Production of thermostable protease enzyme in wastewater sludge using thermophilic bacterial strains isolated from sludge

J. P. Chenel, R. D. Tyagi and R. Y. Surampalli

ABSTRACT

The volume of sludge produced annually is very high and poses serious disposal problems. The traditional methods of sludge disposal produce secondary pollutants. Therefore, the alternate or suitable solution is reuse of sludge in an ecofriendly approach. Biotechnology is an interesting tool to add value to the processes involved in wastewater and wastewater sludge disposal/reuse. In this context, a study was carried out on thermophilic bacterial strains that produce thermostable proteases. The bacterial strains were first isolated from municipal wastewater sludge. In contrast to the conventional strains used in industries, like *Bacillus sp.*, the new strains were Gram-Negative type. In semi-synthetic medium, a maximal protease activity of 5.25 IU/ml (International Unit per ml) was obtained at a pH of 8.2 and at a temperature of 60 °C, which is higher than the stability temperature of 37 °C for a similar protease obtained from the conventional producer *Bacillus licheniformis*. Moreover, growth and protease activity of the strains were tested in wastewater sludge. It is expected that the complexity of sludge could stimulate/enhance the protease production and their characteristics. In conclusion, reuse of wastewater sludge will help to reduce their quantity as well as the value-added products produced will replace chemical products used in industries.

Key words | thermophilic strains, thermostable proteases, wastewater sludge

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INTRODUCTION

The disposal of wastewater sludge (WWS) generated during the treatment of the various municipal and industrial wastewater (WW) is one of the major problems in safe-guarding of the planetary environment. During the last fifty years, people believed that the discharge of WW and WWS in the rivers and the lakes was not detrimental to the environment because the latter had the capacity to self purify and regenerate themselves. However, studies during the last twenty years have contradicted these facts. At the present time, the rivers and the lakes do not have the capacity to absorb all the pollutants which are poured there (Barnabé *et al.* 2003). It thus became imperative to find new methods in order to decrease and/or re-use these wastes. Several of these new methods have been developed in recent past and others are being developed.

Traditionally, the wastewater sludge is land filled, applied to agriculture land as fertiliser or incinerated and a very small part is used for value added products. Recently, technologies have been developed or are under development to produce fuel, biopesticides, biofertilisers or many types of biocatalysts (Barnabé *et al.* 2003). Indeed, certain micro-organisms have the capacity to produce secondary substances at the time of their growth. Tyagi and co-workers (2002) showed that the bacterial strains of *Bacillus sp.* could produce proteolytic enzymes and that certain species as *Bacillus thuringiensis* produced a secondary metabolite to

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serve as insecticide. It was also shown that fungi had also the capacity to produce certain enzymes (Szekeres *et al.* 2004). The WW and WWS are rich in nutrients such as carbon, nitrogen, potassium (Barnabé *et al.* 2003) and can support growth of various micro-organisms to produce many interesting bioproducts.

When the sludge is not land applied, it is generally land filled. On the other hand, if these wastes are converted through microbial fermentation, it is possible to eliminate or stabilize the pathogens, helps to precipitate or degrade toxic chemical compounds (Champagne 2006) and at the same time possibly producing useful secondary substances or value added products.

In Canada, in 2001, 16 836 968 inhabitants were served by water and wastewater treatment facilities (Champagne 2006). Considering a production of biosolids or wastewater sludge of 0.063 kg of dry sludge per person per day, there would be a dry production of 387 166 tons of dry sludge per year. One of the best ways of sludge valorization is bioconversion into proteolytic enzymes. Indeed, in industries these enzymes (the proteases) are produced by cultivating microorganisms in synthetic medium. The cost of the culture medium corresponds to approximately 60–80% of the total production cost of the enzymes (Kumar & Takagi 1999). The sludge is a medium that is available everywhere, in sufficient quantity and allows a good bacterial growth. Thus, the enzyme production cost using wastewater sludge as a raw material could be substantially reduced (Chenel 2004).

The bacterial strains that conventionally used in industries are of *Bacillus sp*. They are mesophilic bacteria, having optimal growth around temperature of 35 °C and the produce proteases which are stable at an average temperature of 37 °C. Moreover, it is also possible to use thermophilic bacteria which have optimal growth temperature of higher than 45 °C. The proteases produced by these bacteria are known as thermostable and are effective at average temperatures of 60 °C. The most important advantages of these proteases are that they are more resistant and hence their effectiveness is prolonged. They better resist the changes in environmental conditions in which they are used.

