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One Factor at A Time based optimization of protease from poultry associated *Bacillus licheniformis*

Ameer Khusro

Department of Plant Biology and Biotechnology, Nungambakkam, Chennai, India.

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ABSTRACT

Bacteria were isolated from poultry farm of Guduvanchery, Tamil Nadu, India and exhibited variable protease activity on skim milk agar plates. Of 10 bacterial isolates screened, *Bacillus licheniformis* strain 018 was observed as a hyperprotease producer and it was further characterized using biochemical and molecular tools. Protease production from the isolate was enhanced by optimizing the culture conditions using One Factor at A Time (OFAT) method. The bacteria exhibited its optimal enzyme activity at pH- 9.0, temperature- 35^{0}C, agitation speed- 130 rpm, incubation time- 24 h, carbon source- casein and nitrogen source- yeast extract. On the other hand, the crude proteases were found to be significantly active and stable at broad range of pH (5.0-9.0) and temperature (30-60^{0}C). To the best of my knowledge this is the first report on the production and enhancement of alkaline protease from poultry farm isolate using OFAT method. Stability of the enzyme at high temperature and pH can be explored for varied industrial applications.

INTRODUCTION

Proteases are one of the most important classes of enzymes for biotechnological interest that account for 60% of total enzyme market. Among various types of proteases, alkaline proteases have extensive applications in industries like laundry detergents, pharmaceutical, food industry, leather processing and proteinaceous waste bioremediation because of their high proteolytic activity and stability under alkaline conditions (Bayoudh *et al*., 2000). Proteases from microbial sources are preferred over the enzymes from plants and animals. The majority of commercial alkaline proteases are produced by bacteria, especially from *Bacillus* sp (Jellouli *et al*., 2009). Extracellular thermoalkaline bacterial proteases are important for the hydrolysis of waste proteins and enable the bacteria to absorb and utilize hydrolytic products (Srinivasan *et al*., 2009; Habib *et al*., 2012) by growing easily under harsh conditions. 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 (Haddar *et al*., 2009). Fermentation medium optimization plays a critical role in enhancing the production yields of the industrially important enzymes. Optimization for enhanced production of enzyme depends upon medium components like carbon source, nitrogen sources, pH, temperature, agitation and incubation time. The optimization of the fermentation medium by one factor at a time (OFAT) is a potential approach in order to analyze the interactive effects of various factors. In the present day scenario, proteases have a great commercial value in biotechnological applications. Although proteases have been identified from different sources for their industrial and biotechnological applications, but still the proteases exploited is very limited to meet all the essentials because the available enzymes could not resist the drastic changes in environmental conditions that is equivalent to industrial demands. Thus, new promising bacterial strains that could survive under harsh environmental conditions could be isolated from diverse sources to enhance the yield of such enzymes. As there are very few research activities on protease production from poultry farm bacteria, hence considering the industrial importance of proteases, the present study was investigated to isolate a new *Bacillus* strain from poultry farm for protease production and to identify the isolate efficient for maximum protease production at different parameters using OFAT method.

* *Corresponding Author*
Ameer Khusro, Department of Plant Biology and Biotechnology, Nungambakkam, Chennai, India. Email: armankhan0301[at]gmail.com

MATERIALS AND METHOD

Sample collection

Poultry faeces soil sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India) in sterile container. Faeces soils were brought to the laboratory in aseptic condition for further analysis.

Screening of bacteria for extracellular protease production

The collected sample was serially diluted and streaked on sterilized skim milk agar plates. The plates were incubated for 24 h at 37 ^{0}C and protease producers were selected by observation of zone of hydrolysis around the colonies. Only the hyperprotease producing isolate was selected for further optimization process.

Organism identification

The isolate showing potential protease production was characterized by cultural, morphological and biochemical analysis following standard procedures according to the Bergey's Manual of Systemic Bacteriology (Sneath, 1994).

Genomic DNA isolation

Two ml of hyperprotease producing bacterial culture was centrifuged at 6000 rpm for 5 min. The supernatant was discarded. One ml of UniFlex™ Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 min at 37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol:chloroform were added and mixed well. The sample was centrifuged at 10,000 rpm for 15 min at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex™ Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 min at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 min till the ethanol evaporates. The pellet was resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at -20ºC.

