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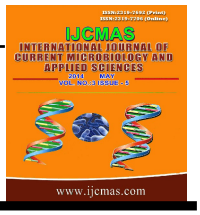

Original Research Article

Media optimization of protease production by *Bacillus licheniformis* and partial characterization of Alkaline protease

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K e y w o r d s

Bacillus licheniformis, Alkaline protease, Growth media optimization

A B S T R A C T

Protease production was enhanced by optimizing the culture conditions. The nutritional factors such as carbon and nitrogen sources, NaCl and also physical parameters like incubation time, pH, inoculum size were optimized for the maximum yield of protease. *Bacillus licheniformis* (MTCC NO. 7053) was optimized with respect to inoculum concentration, temperature, initial medium pH and incubation time, the designed medium for *Bacillus licheniformis* was Rice husk,3%(w/v); Potassium nitrate, 0.75%(w/v); salt solution, 5%(v/v) {$MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4, 0.5%(w/v)}; $FeSO_4.7H_2O$, 0.01%(w/v) and $CaCO_3$, 0.5%. Thus, with above selected carbon and nitrogen sources along with 1% NaCl and 2% inoculum, the maximum protease production (184±0.25U/ml) was obtained in the period of 72 h of incubation at pH-9.0 under 160 rpm when compared to the initial enzyme production(98±0.32U/ml) on basal media with cost effective manner. The crude enzyme extract of the strain was also characterized with respect to temperature, pH, incubation period and different concentrations of casein which was used as enzyme substrate. This study showed that this enzyme has wide range of pH stability from 8 to 11 with optimum activity at pH-10.0.It is thermostable with optimum activity at 65°C (*Bacillus licheniformis*) with 1h incubation of enzyme with 1% casein as its substrate. From the above investigations it is concluded that the enzyme is alkaline protease and thermostable with potential applications in various industrial processes.

Introduction

Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions (Maurer, 2004; Saeki *et al*., 2007). These enzymes find applications in detergents, feather processes, food processing, silk

gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation (Bhaskar *et al*., 2007; Gupta *et al*., 2002; Jellouli *et al*., 2009; Sareen and Mishra, 2008). The majority of commercial alkaline proteases are produced by bacteria, especially *Bacillus* spp. (Jellouli *et al*., 2009). Studies have showed that nutritional factors including sources of carbon and nitrogen can influence protease enzyme production by bacteria. Besides this nutritional factors, physical factors such as inoculum concentration (Kaur *et al*, 1998), temperature, pH (Tobe *et al*, 2005) and incubation time (Yossan *et al.*, 2006) can also significantly affect protease production.

A fermentation medium forms the environment in which the microorganisms live, reproduce and carry out their specific metabolic reactions to produce useful products. Two distinct biological requirements are considered in most of the industrial fermentation processes for medium design where the product is something other than the cell mass itself. First, the nutrient has to be supplied to establish the growth of the microorganism. Second, the proper nutritional conditions have to be provided to maximize the product formation. It is also well established that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH, temperature, dissolved oxygen (DO) and incubation time (Moon and Parulekar, 1993; Razak *et al.*, 1994; Oberoi *et al.*, 2001). The cost of the growth medium is another significant parameter for making the production process industrially viable. Approximately 30-40% of the production cost of the industrial enzyme is estimated to be accounted by the cost of the growth medium (Gessesse, 1997). The purification process of an enzyme also depends on the composition of the growth medium. Therefore, selection of the right medium ingredients and their concentrations optimization after selection of suitable microorganism is imperative for high yield of desirable enzymes by fermentation.

The present investigation was aimed at optimization of growth conditions of *Bacillus licheniformis* (MTCC NO.7053). to enhance the protease enzyme production using submerged fermentation by the selection of medium components for the optimal production of extracellular alkaline protease and to study the biochemical properties of the enzyme.

