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Regular Article

Screening of alkalophilic thermophilic protease isolated from *Bacillus* RV.B2.90 for Industrial applications

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Protease enzyme from *Bacillus* RV.B2.90 was purified, fractionated and tested for various applications. This enzyme could degrade the natural proteins like coagulated egg and blood clot. Blood and natural pigment stain (carrot, beetroot, green leaves, coffee and tea) were removed by this enzyme easily. It exhibited good compatibility with the commercial detergents such as Surf Excel, Tide, Ujala, Ariel and Rin. The keratinolytic activity of the enzyme was evidenced by the complete degradation of feathers. X-ray films treated with the enzyme showed release of protein from the gelatin coating, a pre requisite for recovery of silver. Also showed better stability with surfactants and solvents, which will be advantageous as detergent formulations containing chelating agents like EDTA and in peptide synthesis. The partially purified enzyme showed direct hydrolysis of various natural proteins (BSA, casein and azocasein) applied to clean glass slides followed by denaturation by boiling. *Bacillus* RV.B2.90 immobilization with 3% alginate showed maximum production (2002 U/ml). In continuous production, the beads were stable over 24 hrs X 9 cycles. RV.B2.90 protease highly thermostable and alkalophilic with a variety of activities, has great potential for application in a wide range of industry.

Keywords: *Bacillus* RV.B2.90, alkalophilic, thermophilic, Protease, Industrial applications

Proteases have a wide variety of industrial applications. Proteases constitute world's third largest group of enzymes that find applications in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes (Anwar and Saleemuddin., 1998; Gupta et al., 2002). The largest application of proteases is in detergent industries, as they could remove protein based stains from cloths (Banerjee et al., 1999). For use in detergent formulations, the enzyme must be stable in surfactants, bleaching agents, fabric softeners and various other formulation aids. Proteases are used in stiff and dull gum layer removal from raw silk to improve the softness of the silk. Wool and silk fibers will get a unique finishing when treated with protease. Removal of silver from X-ray films by decomposing the gelatin layer on the films can be achieved by proteases. (Ishikawa et al., 1993). This enzyme can also use in enzyme debriders and contact lens cleaner which is important in biopharmaceutics (Anwar and Saleemuddin., 2000). Proteases are also involved in removing necrotic material and help in natural healing (Sjodahl et al., 2002). Chicken feather is an important poultry waste which contains 90% of protein as keratin. Biological treatment of feather is increased over the past

few decades due to environmental awareness. (Suntornsuk and Suntornsuk., 2003). In this study, we showed the multiple applications of protease isolated from *Bacillus* RV.B2.90

Material and methods

Microorganism and culture conditions

Bacillus cereus RV.B2.90 used in the experiment was previously optimized for protease production (Vijayalakshmi et al., 2010) and purified in our laboratory (Vijayalakshmi et al., 2010). The isolate was maintained in nutrient agar slopes containing 0.1% casein at 4˚C. Medium used for cultivation contained peptone, 5.0 (g/l); yeast extract, 1(g/l); NaCl, 0.5(g/l); casein, 1(g/l); glucose, 1(g/l); KH_2PO_4, 1(g/l); K_2HPO_4, 1(g/l); $MgSO_4$, 0.5(g/l) at constant pH 8.0, grown for 37˚C under static condition for 24 hrs.

Immobilization in alginate

The liquid culture in the flask was centrifuged at 10,000 rpm for 15 min at 4˚C. The cell pellet was washed with sterile saline (0.9% NaCl). It was suspended in sterile saline and used for immobilization. 25 gm% cell suspension was aseptically added to equal volumes of different concentrations of sodium alginate (1, 2, 3, 4 and 5%) in distilled water. The contents were mixed well to get uniform suspension. Beads was prepared by adding the slurry into 0.2M $CaCl_2$ and kept for curing at 4˚C for 48 hrs (Adinarayana et al., 2004).

