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Production of Extracellular Proteases by *Bacillus cereus* (Strain PD1) Grown on pH Adjusted Whey

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Three bacterial strains capable of producing extracellular proteases were isolated based on their ability to digest skimmed milk. One of these strains (designated as PD1) was identified as *Bacillus cereus*. Proteases secreted by PD1 were found to retain activity despite exposure to 65°C. Here, we have characterized the strain PD1 and its extracellular proteases and explored the possibility of recycling whey as a growth medium. Antibiotic sensitivity and carbohydrate utilization tests were performed. The kinetics of growth and protease secretion by PD1 were also established using LB broth or pH adjusted whey. It was found that protease activity in the culture supernatant was induced in the stationary phase of growth. Importantly, the stationary phase culture supernatants of PD1 in whey showed maximal protease activity at pH 8.0 and reaction temperature of 56-65°C. The data indicates that PD1 could prove to be a useful source of industrially relevant proteases. Furthermore, this study suggests a possible method of recycling whey to prevent pollution due to whey disposal.

Key Words: *Bacillus cereus*, Whey, Extracellular protease.

Proteases represent one of the most studied and ubiquitously expressed classes of biocatalysts which play important roles in a myriad of cellular processes[1]. They exhibit remarkable diversity in substrate specificity, mechanism of action and dependence on physico-chemical parameters such as pH, temperature and salt conditions. Proteases have been a subject of great interest due to their potential in industrial applications[2]. Microbes have been the most exploited sources of proteolytic enzymes. A number of microbial proteases have already been commercialized and made an impact in the detergent industry[3]. However, the potential of microbes and their proteases have not been fully tapped in the areas of waste management, biodegradation, and recycling.

Paneer (Indian cottage cheese), in its various forms, is an inseparable component of the Indian food culture. Whey is rich in organic content and minerals, which endows it with high biological and chemical oxygen demands. Whey presents itself as a serious environmental pollutant in the absence of a proper system for its re-utilization or treatment prior to disposal. Small units manufacturing various kinds of dairy products

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including *Paneer* represent a significant share of the Indian food industry. These units operate at the local level and cannot afford the expensive whey valorization technologies. Biodegradation or recycling of whey stands out as the most viable options.

We isolated three protease producing bacterial strains from the soil at IIT Guwahati based on their ability to digest skimmed milk. Here we have presented data on the identification and characterization of one of these strains (designated as PD1). Our data suggest that PD1 could be utilized for large scale protease production by utilizing whey as the growth medium.

MATERIALS AND METHODS

Media

Standard Luria Bertani broth (LB) and LB-agar were purchased from HiMedia (India). For screening of protease producing bacterial strains LB-agar plates containing 1% skimmed milk were used (referred to as LB-sm-agar). For assaying protease activity in cell free culture supernatants, agar gels containing 1% skimmed milk buffered with 10 mM Tris-Cl, pH 8.0 (referred to as Tris-sm-agar) were used. Whey used in this study was collected from the local market. Its pH was adjusted to 8.0 with sodium hydroxide and filter-sterilized before use.

Isolation of protease producing strains

Soil collected from IIT Guwahati campus was suspended in sterile LB and kept in a shaker incubator maintained at 37°C for 15 min. The suspension was kept standing at room temperature until the soil particles settled. A loopful of the turbid supernatant was streaked on an LB-sm-agar plate and incubated overnight at 37°C. The plate was then examined for presence of colonies which showed clear zones of skimmed milk digestion. Based on visual inspection, eight colonies were chosen (PD1 to PD8), and their protease secretion was confirmed by repeated inoculation on LB-sm-agar plates. The pure cultures of these strains were stored as glycerol stocks.

Ammonium sulphate precipitation of proteins from culture supernatant

Fresh 50 ml overnight culture was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a beaker and kept on ice with constant stirring. Small quantities of ammonium sulphate were dissolved until 60% saturated. Following an overnight incubation at 4°C the precipitated protein was pelleted by centrifugation at 13,000 rpm for 30 min at 4°C. The supernatant was discarded. The pellet was dissolved in 1 ml of 10 mM Tris-Cl (pH 8.0) and stored at -20°C until use.

Antibiotic sensitivity and carbohydrate utilization test for strain PD1

An overnight grown culture of PD1 in standard LB was spread on an LB-agar plate and incubated at 37°C for 15 min. Octodisc-GVII-Minus antibiotic discs (HiMedia, India) were then placed on the agar surface followed by further incubation at 37°C until clear zones of inhibition were observed.

