

Full Length Research Paper

# Optimization of cultural conditions for protease production by *Bacillus subtilis* EFRL 01

A.Sattar Qureshi\*, M. Aqeel Bhutto, Imrana Khushk and M.Umar Dahot

Institute of Biotechnology and Genetic Engineering, University of Sindh Jamshoro, Pakistan.

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**Molasses was used as a sole carbon source for the protease production from *Bacillus subtilis* EFRL 01 in batch wise submerged condition. The bacterial culture was grown on mineral medium and maximum production was noted after 8 h of incubation. The effect of different variable such as carbon sources (0.5 and 1.0%), nitrogen sources (0.75), sodium chloride, potassium chloride, zinc chloride (0.5 - 3.0%), pH (3 - 12) and temperature (25 - 55°C) on the protease production was checked. The maximum enzyme production was noted when *B. subtilis* EFRL 01 was grown on mineral medium containing 1.0% molasses, 0.75% peptone and 2.0% sodium chloride when incubated at 45°C for 8 h with initial pH 8.5. The enzyme produced by *B. subtilis* is pH stable and thermostable, which can be utilized in local detergent and leather industry.**

**Key words:** *Bacillus subtilis* EFRL 01, molasses, protease.

## INTRODUCTION

Proteolytic enzymes represent a very important group of industrial enzyme (Al-Shehri et al., 2004). Protease for industrial purposes can be obtained from bacteria, moulds, yeasts and mammalian tissues. At present, huge proportion of commercially available alkaline proteases are produced from *Bacillus sp* (Abdel-Naby et al., 1998; Mabrouk et al., 1999; Mehrota et al., 1999). Proteases have wide range of industrial applications as detergent additives, waste treatment process, medical science, silver recovery, leather and pharmaceutical industries (Anwar and Saleemuddin, 1998; Kumar and Takagi, 1999). Proteases production by micro-organisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, incubation time, agitation and inoculum density (Ferid et al., 2008). Many processes have been developed for the utilization of agro-industrial residue as raw material for the production of bulk chemicals and agro-industrial residues in bioprocess, which provides alternative substrates and solve the

pollution problems (Pandey et al., 2000). This work reports protease production by *Bacillus subtilis* EFRL 01 with appropriate properties. Experiments also described the optimization of cultural conditions for alkalophilic and thermostable protease production using cost effective molasses as a sole energy source.

## MATERIALS AND METHODS

### Microorganism

*B. subtilis* EFRL 01 was isolated from soil from the lawn of Institute of Chemistry, University of Sindh, Jamshoro. The culture was examined for various morphological and biochemical characteristics as per Bergey's Manual of determinative Bacteriology (Holt et al., 1994). The Culture was maintained on nutrient agar medium at 4°C.

### Screening for protease production

The sample culture was spread on casein agar medium containing casein 2.0%; peptone, 0.5% and agar 1.5% and then incubated at 37°C for 24 h. The clear zone of casein hydrolysis (Figure 1) was an indication of protease secretion as reported by Folasade et al. (2005). The isolates were selected on the basis of larger zone on casein agar medium and further confirmed through batch wise submerged fermentation and the best one was selected for further study.

\*Corresponding author. E-mail: [asattar\\_qureshi@yahoo.com](mailto:asattar_qureshi@yahoo.com).  
Tel: +92-22-9239024.



**Figure 1.** Casein hydrolysis by *B. subtilis* at 37°C

#### Growth conditions for enzyme production

The organism was grown on medium containing glucose 0.5%, peptone 0.5%,  $MgSO_4 \cdot 7H_2O$  0.5%,  $KH_2PO_4$  0.5% and  $Fe_2SO_4 \cdot 7H_2O$  0.001% when incubated at 37°C with initial pH 7.5 as reported by various workers (Folasade et al., 2005; Kunamneni et al., 2003) with slight modification. The culture was grown in 250 ml conical flask containing 50 ml of sterilized fermentation medium. The flasks were inoculated with 0.5 ml of culture and grown for 12 h at 37°C. The bacterial biomass was separated from culture broth after an interval of 1 h incubation period through refrigerated centrifuge and further analysis was carried out from culture broth. The effects of carbon sources (0.5 and 1.0%) on enzyme production were determined by replacing glucose by pure carbon sources (fructose, galactose, sucrose, lactose, maltose, citric acid and trisodium citrate), industrial waste (molasses and date syrup) and agricultural waste (sugarcane bagasse and rice husk).

#### Effect of sodium chloride, potassium chloride and zinc chloride on protease yield

The influence of sodium chloride, potassium chloride and zinc chloride (0.5 - 3.0%) on growth and protease production were determined by supplementing these salts in the fermentation medium when incubated at 37°C, with initial pH 7.5 for 8 h with and without ammonium chloride as a nitrogen source.

#### Effect of nitrogen source on protease biosynthesis

Effect of peptone concentration (0.25 - 1.5%) on protease production and growth of microorganism was studied with a range of 0.25 - 1.5%. Various nitrogen sources (casein soluble, casein hydrolyzed, tryptone, corn steep liquor, yeast extract, urea ( $NH_2-CO-NH_2$ ), sodium nitrate ( $NaNO_3$ ), ammonium nitrate, potassium nitrate ( $KNO_3$ ), ammonium chloride and ammonium sulphate) were utilized instead of peptone 0.75% for protease production by *B.*

*subtilis*. In another set of experiment, all nitrogen sources were tested with sodium chloride when incubated at 37°C for 8 h.

