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# Potential application of *Bacillus pseudofirmus* SVB1 extract in effluent treatment

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ABSTRACT:

The wastewater generated from food industries like dairy and meat processing industries are rich in organic compounds and nutrients having high concentrations of BOD, COD and suspended solids. However, most of the conventional techniques used for treatment are not so eco-friendly. The eco-friendly approaches mostly employ biological aid but are usually ineffective in treating high organic load under extreme conditions. This study uses alkaline protease isolated from *Bacillus pseudofirmus* SVB1 to treat effluents from dairy industry and domestic sludge. The results obtained from this study indicate that both crude and partially purified alkaline protease performed significantly well in the treatment of effluents from dairy and domestic sewage. They were also able to effectively lyse both Gram positive and Gram negative bacteria. Both crude and partially purified enzyme procured from SVB1 performed efficiently when compared to the already available commercial enzymes used in this study. The efficiency of the extract reduces the overall cost of treatment, hence can potentially be used for treating effluents, enhancing the economy of the entire process.

## 1. Introduction

The dairy industries generate a considerable amount of wastewater which are rich in organic molecules and nutrients (Farizoglu and Uzuner, 2011; Hena et al., 2015; Hung et al., 2005; Kothari et al., 2013; Shete and Shinkar, 2013). The fat content of these wastewaters are usually significantly high and the proteins present have a low biodegradability coefficient. This leads to high biological oxygen demand (BOD) and chemical oxygen demand (COD), hence posing a threat to the environment. Numerous pretreatment processes are implemented to eliminate oil and grease from the wastewater to prevent complications associated with its discharge. However, the efficiency of the treatment is often compromised due to the reduced cell-aqueous phase transfer rates, sedimentation barrier caused due to the growth of filamentous microorganisms, development and floatation of sludge with poor activity and clogging co-occurring with release of an unpleasant pungent odour. The implementation of pretreatment protocols to hydrolyze and dissolve the conjugated lipids, improves the biological degradation of fatty wastewaters, hence promotes process efficiency.

However, conventional techniques of wastewater treatment which employ biological aid are not suitable to treat such complex effluents (Pell and Wörman, 2011), because of the excessively high salinity and organic load with suspended solids (sand, lime, hair, flesh, dung, etc.). These extreme conditions will most likely have a negative toll on the biological organism being used, thus substantially reducing the efficiency of the entire process. Alkaline protease is extremely stable under excessive alkaline and saline concentration. Moreover, they have been reported to be active under influence of high metal ion concentrations (Chittoor et al., 2016; Yilmaz et al., 2016). Till date a massive amount of work has been done elucidating the degradation of complex biomolecules in effluents using enzymatic hydrolysis. Alkaline protease isolated from *B. subtilis* has been used for the degradation of waste feathers from poultry slaughterhouses (Zaghloul et al., 2011). A formulation comprising proteolytic enzymes from *B. subtilis*, *B. amyloliquefaciens, Streptomyces* sp. and a disulfide reducing agent (thioglycolate) is available commercially (Gupta et al., 2002), which has been greatly used to clear pipes which have been clogged with hair deposits. Although much progress has been made in terms of degradation of organic waste using alkaline protease, there still remains a need to enhance this process. Usage of exteremophilic organisms can combat

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this problem to a large extent.

The degradation of sludge mostly requires long sludge retention time and cannot degrade organic carbon effectively. The first step towards anaerobic digestion is hydrolysis, which is also the rate limiting step (Watson et al., 2004). The Enzymatic treatment can improvise the solubility of the sludge solids, thus enhancing the anaerobic digestion (Diak et al., 2012). This in turn alters the rate limiting step and leads to process efficiency and hence speeds up the system. To improve the efficiency further, large amount of effluent substrate must be available to the microorganism for digestion and breakdown (Roman et al., 2006). This is done by disintegration of large, organic particles into smaller ones, thus multiplying the surface area exposed to the bacteria. Floc structure disruption can also be used to render floc matrix trapped organic material bioavailable. The enhancement of sludge digestion by reduced particle size was supported by the experiment conducted by Onyeche et al. who reported an increase in biogas production and COD removal (Onyeche et al., 2002).

Extracellular enzymes including, protease, lipase and cellulase can lyse pathogens and several other bacteria (Ruggaber and Talley (2006); song and feng (2011); Arora et al., 2014)). Alkaline protease when added to wastewater significantly reduced its coliform and *Salmonella* counts (Parmar et al. (2001)). In another study, alkaline protease isolated from *Bacillus proteolyticus* assisted the lysis of pathogenic strains (Bhaskar et al., 2007). Reduction of the solid content in sewage effluent, decreases the volume of sludge that requires dewatering and disposal. This in turn significantly reduces a part of COD in sludge liquor.

