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Production of alkaline protease by *Bacillus subtilis* using solid state fermentation

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The present study describes the optimization of nutritional and cultural parameters for the production of alkaline protease by *Bacillus subtilis* under solid state conditions. Among cultural conditions, incubation temperature, incubation period and moisture level of the substrate were optimized and it was found that maximum production of alkaline protease was observed at 37°C and moisture to substrate ratio of 1:1 after 48 h of incubation period. Among different nutritional parameters, the effects of different diluents, carbon and nitrogen sources on the enzyme production were studied. Maximum enzyme production (101.23 U/g) was observed when D_2 {(% w/v) $CaCO_3$, 0.05; peptone, 0.1; glucose, 0.1 and yeast extract, 0.1} was used to moisten the substrate. The best carbon source for the production of alkaline protease by *B. subtilis* was found to be sucrose at a concentration of 1%. Similarly, nutrient broth (1.5%) and diammonium hydrogen phosphate (0.1%) were found to be best organic and inorganic nitrogen sources, respectively. It was also found that the maximum protease (126.8 U/g) was produced when 25% (v/w) inoculum was used to inoculate the fermentation flasks.

Key words: Fermentation, carbon, nitrogen, inoculum, incubation temperature.

INTRODUCTION

Microbial proteases are one of the most important classes of biocatalyst from an industrial point of view, occupying a major share of 60% of the total enzyme market (Gupta et al., 2002). These biocatalysts hydrolyze peptide bonds of proteins and hence are classified as hydrolases and are categorized in the subclass peptide hydrolases or peptidases. Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals and microorganisms. The quantities of enzyme produced on commercial scale from plant and animal sources are very limited, both for economic and technical reasons (Uchikoba and Kaneda, 1996; Malathi and Chakraborty, 1991). But the microbial enzymes are not subjected to any of the production and supply limitation, so proteases are mainly produced by microorganisms through fermentation.

Among bacteria, there are number of genera which produce proteases but *Bacillus* remains the organism of choice (Nomoto et al., 1984; Kumar and Takagi, 1999; Mehrotra et al., 1999). *Bacillus* species produce a variety of proteases of which an alkaline protease (subtilisin) and a neutral metalloprotease are extracellular, whereas at least two serine proteases are produced intracellularly (Hirushi and Kadota, 1976; Mantsala and Zalkim, 1980). However, the proteases produced by *Bacillus* are mostly alkaline in nature and are highly stable against extreme temperature, pH and other conditions (Sharma et al., 1980).

Bacteria usually produce proteases by submerged fermentation in shake flasks or fermenters but there are also reports that bacteria produce considerable amount of proteases by solid substrate fermentation (Moo-Young et al., 1983; George et al., 1995; Chinn et al., 2006; Chen-

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Tien et al., 2000). Solid state fermentation (SSF) is the growth of microorganisms for the production of useful products on a predominantly insoluble substrate without a free liquid phase. It has the potential to serve as a production method for microbial products including enzymes, antibiotics, fuel, industrial chemicals and pharmaceuticals and for processing methods such as bioremediation and animal feed enrichment (Pandey et al., 2000). The substrates typically used are agricultural crop and processing residues including wheat bran, soybean meal, sugar cane bagasse, corn stover and residues of coffee, paper and oil processing (Pandey et al., 2000*).* Such substrates are structurally and mutationally complex, often creating a supportive environment as compared to the submerged cultivation method. Pakistan is an agricultural country so it has a wide variety of agro-industrial byproducts which are cheaply available in the market. These agro-industrial by products are good sources of proteins, carbohydrates and minerals needed for the growth of microorganism and synthesis of microbial enzymes (Qadeer et al., 1990).

The selection of ideal agro-industrial byproduct for the enzyme production in a solid state fermentation process depends upon several factors mainly related with cost and availability of the substrate and thus may involve screening of several agro-industrial byproduct (Pandey et al., 2000). Agricultural byproducts such as soybean meal, corn gluten meal, wheat bran, gram husk, rice bran, rice husk, rice polish, sunflower meal and linseed meal have been evaluated for the biosynthesis of protease. *Bacillus horikoshii*, *Bacillus subtilis* and *Bacillus circulans* have been reported to synthesize the maximum alkaline proteases using soybean meal, rice bran and green gram husk, respectively (Joo et al., 2002; Qadeer et al., 1990; Haque et al., 1990; Prakasham et al., 2006).

The present study is concerned with the optimization of process parameters for alkaline protease production by *B. subtilis* IH_{72} under solid state fermentation.

MATERIALS AND METHODS

Microorganism

Bacterial culture of *B. subtilis* IH_{72} was obtained from the culture collection bank of Institute of Industrial Biotechnology, Government College University, Lahore. The culture was revived by transferring to the peptone-yeast extract agar slants which were placed in an incubator at 37°C for 48 h. The culture was maintained by weekly transfers to new slants and was stored in cool laboratory at 4°C.

Preparation of inoculum

Bacterial inoculum was prepared in 250 ml conical flask containing 50 ml of sterilized nutrient broth and was aseptically inoculated with a loopful of bacteria from a fresh slant and allowed to grow at 37°C for 24 h in a rotary shaker. After 24 h of growth, the bacterial culture was used as inoculum.