#### Effect of pH and temperature on protease production

The effect of initial pH on protease production was analyzed by adjusting different initial pH of culture medium ranging from 3 to 12 with 0.1 N HCl or NaOH. The favorable temperature for maximum production was studied with a range of 25 to 55°C.

#### Protease assay

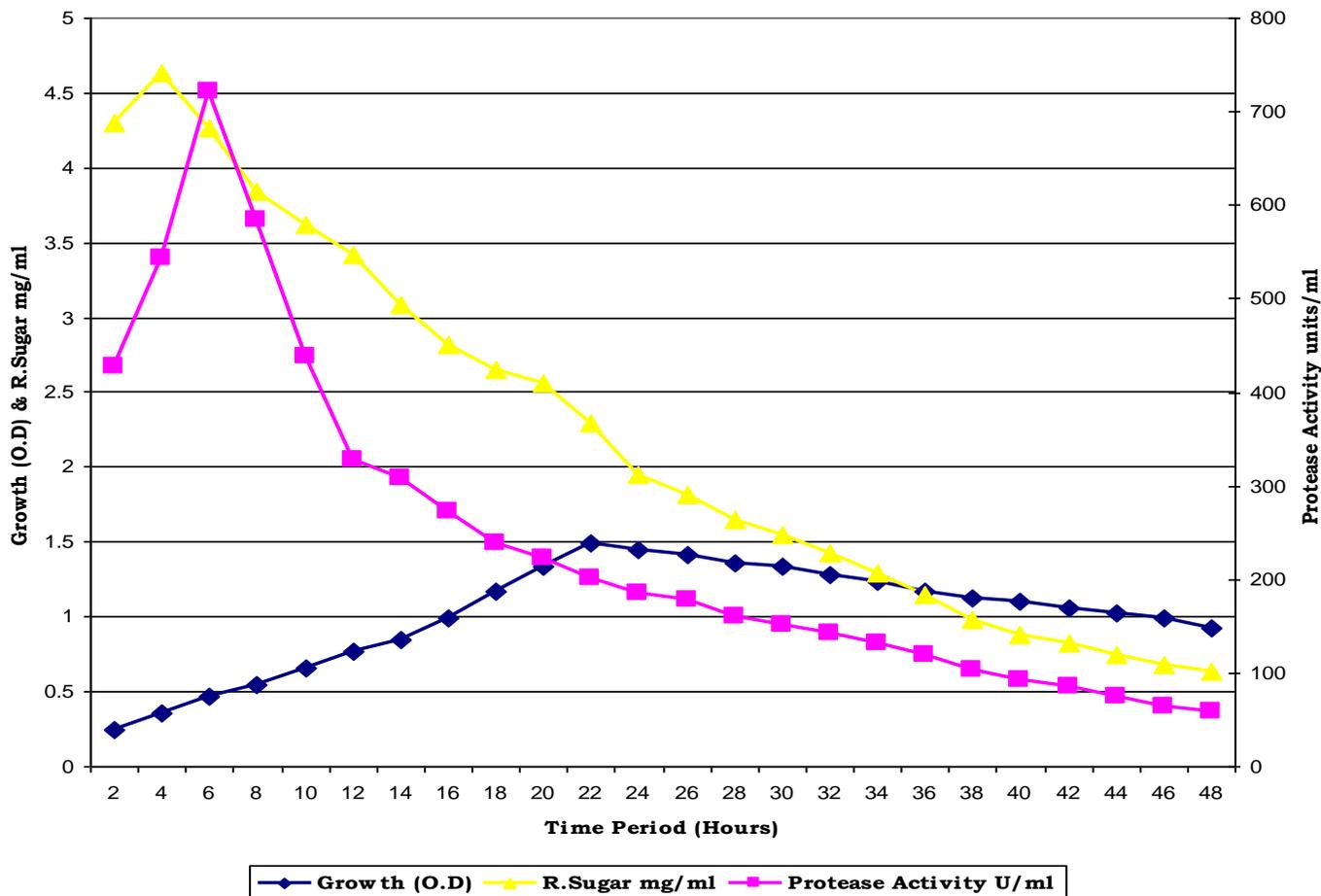
Protease activity was determined by spectrophotometer method as reported by Penner and Ashton (1967). To 0.5 ml culture broth, 0.5 ml of substrate (1% soluble casein) and 1.5 ml sodium phosphate buffer pH 7.6 were added and incubated at 35°C for one hour. After incubation, 2.0 ml of each sample was taken and 2.0 ml of 15% TCA was added and centrifuged for 5 min at 4000 rpm. To 1 ml aliquot, 4.0 ml 0.5 N NaOH and 1 ml Folin-phenol reagent (1:1) were added and then final volume was made up to 10 ml by adding 4.0 ml double distilled water. The absorbance was read at 625 nm. One unit of protease activity was defined as the amount of protease required to catalyze the liberation of 1 µg of tyrosine under the assay conditions. Protein content was determined by Lowry et al. (1951) method.