Immobilization in Polyacrylamide

Cell pellet (0.03 g) was suspended in sterile chilled distilled water. Acrylamide (2.85 g), bis-acrylamide (0.15 g), ammonium per sulphate (10 mg) and 1 ml of TEMED (NNN^1N^1tetra methyl ethylene diamine) were added in 10 ml of 0.2M phosphate buffer. The cell suspension and the phosphate buffer mixture was mixed well and poured into sterile petriplate of 10 cm diameter. The gel was allowed to polymerize and cut into equal size cubes (5 mm^3) and placed in 0.2M phosphate buffer (pH 7.0) and kept in refrigerator for curing. The cubes was washed with sterile distilled water for 2 – 3 times and stored in phosphate buffer for further analysis. (Hemachander et al., 2001)

Immobilization in agar-agar

Cell suspension (0.03 g) was mixed with 18 ml of agar-agar (dissolved in 0.9% saline and autoclaved) at 40˚C mixed well and poured in a sterile petriplate of 10 cm diameter. After solidification the agar was cut into equal blocks (5 mm^3) and added to 0.2 M phosphate buffer (pH 7.0) and kept for curing. Cubes was washed and stored in phosphate buffer. (Li et al., 2008).

Production with batch and continuous process

Alginate beads were added to 50 ml of the production medium in 250 ml Erlenmeyer flask and incubated at 37˚C for 48 hrs. Samples were drawn at regular intervals and assayed for protease activity. In continuous process, every 24 hrs medium was replaced with fresh medium and repeated for several batch until the beads started to disintegrate. Enzyme activity and cell leakage was monitored for each cycle (Adinarayana et al., 2005).

Applications studies of *Bacillus* RV.B2.90

Coagulative and fibrinolytic activity

Partially purified enzyme (200 U) was incubated with coagulated egg and blood clot in 1mM Tris-HCl buffer (pH 8.0) at 37˚C. Control was included with the substrates and buffer alone. The tubes were monitored at regular time intervals (Najafi et al., 2005)

Stain removal

A clean piece of cloth was soaked in blood and other stains including juices of beet root,

carrot, green leaves, coffee and tea. The cloth was then dried and soaked in 2% formaldehyde and washed with water to remove the excess formaldehyde. The partially purified protease (200 U) was dropped on the cloth and incubated at 37˚C. After incubation, each piece of cloth was washed and dried. Controls were put up without enzyme (Rai and Mukherjee., 2010).

Chicken feather degradation

Chicken feathers were collected from local slaughter house. It was thoroughly washed to remove blood and autoclaved. Partially purified protease (200 U) was added to preweighed feather in Tris-HCl buffer (pH 8.0) and maintained at 37˚C. Disintegration of feathers was observed periodically. Loss of weight was determined by filteration through whatman filter paper followed by washing and drying (Won nam et al., 2002).

Metal recovery

Used X-ray films were washed with water and wiped with ethanol. Washed films were dried in oven at 50˚C for 30 min. 1 gm of X-ray film was weighed and incubated with partially purified protease (200 U) and incubated at 37˚C (Shankar et al., 2010). The breakdown of protein coat was measured by increase in turbidity at 660 nm. The protein concentration was determined by Lowry's method. (Lowry H et al., 1951).

Action of protease on solid surface

Clean glass slide and test tubes were washed and wiped with ethanol and oven dried. 1 ml of substrates (1%) BSA and casein were pipetted into the slide and azocasein was added into test tubes followed by flaming over a Bunsen. Slides were immersed in 30 ml of Tris-HCl buffer (pH 8.0) containing 200 U purified protease and incubated at 37˚C. For azocasein, enzyme in buffer was added to the tube. After incubation, slide and tubes were removed and the buffer was analyzed at 280nm (Turner et al., 2005).

Detergent compatibility

The compatibility of enzyme with commercial detergents was tested. Detergents used for test include, Ariel, Surf excel, Tide, Rin and Ujala. The detergents were diluted to 0.7% and the enzymes present in it were deactivated by heating at 95˚C for 10 min. The purified enzyme (200 U) was added to each detergent and incubated at 65˚C for 3 hr. Enzyme activity was measured at every 1 hr. Control without detergent was taken as 100% (Subba rao et al., 2009).

Effect of surfactants and oxidizing agents

The effect of surfactants (SDS, CTAB, Triton X-100 and Tween-80) and oxidizing agents (H_2O_2) at different Concentrations (1, 5 and 10%) was determined by pre incubating enzyme at 60˚C for 1 hr then the residual activity was measured (Mei and Jiang., 2005).

Effect of organic solvents

To check the effect of organic solvents on enzyme activity was determined by incubating the 1 ml of enzyme with 1 ml of organic solvents at a final concentration of 25% at 37˚C for 1 hr and then the residual activity was measured at 60˚C (Xu et al., 2010).

