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Regular Article

Optimal conditions for production of extracellular alkaline protease from a newly isolated *Bacillus subtilis* strain AKRS3

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A new strain of alkaline protease producing *Bacillus* bacterium was isolated from an alkaline soil sample and was characterized. The bacterium was identified as *Bacillus subtilis* based on 16S rRNA sequencing and phylogenetic tree analysis. The organism was coded as *B. subtilis* AKRS3. The growth curve of the organism was elucidated by culturing in a basal medium in shake flasks under ambient shaking conditions. The optimal conditions for alkaline protease production were studied by following the monofactorial methodology. Enzyme yield was found to be optimum close to 24 hours, which coincided with the commencement of the stationary phase of the bacterium. A pH of 9.0, temperature of 37° C and agitation speed of 125 rpm were also identified to be optimum for enhanced enzyme yields in shake flasks. An inoculum of 4% v/v with 24 hrs of age was also identified to be ideal for inoculation. Xylose at 2 g/l concentration and beef extract at 1.5 g/l concentration were preferred by the organism for optimum enzyme productivity in the broth. The organism displayed an NaCl tolerance of upto 1 % as optimum. Fe^{2+} salts at 0.01 % concentration was the preferred metal component in the medium.

Keywords: *B.subtilis* AKRS3, monofactorial methodology, optimization

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms. There is substantial interest in the study of proteolytic enzymes, not only because of the physiological roles they play, but also due to their commercial value and diverse applications in various industrial sectors. Currently, proteases account for approximately 40 % of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. Microbial proteases are among the most important hydrolytic enzymes which have been studied extensively since the advent of enzymology. Microorganisms

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represent an attractive source of proteases, owing to the distinct advantages they offer over plant and animal proteases (Gupta, 2002). Despite the long list of protease-producing microorganisms, only a few are considered as appropriate producers for commercial exploitation, being 'generally regarded as safe' (GRAS), non-toxic and non-pathogenic. Microbial proteases are classified into various groups, depending on whether they are active under acidic, neutral, or alkaline conditions and on the characteristics of the active site group of the enzyme, i.e. metallo- (EC.3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphydryl- (EC.3.4.22), or serine-type. Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo- type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes exploited commercially. A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of the serine type (Kumar and Takagi, 1999).Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B.licheniformis, B.subtilis, B amyloliqui-faciens,* and *B. mojavensis* (Gupta et al. 2002; Kalisz 1988; Kumar and Takagi 1999; Rao et al. 1998).

The present study is aimed at characterizing a newly isolated extracellular alkaline protease producing bacteria belonging to the *Bacillus* sp. and to optimize the medium and parameters required to attain optimal yield of the enzyme, through submerged fermentation in shake flasks.

Materials and methods

Sample collection, isolation and screening:

Samples were collected from the alkaline soils of Ukkadam fish market, Coimbatore, Tamil Nadu, India. The samples were suspended in sterile distilled water by rigorous vortexing. Appropriate dilutions of the solution were pour plated directly in skim milk agar plates containing peptone (0.1% w/v), NaCl (0.5% w/v), agar (2.0% w/v), and skim milk powder (1% w/v) at pH 10.0. After incubation at 37°C for 24 hours, colonies with visible zones of clearance were picked and subjected to secondary screening by radial(zone) diffusion assay in casein agar plates (Wikström, 1981).

Selection of high yielding strains

The proteolytic bacterial strains were tested for yield of alkaline protease by submerged fermentation in shake flasks. For preparation of inoculum, the bacterial isolates were first grown on nutrient agar slants (pH 8.0) for 24 hours. A loopful of the culture was then transferred to sterile nutrient broth (pH 8.0) and allowed to grow at room temperature (28 ± 2 ^{o}C) for 24 hours in a shaking incubator with ambient agitation (100rpm). The culture with cell concentration adjusted to an optical density (at 600 nm) corresponding to approximately 2 mg DCW/ml was used as the inoculum. 45 ml of a sterile basal medium (PYE medium) containing (per litre): 10 g peptone, 5 g yeast extract, 5 g NaCl, 1 g KH_2PO_4,2 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$.7H2O, 0.01 g $MnSO_4.H_2O$ and 0.01 g $FeSO_4$. $7H_2O$ at pH 8.0 was inoculated with 5 ml inoculum in a 250 ml Erlenmeyer flask and incubated for 48 hours at 37^{o}C with reciprocal shaking at 150 rpm. The cells were then harvested by centrifugation at 10,000 rpm for 15 min, and the supernatant was used for further protease assay (Palavesam et al, 2007).

