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Isolation and optimization of alkaline protease producing Bacteria from undisturbed soil of NE-region of India falling under Indo-Burma biodiversity hotspots

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ABSTRACT

A total of 28,000 bacterial isolates were encountered during 2008-2011 from north eastern region of India, which is falling under Indo-Burma biodiversity hot-spots and were preserved in glycerol stock solution at -80°C in deep freezer. 19,834 isolates were biochemically characterized and screened for protease activity in culture conditions further 1,579 isolates were found to be good amount of protease producing bacteria where, the zone of inhibition was observed in the range between 20-45 mm on skim milk agar medium at different pH range i.e. 4.0, 7.0 and 10.0. Out of 1,579 protease producing bacteria, 425 isolates produced protease activity at acidic pH-4.0, 547 bacteria at alkaline pH-10.0 and 607 bacteria at neutral pH-7.0. Compared to acidic and neutral pH, the highest proteolytic activity was exhibited by the isolates which were grown in alkaline medium. In this study, 5 isolates forming larger zone as a result of casein hydrolysis were further studied for quantitative production of extracellular alkaline protease activity. Isolate no. B-2 was observed as a highest potential protease producer bacterium. Upon 16S rRNA analysis, it displayed maximum similarity with *Bacillus* sp. and the sequences were deposited in GenBank database. Different cultural parameters like effect of pH, temperature, time and inoculum sizes were optimized for maximal enzyme production. Maximum yield of enzyme was obtained at a pH of 10.0 with 1 ml of inoculum in the medium after 48 hours of incubation and maintained at a temperature of 37ºC. The present investigation indicates the potential use of these microorganisms as biotechnological tools for various industrial activities.

1. INTRODUCTION

Proteases are one of the most important classes of enzymes, occupying a major share of 60% of total enzyme market [1]. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms and are essential for cell growth and differentiation. Proteases represent one of the three largest groups of industrially important enzymes [2]. The majority of commercial alkaline proteases are produced by bacteria, especially *Bacillus* sp. [3]. Since the first alkaline protease from *Bacillus licheniformis* was commercialized as an additive in detergents in 1960s [4], a number of *Bacillus* derived alkaline proteases have been purified and characterized because of their significant proteolytic activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification and low cost [5,6]. Proteases have variety of functions and important pharmaceutical biotechnological applications [7, 8] like, meat tenderization process [9, 10] infant formula preparation (American Academy of Pediatrics Committee on Nutrition, 1998), leather processing [11], diagnostic waste management and silver recovery [12]. Among different types (acidic, neutral and alkaline) of proteases, alkaline protease play important role as most commonly used industrial enzyme in view of their activity and stability at alkaline pH. Alkaline protease has major application in detergent industry because the pH of laundry detergents remains generally in the range of 9-12. The performance of detergent protease is also influenced by several other factors such as washing temperature and detergent composition. Enzymes from microorganisms that can survive under extreme pH may be useful for commercial applications under highly alkaline reaction conditions, e.g., in the production of detergents [13]. The detergent industry is the largest single market for this enzyme. The enzyme has better resistance to alkali and some other denaturing chemicals in the reaction mixture and has a higher affinity towards proteinaceous substrates. Alkaline proteases produced by *Bacillus pseudofirmus*, *Cohnella thermotolerans* and *Bacillus odysseyi* are of great importance in detergent industry due to their high thermo-

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stability and pH stability which is the most important industrial enzymes accounting for about 60% of total enzyme market [14]. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals [15]. They are commercially important and isolated from various living sources such as plants, animals, bacteria and fungi [16]. With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as clinical, medicinal and analytical chemistry. To meet the current largely increase demand, studies on cost effective production of industrially important enzymes have become the need of today. Proteases from microbial sources are preferred over the enzymes from plant or animal sources since they possess all most all the characteristics desired for their biotechnological applications [17]. The yield of extracellular enzyme is influenced by the physiochemical conditions. Hence, physiochemical parameters are optimized for the maximum production of protease. Therefore, attempt was made to isolate a *Bacillus* species from undisturbed ecosystems which can produce good quality of proteases useful in the detergent and leather industry. A 16S rRNA gene sequence analysis was made for identification of the isolated species of *Bacillus*. This study presents effect of different cultural conditions on production of protease from bacteria isolated from soil.

