Bacillus licheniformis proteases as high value added products from fermentation of wastewater sludge: pre-treatment of sludge to increase the performance of the process

M. Drouin*, C. K. Lai*, R.D. Tyagi* and R.Y. Surampalli**

*Institut National de la Recherche Scientifique, centre Eau, Terre et Environnement, Université du Québec, 490 de la Couronne, Quebec, G1K 9A9, Canada

** US EPA, P.O. Box-17-2141, Kansas City, KS 66117, USA
E-mail: mathieu.drouin@ete.inrs.ca; tyagi@ete.inrs.ca
Phone : (418) 654 2617; Fax : (418) 654 2600

Abstract: Wastewater sludge is a complex raw material that can support growth and protease production by Bacillus licheniformis. In this study, sludge was treated by different thermo-alkaline pre-treatment methods and subjected to Bacillus licheniformis fermentation in bench scale fermentors under controlled conditions. Thermo-alkaline treatment was found to be an effective pre-treatment process in order to enhance the proteolytic activity. Among the different pre-treated sludges tested, a mixture of raw and hydrolysed sludge caused an increase of 15% in the protease activity, as compared to the untreated sludge. The benefit of hydrolysis has been attributed to a better oxygen transfer due to decrease in media viscosity and to an increase in nutrient availability. Foam formation was a major concern during fermentation with hydrolysed sludge. The studies showed that addition of a chemical anti-foaming agent (polypropylene glycol) during fermentation to control foam could negatively influence the protease production by increasing the viscosity of sludge.

Keywords: alkaline protease, Bacillus licheniformis, pre-treatment, wastewater sludge.

INTRODUCTION

Most of the enzymes market is related to hydrolytic type of enzymes such as proteases, lipases and the cellulases (Rao et al., 1998). The proteases alone represent 60% of the total sales of enzymes (Gupta et al., 2005). At present, the largest part of the hydrolytic enzymes market is occupied by the alkaline proteases, mainly because of their use in detergents. The alkaline proteases resulting mainly from Bacillus licheniformis are used in detergents and occupy a large portion of the market (Godfrey and West, 1996). Although use of enzymes has many advantages, the competitiveness of the enzymes compared to the chemicals is limited by their higher production costs. The culture medium is responsible for 30 to 40% of the production costs (Kumar and Parrack, 2003). The use of a less expensive culture medium and concomitant stimulation of protease production/yield would thus considerably lower the production cost. Hence, the use of tertiary matters as alternative substrates, available in large quantities and at less cost, is an interesting option.

Municipal wastewater sludge (a rich source of carbon, nitrogen, phosphorus and other nutrients required for growth and production) can be an interesting low-cost culture medium. Actually, technologies have been developed or are under development to produce biopesticides, biofertilizers or many other value added products from sludge (Barnabé et al., 2003). Drouin et al. (2007) showed that municipal wastewater sludge can be a suitable medium to support growth and protease production by Bacillus licheniformis. In fact, sludge fermentation in fermenters resulted in proteolytic activity comparable to conventional synthetic medium.

The characteristics of sludge, specifically nutrient content plays an important role in the process performance. Drouin et al. (2007) confirmed a concomitant increase in proteolytic activity with sludge suspended solids concentration (SS) from 20 to 35 g SS/L. The maximum proteolytic activity was obtained in a sludge containing
35 g SS/L. However, further increase in SS to 40 g/L resulted in a decrease of proteolytic activity. These results showed that the sludge concentration cannot be increased indefinitely. In highly concentrated sludge, the reduction in enzyme activity was due to either inhibition by the substrate or low oxygen transfer capacity in the culture medium caused by high viscosity associated with high concentrated sludge (Bar et al., 2004).