Materials and Methods

Chemicals

All chemicals (99% purity) used in this study were purchased from Hi-Media Laboratories, Merck (Mumbai, India) and Sigma(U.S.A).

Culture and Growth Condition:

Bacillus licheniformis (MTCC NO.7053) used for this study was obtained from IMTECH, Chandigarh, India. The strain was maintained on nutrient agar slants having pH 7.0 at 35±2°C slants and subcultured for every 15 days.

Preparation of inoculum

A slant culture of *B.licheniformis* was inoculated into 50ml of sterilized media of glucose, 0.5%(w/v); peptone, 0.75%(w/v); salt solution, 5% (v/v)-{($MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4, 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01%(w/v) in 250 ml Erlenmeyer conical flask and incubated at 37^0C for 24 h in a shaker incubator.

Production of enzyme

Protease enzyme production was carried out using standard media glucose, 0.5%(w/v); peptone, 0.75%(w/v); salt solution, 5%(v/v)-{($MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4 0.5%(w/v)}; and $FeSO_4.7H_2O$, 0.01%(w/v) at 160rpm. The culture medium was harvested and was subjected to centrifugation at 10,000 rpm for 20 min to obtain crude extract, which was used as enzyme source. *Bacillus licheniformis* was taken for further optimization studies to enhance the protease production.

Protein estimation

Protein estimation was determined according to the method of Lowry *et al*., (1951), using crystalline bovine serum albumin as standard.

Assay of Alkaline protease enzyme activity

The enzyme activity was determined by using Mc Donald & Chen method (1965). One ml of enzyme was added to 2 ml of casein (1% w/v in 0.1N Glycine – NaOH buffer pH 10) and the mixture was incubated for 15 min at 60^0C.

The reaction was terminated by adding 3 ml of 10% trichloroacetic acid and then centrifuged for 15 min at 10,000 rpm. Then 1 ml of filtrate was mixed with 5ml of alkaline copper reagent and after 15 min, 0.5ml of Folin-ciocalteau reagent was added, up on standing for 30 min the absorbance was read at 700nm. Similarly blank was carried out by replacing enzyme with distilled water. One unit enzyme activity is defined as the amount of enzyme that releases 1µg of tyrosine per ml per min under the assay conditions. The range of concentration 50–250 µg of tyrosine was used as standard.

Optimization of basal media

The basal media used for production of protease was subjected to optimization with respect to different carbon and nitrogen sources along with physical parameters like pH, temperature, salinity, inoculum size and incubation period.

Effect of different carbon sources on protease prodution

Standard media was supplemented with various carbon sources such as glucose, mannose, maltose, soybean meal, wheat flour, sugarcane bagasse, rice bran, rice husk and molasses at various concentrations.

Effect of different nitrogen sources on protease prodution

Standard media was also optimized with different inorganic nitrogen sources such as potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate and organic nitrogen sources like peptone, casein, skim milk powder, beef extract and yeast extract.

Effect of NaCl, pH, inoculum size, incubation period and temperature on protease prodution

The effect of various physical parameters on protease production was assessed by growing bacterial culture of *Bacillus licheniformis* in the standard growth media and varying the salt concentrations from 1% to 3% variation. For optimizing pH, the medium was prepared by varying the pH from 2.0 to 12.0. Effect of varying inoculum size from 0.5% to 5% on protease production was determined.

Similarly, for the investigation of optimal incubation time for protease production, the bacterial culture was inoculated in the growth media and optimized for different incubation periods up to 120h at 160rpm. Samples were withdrawn aseptically for every 6 h intervals and protease activity was determined. Different temperatures (20^0C to 70^0C) were also tested for optimizing the temperature.

Characterization of crude protease enzyme

The crude protease obtained from *B.licheniformis*, which showed the highest potential for proteolytic activity, was further subjected to preliminary characterization study. Therefore, the effects of pH, incubation period, temperature and substrate concentration on enzyme activity were studied. The procedures are outlined in detail below.