For carbohydrate utilization test, cultures of PD1 were freshly grown until the optical density at 660 nm (OD_{660nm}) reached a value of 0.5. The cells were pelleted by centrifugation and washed once and resuspended in sterile phosphate buffered saline (PBS). The suspended cells were then used for carbohydrate utilization test using HiCarbohydrate™ kit (HiMedia, India), according to manufacturer's instructions.

Establishment of growth curve of PD1 in LB or whey

PD1 was grown at 37°C in 3 ml of LB using 30 µl of glycerol stock as an inoculum. Typically, the OD_{660nm} of the culture after 12-14 h was 1.8. Five hundred microlitres of this culture was used to inoculate 50 ml of LB or whey and grown at 37°C with constant shaking at 180 rpm. OD_{660nm} of 1 ml aliquots of this culture was measured at 1 h intervals. The growth kinetic was represented as a graph with OD_{660nm} plotted against time. After measuring OD_{660nm} at each time point, the cells were pelleted by centrifugation and the supernatant was stored in -20°C for further analysis.

Protease activity assays

Agar gel diffusion assay

Ammonium sulphate precipitates or equal volumes of cell free culture supernatants were placed in wells made in Tris-sm-agar plates and incubated overnight at 37°C. The plates were examined for clear zones around the wells. The diameters of the clear zones reflecting the extent of proteolytic activity were measured.

Skimmed milk digestion assay in buffered solution

Ammonium sulphate precipitates or equal volumes of cell free culture supernatants were added to 1 ml of 1% skimmed milk in 10mM Tris (pH 8.0) and incubated at 37°C for 2 h. Five hundred microlitres of 10% TCA was added followed by incubation in dark for 30 min. The precipitated proteins were separated by centrifugation at 10,000 rpm for 15 min. The absorbance of the resultant supernatant was measured at 280 nm (A_{280nm}). 10 mM Tris-Cl (pH 8.0) containing 1% skimmed milk served as blank.

Zymography

Protease activities in the culture supernatants were also demonstrated using zymography with skimmed milk as the substrate. Proteins were resolved in 7.5% non-denaturing PAGE containing 1% skimmed milk. The gels were washed in 2.5% Triton-X 100 to remove SDS followed by overnight incubation in 10 mM Tris-Cl (pH 8.0). The gels were stained with 0.5% Coomassie Brilliant Blue and destained with methanol: glacial acetic acid: water (30:10:60) until clear bands were visible against a blue background.

Thermostability of the extracellular proteases

Aliquots of whey culture supernatants were incubated at different temperatures ranging from 15-95°C for 10 min before assaying for protease activity. The aliquots kept on ice for 10 min served as controls.

Optimum pH and temperature

For determining the optimum pH, aliquots of whey culture supernatants were mixed with 1% skimmed milk solution buffered at different pH levels - 0.1 M sodium acetate (pH 5.0), 0.1 M sodium phosphate (pH 6.0), 0.1 M sodium phosphate (pH 7.0), 10 mM Tris-Cl (pH 8.0), 10 mM Tris-Cl (pH 9.0) and 0.1 M glycine-NaOH (pH 10.0). The mixtures were incubated at 37°C for 2 h and assayed for protease activity. For determining the optimum temperature, aliquots of whey culture supernatants were mixed with 1% skimmed milk buffered with 0.1 mM Tris-Cl (pH 8.0) and incubated at different temperatures ranging from 15-95°C for 2 h before assaying for protease activity.

RESULTS AND DISCUSSION

Based on their ability to digest skimmed milk on LB-sm-agar plates we initially isolated eight bacterial strains (PD1-PD8) capable of extracellular protease production. Three strains, namely, PD1, PD2 and PD5 appeared to secrete higher levels of proteases compared to others and hence were

