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Production optimization and characterization of an alkaline thermostable protease and its application as laundry additive

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Abstract

A number of alkaline proteases having potential industrial applications have been reported. However, the search is still on for better alkaline proteases having better suitability in industrial processes. New microbial strains producing useful enzymes can be isolated from natural habitats. In this regard the present study entitled “Production Optimization and Characterization of an alkaline thermostable protease and its application as laundry additive” was done and the major findings of this study are, alkalophilic organisms producing alkaline thermostable protease(s) were isolated from landfill site and milk industry soil. Isolate AP11 was found Gram positive alkalophile. Optimization studies on protease production from isolate AP11 revealed that the conditions for maximum protease production were 24 hours of incubation, 1% soyabean meal as nitrogen source, pH 10.2 (1% sodium carbonate),1% glucose, inoculum size of 1%,inoculum age of 16 h, Incubation temperature of 25°C and agitation rate of 250 rpm.

Key words: thermostable, protease, optimization, Isolate AP11

1. Introduction

Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in a number of processes. Proteases are the most important industrial enzymes accounting for approximately 60% of the total industrial enzyme market. They have diverse applications in a wide variety of industries, such as in detergent, food, pharmaceutical, leather, silk and recovery of silver from used X-ray films (Gupta *et al*., 2002; Rao *et al*., 2009).

There is a great industrial demand of proteolytic enzymes, with appropriate specificity and stability of pH, temperature and surfactants. Proteases with high activity and stability in high alkaline range and high temperatures are interesting for bioengineering and biotechnological applications. Their major application is in detergent industry because the pH of laundry detergents is generally in the range of 9.0-12.0. Owing to the better cleansing properties of enzyme-based detergents and pollution-alleviating capacity over conventional synthetic detergents (Krik *et al*., 2002; Mukherjee *et al*., 2008, Bhardwaj *et al.*,2014), alkaline proteases have made their way as key ingredients in detergent formulations (Maurer, 2004). Proteases in detergent industries account for approximately 30% of the total world enzyme production (Kumar *et al* 2014).

Though plants and animals also produce extracellular proteases, microorganisms are preferred source of proteases because of their rapid growth, limited space required for their cultivation, longer shelf life and the ease with which they can be genetically manipulated to generate improved enzymes (Rao *et al*., 1998). Although alkaline proteases are produced by a wide range of microorganisms including bacteria, actinomycetes, molds and yeasts, yet bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most predominant source followed by *Pseudomonas* such as *Bacillus thermoruber* (Manachini *et al*., 1988), *Bacillus clausii* (Kazan *et al*., 2005), *Bacillus cereus* (Shafee *et al*., 2005; Joshi *et al*., 2007), *Bacillus licheniformis* (Olajuyigbe and Ajele, 2008), *Bacillus subtilis* (Pillai and Archana, 2008; Ghafoor and Hasnain, 2009), *Bacillus circulans* (Rao *et al*., 2009) and *Pseudomonas*

aeruginosa (Karadzic *et al*., 2004; Khan *et al*., 2006). A considerable number of fungal species are also known to produce extracellular alkaline proteases such as *Conidiobolus coronatus* (Phadatare, 1993), *Trichoderma harzianum* (Dunaevsky *et al*., 2000) and *Aspergillus fumigatus* (Wang *et al*., 2005). Among yeast proteases, *Candida* sp. has been studied in detail (Poza *et al*., 2001).

Most of the available industrial proteases have some limitations and their use highly depends on their stability during isolation, purification and storage in addition to their robustness against solvents, surfactants and oxidants. Although a number of alkaline proteases for their potential application have been characterized and patented, the industry is still in search of efficient alkaline proteases. Isolation of alkaline protease(s) producing bacteria.

2. Materials

2.1 Growth Medium

Composition of Nutrient medium (Horikoshi medium) (Horikoshi and Akiba, 1982).Glucose1.0%, Peptone 0.5%, Yeast extract 0.5%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.02%, Agar 2.0%, Na_2CO_3 1.0 % (pH 10.2).Na_2CO_3 (10% stock solution) is sterilized separately and added to the medium after sterilization.

2.2 Glassware/Plastic ware and chemicals

Borosil and Schott Duran glassware was used throughout the study, which was washed twice with the detergent with final rinsing in distilled water. Tarsons and Laxbro plasticware was used during the study. These were washed twice with detergent, rinsed with distilled water and dried in hot air oven at 70°C prior to use.All the chemicals and reagents used in the study were of high purity obtained from Hi-Media, SD Fine Chemicals, E-Merck, Qualigens and SRL.Casein was purchased from Sigma.

2.3 Soil sample

Soil samples were collected from landfill site and milk industry. The pH of the soil was measured at the site of collection of samples with the help of a pH strip. The samples were brought to the laboratory and processed immediately.

3 Methods

3.1 Isolation of the organisms

1 gram of the soil sample was suspended in 100 ml of sterile distilled water and suitable dilutions were plated on the Horikoshi Medium for the isolation of alkalophiles (Horikoshi and Akibia, 1982).

3.2 Screening of alkaline protease producing bacteria

The isolates were grown on Horikoshi medium plates containing 1% casein and incubated at 37°C for 24 hrs. The protease production was observed by visualization of clear halo zones around the colonies after the plates were flooded with 1 N HCl.

3.3 Protease production in liquid medium

3.3.1 Inoculum preparation

20 ml of HK broth (pH 10.2) was taken in a 100 ml flask. The inoculum was prepared by inoculating it with a loopful of pure culture from the plate and incubated for 20 h at 37°C, 150 rpm.

3.3.2 Submerged fermentation

20 ml of HK broth (pH 10.2) was taken in a 100 ml flask and was inoculated with 1% inoculum of 20 h old culture and incubated in a shaker for 24 h at 37°C, 150 rpm. The culture was centrifuged at 10,000 rpm for 10-15 min. Protease activity was assayed in the cell free supernatant.

3.3.3 Assay for protease enzyme
Protease activity was measured by Lowry method (Lowry *et al*., 1951).
Enzyme units
One unit of protease activity is defined as micromoles of tyrosine liberated per minute per ml of the enzyme.George et al 2014
3.4 Optimization of parameters for alkaline protease production
The effect of various physico-chemical cultural parameters e.g. carbon source, nitrogen source, temperature, pH and agitation rate was studied for optimum protease production in isolate AP11.

3.4.1 Incubation time
Viable cell count and protease activity in the cell free supernatant was measured for isolate AP11 for a period of 0-48 h. For this, 500 ml of HK medium broth was inoculated with 1% inoculum of 20 h old culture of isolate AP11. The inoculated medium was distributed equally (20 ml each) in 24 autoclaved 100 ml flasks. These were incubated at 37°C, 150 rpm. Flasks were taken out at regular intervals (0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 8 h, 10 h, 24 h, 28 h, 32 h and 48 h) in duplicates and with these growth curve was plotted by measuring cell count which was done by plating suitable dilutions on HK medium plates for each time interval. Enzyme activity was also measured in the cell free supernatant for each time interval.

3.4.2 Nitrogen sources
Various organic nitrogen sources i.e. casein, soyabean meal, tryptone, gelatin, peptone and yeast extract were added to the HK medium at a concentration of 1% (instead of 0.5% peptone and 0.5% yeast extract in HK medium) for the production of alkaline protease. Medium was inoculated with 1% inoculum of 20 h old culture of isolate AP11. Enzyme was harvested after 24 h of incubation and activity was determined.

3.4.3 Concentration of nitrogen source
Different concentrations of nitrogen source (soyabean meal) viz. 0.5%, 1%, 1.5%, 2%, 2.5% and 3% were used in the medium to determine the effect of concentration of nitrogen source on the production of alkaline protease. Enzyme was harvested after 24 h of incubation.

3.4.4 pH
The effect of pH was studied by adding different concentrations of Na_2CO_3 in the medium i.e. 0% (pH 6.8), 0.25% (pH 7.0), 0.5% (pH 9.3), 1.0% (pH 10.2), 1.5% (pH 10.8), 2.0% (pH 11.2), 2.5% (pH 11.6), 3.0% (pH 11.8) and inoculating with 1% inoculum of 20 h old culture of isolate AP11. The inoculated flasks were incubated at 37°C under shaking conditions (150 rpm) and the enzyme from respective flask was harvested after 24 h of incubation for protease assay.

3.4.5 Carbon source
Various carbon sources i.e. glucose, maltose, sucrose and starch were added to the medium at a concentration of 1% and their effect on alkaline protease production was studied. Enzyme was harvested after 24 h of incubation.

3.4.6 Inoculum size
The medium was inoculated with different inoculum sizes (0.1%, 1.0%, 5.0% and 10.0%) of 20 h old culture and their effect on the protease production was studied after 24 h of incubation.

