ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

16S rDNA Based Identification of Alkaline Protease Producing Alkaliphilic Bacillus Sp Isolated From Dairy Industry Soil And Evaluation of the Enzyme Potential In Detergent Formulation

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ABSTRACT

A strain of *Bacillus sp* was isolated from dairy industry soil with pH 9.86. The isolate being moderately halophilic was able to produce extracellular alkaline protease at pH ranging from 8-11, and temperature 25-50^{0}C. On the basis of 16S rDNA sequencing results, isolated sp. was confirmed as *Bacillus flexus* having 99% identity with the related sequences of exiting strain *viz*., *Bacillus flexus* Accession no. JN033557.1, FJ948078.1, EF157300.1 retrieved from the databases Genebank, NCBI and Ribosomal Database Project.

Keywords: *Bacillus flexus*, alkaline protease, detergent formulation

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ISSN: 0975-8585

INTRODUCTION

Alkaliphilic *Bacillus* species are appealing candidates for the production of extracellular enzymes that include alkaline amylase, protease and carboxymethyl cellulase which are active and stable at high pH [7]. Proteases that are functional at broad ranges of pH and temperature in a better manner are greatly preferred since they can withstand rigorous industrial production processes [20]. Alkaline proteases (EC.3.4.21-24, 99) are active between neutral to alkaline pH and are of immense interest due to their widespread application in the detergents, food, pharmaceuticals, chemicals, leather, paper & pulp and silk industries [14]. Nowadays they are considered as significant key-ingredients in detergent formulations [18]. Several microbial strains such as *Bacillus pseudofirmus, Bacillus subtilis, Bacillus licheniformis* and *Bacillus cereus* have been isolated from soil and screened for their application and performance at industrial scale with specific catalytic role [19, 22, 3]. *B. flexus, B. cereus, B. pseudofirmus, B. pseudoalkalophilus,* have been found to produce protease at alkaline pH 10 and make up a valuable tool in industrial enzyme production [24]. A facultative alkaliphilic protease producing gram positive, rod shaped *Bacillus flexus* EMGA5, isolated from mangrove soil and identified by 16S rDNA sequence analysis has been found able to grow at pH 7.2 to 10.5, thus being potentially applicable for a significant role in buffering capacity and membrane H^+ conductance [11]. Among other application, a facultative alkaliphilic *Bacillus flexus* FPB17 isolate has been reported [17] from alkaline lake soils of north Gujarat, India for production of alkaline phosphatase enzyme along with another alkaliphilic *Bacillus flexus* XJU-3 [28] for alkaline amylase production and it being alkalitolerant they had better enzyme properties compared to previously reported *Bacillus sp*. Alkaliphilic *Bacillus sp.* have attracted much interest not only due to their ability to produce extracellular enzymes that are active and stable at high pH [12] but their biodegradation potential as well. *Baciilus flexus* XJU-4 strain has been reported [15] for degradation of 3 nitrobenzoate could be enzymatically coupled for bioremediation of soil and industrial effluents contaminated with toxic nitroaromatic compounds. Recently *B. flexus* SSZ01was reported for biodegradation of microcystin-RR and could be contributed to the self purification of the ecosystem from such potent toxins [2]. In the aforesaid context, the present study attempts to characterize a potent bacillus isolate from dairy house soil, by its identification followed with 16S rDNA sequencing and evaluation of its application potential for detergent industry.

MATERIALS AND METHODS

Screening of bacteria

Alkaline soil samples with a pH 9.86 were collected from dairy industry soil of U.P. and suspended in sterile water (10g/90ml) and agitated on an orbital shaker at 200rpm for 72 h. Soil suspension was serially diluted and spread on alkaline agar medium containing 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 1% Na_2CO_3 and 1.5% agar, pH 10.5 [7]. The plates were incubated at 37^0C for 24-48 h. Pure colonies were developed into single colony and screened on skimmed milk agar medium containing 10% skimmed milk [8] and incubated at 37^0C for 24-72 h. A translucent zone of skim milk hydrolysis around the colonies gave a clear indication of protease producing organisms.

Pure culture of isolates were developed from single bacterial strains and maintained on alkaline agar slant at 4^0C.