## RESULTS AND DISCUSSION

In order to identify best protease producer, different bacterial strains (*Staphylococcus epidermidis*, *Bacillus megaterium*, *Bacillus cereus* and *Bacillus subtilis*) were tested for extracellular protease production on casein agar medium (Alagarsamy et al., 2005; Chi et al., 2007; Folasade et al., 2005), *B. subtilis* EFRL 01 was found to be the best strain among all tested in the experiments. *Bacillus* sp. is extensively exploited for the production of various industrial enzymes. In the present study, *B. subtilis* produced protease with better properties required for several industrial processes such as pH stable and thermostable (data not shown). According to current results, *B. subtilis* was selected for further optimization of cultivation conditions.

#### Effect of time course of fermentation on protease production

Effect of time course on biosynthesis of protease by *B. subtilis* was checked and data is presented in Figure 2. The maximum protease secretions were noted after 6 h of incubation with 1.0% glucose as substrate at 37°C. Wellington et al. (2004) have reported that the maximum protease production was achieved in 9 h using trisodium citrate as substrate by thermophilic *Bacillus* sp. The earlier secretion of proteases suggests the presence of enriched medium, which is required for the growth of microorganism and protease production. The decline in medium pH might be due to acid production by utilization of glucose (data not shown).



**Figure 2.** Effect of incubation time on growth and protease production by *B. subtilis* at 37°C. The results shown are the mean of triplicate experiment.

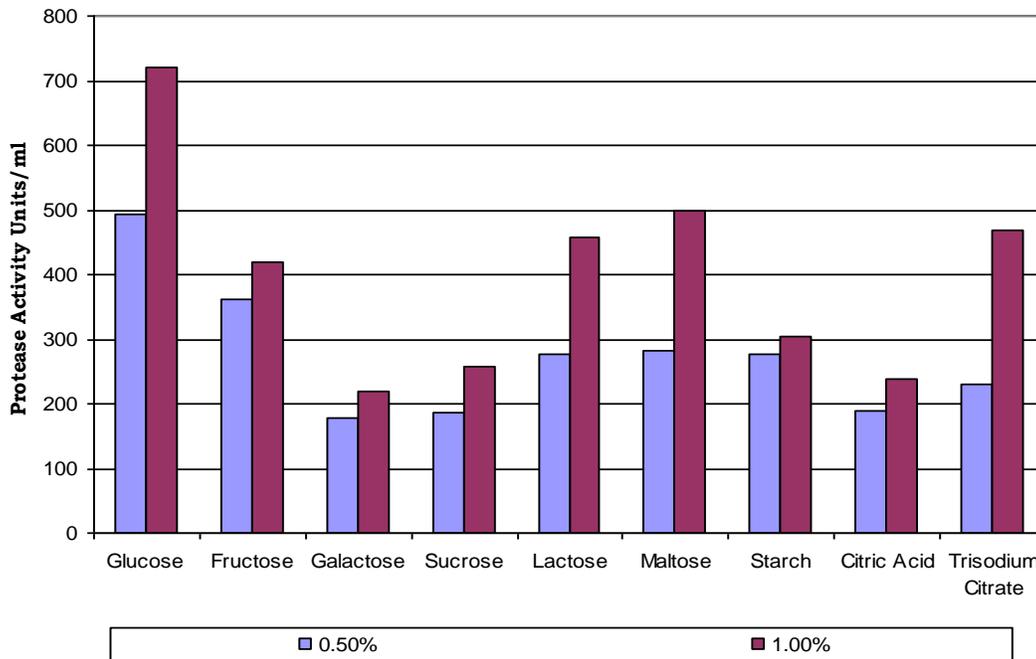
### Effect of carbon source on protease production

To optimize the cultivation conditions yielding maximum extracellular protease, *B. subtilis* was grown on various carbon sources at optimum pH, temperature and incubation time period in respect to protease production, bacterial growth, protease activity, total sugar, reducing sugar and total proteins were observed (Figures 3 and 4). The maximum protease secretion was noted when *B. subtilis* grown on 1.0% molasses mineral medium by at 37°C for 8 h with initial pH 7.5. The superior effect of molasses in enzyme production may be due to presence of growth promoting substances in enough quantity covering the requirement of bacterial growth along with biosynthesis of protease in both concentrations (0.5 and 1.0%). Molasses is a comparatively economical and cost effective energy source on large scale fermentation. The various researchers in the field (Ferid et al., 2008) have reported protease production by agro industrial waste. The utilization of agro industrial waste not only fulfills the requirement as a substrate for the production of several value added products, but also reduce the pollution.