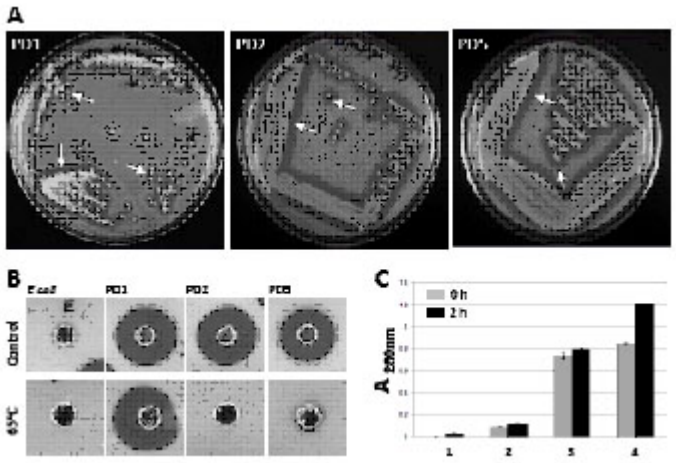

Fig. 1. A. Pure cultures of PD1, PD2 and PD5 streaked on LB-sm-agar plates. Zones of skimmed milk digestion produced by growing colonies have been indicated by white arrows. B. Top panel- Proteins in culture supernatants of PD1, PD2 and PD5 produce clear zones in Tris-sm-agar plates. Bottom panel- Culture supernatant proteins of PD1 retain their protease activity despite prior exposure to 65°C for 10 min. C. Demonstration of protease activity in culture supernatant proteins from PD1 using 1% skimmed milk solution buffered with 10 mM Tris-Cl (pH 8.0). Bars represent mean ± SD of A_{280nm} measurements (n=3). 1, buffer alone; 2, buffer + PD1 culture supernatant protein; 3, skimmed milk alone; 4, skimmed milk + PD1 culture supernatant protein.

chosen for further studies. Pure cultures of PD1, PD2 and PD5 streaked on LB-sm-agar plates and maintained overnight at 37°C yield colonies which show clear zones of skimmed milk digestion (Fig. 1A). Ammonium sulphate precipitated proteins from culture supernatants of PD1, PD2 and PD5 grown in LB showed protease activity in agar gel diffusion assays on Tris-sm-agar plates. Proteins from cell free culture supernatant of *E. coli* did not show such activity (Fig. 1B, top panel). This demonstrated that the strains PD1, PD2 and PD5 were capable of producing extracellular proteolytic enzymes. The precipitated proteins were incubated at 65°C for 10 min before testing their activity on Tris-sm-agar plates. It was found that PD1 samples retained their activity, while PD2 and PD5 samples showed no activity (Fig. 1B, bottom panel). This observation encouraged us to focus on PD1 for further studies. Proteins from culture supernatant of PD1, harvested by ammonium sulphate precipitation, were also tested for proteolytic activity using a 1% solution of skimmed milk buffered with 10 mM Tris-Cl (pH 8.0). As shown in Fig. 1C, the PD1 supernatant proteins caused an appreciable digestion of skimmed milk over a period of 2 h.

The strain PD1 was found to be sensitive to a panel of commonly used antibiotics, namely, Ampicillin, Chloramphenicol, Tetracycline, Gentamycin, Kanamycin, Co-trimoxazole, Amikacin and Streptomycin (Fig. 2A). It was found to be catalase positive based on the observed effervescence following addition of 0.3% solution of hydrogen peroxide to the growing colonies (data not shown). Carbohydrate utilization tests revealed its ability to utilize maltose, dextrose, trehalose, glycerol, salicin, and ribose (Fig. 2B). The task of ascertaining the identity of this strain based on 16S rDNA sequence was outsourced to Vimta Labs, Hyderabad, India. Based on the sequence data, the strain PD1 was identified as *Bacillus cereus* (data not shown).

Five hundred microlitres of an overnight culture of PD1 (OD_{660nm} of 1.8) was inoculated in 50 ml of LB and the growth kinetics was established by measuring OD_{660nm} of the growing culture at 1 h intervals (Fig. 3A). The growth reached the mid-log phase at 3 h post inoculation and entered the stationary phase typically after 8-10 h, when the OD_{660nm} of the culture was around 2.5. It was found that the culture remained in the stationary phase even until 24 h post inoculation. The 3, 6, 12 and 24 h cultures were sampled and equal volumes of cell free culture supernatants were tested for protease activity in Tris-sm-agar plates. It was found that 6, 12 and 24 h samples showed large