PCR amplification and sequence of 16S rRNA

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and universal primers. The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining.

Sequencing of PCR product and BLAST search

The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers were also used for sequencing.

The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/. The sequences were submitted to GenBank and the following accession number was assigned for the isolate; *Bacillus licheniformis* strain 018 - KC342225

Protease production

Extracellular protease production was carried out using basal media - Glucose, 0.5% (w/v); Peptone, 0.75% (w/v); Salt solution- 5% (v/v) {($MgSO_4.7H_2O$, 0.5% (w/v); KH_2PO_4 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01% (w/v) at 160 rpm. The culture medium was harvested and was subjected to centrifugation at 8,000 rpm for 10 min to obtain crude enzyme source. The isolate was taken for further optimization studies to enhance the protease production using OFAT method.

Extracellular Protease assay

One ml of enzyme was added to 2 ml of casein (1% w/v in 0.1N Glycine – NaOH buffer pH 10) and the mixture was incubated for 15 min at 60^{0}C. The reaction was terminated by adding 3 ml of 10% trichloroacetic acid reagent and then centrifuged for 15 min at 10,000 rpm.

Then 1 ml of filtrate was mixed with 5 ml of alkaline copper reagent and after 15 min 0.5 ml of Folin-ciocalteau reagent was added. After 30 min of incubation the absorbance was read at 700 nm. Similarly blank was carried out by replacing enzyme with distilled water.

One unit enzyme activity is defined as the amount of enzyme that releases 1µg of tyrosine per ml per min under the assay conditions. Tyrosine at the concentration of 50–250 µg was used as standard.

Estimation of total soluble protein

Estimation of total extracellular protein was performed through Bradford test (Bradford, 1976). Bradford method is a simple and rapid method to estimate the protein content in a sample based on the ability of protein to bind with the dye Coomassie Brilliant Blue G250.

The unbound dye has an absorption maxima of 495 nm, on binding with the protein, the absorption maxima becomes 595 nm. Thus from the absorbance at 595 nm, the protein in the sample solution can be estimated. Bovine serum albumin (BSA) was used as standard. Hundred microliter of bacterial supernatant was pipetted out into test tubes. Volume of the tubes was made up to 1 ml using sterilized distilled water.

Five millilitre of the Bradford reagent were added to all the tubes and mixed thoroughly. One ml of distilled water with 5 ml of Bradford reagent was used as blank. Absorbance at 595 nm was recorded against blank. Protein content per millilitre of test samples was determined against the standard curve.

Optimization of process parameters by OFAT (One factor at a time) method and growth kinetics of bacteria

In order to study the growth kinetics and protease activity of bacteria, the organism was grown in the liquid media. Growth was estimated by measuring optical density of culture broth at various parameters. Using OFAT method, the production media was optimized with various fermentation parameters like pH, temperature, agitation, incubation period, additional carbon sources and nitrogen sources after working out a series of experiments for the bacterial strain. Effect of pH on protease activity was assessed by cultivating the isolate (1% inoculum) in the production media of varied pH ranging from 5-10. The influence of pH on protease activity over casein was investigated in 100 mM phosphate buffer with desired pH. The influence of different fermentation temperature such as 30°C, 35°C, 40°C, 45°C and 50°C on protease activity was evaluated under optimized pH (using 100 mM phosphate buffer) of media for the isolate (1% inoculum).

The fermentation medium with optimized pH was incubated at optimized temperature in order to study enzyme activity at different agitation rate. The fermentation was carried out at varying agitation speed such as 120 rpm, 130 rpm, 140 rpm, 150 rpm and 160 rpm in an orbital shaking incubator. The bacterial inoculum (1%) was added to 50 mL of fermentation medium in 250 mL of Erlenmeyer flasks.

The flask was incubated at optimized pH, temperature and agitation for 12-96 h. The effect of various carbon and nitrogen sources on the extracellular protease production was studied at optimized parameters.