Effect of pH on activity of protease

The effect of pH on the proteolytic activity of crude alkaline protease from strain S8 and *B.licheniformis*, was determined by assaying the enzyme activity at different pH values ranging from 2.0 to 12.0 using the following buffer systems: KCl-HCl (pH 2.0), Citrate (pH 3.0 to 6.0), phosphate (pH 7.0), Tris–HCl (pH 8.0 to 9.0) and Glycine–NaOH (pH 10 to12).

Effect of incubation period on activity of protease

The effect of incubation period was determined by incubating the reaction mixture with enzyme at pH 10.0 at different incubation periods ranging from 5 minutes to 70 minutes with an interval of about 5 minutes.

Effect of temperature on activity of protease

The effect of different temperatures ranging from 20^0C to 100^0C with an interval of 5^0C was carried out by incubating the reaction mixture with enzyme at pH 10.0 for 1hr.

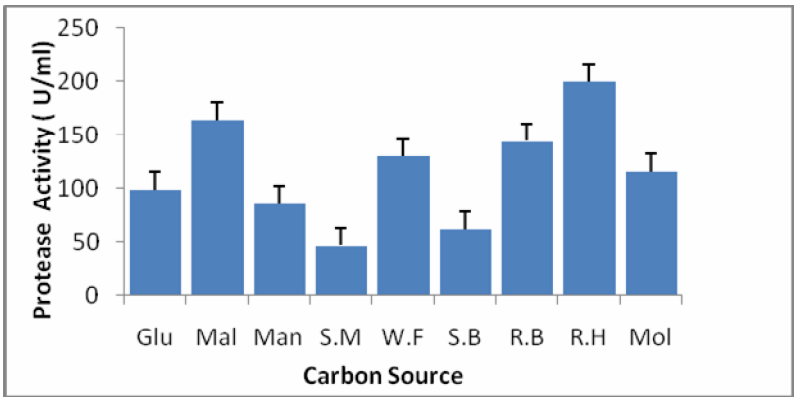
Effect of substrate concentration on activity of protease

Casein was used as substrate for enzyme assay. Different concentrations of casein (1% to 5%) in Glycine-NaOH buffer pH 10.0 was used as enzyme substrate with the above mentioned parameters to determine optimum concentration of substrate.

Results and Discussion

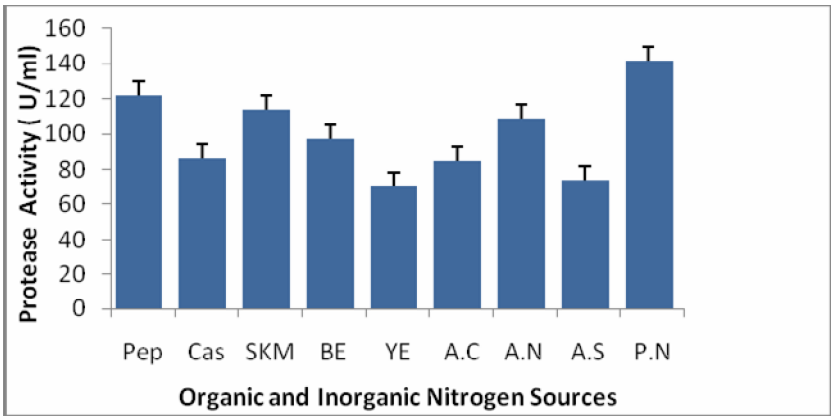
Production of proteases of commercial significance by employing a suitable organism and economical growth medium has been a worth praising achievement in the field of fermentation biotechnology. Protease production is an inherent capacity of all microorganisms; and large numbers of bacterial species are known to produce alkaline proteases (Gupta *et al.*, 2002). Among various bacteria, the *Bacillus* species are most significant and specific producers of alkaline proteases (Priest, 1977; Ward, 1985).