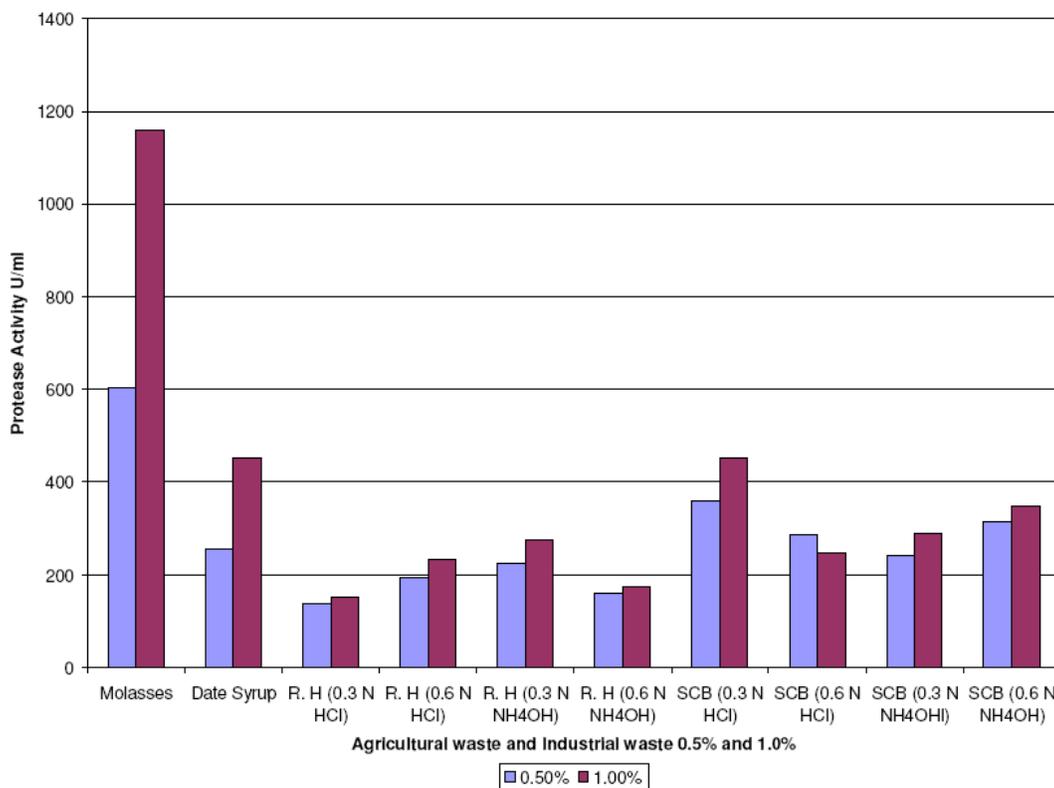
### Effect of nitrogen source on protease production

The effect of different nitrogen sources (0.75%) on protease production was observed and results are presented in Figure 5. Optimum protease production by *B. subtilis* was achieved when grown on 0.75% ammonium chloride molasses mineral medium in comparison to other nitrogen sources. Similar results are reported by Usama et al. (2005) in the case of protease production by *Teredinobacter turnirae* grown on ammonium chloride.

Figure 6 shows the effect of different nitrogen sources with 2.0% sodium chloride on biosynthesis of protease by *B. subtilis* using molasses mineral medium incubated at 37°C for 8 h. Maximum protease production was achieved with 0.75% peptone and 2.0% sodium chloride (2214 Units/ml), which was higher than control (1517 Units/ml). Many researchers (Al-Shehri et al., 2004; Prakasham et al., 2006) have observed that complex nitrogenous compounds supported better protease secretion in a better way than when compared to inorganic nitrogen compounds. This observation is in agreement with the result reported by Al-Shehri et al. (2004) in the

