

## Identification of Alkaline Protease Producing *Bacilli* from Sludge of Bactofuge and Separator Using Culture–Based and Molecular Techniques

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### ABSTRACT

Dairy factories produce high volume of sludge from bactofuge and separator. Meantime, global demand for the proteases is increasing. Recently, utilization and conversion of the waste materials into value added product is a sustainable process. The objective of this study was to investigate the potential of bactofuge and separator sludge to produce alkaline protease enzymes. Total viable aerobic and anaerobic counts were determined on Plate Count Agar at 37 and 50°C for both types of sludge. Lactobacillus count in MRS Agar plates corresponded to  $3.12 \pm 0.25 \log \text{CFU mL}^{-1}$  for sludge of bactofuge and  $3.085 \pm 0.2 \log \text{CFU mL}^{-1}$  for sludge of separator. Mold and yeast had population levels of  $2.3 \pm 0.1 \log \text{CFU mL}^{-1}$  for bactofuge and  $2.08 \pm 0.1 \log \text{CFU mL}^{-1}$  for separator. Proteolytic bacteria were isolated from dairy sludge using Skim Milk Agar media. A clear zone of Skim Milk hydrolysis indicated protease-producing organisms. Different cultural parameters (temperature, pH, thermal shock, and kind of sludge) were optimized for maximal enzyme production. Maximum proteolytic activity was observed at 37°C ( $P < 0.05$ ). Isolated alkaline protease producing *Bacilli* were identified by Polymerase Chain Reaction (PCR). The species were identified as *Bacillus cereus* strain zk2, *Bacillus* sp. cp-h71, *Bacillus thuringiensis* strain ILBB224, and *Bacillus* sp. Bac6D<sub>2</sub>.

**Keywords:** *Bacillus*, Dairy sludge, Polymerase Chain Reaction, Proteolytic activity.

### INTRODUCTION

Spore-forming bacteria play an important role in quality maintenance of milk. In particular, spore-forming *Bacillus* spp. (Ozer and Yaman, 2014). Bactofugation is a commercial and effective method for physical removal of bacteria, spores and somatic cells from milk (Gesán Guiziou, 2010). Centrifugation is applied to separate components (microorganisms, fat globules, insoluble proteins, etc.) that have distinct densities compared to dispersing phase (Gesán Guiziou, 2010). The sludge can compose 0.05–0.1% of the milk volume, and contains 14–16% dry matter comprising nitrogen (6–8%), fat (0.25–0.35%), lactose

(4.7%), and non-milk substances (1.5–3%) (McCarthy, 2011).

Proteases are enzymes that catalyze the degradation of proteins (Hosseini *et al.*, 2016). Proteases are the most important industrial ones that perform a wide diversity of functions and have various important biotechnological applications. They constitute two-thirds of the total enzymes used in various industries and account for at least a quarter of the total global enzyme production (Mukesh Kumar *et al.*, 2012). Today proteases account for at least 60–65% of the total global enzyme market. It is reported that the global proteolytic enzyme demand will increase dramatically to 1.5–2.5 billion Dollars in the near future (Saran *et al.* 2007).

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Alkaline proteases (EC: 3.4.11-19) are one of the important proteases in industry with many applications including food industry, feeds modification, preparation of organic fertilizers, waste treatment, peptide synthesis, detergent, leather finishing, silk industry, brewing pharmaceuticals, diagnostic reagents, and silver recovery from X-ray/photographic films (Rathod and Pathak, 2016).

Although proteolytic enzymes can be obtained from plants and animals, microorganisms are preferred source of enzymes in industrial application because of their easy genetic manipulation, high efficiency, limited growing space requirement, wide biochemical variety and desirable characteristics of produced enzyme, such as stability that make them suitable for application of biotechnology (Hosseini *et al.*, 2016 ; Saran *et al.*, 2007). Bacterial proteases are mainly extracellular, easily produced in larger quantities, thermo stable, and active in a broad pH range of 4.0-12.0 (Asker *et al.* 2013).

Among bacteria, *Bacillus* sp. are producers of extracellular alkaline proteases specifically (Mukesh Kumar *et al.*, 2012 ) that contribute to the global market for industrial enzymes, because protease from *Bacillus* has been purified and characterized, and significant activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification and its low cost have been demonstrated .

Bioactive peptides are typically short amino acid sequences of 2–20 amino acid residues, which are generated by the hydrolysis of proteins (Bah *et al.*, 2016). Alkaline proteases from *Aspergillus flavus*, *Bacillus pseudofirmus* SVB1, and *Pseudomonas aeruginosa* PseA have demonstrated potential of peptide synthesis. Alkaline proteases from *B. pumilus* strain CBS and *Streptomyces* sp. strain AB1 are also in use in peptide synthesis in low water systems (Furhan and Sharma, 2014).

Elastoterase, a preparation with high elastolytic activity from *Bacillus subtilis*

316M, was also immobilized on a bandage for remedial application in the therapy of burns and purulent lesion, furuncles, carbuncles, and deep abscesses (Furhan and Sharma, 2014). Furthermore, *Bacillus* spp. have been known as being safe to humans (Furhan and Sharma, 2014).