Approximately 1% (w/v) of carbon sources (Glucose, Casein, Xylose, Sucrose, Lactose and Mannose) and nitrogen sources (Peptone-Pep, Yeast extract-YE, Beef extract-BE, Tryptone-Tryp, KNO_3, Ammonium sulphate- Asp and Ammonium chloride- ACl)) were added separately to the production media. After 24 h of incubation, cultures were centrifuged at 6000 g for 10 min at 4°C and the supernatants were collected. The extracellular protease activity was estimated as described earlier.

Estimation of biomass (Analytical study)

Two millilitre sample was collected in a pre-weighed eppendorf tube and centrifuged at 8000 rpm for 10 min. Supernatant was discarded and the pellet was washed thrice with sterile distilled water, followed by drying the pellets at 95°C till constant weight and expressed in dry cell weight (mg/mL).

Partial characterization of crude protease

Temperature stability

The temperature stability of the enzyme was studied by using crude enzyme. To evaluate thermal stability, the enzyme solution was incubated at temperatures of 30-70^{0}C for up to 4 h. The relative enzyme activity was recorded at 1 h interval during 4 h incubation. The enzyme activity was determined as described earlier. The relative activity was estimated taking original activity as 100%.

pH stability

pH stability was measured by incubating the enzyme at pH 5 to 10 in different buffers (0.1 M) such as Sodium phosphate (pH 5.0 to 7.0), Tris-HCl (pH 8.0, 9.0) and Carbonate-bicarbonate (pH 10.0). To evaluate the stability of the enzyme at each pH, the crude enzyme was incubated into the respective buffer over a pH range of 5.0 – 10.0 for up to 4 h at optimum temperature. The relative enzyme activity was determined at 1 h interval during the 4 h period of incubation. The enzyme activity was determined as described earlier. The relative activity was estimated taking original activity as 100%.

Antibiotic susceptibility

The growth behaviour of the isolate was studied in the presence of a range of antibiotics. The antibiotics used in this study were Ampicillin (AMP- 10 µg), Kanamycin (K- 30 µg), Nalidixic acid (NA- 30 µg), Streptomycin (S- 10 µg), Cephotaxime (CTX- 30µg) and Penicillin (P- 10 µg).

Phylogenetic tree analysis

Phylogenetic relationship of the isolate with other *Bacillus* species was inferred from phylogenetic comparison of the 16S rRNA sequences.

The partial 16S rRNA sequences were retrieved on NCBI server using BLAST tool. Sequences similar to isolate sequences were downloaded in FASTA format from NCBI server. Phylogenetic tree was inferred using the Neighbor-Joining (NJ) algorithm in Molecular Evolution Genetic Analysis (MEGA) software version 4.0 (Tamura *et al.*, 2007). The evolutionary history was inferred using NJ method.

Statistical analysis

All the analytical experiments were conducted in triplicates and data presented is mean ± SD.

RESULTS

Isolation and screening of protease producing bacteria

Ten bacteria were isolated from faeces soil samples of poultry farm, collected from Guduvanchery. These isolates were further screened for extracellular protease production. Of 10 isolates, 1 showed the production of hyperprotease on skim milk agar plate (Figure not shown). This protease positive isolate was further selected for cultural, morphological and biochemical characteristics.

Morphological characterization and bacterial identification

The cultural, morphological and biochemical characteristics of the isolate was studied (Table 1). The bacterial culture showed white and spreading type colonies. The microscopic observation showed the morphology of culture as rod shaped with endospore. The isolate was identified as *Bacillus* sp. based on the taxonomical characteristics. Genomic DNA of the isolate was visualized under UV. In the present study, 16S rRNA gene sequencing of the isolate was investigated. The isolate was

identified as *Bacillus licheniformis* strain 018 (Accession number - KC342225) by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST.

Table 1: Morphological and Biochemical characteristics.