Keeping in mind the above factors, the potential of using alkaline protease from *B. pseudofirmus* SVB1 for the treatment of dairy and domestic effluents was investigated with respect to the removal of COD, BOD and total solids (TS). The effect of this alkaline protease on human and plant pathogens has also been studied.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The chemicals and reagents used are tabulated in Table 1.

### 2.2. Microorganisms and culture conditions

From the premises of tannery industry *Bacillus pseudofirmus* SVB1 was isolated (Sen, 2010). Every 2–3 weeks cultures were regenerated on freshly prepared nutrient agar plates from the frozen stock culture. To test the antimicrobial activity of SVB1 *Bacillus coagulans* NRRL NRS 609 (Gram-positive) and *Pectobacterium carotovorum* MTCC1428 (Gram-negative), *Serratia marcescens* MTCC 97 (Gram-negative) and *Wautersia eutropha* NRRL B-2804 (Gram-negative) were used. Except SVB1 all the species were grown in nutrient broth/agar at 28 °C and pH 7.0.

### 2.3. Inoculum preparation and alkaline protease production

To 50 ml of LB broth, (pH 9.5) a loop full of sterile culture was added in a 250 mL Erlenmeyer flask. The system was then incubated on a shaker incubator at 28 °C for 10 h 1 mL of this culture ($A_{600}$~1 OD) was added to 100 mL having an initial pH of 9.5. The only carbon and nitrogen source provided was casein. At a regular interval samples were withdrawn and growth of the microorganism was measured. The culture was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant or the cell free extract was used as the crude preparation of alkaline protease for this study. Partially purified alkaline protease (PPE) was obtained from acetone fractionation. (Sen et al. (2011)).

### 2.4. Effluent treatment

The two different effluents collected were allowed to settle for 12 h. After this pretreatment, 98 ml of each effluent was added to 2 ml of partially purified and crude alkaline protease from SVB1, commercial protease P-2380/P-8038 and active cells of *Bacillus pseudofirmus* SVB1 in log phase ($A_{600}$~1.0). The negative controls used were distilled water and distilled water/borax-NaOH buffer (pH 10.0). Sterile distilled water was added to adjust the volume of control and experimental flaks to 100 ml. They were then incubated in a shaking incubator at 30 °C. The samples were subjected to total solid (TS), chemical oxygen demand (COD) and biological oxygen demand (BOD) assay after and before a 90 h incubation period.

### 2.5. Domestic sewage sludge solubilization

Well homogenized sludge (35 ml) collected from the domestic sewage treatment plant located at IIT Guwahati was taken in 250 ml Erlenmeyer flasks. With respect to their concentration initial total suspended solids (TS) in both the flasks were equalized. Alongside the sludge, 10 ml of crude alkaline protease (185 U/ml, specific activity 36.9 U/mg) was added. Sterile distilled water was added to adjust the final volume to 100 ml and incubated in a shaking incubator at 30 °C. To study the change in sludge volume with respect to time, a control flask was used which contained distilled water instead of the enzyme.

### 2.6. Antibacterial activity

#### 2.6.1. Colony forming units count

Besides solubilizing sludge, alkaline protease might be able to reduce the pathogen load significantly during sewage treatment. To determine the effect of alkaline protease treatment on pathogenic strains in effluent sample, microbial count was performed by enumeration of the colony forming unit (cfu) per ml of the effluent, before and after the treatment. To obtain developed colonies, from each experimental flask 100 μl of serially diluted domestic sewage samples were plated in triplicate on nutrient agar plate and incubated at 37 °C for 24 h. This was done to enumerate the total viable colony count in terms of colony forming units per mL (cfu/mL).

#### 2.6.2. Plate studies on zone of inhibition

Disc diffusion method was used to investigate the antibacterial activity of crude alkaline protease against *Pectobacterium carotovorum* MTCC 1428, *Serratia marcescens* MTCC 97 and *Wautersia eutropha* NRRL B-2804. To ensure confluent growth, a sterile cotton swab was used to streak the microbial suspensions in their mid log phase ($A_{600} \approx 0.5$) over the agar surface. 10 μl aliquot of crude alkaline protease from SVB1 was spotted on Whatman No. 1 paper discs (6 mm) and then aseptically placed on the agar plate surface, and incubated at 30 °C for 12 h.