2. MATERIALS AND METHODS

2.1 Isolation and screening of alkaline protease producing bacteria

A total of 28,000 bacterial isolates were encountered from north eastern region of India, (87°32′ E-97°52′ E longitude and 21°34′ N-29°50′ N latitude), falling under Indo-Burma biodiversity hot-spots and were preserved in glycerol stock solution at -80°C in deep freezer. The isolates were plated on nutrient agar (HiMedia, India) medium and again inoculated to skim milk medium. The inoculated plates were then incubated at 37°C in BOD (Biological Oxygen Demand) incubator (ORBITEK BOD350L, Scigenics Biotech, India) for 24 hours for optimum growth. Cultures were grown on nutrient agar plates and pre-screened by inoculation in skim milk agar (HiMedia, India) medium of different pH range i.e. 4.0, 7.0 and 10.0 and incubated at 37°C for 72 hours to observe the zone of proteolysis which indicates proteolytic activity. Amongst the protease producing bacteria, 425 isolates produced protease activity at acidic pH-4.0, 547 bacteria at alkaline pH-10.0 and 607 bacteria at neutral pH-7.0. The highest proteolytic activity was exhibited by the isolates which were grown in alkaline medium were selected for further studies. Out of 547 isolates from pH-10.0, 5 isolates were used in present study for thorough investigation as it showed most prominent zone around the colony. Hydrolysis is expressed as diameter of clear zone in millimeter (mm). The bacterial isolates with prominent zones of proteolysis were further processed. The most potent protease positive 5 strains were selected for quantitative test of protease activity.

2.2 Molecular characterization

2.2.1 Isolation of genomic DNA and amplification by 16S rRNA

For the molecular characterization, genomic DNA was isolated by Scott Newman's method [18]. 16S rRNA sequence was amplified from genomic DNA and was amplified using universal primer (Integrated DNA Technology, India) 536F 5′-GTGCCAGCAGCCCGGGTR ATA-3′ and 1488R 5′-GGTTACCTTGTTACGACTTCACC-3′ [19]. For the polymerase chain reaction (PCR), a total of 50 µl of PCR reaction mixture was prepared having 5 µl of 1X *Taq* buffer, 5 µl of 200 µM of each deoxynucleotides, 1.5 µl of 0.3 µM of each forward and reverse primer, 0.25 µl of 5U *Taq* DNA polymerase and 2 µl of genomic DNA extract. Amplification of DNA for 50 µl reaction was carried out under the following condition. The PCR condition was set for 28 cycles with initial denaturation at 95°C for 5 minutes then final denaturation of 95°C for 1 minute, annealing at 55ºC for 1 minute and final extension at 72ºC for 2 minutes using Applied Biosystem Veriti 96 well Thermal cycler. Amplified PCR product was detected with standard agarose gel electrophoresis (Elchrom Scientific GEPS 200/2000, Switzerland) and quantification of PCR product was done with spectrophotometer (Mecasys optizen pop UV/VIS Spectrophotometer, South Korea). Sequencing of the quantified 16S rRNA PCR product was conducted at National Centre for Cell Science (NCCS), Pune, India. The sequences were analyzed using the gapped BLASTN (http://www.ncbi.nlm.nih.gov) search algorithm for correct identification of the bacteria and the sequences obtained in this study were deposited in the GenBank database.

2.3 Inoculum preparation

A loopful of culture was inoculated into 10 ml of Luria Bertani broth medium (HiMedia, India) and incubated at 37°C for 24 hours. After this, 100 µl of the bacterial culture was inoculated to 20 ml of production medium (production medium composed of glucose 0.5 gm, peptone 0.75 gm, $MgSO_4$ 0.5 gm, KH_2PO_4 0.5 gm, $FeSO_4$ 0.01 gm in 500 ml distilled water) and kept in a refrigerated incubator shaker (C24KC, New Brunswick Scientific Classic Series, NJ, USA) at 140 rpm for 72 hours at 37°C. At the end of the fermentation process, the fermented broth was centrifuged at 10,000 rpm in a centrifuge (5430R, Eppendorf, Germany) for 10 minutes and the supernatant was separated and used as crude enzyme source for the assay of protease production.