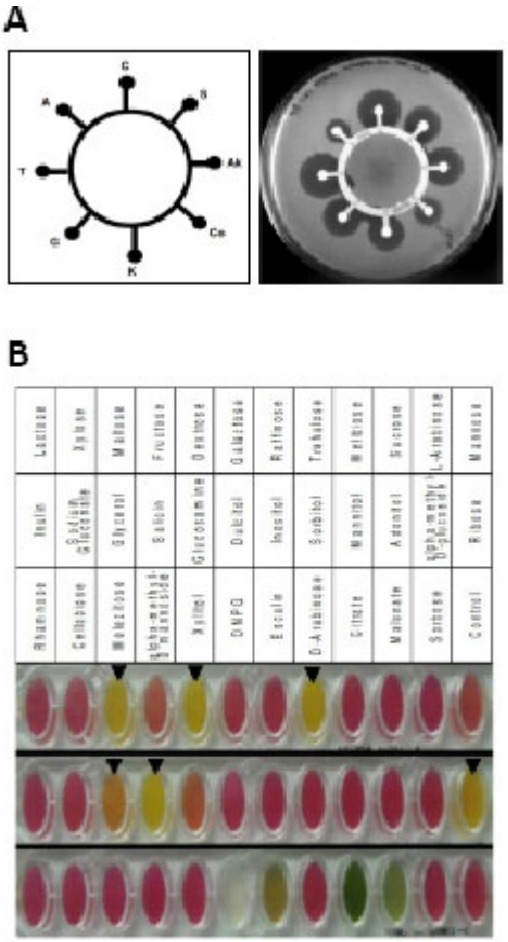

Fig. 2. Antibiotic sensitivity and carbohydrate utilization test. **A**. Result of the antibiotic sensitivity test using Octodisc-GVII-Minus, HiMedia, India. Left panel is a schematic showing locations of the antibiotic discs on the plate. C- Chloramphenicol, S- Streptomycin, Ak- Amikacin, Co- Cotrimoxazole, K- Kanamycin, G- Gentamycin, T- Tetracycline, A- Ampicillin. Right panel shows zones of growth inhibition around the antibiotic discs. **B**. Result of the carbohydrate utilization test using HiCarbohydrate™ kit (HiMedia, India). A color change from red to yellow implies utilization of the carbohydrate (indicated by black triangles). Top panel shows a grid specifying the location of each carbohydrate.

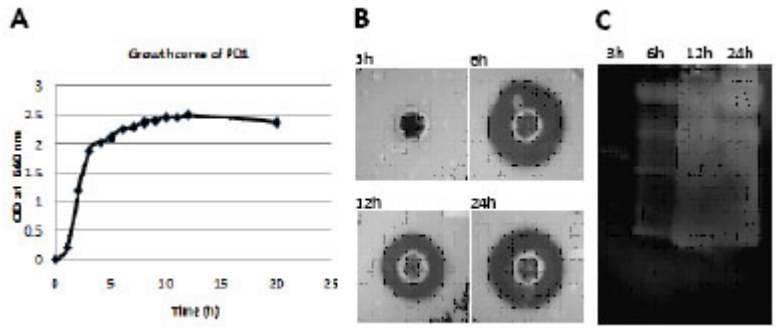

Fig. 3. Time dependent growth and extracellular protease production by PD1 grown in LB. A. Growth curve of PD1. Data shown is representative of three independent experiments. B. Qualitative assessment of protease activity in 50 µl of cell free culture supernatants of PD1 in LB at the indicated time points using Tris-sm-agar plates. C. Assessment of protease activity in cell free culture supernatant at the indicated time points using zymography with skimmed milk as substrate.

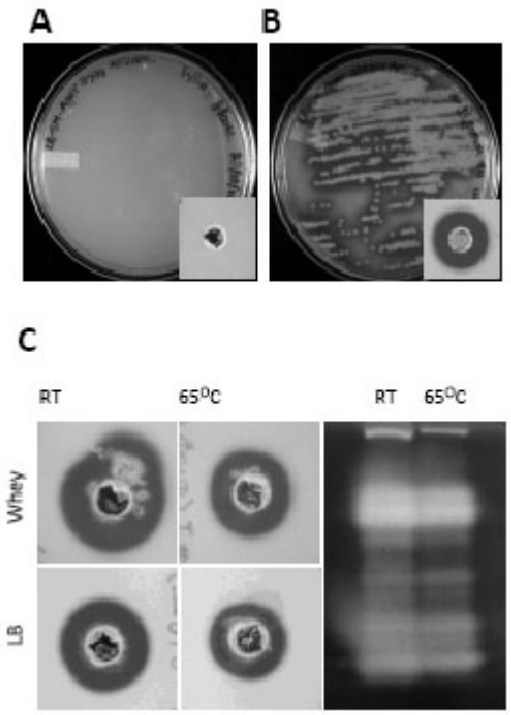

Fig. 4. Growth and extracellular protease production by PD1 in whey. The processed whey, either alone (control) or following inoculation with PD1, was kept overnight in a 37°C shaker incubator. Loopful of the control and inoculated cultures was streaked on a freshly prepared LB-sm-agar plates and maintained at 37°C for 12 h. A. Image of the control LB-sm-agar plate streaked with a loopful of uninoculated whey (Control). B. Image of the LB-sm-agar plate streaked with a loopful of culture of PD1 grown in whey. The insets in A and B show the results of protease activity assays of control and inoculated culture supernatants performed in Tris-sm-agar plates. The data shown is a representative of three independent experiments. **C**. Effect of high temperature on the protease activity in whey culture supernatant. Equal volumes of whey culture supernatants were exposed to room temperature (RT) or 65°C prior to analyzing the protease activity on Tris-sm-agar plates and by zymography.

clear zones of skimmed milk digestion with negligible clearing observed with the 3 h sample (Fig. 3B). These samples were also tested using zymography with skimmed milk as substrate. The zymography results shown in Fig. 3C were in complete agreement with the protease activities observed in Tris-sm-agar plates. These data clearly suggest that protease activity in the culture supernatant is induced as the culture transits the log phase and enters into a stationary phase.