Tests	strain 018
Surface	Smooth
Colony colour	White
Opacity	Opaque
Gram reaction	Gram (+)
Cell shape	Rod
Endospore	+
Motility	+
Glucose test	+
Lactose test	+
Mannitol test	+
Starch hydrolysis	+
Tween 80	-
Casein	+
Indole	-
Methyl Red	+
Voges Proskauer	+
Citrate utilization	+
Urease test	-
Catalase test	+
Oxidase test	+
Aerobic growth	+

'+' = Positive; '-' = Negative

Effect of pH on protease activity

Protease activity was markedly affected by change in pH. pH played a major role in the secretion and production of protease by the isolate (Figure 1a). During optimization study pH was changed from 5 to 10 for the isolate. Maximum protease activity of 61.891 U/mL was estimated by strain 018 at alkaline pH (pH 9).

Effect of temperature on protease activity

The fermentation temperature also plays a very important role in the production of protease by the isolate. Figure 1b shows the extracellular protease activity of the isolate at different temperatures under optimized pH of the isolate. Maximum enzyme activity of 60.552 U/mL was observed by the isolate at 35°C.

Effect of agitation speed on protease activity

The influence of varying agitation for protease activity was evaluated in Figure 1c. Protease activity for strain 018 (62.91 U/mL) was found to be maximum at 130 rpm. The agitation speed lower and higher than 130 rpm affected the enzyme activity for the isolate.

Effect of incubation time on protease activity

Time period plays a very critical role in the protease production. In the present investigation protease activity was determined from 12- 96 h. The obtained results indicated that the highest yield of extracellular protease was 65.86 U/mL after 24 h of incubation. After 24 h, the protease production was drastically decreased up to 96 h in a constant manner (Figure 1d).

Effect of carbon and nitrogen sources

The effect of various carbon and nitrogen sources on extracellular protease production is shown in Figure 1e and 1f respectively. The isolate showed enhancement in the enzyme production when grown in the media supplemented with 1% (w/v) casein and yeast extract.