#### 2.6.3. Scanning electron microscopy (SEM) studies

After treatment with the various alkaline proteases mentioned above, the bacterial cells were analyzed to distinguish changes caused in their morphology due to the enzymatic treatment. 100 μl crude alkaline protease from SVB1 was added to serially diluted ($10^{-4}$) mid log phase ($A_{600} \approx 0.5$) cultures of *Bacillus coagulans* (NRRL NRS 609), *Pectobacterium carotovorum* MTCC 1428, *Serratia marcescens* MTCC 97 and *Wautersia eutropha* NRRL B-2804. These were then incubated at 30 °C for 24 h. After incubation, the cells were washed twice with distilled water after centrifugation at 5000 rpm for 15 min. The cell pellets thus obtained were transferred onto the SEM stub. The pellets were dehydrated in water–alcohol solutions at varying alcohol concentrations (30%, 50%, 70%, 80%, 90% and 100%) for 10 min. These samples were then dried and analyzed using a SEM (LEO 1430VP).

### 2.7. Analytical techniques

#### 2.7.1. Determination of TS, BOD, COD and settleability

For determination of TS, 2 ml of the effluent sample was well mixed

**Table 1**
Chemicals and Reagents and their respective sources.

| Chemicals and Reagents | Source |
|---|---|
| Salts and Media Constituents | Central Drug House, India |
| TCA, Tris, HCl, Borax | Merck, India |
| Biological grade Casein, Agar | Himedia, India |
| Commercial Protease Enzymes (P5380, P4860, P8038, P5459) | Sigma Aldrich |
| Domestic Sewage | IIT Guwahati sewage treatment plant |
| Dairy Effluent | Central Dairy, Guwahati, Assam, India |

**Table 2**
Characteristic of effluents from dairy and domestic sewage were studied in the present work.

| Parameter | Dairy effluent | Domestic waste |
|---|---|---|
| pH | 7.1 ± 0.2 | 7.1 ± 0.3 |
| TS (mg/l) | 5000 ± 400 | 181 ± 9 |
| COD (mg/l) | 1850 ± 40 | 154 ± 8 |
| BOD (mg/l) | 860 ± 31 | 88 ± 4 |

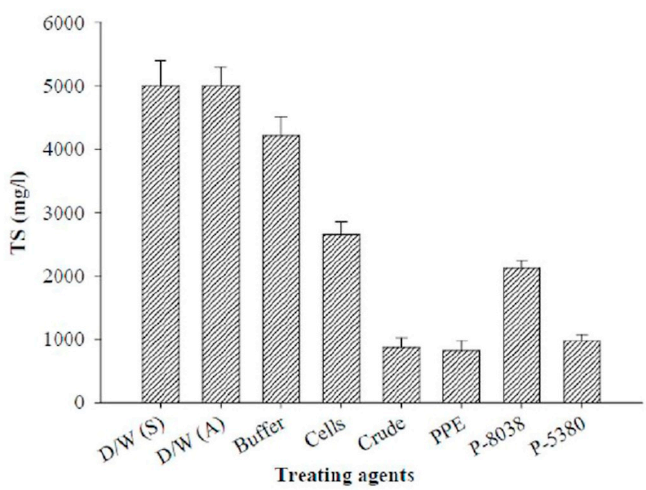


**Fig. 1.** Reduction in TS of the dairy effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30 °C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

and centrifuged at 7000 g for 10 min. The supernatant was discarded and the residue was washed with 2 ml of water and centrifuged again for 10 min. The solids were then transferred to a pre-weighed petri dish and were maintained at 105 ± 2 °C till they completely dried. The BOD and COD were determined using standard techniques (APHA/AWWA/WEF, 2012).

Settleability of the domestic sewage was determined by measuring the volume of settled solids. 100 mL of sludge sample was taken in measuring cylinder and was allowed to settle at room temperature for 2 h. The volume was recorded and presented as milliliters of settled solids per 100 mL total sample.

## 3. Results and discussion

### 3.1. Characteristics of effluents

The comparison of physicochemical analysis of untreated effluents from dairy and domestic sewage are shown in Table 2. Maximum

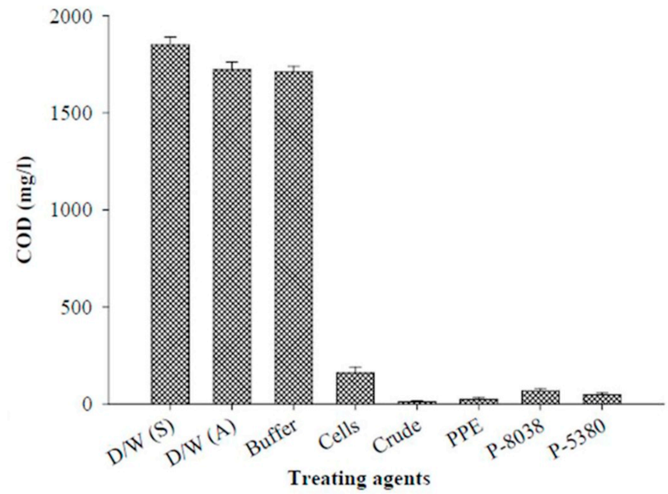


**Fig. 2.** Reduction in COD of the dairy effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30 °C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