Protease Assay:

The protease activity was measured as described by Meyers and Ahearn (1976) with some modifications (1977). 0.5 ml of glycine -NaOH buffer (pH 10, 0.2 M) was added to 0.5 ml of appropriately diluted enzyme and was incubated with 1 ml of 1% casein solution (prepared in glycine - NaOH buffer, pH 10) for 15 min at 60 °C. The reaction was stopped by the addition of 4 ml of 5% (v/v) trichloroacetic acid. The contents were centrifuged after 1 h at 3000 x *g* for 10 min and the supernatant was used for measuring protease activity on the basis of color change. 5 ml of 0.4 M sodium carbonate solution was added to 1 ml of the supernatant and was kept for 10 min. Folin's Ciocalteau Phenol reagent of 1:1 dilution (1 N) was added and kept in dark for 30 min. The color change was determined at 660 nm. One unit of protease was defined to be equivalent to the amount of enzyme required to release 1µg/ml/min of tyrosine under standard assay conditions.

Identification of the microorganism:

Various cultural, morphological, physiological and biochemical properties of the highest yielding bacterial isolate among the ones which were subjected to screening and selection, were studied. Identification was done in accordance with the guidelines provided by *Bergey' manual of systematic Bacteriology* (Hensyl, 1989).

Identification at species level was accomplished by amplification and analysis of 16SrRNA of the selected isolate. The bacterium was grown in nutrient broth at room temperature on a rotary shaker with ambient shaking (100 rpm). Genomic DNA from the isolate was extracted by using genomic DNA purification kit. The 16S rRNA genes were amplified from genomic DNA by polymerase chain reaction with two primers of 16SrRNA Forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and 16S rRNA Reverse primer 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The cycle sequencing reaction was performed using Big Dye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (from Applied Biosystems, P/N: 4337457). The sequencing reaction - mix was prepared by adding 1 µl of Big Dye v3.1, 2 µl of 5x sequencing buffer and 1 µl of 50% DMSO. To 4 µl of sequencing reaction mix was added 4 Pico moles of primer (2 µl) and sufficient amount of plasmid. The constituted reaction was denatured at 95°C for 5 minutes. Cycling began with denaturing at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension for 4 minutes at 60°C and cycle repeated for a total 30 cycles in a MWG thermo cycler. The reaction was then purified on sepheadex plate (Edge Biosystems) by centrifugation to remove unbound labeled and unlabelled nucleotides and salts. The purified reaction was loaded on to the 96 capillary ABI 3700 DNA analyzer and electrophoresis was carried out for 4 hours. This 16S rRNA sequencing work was carried out in Synergy Scientific Services, Chennai.

In order to construct the phylogenetic tree, complete 16S rRNA sequences were determined and compared to 16S rRNA sequences with those of other *Bacillus* bacteria. The sequences were subjected to homology search using BLAST programme of the National Center for Biotechnology Information (NCBI). Neighbor joining trees were constructed using EMBOSS (Uyar et al., 2011).

Elucidation of growth curve

The growth pattern of the bacterial isolate was ascertained by culturing the same in a basal medium containing (per litre) 8 g Dextrose (D- Glucose), 10 g Peptone, 0.5 g $MgSO_4$. $7H_2O$, 0.5 g KH_2PO_4 and 0.01 g $FeSO_4.7\ H_2O$ of pH 7.0 at 37°C in a shaking incubator with ambient shaking (100 rpm). Samples were collected at 12 hour intervals and tested for biomass concentration, total

protein content (Lowry et al., 1951) and enzyme activity (Meyers and Ahearn, 1977)

Optimization of fermentation medium and parameters:

Optimization of various physical parameters (incubation period, pH, temperature, agitation speed, inoculum age and inoculum density) and medium constituents (carbon source, nitrogen source, sodium chloride concentration and metal ion requirements) was accomplished through the traditional monofactorial (one factor at a time) approach involving multiple trials in 250 ml Erlenmeyer flasks containing basal medium, filled to 20 % capacity (50 ml).