Fermentation technique

Solid state fermentation was carried out for the production of alkaline protease from *B. subtilis*. Five grams of wheat bran and five grams of soybean meal contained in 250 ml Erlenmeyer flask was moistened with 10 ml of distilled water. (the distilled water was used instead of tap water as the tap water may contain some metals/ contaminant that may inhibit the growth of microorganism). The flasks were cotton plugged and sterilized in an autoclave at 15 lbs/inch^2 pressure (121°C) for 15 min. After sterilization, the medium was cooled at room temperature and was inoculated with 1.0 ml of the bacterial inoculum as prepared earlier. The flasks were vigorously shaken to distribute the inoculum uniformly in the medium and were incubated statically at 37°C for 48 h. During incubation, the flasks were shaken twice a day for achieving homogeneity. The fermentation batches were run in triplicate and the mean of three was reported in the results. During all the experiments, calibrated glass ware and analytical grade chemicals were used.

Diluents

Seven different diluents were used to moisten the agro-industrial byproducts for fermentation studies. The composition of the diluents is as follows:

D_1: (%w/v) $NaNO_3$, 0.2; KH_2PO_4, 0.1; $MgSO_4.7H_20$, 0.05; KCl, 0.05; $ZnSO_4.7H_20$, 0.01; $FeSO_4.7H_20$, 0.01.
D_2: (%w/v) $CaCO_3$, 0.05; peptone, 0.1; glucose, 0.1; yeast extract, 0.1.
D_3: (%w/v) NaCl, 0.05; beef extract, 0.15; peptone, 0.1; glucose, 0.1.
D_4: (%w/v) $CaCl_2$, 0.3; $MgSO_4.7H_20$, 0.3; KH_2PO_4, 0.2; urea, 0.3; $(NH_4)_2SO_4$, 0.15.
D_5: (%w/v) yeast extract, 0.1 glucose, 0.33; $CaCO_3$, 0.1; peptone, 0.3.
D_6: (%w/v) glucose, 1; peptone, 0.5; yeast extract, 0.5; KH_2PO_4, 0.1; $MgSO_4$. $7H_20$, 0.05; Na_2CO_3, 1.
D_7: Distilled water.

The pH of all the diluents was adjusted to 7.0 with 0.1 N NaOH/HCl.

Extraction of enzyme

After 48 h of incubation, 40 ml of distilled water/buffer was added to each flask and the flasks were rotated on rotary shaker for one hour at a speed of 200 rpm for the extraction of protease from fermented substrate, After shaking, the contents of flasks were filtered through Whatman filter paper No.44. The filtrate was used for enzyme assay.

Assay of protease

The activity of protease was assayed by the method of McDonald and Chen (1965). To 1.0 ml of the enzyme extract in the test tube, 4.0 ml of casein solution was added; the reaction mixture was incubated at 40°C for 30 min. The residual protein was precipitated by adding 5.0 ml of TCA solution. The content of the tubes were centrifuged and 1.0 ml of supernatant was mixed with 5.0 ml of alkaline reagent. After 10 min, 0.5 ml of Folin and Ciocalteau reagent was added; as a result, blue color was produced. The optical density of the mixture was read at 700 nm on a UV/VIS spectrophotometer (Cecil-CE7200-series, Aquarius, UK).

One unit of protease activity is defined as “the amount of enzyme required to produce an increase of 0.1 in optical density under defined conditions”.

RESULTS AND DISCUSSION

Effect of incubation temperature

The production of alkaline protease from *B. subtilis* IH_{72}

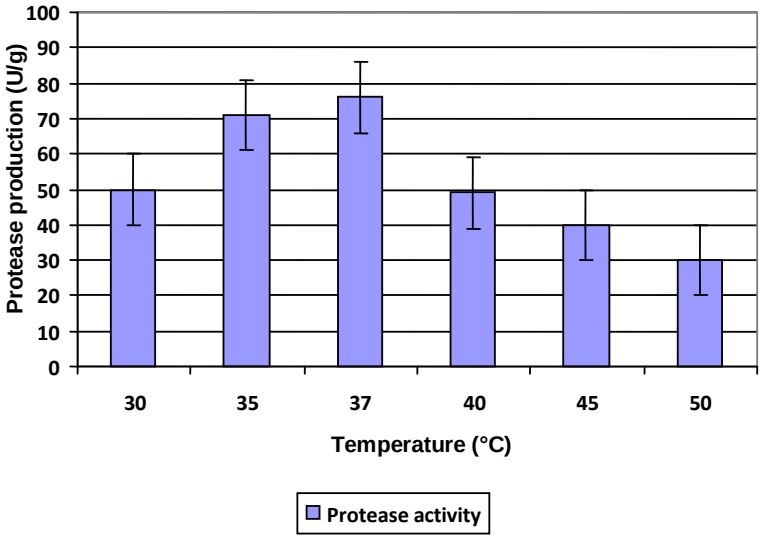

Figure 1. Effect of incubation temperature on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation period= 48 h, diluent distilled water, pH of diluent = 7, inoculum size 10%). Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.

was studied at different incubation temperatures ranging from 30 to 50°C (Figure 1). It was observed that there was an increase in the production of alkaline protease when the incubation temperature was raised from 30 to 37°C where the enzyme production was maximum (74.0 U/g). Above 37°C and up to 50°C, there was a marked decrease in the biosynthesis of alkaline protease by *B. subtilis*.

Higher temperature is found to have some adverse effects on metabolic activities of microorganisms. The enzyme is denatured by losing its catalytic properties due to stretching and breaking of weak hydrogen bonds within enzyme structure by increasing the temperature (Conn et al., 1987). Most of the bacteria give maximum growth and production of protease at the temperature range of 30 to 40°C (Joo et al., 2002).