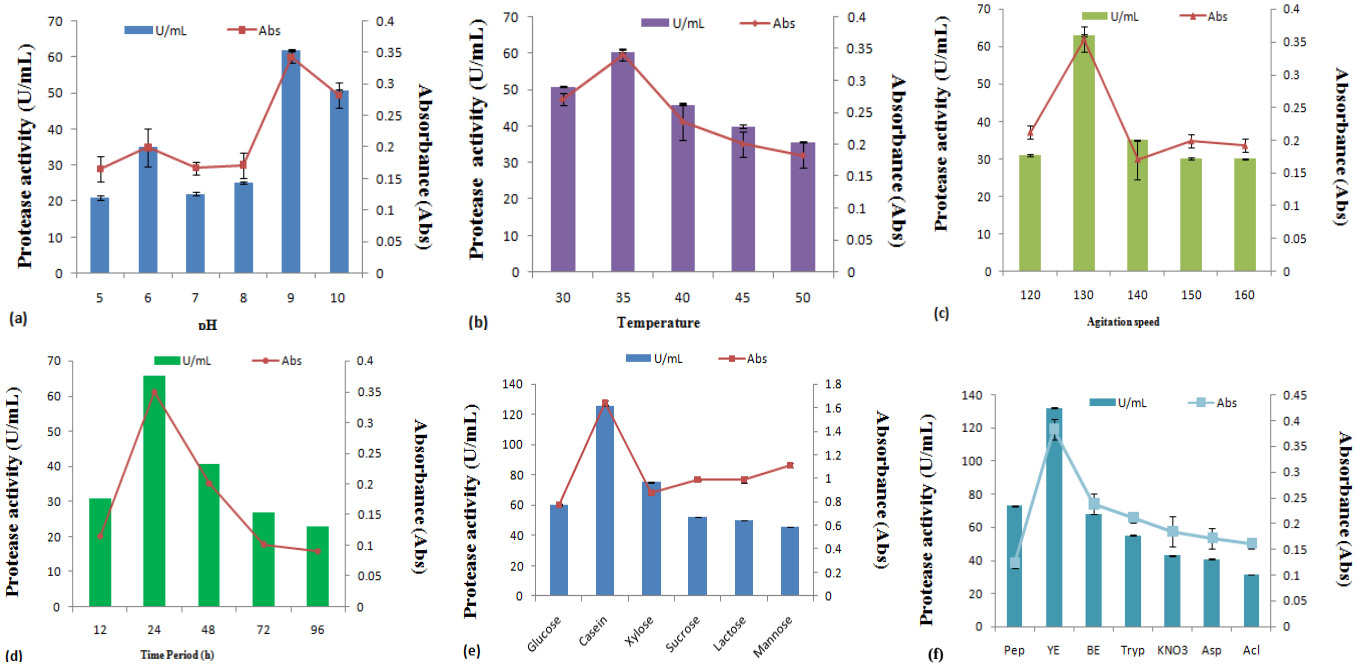

Fig. 1: Effect of various process parameters on protease production. *Bacillus licheniformis* strain 018 was showing maximum protease activity at pH 9 (a), at 35°C (b), at 130 rpm (c), after 24 h of incubation (d), in the presence of casein as sole carbon source (e) and in the presence of yeast extract as sole nitrogen source (f).

Table 2: Effect of culture conditions for extracellular protease activity and biomass production from *Bacillus licheniformis* strain 018 in shake-flask cultivations.

Culture conditions	Protease activity (U/mL) (Mean±SD)	Relative activity (%)	Total soluble protein (mg/mL) (Mean±SD)	Dry weight of biomass (mg/mL) (Mean±SD)
		***Bacillus licheniformis* strain 018**		
pH				
5	21.012±0.51	33.94	0.321±0.02	0.4±0.02
6	35.031±0.35	56.59	0.467±0.01	2.0±0.01
7	22.036±0.51	35.6	0.359±0.02	0.63±0.01
8	25.191±0.42	40.7	0.413±0.01	0.65±0.03
9	61.891±0.35	100	0.458±0.03	1.2±0.01
10	50.781±0.26	82.04	0.296±0.021	1.3±0.01
Temperature (^oC)				
30	50.782±0.12	83.86	0.15±0.01	1.2±0.02
35	60.552±0.33	100	0.304±0.02	1.8±0.02
40	45.862±0.21	75.74	0.589±0.04	1.6±0.01
45	40.001±0.35	66.06	0.411±0.01	1.5±0.02
50	35.782±0.21	59.09	0.4±0.01	1.5±0.01
Agitation speed (rpm)				
120	31.021±0.22	49.31	0.216±0.03	1.3±0.03
130	62.91±0.35	100	0.477±0.04	2.0±0.01
140	35.001±0.13	55.63	0.3110.01	1.3±0.01
150	30.10±0.24	47.84	0.211±0.01	0.6±0.02
160	30.08±0.11	47.63	0.21±0.02	0.7±0.01
Incubation time (h)				
12	30.97±0.23	47.02	0.112±0.03	0.8±0.02
24	65.86±0.12	100	0.452±0.02	2.2±0.01
48	40.605±0.21	61.66	0.381±0.01	1.8±0.01
72	26.896±0.31	40.83	0.211±0.01	1.9±0.03
96	22.811±0.33	34.63	0.0677±0.02	1.6±0.02
Carbon source				
Glucose	60.523±0.31	47.71	0.321±0.02	1.4±0.01
Casein	125.752±0.35	100	0.432±0.01	2.5±0.01
Xylose	75.123±0.25	59.73	0.311±0.02	1.9±0.02
Sucrose	52.321±0.28	41.6	0.221±0.01	1.6±0.02
Lactose	50.023±0.11	39.77	0.178±0.01	1.8±0.01
Mannose	45.541±0.21	36.21	0.213±0.03	1.1±0.02
Nitrogen source				
Peptone	72.976±0.22	55.15	0.115±0.02	0.8±0.02
Yeast extract	132.321±0.11	100	0.338±0.03	2.7±0.02
Beef extract	68±0.17	51.39	0.221±0.01	2.0±0.01
Tryptone	55.231±0.16	41.74	0.117±0.01	1.3±0.03
KNO_3	43.001±0.12	32.49	0.222±0.03	1.6±0.01
Ammonium sulphate	40.875±0.15	30.89	0.117±0.01	1.1±0.02
Ammonium chloride	31.31±0.1	23.66	0.224±0.02	0.6±0.035

Biomass estimation

Dry cell weight (DCW, mg/mL) was analyzed for the isolate at different parameters (pH, temperature, agitation speed, incubation time, carbon and nitrogen sources). The results indicate good biomass production for protease production. The biomass production was more for the isolate at optimum condition of selected parameters (Table 2).