The objective of this research was to identify alkaline protease producing *Bacilli* from sludge of bactofuge and separator with the help of culture –based and molecular techniques.

## MATERIALS AND METHODS

### Preparation of Samples

Samples of dairy sludge were collected from Pegah Factory in Mashhad, Iran. Samples were collected in clean sterile plastic containers and transferred to the laboratory under refrigeration. Culturing analyses were performed in the shortest period after arrival (Standard Iran No. 326).

### Culture-Dependent Approach

Sludge samples were diluted in sterile Ringer solution in a dilution of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and then plated on Plate Count Agar (PCA) (Mhone *et al.*, 2011). The counts for total aerobic bacteria were measured using the surface spread technique. Plates were incubated at 37 and 50°C for 24 hours. The counts for total anaerobic bacteria were determined using the pour plate method. Briefly, 1 mL of each of ten-fold dilutions of the sample (sludge of bactofuge and separator) was added onto sterile petri dishes in duplicate and mixed with 15 mL of molten Plate Count Agar (PCA) cooled to approximately 45°C. The set agars were incubated aerobically at 37 and 50°C for Mesophilic and thermophilic bacteria, respectively, and were counted after 24 hours.

Sludge samples were diluted similarly as described above. Agarified plates of MRS

were used for Lactobacillus counting. Plates were incubated at 37°C for 48-72 hours.

Agarified plates of Sabouraud Dextrose Agar (SDA) were used for this purpose. After dilution and culture, plates were incubated at 25°C for 24-48 hours.

Samples were suitably diluted with ringer solution and heated (15 minutes, 80°C) in a water bath to thermal shock (Harrigan, 1998 and Allaf, 2011). Heated and unheated samples, plated on the Skim Milk Agar with different pH of 9 and 10. The plates were incubated at 37 and 50°C for 24 hours. A clear zone of Skim Milk hydrolysis indicated protease producing organisms (Baur *et al.*, 2015). The strains with proteolytic activity were selected for further study.

After identification by using biochemical tests (Gram staining and catalase test), isolated strains were selected for further identification and stored as frozen at -80°C in BHI (Brain Heart Infusion) broth containing 20% glycerol (Edalatian *et al.*, 2012). The variables in our study included, Source of samples (sludge) in 2 levels: Bactofuge and separator; Types of samples in two levels: Heated and unheated samples; pH in 2 levels: 9 and 10; Incubation temperature in 2 levels: 37 and 50°C.

A parameter, relative halo size, was determined as follows (Adav *et al.*, 2009):

$$\text{Relative halo size} = \frac{D_{\text{halo}} - D_{\text{colony}}}{D_{\text{colony}}} \times 100$$

Where,  $D_{\text{halo}}$  is the Diameter of the halo formed around the colony (mm), and  $D_{\text{colony}}$  is the colony Diameter (mm)

### Molecular Identification

Strains were incubated at 37°C for 16 hours (Molva *et al.*, 2009) in 200 rpm. Then, DNA were extracted according to procedure of Denazist extraction DNA kit, Iran.

The primers used for the amplification of the 16S *rRNA* genes were 27FYM (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-GGTTACCTGTTACGACTT-3'), based on the conserved regions of the 16S *rRNA* gene. PCR was performed in 25 µL

reaction containing 1 µL of each the cell-free extracts, 12.5 µL Tag 2X Master Mix containing 1.5 mM MgCl<sub>2</sub> and 0.5 µL of each primer (Edalatian *et al.*, 2012).

PCR was carried out in a programmable heating incubator using an initial denaturation step at 95°C for 5 minutes followed by 35 cycles of 30 seconds at 95°C, 40 seconds at 58°C and 30 seconds at 72°C per cycle. Finally, a 10 minute extension at 72°C was performed.

Amplification products from PCR were subjected to electrophoresis in 1% agarose gels with syber green in 1X TBE buffer (Hermet *et al.*, 2014) for 30 minutes and 95V. Bands were visualized under UV light.

PCR products were purified and sequenced by MacroGen Sequencing Service (Korea) (Davati *et al.*, 2015). Sequences were comprised with those available in NCBI databases (National Center for Biotechnology Information, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

### Statistical Analysis

Each experiment was conducted in duplicate. All experimental data were analyzed using the General Linear Model (GLM) and Analysis Of Variance (ANOVA) procedure in order to assess the effects of incubation temperature (37 and 50°C), pH (9 and 10), heat shock (heated and unheated samples), type of sludge (sludge from bactofuge and separator) and their interactions using the Minitab software (Version 16).

## RESULTS AND DISCUSSION

### Total Microbial Count

Total count was determined on Plate Count Agar in both aerobic and anaerobic bacteria at 37 and 50°C for both types of sludge. Results are presented in Table 1.