2.4 Protease enzymatic assay

Protease assay was determined by Caseinolytic method [20]. Protease activity was assayed by using 2 ml of 2% casein in 0.65 ml of alkaline phosphate buffer. Casein solution was incubated with 0.5 ml of properly diluted enzyme at 37°C for 30 minutes. After 30 minutes, 200 µl of NaCl was added and then the reaction was terminated by addition of 5 ml of 5% TCA. The solution mixture was centrifuged at 10,000 rpm for 10 minutes. After centrifugation 0.1 ml of the sample was taken and the volume was made up to 1 ml with distilled water. And 5 ml of solution-C (Solution A- In 50 ml distilled water, 0.2 gm NaOH

was dissolved and 1 gm of Na_2CO_3 was added, Solution B- 0.5% $CuSO_4$ in 1% sodium potassium tartarate, Solution C- mixed 50 ml of solution A + 1 ml of solution B) was added and incubated at room temperature for 10 minutes. These were preceded by the addition of 0.5 ml Folin Ciocalteu's reagent and incubated in dark condition for 30 minutes. The colour developed was read at 660 nm against a reagent blank prepared in the same manner. One unit of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1 µg tyrosine/ml/minute from casein under specified assay conditions.

2.5 Process optimization for maximum protease production:

2.5.1 Optimization of enzyme production at different pH

Flasks with broth containing the optimum concentration of substrate and carbon source were taken and the pH of the broth was adjusted to 7.0, 8.0, 9.0, 10.0 and 11.0 in different flasks using 1N NaOH and sterilized. The cultures were inoculated and incubated at 37°C. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source.

2.5.2 Optimization of enzyme production at different temperature

Production medium at pH 7.0 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures of 30, 35, 40, 45 and 50ºC for 24 hours. At the end of incubation period, the cell free culture filtrate is obtained and used as enzyme source.

2.5.3 Optimization of enzyme production at different time period

Production medium at pH 7.0 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different time period of 24, 48, 72, 96 and 120 hours. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source.

2.5.4 Optimization of enzyme production with different Inoculum size

Production medium at pH 7.0 was inoculated with overnight grown selected bacterial strain. The broth was incubated with different volume of strain i.e. 0.1 ml, 0.5 ml, 1 ml, 1.5 ml and 2 ml. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source.

3. RESULTS AND DISCUSSION

3.1 Isolation and screening

A total of 28,000 single isolated colonies were inoculated on nutrient agar medium and pure culture of bacterial colonies were obtained by repeated streaking after which transferred to alkaline skim milk plate for screening of protease producing bacteria. Screening of protease producing microorganism usually involves growth on the medium that contain protein as a selective substrate and in the present study skim milk was used as the substrate. Following inoculation and incubation of the agar plate, organisms secreting proteases exhibited a zone of proteolysis which was demonstrated by clear area surrounding the microorganism's growth. Out of the 547 isolates of alkaline proteases, 5 bacterial colonies which developed a translucent zone on alkaline skim milk agar and which showed highest proteolytic activity were selected for further investigation and were designated as B-1, B-2, B-3, B-4 and B-5. The protease producing strains with 25-40 mm diameter of proteolytic zone were characterized and further processed for detailed studies. Among protease producing isolates, B-2 exhibited best proteolytic ability by distinctly forming clear zone around the colonies. The zone formation around the bacterial colony indicated the protease positive isolates which was due to hydrolysis of casein as shown in Fig 1. Optimization of the culture condition is necessary in the selection of the bacterial source for industrial exploitation of their extracellular enzymes. In the present investigation, skim milk powder was used as the selective substrate, in which the isolated organisms were streaked. All the 5 bacterial isolates produced clear zone in the medium and hence was confirmation of protease production. All the positive bacterial cultures were further analyzed for protease production by fermentation process.

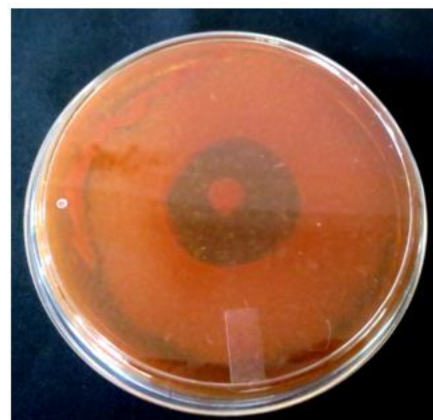

Fig. 1: Zone of casein hydrolysis by B-2 on Alkaline skim milk agar plate.

3.2 Molecular characterization and identification of bacterial isolates

Good quality genomic DNA was isolated without any signs of smearing when observed in gel documentation system (Vilber Lourmat, France). The yield of genomic DNA varied from 60 to 140 ng/µl. PCR amplification of ribosomal DNA was carried out with universal forward and reverse primers of 16S rRNA and produced a fragment of approximately 800-1000 bp (Fig 2). This size corresponded to the expected size as compared to the other bacteria as earlier reported [21].