Bacterial identification using 16SrDNA Gene sequencing

Isolated culture of most promising isolate with prominent zone of clearance was identified based on the morphological and biochemical characterization and the same isolate was send to LacZene BioSciences (Delhi) India for authentication. Genotypic identification was carried out by 16S rDNA analysis in which, the isolated DNA was amplified using the upstream primer: 5'-GAGAGTTTGATCCTGGCTGGCTCAG-3' and the downstream primer: 5'-AAGGAGGTGATCCAGCCGCA-3'. Amplification of DNA was carried out under the following conditions: denaturation at 94° C for 5 min followed by 30 cycles of 94° C for 30 s, 52° C for 30 s, 72° C for 1.5 min and final extension at 72° C for 10 min [3]. Amplified PCR products of bacterial isolate was analyzed by electrophoresis with 1 % agarose gel run at 7 V/cm (90 min). After electrophoresis the gel was stained with ethidium bromide and further visualized and photographed under UV light. Further PCR product was sequenced subsequently and analyzed with the GenBank database using BLASTN program (NCBI) and Ribosomal Database Project release 10 (RDP).

Protease assay

Protease activity was assayed by modified procedure [25] using 1% Casein in .05 M sodium phosphate buffer (pH 8) as substrate. The absorbance was measured against a suitable blank at 660 nm. One unit of alkaline protease activity was defined as the amount of the enzyme able to produce 1g tyrosine ml^{-1} min^{-1} and expressed as Unit/ml of enzyme.

Protein Quantification

Protein quantity was determined by Lowry et al. Method [10] and Bovine serum albumin was used as standard.

Effect of incubation on enzyme production

The protease production was determined in the basal medium following an incubation of 24, 48, 72, 96, 120 h. The culture was incubated at 37^0C. The reaction mixture was centrifuged at 10,000 rpm at 4^0C for 10 min and protease activity was checked in the cell free extract.

Effect of pH and temperature on enzyme production

The effect of pH was determined in alkaline broth under different pH (6, 7, 8, 9, 10, 11, and 12) and the effect of temperature was determined by incubating the production medium for different temperature (25-60^0C). The culture was incubated at 37^0C for 72 h. Subsequently, the reaction mixture was centrifuged at 10,000 rpm at 4^0C for 10 min and protease activity was checked in the cell free extract.

Effect of different carbon sources on enzyme production

In order to find the optimum carbon source for enzyme production, six carbon sources (1%): glucose, galactose, lactose, maltose, sucrose and starch were selected and added to alkaline broth. The culture was incubated at 37^{0}C for 72 h. The reaction mixture was centrifuged at 10,000 rpm at 4^{0}C for 10 min and protease activity was checked in the cell free extract.

Effect of different nitrogen sources on enzyme production

In order to optimize the nitrogen sources for enzyme production, six different nitrogen sources (1%): casein, yeast extract, beef extract, peptone, tryptone and glycine were selected and added to alkaline broth. The culture was incubated at 37^{0}C for 72 h. The reaction mixture was centrifuged at 10,000 rpm at 4^{0}C for 10 min and protease activity was checked in the cell free extract.

Application studies of protease in detergent industry

Effect of Ionic and anionic surfactants on protease activity

The effect of surfactants on protease activity was investigated 1% of the ionic and nonionic surfactants such as Tween 20, Triton X 100 and SDS respectively, were added to the enzyme solution and incubated for 30 min. Enzyme activity was measured by standard assay method.

Effect of locally available commercial detergents on protease activity and its compatibility

Locally available commercial detergents (Rin, Surf excel, Ariel, Tide, Wheel, Nirma) were used to see their effect on protease activity. Detergent solutions were prepared at 0.7% (w/v) concentration in distilled water and boiled for 10 min to denature the enzyme present in the solution. Enzyme was mixed with equal volume of each detergent solution and incubated for 1 h at 60^{0}C and assayed for residual protease activity under standard conditions. The enzyme activity of control sample (without any detergent) was taken as 100%.

RESULT AND DISCUSSION

Screening of bacteria

Proteases are degradative enzymes which spilt the peptide bonds releasing oligopeptides or free amino acids [5]. Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* which happens to be an ideal source because of their rapid growth and limited space required for cultivation [6]. Out of the 79 isolates, 11 *Bacillus* strains were scored and confirmed for alkaline protease in plate assay. Among these *Bacillus sp* BC0016 exhibited better proteolytic ability as determined by hydrolysis of casein on skimmed milk agar plate (Fig. 1) compared to other isolates and selected for further studies.