The present study concerns with the optimization of the growth media and



Glu: Glucose, Mal: Maltose, Man: Mannose, S.B: Sugarcane Bagasse, R.B: Rice bran, R.H: Rice husk, Mol: Molasses

Fig.1. Effect of different Carbon sources on protease production in *Bacillus licheniformis*. The bars indicate the standard deviation of three replicates analyzed.



Pep: Peptone, Cas: Casein, SKM: Skim milk powder, BE: Beef extract, YE: Yeast extract, A.C: Ammonium chloride, A.N: Ammonium nitrate, A.S: Ammonium sulphate, P.N: Potassium nitrate.

Fig.2. Effect of different Organic and Inorganic nitrogen sources on protease production in *Bacillus licheniformis*. The bars indicate the standard deviation of three replicates analyzed.

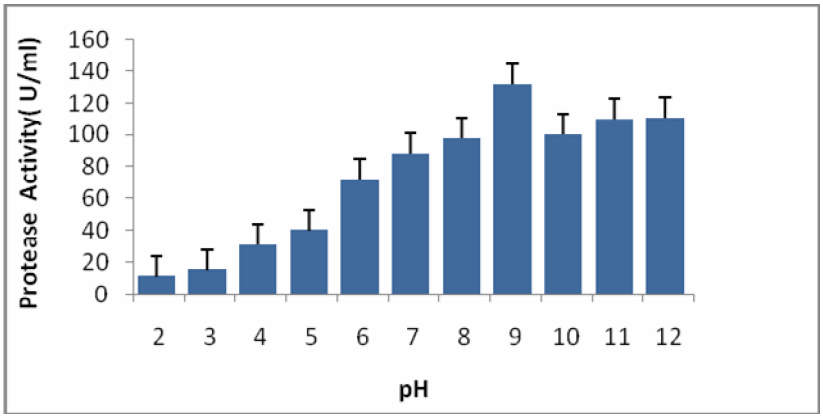


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Fig.3: Effect of pH on protease production in *Bacillus licheniformis*. The bars indicate the standard deviation of three replicates analyzed.

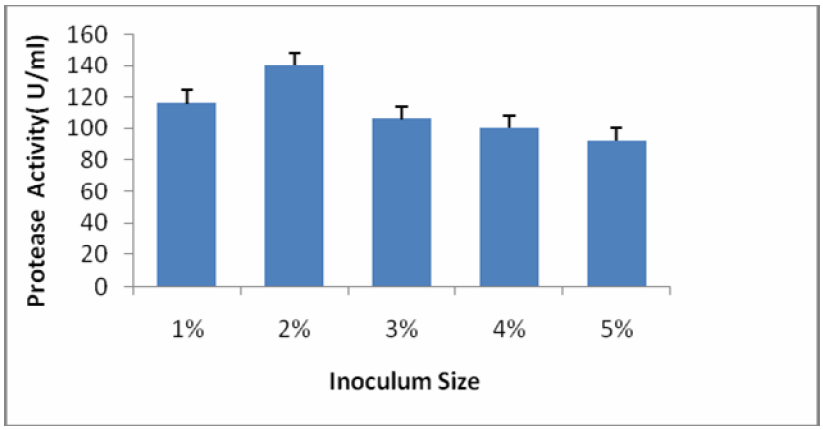

Fig.4. Effect of different inoculum concentrations on protease production in *Bacillus licheniformis*. The bars indicate the standard deviation of three replicates analyzed.