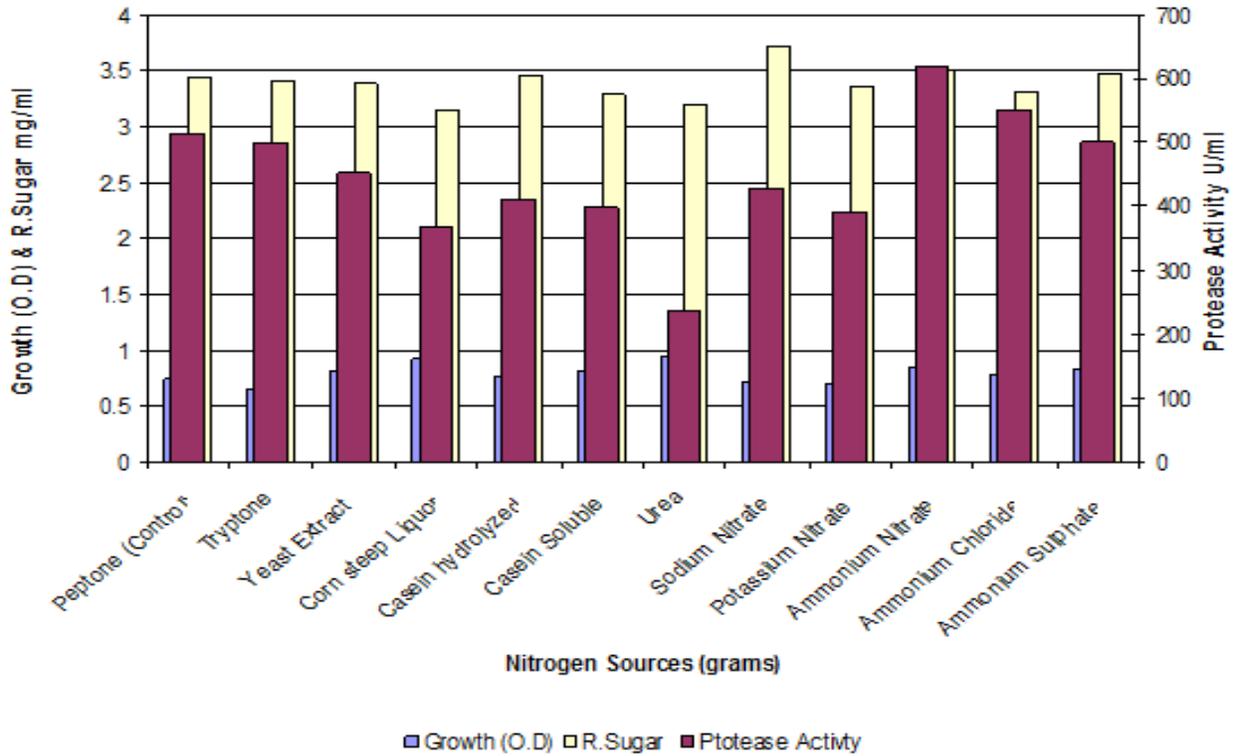


**Figure 3.** Effect of pure carbon sources (0.5 and 1.0%) on protease production by *B. subtilis* at 37°C. The results shown are the mean of triplicate experiment.

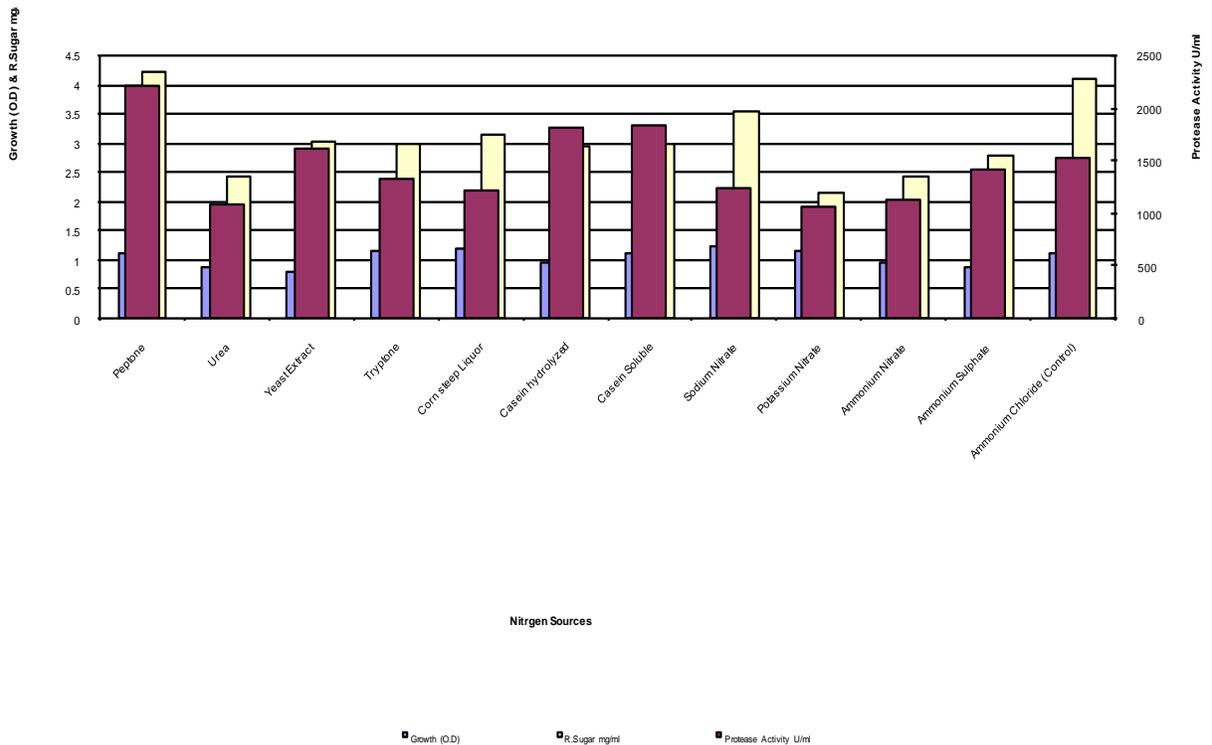


**R. H:** Rice Husk, **SCB:** Sugarcane bagasse, **N:** Normal solution  
 The results shown are the mean of triplicate experiment.