To study the effect of incubation time on alkaline protease production, submerged fermentation was carried out in shake flasks for varying periods of time (12 hrs- 96 hrs) and the enzyme secreted into the broth was assayed. The effect of agitation speed was assessed by incubating the inoculated medium under static and shaking conditions for the optimal incubation period. The speed of agitation under shaking conditions was varied from 100rpm to 150 rpm. The effect of inoculum age on alkaline protease production was studied by inoculating 45 ml of the basal medium with 5 ml of inoculum containing cells which were grown for varying time periods ranging from 12 hours (overnight) to 48 hours. The effect of inoculum density was ascertained by varying the percentage of inoculum (grown to the optimal age) added to the fermentation medium. The effect of temperature and pH on alkaline protease production was determined by incubating the inoculated medium at varying temperatures (ranging from 32^{o}C to 50^{o}C) and pH (ranging from 6.0 to 12.0).

The medium composition was optimized by studying the effect of various carbon sources (glucose, starch, sucrose, maltose and xylose) and nitrogen sources (beef extract, casein, yeast extract, peptone and soybean meal) at varying concentrations. The effect of changing concentrations (0 to 5 %) of sodium chloride and requirement for low levels (0.1 %) of metal ions (Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+}) was also studied.

Results and discussion

Isolation, screening and selection

Six microbial strains were isolated by using basic microbiological techniques of serial dilution, spread plate and quadrant streak method on skim milk agar plates. Among these, the bacterial isolates were shown to exhibit better proteolytic ability by hydrolysis of casein. A single bacterial isolate that showed the highest extracellular protease activity was selected subsequent to fermentation studies using basal medium in shake flasks.

Identification of the microorganism

One of the high yielding bacterial isolates selected through screening was identified to belong to *Bacillus* sp., based on microbiological and biochemical tests. The microbiological physiological characteristics of selected isolate are shown in Table 1.

Table 1: Microbiological & Biochemical Analysis for identification of Microorganism.

Sl. No.	Test	Result
1.	Gram staining	+
2.	Spore staining	+
3.	Catalase test	-
4.	Hydrolysis of Casein	+
5.	Methyl Red test	-
6.	Indole Production test	-
7.	Voges Proskauer test	-
8.	Citrate Utilization test	-
9.	Nitrate Reduction test	-
10.	Urease test	-

Earlier investigators had isolated and identified *B.subtilis, B.megaterium, B.sphaericus, B.thuringiensis and B.pumilus* (Beyatli and Aslam, 2002), from 6 different

soil samples. This shows that Bacillus genera are widespread among the bacterial types in soil and agrees with the results of this study.

16SrRNA sequencing and analysis

The 16S rRNA gene partial sequences for the isolate were elucidated and deposited in the NCBI- GENBANK with accession number HQ625025.1. The partial sequence of 16S rRNA of the isolated bacterium was aligned and compared with the sequence of other *Bacillus* species to form the similarity matrix. A distance-based neighbor-joining tree was constructed with the Gamma proteobacteria-related sequences from this study and reference sequences from the GenBank database (Uyar et al., 2011). Calculated by number of base differences, the highest level of similarity observed was with that of *Bacillus subtilis*. Accordingly, the isolated strain was coded as *B. subtilis* AKRS 3.