Effect of temperature and pH on the stability of crude alkaline protease

Stability is one of the most important factors in studying characteristics of enzyme. Crude protease obtained from strain 018 was more stable till 60^0C for 4 h of incubation and retained upto 67% of the activity. The enzyme stability was reduced significantly above 60 ^{0}C (Figure 2a).

The effect of pH on stability of protease from the isolate is shown in Figure 2b. The crude protease obtained from strain 018 was stable at a wide range of pH from 5.0 to 9.0. The isolate was found stable upto pH 9.0 for 4 h of incubation. About 60% of activity was retained by strain 018 at pH 9.0.

Antibiotic susceptibility test and Phylogenetic tree of the isolate

The isolate was found to be susceptible to all the antibiotics tested here (Data not shown). A Neighbor- joining tree of isolate's 16S rRNA sequences, including different strains of *Bacillus* species, clustered all the isolates belonging to the previously identified species to the corresponding species (Figure 3).

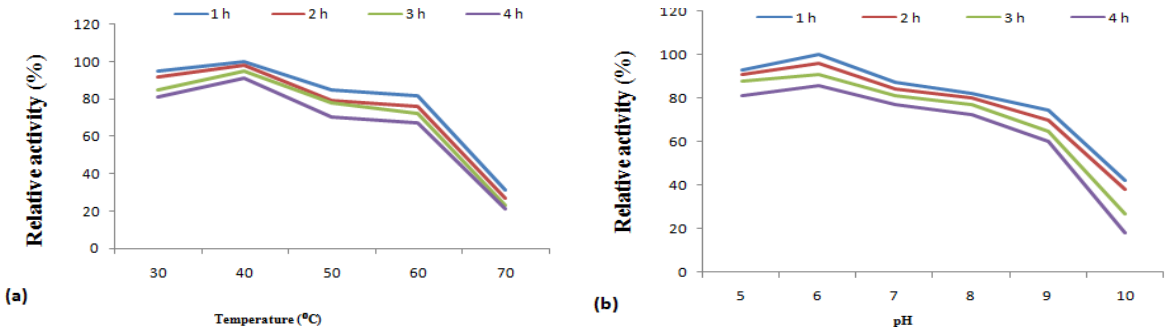

Fig. 2: (a) Effect of temperature on stability of crude protease. The crude protease obtained from the isolate was found to be thermo-stable upto 60℃ for 4 h of incubation. Further, there was drastic decrease in the stability of the enzyme as the temperature rises up to 70℃. (b) Effect of pH on stability of crude protease. The crude enzyme obtained from strain 018 was showing maximum stability up to pH 9 for 4 h of incubation. As the pH was increased further, the stability was lost drastically.