ISSN: 0975-8585

Fig. 1 Microphotograph of representative bacterial isolate BC0016 and qualitative screening on Skimmed milk agar showing a transluscent zone around colony

Bacterial identification

The physiological and biochemical observation has been summarized in (Table 1). The partial 16S rDNA gene sequences (1423 bp) of the isolated strain revealed that *Bacillus sp* BC0016 was closely related to type strain of *Bacillus flexus* listed in the National center for Biotechnology information (NCBI) Genebank (Accession no. JN033557.1). The sequence was submitted under accession number JX855288.1, at NCBI. The phylogenetic analysis of 16S rDNA partial sequences of *Bacillus flexus* isolates has been summarized in (Fig. 7). Although morphological and biochemical identification (Table 1) of the isolate indicated that the organism belongs to *Bacillus* group however, this identification alone is not enough, hence, supporting experiments were planned to determine the phylogeny at species level. At species level the best way of identifying bacteria is through a more advanced and time extensive technology using 16S rRNA gene sequences or 16S rDNA [26].

Table 1 Phenotypic and biochemical characterization of *Bacillus flexus*

TEST	Observation
Colony morphology	smooth
color	pink
Culture growth temperature (^{0}C)	Ranging 25-50^{0}C
Gram staining	+
Endospore staining	Spore forming
Motility test	+
Indole production	+
Methyl red test	-
Voges-proskauer test	-
Citrate utilization test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Urease test	-
Oxidase test	+
Catalase test	+
Nitrate utilization test	-

+, positive; -, negative

Effect of incubation on enzyme production

Microorganisms show a considerable variation in enzyme production at different incubation periods which can be correlated to different phases of growth, hence, it is very essential to determine the optimum incubation period at which an organism reveal potential growth concomitant with enzyme production. The effect of incubation period (24-120 h) on the production of alkaline protease elaborated maximum activity (10.78 U/ml) at 72 h of incubation (Fig. 2). However, an increase in incubation period beyond 72-120 h, revealed a gradual decline in the activity.

Effect of pH and temperature on enzyme production

Since microorganisms show considerable variation at different incubation period, it is essential to detect the optimum period for maximum protease production [27]. pH and temperature are important factors affecting growth and yield of the organism was incubated at different the microorganisms. In the present study the bacterial isolate showed maximum protease production (9.90 U/ml)) at pH 9.0 (Fig. 3) and 11.06 U/ml at temperature 35^0C (Fig. 4). The maximum protease production by *Bacillus flexus* was obtained at 72 h of incubation at pH 9. The pH of medium strongly affects enzymatic process and transport of compound across the membrane. The optimum pH for protease production has been reported between 8-12 for potential application in fields of detergent formulation, leather industry, sliver recovery, degumming of silk *etc* [9]. The optimum pH for production of alkaline protease in the present study was found to be 9. Several workers have reported that the *Bacillus sp* gave maximum protease production at pH 9 [19, 23]. The bacterial isolate preferred the temperature range 35-37^0C for protease production giving maximum yield (11.06 U/ml) at 35^0C, which is also reported by [4, 19].

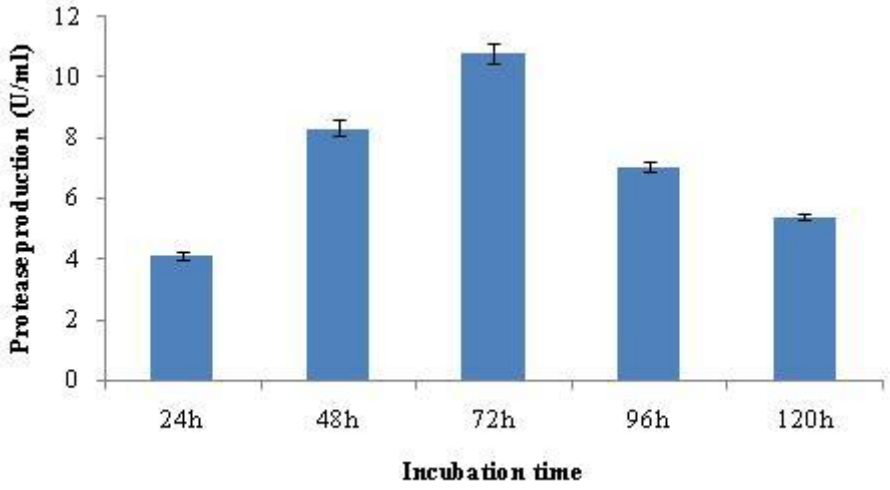

Fig. 2 Effect of incubation period on alkaline protease production in terms of U/ml by BC0016