Results and discussion

Immobilization in alginate

The stability of beads was determined with different percentage of sodium alginate (1%, 2%, 3%, 4% and 5%). Protease activity and cell leakage was monitored for 24 hrs. Alginate concentration played an important role in protease production and cell leakage. This study showed that enzyme production decreased with increase in alginate concentration, which may be due to the nutrient and oxygen limitation. Alginate at 3% was found to be the best optimum concentration for enzyme production. This

result is in accordance with previous studies (Elibol and Moreira., 2003; Adinarayana et al., 2004) that showed 3% alginate has optimum for enzyme production by *Teredinobacter turnirae* and *Bacillus sps* respectively (Fig. 1).

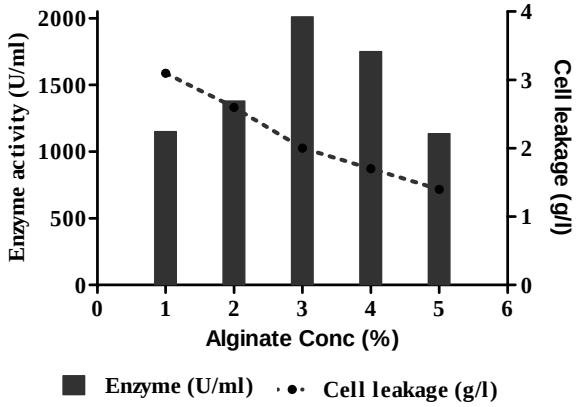

Fig.I. Immobilized RV.B2.90 cells: Influence of % of sodium alginate on protease activity and cell leakage.

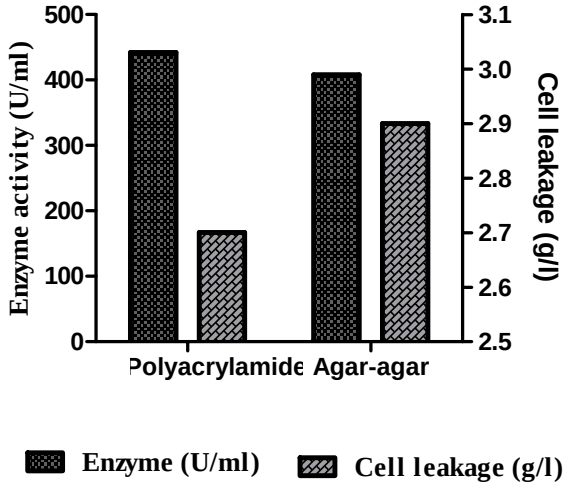

Fig.II. Comparison of enzyme activity and bead stability in Polyacrylamide and agar-agar immobilized RV.B2.90 cells.

Immobilization in Polyacrylamide and agar-agar

Enzyme was entrapped in Polyacrylamide gel and agar-agar, protease production was found maximum at 12th hr, followed by decline. Production was lesser compared to alginate beads (Fig. 2). Similar report (Adinarayana et al., 2005) with other matrices like Polyacrylamide and agar-agar showed lesser production compared to alginate. In Some studies cells have been immobilized in Polyacrylamide. Viable cells were found to be destroyed by acrylamide toxicity and the heat of polymerization (Hemachander et al., 2001).

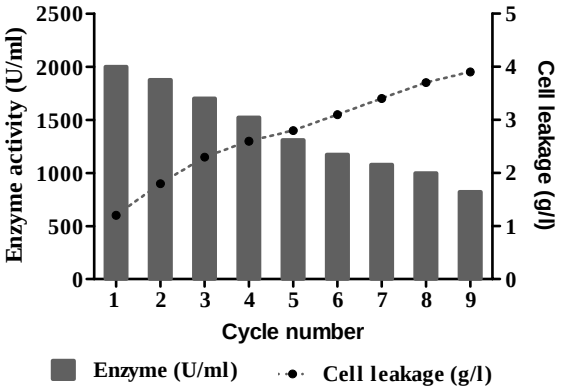

Fig.III. Continuous enzyme production by sodium alginate immobilized RV.B2.90.

Table I. Comparison of protease activity of RV.B2.90 free vs. immobilized cells.

Matrix	Fermentation Time (hr)	Protease activity (U/ml)
Free Cells	48	850
Immobilized Cells	48	2002

Continuous process

Continuous fermentation was carried out with optimum alginate concentration (3%) for 9 days until the beads disintegrated. Maximum enzyme production seen in first cycle (2002 U/ml) with cell leakage of 1.2 g/l. Protease production showed reduction in each cycle with increase in cell leakage.

