at 40^0C. The reaction was terminated by adding 2 ml of 10% TCA (Trichloroacetic acid). The mixture was centrifuged and collected the supernatant. From that supernatant 0.5ml enzyme was taken and 5 ml of 0.5M Na_2CO_3 and 1 ml of 1N Folin-Ciocalteau's reagent were added. In addition, these tubes were incubated for 30 minutes for dark color development. The proteinase activity was prepared along with test. The color was read against the reagent blank at 660nm. The proteinase activity was expressed as micromols of tyrosine released per minute per ml.

Estimation of soluble protein:

Soluble protein was analyzed by Lowry method (Lowry *et al.*, 1951). To 1 ml of diluted supernatant solution, 5 ml of 1% $CuSO_4$ was added and the tubes were shaken well and incubated for 10 minutes. To this 0.5 ml of Folin-Ciocalteaus reagent (1:1) was added and mixed well. A reagent blank was also prepared. These tubes were kept in dark for 30 min for color development. The color developed was read against a reagent blank at 640 nm. The proteins liberated were compared with standard graph of bovine serum albumin.

Estimation of biomass:

Biomass can be estimated by dry weight method. A known volume of culture broth was taken in pre-weighed crucible, dried in an oven at 103^0C for 3 hours until a constant weight was obtained.[Biomass = (Initial weight-Final weight)/ Initial weight].

Production of alkaline proteinase at different temperature:

Fermentation experiments were carried out at different growth temperatures 10, 20, 25, 30, 35 and 40 in modified Ree's media. After autoclaving and cooling, pH of all media was adjusted to 10 by the addition of volume sterile 10% Na_2CO_3. Experiments were performed taking 50ml each of the above- described media in 250ml Erlenmeyer flasks. 5% of an 18hours old inoculum raised in the basal medium was used as inoculum.

The inoculated flasks were kept on an environment shaker (New Brunswick – make 4380) at 150 rpm for 96 hours at the different temperatures. Samples were removed at every 24 hours and estimated for pH and biomass. The culture broths were centrifuged at 40^0C in a cold centrifuge (Himac) with 6000 rpm for 15 minutes. In the cell free supernatant, enzyme activity at temperature 40^0C and soluble protein were estimated.

Production of alkaline proteinase at different pH:

The growth media pH was adjusted as 6, 7, 8,9,10 and 11 and the culture was grown in above media. All other steps were followed have given earlier.

RESULTS AND DISCUSSION

The influence of bioparameters on enzyme production *i.e.* proteinase production from a *Bacillus* sp-26 were done. The incubation temperatures were taken at 10, 20, 30, 35 and 40^0C as the earlier reports suggested that this culture is a psychrophilic one. The psychrophilic organism can grow at 5-25^0C and they will be able to have cell multiplication at 0^0C (Margresin and Schinner, 1994). Moreover, the enzyme-producing organism will have optimum temperature between 10-20^0C than their growth temperature. Based on these data the present study has been designed.

Influence of temperature on proteinase production:

The results pertaining to the above experiments are given in Fig. 1, 3 and 4. When the culture was grown at 10^0C the soluble protein level increased slowly, where as biomass production was very negligible. At temperatures 30, 35 and 40^0C, there was an increase in soluble protein level up to 48 hours and the biomass production increased considerably at higher temperatures.

The level of proteinase produced from this culture that was grown at different temperature was given in Fig. 1. The enzyme assay temperature was maintained at 40^0C and pH at 10. The results clearly indicated the enzyme production was more at 30^0C and as the temperature grows