3.4.7 Inoculum age
The medium was inoculated with 1% inoculum of different ages viz. 7 h, 16 h, 20 h and 24 h and enzyme activity was estimated after 24 h of incubation.
3.4.8 Incubation temperature
The effect of incubation temperature was studied by incubating the inoculated medium flasks at different incubation temperatures i.e. 25°C, 30°C, 37°C and 42°C. Enzyme activity was estimated in the cell free supernatant after 24 h of incubation.

3.4.9 Agitation rate
Alkaline protease production from isolate AP11 was studied at different agitation rates of 0 rpm (no agitation), 100 rpm, 150 rpm, 200 rpm and 250 rpm. The medium flasks were inoculated and incubated rotary shakers set at different agitation rates. The protease assay was done after 24 hours.

3.4.10 Optimization of protease production using response surface methodology
Based on the results of the one-variable-at-a-time experiments, three variables were chosen viz. soyabean meal concentration, Na_2CO_3 concentration (pH) and incubation temperature to optimize the production of alkaline protease from isolate AP11 using response surface methodology (RSM). In this regard, Design-Expert 8.0.2 Trial version was used to design the experiment. The optimum value for each parameter was used as the central value. A total of 20 experiments with different combinations of the selected parameters were performed so as to get the conditions optimized for maximum production of the enzyme.
* All the experiments were done in duplicates and average values were taken for the final calculation of results.

3.5 Characterization of alkaline protease produced by isolate AP11
The alkaline protease of the isolate AP11 was produced under optimum conditions. This enzyme was used to study the effect of various parameters on enzyme activity.

3.5.1 Effect of temperature
The optimal temperature for enzyme activity was determined by carrying out enzyme reactions at different temperatures in the range of 30°C-70°C. The thermal stability of the protease was determined in the temperature range of 40°C-60°C by measuring the residual protease activity at each temperature. The enzyme was incubated at respective temperatures for four hours and aliquots were withdrawn sequentially at different time intervals (0 min, 5 min, 10 min, 15 min, 30 min, 60 min, 120 min, 180 min and 240 min) and residual protease activity was measured under standard assay conditions.

3.5.2 Effect of pH
The optimum pH of the enzyme was determined by preparing the substrate (0.6% casein) as well as suitable enzyme dilutions in the buffers of different pH values i.e. pH 7, pH 8, pH 9.2, pH 10, and pH 11 and performing the assay under standard conditions. The assay was done at 60°C. The pH stability of protease was determined by suitably diluting the enzyme in the buffers of different pH values i.e. pH 8, pH 9.2, pH 10, and pH 11 and then incubating the enzymes at room temperature for four hours. Aliquots were withdrawn at different time intervals (0 min, 10 min, 20 min, 30 min, 60 min, 120 min, 180 min and 240 min) and residual activity was measured. Buffers used were phosphate buffer (pH 7 and pH 8), carbonate-bicarbonate buffer (pH 9.2 and pH 10), and sodium bicarbonate-sodium hydroxide buffer (pH 11).

3.5.3 Effect of substrate concentration

The effect of different concentrations of casein on alkaline protease activity was determined by varying the concentration of casein i.e. 1 mg/ml to 10 mg/ml. To obtain a typical hyperbolic curve, substrate concentration of 0.2 to 2 mg/ml was used and the effect on enzyme activity was studied. After that, a double reciprocal curve was plotted (Lineweaver-Burk plot) to calculate the Km and Vmax of enzyme.

3.5.4 Effect of inhibitors

The effect of inhibitors on the activity of protease from isolate AP11 was studied by incubating the enzyme at 30°C for 30 min in the presence of inhibitors viz. phenylmethylsulfonyl fluoride (PMSF) and β-mercaptoethanol at a final concentration of 1 mM, 5 mM and 10 mM. For control, equal amount of enzyme was diluted in carbonate-bicarbonate buffer (pH 9.2, 50 mM) and incubating at 30°C for 30 min. The residual protease activity (with respect to control) was measured by performing the assay under standard conditions.

3.5.5 Effect of metal ions

The effect of metal ions on alkaline protease activity was studied by incubating the enzyme at 30°C for 30 min in the presence of metal ions viz. Fe^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, K^{+}, Co^{2+}, Zn^{2+} at a final concentration of 1 mM, 5 mM and 10 mM. Simultaneous control was also run which lacked the ion. The residual protease activity (with respect to control) was measured by performing the assay under standard conditions.

3.5.6 Studies on compatibility of alkaline protease with detergents

The compatibility of alkaline protease from AP11 with some commercial laundry detergents viz. Wheel, Rin, Ariel and Tide was assessed. Detergents solutions were prepared at a concentration of 0.25% and then heated to destroy the indigenous protease activity. After cooling, the enzyme was added to the detergent solutions to prepare a suitable dilution of the enzyme and the mixture was pre-incubated at 30°C for 30 min. Thereafter, alkaline protease activity was measured.

3.6 Stain removal activity of alkaline protease (De-staining properties)

The blood stain removal activity of alkaline protease was determined by dipping 5 cm × 5 cm piece each of artificially blood stained cotton and muslin cloths in 50 ml of detergent solution supplemented with enzyme for 30 minutes at room temperature. A simultaneous control was run in which the stained cloths were dipped in detergent solution without the addition of enzyme. Before the addition of enzyme, the detergent solutions were boiled and then cooled to destroy the indigenous protease activity.

*All the experiments were done in triplicates and average values were taken for the final calculation of results.

4. Results and discussion

4.1 Isolation of alkalophiles

In the present study soil samples were collected from the landfill site and milk industry and 51 alkalophilic organisms were isolated from these samples on Horikoshi medium as described in section 3.2. The isolates were able to grow at pH 10, although some of the soil samples had a neutral pH. This is well known that the occurrence of alkalophilic microorganisms is independent of the pH of sample origin. Horikoshi (1991) reported that frequency of alkalophilic microorganisms in neutral soil samples can be upto $1/10^{th}$ of the population of the neutrophilic microorganisms (Horikoshi, 1999; Zhao *et al*., 2006).

4.2 Screening of alkaline protease producing bacteria

All the isolates were screened for the production of proteases on Horikoshi medium plates containing casein as described in section 3.2. Out of 51, 14 isolates were found to be alkaline protease producers (Table 4.1).

Table 4.1: Screening of alkalophilic isolates for protease production

Isolate number	Alkaline protease production	Isolate number	Alkaline protease production
1	_	26	_
2	+	27	+
3	_	28	_
4	_	29	_
5	+	30	_
6	_	32	++
7	_	33	_
8	_	34	_
9	_	35	+
10	+	36	+++
11	+++	37	_
12	_	38	_
13	_	39	_
14	_	40	+
15	_	41	_
16	_	42	_
17	_	43	_
18	+	44	+
19	_	45	_
20	_	46	+ +
21	_	47	+

22	_	48	_
23	_	49	_
24	_	50	_
25	+	51	_

4.2.1 Selection of isolate for alkaline protease production

Out of the 14 protease producers, two isolates viz. isolate numbers 11 and 36 were giving the maximum hydrolysis of casein as visualized by zone diameter to colony diameter ratio (Fig. 4.1).

Both the isolates were analyzed for the enzyme activity by submerged fermentation in Horikoshi medium. Isolate no. 11 was found to produce more activity than isolate no. 36 when assayed at 60°C and pH 9.2 (Table 4.2). This isolate was selected for further studies and it was designated as isolate AP11.

Table 4.2: Submerged fermentation of isolate no. 11 and 36

Isolate number	Enzyme activity (Units/ml)
11	21.6
36	17.9

4.3 Morphological characterstics of isolate ap11:

Broth culture was used for detecting the Gram behavior of isolate AP11 which revealed that it is Gram positive, rod-shaped, present singly or in pairs. It produced white, smooth, translucent, low convex colonies. The physiological characteristics of isolate AP11 showed it to be an alkalophile growing at pH 10 and the organism was a mesophile as it was not able to grow at temperatures above 40°C. The organism was spore forming as determined by spore staining technique. These preliminary results indicate that isolate AP11 is an alkalophile and most probably a *Bacillus* sp.

4.4 Optimization of alkaline protease production

The production of alkaline protease from isolate AP11 was optimized with respect to different parameters in the submerged culture:

4.4.1 Effect of incubation time on enzyme production and growth

Kinetics of bacterial growth and protease production were investigated by inoculating isolate AP11 in Horikoshi medium at pH 10.2 (1% Na_2CO_3). The organism showed a typical bacterial growth curve. No extracellular proteolytic activity was observed during the early- and mid-exponential growth phase. The enzyme production started with the onset of stationary phase and it reached its maximum after 24 hours. On further incubation, the enzyme production decreased (Fig. 4.2).