Within 9 days the alginate beads were completely disintegrated. The enzyme production and the cell leakage were found to be 821 U/ml and 3.9 g/l respectively at final cycle (Fig. 3). These results are in accordance with Adinarayana et al 2004 with *Bacillus subtilis*. The comparative production of free cells and immobilized cells has been listed in Table. 1.

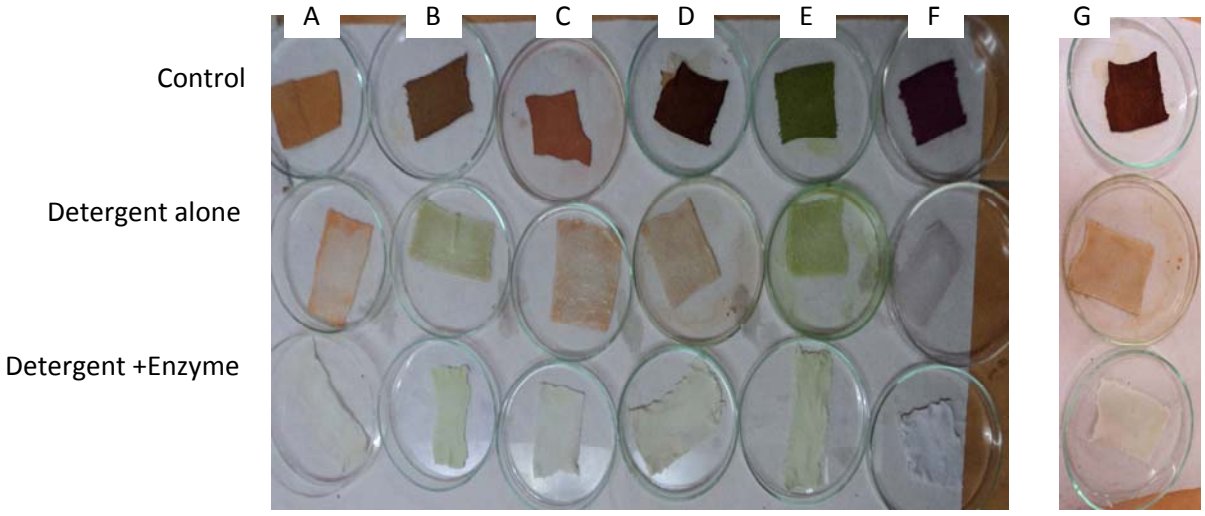

Fig.IV. Degradation of natural colors, compounds and blood by RV.B2.90 enzyme vs. detergents. A- Carrot, B- tea, C- commercial tomato sauce, D- coffee, E – green leaves, F- beetroot and G- blood stain.

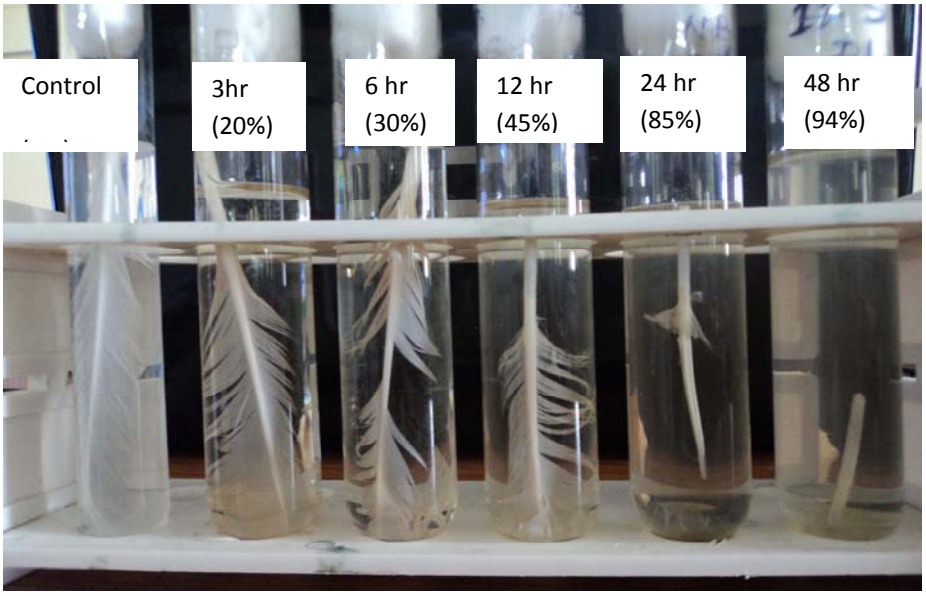

Fig.V. Degradation of feather by partially purified RV.B2.90 protease at 37˚C – influence of period of incubation.