Growth curve elucidation

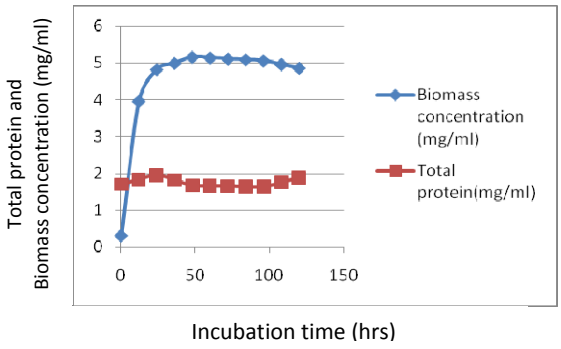

Fig- 1: Growth curve elucidation

The growth curve of the organism did not exhibit a lag phase over the chosen interval. The log phase was found to extent up to 24 hours. The stationary phase was found to extent from approximately 24 hours to 96 hours. The biomass concentration was found to decline beyond 96 hours. The total protein content was found to increase up to 24 hours and subsequently declined further beyond.

Optimization of fermentation medium and parameters

Effect of incubation time

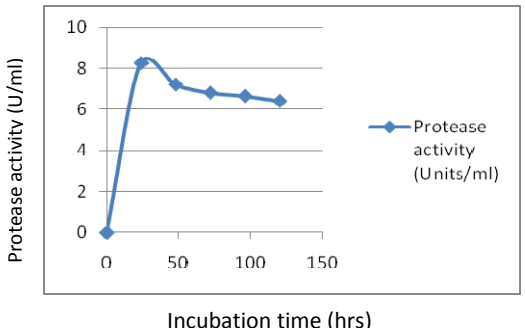

Fig- 2: Effect of Incubation time

The enzyme activity was found to be the maximum between24 to 48 hours. This corresponds to the transition of microbial growth curve from log phase to stationary phase. The enzyme activity was found to decline beyond 24 hours, presumably due to normal effects such as enhanced sporulation and accumulation of toxic inhibitory metabolites in the medium during the stationary phase of growth in *Bacillus* sp. The optimal incubation period for other *Bacillus* bacteria range from 6 to 8 hrs in case of *B. subtilis* 168 (Longo et al., 1999), 18 hours for *Bacillus* sp. SSR- 1 (Singh et al., 2001), 24 hours for *B.mojavensis* (Beg et al., 2002), 48 hrs for *B. licheniformis* ATCC 21415 (Mabrouk et al., 1999), 60 to 72 hrs for *Bacillus* sp. K2 (Hameed et al., 1999) and 72 to 96 hrs (Oberoi et al, 2001; Puri et al. 2002)

Effect of pH

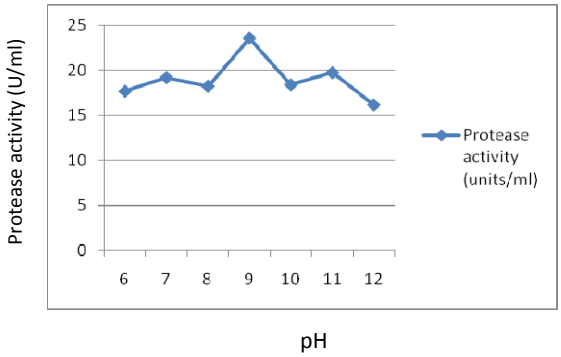

Fig- 3: Effect of pH

The enzyme activity in the culture supernatant was found to increase with increasing pH up to an optimum of 9.0, beyond which the activity was found to decline. This could be indicative of the alkalophilic nature of the microorganism. Hence, in all further studies the pH of the medium was adjusted to 9.0. The optimal pH for other *Bacillus* bacteria reported in literature are pH 7.0 for *Bacillus* sp. K2 (Hameed et al., 1999), *Bacillus* sp. RGR- 14 (Oberoi et al., 2001; Puri et al.,2002), *B. licheniformis* ATCC 21415 (Mabrouk et al, 1999) and *B. mojavensis* (Beg et al., 2002), pH 9.5 – 9.6 for *Bacillus* sp. P-2 (Kaur et al., 2001) and *B. pumilis* MK6-5 (Kumar, 2002) and pH 10.0 – 10.5 for *Bacillus* sp. JB99 (Johnvesly and Naik, 2001), *Bacillus* sp. IS- 3 (Purva et al., 1998), *Bacillus* sp. SSR-1 (Singh et al, 2001) and *Bacillus brevis* MTCC B0016 (Banerjee et al, 1998).