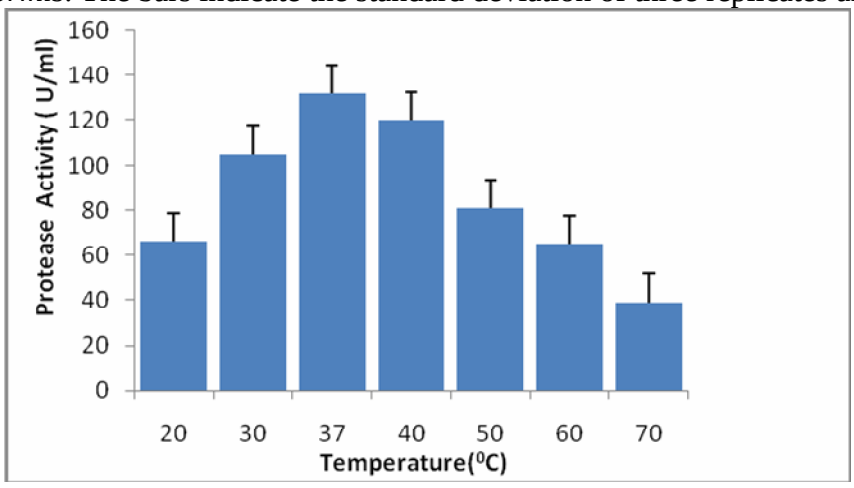

Fig.5. Effect of different temperatures on protease production in *Bacillus licheniformis*. The bars indicate the standard deviation of three replicates analyzed.

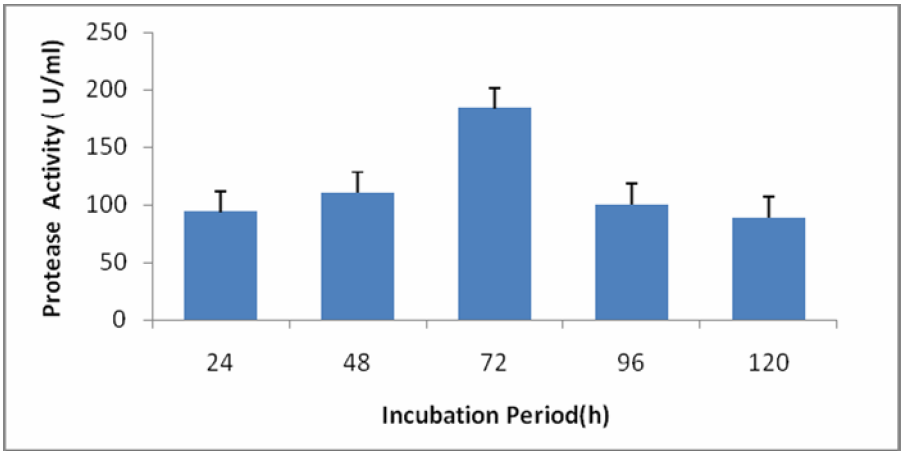

Fig.6: Effect of different incubation periods on protease production in *Bacillus licheniformis*. The bars indicate the standard deviation of three replicates analyzed.

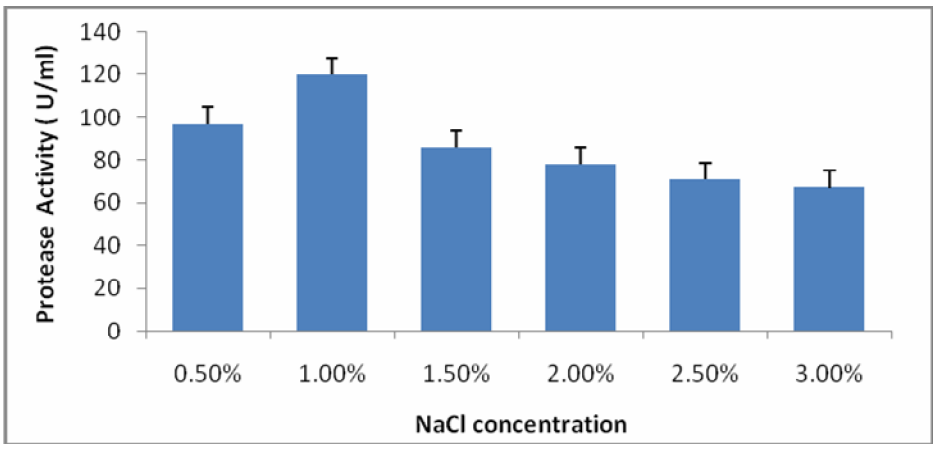

Fig.7. Effect of different NaCl concentrations on protease production in *Bacillus licheniformis*. The bars indicate the standard deviation of three replicates analyzed.