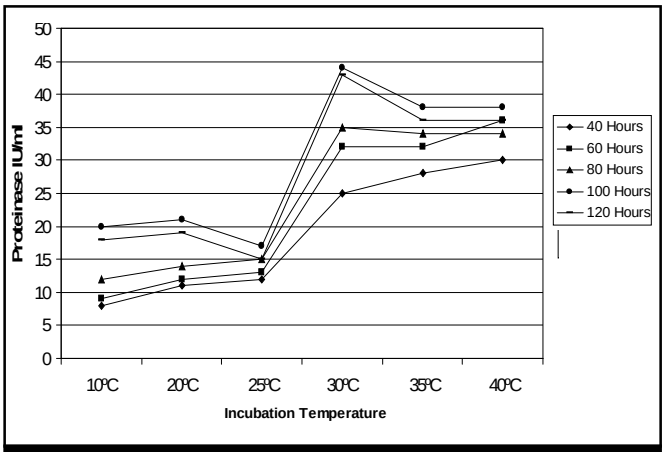

Fig. 1 : Proteinase levels from *Bacillus* spp. grown at different incubation temperature

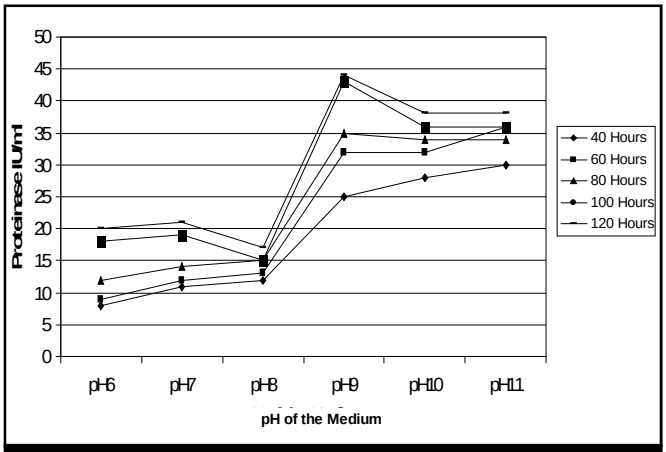

Fig. 2 : Proteinase levels from *Bacillus* spp. grown at different media pH

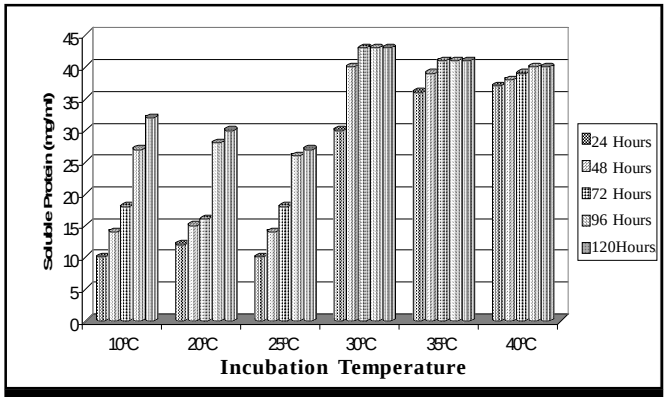

Fig. 3 : Level of solubel protein at different incubation temperature

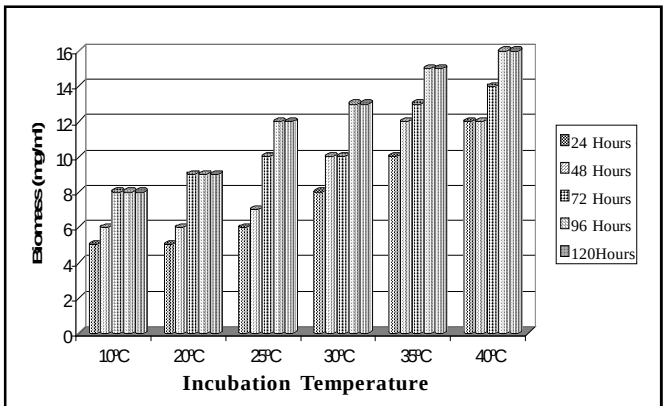

Fig. 4 : Level of biomass at different incubation temperature

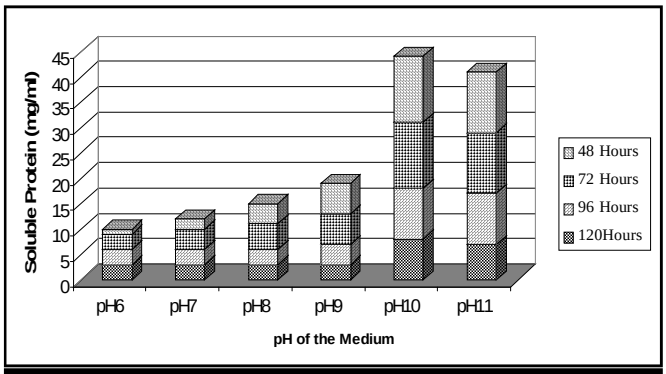

Fig. 5 : Level of soluble protein at different pH condition

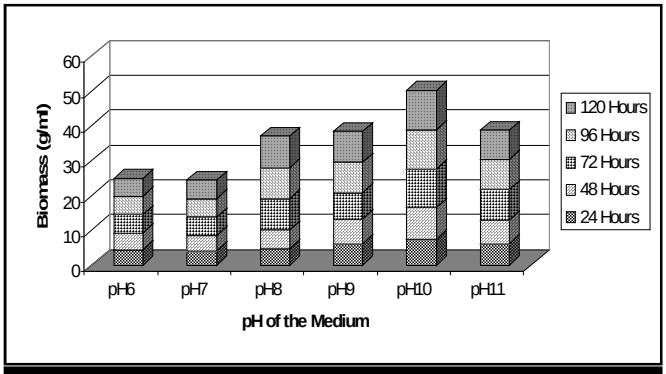

Fig. 6 : Level of biomass at pH condition

up, the production decreased. At higher temperatures like 40^{0}C the enzyme production was affected.

Current results confirmed the general theory that when psychrophilic / psychotropic organisms were grown at low temperature, biomass production will mew minimum with high enzyme activity. This enhanced enzyme activity could be to met the metabolic activities in that low temperature (Margresin *et al.*, 1994).

Temperature is one of the crucial parameters that have to control during the growth of the microorganisms, since it influences the growth and energy metabolism. Low temperature imparted stress to the organisms and subsequent effect on synthesis of protein. They also

produce cold shock protein and they might be required for low temperature adoption (Gessey and Morita, 1979).

In the current investigation an increase in enzyme production occured at 10^0C and then decreased up to 25^0C and after 30^0C, there was an increase. This shifting clearly indicates that *Bacillus* sps-26 would be producing a set of proteinase at low temperature then switched over to another set of proteinase at higher temperature.