The industrial enzymes represent a major part of the global enzymes market and their production is rapidly increasing. The enzyme production in 1982 was of 375 M$, reached 720 M$ in 1990, 1 billion $ in 1994, 1.9 billion $ in 1996, 2.5 billion in 2001 and approximately 2.8 billion in 2002 (Tyagi 2004). And the proteases account approximately 60% of this market (Gupta *et al.* 2002).

One of the major use of alkaline proteases is in detergents. In addition to detergents, the slaughter-houses, the tanneries are the sectors for use of these proteases. They could be useful in maintenance of apparatuses like in the production of a marketable product. The production of thermostable proteases using wastewater sludge as raw material would make it possible to market them at low production cost. At the same time, it would be possible to reduce the quantity of sludge for final disposal. The use of these proteases would also make it possible to reduce the use of harmful chemicals for the environment because these chemicals can be replaced by these newly produced proteases. Thus, the objective of the present work is to demonstrate the effectiveness of the production of thermostable proteases by thermophilic bacteria using municipal wastewater sludge as a culture medium.

MATERIALS AND METHODS

Collection of sludge samples

Sludge samples were obtained from three different locations: municipal wastewater treatment plants of Jonquière, Black Lake and Communauté Urbaine de Québec. Sludge samples were stored in a cold chamber at 4 °C for future use (within two weeks to minimise sludge degradation).

Isolation of new bacterial strains

The bacterial strains were isolated from three wastewater sludge: Jonquière, Black Lake and Communauté Urbaine de Québec. Each sludge sample was inoculated on nutrient agar plates and incubated at 50 °C for 24 hours. All bacteria were purified on nutrient agar plates.

Determination of optimal temperature for bacterial growth, enzyme production and stability

To determine the optimal temperature for bacterial growth and enzyme production, the bacterial isolates were

inoculated into 100 mL of semi-synthetic medium in 500 mL Erlenmeyer flasks and incubated for a period of 24 hours at 40, 50 and 60 °C in an orbital incubating shaker at 200 rpm. Composition of semi-synthetic medium (g/L) is: Soy flour, 15; dextrose, 5; corn starch, 5; KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.02; $CaCO_3$, 1. Samples of 0.5 mL were drawn at three hours intervals to determine viable cell count and protease activity.

To determine the optimal temperature for enzyme activity, broth samples (grown at 50 °C) were drawn at the end of 24 hours. This sampling time was chose to leave enough time for bacteria to grow and begin the production of proteases. The enzyme activity was determined at 40, 50, 60 and 70 °C. The enzyme activity test was performed by the modified Kunitz method (Kunitz 1947). In assay tubes, 5 mL of casein solution (1.2% casein in borate buffer pH 8.2) was incubated in a shaker water bath for 5 minutes. One mL of the appropriately diluted enzyme sample was added to the casein solution and incubated for 10 minutes. The reaction was terminated by adding 5 mL of trichloroacetic acid (TCA) and the solution (total volume of 11 mL) was left for 30 minutes in the water bath at same temperature. A blank (control) sample was also prepared by adding TCA prior to the enzyme addition. All samples and blanks were filtered with a vacuum pump using 0.45 μm filter. The absorbance of the filtered samples was measured at 275 nm. The temperature stability of the enzyme was tested at different temperature. It was varied from 40 °C to 70 °C.

Total cell count (TC)

Each broth sample taken from each Erlenmeyer flask was diluted from 10^3 to 10^7 times and 100 μL of these dilutions were inoculated on nutrient agar medium. After 24 hours of incubation at 50 °C the cell count was performed as colony forming units (CFU). Only those plates were counted which contained 30 to 300 CFU with a percent error of ±5%.

Bacterial growth and enzyme production in fermentor

Fermentation experiments were conducted in a 15 L bench scale bioreactor equipped with accessories and automatic control systems for dissolved oxygen, pH, antifoam, agitation speed, aeration rate and temperature.

The fermentation was conducted at pH 7.0 (controlled automatically by using 2N NaOH or 2N H_2SO_4) and at a temperature of 50 °C for 48 hours. The dissolved oxygen level was kept above 20% of saturation by varying agitation speed (300–500 rpm) and airflow rate (2.0–5.0 L/min). This ensured that the DO level was above critical level of 20% for protease production (Beg *et al.* 2003).