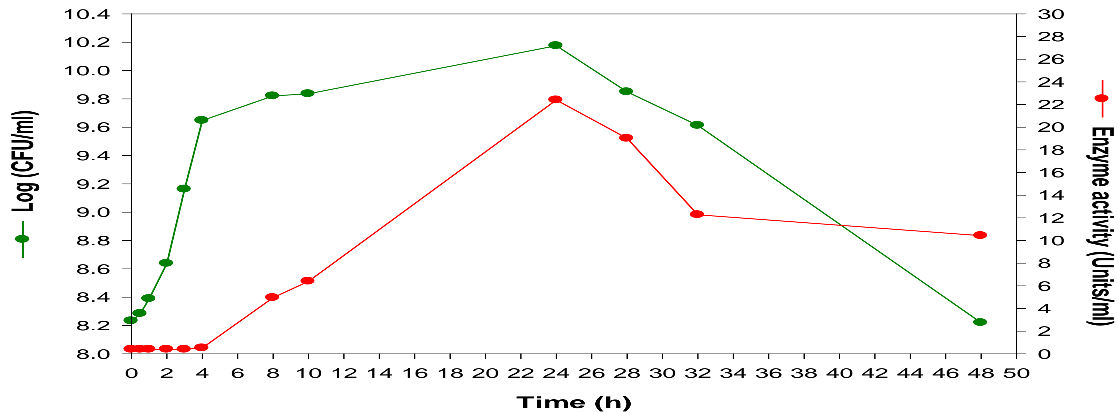

Fig. 4.2 Production of protease from alkalophilic isolate AP11 at different time intervals.

Similar results have been shown with *Bacillus subtilis* (Dutt *et al*., 2008), *Bacillus cereus* (Joshi *et al*., 2007), *Chryseobacterium taeanense* TKU001 (Wang *et al*., 2008) and *Halobacillus karajensis* (Heidari *et al*., 2009) where the maximum extracellular protease production was seen in the stationary phase.

4.4.2 Effect of different nitrogen sources

Nitrogen is metabolized to produce primarily amino acids, nucleic acids, proteins and cell wall components. The nitrogen sources have regulatory effect on enzyme synthesis. Therefore the production of proteases is highly dependent on nitrogen source available in the medium (Chu *et al*., 1992; Patel *et al*., 2005).

Effect of different nitrogen sources on the production of protease with isolate AP11 was analyzed. Different nitrogen sources viz. casein, soyabean meal, tryptone, gelatin, peptone and yeast extract were added to the HK medium (instead of 0.5% peptone and 0.5% yeast extract in HK medium). Protease production could be seen with most of the nitrogen sources except yeast extract. Maximum protease activity was observed with soyabean meal. However, comparable enzyme production occurred with casein and peptone also (Fig 4.3).

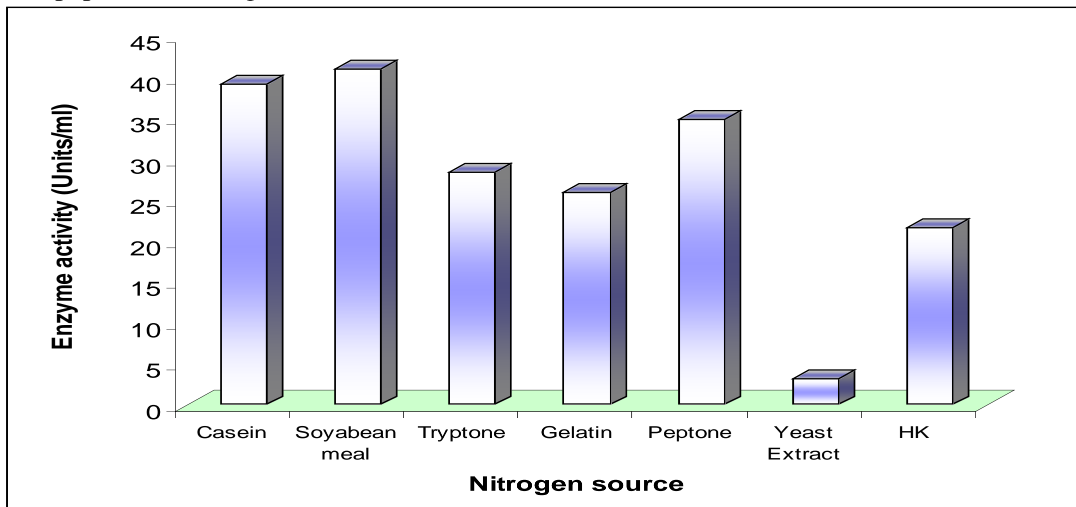

Fig. 4.3 Production of protease from alkalophilic isolate AP11 with different nitrogen sources.

In literature, different nitrogen sources like soyabean meal, casein, gelatin, peptone, yeast extract and tryptone have been reported best for the optimal production of bacterial alkaline proteases (Laxman *et al*.,

2005; Thomas *et al*., 2007; Chu, 2007; Joshi *et al*., 2007; Enshasy *et al*., 2008; Ghafoor and Hasnain, 2009). Soyabean meal was usually recognized as a potentially useful and cost-effective medium ingredient, because it is largely produced as a by-product in oil extraction industry. Moreover, chemical analysis showed that it is composed of approximately 40% protein and is rich in other organic and inorganic components. Therefore, soyabean meal has been used for the cost-effective production of extracellular proteases by many authors (Joo *et al*., 2002; Joo and Chang, 2005; Tari *et al.,* 2006).

Soyabean meal is an inexpensive and readily available substrate. Therefore, for further experiments soyabean meal was used as nitrogen source in this study.

4.4.3 Effect of soyabean meal concentration

Since soyabean meal was the most favourable nitrogen source for the enzyme production, it was necessary to study the effect of its concentration on the enzyme production. The concentration of soyabean meal was varied (0.5%-3%) and its effect was studied on enzyme production. The protease production was highly dependent on the availability of nitrogen source which was clear from the fact that at low soyabean meal concentration, very low enzyme production was observed. Maximum enzyme production was observed with 1%-1.5% soyabean meal concentration. No increase in enzyme production was seen with further increase in the concentration of soyabean meal (Fig 4.4).

These results are in agreement with Saurabh *et al*. (2007) and Ibrahim *et al*. (2009) where 1.5% and 2% of soyabean meal was shown to be the best for the production of proteases from *Bacillus* sp. SBP-29 and *Bacillus halodurans* respectively. Maximum protease production from *Streptomyces avermectinus* NRRL B-8165 has been reported with 0.5% of soyabean meal (Ahmed *et al*., 2008) whereas with isolate AP11 very less amount of protease could be produced with 0.5% of soyabean meal.

For further experiments 1% soyabean meal was used as nitrogen source in this study.

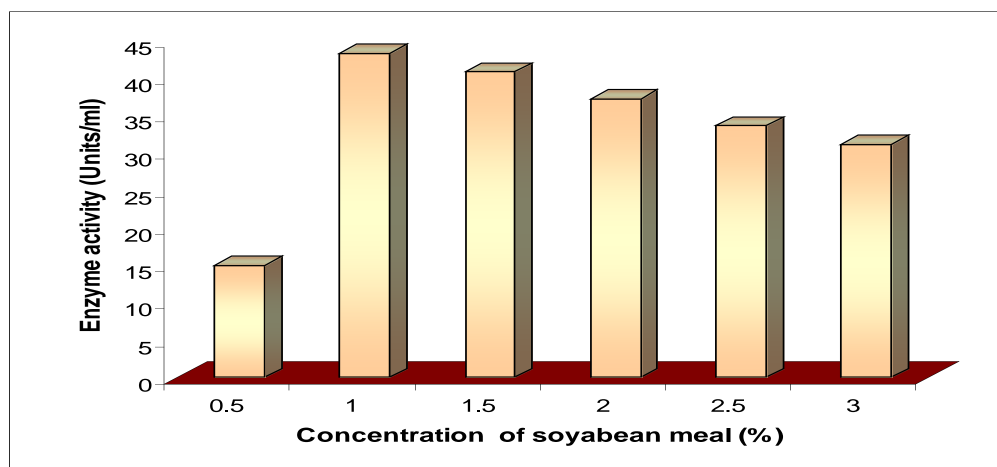

Fig. 4.4 Production of protease from alkalophilic isolate AP11 with different concentrations of soyabean meal.

4.4.4 Effect of pH

Isolate AP11 is an alkalophilic organism. Therefore the pH of the growth medium is expected to effect the production of the enzyme. The enzyme production was done at different pH levels by adding different amounts of sodium carbonate in the medium i.e. 0.25% (pH 7.0), 0.5% (pH 9.3), 1.0% (pH 10.2), 1.5% (pH 10.8), 2.0% (pH 11.2), 2.5% (pH 11.6) and 3.0% (pH 11.8). Since the organism is alkalophilic,

negligible growth and enzyme production was observed in the neutral pH range. Maximum enzyme production was observed with 0.5%-1% sodium carbonate (pH~9.3-10.2). However, enzyme production decreased with further increase in pH (Fig 4.5).