Effect of Temperature

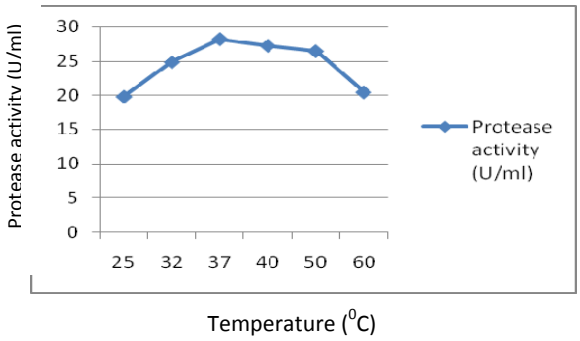

Fig- 4: Effect of Temperature

Enzyme activity was found to increase with increase in incubation temperature upto 37°C and then declined further beyond. Thus, the optimum temperature for alkaline protease production by the bacterium was determined to be 37°C.In literature, optimum temperature was reported to be 30°C for *B. sphaericus* (Singh et al, 2001), 35°C for *B. pumilis* MK6-5 (Kumar, 2002), 37°C for *Bacillus* sp. K2 (Hameed et al., 1999), 40°C for *Bacillus* sp. SSR- 1 (Singh et al., 2001), 50°C for *B. mojavensis* (Beg et al., 2002) and 55°C for *Bacillus* sp. JB99 (Johnvesly and Naik, 2001).

Effect of agitation speed

Agitation influences the availability of nutrients for growth and product formation by the microorganism. Levels of agitation also influence the pH and dissolved oxygen concentration in the broth, which in turn influences the yield of enzyme. Under static conditions, the enzyme activity was substantially low. The protease activity was found to increase up to a speed of 125 rpm and eventually started declining. Thus an agitation sped of 125 rpm was considered optimum for fermentation.

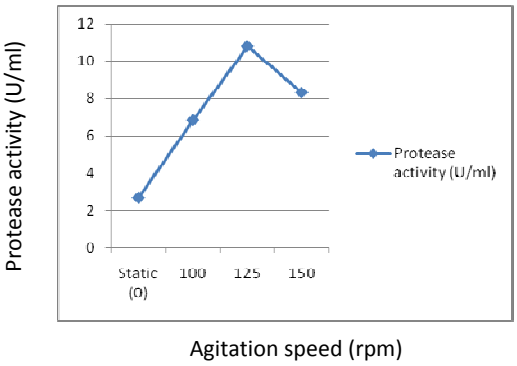

Fig- 5: Effect of Agitation speed

Effect of inoculum age

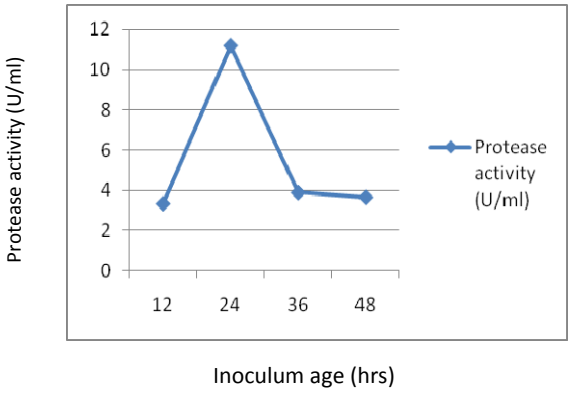

Fig- 6: Effect of Inoculum age

The age of the inoculum influences the pattern of growth and product formation by the microorganism. An inoculum age of 24 hours was found to be optimum for alkaline protease production by the present bacterial isolate.

Effect of inoculum density

The cell density of the culture used to inoculate the fermentation medium determines the duration of the initial growth stages. A very low inoculum density could imply extended lag and log phases, leading to delay in attainment of stationary phase, hence adversely affecting the production of secondary metabolites (like extracellular enzymes). However, a very high inoculum density could also lead to growth inhibition due to presence of excessive concentrations of toxic, inhibitory metabolic substances. Thus a moderate inoculum density of the order of 4 % was found to be sufficient for optimal enzyme production by the microorganism.