**Table 1.** Total microbial count in Plate Count Agar as log CFU per mL.

| Kinds of sludge      | Separator |      |         |       | Bactofuge |       |         |      |
|----------------------|-----------|------|---------|-------|-----------|-------|---------|------|
|                      | Anaerobic |      | Aerobic |       | Anaerobic |       | Aerobic |      |
| Culture conditions   | 50°C      | 37°C | 50°C    | 37°C  | 50°C      | 37°C  | 50°C    | 37°C |
| Temperature          | 50°C      | 37°C | 50°C    | 37°C  | 50°C      | 37°C  | 50°C    | 37°C |
| Counting of colonies | 1.85±     | 4.06 | 2.11±   | 4.46± | 3.23      | 5.69  | 5±1     | 6.29 |
|                      | 0.12      | ±1.2 | 0.07    | 0.025 | ±0.67     | ±0.99 |         | ±1.5 |

Few microbial studies of raw milk and processed milk have been undertaken and, to our knowledge, this is the first microbiological description of sludge of bactofuge and separator, but a number of reports is published on microbiological description of raw milk and processed milk. Ozer and Yaman (2014) identified main groups of aerobic mesophilic microorganisms in raw milk which contains:

Spore-formers: *Bacillus* spp-Micrococci: *Micrococcus* and *Staphylococcus*.

Gram-positive rods: *Microbacterium*, *Corynebacterium*, *Arthrobacter* and *Kurthia*.

Streptococci: *Enterococcus*, *Streptococcus*, *S. Agalactiae*, *S. Dysgalactiae*, *S. Ueberis*.

Gram-negative rods: *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Enterobacter*, *Klebsiella*, *Aerobacter*, *Escherichia*, *Serratia*, *Alcaligenes*.

The total count for raw milk differed significantly ( $P < 0.05$ ) with the lowest ( $5.6 \pm 4.7 \log_{10}$  CFU mL<sup>-1</sup>) and highest ( $6.7 \pm 5.8 \log_{10}$  CFU mL<sup>-1</sup>) recorded from Marirangwe and Nharira, respectively. The mean total count of processed milk ( $6.6 \pm 6.0 \log_{10}$  CFU mL<sup>-1</sup>) were marginally higher than raw milk ( $6.4 \pm 5.6 \log_{10}$  CFU mL<sup>-1</sup>) but not significant ( $P > 0.05$ ) (Mhone *et al.*, 2011). Therefore, total count in sludge of bactofuge will be affected by microbial load of raw milk and is similar to it, because raw milk is transferred directly into bactofuge, but in separator, total count is lower.

In another study (Yavarmanesh *et al.*, 2007) conducted on predicting the microbiological quality of raw milk, total count of mesophilic bacteria reported approximately between 8-6 log cfu mL<sup>-1</sup> that is similar to total mesophilic count in sludge of bactofuge.

### Lactobacillus Count

Lactobacillus count in MRS Agar plates corresponded to  $3.12 \pm 0.25 \log$  CFU mL<sup>-1</sup> for sludge of bactofuge and  $3.085 \pm 0.2 \log$  CFU mL<sup>-1</sup> for sludge of separator.

In a study performed at the dairy factory in the routine cheese production, presumptive coccus LAB contained between 7.5 and 8.9 log CFU g<sup>-1</sup>, while presumptive rod LAB were in the range 6.2-8.2 log CFU g<sup>-1</sup> (Settanni *et al.*, 2013).

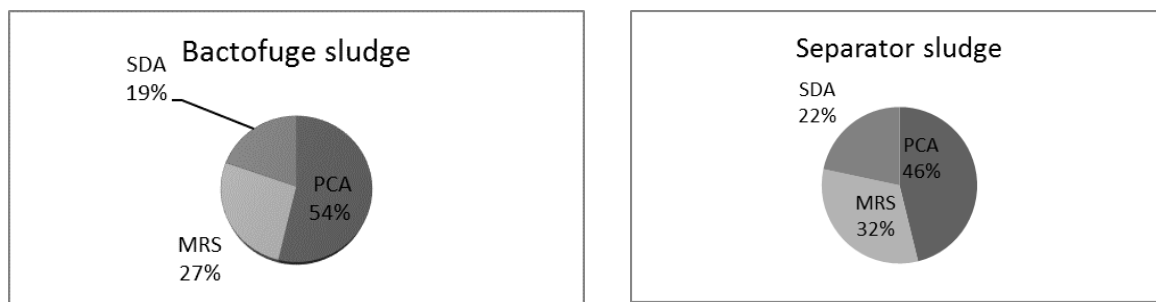
### Mold and Yeast Count

Sabouraud Dextrose Agar plates had population levels  $2.3 \pm 0.1 \log$  CFU mL<sup>-1</sup> for sludge of bactofuge and  $2.08 \pm 0.1 \log$  CFU mL<sup>-1</sup> for sludge of separator.

In another study conducted on microbiological contamination sources on swelling of Iranian yoghurt drink, total count in raw milk from three different factories were reported as  $3 \times 10^6$ ,  $3.4 \times 10^7$ , and  $4.5 \times 10^6$  (CFU mL<sup>-1</sup>). Yeasts count  $3 \times 10^3$ ,  $3.8 \times 10^4$  and  $2.6 \times 10^5$  CFU mL<sup>-1</sup> were reported (Mehrban Sang Atash *et al.* 2011). Total count in yoghurt drink was similar to sludge of bactofuge, but yeast count showed higher amounts than those in our study.