Meanwhile, wastewater sludge is a complex and heterogeneous material containing different types of organic matter that has been classified as easily biodegradable, biodegradable, difficult to biodegrade and non-biodegradable (Tirado-Montiel et al., 2003). Different techniques have been tested to increase the biodegradability of difficult-to-biodegrade material that constitutes wastewater sludge (Ben Rebah et al., 2001). Several methods of sludge pre-treatment such as thermal and chemical treatments by acidification, alkaline and peroxide hydrolysis have been introduced in our laboratory (Barnabé, 2004). Among these methods, it has been demonstrated that thermal alkaline sludge hydrolysis is a suitable way to break larger molecules into smaller ones and thus improved the rheology of sludge, providing better oxygen transfer and better nutrient assimilation for Bacillus thuringiensis based biopesticide production (Barnabé, 2004; Bar et al., 2004; Yezza et al. 2005). The main objective of this study was to determine the potential of using different thermo-alkaline pre-treated sludges for the production of proteases by Bacillus licheniformis. The study was conducted in fermentors under controlled parameters.

MATERIAL AND METHODS

Bacillus licheniformis strain

Bacillus licheniformis ATCC 21424 (Bl) was used for all the experiments in this study. An active culture was maintained by streak inoculating nutrient agar plates (3 g/L of beef extract, 5 g/L peptone and 15 g/L agar) and incubating at 35°C for 48 h. The plates were stored at 4°C for later use.

Sludge samples and medium composition

The wastewater secondary sludge samples were collected from Quebec municipal wastewater treatment plant (Communauté Urbaine de Québec, Quebec, Canada). The experiments were conducted with settled sludge at SS concentration of 35 g/L.

Sludge pre-treatment

Four different types of sludge were used in this study.

Raw sludge (RAW): Secondary sludge was settled by gravity settling to increase the suspended solids concentration in order to obtain the desired SS concentration.

Thermal alkaline hydrolysis of sludge (TAH): the sludge was concentrated by centrifugation (> 60 g/L suspended solids concentration). The pH was adjusted to 10 by adding sodium hydroxide. Thermo-alkaline pre-treatment was conducted in 10 L working volume hydrolysers at 100 rev/min stirrer speed and 140 °C for 30 min. Thermal energy was conveyed into the sludge by direct steam injection. Steam injection provided direct thermal contact with the sludge; however, it increased sludge volume, owing to condensation of a part of the injected steam and reducing the suspended solids (SS) concentrations to around 40 g/L. The concentration of sludge was further adjusted to the desired value.

Mix of raw and hydrolysed sludge (MIX): Mixed sludge contained an equal amount of raw sludge (50%) and the thermo-alkaline treated sludge (50%).

In-situ hydrolysis (ISH): The desired suspended solids concentration was adjusted; the pH was brought to 10 followed by sterilization in fermentor at 121°C for 30 minutes. This way the sludge was hydrolyzed as well as sterilised in one step.

Inoculum preparation

An inoculum prepared in two steps was found to be optimal in a previous study (Drouin et al., 2007). A starting culture was prepared in 100 ml of nutrient broth whose composition was as follows (g/L): beef extract - 3, peptone - 5. The preparation was carried out in an Erlenmeyer of 500 ml. The medium pH was adjusted to 7.5.
by addition of 4N NaOH or 4N H₂SO₄ and then autoclaved (121°C, 15 min). The sterilised medium was inoculated with a loopful of *B. licheniformis* grown and stored on nutrient agar. The Erlenmeyers were incubated at 35°C under a constant agitation of 250 revolutions per minute (rpm) for 12 h in an incubator-shaker (Lab-Line, Dubuque, Iowa, USA). A 2% (v/v) of this broth (first stage inoculum) was used as seed culture to inoculate 500 ml Erlenmeyer flask containing 150 ml of the same medium as the fermentation substrate (raw or pre-treated sludge) (second stage inoculum). The suspended solids concentration used to grow the inoculum was 15 g/L. The flasks were then incubated in a rotary shaking incubator at 35°C and at 250 rpm for 12 hours. Finally, a 4.5% (v/v) of inoculum volume of the actively growing cells of the pre-culture was transferred to the fermentor.