Extensive experimental evidences have been postulated that the expression of proteinase production in different temperature would be decreased or increased according to the mRNA content exceeding for the particular enzyme (Sato *et al.*, 1994). Rate of protein synthesis or mRNA activities were controlled by external temperature (Margresin and Schinner, 1994 and Rao *et al.*, 1998). Present studies revealed that for *Bacillus* sps-26 an optimum growth temperature was 40^0C; but the enzyme production was at 30^0C.

Influence of pH on proteinase production:

The results of the effect of pH on the growth and production of enzyme by Bacillus culture at different media pH are given in Fig 2, 5 and 6. The level of soluble protein was low at low pH and it increased at pH 10 and 11.Similar conditions were observed in biomass production too.

Maximum enzyme production was occurred at pH 10. At pH 6, 7, 8, the production was very low. Beyond these pH values the enzyme level increased but at pH 11again it decreased. Earlier reports also confirmed the above results (Sandhia and Prema, 1998). The initial pH of the media influenced expression of many enzyme systems and their transport across the cell membrane (Moon and Parulekar, 1991).

There was a close relationship between proteinase synthesis, utilization of nitrogenous compounds and pH variation (Reilly and Day, 1983). In *B.stearothermophilus* better growth and proteinase production occur at pH 5 (Razak *et al.*, 1994). Fungi have proteinase production towards acidic pH and in others, alkalophilic species were in alkaline condition.

From the above studies, it was concluded that the temperature 30^0C and pH 10 were the optimal bioparameters for peak production of proteinase by *Bacillus* sp-26.

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