### Comparison between the Three Media

Lactobacillus strains in sludge constituted 27-32% and mold and yeast constituted 20-21% of total (mesophilic and thermophilic) count (Figure1).

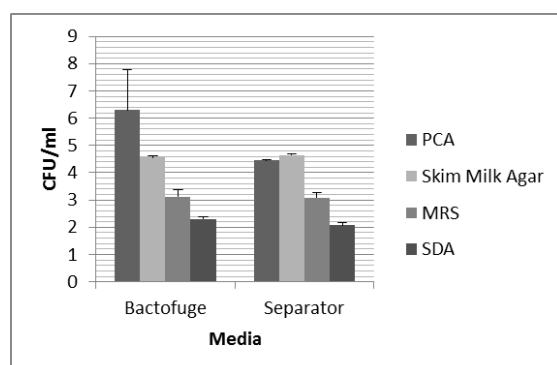


**Figure 1.** Comparison between the growth potential of bacteria in the sludge among three media including PCA and MRS and Sabouraud Dextrose Agar.

Almost all bacteria of milk (especially in separator) can grow in Skim Milk Agar (Figure 2) because this medium contains large amounts of casein similar to milk. Colony count in Skim Milk Agar was  $4.55 \pm 0.07$  for sludge of bactofuge and  $4.64 \pm 0.05$  for sludge of separator. Lactobacillus, mold and yeast counts in sludge of bactofuge were not much different with separators sludge but total count in sludge of bactofuge was more than separators sludge because the aim of bactofuging is reduction of microbes and spores from milk that reduce microbial load about 2 logarithmic units.

### Proteolytic Activity of Isolates

After completion of purification and biochemical tests, four isolates were screened and selected for molecular identification. Biochemical characteristics of the isolated strains are indicated in the Table 2. As



**Figure 2.** Distribution of mesophilic bacteria of sludge in different media.

relative halo size increases, the proteolytic activity yielded by colony unit volume increases too (Adav *et al.*, 2009).

The genus “*Bacillus*” is an important source of industrial alkaline proteases and is probably the only genus being commercialized for alkaline protease production (Furhan and Sharma, 2014). In Ercolini *et al.* (2009) study, only 13 Gram-negative isolates from raw milk displayed proteolytic activity on Milk Agar. Among gram negative isolates, the proteolytic strains were mainly *Pseudomonas* spp. These strains displayed the activity at two temperatures of 7 and 20 °C. Another study that was conducted on proteolytic of enzymes activity in stored aerobic granular sludge determined that phylogenetic analysis of 16S rDNA sequences demonstrated that the isolates were members of three principal groups: Proteobacteria, Enterobacteriaceae, and Firmicute. The most abundant isolates were Proteobacteria (70%), particularly of the *Pseudomonas* genus (Adav *et al.*, 2009). *Pseudomonas* spp. grow at low temperatures, but our study was not performed at low temperatures; so, any of the isolates were not *Pseudomonas*. (Figure 3).

Crystal morphologies of *B. thuringiensis* strains were found either spherical or spherical and irregular-shaped by phase contrast microscopy (Molva *et al.*, 2009).

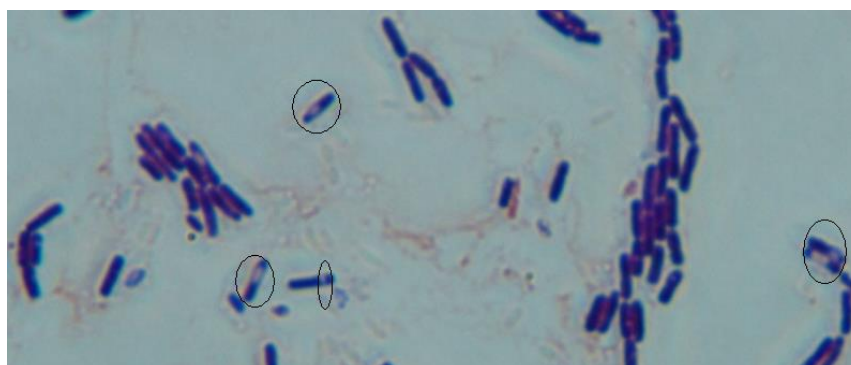
Results of the statistical analysis are shown in Table 3. Accordingly, the effect of temperature at 37°C was significant on enzyme activity ( $P < 0.05$ ) and on halo diameter ( $P < 0.01$ ). As a result, the mesophilic bacteria are preferred compared