Coagulative and fibrinolytic activity

Partially purified enzyme was incubated with the coagulated egg and blood clot at 37°C. The insoluble form of the coagulated egg white and fibrin blood clot were solubilized. The hydrolytic activity of the RV.B2.90 protease on blood clot, egg protein etc., indicate its significance in industrial application and also in medicine and waste water treatment (Najafi et al., 2005).

Stain removal

In removing blood and natural pigment stain from cloth, this protease showed greater efficiency. Due to its higher potential in removing stains from cloth it could be used in detergents as powder or solution. The washing efficiency of detergents was remarkably increased with addition of the enzyme (7 mg/ml). The protease enzyme was very effective compared to the enzymes reported earlier with respect to the short times required for complete removal of stains (Fig. 4). Some have reported (Anwar and Saleemuddin., 1998) the effectiveness of protease on blood stain removal from cloth in the presence and absence of detergents.

Chicken feather degradation

The degradation of native chicken feathers was performed by incubation with crude protease with 0.1% mercaptoethanol for 24 h at 37°C. Interestingly, complete degradation of feathers was obtained within 48 hrs. The solubilisation of chicken feathers may be explained by the presence of the keratin-degrading enzymes secreted by the strain (Fig. 5). Poultry feather consists of mainly more than 90% keratin, which is not easily degraded by proteases reported earlier. On comparison RV.B2.90 protease was found to be very powerful active at extreme conditions and solublizing the feather completely. Time required also was remarkably very short (85% in 24 hrs). There are reports on complete feather degradation occurring within 24 hrs of incubation. (Haddar et al., 2010). It is suggested that keratin lysing enzymes may be involved in disulfide bond reduction (Won Nam et al., 2002).

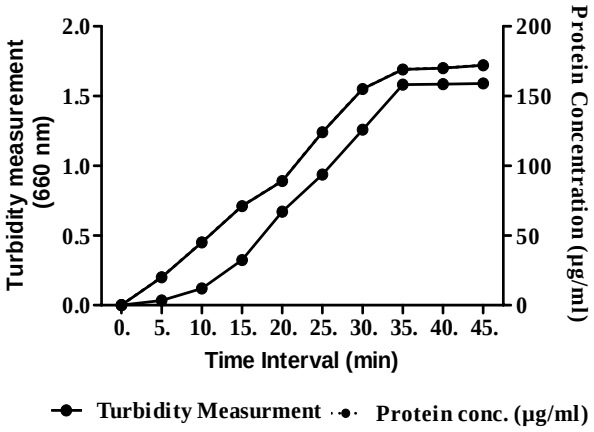

Fig.VI. Removal of gelatin coating from X-ray film using RV.B2.90 protease enzyme

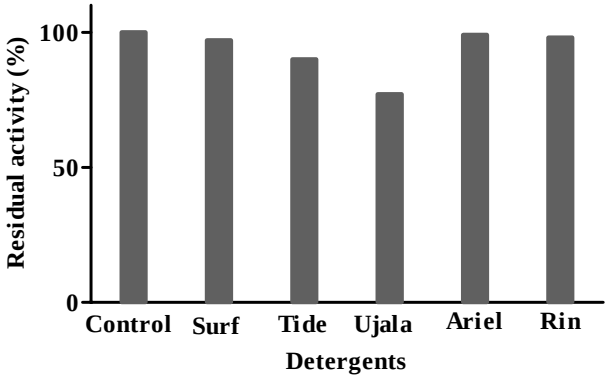

Fig.VII. Effect of commercial detergents on activity and stability on RV.B2.90 protease.

Metal recovery

To determine the efficiency to hydrolyze the gelatinous coating on X-ray film, 200 U of partially purified protease was incubated with 1 gm of used X-ray films. Hydrolysis was complete within 30 min (Fig. 6). Masui et al. (1999) reported 60 min for the complete hydrolysis of gelatin layer. Concentration of protein was directly proportional to the rate of gelatin hydrolysis 169-172 µg/ ml. The enzyme when added to X-ray films was found to remove the layer of gelatin totally and film became transparent (Shankar et al., 2010).