Our observation on increased protease production by PD1 in the stationary phase is consistent with the existing literature. Production of extracellular proteases by several species of the *Bacillus* group have been described earlier and studied extensively. The importance of these protease producing strains and their enzymes stems from their applicability in various industrial processes; especially those that involve degradation of protein rich substrates[1,2,4]. Plenty of reports on the production of extracellular proteases by strains of *Bacillus cereus* in particular and their possible use in deproteinization of shrimp waste and raw feather, dehairing of hide, and in detergents as supplements have been published[5-8]. In view of the skimmed milk degrading property of PD1 it was of our interest to explore whether the strain could be useful in mitigating pollution caused by whey disposal.

Whey has an acidic pH and does not support the growth of PD1. Hence, the pH of whey was adjusted to 8.0 using sodium hydroxide and sterilized by filtration. The pH adjusted sterile whey was inoculated with PD1 and maintained overnight in a 37°C shaker incubator. A loopful of the overnight culture streaked on an LB-sm-agar plate resulted in numerous colonies (Fig. 4B). Notably the colonies also showed clear zones of skimmed milk digestion. One of these colonies was chosen and the identity of this strain was confirmed based on 16S rDNA sequence. The report from Vimta Labs (Hyderabad, India) again confirmed its identity to *Bacillus cereus*. No colonies were obtained with an uninoculated control culture (Fig. 4A). When aliquots of culture supernatant of PD1 grown in pH adjusted whey were added to wells created in Tris-sm-agar plates, clear zones of skimmed milk digestion were observed (Fig. 4B, inset). Supernatant from uninoculated whey, which served as a control, did not result in a clear zone of

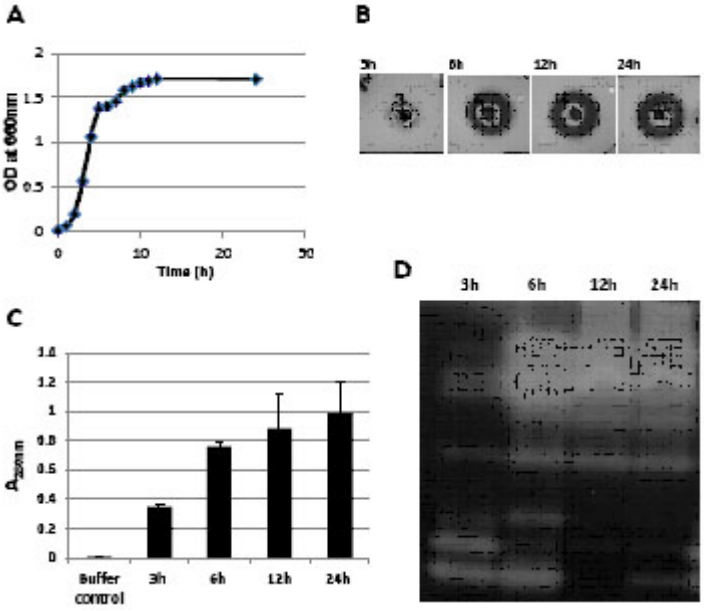

Fig. 5. Time dependent growth and extracellular protease production by PD1 grown in whey. A. Growth curve of PD1. Data shown is representative of three independent experiments. B. Qualitative assessment of protease activity in 50 µl of cell free whey culture supernatants of PD1 at the indicated time points using Tris-sm-agar plates. C. Protease activities of culture supernatants harvested at indicated time points analyzed with buffered skimmed milk solution. D. Zymographs of culture supernatants harvested at the indicated time points.

protein digestion (Fig. 4A, inset). These experiments clearly demonstrated the ability of PD1 to produce extracellular proteases when grown in pH adjusted whey alone as the growth medium. Furthermore, the cell free whey supernatant was found to retain proteolytic activity despite exposure to 65°C for 10 min when tested in Tris-sm-agar plates or by zymography (Fig. 4C), suggesting that one or more of the constituent proteases were likely to be thermostable.