**Table 2.** Biochemical characteristics of isolated bacteria.

| Halo diameter | Relative halo size (Enzyme activity) | Shape                         | Gram staining | Catalase test | Heat shock (80°C for 15 minutes) | Incubation temperature | pH | Type of sludge                      |
|---------------|--------------------------------------|-------------------------------|---------------|---------------|----------------------------------|------------------------|----|-------------------------------------|
| 14±0.5        | 10.7±1.35                            | Spore-forming <i>Bacillus</i> | +             | -             | -                                | 37°C                   | 9  | Bactofuge                           |
| 13±0.5        | 8±1.35                               | Spore-forming <i>Bacillus</i> | +             | +             | -                                | 37°C                   | 9  | Bactofuge                           |
| 6±3           | 20±10                                | Spore-forming <i>Bacillus</i> | +             | +             | -                                | 37°C                   | 10 | Bactofuge                           |
| 9±4.5         | 11±6.5                               | Spore-forming <i>Bacillus</i> | +             | +             | +                                | 37°C                   | 9  | Separator                           |
| 4             | 37.5                                 | Spore-forming <i>Bacillus</i> | +             | +             | -                                | 37°C                   | 9  | <i>Bacillus cereus</i> <sup>a</sup> |

<sup>a</sup> *Bacillus cereus* is index for protease activity.



**Figure 3.** View of Gram-positive sporulated bacteria under a microscope. Spores are shown with the circle.

to thermophilic bacteria for proteolytic activity in milk. The effect of pH 9 was significant only on halo diameter ( $P < 0.05$ ). Most proteolytic bacteria were isolated from unheated samples, but effect of heat treatment was not significant. There was no difference between sludge of bactofuge and separator in terms of statistical analysis for isolating proteolytic bacteria.

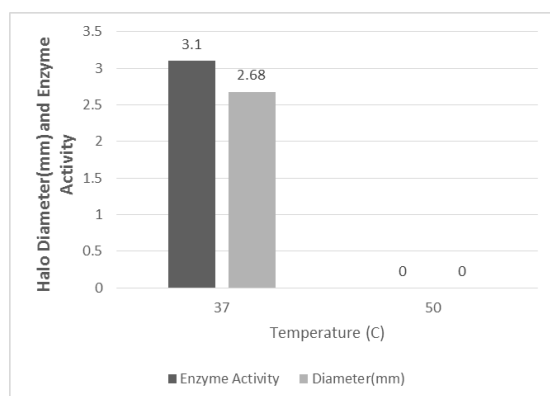
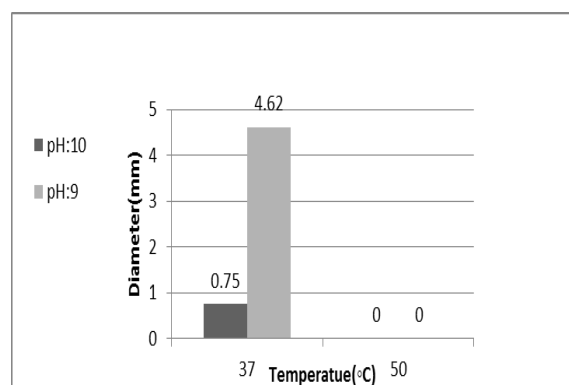
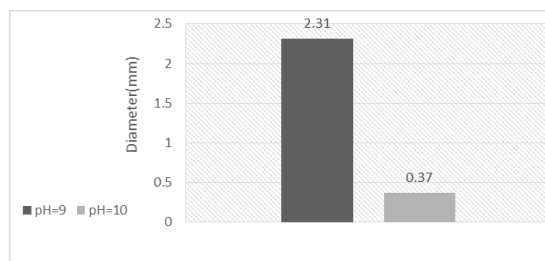
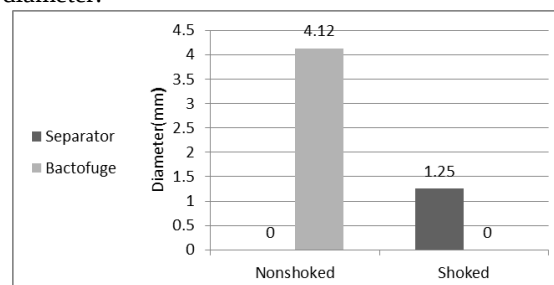
In Table 3, interactive effects between this four treatments are given in addition to the effects of incubation temperature, pH, heat shock, and sludge alone. Also, graphs were plotted for those treatments that  $P$ -values were significant in Figures 4 to 11. Effects of incubation temperature (37 and 50°C) on halo

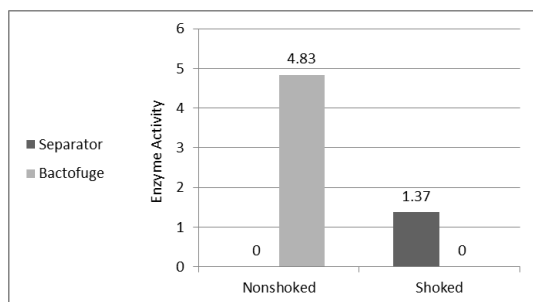
diameter and enzyme activity are shown in Figure 4. Protease activity at 37°C was significantly ( $P < 0.05$ ) higher than 50°C. Interaction effects of incubation temperature and pH (9 and 10) on halo diameter are shown in Figure 5. Halo diameter at 37°C and pH 9 was significantly ( $P < 0.05$ ) higher than others. Effects of pH on halo diameter are given in Figure 6. Interaction effects of heat shock (heated and unheated samples) and type of sludge (sludge from bactofuge and separator) on halo diameter and enzyme activity are shown in Figures 7 and 8. Interaction effects of incubation temperature, heat shock, and type of sludge on halo