Systematic study coupled with molecular study is well established to reveal the true identity of the organisms. Since the organisms were potentially identified by 16S rRNA method, the partial 16S rRNA sequences of all alkaline protease producing bacteria were compared with other strains of bacteria from the similarity matrix, calculated by the number of base differences, depending upon the highest level of similarity (Table 3). Many workers have characterized and identified novel strain by 16S

rDNA analysis [22, 23]. Although 16S rDNA does not give authentic identification and differentiation of closely related *Bacillus* sp. such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus* etc., it can be used as preliminary relatedness. The need for developing a tool for identifying *Bacillus* sp. arose due to two reasons: (i) more than 50% of the 16S rDNA sequences deposited in the databases were annotated and identified only as *Bacillus* sp. (ii) phylogenetic tree construction [24]. The amplified PCR product of representatives isolates were identified and sequenced. A BLASTN analyses were carried out through GenBank (http://www.ncbi.nlm.nih.gov) revealed that all the isolates were member of same genera which belong to the group *Bacillus* sp. to which accession number mentioned in Table 2. The 16S rRNA analysis of all the bacterial isolates demonstrated 100% sequence similarity with the blast hit as mentioned in Table 3. The sequence length (bp), G+C% content, A+T% content and Tm (melting temperature) of all the bacterial isolates is indicated in Table 2.

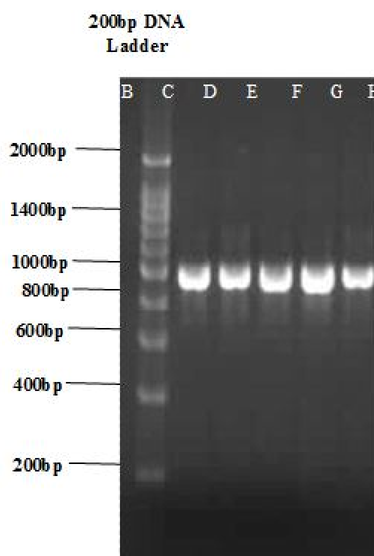

Fig. 2: 16S rDNA-PCR amplification of alkaline protease producing bacteria. A= B-1; B= B-2; C=B-3; D=B-4; E=B-5

3.3 Nucleotide sequence accession number

The partial 16S rRNA sequences for strains B-1, B-2, B-3, B-4 and B-5 are deposited in GenBank under accession numbers KJ562181, KM010238, KM010239, KM010243 and KM588107 respectively.

3.4 Enzyme activity

The objective of the present investigation was to select the bacterial strains with alkaline protease producing ability. Without optimization when the organisms grown in the production medium (pH 7.0) at 37ºC for 48 hours in a shaker, the bacterial isolate, B-2 produced 68.05 IU/ml enzyme as shown in Table 1 and Fig 3. 5 bacterial isolates in alkaline medium were checked for quantitative test of extracellular protease in liquid medium and were found to produce proteases at varying levels from 50.26 IU/ml to 75.51 IU/ml at alkaline pH. Several investigations have been done for screening new isolates for protease production. Other works reported that *Bacillus anthracis* S-44 and *Bacillus cereus* S-98 exhibited their maximum ability to biosynthesize proteases within 60 hours incubation period since the productivity reached up to126.09 units/ml for *Bacillus anthracis* S-44 and 240.45 units/ml for *Bacillus cereus* S-98 [25]. Several investigations done in alkaline protease enzyme production by *Bacillus* sp. has been previously reported 26, 27, 28, 29, 30.

Table 1: Quantitative Enzymatic Assay for alkaline Protease.

Strain code	Protease IU/ml
B-1	61.75
B-2	68.05
B-3	60.21
B-4	63.40
B-5	65.39

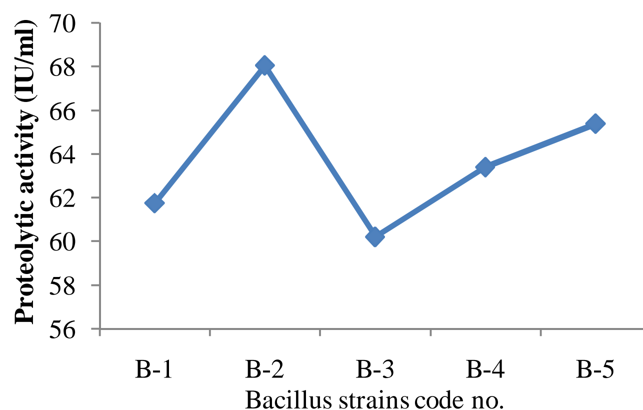

Fig. 3: *Bacillus* strains showing protease activity in alkaline medium.