RESULTS AND DISCUSSION

Isolation of new bacterial strains

Two new thermophilic strains which produced thermostable proteases were isolated from municipal wastewater sludge. They showed excellent growth at 55 °C on nutrient agar medium. Preliminary experiments showed that these two strains were non-spore forming Gram-negative bacteria; in contrast to conventional enzyme producing strains that are spore-forming Gram-positive bacteria (Burhan *et al.* 2003).

Shake flasks experiments – growth of isolated strains

Figure 1 showed the growth profiles (total cell count – TC) of the two different strains at three temperatures (45, 55 and 65 °C) for 48 hours of fermentation in semi-synthetic medium. It is evident from these results that 55 °C was the optimal growth temperature. Indeed, a higher cell concentration was observed at 55 °C.

The growth profile of the strain AC at 45 °C followed the same pattern as 55 °C, but the total cell count (TC) was lower. Meanwhile at 65 °C, there was a longer lag phase and the total cell count was lower at 10^6 at 45 °C in contrast to 10^7 CFU/ml at 55 °C. Normally, thermophilic bacteria can tolerate a temperature range of 45 to 70 °C (Deacon 2004), but the maximum for these isolated bacterial strains was around 55 °C. High temperature can inactivate important enzymes needed in bacterial cell division, like DNA polymerase and all the enzymes used in DNA duplication (Snyder & Champness 1997). The isolated strains were aerobic and in general required a dissolved oxygen concentration (DO) superior to 20% of saturation. At this concentration, the

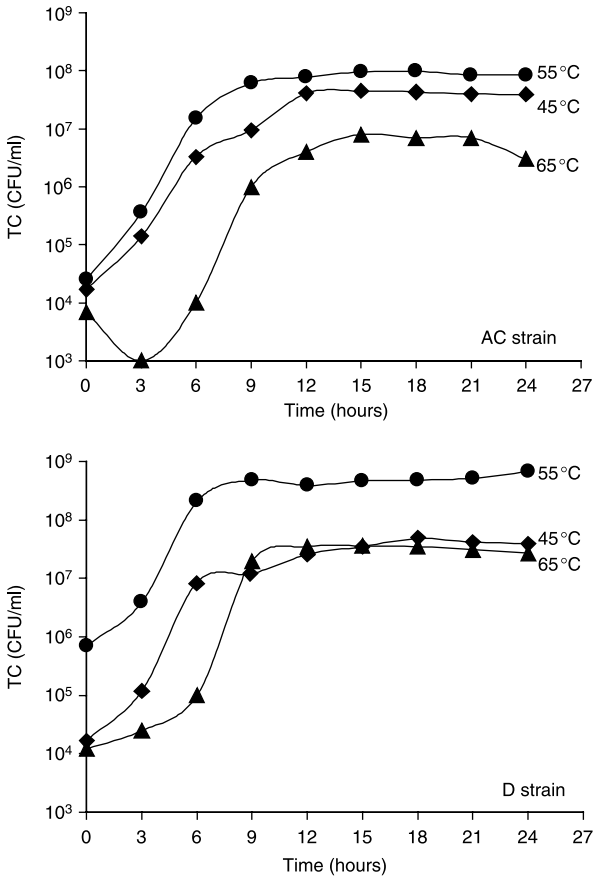

Figure 1 | Growth profile for the two strains named AC and D at different temperatures (45, 55 and 65 °C) in shake flask experiments employing semi-synthetic medium.

growth rate was lower as there was not enough oxygen in contact with the bacteria. It is not possible to conduct shake flask experiments at controlled DO as environmental conditions are uncontrollable (van Suijdam *et al.* 1978). Therefore, at higher temperature (65 °C), DO probably decreased below 20% due to increased demand of DO resulting from growth of the bacteria and low oxygen solubility at higher temperature (Zumdahl 1998). Thus, lower growth of AC strain at 65 °C may be due to a lack of dissolved oxygen in the medium, which should be tested in fermentor under controlled condition of DO.

Similar growth profiles were observed for D strain (Figure 1) with optimum temperature at 55 °C. Growth rate and maximum total cell count were higher at this temperature than at 45 and 65 °C. Also, 65 °C showed the least TC of the two strains.