**Table 3.** Analysis of variance and least squares means for halo diameter and enzyme activity.

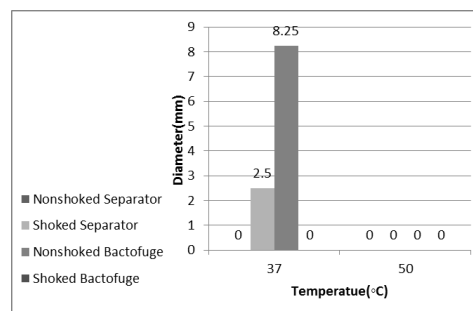
| Source                      | Diameter |         |         | Enzyme Activity |         |          |
|-----------------------------|----------|---------|---------|-----------------|---------|----------|
|                             | Mean     | SE Mean | P       | Mean            | SE Mean | P        |
| Incubation temperature (°C) |          |         |         |                 |         |          |
| 37                          | 2.6875   | 0.5173  | 0.002** | 3.1063          | 1.0158  | 0.046*   |
| 50                          | 0.0000   | 0.5173  |         | 0.0000          | 1.0158  |          |
| pH                          |          |         |         |                 |         |          |
| 9                           | 2.3125   | 0.5173  | 0.018*  | 1.8563          | 1.0158  | 0.0679ns |
| 10                          | 0.3750   | 0.5173  |         | 1.2500          | 1.0158  |          |
| Shock                       |          |         |         |                 |         |          |
| Shocked                     | 0.6250   | 0.5173  | 0.067ns | 0.6875          | 1.0158  | 0.246ns  |
| Non shocked                 | 2.0625   | 0.5173  |         | 2.4188          | 1.0158  |          |
| Sludge                      |          |         |         |                 |         |          |
| bactofuge                   | 2.0625   | 0.5173  | 0.067ns |                 | 1.0158  | 0.246ns  |
| separator                   | 0.6250   | 0.5173  |         |                 | 1.0158  |          |
| Temperature×pH              |          |         | 0.018*  |                 |         | 0.679ns  |
| Shock×Sludge                |          |         | 0.002** |                 |         | 0.046*   |
| Temperature×Shocked×Sludge  |          |         | 0.002** |                 |         | 0.046*   |
| pH×Shock×Sludge             |          |         | 0.002** |                 |         | 0.0679ns |
| Temperature×pH×Shock×Sludge |          |         | 0.018*  |                 |         | 0.0679ns |

\*: Significant ( $P > 0.05$ ); \*\*: Significant ( $P > 0.01$ ), ns: Not significant.

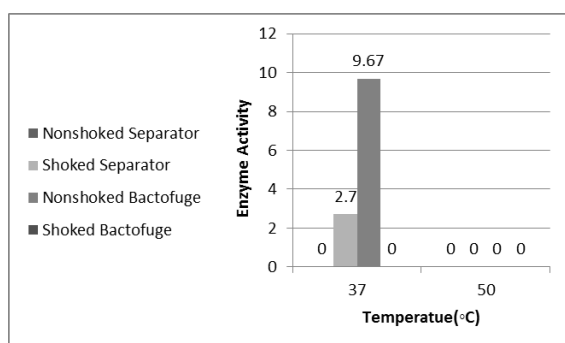
**Figure 4.** Effects of incubation temperature (37 and 50°C) on halo diameter and enzyme activity.**Figure 5.** Interaction effects of incubation temperature (37 and 50°C) and pH (9 and 10) on halo diameter.**Figure 6.** Effects of pH (9 and 10) on halo diameter.**Figure 7.** Interaction effects of heat shock (heated and unheated samples) and type of sludge (sludge from bactofuge and separator) on halo diameter.



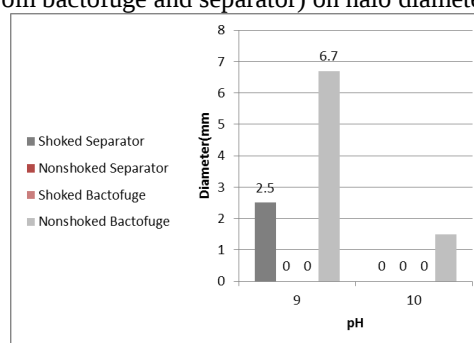
**Figure 8.** Interaction effects of heat shock (heated and unheated samples) and type of sludge (sludge from bactofuge and separator) on enzyme activity.



**Figure 9.** Interaction effects of incubation temperature (37 and 50°C), heat shock (heated and unheated samples) and type of sludge (sludge from bactofuge and separator) on halo diameter.