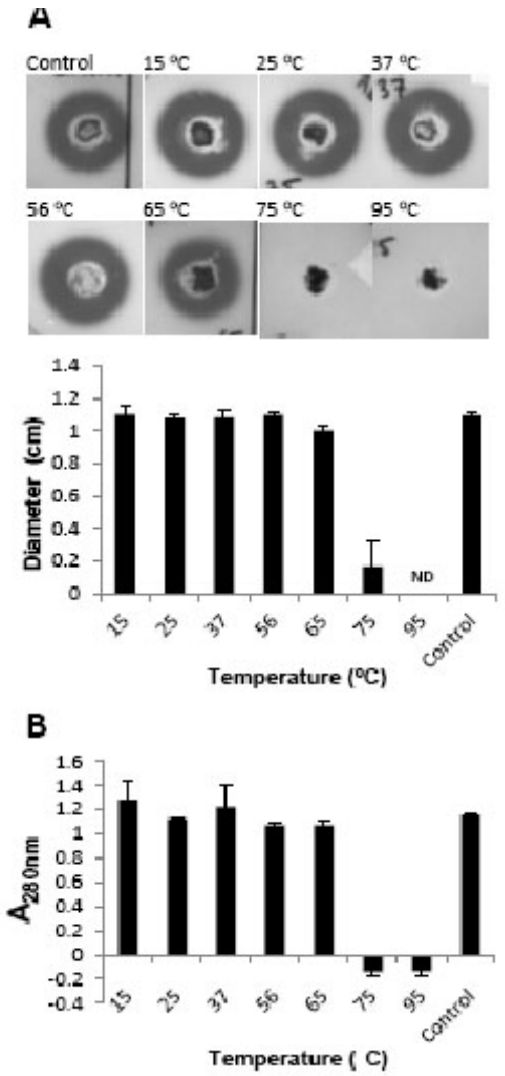

Fig. 6. Thermostability of proteases in whey culture supernatant of PD1. A. Following exposure to indicated temperatures the cell free culture supernatants were added to wells created in Tris-sm-agar plates. After incubation at 37°C for 12 h, the plates were observed for zones of skimmed milk digestion. The diameters of the zones were measured and plotted against temperature. The bottom panel is a graphical representation of the data. Bars represent mean ± SD of the diameters of the clear zones. B. Following exposure at indicated temperatures the cell free culture supernatants were added to tubes containing 1% skimmed milk buffered at pH 8.0. The protease activity was then assayed as described in materials and methods. Bars represent mean ± SD of A_{280nm} measurements.

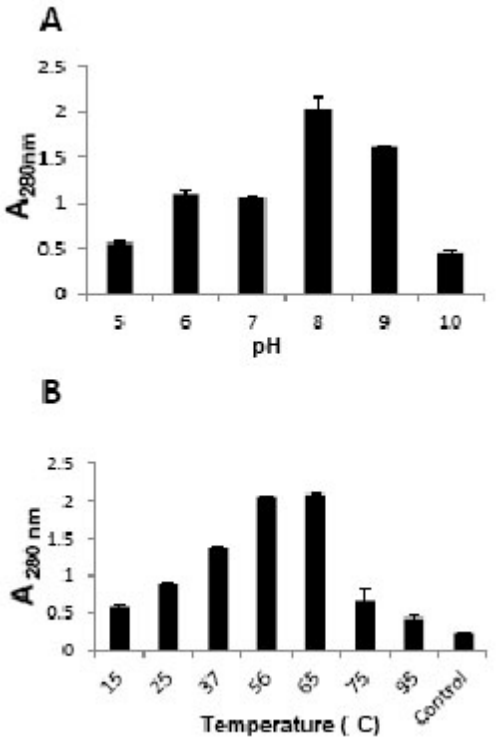

Fig. 7. Determination of optimum pH and temperature. A. Cell free culture supernatants were added to 1% skimmed milk solution buffered at indicated pH using different buffers. The mixtures were maintained at 37°C for 2 h and assayed for protease activity as described in materials and methods. B. Cell free culture supernatants were added to 1% skimmed milk solution buffered at pH 8.0 and incubated at indicated temperatures for 2 h. The extent of skimmed milk digestion was determined as described in materials and methods. In both A and B the bars represent mean SD of A_{280nm} measurements (n=3).