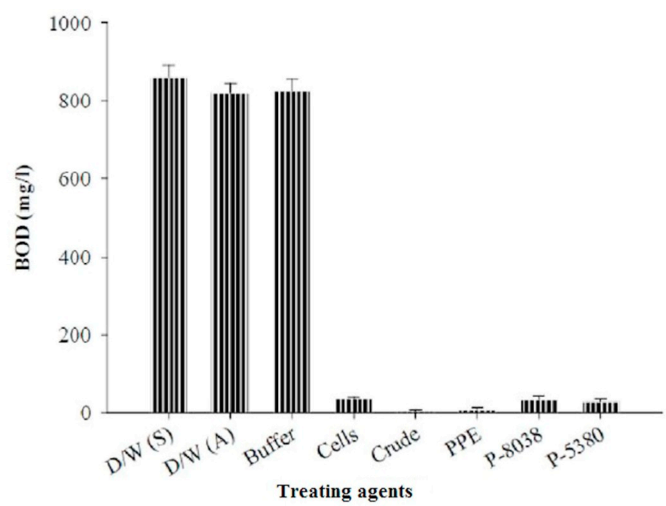


**Fig. 3.** Reduction in BOD of the dairy effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30 °C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

organic load was observed in dairy effluents. Domestic wastewater also contained a significant amount of organic load (both dissolved and suspended matter). On the contrary the industrial effluents contained much more organic load. TS, COD and BOD reduction by using various treating agents as mentioned in 2.4.

### 3.2. TS, COD and BOD reduction in dairy effluent

The reduction of TS, COD and BOD in dairy effluent with different treating agents (live cells, partially purified enzyme and commercially available purified enzymes) is represented in Figs. 1–3.

The partially purified alkaline protease from SVB1 resulted in maximum reduction in TS (from 5000 to 1000 mg/l) and the crude alkaline protease from SVB1 was a close second. P-5380 also effectively reduced TS to 19%. However, in presence of the viable cells of SVB1 and P-8038, TS decreased only to 53% and 42%, respectively. The low

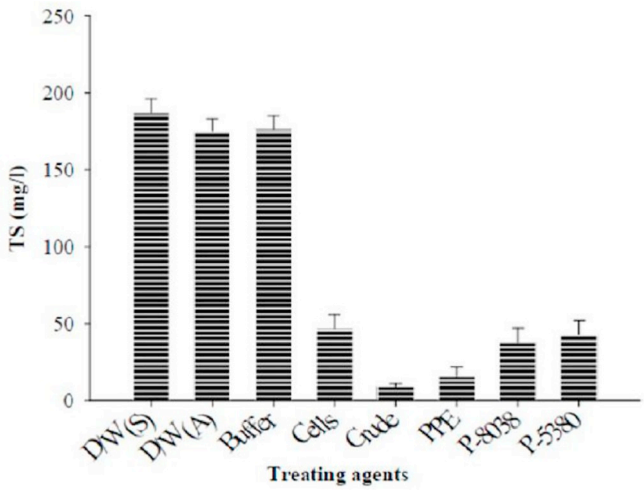


**Fig. 4.** Reduction of TS of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30 °C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

efficiency of the first may be due to the increase in biomass. The crude alkaline protease from SVB1 resulted in maximum reduction of COD after treatment (reduced to 0.6%) (Fig. 2).

The other alkaline proteases (partially purified from SVB1, P-8038 and P-5380) could also reduce COD to a large extent (1.3, 3.6 and 2.5% of the COD before treatment, respectively). In all the experimental flasks there was remarkable reduction of BOD (less than 4% of the original BOD). However, among the samples studied, crude alkaline protease was the most efficient (Fig. 3).

Typically the wastewater generated form the dairy industries rarely contain any toxic chemicals, like the ones listed under EPA's Toxic Release Inventory. However, the effluent contains significant concentration of dissolved organic compounds like whey proteins, lactose, fat and minerals, which generates foul smell on decomposing. (Mukhopadhyay et al., 2003).