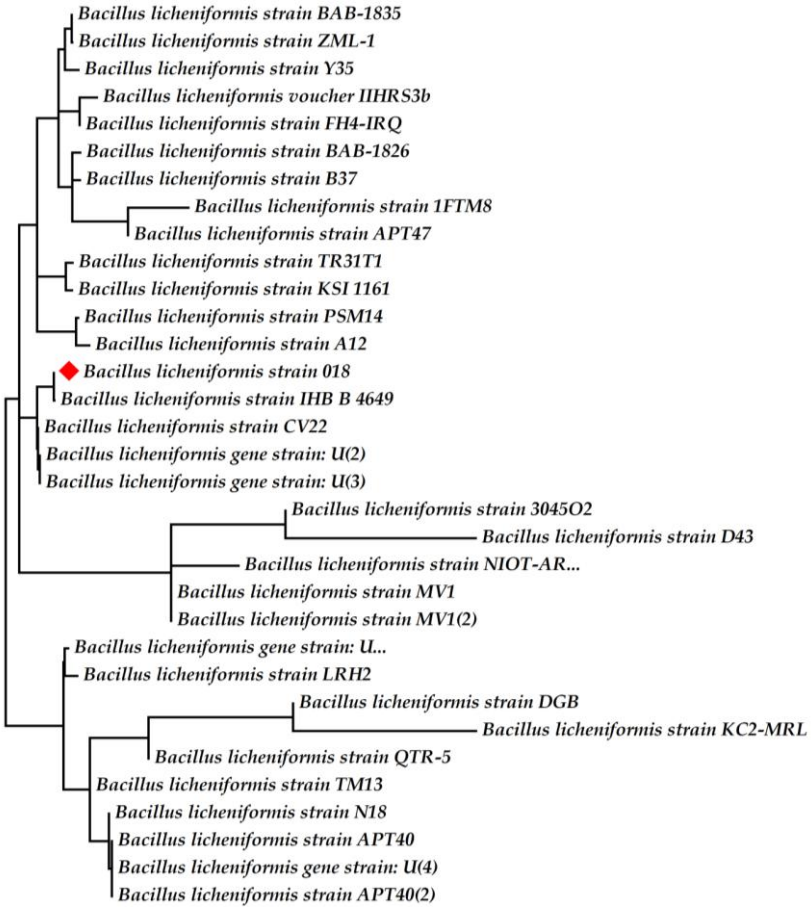

Fig. 3: Phylogenetic tree based on a comparison of the 16S ribosomal RNA sequences of *Bacillus* isolate and some of their closest phylogenetic relatives. The isolate characterized in the present study is indicated in red bullet.

DISCUSSION

Members of the genus *Bacillus* produce large variety of extracellular enzymes, of which proteases play an important role at industrial scale. In the primary step of this investigation, the hyperprotease producing *Bacillus* sp. was isolated from the collected sample by screening procedure on skim milk agar plate.

The culture conditions were found to have profound influence on extracellular protease production (Table 2). In the present study the changes in pH from 5-10 caused change in the protease activity of the isolate. *B. licheniformis* strain 018 was showing maximum enzyme activity at pH 9. The study strongly favours the finding of Kumar *et al*. (1999), Khusro (2015) and Pastor *et al*. (2001) who demonstrated that *Bacillus* sp. had shown maximum protease production at high pH. Similar results were also obtained by Almas *et al*. (2009). It is clear from the present investigation that strain 018 can be used at large scale in food, tannery and detergent industries because of its alkaline tolerant nature. pH played an important role in the production of extracellular protease by each isolate. This is because substrate

binding and catalysis are often dependent on charge distribution on both substrate and in particular enzyme molecules. pH markedly affects the metabolic pathways of microorganisms. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the microorganisms thus lowering their overall metabolic activity (Willey *et al*., 2008).

Temperature had profound effect on protease production. The isolate showed maximum protease activity at 35^0C. Enzyme activity was 100% at 35^0C for strain 018. The finding of this present study supports the application of the isolate in waste water treatment. The isolate can be used in the bioremediation of wastewater treatment. However our study was against the finding of Khan *et al*. (2011) who found that protease produced by *Bacillus* sp. gave the best activity at 50^0C. The variations among our findings and the previous reports may be because of the source of the isolation and types of bacterial strain. In order to convert casein substrate into product, enzyme must collide with and bind to the substrate at the active site. Increasing or decreasing the temperature of a system will increase or decrease the number of collisions of enzyme and substrate per unit time. Thus, within limits, the rate of the reaction will change. For the mesophilic bacteria, as the temperature of the system increases, it causes thermal denaturation of the protein and enzymes. Thus too much heat can cause the rate of an enzyme catalyzed reaction to decrease because the enzyme or substrate becomes denatured and inactive.