**Figure 10.** Interaction effects of incubation temperature (37 and 50°C), heat shock (heated and unheated samples) and type of sludge (sludge from bactofuge and separator) on enzyme activity.



**Figure 11.** Interactions effects of pH (9 and 10), heat shock (heated and unheated samples) and type of sludge (sludge from bactofuge and separator) on halo diameter.

diameter and enzyme activity are given in Figures 9 and 10. Interaction effects of pH, heat shock and type of sludge on halo diameter are shown in Figure 11. Alkaline proteases from *Aureobasidium pullulans*, *Yarrowia lipolytica*, *Issatchenkia orientalis* and *Cryptococcus aureus* with optimum pH of 9-10 and optimum temperature of 45-50°C have been reported for their excellent bioactive peptide production properties (Furhan and Sharma, 2014).

Gaur et al. (2014) found strain P-O2 as the best protease producer and it was identified as *Bacillus* sp. The isolated strain showed maximum protease production at 60°C, pH of 9.0 within 48 hours of incubation.

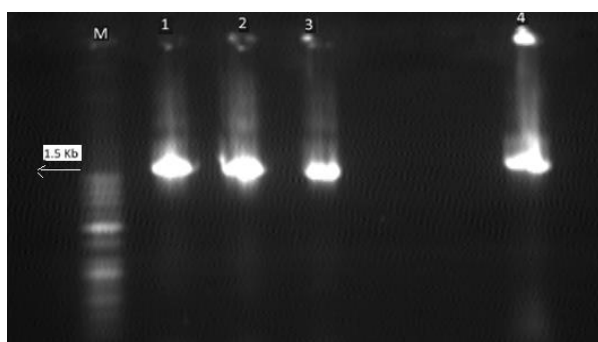
### Amplification of 16S rDNA Genes

In order to ensure creation of the band 1,500 bp, PCR products were

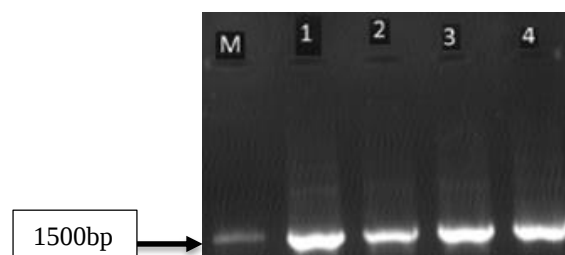
electrophoresed with a control sample that is shown in Figures 12 and 13.

### Sequencing Results

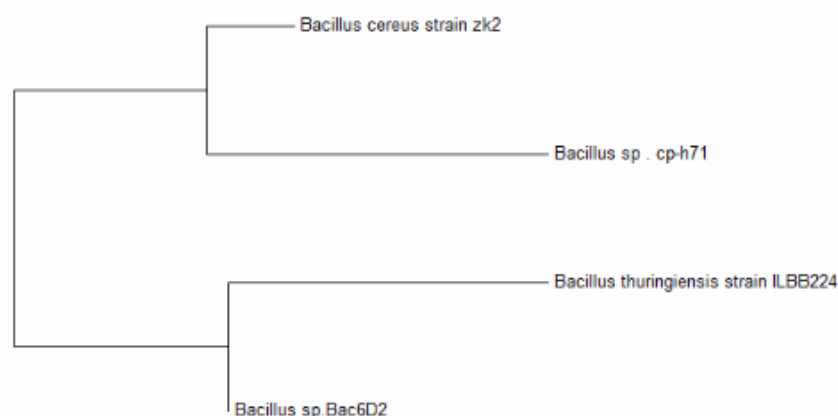
Molva et al. (2009) presented that *B. cereus* and *B. thuringiensis* strains were found to produce extracellular enzymes, respectively, gelatinase (83 and 91%), DNase (83 and 77%), lecithinase (83 and 95%), protease on Skim Milk Agar (100 and 100%), protease on Milk Agar (100 and 91%), protease on Casein Agar (83 and 77%), xylanase (100 and 45%), and cellulase (0 and 41%), and amylase (83 and 27%). The DNA sequence of the *B. cereus* thermolysin-like enzyme has a high degree of homology to the sequences of other metalloproteases, especially *B. thuringiensis* (Ghorbel et al., 2003). Alkaline protease has been reported from *Bacillus* sp. MPTK 712,



**Figure 12.** The PCR products on the gel electrophoresis (M: Ladder 100 plus).



**Figure 13.** The PCR products (1, 2, 3 and 4) along with M: The band equal to 1,500 bp on the gel electrophoresis.



**Figure 14.** Dendrogram of similarity of the different typing patterns clustered by Chromas, BioEdit and Mega 7.

**Table 4.** Identification of bacterial strain on the basis of 16S rRNA gene sequence similarity.

| Strain code | Accession no | % Similarity/Blast hit | Bacterial name                               |
|-------------|--------------|------------------------|----------------------------------------------|
| Bact1       | JQ773351.1   | 95%                    | <i>Bacillus cereus strain zk2</i>            |
| Bact2       | EU719665.1   | 98%                    | <i>Bacillus sp. cp-h71</i>                   |
| Bact3       | KT340483.1   | 97%                    | <i>Bacillus Thuringiensis strain ILBB224</i> |
| Bact4       | KF496112.1   | 88%                    | <i>Bacillus sp. Bac6D2</i>                   |