The components of the dairy effluents mainly consist of the raw

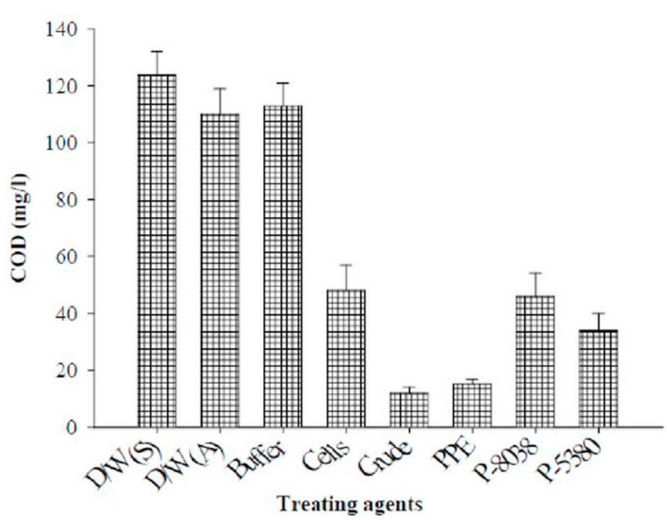


**Fig. 5.** Reduction of COD of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30 °C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

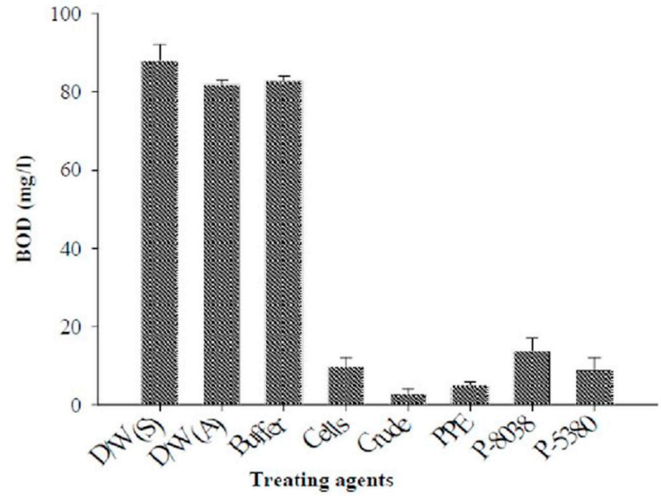


**Fig. 6.** Reduction of BOD of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30 °C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

materials used, mixed with processing water. These raw materials are lost subsequently at each step of the industrial process (handling, processing and cleaning). Thus, the effluents before being discharged into the natural waterbodies should be carefully treated because of the high oxygen demand. The primary treatment of the dairy effluent can be carried out by an anaerobic hybrid reactor which resulted in 95% removal of COD (Rajesh Banu et al., 2008). In a study conducted earlier, the efficiency of COD removal achieved was 90% higher in a series of batch reactors (Prokopov et al., 2014). Previous studies used lipases and protease to treat dairy effluent, primarily to eliminate high concentration of lipid/fat and they demonstrated positive results (Lamas et al., 2001; Leal et al., 2006). However, in this study the extremophilic nature of the alkaline protease has attributed to its higher efficiency in treating dairy waste water.

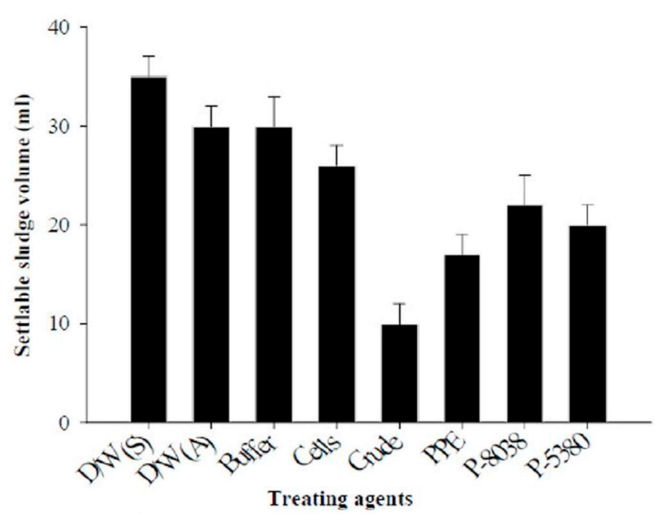


**Fig. 7.** Reduction of settlable sludge volume (SSV) of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30 °C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

**Table 3**
Microbial count (cfu/ml) of the domestic sewage effluent after treatment with different treating agents.

| Sl. No. | Treating agents | Microbial count (cfu/ml) |
|---|---|---|
| 1 | D/W (S) | $15 \times 10^4 \pm 0.02 \times 10^4$ |
| 2 | D/W (A) | $18 \times 10^4 \pm 0.03 \times 10^4$ |
| 3 | Buffer | $17 \times 10^4 \pm 0.01 \times 10^4$ |
| 4 | Cells | $12 \times 10^3 \pm 0.03 \times 10^3$ |
| 5 | Crude | $4 \times 10^2 \pm 0.05 \times 10^2$ |
| 6 | PPE | $6 \times 10^2 \pm 0.04 \times 10^2$ |
| 7 | P-5380 | $10 \times 10^2 \pm 0.09 \times 10^2$ |
| 8 | P-8038 | $9 \times 10^2 \pm 0.06 \times 10^2$ |