Effect of incubation period

Figure 2 shows the effect of incubation period on production of protease by *B. subtilis* IH_{72} under solid state fermentation. Fermentation experiments were carried out for different time intervals ranging from 24 to 120 h. After 24 h of incubation, the amount of protease production was 59.0 U/g and it was increased with increase in the incubation period and reached maximum (75.0 U/g) after 48 h of incubation. Further increase in the incubation period resulted in the decreased enzyme production.

The incubation period is directly related with the production of enzyme and other metabolites up to certain extent. After that, the enzyme production starts to decrease which can be attributed to the decreased supply of nutrients to microorganisms (Romero et al., 1998). *Bacillus* species are known to produce maximum amount of proteases in the stationary phase or post exponential phase of their growth (Atalo and Gashe, 1993; Sharipova et al., 2000). However, some other *Bacilli* synthesize proteases during the exponential growth phase, but it mainly depends on the composition of the medium and some other factors. It was also reported that *Bacillus* sp. produce maximum alkaline protease after 48 h of incubation in alkaline medium (Fujiwara and Yamamota, 1987).

Effect of moisture level

The correlation between enzyme production and water content of solid state fermentation is a critical factor and was studied by adding different quantities of distilled water to the substrate. A ratio of 1:1 (w/v) (100%) between substrate and diluent was found best for the maximum enzyme production (77.1 U/g) as shown in Figure 3. Above this level, there was no further increase in the enzyme production due to the formation of a semi- solid and pasty substrate. So, with 1:1.5, 1:2 and 1:2.5 (150, 200 and 250%) substrate to moisture ratio, the yield of alkaline protease was 65.2, 42.0 and 36.8 U/g, respectively (Figure 3).

It might be due to the fact that less quantity of diluent was insufficient to fulfill the moisture requirements of the organism. By increasing the quantity of diluent, the conditions became suitable for bacterial growth and enzyme production. Further increase in moisture level resulted in the formation of a paste and the conditions became anaerobic, which were not favourable for the growth of organism and hence the enzyme production was decreased.

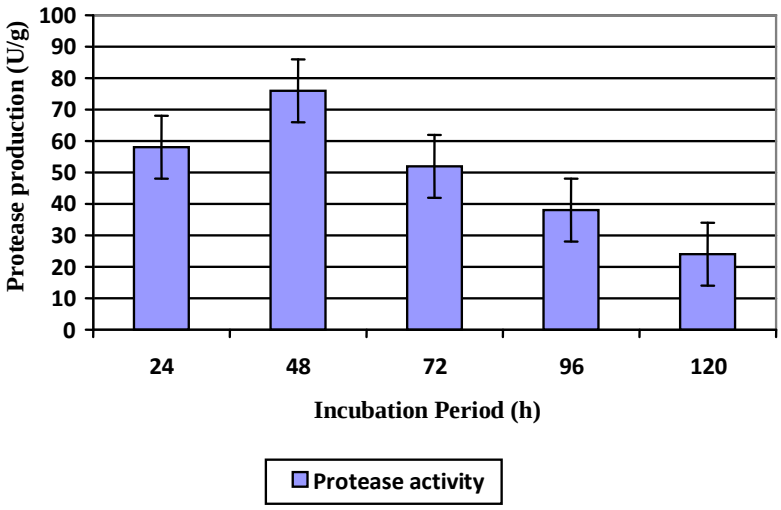

Figure 2. Effect of incubation period on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation temperature = 37°C, diluent = distilled water, pH of diluent 7.0, inoculum size = 10%). Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.

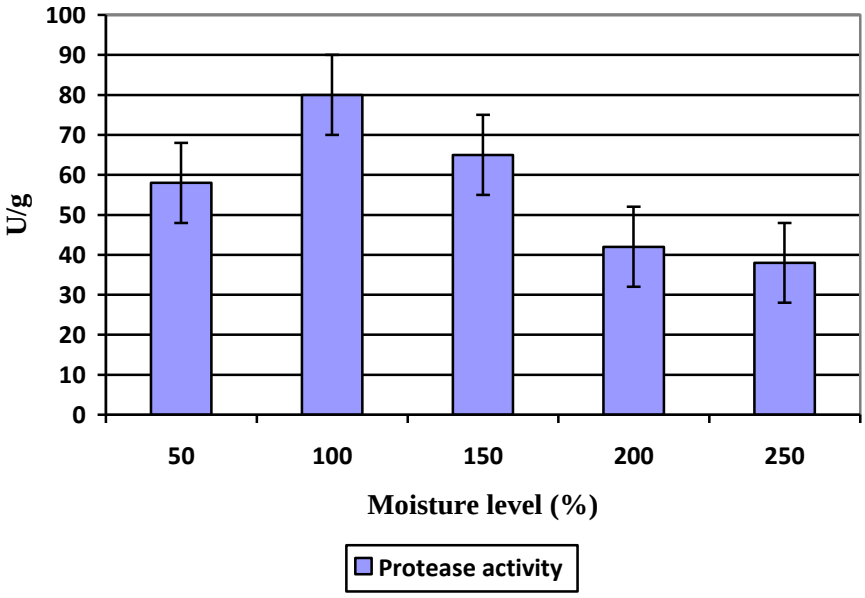

Figure 3. Effect of moisture level on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (*incubation temperature = 37°C, incubation period= 48 h, diluent = distilled water, pH of diluent = 7.0, inoculum size = 10%). Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.