3.4.1 Effect of pH on protease production

From the pH optimization study, shown in Fig 4, it was clearly observed that B-2 was able to produce 75.51 IU/ml at pH 10.0. It was observed that there was gradual increased in protease production from pH 7.0-10.0, subsequently the production capacity was shown a downward trend after pH 10.0. However majority of microorganisms produced alkaline proteases show growth and enzyme production under alkaline condition [31, 32, 33].

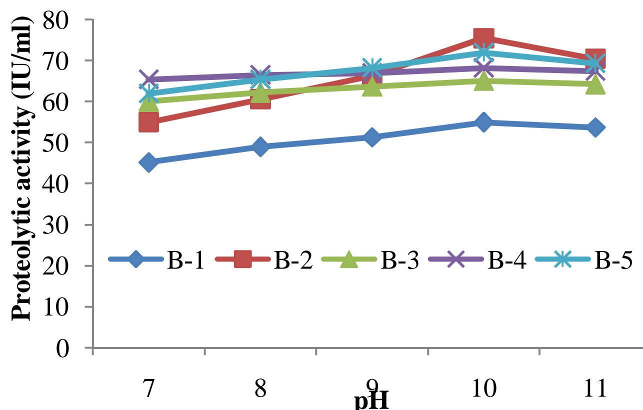

Fig. 4. Effect of pH on protease production of 05 strains.

Table 2: Identification and sequence details of bacterial species on the basis of 16S rRNA gene sequence similarity.

Strain code	GenBank Accession no.	Bacterial name	Sequence length (bp)	G+C % content	A+T % content	Tm
B-1	KJ562181	*Bacillus* sp.	749	53.1	46.9	80.8
B-2	KM010238	*Bacillus* sp.	649	53.6	46.4	80.8
B-3	KM010239	*Bacillus* sp.	1507	55.1	44.9	82.1
B-4	KM010243	*Bacillus* sp.	844	53.2	46.8	80.9
B-5	KM588107	*Bacillus* sp.	717	53.1	46.9	80.7

Table 3: Sequence identities of bacterial strains based on nucleotide–nucleotide BLAST (BLASTN) of the NCBI.

Bacterial strain	Query coverage (%)	Score (%)	Identity (%)	E-value	99% Similarity/blast hit
B-1	100	1384	100	0.0	KJ562181.1
B-2	100	1199	100	0.0	KM010238.1
B-3	100	2784	100	0.0	KM010239.1
B-4	100	1559	100	0.0	KM010243.1
B-5	100	1325	100	0.0	KM010244.1

3.4.2 Effect of incubation temperature on protease production

Enzyme activity recorded at different temperatures revealed that between a range of 30ºC-50ºC the enzyme production was found to be maximum at 40ºC (Fig 5), which shows that the isolate was temperature dependent for the enzyme production. In another study, it was reported that protease production by *Bacillus laterosporous* was best at 40ºC [34]. The present finding recorded 40°C as optimum temperature, which is in agreement with earlier findings of VijayAnand et al. [35] and Fulzele et al. [36], optimum temperature for protease was 40°C. El-Safey and Abdul-Raouf [37] also reported the same findings for protease enzyme from *Bacillus subtilis*. In earlier studies of Rahman et al. [38], they pointed out that temperature influences the secretion of extracellular enzyme possibly by changing the physical properties of the cell membrane.

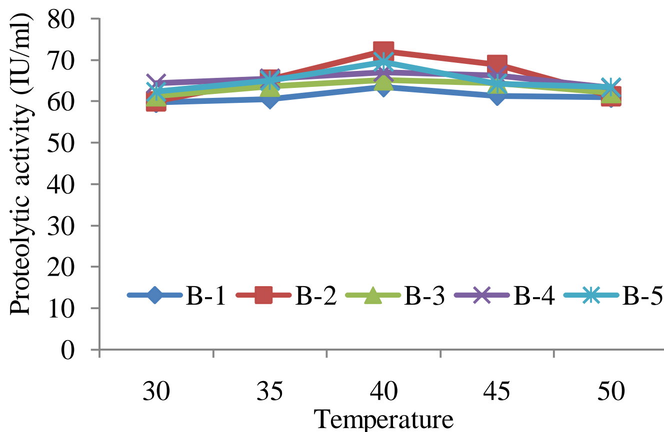

Fig. 5: Effect of temperature on protease production of 05 strains.