ISSN: 0975-8585

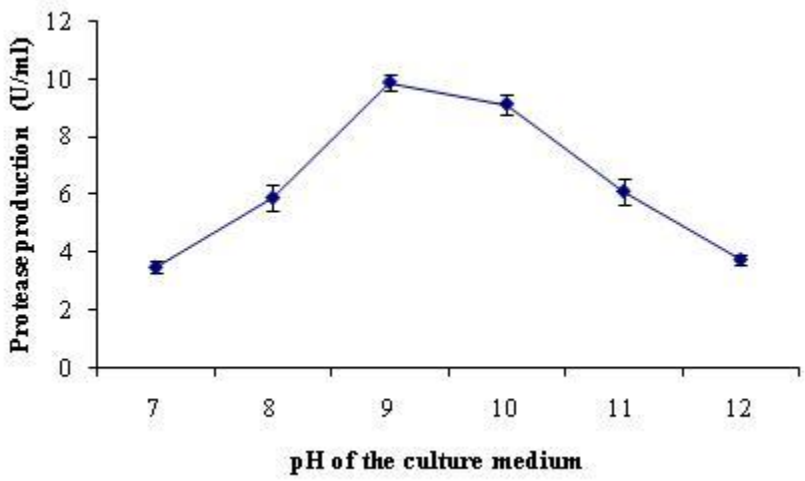

Fig. 3 Effect of pH on alkaline protease production by BC0016. The values of enzyme activity represent averages of at least three experiments

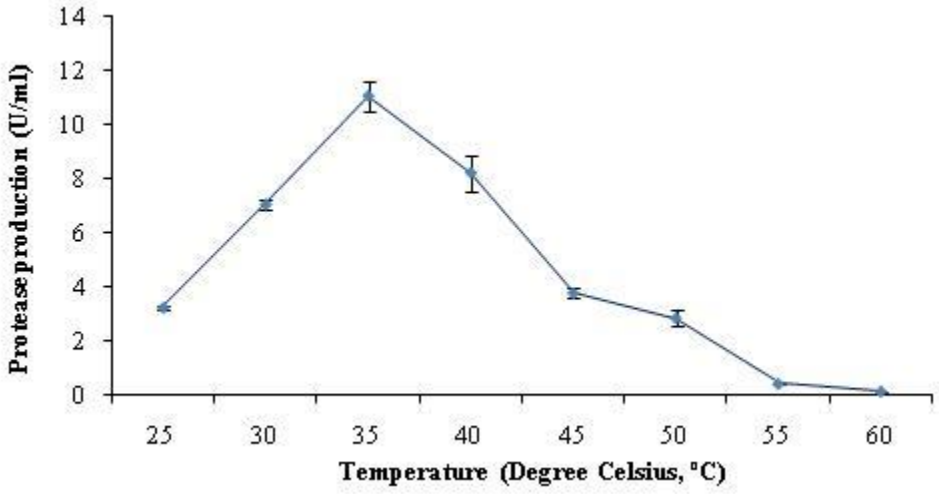

Fig. 4 Effect of temperature on alkaline protease production by BC0016. The values of enzyme activity represent averages of at least three experiments

Effect of carbon sources on enzyme production

Different carbon sources such as glucose lactose, sucrose, maltose, starch and galactose were tested for protease production. Various carbon sources were inoculated in the basal medium with 1% inoculum and incubated at 35^0C for 72 h at 200rpm. Among them, galactose (18.92 U/ml) was found to be the best carbon source (Fig. 5) giving approximately 1.74 fold higher activity than glucose (10.85 U/ml) which was also reported by [16]. Moderate to good amount of protease activity has been reported in the presence of lactose and galactose in *Bacillus* sp. strain CR-179 [21].