Different Bacillus spp. have been reported to produce alkaline protease in alkaline range, e.g. *Bacillus* sp. isolate K30 (Naidu and Devi, 2005) at pH 9.0, *Bacillus brevis* at pH 10.5 (Banerjee *et al*., 1999), *Bacillus cereus* MTCC 6840 (Joshi *et al*., 2007) and *Bacillus* sp. APP1 (Chu, 2007) at pH 9.0 and *Bacillus licheniformis* at pH 10.0 (Enshasy *et al*., 2008).

For further experiments 1% sodium carbonate was used in this study.

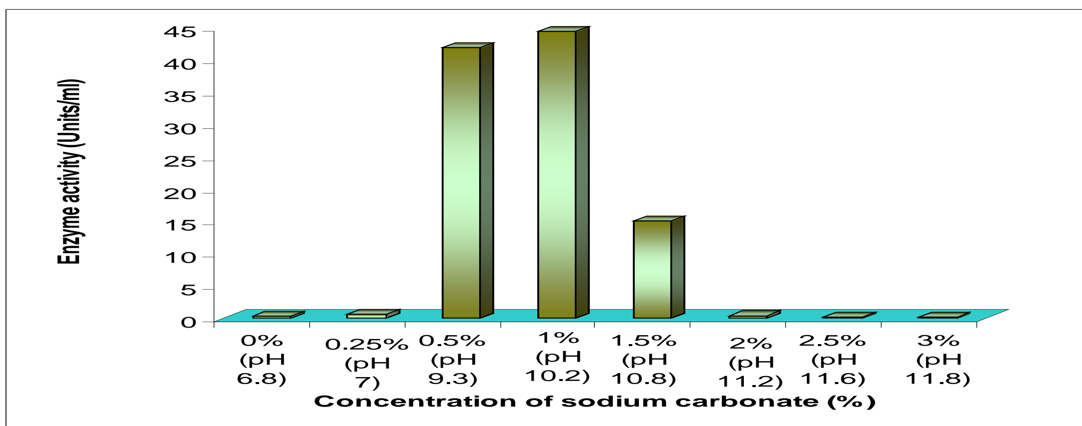

Fig. 4.5 Production of protease from alkalophilic isolate AP11 at different concentrations of sodium carbonate (pH range 6.8-11.8).

4.4.5 Effect of different carbon sources

The protease production was done with different carbon sources viz. glucose, maltose, sucrose and starch at a concentration of 1%. Maximum enzyme production was observed with glucose. However, enzyme production was comparable in other carbon sources as well (Fig 4.6).

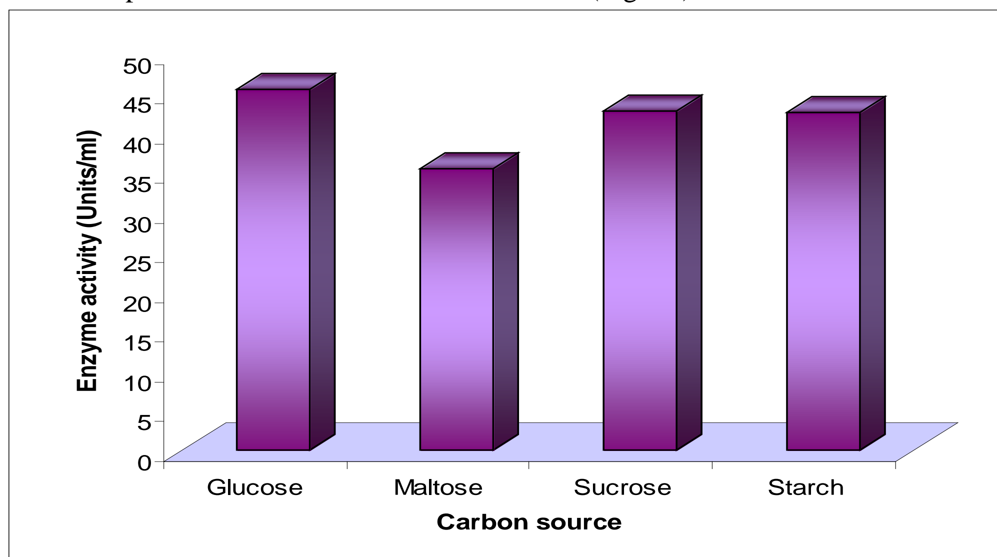

Fig. 4.6 Production of protease from alkalophilic isolate AP11 with different carbon sources.

Different carbon sources like glucose, starch, maltose and sucrose have been reported to be the best for the production of proteases (Johnvesly *et al*., 2001; Frikha *et al*., 2005; Salleh *et al*., 2005; Joshi *et al*., 2007; Dutt *et al*., 2008; Gupta *et al*., 2008; Ahmed *et al*., 2008; Ghafoor and Hasnain, 2009). Alike isolate AP11, 1% glucose has been reported to be the best for the production of alkaline proteases from *Bacillus subtilis* AG-1 (Ghafoor and Hasnain, 2009) and *Bacillus licheniformis* (Enshasy *et al*., 2008).

For further experiments glucose was used as carbon source in this study.

4.4.6 Effect of inoculum size

To ensure a high production of enzyme in the limited volume of medium, the bacterial inoculum size should be controlled. Small inoculum size leads to higher surface area to volume ratio as well as improved distribution of dissolved oxygen resulting in increased enzyme production. However, if the inoculum size is too small, insufficient number of bacteria would lead to reduced amount of secreted protease. Conversely, higher inoculum size results in reduced dissolved oxygen and increased competition towards nutrients. Hence, isolate AP11 was grown for 20 h and it was used as starting culture for inoculation in different sizes (0.1%, 1.0%, 5.0% and 10.0%). Inoculum size of less than 1% was inadequate for protease production whereas inoculum size of 1% and above could produce comparable amount of enzyme (Fig 4.7).

Inoculum size of 1% has been reported to be best for the production of alkaline protease by *Bacillus subtilis* EAG-2 and *Bacillus subtilis* AG-1 (Ghafoor and Hasnain, 2009). However, inoculum sizes of 4% and 5% have been reported to be optimum for the production of proteases from *Bacillus cereus* strain 146 (Shafee *et al*., 2005) and *Bacillus subtilis* strain Rand (Abusham *et al*., 2009) respectively.

For further experiments 1% inoculum was used in this study.

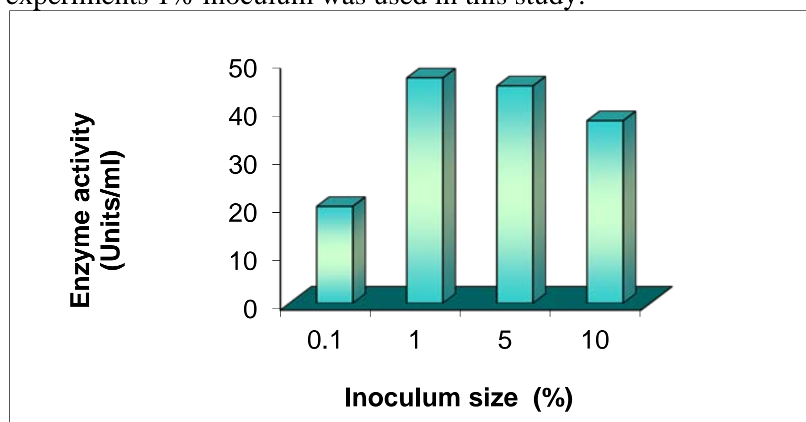

Fig. 4.7 Production of protease from alkalophilic isolate AP11 with different inoculum sizes.

4.4.7 Effect of inoculum age

Like inoculum size, the inoculum age (physiological state of cells) can also be an important parameter for the enzyme production. The enzyme production was done by inoculating the medium with 1% inoculum of different ages viz. 7 h, 16 h, 20 h and 24 h (The inoculum was prepared by inoculating HK medium with a loopful of culture from the plate). Maximum enzyme production was observed with 16 h inoculum. Comparable enzyme production was observed with 20 h and 24 h inoculum (Fig 4.8).

For further experiments 16 h inoculum was used in this study.

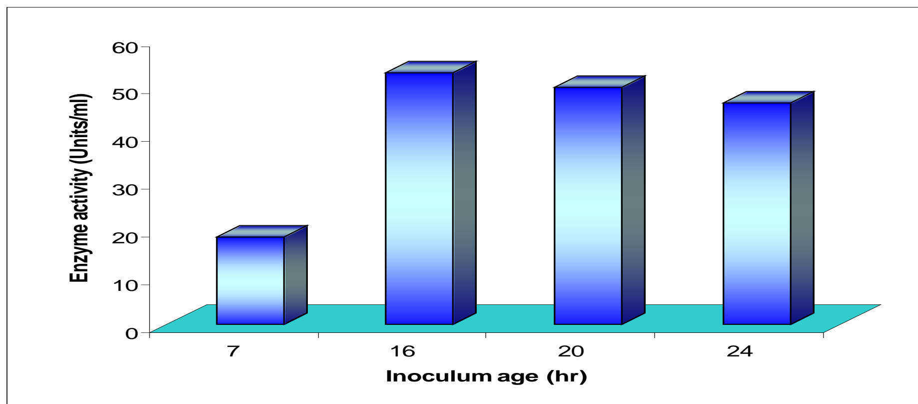

Fig. 4.8 Production of protease from alkalophilic isolate AP11 with different inoculum sizes.