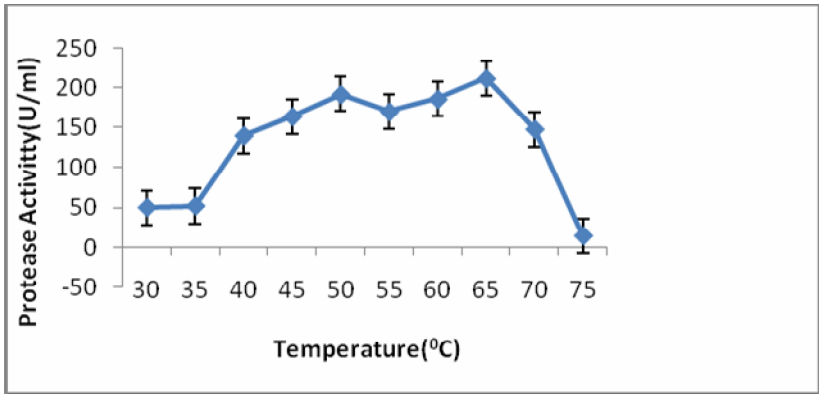

Fig.8: Activity of protease from *Bacillus licheniformis* at different temperatures.

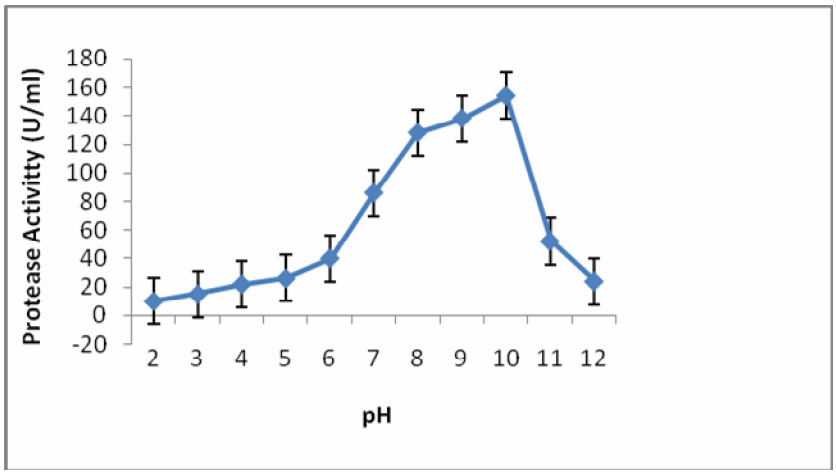

Fig.9: Activity of protease from *Bacillus licheniformis* at different pH.

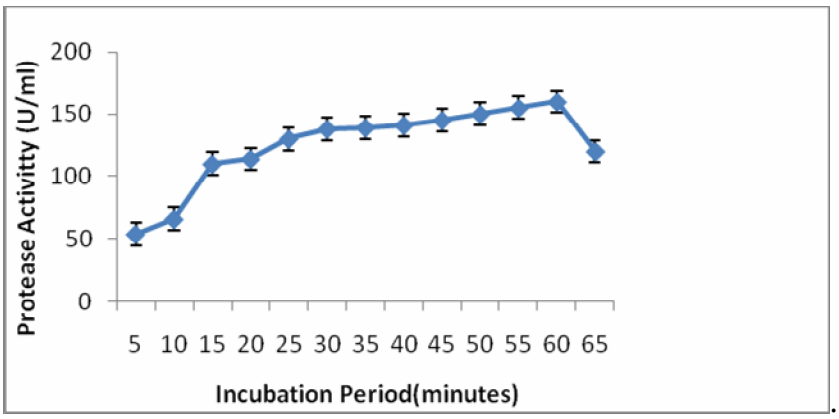

Fig.10: Activity of protease from *Bacillus licheniformis* at different Incubation Periods of Enzyme mixture.