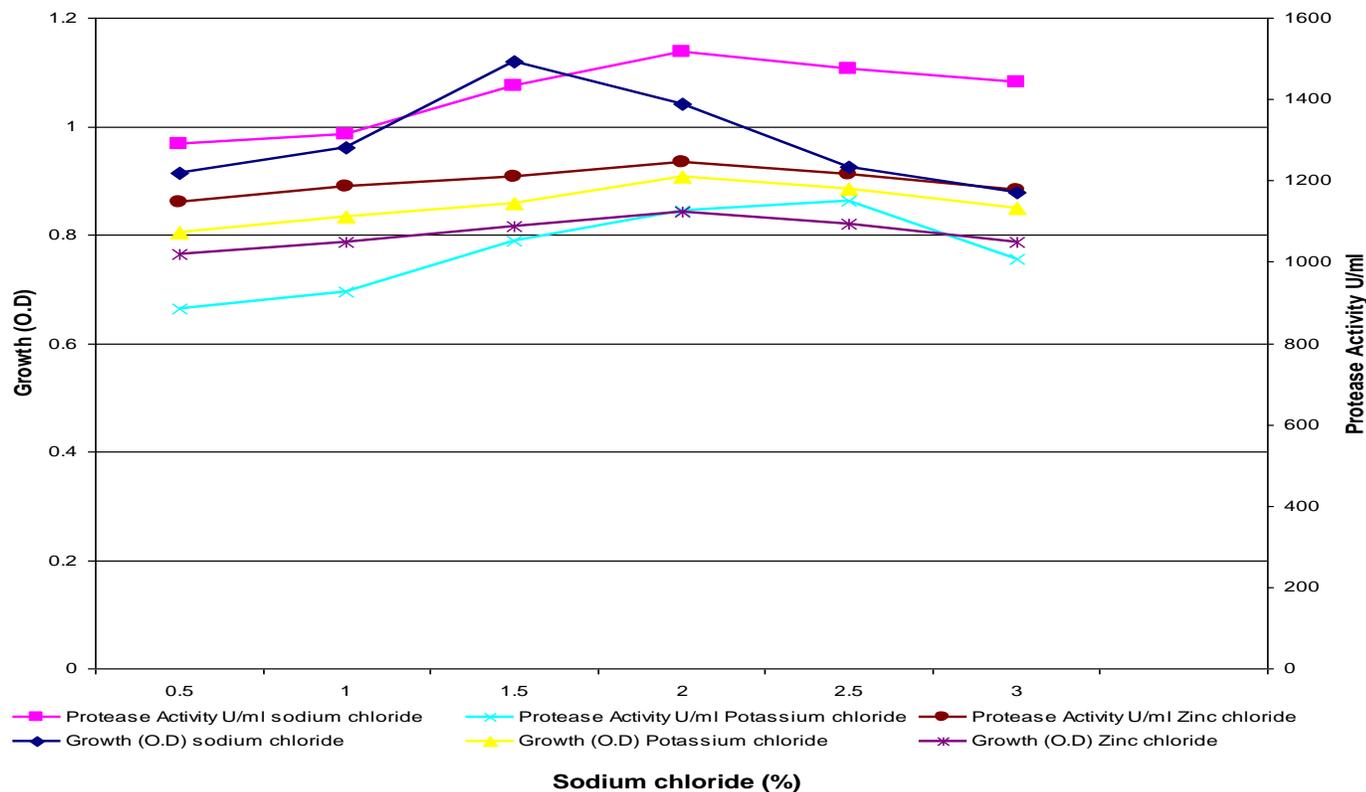
**Figure-4:** Effect of industrial and agricultural waste (0.5 & 1.0 %) as a carbon source on protease production by *Bacillus subtilis* at 37°C



**Figure 5.** Effect of nitrogen on protease production by *B. subtilis* at 37°C. The results shown are the mean of triplicate experiment.



**Figure 6.** Effect of nitrogen sources with 2% sodium chloride on protease production by *B. subtilis* at 37°C. The results shown are the mean of triplicate experiment.



**Figure 7a.** Effect of sodium chloride, potassium chloride and zinc chloride with ammonium chloride on protease production by *B. subtilis* at 37°C. The results shown are the mean of triplicate experiment.

case of protease production by *Bacillus licheniformis* grown on peptone.

#### Effect of sodium chloride, potassium chloride and zinc chloride on protease production

Figure 7a and b showed biosynthesis of protease by *B. subtilis* when grown on sodium chloride, potassium chloride and zinc chloride (0.5 - 3.0%) with and without ammonium chloride in molasses mineral medium at 37°C for 8 h. It was observed that the protease production was enhanced by addition of 2.0% sodium chloride with ammonium chloride from 1260 Units/ml to 1517 Units/ml. Very low amount of protease was secreted by *B. subtilis* when medium contained potassium chloride and zinc chloride with and without ammonium chloride; it may be due to inhibitory effect of these salts. Similar results are reported (Hamid et al., 2007; Patel et al., 2006) in the case of protease production by *Bacillus* sp. and *Salinivibrio* sp.