Other workers have also reported that 1:1.5 ratio of moisture content to substrate was better for yield of protease (Malathi et al., 1989; Malathi and Chakraborty, 1991). Maximum protease production under solid state fermentation by *B. circulans* has been reported when the moisture level was kept at 18.8% in the substrate (Prakasham et al., 2006).

Effect of different diluents

Different diluents such as D_1 - D_7 containing different nutrients were used for moistening the fermentation substrate (Figure 4). Maximum enzyme production (101.2 U/g) was observed when D_2 {(% w/v) $CaCO_3$, 0.05; peptone, 0.1; Glucose, 0.1 and yeast extract, 0.1} was used as a

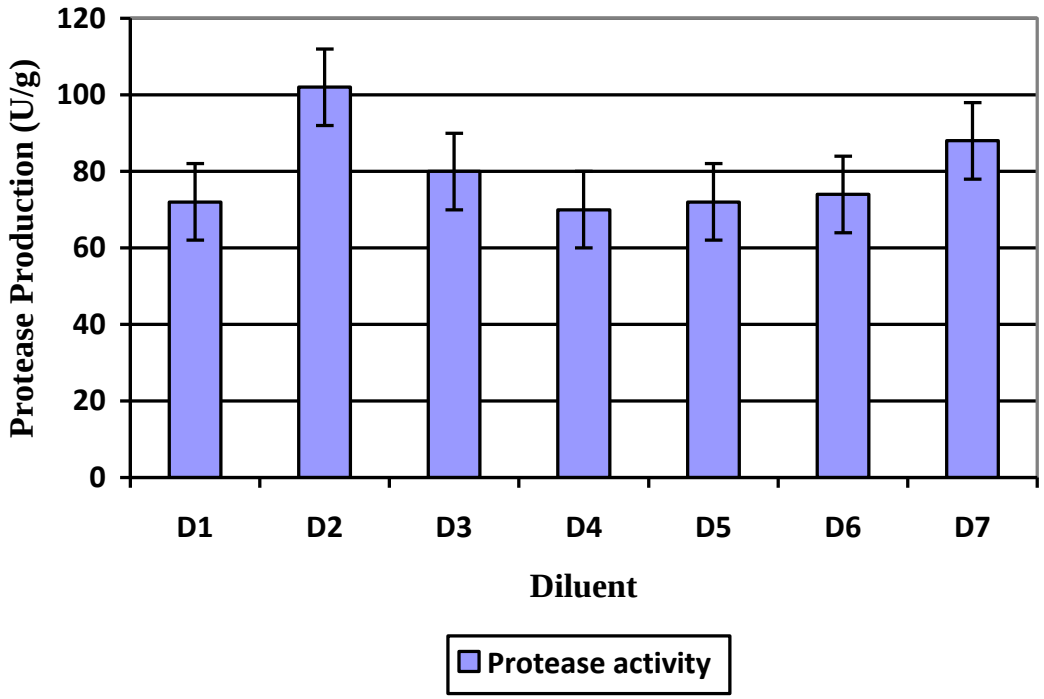

Figure 4. Effect of different diluents on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (*incubation temperature 37°C, incubation period = 48 h, moisture level = 100%, pH of diluent 7.0, substrate wheat bran and guar meal, inoculum size = 10%). Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.

diluent. The synthesis of enzyme however, was decreased in the order of $D_7 > D_3 > D_6 > D_1 > D_5 > D_4$. The diluent D_2 containing yeast extract, peptone and glucose had a positive influence on the productivity of protease. It indicates that the organism required additional nutrients for its growth. All the deficient nutrients from the substrate were supplied by the diluent D_2 for growth of the organism and production of the enzyme. It has also been reported that diluent with similar composition ($CaCO_3$, glucose, yeast extract and peptone) was found to be best for the maximum protease production by *Aspergillus niger* (Chakraborty et al., 1995). It also seems that the nutrients present in other diluents may not be sufficient or may have an inhibitory action on the growth of the organism and subsequently on the enzyme production; so it had less production of the enzyme (Battaglino et al., 1991).

The effect of initial pH of the diluent (7.0 to 10.5) on the production of alkaline protease by *B. subtilis* IH_{72} was also studied (Figure 5). The results showed that there was a gradual increase in the amount of protease synthesis with increase in diluent pH above 7.0 and maximum production of protease (105.7 U/g) was observed at a pH of 9.5. As the pH of diluent was increased beyond 9.5, there was a marked decrease in the production of protease, that is, from 105.69 to 47.2 U/g.

The production of the enzyme is greatly influenced by pH of the fermentation medium. Changes in the medium pH causes denaturation of enzyme resulting in the loss of catalytic activity. The pH also has a marked effect on the type and amount of enzyme produced by the microorganism. One organism may secrete variable amount and types of enzymes depending upon the pH and composition of medium (Kubackova et al., 1975) and most of the *Bacillus* species are known to grow in the fermentation medium having alkaline pH (Szumacher et al., 2004). Atalo et al. (1993) has also reported that maximum protease enzyme production was obtained from *Bacillus* species at pH 9.5.