**Fermentation procedure**

The experiments were carried out in two fermentors (15 L) (Biogénie Inc, Quebec, Quebec, Canada) equipped with agitation, aeration, temperature, pH, foam and dissolved oxygen (DO) control systems. The fermentors were filled with 10 L of culture medium, 20 ml solution of polypropylene glycol (Sigma-Aldrich, Canada) was added to fermentor as anti-foam agent during sterilization. Sterilization was carried out in situ at 121°C for 30 minutes. After cooling to 35°C, the fermentors were inoculated. Fermentations were carried out at 35°C for 48 h. Agitation was controlled between 200 and 500 rpm whereas an aeration rate between 2 – 6 litre per minute (LPM) was used to keep the DO level above 20% of saturation. The pH was automatically controlled at 7.5 using either sterile solutions of sodium hydroxide (NaOH, 4 M) or sulphuric acid (H₂SO₄, 3 M) through computer controlled peristaltic pumps. The foam was controlled by a mechanical foam breaker or by the addition of a solution of 20% (v/v) polypropylene glycol (PPG). Samples were drawn from the fermentor at regular intervals to determine cell and spore counts and protease activity.

**Analytical**

**Cell and spore counts** To determine viable cell and spore count of *B. licheniformis*, the samples serially diluted with sterile saline solution (0.85% w/v NaCl). The appropriately diluted samples (0.1 ml) were plated on TSA plates and incubated at 35°C for 24 h to form fully developed colonies. For spore count, the appropriately diluted samples were heated in a silicone bath at 80°C for 10 min and then chilled on ice for 5 min. The cell and spore counts were estimated by counting colonies grown on nutrient agar medium. For all counts, the average of at least three replicate plates was used for each count. For enumeration, 30 to 300 colonies were enumerated per plate. The results were expressed as colony forming units per ml (CFU/ml). In order to establish the reliability and reproducibility of the plate count technique, 10 independent samples were drawn (at the same time) from the shake flask experiment and were serially diluted and plated. Each dilution was plated in three different plates and the colonies were counted and the standard deviation was calculated. The calculated standard deviation was 6%.

**Protease activity assay** Protease activity was determined according to Kunitz (1947) with minor modifications. Samples collected from the fermentor were centrifuged at 7650 g for 20 min at 4°C. The supernatant was appropriately diluted with borate buffer, pH 8.2. Alkaline protease activity was assayed by incubating 1 ml of properly diluted enzyme solution with 5 ml of casein solution (1.2% w/v) (Sigma-Aldrich, Canada) for 10 minutes at 37°C in a constant temperature water bath. The reaction was terminated by adding 5 ml of 10% trichloroacetic acid (TCA). This mixture was incubated for 30 min in order to precipitate the total non-hydrolysed casein. Parallel blanks were prepared with inactivated casein. At the end of this incubation period, the samples as well as the blanks were filtered using Whatman paper 934-AH (Whatman Inc, USA). The absorbance of the filtrate was measured at 275 nm using a Cary 100 Bio UV-Visible spectrophotometer (Varian Techtron Pty. Ltd., Australia). Validation of the results was established by treating a standard enzyme solution of known enzymatic activity, in the same way and under the same conditions. One protease activity unit was defined as the amount of enzyme required to liberate 1 μmole (181μg) of tyrosine from casein per minute at pH 8.2 and 37°C. Statistical treatment of the results showed a maximal deviation of 5%.
RESULTS AND DISCUSSION