4.4.8 Effect of temperature

Temperature is a critical parameter for all the cell functions. It has been shown that there is a link between enzyme synthesis and energy metabolism in bacteria and this is controlled by temperature and oxygen uptake (Frankena *et al*., 1986). Moreover, for extracellular enzymes, temperature has been found to influence their secretion, possibly by changing the physical properties of the cell membrane (Rahman *et al*., 2005).

The optimum temperature for the production of protease from isolate AP11 was investigated from 25°C-42°C. The medium was inoculated with isolate AP11 and incubated at different temperatures viz. 25°C, 30°C, 37°C and 42°C. Since the organism is a mesophile, very low growth as well as low amount of protease production was observed at higher temperature (42°C). Maximum protease production was observed at 25°C. The enzyme production decreased with the increase in temperature. At 37°C, it decreased by 60% (Fig. 4.9).

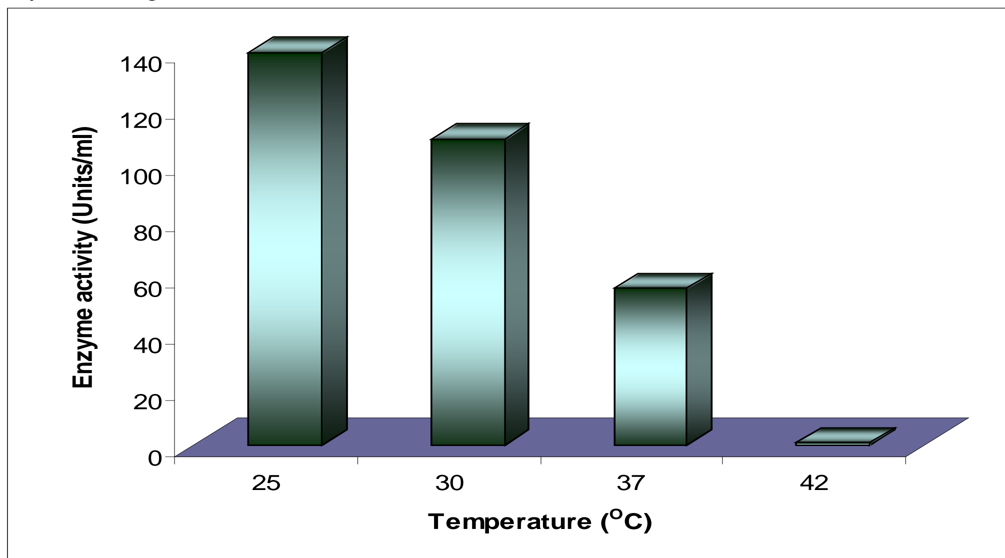

Fig. 4.9 Production of protease from alkalophilic isolate AP11 at different temperatures.

The alkaline protease production from bacteria showed wide range of variations with respect to incubation temperature. *Bacillus* sp. isolated from glaciers showed maximum protease production at 7°C (Gordon, 1982) while *Bacillus subtilis* strain Rand (Abusham *et al*., 2009) and *Bacillus halodurans*

(Ibrahim *et al*., 2009) showed maximum protease production at 37°C. *Bacillus clausii* (Kumar *et al*., 2004) was reported to produce alkaline protease at 42°C, *Bacillus* sp. APP1 (Chu, 2007) at 50°C, *Bacillus* sp. K-30 (Naidu and Devi, 2005) and *Thermomicrobium* sp. KN-22 at 70°C (Murao *et al*., 1991).

Although isolate AP11 was able to grow well in the range of 25°C-37°C, it preferred lower mesophilic temperature for enzyme synthesis with maximum production being recorded at 25°C. Similar results have been reported with *Bacillus cereus* (Joshi *et al*., 2007) and *Bacillus circulans* (Jaswal *et al*., 2008) where maximum protease production has been shown to be at 25°C. This is slightly higher than optimum temperature of alkaline protease producing psychrophilic *Bacillus cereus* sp. (Shi *et al*., 2005) and *Pedobacter cryoconitis* (Margesin *et al*., 2005).

For further experiments 25°C was used as incubation temperature in this study.

4.4.9 Effect of aeration and agitation

Microorganisms vary in their oxygen requirements. In particular, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. Moreover, the variation in the agitation speed has been found to influence the extent of mixing in the shake flasks thereby affecting the nutrient availability (Nascimento and Martins, 2004).

The protease production was done at different agitation rates viz. 0 rpm (without agitation), 100 rpm, 150 rpm, 200 rpm and 250 rpm. Results revealed that agitation is must for the enzyme production. The enzyme production increased when agitation was increased. Maximum enzyme production was seen at the agitation rate of 250 rpm (Fig. 4.10).

At lower agitation rates, insufficient aeration and nutrient uptake perhaps causes inability of bacteria to grow efficiently and at optimum agitation, aeration of medium is increased which leads to sufficient supply of dissolved oxygen in the medium (Kumar and Takagi, 1999). Nutrient uptake by bacteria will also be increased at higher agitation rates (Beg *et al*., 2003) resulting in increased enzyme production. In most of the cases, an agitation rate of 200 rpm has been reported to be the best for the production of proteases (Jaswal *et al*., 2008; Maal *et al*., 2009; Ibrahim *et al*., 2009). In some cases, higher agitation rates have been shown to decrease the enzyme production which can be due to the denaturation of the enzyme caused by higher agitation rates (Lee *et al*., 2002). Moreover, excessive agitation and aeration may lead to cell lysis (Darah *et al*., 1996). However, agitation rates as high as 400 rpm have been reported to be good for the production of alkaline proteases as in *Bacillus circulans* (Jaswal *et al*., 2008).

For further experiments agitation rate of 250 rpm was used in this study.

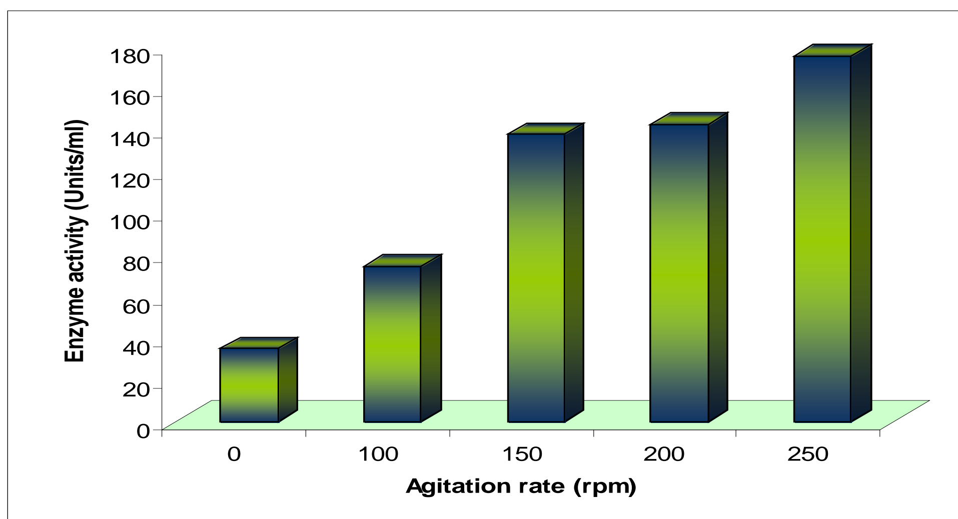

Fig. 4.10 Production of protease from alkalophilic isolate AP11 at different agitation rates.

4.4.10 Optimization of protease production using response surface methodology

As has been discussed in the previous sections, some factors greatly influenced the production of protease from alkalophilic isolate AP11. However, the used optimization procedures involved altering of one parameter at a time keeping all other parameters constant. This enables one to assess the impact of one particular parameter on the process performance and cannot provide information about the mutual interactions of the parameters (Beg *et al*., 2003). The use of statistical approach involving response surface methodology (RSM) has gained a lot of impetus for the optimization and understanding the interactions among the various physico-chemical parameters. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of studied factors for desirable responses (de Coninck *et al*., 2000). Hence, RSM is an efficient strategic experimental tool by which the optimal conditions of a multivariable system may be determined. RSM has been successfully applied in many areas of biotechnology, such as the protease production (Dutta *et al*., 2004; Khan *et al*., 2006; Hajji *et al*., 2008; Jaswal *et al*., 2008; Reddy *et al*., 2008), α-amylase production (Kunamneni *et al*., 2005) and neomycin production (Adinarayana *et al*., 2003). Therefore in the present study Design-Expert 8.0.2 Trial version was used to design the experiment for the optimization of alkaline protease production by isolate AP11 using response surface methodology.