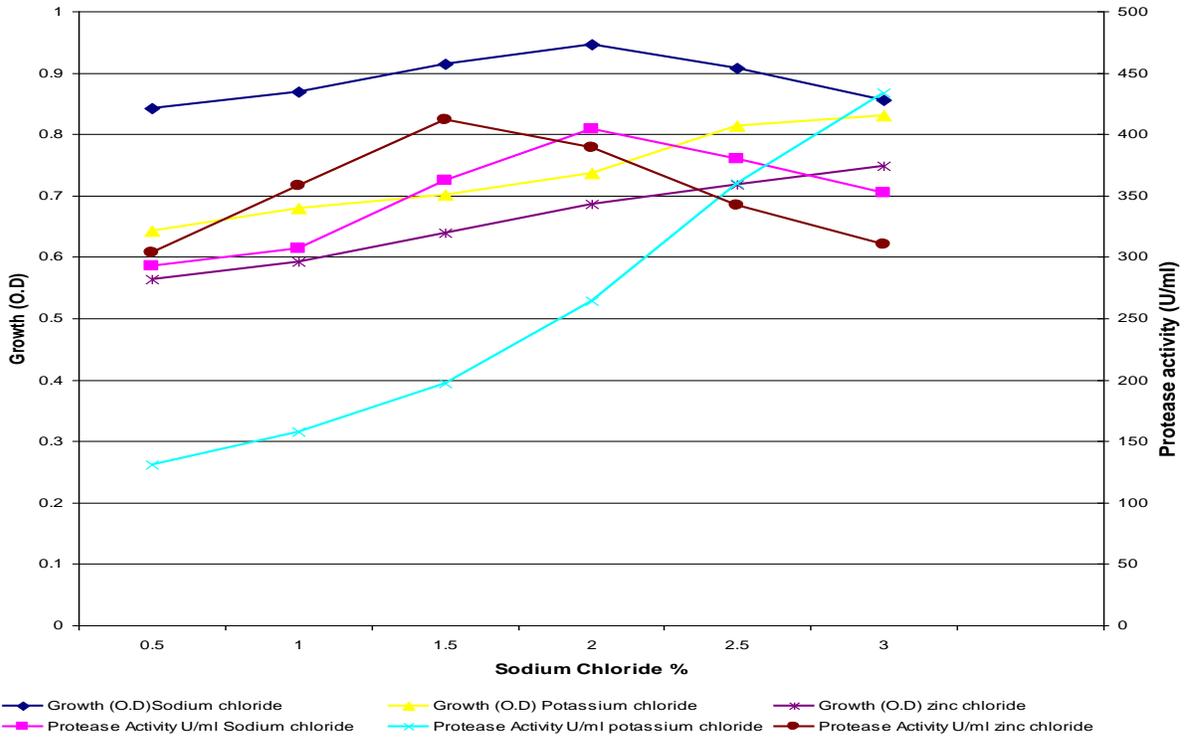
#### Effect of initial pH on protease production

The influence of initial pH on production of protease by *B. subtilis* was determined in the pH range of (3 - 12).

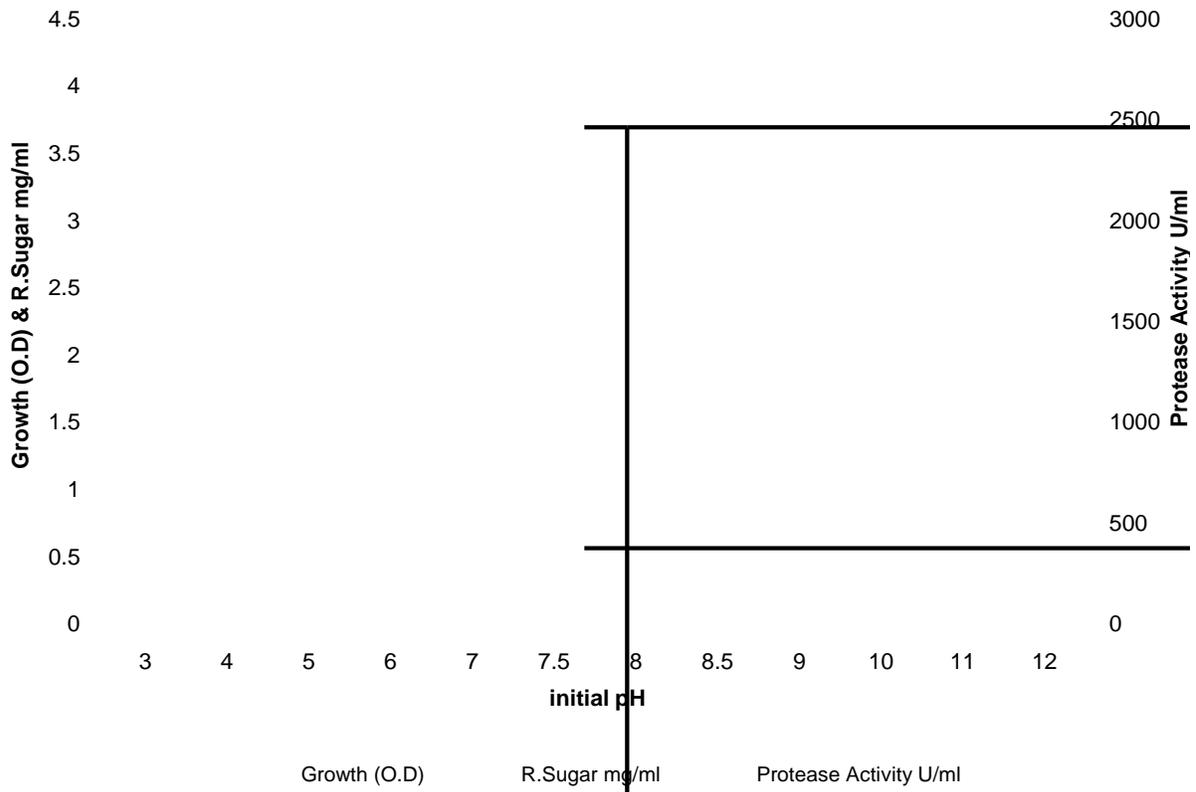
Medium was adjusted to required pH with the addition of 0.1 N HCl or 0.1 N NaOH. Protease synthesis increased with increase of initial pH of medium and reached maximum at pH 8.5, whereas below and above this level of pH, yield was low (Figure 8). The pH dependent changes in the amount of enzyme production might have been due to pH control over the growth of bacteria or pH dependent control of protease gene expression (Young et al., 1996). Al-Shehri et al. (2004) have reported the protease production by *B. licheniformis* at pH 8.0, Chi et al. (2007) at pH 9.0 by yeast *Aureobasidium pullulans*, Prakasham et al. (2006) at pH 9.0 by *Bacillus* sp., Ikram-ul-Haq and Hamid (2006) at pH 9.0 by *B. subtilis*, Folasade et al. (2005) at pH 8.0 by *Bacillus* sp. and Moreira et al. (2003) at pH 8.0.