Evolution of viable cells and spore counts, proteolytic activity and viscosity during the fermentation course in raw sludge and in the pre-treated sludges are illustrated in Figure 1. The values of various parameters for the four types of sludge are reported in Table 1. Different pre-treatment had a significant impact on the growth of BI and proteolytic activity. In case of raw and hydrolysed sludge, the exponential growth phase lasted during the first 18 h. In mixed and in-situ hydrolysed sludge, the exponential growth phase was shorter, lasting until 15 h (Fig 1). The highest maximum specific growth rate was obtained in the MIX sludge, whereas the minimum value was recorded in raw sludge. The maximum cell count was observed in raw sludge. The profiles of viable spores obtained showed that there was no significant increase in the spore counts during the exponential phase. In all kinds of sludge, the beginning of sporulation phase coincided with the end of exponential growth phase or beginning of the stationary phase (Fig. 1). The maximum spore count was observed in mixed sludge. The maximum protease activity (13.0 I.U./mL) was observed in the mixed sludge. The minimum value (9.8 I.U/mL) was obtained in TAH sludge. For all types of pre-treated sludge, practically no protease activity was observed during the exponential growth phase. The increase in the proteolytic activity coincided with the transition from exponential growth phase to stationary phase and continued increasing until 36-42 h, followed by a slight decrease. However, in raw sludge, the production of proteases began earlier (9 h) whereas in the pre-treated sludge the production of protease was delayed (12 h). For all types of sludges tested, the viscosity decreased during the fermentation (Fig 1). The highest viscosity values were obtained in TAH sludge and the lowest in the IS (in-situ hydrolysed) sludge.

The results showed that there is no direct relation between cell count and proteolytic activity, as reported by Drouin et al. (2007). Even if the maximum cell counts were obtained in raw sludge, the highest proteolytic activity values were obtained in MIX pre-treated sludge. There is also no clear relation between spore count and proteolytic activity. In all media, the production of protease activity begins when the cells have already entered in sporulation phase (Fig. 1). Many workers suggested an indirect relation between the sporulation and proteases production (Kumar et al., 1999; Mabrouk et al., 1999; Gupta et al., 2000). However, Fig. 1 reveals that increase in protease activity continued even after the spore count reached plateau. In fact a substantial increase in protease activity could be noticed after cessation of spore count increase, implying a complex relation between spore production and protease activity.
Evolution of total count (TC), viable cells (VS) (a), proteolytic activity (b) and viscosity (c) during Bacillus licheniformis growth in different pre-treated sludges.

Table 1. Bacillus licheniformis process performance in pre-treated and raw sludge

<table>
<thead>
<tr>
<th>Sludge type</th>
<th>Total count max (x 10^9 UFC/mL)</th>
<th>Spore count max (x 10^7 UFC/mL)</th>
<th>μm (h⁻¹)</th>
<th>Proteolytic activity (U.L./mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW</td>
<td>3.34</td>
<td>9.57</td>
<td>0.33</td>
<td>10.4</td>
</tr>
<tr>
<td>TAH</td>
<td>1.27</td>
<td>8.20</td>
<td>0.36</td>
<td>9.8</td>
</tr>
<tr>
<td>MIX</td>
<td>3.21</td>
<td>10.20</td>
<td>0.44</td>
<td>13.0</td>
</tr>
<tr>
<td>ISH</td>
<td>2.23</td>
<td>9.39</td>
<td>0.47</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Thermo-alkaline pre-treatment of sludge causes hydrolysis of proteins, polysaccharides, lipids and other intracellular macromolecules secreted from disrupted sludge cells and significantly enhances the amount of dissolved organic matter from wastewater sludge. This phenomenon makes the carbohydrates, proteins and other nutrients accessible for Bl growth. Thus, difficult-to-degrade compounds present in sludge were transformed into easily degradable ones. Furthermore, enhanced nutrient availability and biodegradability in pre-treated sludge favoured the growth of the bacteria (Yezza et al., 2005). This could explain the higher maximum specific growth rate values calculated in MIX and IS pre-treated sludge, in comparison with the raw sludge (Table 1).