Encouraged by these findings we performed experiments to study the growth kinetics of PD1 in pH adjusted whey and to characterize the proteases present in the whey culture supernatant. Using a methodology similar to the one used for studying the growth kinetics of PD1 in LB broth, a typical growth curve was established. As shown in Fig. 5A the culture reached the mid-log phase at 5 h (OD_{660nm} of around 0.8) and the stationary phase at around 12 h (OD_{660nm} of around 1.6) post inoculation . In a manner similar to the cultures of PD1 in LB, the whey culture supernatants from the stationary phase showed higher levels of protease activity compared to those from the mid-log phase (Fig. 5B and 5C). Interestingly, zymography experiments revealed several bands of protease activity which were differentially modulated as the culture progressed from the mid log phase to stationary phase (Fig. 5D). The data indicates that the protease activity in the whey culture supernatant increases as the culture transits from mid-log phase to stationary phase. 50 µl aliquots of cell free culture supernatants were exposed to different temperatures ranging from 15-95°C for 10 min and their protease activities were determined with Tris-sm-agar plates and buffered solution of skimmed milk. As shown in figure 6A, exposure of the cell free supernatants to a temperature as high as 65°C did not result in any reduction in the zones of skimmed milk digestion on Tris-sm-agar plates. Similar results were obtained when the protease activity was measured based on digestion of skimmed milk in buffered solution (Fig. 6B). Determination of protease activity at different pH (ranging from 5 to 10) revealed a peak activity at pH 8.0 (Fig. 7A). At pH 8.0, maximum protease activity was observed at temperatures of 56 and 65°C when assayed with buffered skimmed milk solutions (Fig. 7B). These data indicate that the proteases secreted by PD1 in whey culture medium are thermostable enzymes with an optimum pH of 8.0.

Physical[9], chemical[10, 11], electrochemical[12] and bioreactor based[13-15] methods of decreasing the biological and chemical oxygen demands of whey have been reported in the literature. While these methods have been shown to be effective in significantly reducing the pollutant load, there has been an increased appreciation of the use of microbes for proper management of whey and dairy wastewaters[16]. Whey, although a byproduct, is rich in Lactose and proteins, which endows it with nutritive value. Several investigators have successfully utilized whey as a growth substrate or an additive for culturing strains of yeast and other microbes for manufacture of value added products. In an interesting study, Rodriguez *et al.*, have shown the utility of cheese whey as a carbon source and an inducer for production of penicillin acylase by a recombinant *E. coli*[17]. Several others have proposed the utility of whey for production of organic solvents[18, 19], biopolymers[20-22] and organic acids[23, 24] using fermentation technology. Romero *et al*., have reported the production of extracellular proteases from *Serratia marcescens* using whey as a growth substrate[25]. On similar lines, the strain PD1 identified in this work may prove to be useful in tackling the problem of whey disposal. The fact that PD1 produces extracellular proteolytic enzymes when grown in whey should provide impetus for feasibility studies on the use of PD1 for whey bioremediation.

CONCLUSION

We conclude that the *Bacillus cereus* strain PD1 can grow in pH adjusted whey and produce extracellular proteases with thermotolerant properties and optimum activity at pH 8.0. This work shows that the strain PD1 can be useful as a source of proteases for applications in industrial processes involving digestion of protein rich substrates. It also suggests a possible strategy for mitigating pollution due to whey disposal by recycling whey as a growth medium.