ISSN: 0975-8585

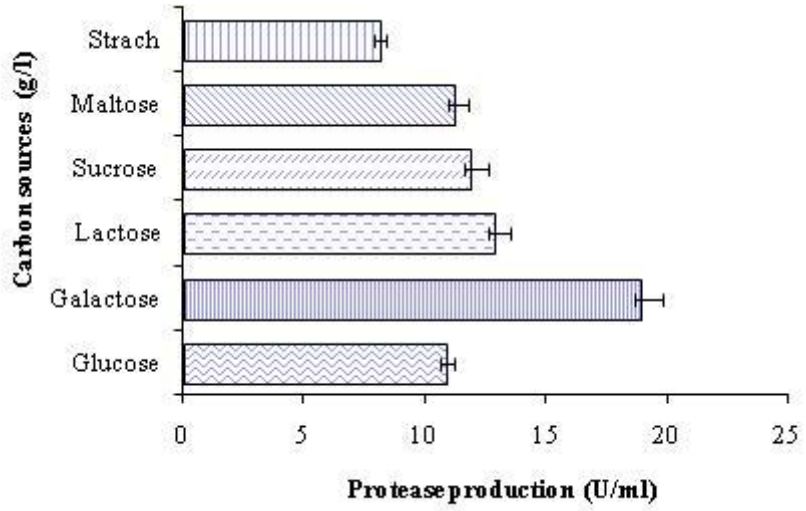

Fig. 5 Effect of carbon sources on alkaline protease production by BC0016 at pH 9 and 35^{0}C during 72h incubation. Glucose of the production medium was replaced with various carbon sourc4es at 10 g/l and fermentation was carried out under shaking (200 rpm)

Effect of nitrogen sources on enzyme production

Yeast extract and peptone served as nitrogen sources in the basal alkaline broth medium for protease production and maximum protease production was achieved when casein was used to replace both the nitrogen sources. Among different nitrogen sources tested for alkaline protease production by *B. flexus*, casein exhibited prominent effect on protease production. The enzyme yield in the presence of casein was 12.96U/ml which was approximately 1.87 fold higher compared to yeast extract (6.92 U/ml) and 1.32 fold higher than peptone (9.76 U/ml) in Fig. 6. It has been previously reported by [9] and a moderate increment in the enzyme activity in the presence of casein was also reported by [1].

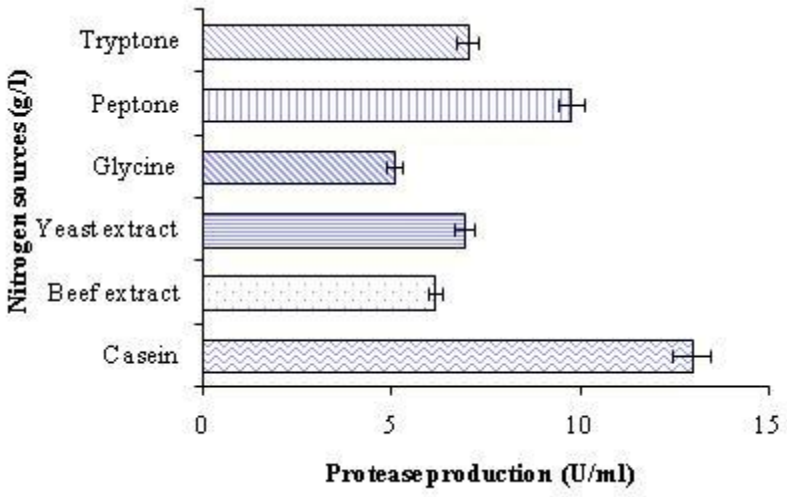

Fig. 6 Effect of nitrogen sources on alkaline protease production by BC0016 at pH 9 and 35^{0}C during 72h incubation. Yeast extract and Peptone of the production medium were replaced with various nitrogen sourc4es at 5g/l and fermentation was carried out under shaking (200 rpm)