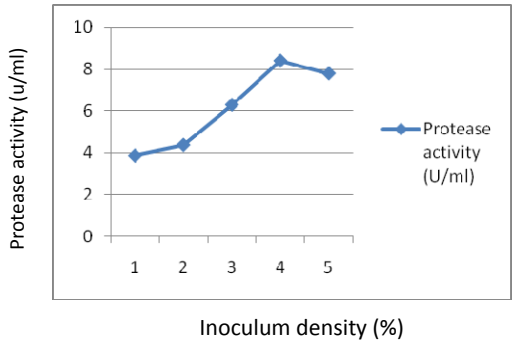

Fig- 7: Effect of Inoculum density

Effect of carbon source

Production of protease is highly dependent on the both carbon and nitrogen source available in the medium. Among the 8 carbon sources tested, the optimal enzyme activity was attained with Xylose. A lower enzyme yield with other easily metabolizable sugars is a possible indication of catabolite repression effects. Xylose being a less easily metabolized carbon source, presents negligible repression on product formation and hence, the organism utilizes the protein source provided in the medium to yield a high concentration of enzyme at the end of optimal incubation period. Further studies indicated that addition of xylose at an initial concentration of 2 g/l is desirable for optimum enzyme activity. Reports from literature on preferred/optimized carbon sources for various *Bacillus* bacteria include glucose for *Bacillus* sp. IS- 3 (Purva et al., 1998), *B.mojavensis*(Beg et al., 2002) and *B. sphaericus*(Singh et al., 2001), citric acid for *Bacillus* sp. JB99 (Johnvesly and Naik, 2001), glycerol for *Bacillus* sp. K2 (Hameed et al., 1999), starch for *Bacillus* sp. RGR- 14 (Oberoi et al., 2001; Puri et al., 2002) and lactose for *B. brevis* MTCC B0016 (Banerjee et al., 1999).

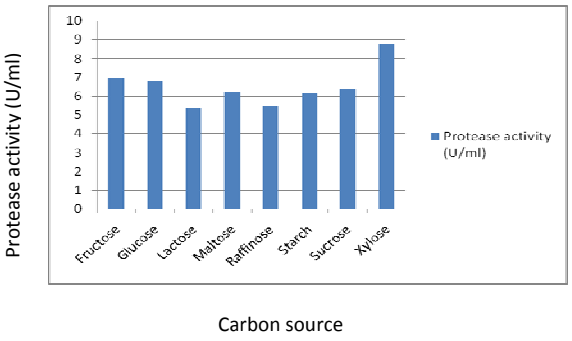

Fig- 8: Effect of Carbon source

Effect of nitrogen source

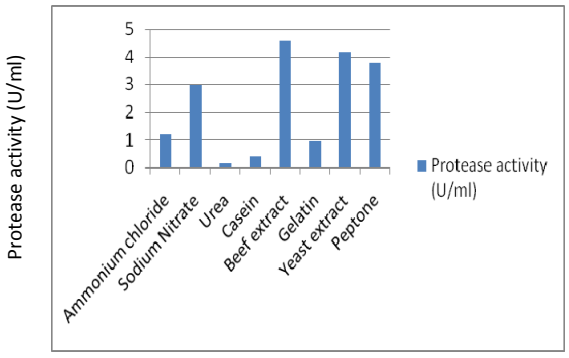

Fig- 9: Effect of Nitrogen source

It is well documented in the literature that nitrogen is metabolized to produce primarily amino acid, nucleic acid, protein and cell wall components. These nitrogen sources have regulatory effect on the enzyme. Comparative fermentation trials with 8 different organic and inorganic nitrogen sources indicated that organic nitrogen sources are more suitable over inorganic sources for enzyme production. Among the inorganic nitrogen sources, the highest enzyme activity was attained with beef extract at an initial concentration of 1.5 g/l. Reports suggest the choice of various nitrogen sources like soybean meal for *Bacillus* sp. IS- 3 (Purva et al., 1998), Potassium nitrate for *Bacillus* sp. JB99 (Johnvesly and Naik, 2001), Peptone and yeast extract for *Bacillus* sp. P-2 (Kaur et al., 2001), Casein or casaminoacids for *B. mojavensis* (Beg et al., 2002), biopeptone and yeast extract for *B. sphaericus* (Singh et al., 2001), casein hydrolyzate and gelatin for *Bacillus* sp. K2 (Hameed et al., 1999) as optimum.