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REFERENCES

1. Rao, M. B., Tanksale, A. M., Ghatge, M. S., Deshpande, V. V. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.*, 1998; **62**(3): 597-635.
2. Gupta, R., Beg, Q. K., Lorenz, P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.*, 2002; **59**(1): 15-32.
3. Kumar, C. G., Takagi, H. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnol. Adv.*, 1999; **17**(7): 561-94.
4. Fujinami, S., Fujisawa, M. Industrial applications of alkaliphiles and their enzymes-past, present and future. *Environ. Technol.*, 2010; **31**(8-9): 845-56.
5. Kim, S. S., Kim, Y. J., Rhee, I. K. Purification and characterization of a novel extracellular protease from Bacillus cereus KCTC 3674. *Arch. Microbiol.*, 2001; **175**(6): 458-61.
6. Nilegaonkar, S. S., Zambare, V. P., Kanekar, P. P., Dhakephalkar, P. K., Sarnaik, S. S. Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. *Bioresour. Technol.*, 2007; **98**(6): 1238-45.
7. Manni, L., Jellouli, K., Ghorbel-Bellaj, O., Agrebi, R., Haddar, A., Sellami-Kamoun, A., Nasri, M. An oxidant- and solvent-stable protease produced by *Bacillus cereus* SV1: application in the deproteinization of shrimp wastes and as a laundry detergent additive. *Appl. Biochem. Biotechnol.*, 2010; **160**(8): 2308-21.
8. Ghosh, A., Chakrabarti, K., Chattopadhyay, D. Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. *J. Ind. Microbiol. Biotechnol.*, 2008; **35**(8): 825-34.
9. Minhalma, M., Magueijo, V., Queiroz, D. P., de Pinho, M. N. Optimization of "Serpa" cheese whey nanofiltration for effluent minimization and by-products recovery. *J. Environ. Manage.*, 2007; **82**(2): 200-6.
10. Savant, V. D., Torres, J. A. Chitosan-based coagulating agents for treatment of cheddar cheese whey. *Biotechnol. Prog.*, 2000; **16**(6): 1091-7.
11. Rivas, J., Prazeres, A. R., Carvalho, F., Beltrán, F. Treatment of cheese whey wastewater: combined coagulation-flocculation and aerobic biodegradation. *J. Agric. Food Chem.*, 2010; **58**(13): 7871-7.
12. Shee, F. L., Angers, P., Bazinet, L. Precipitation of cheddar cheese whey lipids by electrochemical acidification. *J. Agric. Food. Chem.*, 2005; **53**(14): 5635-9.
13. Torrijos, M., Sousbie, P., Moletta, R., Delgenes, J. P. High COD wastewater treatment in an aerobic SBR: treatment of effluent from a small farm goat's cheese dairy. *Water Sci. Technol.*, 2004; **50**(10): 259-67.
14. Bezerra, R. A. Jr., Rodrigues, J. A., Ratusznei, S. M., Zaiat, M., Foresti, E. Effects of feed time, organic loading and shock loads in anaerobic whey treatment by an AnSBBR with circulation. *Appl. Biochem. Biotechnol.*, 2009; **157**(2): 140-58.
15. Saddoud, A., Hassaïri, I., Sayadi, S. Anaerobic membrane reactor with phase separation for the treatment of cheese whey. *Bioresour. Technol.*, 2007; **98**(11): 2102-8.
16. Damirel, B., Yenigun, O., Onay, T. T. Anaerobic treatment of dairy wastewaters: a review. *Process Biochemistry*, 2005; **40**: 2583-95.
17. De León-Rodríguez, A., Rivera-Pastrana, D., Medina-Rivero, E., Flores-Flores, J. L., Estrada-Baltazar, A., Ordóñez-Acevedo, L. G., de la Rosa, A. P. Production of penicillin acylase by a recombinant *Escherichia coli* using cheese whey as substrate and inducer. *Biomol. Eng.*, 2006; **23**(6): 299-305.
18. Huang, Y., Yang, S. T. Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. *Biotechnol. Bioeng.*, 1998; **60**(4): 498-507.
19. Altaras, N. E., Etsel, M. R., Cameron, D. C. Conversion of sugars to 1,2-propanediol by *Thermoanaerobacterium thermosaccharolyticum* HG-8. *Biotechnol. Prog.*, 2001; **17**(1): 52-6.
20. Ahn, W. S., Park, S. J., Lee, S. Y. Production of Poly(3-hydroxybutyrate) by fed-batch culture of recombinant *Escherichia coli* with a highly concentrated whey solution. *Appl. Environ. Microbiol.*, 2000; **66**(8): 3624-7.
21. Koller, M., Bona, R., Chiellini, E., Fernandez, E. G., Horvat, P., Kutschera, C., Hesse, P., Braunegg, G. Polyhydroxyalkanoate production from whey by *Pseudomonas hydrogenovora*. *Bioresour. Technol.*, 2008; **99**(11): 4854-63.
22. Hernàndez-Balada, E., Taylor, M. M., Phllips, J. G., Marmer, W. N., Brown, E. M. Properties of biopolymers produced by transglutaminase treatment of whey protein isolate and gelatin. *Bioresour. Technol.*, 2009; **100**(14): 3638-43.
23. El-Samragy, Y. A., Khorshid, M. A., Foda, M. I., Shehata, A. E. Effect of fermentation conditions on the production of citric acid from cheese whey by *Aspergillus niger*. *Int. J. Food.*

Microbiol., 1996; **29**(2-3): 411-6.

24. Lee, P. C., Lee, W. G., Kwon, S., Lee, S. Y., Chang, H. N. Batch and continuous cultivation of *Anaerobiospirillum succiniciproducens* for the production of succinic acid from whey. *Appl. Microbiol. Biotechnol*., 2000; **54**(1): 23-7.

25. Romero, F. J., Garcia, L. A., Salas, J. A., Diaz, M., Quiros, L. M. Production, purification and partial characterization of two extracellular protease from Serratia marcescens grown in whey. *Process Biochemistry*, 2001; **36**: 507-15.