ISSN: 0975-8585

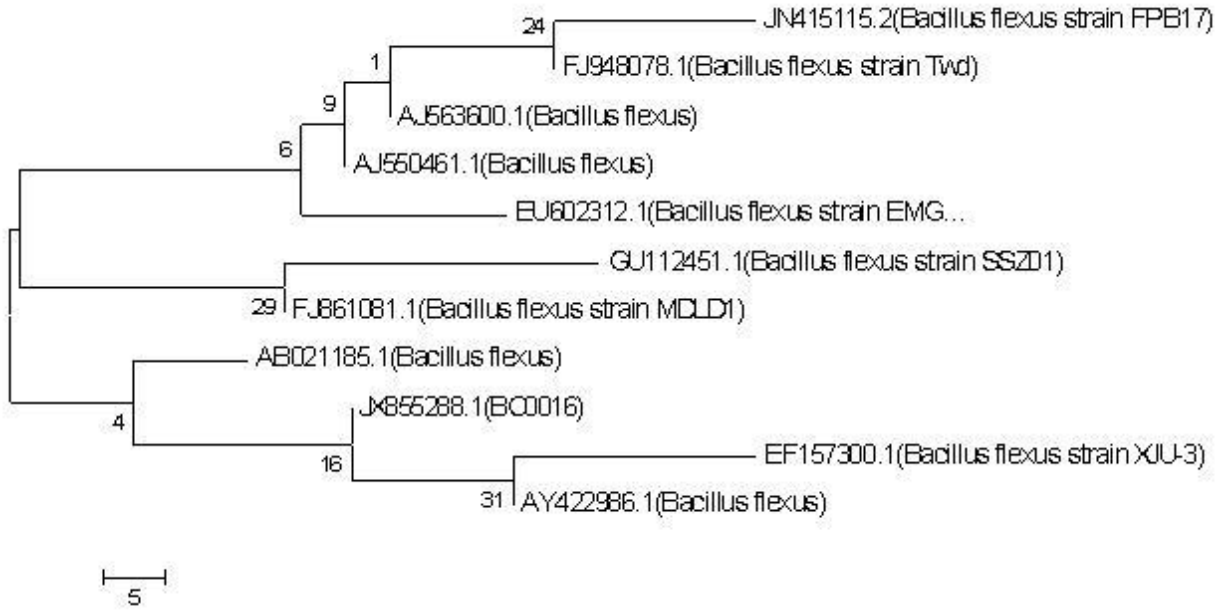

Fig. 7 Phylogenetic analysis of *Bacillus flexus* isolates from based on partial nucleotide sequences of 16S rDNA. The tree was constructed using the neighbor-joining method. Percentages at nodes represent levels of bootstrap support from 1000 resampled datasets. Bootstrap values less then 50% are not shown

Effect of Ionic and anionic surfactants on protease activity

Surfactant stable protease from *Bacillus sp*. was reported by many workers [13]. In the present study the enzyme showed stability as well as an increment in residual activity not only in the presence of nonionic and anionic as well as locally available detergents. The protease stability from the isolated *Bacillus flexus* was studied in the presence of both nonionic surfactants (Tween 20, Triton X 100) and anionic surfactant (SDS). The nonionic surfactants Tween 20 retained 220% protease activity while anionic surfactant (SDS) retained 173% protease activity in Table 2.

Table 2 Effect of surfactants and locally available detergent

Surfactants/Detergents	Concentration	Residual activity
Control	1%	10.67 (100%)
SDS	5%	13.60 (127%)
Triton X 100	5%	18.50 (173%)
Tween 20	5%	23.49 (220%)
Aerial	0.7%	27.92 (261.67%)
Surl excel	0.7%	7.91(74.13%)
Tide	0.7%	9.50 (89.04%)
Rin	0.7%	8.56 (80.22%)
Wheel	0.7%	5.35(50.14%)
Nirma	0.7%	7.80 (73.10%)

Effect of locally available commercial detergents on protease activity and its compatibility

Currently detergent stability plays a vital role in detergent formulation to improve the washing efficiency. The stability pattern of alkaline protease in the presence of

commercially available detergents also reveals its possible application in detergent formulation [23]. The protease activity was variably increased between 150-260% in the presence of 0.7% (w/v) concentration of commercially detergent. Results revealed 2.67 fold greater activity in the presence of Ariel when compared to control, showing the great potential of the enzyme especially in the detergent formulation (Table 2).

CONCLUSION

Overall findings of this study revealed that the isolate *Bacillus sp* BC0016 was a novel strain identified as *B. flexus.* The organism was isolated from dairy industry soil located in U.P. (India) and identified through 16S rDNA gene sequencing; further the sequence was submitted to NCBI under accession number JX855288.1. Different physico chemical and cultural parameters greatly influenced the growth and protease production of the isolated strain. Enzyme also showed significant potential for the detergent formulation due to its stability against nonionic, anionic surfactants and commercially available detergents. The production process can be scaled up for commercial use, evaluation of the purified enzyme and its further characterization.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. S. Saxena, Dept. of Biotechnology, BBA University, Lucknow 226025 (U.P.), India for providing laboratory facilities and also support by research grants from UGC, New Delhi, India.

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