Based on the results of the one-variable-at-a-time experiments the effect of three variables, soyabean meal concentration, Na_2CO_3 concentration (pH) and incubation temperature, which significantly influenced the alkaline protease production, was studied using the RSM. The optimum value for each parameter was used as the central value. A total of 20 experiments with different combinations of the selected parameters were performed (Table 4.3) so as to get the conditions optimized for the maximum production of the enzyme.

Table 4.3 Experimental design used in RSM studies by using three independent variables

Standard	Run	Block	Factor 1 A: Temperature (°C)	Factor 2 B: Sodium carbonate concentration (%)	Factor 1 C: Soyabean meal concentration (%)	Response 1 Activity Units/ml
1	5	Block 1	20.00	0.70	0.70	58.33
2	12	Block 1	30.00	0.70	0.70	85.55
3	6	Block 1	20.00	1.30	0.70	98.19
4	9	Block 1	30.00	1.30	0.70	88.89
5	3	Block 1	20.00	0.70	1.30	158.47
6	1	Block 1	30.00	0.70	1.30	97.22
7	10	Block 1	20.00	1.30	1.30	139.03
8	7	Block 1	30.00	1.30	1.30	77.78
9	4	Block 1	25.00	1.00	1.00	166.25
10	11	Block 1	25.00	1.00	1.00	191.53
11	8	Block 1	25.00	1.00	1.00	159.44
12	2	Block 1	25.00	1.00	1.00	155.55
13	16	Block 2	16.59	1.00	1.00	45.83
14	14	Block 2	33.41	1.00	1.00	137.5
15	13	Block 2	25.00	0.50	1.00	188.61
16	19	Block 2	25.00	1.50	1.00	112.5
17	17	Block 2	25.00	1.00	0.50	46.53
18	15	Block 2	25.00	1.00	1.50	165.28
19	18	Block 2	25.00	1.00	1.00	172.08
20	20	Block 2	25.00	1.00	1.00	160.42

*All the experiments were done in duplicates and average values were taken for the calculation of enzyme activity at each point.

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The predicted value of the enzyme production with the optimized values for the parameters by RSM was similar to the activity achieved after optimization with one variable at a time. Probably the range of the variables selected did not allow any significant interaction and some more parameters have to be tried by RSM. Therefore, further studied are required in this regard.

4.5 Characterization of alkaline protease

The alkaline thermostable protease from alkalophilic isolate AP11 was produced under the conditions optimized in section 4.4. This crude enzyme was characterized for different parameters:

4.5.1 Temperature optima

The effect of temperature on the activity of alkaline protease from alkalophilic isolate AP11 was studied at temperatures ranging from 30°C-70°C by varying the reaction temperature. The results revealed that optimum activity of the enzyme was at 60°C. However, further increase in incubation temperature drastically reduced the enzyme activity whereas at 50°C it was able to retain 85% of its activity (Fig. 4.11). A number of alkaline proteases in literature have been shown to be active in the temperature range of 50°C-70°C (Kazan *et al.*, 2005; Hajji *et al.*, 2007; Almas *et al.*, 2009; Akel *et al.*, 2009; Rao *et al.*, 2009; Rai *et al.*, 2010).

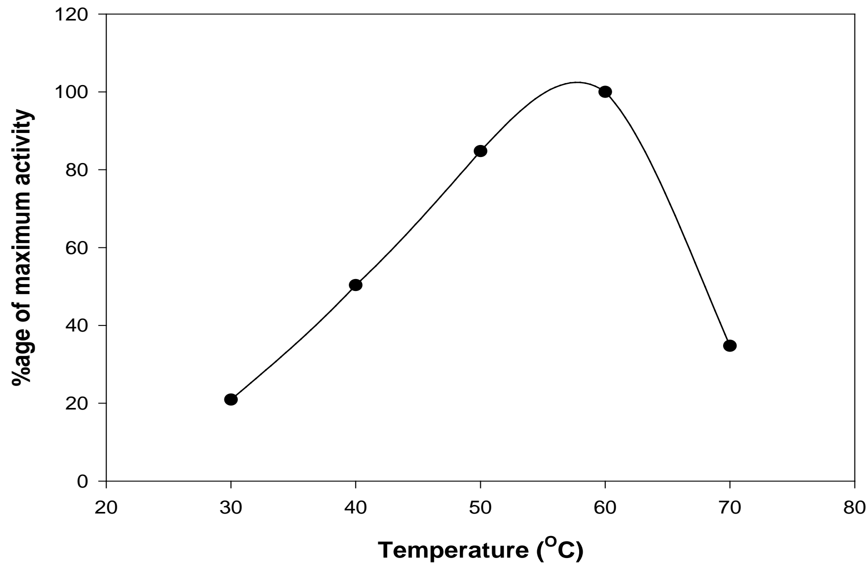

Fig. 4.11 Activity of protease from alkalophilic isolate AP11 at different temperatures.

4.5.2 Temperature stability

The thermostability of protease was examined in the temperature range of 40°C-60°C. The enzyme was incubated at respective temperatures and aliquots were withdrawn sequentially at different time intervals and residual activity was measured under standard assay conditions. The enzyme was stable for 15 min at all the temperatures. It could retain 75%, 72% and 68% of activity at 40°C, 50°C and 60°C respectively. However, further incubation at all the temperatures led to a decrease in enzyme activity (Fig. 4.12). Similar temperature stability patterns have been reported by alkaline proteases from *Bacillus* sp. L21 (Genckal and Tari, 2006), *Bacillus* strain HUTBS71 (Akel *et al.*, 2009), *Bacillus clausii* GMBAE 42 (Kazan *et al.*, 2005), *Bacillus* strain SAL1 (Almas *et al.*, 2009), *Paenibacillus tezpurensis* sp. nov. AS-S24-II (Rai *et al.*, 2010) and *Bacillus licheniformis* Lbbl-11 (Olajuyigbe and Ajele, 2008).

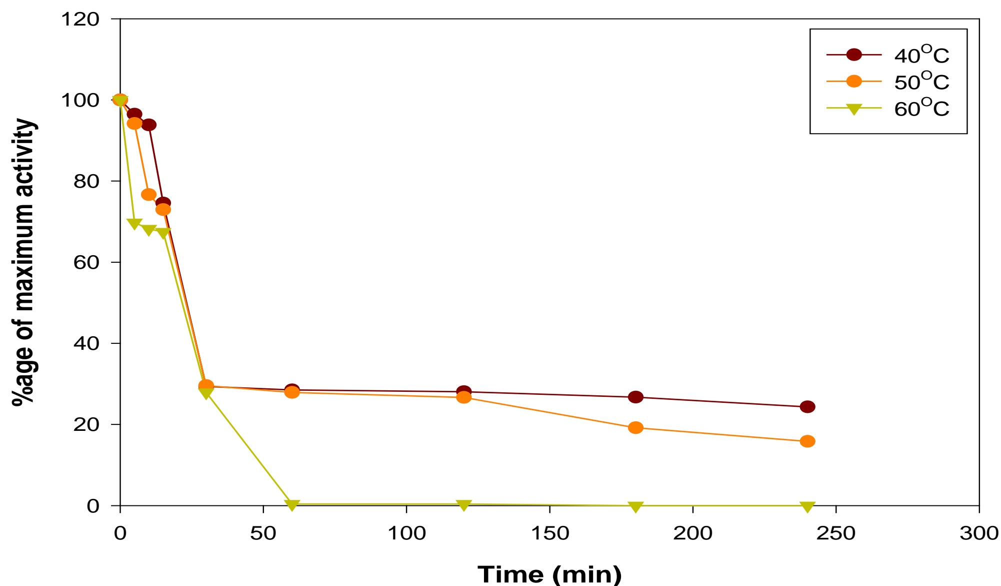

Fig. 4.12 Temperature stability of protease from alkalophilic isolate AP11 at different temperatures

4.5.3 pH optima

The optimal pH for enzyme activity was determined by preparing the substrate as well as suitable enzyme dilutions in buffers of different pH (pH 7, pH 8, pH 9.2, pH 10, and pH 11) (as explained in section 3.3.2) and performing the enzyme assay under standard conditions. The enzyme was active over a wide pH range. The optimum pH of the enzyme was observed to be at pH 9.2. The enzyme could retain 75% of its activity at pH 10 and 67% of its activity even at pH 11. At lower pH values, lesser enzyme activity was observed but 55%-60% of the activity could be observed even in the neutral pH range (pH 7- pH 8) (Fig. 4.13). Alkaline proteases from various organisms have been reported e.g. optimum pH of 8 has been reported for *Bacillus licheniformis* Lbbl-11 (Olajuyigbe and Ajele, 2008), pH 9 for *Bacillus* strain APP1 (Chu, 2007) and *Halobacillus karajensis* (Heidari *et al.*, 2009), pH 10 for *Pseudomonas aeruginosa* strain K (Rahman *et al*., 2006) and 11 for *Bacillus* sp. L21 (Genckal and Tari, 2006).