Effect of different extraction buffers

Extraction of protease from fermented substrate with different buffers was studied and the results are presented in Figure 6. Maximum enzyme extraction (88.9 U/g) was achieved when distilled water was used. The protease yield by extraction with phosphate buffer, acetate buffer, borate buffer and Tris buffer was 86.5, 72.5, 50.6 and 42.4 U/g, respectively. Less extraction of enzyme with buffers might be due to the inhibitory action of the chemicals present in the buffers. The buffers might also have affected the active site of enzyme or they might have changed the enzyme structure. Ammar et al. (1997) also reported similar finding and found distilled water as a best extractant. Bidochka and Khachatourians (1998) however, reported that protease extraction was maximum with citrate buffer.

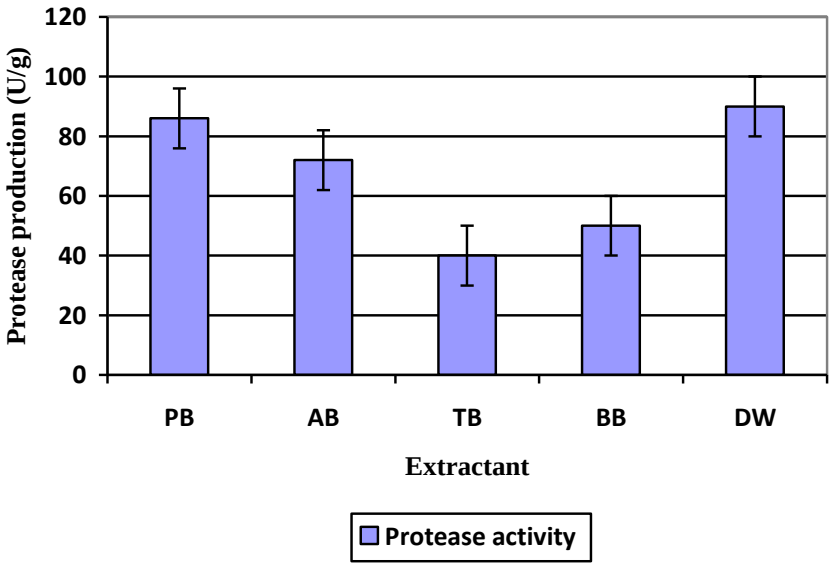

Figure 5. Extraction of alkaline protease from substrate with different buffers. Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean. PB = Phosphate buffer; AB = acetate buffer; TB = Tris buffer; BB = borate buffer; DW = distilled water.

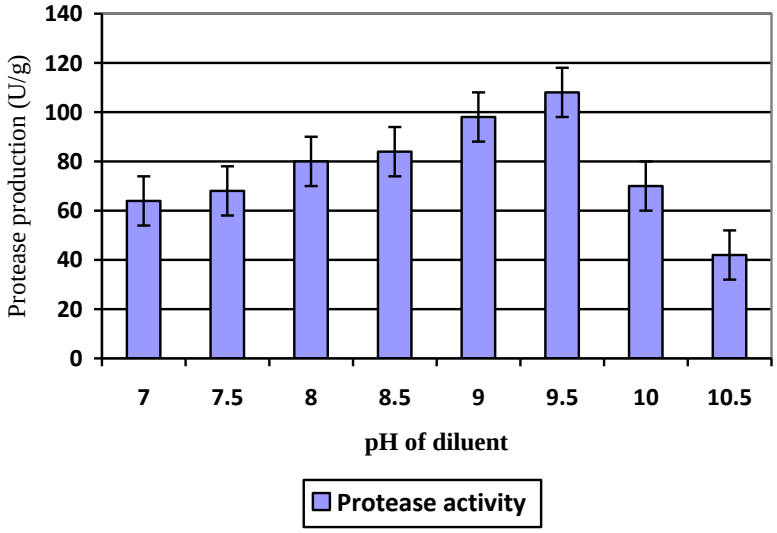

Figure 6. Effect of pH of diluent on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation temperature = 37°C, incubation period = 48 h, moisture level = 100%, diluent D_2, substrate = wheat bran and guar meal, inoculum size = 10%). Each value is a mean of three replicates. Y- error bars indicate the standard error from the mean.

Effect of different carbon sources

Different carbon sources such as sucrose, starch, maltose, fructose, glucose, and lactose were added to the fermentation substrate to study their effect on the production of proteases by *B. subtilis* IH_{72} (Figure 7). The synthesis of alkaline protease was found maximum (108.0 U/g) with sucrose as a carbon source in the medium. It was decreased with starch (82.0 U/g), glucose (100.20 U/g), maltose (87.0 U/g), fructose (85.75 U/g) and lactose (84.87 U/g). Minimum production of alkaline protease was obtained when the substrate was supplemented with lactose (84.87 U/g).

It has been noticed that the absence of proper carbohydrate (c-source) in the medium results in a dramatic decrease in enzyme production (Gajju et al., 1996), so a