Shake flasks experiments – Protease activity of isolated strains

Figure 2 shows the variation of protease activity (PA) of the two isolated strains tested at different temperatures. Out of the four temperatures tested (40, 50, 60 and 70 °C), 60 °C was found to be the optimal for maximum enzyme activity irrespective of strain.

Enzymes with high thermostability are more viable and have a better efficiency (De Azeredo *et al.* 2004). Activity of these proteases decreased at a temperature of 50 °C. At higher temperature (70 °C), the protease activity was also lower. It is a well known fact that at higher temperatures, protein conformation changes or degraded (Johnvesly & Naik 2001) and hence causing a decrease in protease activity. Thus, the optimal temperature for growth and enzyme activity for all the strains was same and could be due to similar characteristics of proteases produced by the two different strains.

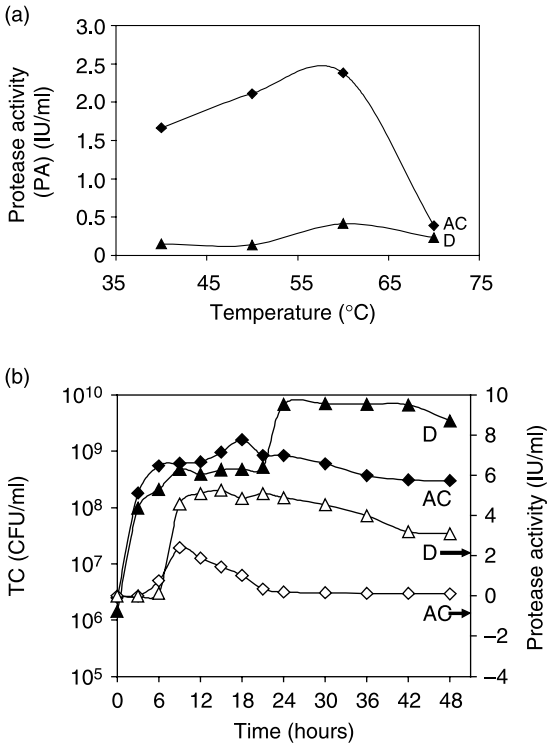

Figure 2 | a) Protease activities (PA) (IU/ml) of two strains AC and D during shake flask experiments using semi-synthetic medium at different temperatures (40, 50, 60 and 70 °C). b) Growth (55 °C) and protease activity (60 °C) profiles for strains AC and D in 15 L fermentor using semi-synthetic medium.

Bench scale study – semi-synthetic medium

Figure 2 b shows profiles of TC and PA of different isolated strains obtained in bench scale fermentor study using semi-synthetic medium. The total cell count increased up to 10^9 CFU/ml for AC strain and almost 10^{10} CFU/ml for D strain.

There was a significant difference between shake flask (Figure 1) and bench scale fermentor experiments (Figure 2 b). D strain showed lower protease activity in shake flask experiments (0.3 IU/ml), however, the activity appreciably increased in fermentor (5.3 IU/ml). AC strain showed a maximum protease activity of 2.4 IU/ml in fermentor which was lower than PA activity (3.2 IU/ml) in shake flask experiments. At present we have no explanation as why the PA was lower in fermentor than in shake flask and more research is required (specifically on enzyme nature produced in fermentor and shake flask) to provide a suitable explanation. Hence, bench scale fermentor experiments were necessary to establish exact protease activity of the strains. Further, scale-up studies have shown better results (increased protease activity) due to controlled environmental conditions (Hempel & Dziallas 1999).

Bench scale study – sludge medium

The fermentor experiments were conducted using CUQ (Communauté Urbaine de Québec) wastewater sludge (25 g/L of total solids) as a sole raw material for growth of two isolated strains under identical conditions as those used for semi-synthetic medium. All the operation parameters were the same. Growth was conducted at 55 °C and the enzyme activity was measured at 60 °C. Growth and protease activity profiles of the two strains are presented in Figure 3.

In comparison to semi-synthetic medium (Figure 2 b) a higher cell concentration for AC strain (Figure 3) but less cells concentration for D strain was observed in wastewater sludge. For AC strain, in semi-synthetic medium, a maximum cell concentration of 1.60×10^9 CFU/ml whereas a cell concentration of 7.00×10^9 CFUml in wastewater sludge was observed. For the D strain, a maximum cell concentration of 7.00×10^9 CFU/ml in semi-synthetic medium and 3.10×10^9 CFU/ml in wastewater sludge was noticed.