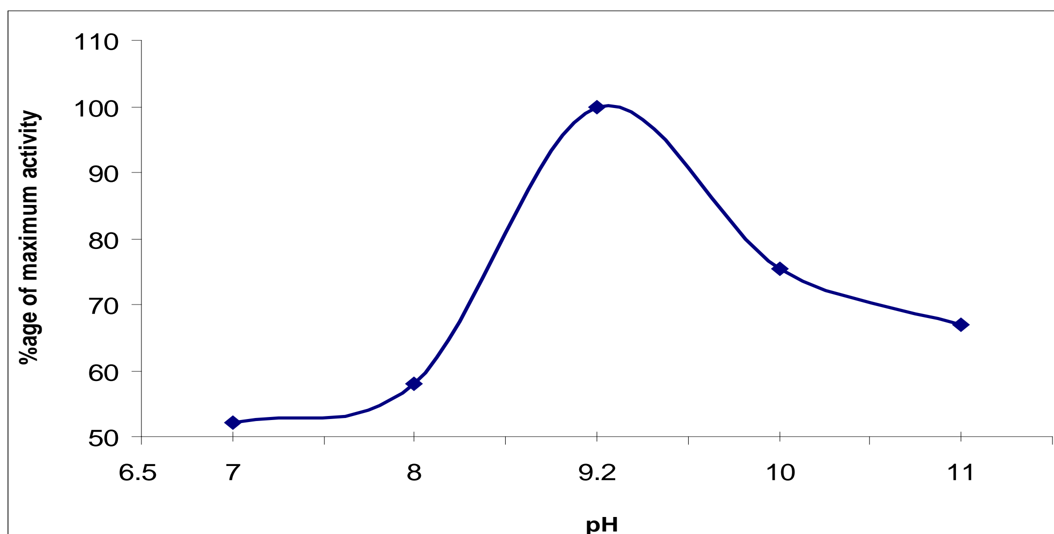

Fig. 4.13 Activity of protease from alkalophilic isolate AP11 at different pH.

4.5.4 pH stability

The pH stability of protease was examined by suitably diluting the enzyme in buffers of different pH values (pH 8, pH 9.2, pH 10, and pH 11) and then incubating the enzymes at room temperature. Aliquots were withdrawn at different time intervals and residual activity was measured under standard assay conditions. The enzyme was highly stable at all pH levels. It could retain 95% of its activity at pH 9.2 after 4 hours. 88% and 82% of the activity was retained after 4 hours even at pH 10 and 11 respectively (Fig. 4.14). The stability of the enzyme in the alkaline range indicates their potential use in detergent formulation. Most of the alkaline proteases have been reported to show pH stability patterns similar to the protease from isolate AP11. Protease from *Bacillus circulans* was stable at pH 10.5-11.5 for 1 hour (Rao *et al*., 2009). Similar pH stability patterns have been reported by alkaline proteases from *Pseudomonas aeruginosa* san-ai strain (Karadzic *et al*., 2004), Bacillus sp. K-30 (Naidu and Devi, 2005), *Virgibacillus pantothenticus* (Thomas *et al*., 2007), *Bacillus subtilis* (Pillai and Archana, 2008), *Bacillus* strain SAL1 (Almas *et al*., 2009), *Paenibacillus tezpurensis* sp. nov. AS-S24-II (Rai *et al*., 2010).

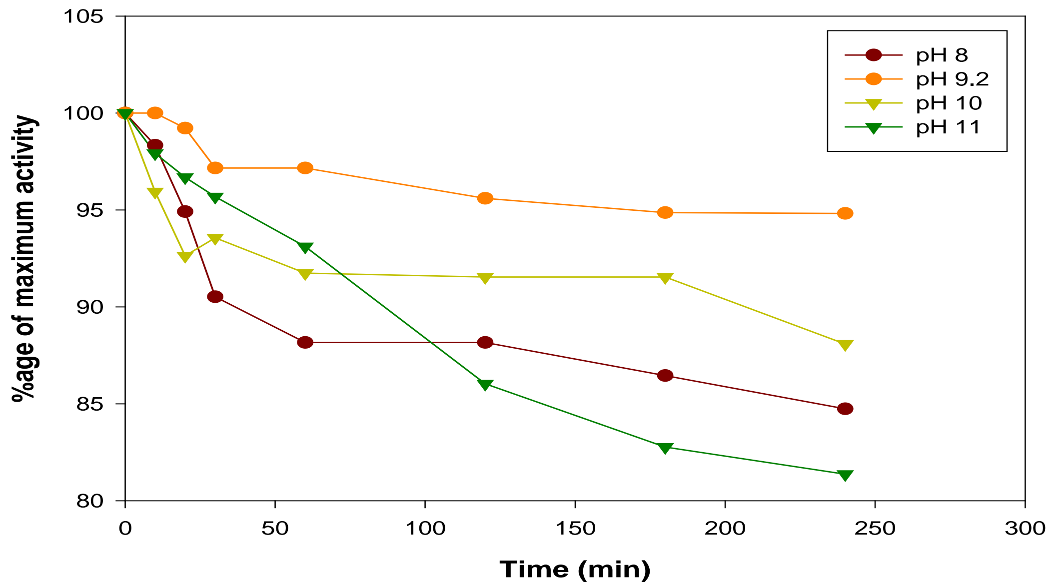

Fig. 4.14 pH stability of protease from alkalophilic isolate AP11 at different pH values

4.5.5 Effect of substrate concentration

The effect of substrate concentration was studied by using varying concentrations of casein (1 mg/ml to 10 mg/ml). A typical hyperbolic curve was obtained in the concentration range of 0.2 mg/ml to 2 mg/ml. A double reciprocal curve was plotted that revealed Km of the enzyme as 2.5 mg/ml of casein (Fig. 4.15). Elsewhere, Thangam and Rajkumar (2002) reported Km value of 1.66 mg/ml for alkaline protease from *Alcaligenes faecalis*, Rao *et al*. (2009) reported Km value of 0.597 mg/ml in *Bacillus circulans*, Madan *et al*. (2002) reported Km value of 3.7 mg/ml in *Bacillus polymyxa*, Kazan *et al*. (2005) reported Km value of 1.8 mg/ml in *Bacillus clausii* GMBAE 42 and Rai *et al*. (2010) reported Km value of protease as 0.227 mg/ml in *Paenibacillus tezpurensis* sp. nov. AS-S24-II all using casein as substrate. This suggests that even with same substrate, alkaline protease of different *Bacillus* sp. show variation in their enzymic rates.

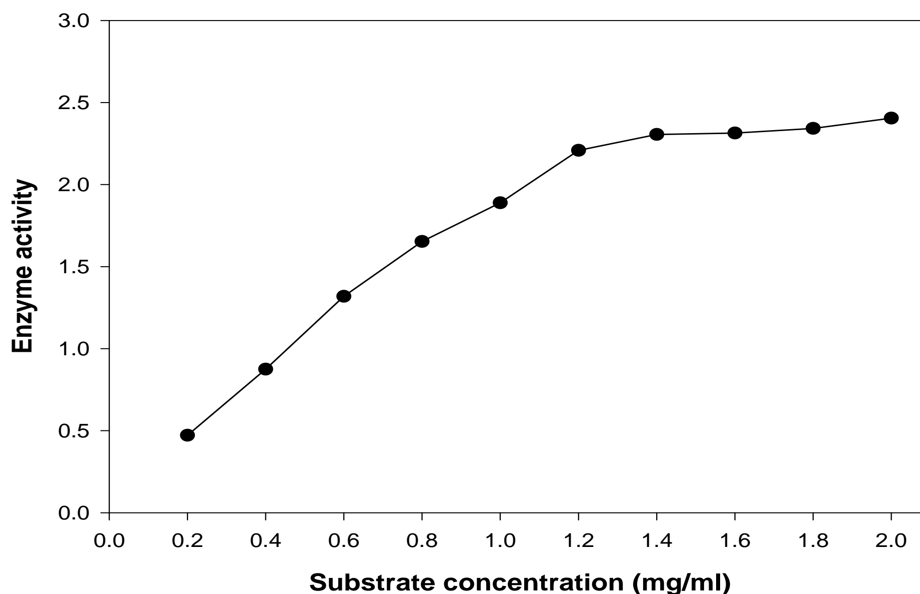

Fig. 4.15 Activity of protease from alkalophilic isolate AP11 at different substrate concentrations