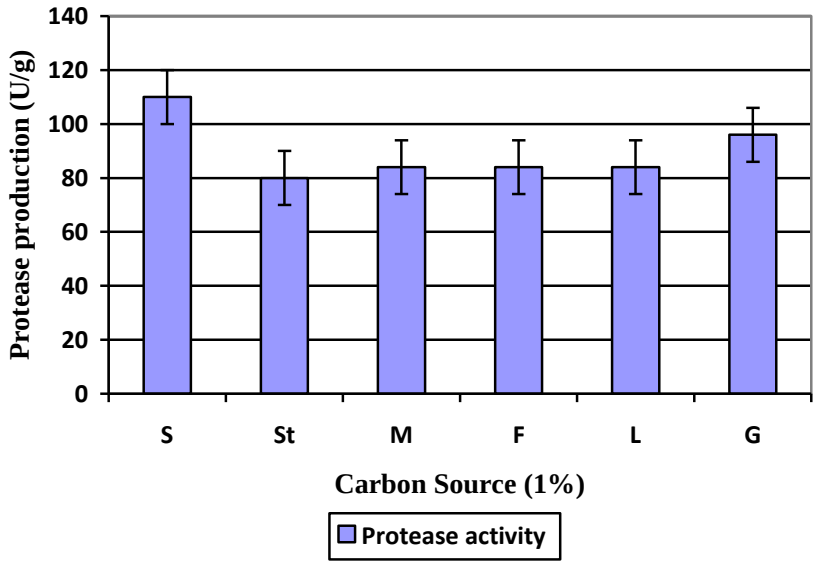

Figure 7. Effect of different carbon sources on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation temperature = 37°C, incubation period= 48 h, moisture level = 100%, diluent = D_2, pH of diluent = 9.5, substrate wheat bran and guar meal, inoculum size = 10%). Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean. S = Sucrose; St = starch; M = maltose; F = fructose; L = lactose; G = glucose.

c-source is always an essential component of fermentation medium. The reason for obtaining highest yield of protease by using sucrose as a carbon source may be due to the secretion of the enzyme invertase by the microorganism which causes the breakdown of sucrose to glucose and fructose which then can be easily used by the microorganism for growth and enzyme production. Sutar et al. (1992) found sucrose, glucose and fructose to be equally good as carbon sources for protease production by *Condiobolus coronatus*. It has been also reported that there was maximum yield of alkaline protease in the presence of lactose, maltose, sucrose and fructose obtained from different protease producing *Bacillus* species (Phadatare et al., 1993; Sen and Satyanarayana, 1993; Naidu and Davi, 2005).

Different concentrations of sucrose ranging from 0.5 to 2.0% were added to the culture medium and their effect on the production of protease was also observed (Figure 8). It was found that increasing the concentration of sucrose up to 1%, the production of alkaline protease was increased and reached maximum (108.8 U/g). Above 1% sucrose concentration, the enzyme production was decreased which might be due to the catabolic repression. The concentration less than 1% did not show more promising results due to deficiency of carbon source that is needed by bacteria.

Effect of different nitrogen sources

Effect of different organic nitrogen sources such as corn steap liquor, beef extract, yeast extract, nutrient broth, meat extract, peptone and urea on the production of protease was studied (Figure 9). Maximum production of the enzyme (116.75 U/g) was observed when nutrient broth was used as an organic nitrogen source. All other nitrogen sources showed lesser production of protease.

It has been frequently described that in a defined medium, a nitrogen source must be present for the enzyme to be produced. In microbial cells, nitrogen (both organic and inorganic form) is metabolized to produce primarily amino acid, nucliec acid, proteins and cell wall components. Alkaline protease production heavily depends on the availability of both carbon and nitrogen sources in the medium as they have regulatory effects on the enzyme synthesis (Chu et al., 1992; Moon and Porulekar, 1991). Nutrient broth consists of peptone and yeast extract which are both very efficient and widely used nitrogen sources for the production of protease by *Bacillus* species (Johnvesly and Naik, 2001; Prakasham et al., 2006).

The quantity of nitrogen in the culture medium is a crucial factor for the production of enzyme by microorganisms. Different amounts of nutrient broth ranging from 0.5 to 2.0% were added to the culture medium to optimize their concentration for the production of protease by *B. subtilis* (Figure 10). The protease production is directly related to the type and amount of nitrogen source in the fermentation medium (Rao et al., 1998). It was found that highest production of protease (117.0 U/g) was achieved when 1.5% nutrient broth was used to supplement the culture medium. Results also indicated that by increasing the concentration of nutrient broth up to 1.5%, the production of alkaline protease was increased; however, the

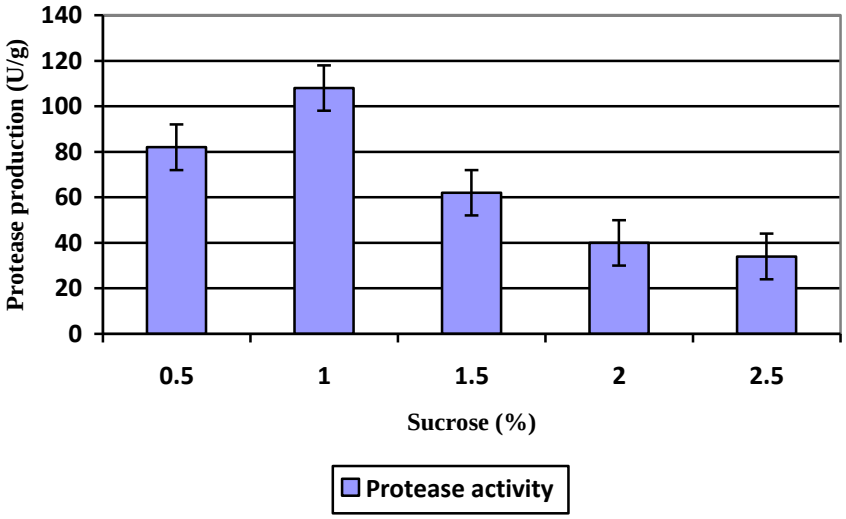

Figure 8. Effect of amount of sucrose on the production of alkaline protease by *B. subtilis* IH72 using solid state fermentation (incubation temperature = 37°C, incubation period = 48 h, moisture level = 100%, diluent = D_2, pH of diluent = 9.5, substrate = wheat bran and guar meal, carbon source = sucrose, inoculum size = 10%). Each value is a mean of three replicates. Y- error bars indicate the standard error from the mean.