#### Effect of temperature on protease production

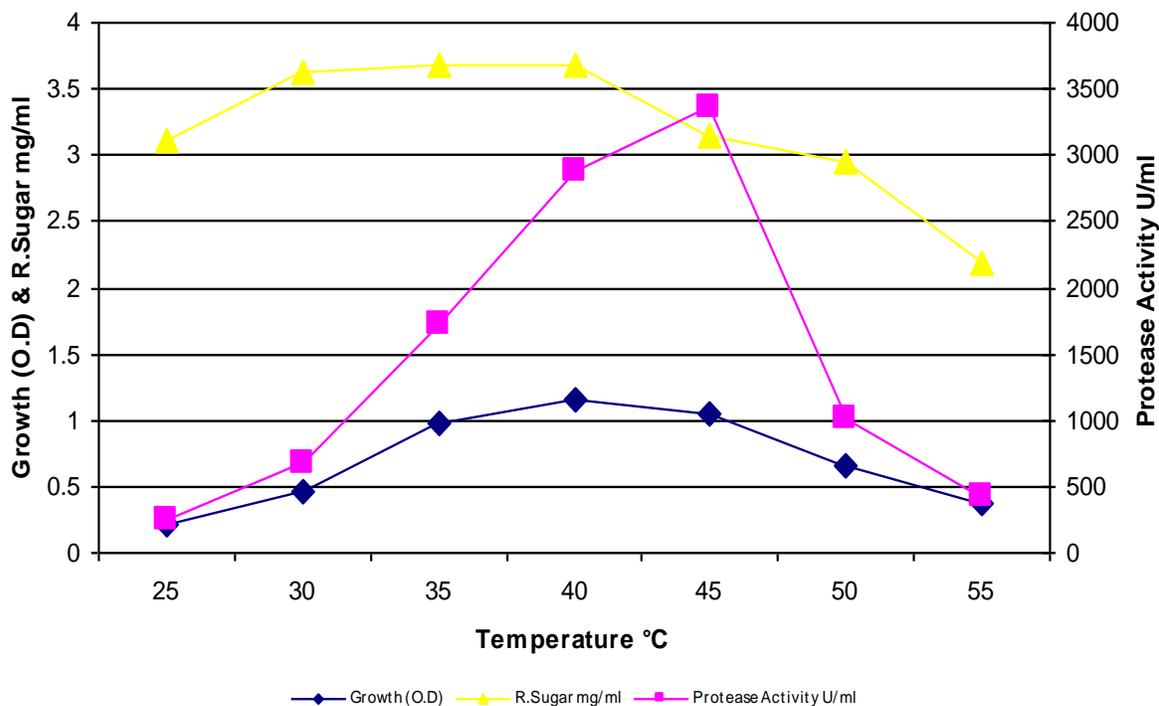
Protease production was investigated in a temperature range of 25 to 55°C by *B. subtilis* when grown on mineral medium incorporated with 1.0% molasses, 0.75% peptone and 2.0% sodium chloride and incubated for 8 h with initial pH of medium 8.5. Maximum proteases activities were produced at 45°C (Figure 9). Many investigators have studied the correlation between protease secretions with temperature but this depends on



**Figure 7b.** Effect of sodium chloride, potassium chloride and zinc chloride without ammonium chloride on protease production by *B. subtilis* at 37°C. The results shown are the mean of triplicate experiment.



**Figure 8.** Effect of initial pH on protease production by *B. subtilis* at 37°C. The results shown are the mean of triplicate experiment.



**Figure 9.** Effect of temperature on protease production by *B. subtilis*. The results shown are the mean of triplicate experiment.

the type of organism and culture conditions. Temperature affects all the physiological activities in a living cell and it is an important environmental factor to control the growth, microbial activities, normal functioning of enzyme and many enzymes control the nutritional requirement of the cell and subsequently its composition (Van Demark and Batzing, 1987). Al-Shehri et al. (2004) have reported the maximum protease secretion at 50°C with *B. licheniformis*, Chi et al. (2007) at 45°C with yeast *A. pullulans* and Camila et al. (2007) at 50°C by thermophilic *Bacillus* sp.

## Conclusion

There are few reports in the literature on biosynthesis of proteases by *B. subtilis* and other microorganisms using molasses as sole energy source. Molasses is sugar industry by product and can efficiently be utilized for the commercial production of proteases and other valuable products. The present work reports the production of proteases by *B. subtilis* EFRL 01 using molasses as sole carbon source. The present results suggest that molasses contain growth regulators and minerals required for proper growth and secretion of proteases. Although several proteases from *Bacillus* sp. have been reported in the literature, this enzyme may become attractive alternative of available proteases in local detergent and leather industry.

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