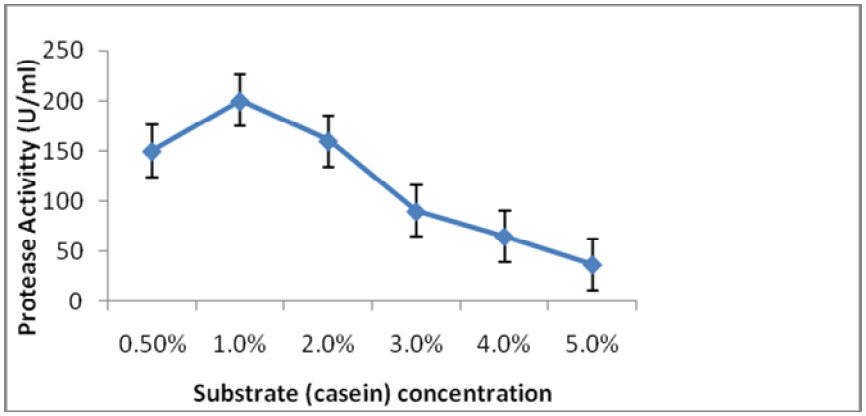

Fig.11: Activity of protease from *Bacillus licheniformis* at different concentrations of substrate (casein).

the charcterizaton of the crude protease. The standard media was optimized with different carbon and nitrogen sources. Among the different carbon and nitrogen sources studied, addition of rice husk (Fig.1) and potassium nitrate (Fig.2) to the basal media showed highest protease production rate. Similar results were reported by Dahot, Ishtiaq Ahmed *et al.*, (1993). A decrease in enzyme production was observed at lower and higher concentrations. The results indicated that proper concentration level of rice husk and potassium nitrate played a significant role in enhancing the production of alkaline protease and growth of the *Bacillus licheniformis*. Repressed growth and enzyme production at higher concentration of the substrates might be due the catabolic repression, or substrate inhibition, a traditional property of batch fermentation processes.

In the present study of different physical factors influencing protease production showed maximum production at pH 9.0 (Fig.3),with 2% inoculum (Fig.4) and optimum growth rate at 37^0C (Fig.5) for incubation period of 72h (Fig.6). In the present

study, maximum protease production was observed at 2% inoculum. This result was similar to *Bacillus brevis* reported by Odu and Akujobi, 2012. There was a reduction in protease production when inoculum size was reduced to 0.2%. This may be due to insufficient number of bacteria, which would lead to reduced amount of enzyme production. Higher inoculum size may have resulted reduced dissolved oxygen and increased competition towards nutrients. Similar results were also reported in *Bacillus odyssey* with 72 h incubation period by Sandhya and Tambekar, 2013. Presence of NaCl (1%) in the medium enhanced protease production (Fig.7). The enhancing effect of sodium on bacterial alkaline protease has been reported rarely. Chandrasekaran and Dhar, 1983 observed the beneficial effect of sodium chloride on alkaline protease production. An increased salt concentration creates change in the lipid composition of cell membrane. Hence, the growth rate decreases causing reduced enzyme production. Presence of calcium carbonate in the medium increased the production of protease. These results were in agreement with the earlier findings with Benarjee *et al.*, (1999), they showed enhancement of protease activity in the presence of metal ions and it was suggested that these metal ions increased stability of proteases.

Partial characterization studies of the crude enzyme showed that, this enzyme is thermostable with an optimum temperature of 65^0C (Fig.8) at pH 10.0 (Fig.9) with 1h incubation (Fig.10) of enzyme reaction mixture with casein (1%) as substrate (Fig.11).

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