**Table 4**
Zone of clearance (mm) of the pathogenic microorganisms studied.

| Pathogen | Zone of Clearance (mm) |
|---|---|
| *P. carotovorum* | 25 |
| *S. marcescens* | 21.5 |
| *W. eutropha* | 28 |

### *3.3. TS, COD and BOD reduction and sludge solubilization in domestic sewage*

The reduction in TS, COD and BOD of domestic sewage indicated the extent to which the different treating agents (viable cells, partially purified enzyme and commercially available purified enzymes) solubilized the sludge. The results have been illustrated in Figs. 4–7.

The maximum reduction in TS was achieved with crude enzyme from SVB1 (Fig. 4). TS reduction via usage of partially purified alkaline protease from SVB1 was seen to give positive results, though not comparable to the crude extract. P-8038, P-5380 and the viable cells of SVB1 also showed a high efficiency in reducing TS to 20, 23 and 25%, respectively. On the contrary, crude alkaline protease from SVB1 achieved maximum reduction in COD (9.7%) (Fig. 5). The efficiency of the partially purified alkaline protease from SVB1 in COD reduction (12%) was appreciable enough, while the other alkaline protease *viz.*, P-8038 and P-5380 were not that efficient (37 and 27%, respectively). Use of crude alkaline protease led to most efficient decrease of BOD (3.4%) when compared to the other experimental cases (Fig. 6). The sludge settleablity volume (SSV) has been reduced to less than 10 mL by the crude protease extract from SVB1, while the partially purified extract reduced the SSV to almost 15 mL (Fig. 7). However, the other enzymes and live cells used in the study struggle to make a significant impact in reducing SSV.

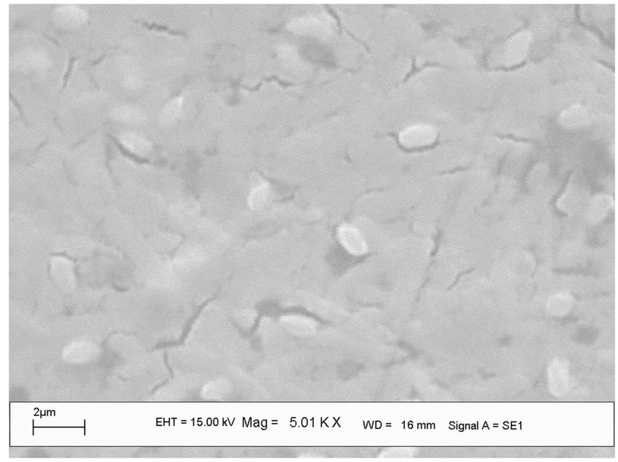


**Fig. 8(b).** P. carotovorum treated with the alkaline protease from SVB1.

These results demonstrate that a primary purification step isn't necessary for reducing sludge volume, hence reducing the treatment cost. Thus, explaining the potential of alkaline protease used in this study. The abundance of protein in sewage sludge and its capacity to bind flocs and form matrices has already been illustrated (Charles et al., 2017; Frølund et al., 1995). The elimination of protein with alkaline protease weakens the floc, which elucidates the function of proteases in solid settling (Higgins and Novak, 1997).

### *3.4. Antibacterial activity*

The subsequent utilization of the sewage sludge requires efficient implementation of pathogen reduction techniques to eliminate the risks of disease transmission (Dumontet et al., 1999). However, there still remains a considerable amount of risk as the anaerobic and aerobic sludge digestion processes in sewage treatment plants cannot destroy pathogens effectively (Parmar et al., 2001). Alkaline proteases used for treating dairy and domestic sewage also have a lytic activity on certain microorganisms hence reducing the cfu count in the treatment streams. Table 3 gives the microbial count in terms of colony forming unit (cfu/ml) after treatment with the treating agents as mentioned in 2.4.