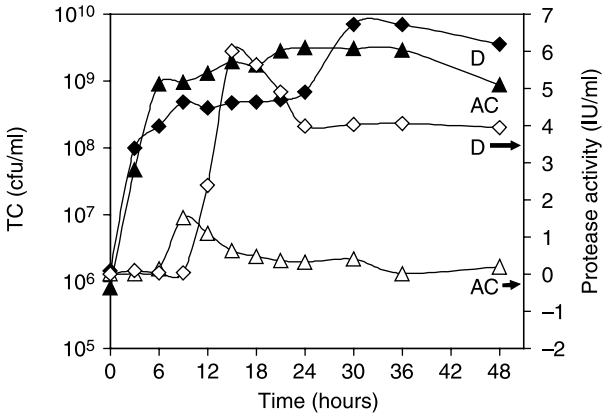

Figure 3 | Growth and protease activity profiles for isolated strains AC and D in 15 L fermentor using municipal wastewater secondary sludge (25 g/L) at an optimal growth temperature of 55 °C and 60 °C for the protease activity.

The degradable organic compounds present in semi-synthetic medium and wastewater sludge are in different forms. The sludge contains, the so called, easily biodegradable, biodegradable and difficult to biodegrade organic matter (Tirado *et al.* 2001). Two bacterial strains may have different capacity to degrade these organic materials and hence may assimilate nutrient compounds differently. However, results show that in wastewater sludge, the available nutrients are easy to assimilate by AC strain because of better growth in this medium. On the contrary, for D strain, the nutrients are easier to assimilate in semi-synthetic medium in comparison to wastewater sludge.

Figure 3 shows that even if the D strain has less cell count in wastewater sludge, the protease activity produced is slightly higher. A greater secretion of enzymes in complex media (municipal wastewater sludge) could be due to poor accessibility of various nutrients, which would otherwise be easily assimilated. Further, these enzymes could break big complex molecules to simpler ones and release them in the medium (from sludge solid phase to liquid phase) enhancing accessibility. Hence, a more complex medium stimulates the bacteria to produce high enzyme activity. On the other hand, AC strain shows less protease activity in wastewater sludge. This could be due to the nature of bacterial strain itself. This bacterium may not have the metabolic capacity to produce the enzyme suitable for this type of medium. Possibly, the bacterium used all the easily biodegradable compounds in the medium for its growth but possess a less efficient enzyme production mechanism. This

is why the protease activity is lower in wastewater sludge, where the nutrients are very complex, than in the semi-synthetic medium.

Banik and Prakash (2004) showed that conventional laundry detergents like Tide (Procter and Gamble) have a protease activity of 57.5 IU/g of granulated proteases. Generally, these granules are mix during formulation with powder detergents in a ratio of 2.3 miligrams of granules per grams of whole detergents. So, in each kilograms of detergent, there is 132.25 IU of proteases.

As announced in the Population Census of 2001 (Statistic Canada 2007), the population of Canada reached 32 730 213 inhabitants and counted 8 170 805 families. Each family uses 0.5 kg of powder detergent, two times per week and 52 weeks per year. So, the Canadian population uses 4.25×10^8 kg of detergent powder during one year and knowing that in each kilogram of powder there is 132.25 IU of proteases (Banik & Prakash 2004), there is a need of 5.62×10^{10} IU of proteases per year. In this study, 6 IU are produced in each mL of fermented sludge (with 25 g/L of suspended solids). Thus to produce 5.62×10^{10} IU per year an approximate amount of sludge required will be 9.37×10^6 litres or 234.25 tons of dry sludge.

CONCLUSION

The production of proteases or any other value-added products is possible by using wastewater sludge. Wastewater and wastewater sludge are produced everywhere on Earth and can be converted into many types of products, it depends on the location and needs of the population. The quantity of biosolids produced by treatment plants can now be reduced by reuse of them. Eventually, all biosolids can be recycled into new eco-friendly products like biopesticides, bioplastics, biocatalysers or in any kind of biogas. Therefore, pollutant chemical products should be replaced by these new bioproducts and in this way reduce the pollutants released into the environment.

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