The pre-treated sludge also had significant impact on proteolytic activity. At the beginning of fermentation, Bl consumes easily assimilable substrates contained in sludge. The presence of these substrates can have an inhibiting (during exponential growth phase) effect on protease production. When easily assimilable substrates were consumed, the inhibition on enzyme production was alleviated (Yezza et al., 2005). The proteases were eventually produced and secreted in the medium by the bacterial cells. The highest amount of easily assimilable substrate present in hydrolysed sludge could explain the early production of proteases (9 h) in raw sludge, as compared to pre-treated sludge where the protease production was delayed (12 h).
Additionally, as discussed earlier, sludge is a complex and heterogeneous material containing difficult to biodegrade organic matter. Thermo-alkaline pre-treatment of sludge can cause the hydrolysis of non-biodegradable matter into more biodegradable forms. Enhanced nutrient biodegradability in pre-treated sludge can stimulate the bacteria to produce higher amount of proteases. This could explain why the proteolytic activity values obtained were higher in MIX and ISH pre-treated sludge, in comparison to raw sludge. However, further systematic study is needed to understand the exact role of hydrolysis on nutrient availability and their quality. Meanwhile, Brar et al. (2004) showed that after sludge thermal-alkaline hydrolysis, the viscosity substantially decreased which simplified mixing and enhanced oxygen transfer. As the viscosity decreases, air in the fermentor fluid becomes better distributed in the form of small bubbles throughout the process (Yezza et al., 2005). The benefit of small bubbles is slow rising velocities which keep air bubbles in the liquid longer, allowing more time for the oxygen to dissolve. Calik et al. (2000, 2004) showed that oxygen transfer is a key parameter for the production of protease. In ISH sludge, lower viscosity, combined with a better nutrient availability, could have contributed to a better protease production, in comparison with the raw sludge.

During the fermentation, the combined effect of agitation and aeration can contribute to foam formation. With MIX sludge, the formation of foam was particularly significant and difficult to control during fermentation. Formation of foam was further intensified with the use of TAH sludge. In case of TAH sludge, the addition of a large quantity of anti-foam agent (PPG) was necessary from the beginning and it has a great impact on the viscosity of the medium. This explains why viscosity increased during the first hours of fermentation (Fig. 1). The addition of anti-foam was also necessary throughout the fermentation, which explains why viscosity remained much higher than in raw sludge throughout the fermentation, whereas Brar et al. (2004) showed low viscosity in thermo-alkaline hydrolysed sludge. The higher viscosity of TAH sludge would have caused to decrease the capacity of transfer of oxygen of the medium, which would explain the low values of proteolytic activities obtained in TAH sludge. Similarly, With MIX sludge, the addition of anti-foam agent could explain why the viscosity values were slightly higher in comparison to those measured in raw-sludge.

A better aeration and agitation strategy should be followed in order to minimize foam formation and to reduce the need of adding chemical anti-foaming agent during the fermentation especially when pre-treated sludge is used as a raw material. A repetition of these experiments with a better oxygen control strategy is therefore required in order to evaluate the real potential of pre-treated sludge. Furthermore, operation parameters (pH, time of treatment) of thermo-alkaline hydrolysis and other sludge pre-treatment methods (oxidative, ultrasonication, ozonation) need to be investigated.

CONCLUSIONS

The thermo-alkaline pre-treatment of sludge was found to be promising to enhance the proteolytic activity. A mixture of raw and hydrolysed sludge and in situ pre-treated sludge resulted in an appreciable increase of protease activity compared to the untreated or raw sludge. The increase of proteolytic activity in mixed sludge was mainly due to an enhanced nutrient availability and biodegradability in the sludge. For the in situ pre-treated sludge, the decrease in viscosity and hence better oxygen transfer could also have played a role in an increase of proteolytic activity. The addition of excessive anti-foam agent (PPG) during the fermentation had a great negative impact on proteolytic activity during the fermentation.

ACKNOWLEDGEMENTS

Sincere thanks are due to the Natural Science and Engineering Research Council of Canada (Grant A 4984, strategic grant and Canada Research Chair) for their financial support. The views and opinions expressed in this paper are those of the authors and should not be construed as the opinions of the U.S. Environmental Protection Agency.
REFERENCES