Aeration is one of the effective factors for the improvement of aerobic fermentation. The present experiments were carried out under the variation of shaking speed ranging from 120-160 rpm in order to optimize the aerobic condition in shake flask cultivation. According to the present context results, maximum protease activity by strain 018 was observed at 130 rpm. The reason behind this may be the uniform distribution of nutrient and supplied oxygen for the cultivation of this particular strain in the fermentation medium. Incubation period is one of the most important parameter in metabolic activity and growth of bacteria. The results obtained in this study indicated that highest yield of protease by the isolate was obtained at 24 h of incubation. A decline in protease activity afterwards was probably due to depletion of nutrients in the fermentation medium of the microorganism, causing stressed and unfavourable conditions for the bacteria resulting in reduction of enzyme activity. Our reports were against the findings of Khan *et al*. (2011) who demonstrated maximum protease activity by *Bacillus* sp. at 36 h of incubation. Findings of the corresponding experiments revealed that the time course of enzyme activity varies with the source of isolation, strains used, genetic makeup of strains and cultivation conditions. Incubation beyond the optimum time course was generally accompanied by a decrease in the growth rate and enzyme productivity, which gradually declined after 24 h of incubation. The decreased activity in the later phase of growth was probably due to catabolite repression by readily metabolizable substrate glucose (Lin *et al*., 1998). Carbon is the essential element for the growth and metabolism of bacteria. The enzyme production is stimulated in the presence of various carbon sources. In the present context, optimal level of extracellular protease from the isolate was recorded when casein was used as a sole carbon source. Lakshmi *et al.* (2014) recorded maximum protease production from *Bacillus* sp. in the presence of molasses. Various organic and inorganic nitrogen sources were tested to estimate the maximum protease production from the isolate. Yeast extract was found to be the best nitrogen source for the isolate in order to achieve maximum production of protease. The present investigation supports the finding of Vanitha *et al.* (2014) who demonstrated yeast extract as a potential nitrogen source for protease production. Yeast extract has been reported to play an important role in enzyme production due to the presence of essential elements and growth factors (Porsuk *et al*., 2013). In the present investigation crude protease obtained from strain 018 was more stable up to 60^0C for 4 h of incubation and retained upto 67% of the activity. Our study was against the finding of Abusham *et al*. (2009) who demonstrated that protease obtained from bacteria was more stable at 50^0C. Thus, the results concluded that the crude enzyme is moderately temperature stable. Significant enzyme stability at higher temperatures would be important for several industrial processes. The industrial importance of an enzyme will be more when the effect of temperature input on its optimal activity is less.

The most desirable characteristic of the isolate of present investigation was steady stability at alkaline pH. The crude protease obtained from the isolate showed stability at a wide range of pH from 5.0 to 9.0. The outcome of the present study is similar to the finding of Almas *et al*. (2009) who demonstrated stability of protease at pH 9. Many of the microorganisms have been found to produce protease with pH optima near neutral region but with high stability in alkaline conditions. Stability at alkaline pH values may be due to charged amino acid residues. The enzymes stable in alkaline conditions were characterized by a decreased number of acidic residues and an increased number of arginines (Hakulinen *et al*., 2003). The isolate of the present context could be a good source for biotechnological applications at industrial scale because of the alkali stability nature of protease. 16S rRNA gene sequences to study bacterial phylogeny and taxonomy have been by far the most common molecular marker. 16S ribosomal RNA-based molecular identification could achieve identification because of the presence of species-specific variable regions. This molecular approach has been extensively used for bacterial phylogeny, leading to the establishment of large public-domain databases and its application to bacterial identification, including that of environmental and clinical uncultured microorganisms, unique or unusual isolates and collections of phenotypically identified isolates (Drancourt *et al*., 2000).

CONCLUSION

The results obtained from the present investigation indicate enhanced production of alkaline protease from *Bacillus licheniformis* strain 018 under optimized medium components and

culture conditions by using traditional OFAT method. The most significant findings of this study were the ability of the isolate to produce maximum level of extracellular alkaline protease after 24 h of incubation, and the stability of enzyme at high temperature and pH. The best process parameters were found to be casein as the sole carbon source and yeast extract as nitrogen source. The isolate of the present context could be a good alternative of the commercial strains in tanneries, pharmaceutical and food processing industries as biotechnological and physiological aspects due to the thermo-alkaline nature of protease obtained. Further studies are in progress in order to purify the alkaline protease from the isolate and the scaling up of the enzyme for commercial applications.

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