The colony count from the effluent before and after treatment was found to be similar. Its effect on different potential human and plant pathogens were studied. In all three plates a clear zone of clearance could be seen, the diameter of these zones have been tabulated in Table 4. This study also demonstrates how alkaline protease from SVB1

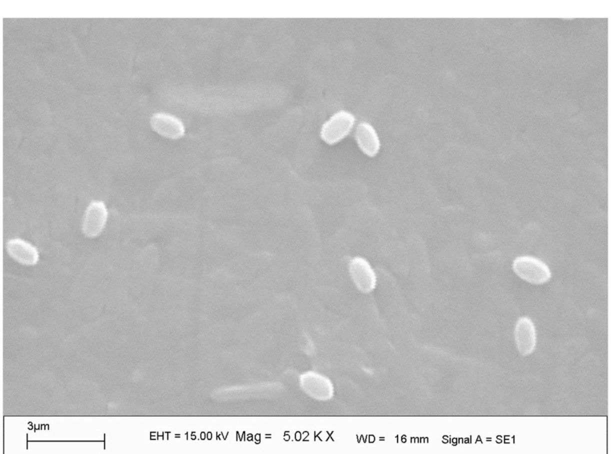


**Fig. 8(a).** Untreated P. carotovorum.

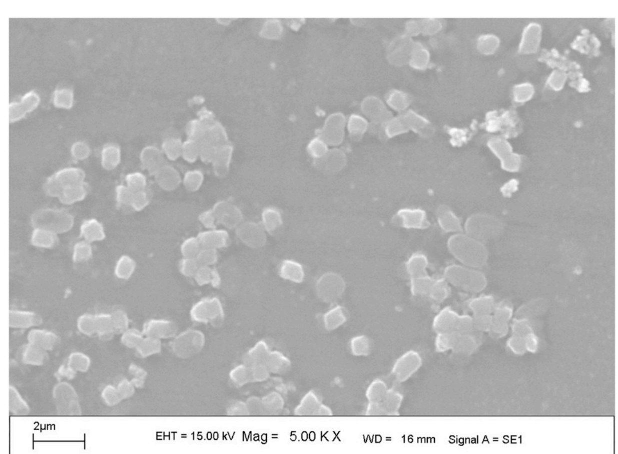


**Fig. 8(c).** Untreated *S. marcescens*.

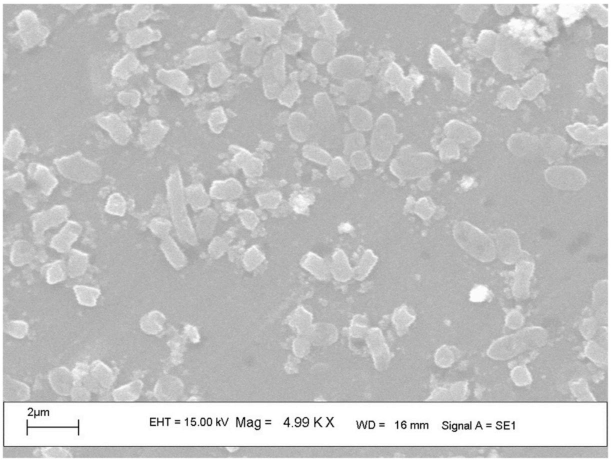


**Fig. 8(d).** *S. marcescens* treated with the alkaline protease from SVB1.

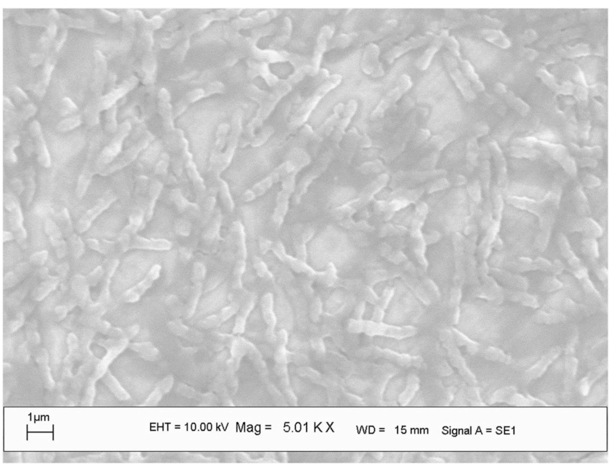


**Fig. 8(e).** Untreated W. eutropha.

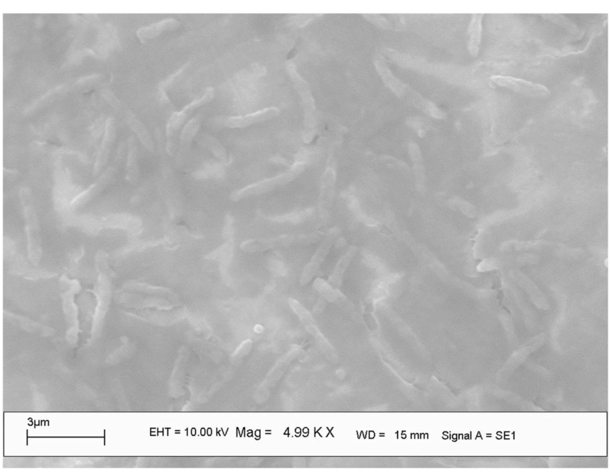


**Fig. 8(f).** W. eutropha treated with the alkaline protease from SVB1.

strongly inhibits the growth of pathogenic microorganisms, *P. carotovorum*, *S. marcescens* and *W. eutropha*.