which has been isolated from dairy sludge. It has been reported that *Bacillus clausii* can be used profitably for large-scale production of alkaline protease to meet the present day demand of the industrial sector (Furhan and Sharma, 2014). In a study about protease producing bacteria from *Rhizosphere* soil, out of 20 bacterial strains screened, maximum protease producing strain was selected and identified as *Bacillus* (Patil *et al.*, 2015). In another study about alkaline protease producing bacteria, results showed that all the isolated bacterial strains were aerobic and

only 2 bacterial isolates were gram negative, while the rest of the isolates were gram positive (Saxena *et al.*, 2014). Numerous salt-tolerant proteases have been identified from the genera of *Pseudoalteromonas*, *Enterococcus*, *Bacillus* and *Alcaligenes* (Gohel and Singh, 2013). Alkaline proteases are also produced by some rare microorganisms. *Kurthiaspiroforme*, a spiral shaped gram-positive bacterium possessing a distant relationship to genus *Bacillus*, was reported to produce alkaline proteases. Mesophilic streptomycetes isolated from



Egyptian habitats have been reported showing protease activity (Furhan and Sharma, 2014). Ellaiah *et al.* (2002) reported that among the bacteria, this enzyme was produced mainly by many members belonging to genus *Bacillus*, especially, *B.licheniformis*, *B. Horikoshii*, *B. Sphaericus*, *Bacillus furmis*, *Bacillus alcalophilus*, and *Bacillus subtilis* (Akcan and Uyar, (2011). Mir Mohammad Sadeghi *et al.* (2009) reported that among the bacteria, many species of the genus *Bacillus* were widely used in industrial production of proteases. Hosseini *et al.* (2016) report that microbial alkaline proteases for industrial uses are produced and studied mainly from *Bacillus* and *Streptomyces* species.

## CONCLUSIONS

Sludge of bactofuge and separator possess alkaline protease-producing bacteria. All the isolated bacteria were identified as *Bacillus*, in agreement with the results of Mukesh Kumar *et al.* (2012) and based on other studies, among bacteria, *Bacillus*, in particular, produce extracellular alkaline protease. extracellular alkaline protease. In this study, the objective was to identify mesophilic and thermophilic alkaline protease producing bacteria, but more research needs to be done on these two sludge for extraction of alkaline protease or lipase to optimize use of these wastes.

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## شناسایی باسیلوس های مولد پروتئاز قلیایی حاصل از لجن باکتوفیوژ و سپراتور با استفاده از تکنیک های مبتنی بر کشت و مولکولی

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### چکیده

کارخانه های صنایع لبنی، حجم بالایی لجن از باکتوفیوژ و سپراتور تولید می کنند و این در حالی است که تقاضای جهانی برای استفاده از پروتئاز ها رو به افزایش است. اخیرا استفاده و ذخیره مواد ضایعاتی به محصولاتی با ارزش افزوده فرایندی پایدار است بنابراین هدف از این مطالعه بررسی پتانسیل این لجن برای تولید آنزیم های پروتئاز قلیایی بود. بار میکروبی کل بر روی محیط کشت Plate Count Agar برای باکتری های هوازی و بی هوازی اختیاری در دمای  $37^{\circ}\text{C}$  و  $50^{\circ}\text{C}$  در هر دو نوع لجن تعیین شد. شمارش لاکتوباسیلوس ها در محیط کشت MRS Agar برای لجن باکتوفیوژ و سپراتور به ترتیب برابر با  $3.12 \pm 0.25 \log \text{CFU/ml}$  و  $3.08 \pm 0.2 \log \text{CFU/ml}$  بود. کپک و مخمر سطح جمعیتی برابر با  $2.3 \pm 0.1 \log \text{CFU/ml}$  و  $2.08 \pm 0.1 \log \text{CFU/ml}$  برای این دو نوع لجن داشت. باکتری های پروتئولیتیک از لجن لبنی با استفاده از Skim Milk Agar جداسازی شدند. هاله ی روشن در اطراف کلنی نشانگر ارگانوسم تولید کننده ی پروتئاز در این محیط کشت است. پارامترهای مختلفی (دما، pH، شوک حرارتی و نوع لجن) برای تولید حداکثر تولید آنزیم بهینه شدند. بیشترین فعالیت پروتئولیتیک در دمای  $37^{\circ}\text{C}$  با ( $P < 0.05$ ) مشاهده شد. جدایه های *Bacillus* های مولد پروتئاز قلیایی به وسیله ی واکنش های زنجیره ای پلیمرز (PCR) شناسایی شدند. باکتری های شناسایی شده شامل *Bacillus thuringiensis* strain ، *Bacillus* sp . cp-h71 ، *cereus* strain zk2 ILBB224 و *Bacillus* sp. Bac6D2 بودند.