Effect of NaCl

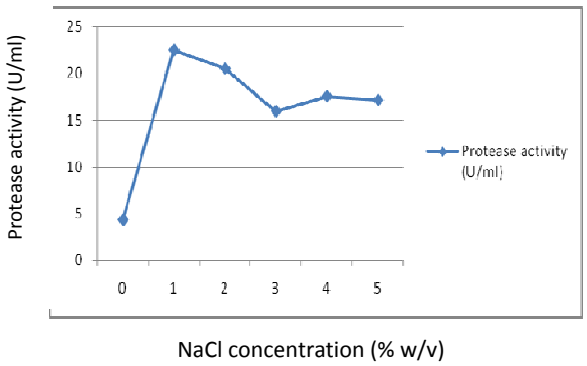

Fig- 10: Effect of NaCl

The influence of NaCl on enzyme productivity was determined, since the source from which the organism was isolated was saline in nature. An NaCl concentration of 1 % w/v was found to be ideal for optimum enzyme yield. Earlier reports suggest that NaCl at 2 % concentration was found to be tolerated by *Bacillus firmus* MTCC 7728 (Rao and Narasu, 2007)

Effect of metal ions

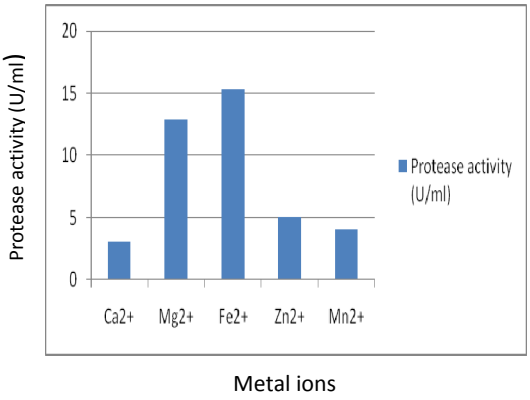

Fig- 11: Effect of metal ions

Metal ions serve as cofactors required for activity of various enzymes which are critical for growth and product formation. Among the various metal ions tested, Fe^{2+} was found to be most essential for optimal enzyme production in the present case. It has been reported that Ca^{2+} and Mg^{2+} ions had effects on protease production by *Bacillus pumilus* (Feng et al., 2001). Nevertheless, inhibitory effect of Cu^{2+} and Li^{2+} on proteases has also been suggested for *Bacillus subtilis*PE-11(Adinarayana et al., 2003).

Conclusions

A total of 6 isolates were obtained from the soil sample used for the study, of which the high yielding bacterial isolate belonging to the *Bacillus* sp. was selected and characterized. The bacterium was identified to be *Bacillus subtilis* based on 16S rRNA sequencing and analysis. The organism was coded as *B. subtilis* AKRS 3 and subsequently studied for analyzing the effects of fermentation parameters and medium composition on yield of alkaline protease. The growth curve of the organism grown in basal medium showed that the stationary

phase of growth commenced close to 24 hours, which was also the incubation time for optimum enzyme activity in the culture supernatant. A pH of 9.0, temperature of 37 ^{0}C and agitation speed of 125 rpm was also identified to be optimum for enhanced enzyme yields in shake flasks. An inoculum of 4% v/v with 24 hrs of age was also identified to be ideal for inoculation. Xylose at 2 g/l concentration and Beef extract at 1.5 g/l concentration were preferred by the organism for optimum enzyme productivity in the broth. The organism displayed an NaCl tolerance of upto 1 %as optimum. Fe^{2+} salts at 0.01 % concentration was the preferred metal component in the medium.

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