Enzymes like lysozyme, which helps in lysing the gram-positive bacteria by cell wall lysis have negligible effect on gram-negative bacteria owing to the presence of an outer envelope hence difficult to hydrolyze (Slein and Logan, 1967). Pavlova et al. reported that vegetative cell walls of gram-negative organisms could be lysed by enzyme complexes isolated from thermophilic soil organisms (Pavlova et al., 1987). Alkaline protease isolated from *Bacillus* sp. has been shown to efficiently lyse *E. Coli* (Dean and Ward, 1991). In conclusion, alkaline protease is a potent enzyme in lysing pathogens, thus making it a suitable alternative to treat wastewater. The changes in morphological characteristics of the protease treated organisms (*B. coagulans*, *P. carotovorum*, *S. marcescens* and *W. eutropha*), as revealed by SEM are presented in (Fig. 8a, 8b, 8c, 8d, 8e, 8f, 8g and 8h).

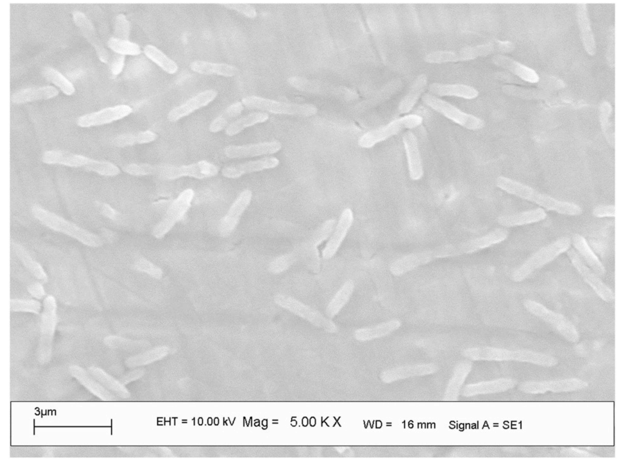


**Fig. 8(g).** Untreated B. coagulans.

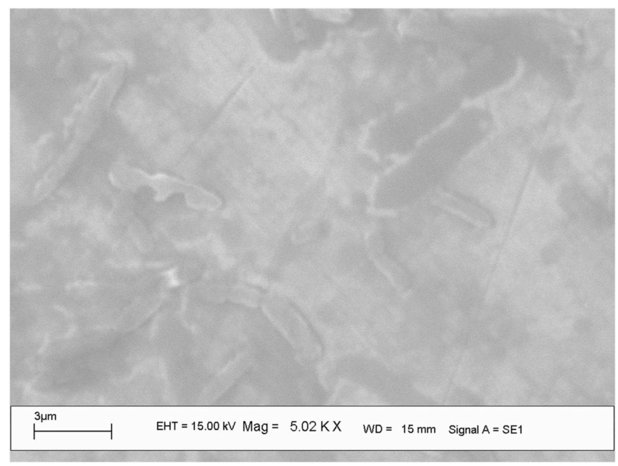


**Fig. 8(h).** B. coagulans treated with the alkaline protease from SVB1.

## 4. Conclusions

The work presented here helps draw certain definitive conclusions with respect to waste water/effluent treatment. Both partially purified and crude alkaline proteases performed remarkably well in the treating effluents from dairy and domestic sewage. The reduction of TS, COD and BOD in all the effluents was achieved within 48 h using the same enzyme of varying purity and 72 h with the viable cells of SVB1. The control flask did not show any significant reduction in TS, COD and BOD even after 90 h of incubation.

The results showed that the alkaline protease form *Bacillus pseudofirmus* SVB1 efficiently disintegrated the sludge structure thus improving the degradation of organic matter in the digestion step, leading to better dewater ability, better compaction and less volume resulting in reduced transport costs and easier handling of the sludge after dewatering step. Both partially purified and crude enzymes performed efficiently in reducing microbial count in domestic sewage. The enzyme was also able to lyse and inhibit growth of both Gram positive and

Gram negative species (*B. coagulans*, *P. carotovorum*, *S. marcescens* and *W. eutropha*).

The combined effect of *Bacillus pseudofirmus* SVB1 extract from SVB1 in pathogen reduction, solids reduction and improved dewatering (solids settling) hence proves that it can be a potential agent for sewage sludge processing.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101250.

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