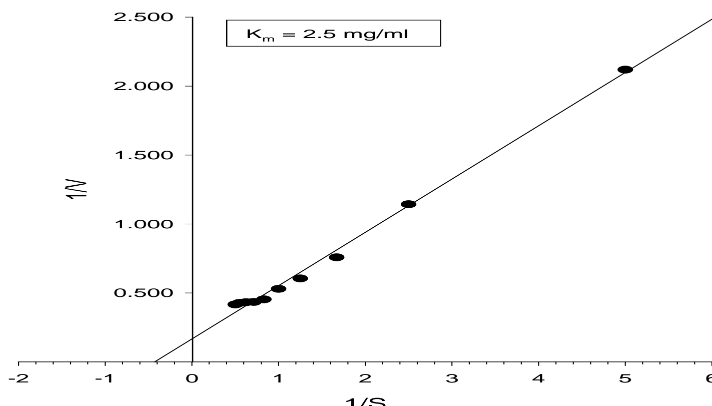

Fig. 4.16 Lineweaver's Burk plot for protease using casein as substrate

4.5.6 Effect of inhibitors

Effect of various inhibitors on protease activity is an important criterion. It has also been used for classifying the proteases (North, 1982). The effect of inhibitors on the activity of protease from isolate AP11 was analyzed. The enzyme was incubated in the presence of phenylmethylsulfonyl fluoride (PMSF) and β-mercaptoethanol at a final concentration of 1 mM, 5 mM and 10 mM. β-mercaptoethanol had no effect on the enzyme activity at all the concentrations. However, the enzyme activity was completely inhibited with PMSF (10 mM). This indicates the existence of a serine residue at the active site. Consequently, the enzyme can be considered as a serine alkaline protease (Table 4.4).

Similar inhibition of protease with PMSF has been reported by Rahman *et al*. (1994), Karadzic *et al*. (2004), Kazan *et al*. (2005), Vidyasagar *et al*. (2006), Hajji *et al*. (2007), Pillai and Archana (2008), Akel *et al*. (2009) and Rai *et al*. (2010).

Table 4.4 Effect of inhibitors on the activity of protease from alkalophilic isolate AP11

Inhibitors	Concentration (mM)	Enzyme activity (Units/ml)	Residual Activity (%)
Control		174.7	100
PMSF	1	166.37	95.23
	5	162.82	93.2
	10	2.88	1.65
β-mercaptoethanol	1	173.77	99.47
	5	180.55	103.35
	10	184.87	105.82

4.5.7 Effect of metal ions

The effect of metal ions on the enzyme activity was analyzed by incubating the enzyme in the presence of different metal ions at a final concentration of 1 mM, 5 mM and 10 mM. Cu^{2+} completely inhibited the

enzyme activity even at a concentration of 1 mM whereas Co^{2+} and Zn^{2+} inhibited the enzyme at higher concentration (10 mM). Mg^{2+}, K^{+} and Na^{+} marginally enhanced the activity (Table 4.5).

Table 4.5 Effect of various metal ions on the activity of protease from alkalophilic isolate AP11

Metal ion	Concentration (mM)	Enzyme activity (Units/ml)	Residual Activity (%)
Control		173.5	100
Fe^{2+}	1	150.65	86.83
	5	146.74	84.58
	10	137.82	79.44
Ca^{2+}	1	171.89	99.07
	5	162.15	93.46
	10	158.09	91.12
Cu^{2+}	1	0	0
	5	0	0
	10	0	0
Mg^{2+}	1	154.05	88.79
	5	188.09	108.41
	10	200.25	115.42
Mn^{2+}	1	188.09	108.41
	5	182.69	105.3
	10	174.31	100.47
Na^{+}	1	159.1	91.7
	5	189.48	109.21
	10	198.28	114.28
K^{+}	1	180.7	104.15
	5	191.89	110.6
	10	188.68	108. 75
Co^{2+}	1	177.49	102.3
	5	145.14	83.87
	10	99.93	57.6
Zn^{2+}	1	176.69	101.84
	5	166.3	95.85
	10	55.97	32.26

Varying effects of metal ions have been reported on the activity of alkaline proteases. Like protease of isolate AP11, Cu^{2+} has been shown to completely inhibit the protease activity in *Halogeometricum borinquense* strain TSS101 (Vidyasagar *et al*., 2006) and *Paenibacillus tezpurensis* sp. nov. AS-S24-II (Rai *et al*., 2010). However, Cu^{2+} has been shown to stimulate the activity in *Bacillus clausii* GMBAE 42 (Kazan *et al*., 2005). Various other ions like Mn^{2+}, Na^{+} and Ca^{2+} have been shown to enhance the protease activity in *Bacillus* strain HUTBS71 (Akel *et al*., 2009).

4.6 Application prospects of alkaline protease of isolate ap11

The alkaline protease of isolate AP11 was tested for its applicability in the detergent industry.

4.6.1 Compatibility with detergents

The high activity and stability of protease from isolate AP11 in the alkaline pH range makes it useful for its application as a detergent additive. To check the compatibility and stability of the alkaline protease towards detergents, the enzyme was diluted in the presence of various commercial laundry detergent solutions (The detergent solution was boiled to inactivate the indigenous enzymes.) and preincubated for 30 min at 30°C and the residual enzyme activity was estimated. The results showed that the enzyme is compatible with most of the detergents and it showed maximum compatibility with Aerial and Rin (Fig. 4.16). This makes alkaline protease from isolate AP11 a potential candidate for use in detergent industry.

The alkaline proteases from other organisms have been reported to show compatibility with different commercial detergents to different levels. Alkaline protease from *Aspergillus niger* has been reported to show 80%-90% compatibility with various detergents. However, contradictory to alkaline protease from isolate AP11, it could retain only 23% of its activity with Aerial (Devi *et al*., 2008). Madan *et al*. (2002) reported compatibility of alkaline protease from *Bacillus polymyxa* in the range of 20%-84.5% with various detergents. Proteases from *Bacillus* sp. APR-4 (Kumar and Bhalla, 2004) and *Paenibacillus tezpurensis* sp. nov. AS-S24-II (Rai *et al*., 2010) have also been reported to be compatible with various detergents.

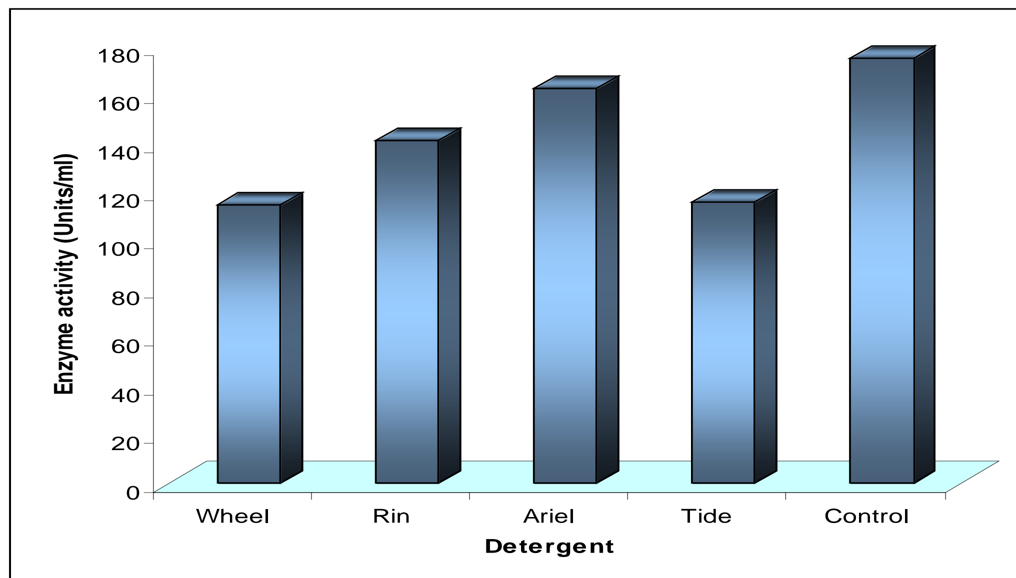

Fig. 4.17 Compatibility of alkaline protease from AP11 with commercial detergents

4.6.2 Destaining properties

To check the use of alkaline protease from isolate AP11 as a laundry additive, its stain removal efficiency was tested. Two pieces each of cotton and muslin cloth artificially stained with blood were dipped in detergent solution without enzyme and detergent solution supplemented with enzyme (The detergent solution was boiled to inactivate the indigenous enzymes.) and incubated for 30 minutes at room temperature. The results (Fig. 4.18) showed complete removal of stain from the cloth pieces dipped in detergent solution supplemented with enzyme whereas bloodstain was not completely removed from the cloth pieces dipped in detergent solution without enzyme.

Similar results were observed with proteases from *Pseudomonas aeruginosa*, *Bacillus subtilis* PE-11 and *Bacillus circulans* were able to remove blood stain from cotton cloth pieces (Adinarayana *et al*., 2003; Najafi *et al*., 2005; Rao *et al*., 2009).

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