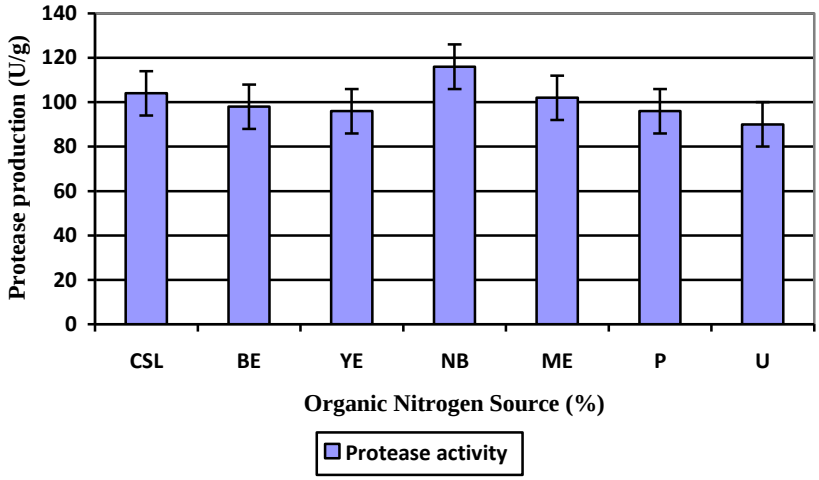

Figure 9. Effect of different organic nitrogen sources on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation temperature = 37°C, incubation period = 48 h, moisture level = 100%, diluent = D_2, pH of diluent = 9.5, substrate = wheat bran and guar meal, carbon source = sucrose, inoculum size= 10%). Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean. CSL = Corn steep liquor; BE = beef extract; YE = yeast extract; NB = nutrient broth; ME = meat extract; P = peptone; U = urea.

production of enzyme was decreased by increasing concentration of nutrient broth more than 1.5%.

Effect of different inorganic nitrogen sources such as ammonium nitrate, ammonium acetate, ammonium chloride, sodium nitrite and diammoniurn hydrogen phosphate on the production of protease by *B. subtilis* IH_{72} was also observed (Figure 11). The highest production of protease (121.4 U/g) by *B. subtilis* was observed when diammonium hydrogen phosphate (as it is already well established that a concentration of 0.1% is best) was added to the culture medium as an inorganic nitrogen source. Other inorganic nitrogen sources such as ammonium nitrate, ammonium acetate, ammonium chloride and sodium nitrite gave less production of the enzyme, that is,

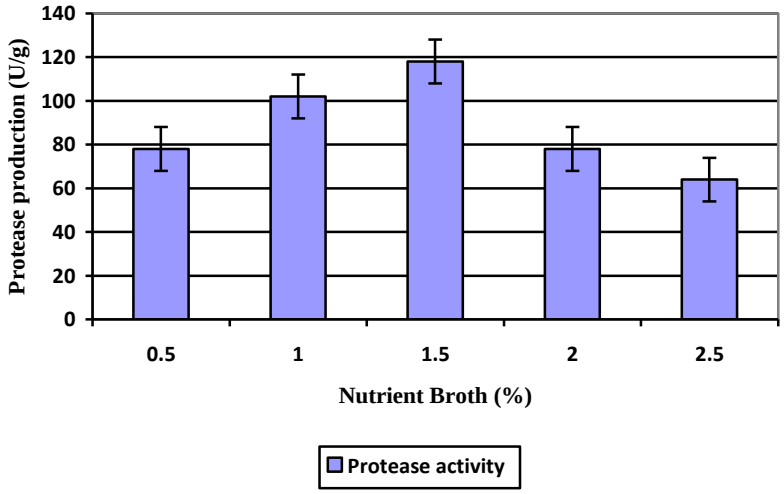

Figure 10. Effect of amount of nutrient broth on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation temperature = 37°C, incubation period 48 h, moisture level = 100%, diluent = D_2, pH of diluent, = 9.5, substrate wheat bran and guar meal, carbon source = sucrose, nitrogen source nutrient broth, inoculum size 10%). Each value is a mean of three replicates. Y- error bars indicate the standard error from the mean.