3.4.3 Effect of time period on protease production

Enzyme synthesis is related to cell growth and therefore there is a co-relation between incubation period and enzyme production [39]. The graph of protease production proceeded gradually reaching a maximum value at 48 hours for all the isolates (Fig 6). Under most growth conditions, *Bacillus* species produce extracellular protease during the post exponential growth phase [40]. Further incubation resulted in a gradual decline in the enzyme production. This decline might be due to cessation of enzyme synthesis together with auto proteolysis. Similar findings were also reported by previous workers [41] in which maximum enzyme production were observed at 48 hours of growth.

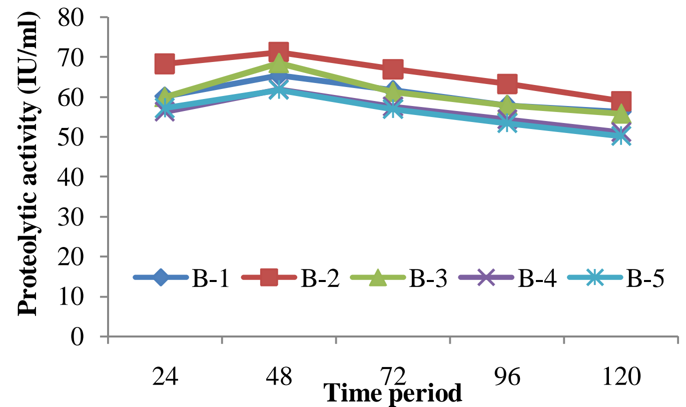

Fig. 6: Effect of time period on protease production of 05 strains.

3.4.4 Effect of inoculum sizes on protease production

Results from the present study showed that optimum inoculum size of the bacterial isolate for protease production was 1 ml from overnight culture broth (Fig 7).

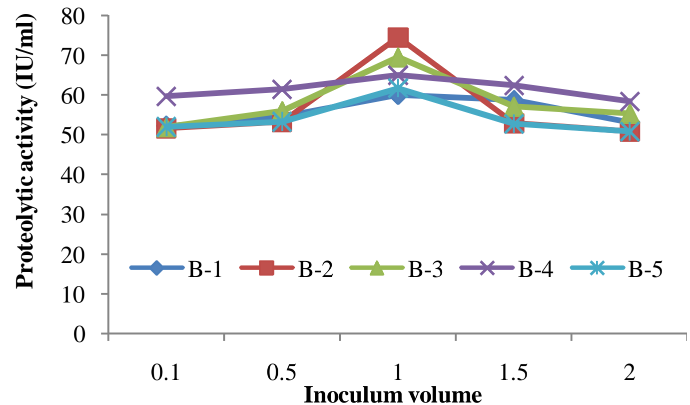

Fig.7: Effect of inoculum volume on protease production of 05 strains.

The less protease production in small inoculum sizes of 0.1 ml and 0.6 ml may be due to insufficient number of bacteria, which would have lead to reduced amount of secreted protease and the decrease even though luxurious growth was observed in higher inoculum size of 2.0 ml, may have resulted due to reduce dissolved oxygen

and increased competition towards nutrients. A similar observation has been reported by some authors [37], where an optimum inoculum size was 1 ml from stock suspension for maximum protease.

4. CONCLUSIONS

Several bacterial strains secreting alkaline proteases were screened and isolated from soil. *Bacillus* strains exhibited prominent clear zones around the colonies on alkaline skim milk agar plates indicating that it secretes significant amount of proteases, and this organism was selected for further optimization of the extracellular protease production. *Bacillus* strains were identified by aligning partial 16S rRNA sequence with the GenBank database. From the present study it can be concluded that amongst the 05 alkaline protease producing isolates, B-2 produced maximum amount of alkaline protease. The optimum pH, temperature, time of incubation and inoculum size for protease production was determined as pH-10, 37ºC, 48 hours and 1 ml respectively. Proteases produced by *Bacillus* species are by far the most important group of enzymes being industrially exploited. The results presented here are in agreement with the literature, as several *Bacillus* species are known to be good alkaline protease producers and have been widely used in the detergent industry. Use of proteases in detergent will help in formulating efficient detergents for removal of various stains, so that volume of detergents used will be very much reduced which in turn could provide a safer environment in the pollution loaded world.

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