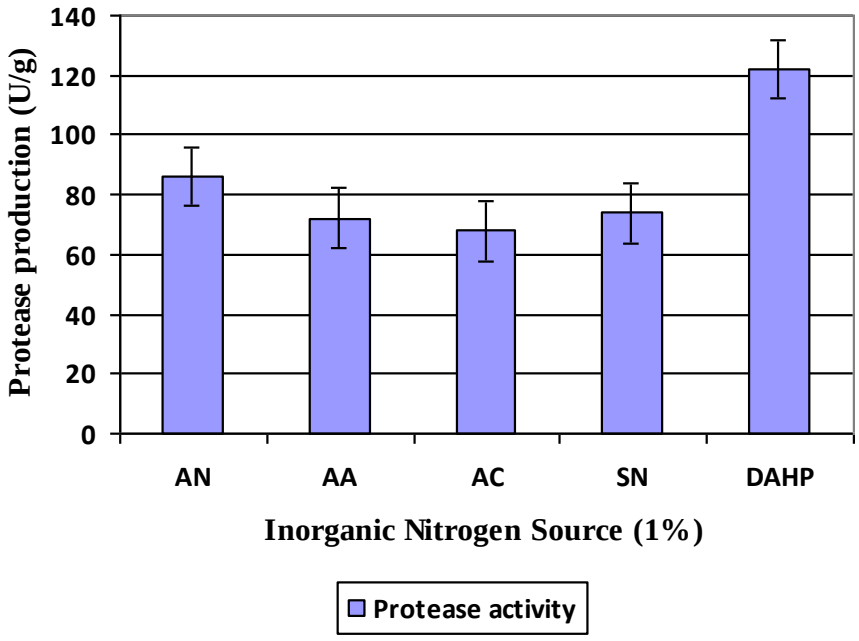

Figure 11. Effect of different inorganic nitrogen sources on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation temperature = 37°C, incubation period = 48 h, moisture level = 100%, diluent = D_2, pH of diluent = 9.5, substrate wheat bran and guar meal, carbon source = sucrose, nitrogen source nutrient broth, inoculum size = 10%). Each value is a mean of three replicates. Y- error bars indicate the standard error from the mean. AN = Ammonium nitrate; AA = ammonium acetate; AC = ammonium chloride; SN = sodium nitrite; DAHP = Di-ammonium hydrogen phosphate.

85.45, 71.4, 68.85 and 73.35 U/g, respectively.

The inorganic nitrogen is present in simple form as compared to the complex organic nitrogen, so it is readily available to microorganisms for the protein synthesis. Thus, inorganic nitrogen source must be present in medium for growth of microorganisms. Alkaline protease from

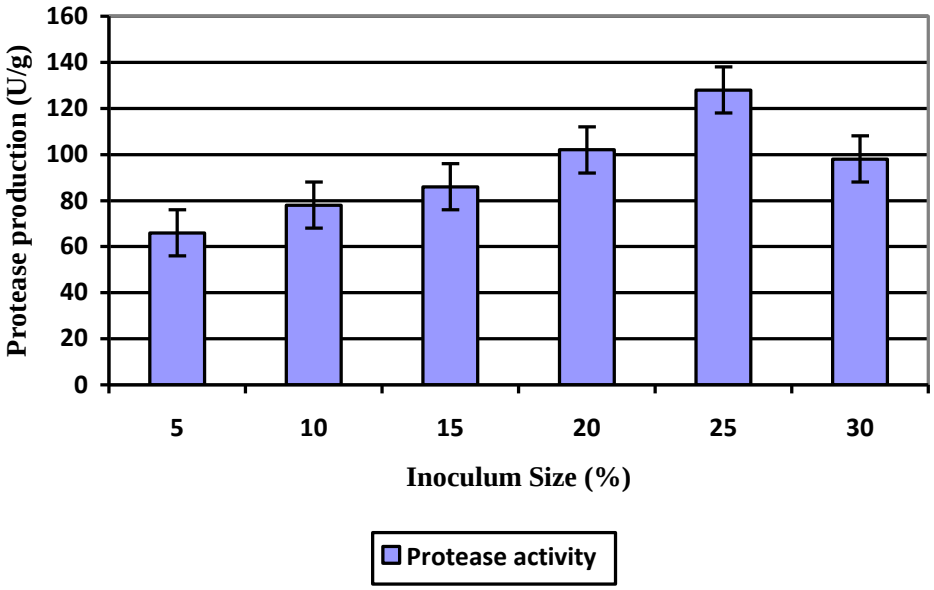

Figure 12. Effect of inoculum size on the production of alkaline protease by *B. subtilis* IH_{72} using solid state fermentation (incubation temperature = 37°C, incubation period = 48 h, moisture level = 100%, diluent = D_2, pH of diluent = 9.5, substrate = wheat bran and guar meal, carbon source = sucrose, nitrogen source = nutrient broth). Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.

Conidiobolus coronatus was also found maximum when diammonium hydrogen phosphate was used as inorganic nitrogen source (Laxman et al., 2005). Sinha and Satyanarayana (1991) noticed the ammonium nitrogen associated with regulation for protease production in thermophilic *Bacillus licheniformis*.

Effect of size of inoculums

Different sizes of inoculum ranging from 5 to 30% (v/w) were added to the fermentation flasks to study their effect on the production of protease by *B. subtilis* IH_{72} (Figure 12). It was found that maximum amount of protease (126.8 U/g) was produced when 25% (v/w) inoculum was used to inoculate the medium. However, further increase in the size of inoculum resulted in the lower yield of protease. The inoculum size less than 25% also gave the less yield of protease. The size of inoculum has great influence on the production of proteases and an appropriate inoculum size is essential for maximum production of enzyme. The decrease in enzyme production by increasing inoculum size beyond 25% is due to the fact that microbial cells started to consume the nutrients rapidly for their growth purposes. Moreover, toxins are also produced in fermented culture media by death of older cells (Carlile et al., 2001). Prakasham et al. (2006) have also reported that inoculum size of 26.2% was the most significant for protease production by *B. circulans.*

Conclusion

It is concluded from the present studies that *B. subtilis* IH_{72} was a good producer of alkaline protease in solid state fermentation. Culture conditions such as incubation temperature, incubation period, diluent pH, etc. had a profound effect on the production of enzyme. It was also found that suitable concentration of carbon and nitrogen sources resulted in significant rise in the protease production by *B. subtilis.*

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