Protein removal from solid surface

The buffer from the post-cleaning wash was measured at 280 nm (tyrosine, tryptophan and phenylalanine). In post cleaning wash, measuring absorbance at this wavelength revealed the presence of released peptide/protein fragments. Buffer with enzyme wash showed increased absorbance and the values were negligible in control tubes without enzyme. Maximum removal was obtained with casein followed by azocasein (Table. 2). As the test proteins was heat fixed on the solid surface it structurally weakened the proteins, denatured and facilitated the enzymatic breakdown of the unfolded proteins. This resulted in the extensive shedding of peptides in to the wash buffer. Increased absorbance at 280 nm clearly showed the efficiency of the enzyme based cleaning on heat fixed proteins on glass surfaces (Turner et al., 2005).

Table II. Breakdown and clearance of natural proteins applied on solid surface by RV.B2.90 enzyme at 37˚C, 30 min.

Protein substrates (10,000 µg)	Solution used for wash	Absorbance at 280 nm	µg protein per ml in the washing
BSA	Buffer alone	<=0.01	Negligible
	Buffer + Enzyme	0.511	2792
Casein	Buffer alone	<=0.01	Negligible
	Buffer + Enzyme	0.852	4656
Azocasein	Buffer alone	<=0.01	Negligible
	Buffer + Enzyme	0.793	4333

Table III. Effect of surfactants and oxidizing agents on RV.B2.90 enzyme activity

Surfactants and Oxidizing agents	**Residual Activity (%)**		
	1%	**2.5%**	**5%**
Control	100	100	100
SDS	100	83	70
CTAB	100	91	80
Triton X-100	100	94	85
Tween – 80	100	95	82
H_2O_2	100	96	89

Table IV. Effect of solvents on RV.B2.90 enzyme activity

Solvents	Residual activity (%)
Control	100
Octane	52
Methanol	83
Isoproponal	99
Ethylene glycol	91
Xylene	95
Butanol	89
Ethanol	58
DMSO	80
Hexane	58

Detergent compatibility

Partially purified enzyme was incubated with commercial laundry detergents which were previously deactivated for endogenous enzyme. Protease from *Bacillus* RV.B2.90 showed excellent stability with all the tested detergents includes Ariel, Rin, Surf excel, Tide and Ujala with 99, 98, 97, 90 and 77% of residual activities respectively (Fig. 7). Thus, this protease showed a greater compatibility with commercial detergents.

Effect of Surfactants

The stability and compatibility of novel microbial proteases are important criteria for addition in detergent preparations. (Kumar and Takagi., 1999). Surfactants like SDS, CTAB, Trition X-100 and Tween 80 at a concentration of 1, 2.5 and 5% was incubated with 100µg of enzyme for 30 min at 65˚C. It was found that in the presence of non ionic surfactant like Tween 80 and Triton X-100 at 1% it did not affect the enzyme activity (100%) and at 2.5% and at 5% it showed slight reduction of the activity (95, 94 and 82, 85 respectively). With CTAB it showed good stability and there was not much reduction in activity. Regarding anionic surfactant (SDS) at 5% it could retain 70% of activity (Table. 3). There are similar reports (Wang and Yeh., 2006), in the case of *Bacillus subtilis* giving higher activity with non ionic surfactant and reduction with ionic surfactant. It showed good stability with hydrogen peroxide, which is similar to Joo et al., 2005 showed 110% of activity at 5% by *Bacillus clausii*. For protease based detergent formulations, the enzyme should have the capability to tolerate oxidizing agents and surfactants. Currently used enzymes lack the stability to withstand bleaching chemicals. Thus research with advanced techniques like protein engineering and gene technology for improving the stability of enzyme are undertaken (Mei and Jiang., 2005). As this enzyme is highly stable to oxidizing agents and surfactants it is an excellent candidate for industrial applications.

Effect of organic solvents

Previous reports have pointed out that peptide synthesis will be improved by the addition of organic solvents. But, enzymes will usually be inactivated in the presence of organic solvents. Various organic solvents like methanol, isoproponal, xylene, butanol, ethanol, DMSO and hexane (10%) are used to test the stability of enzyme. Partially purified enzyme (100 µg) in 1mM Tris-HCl buffer (pH 10.0) was incubated with the respective solvents (3 ml) for 1 hr at 65˚C. After incubation the residual activity was measured. Sample without any solvent was taken as 100%. Better stability was seen with isoproponal, xylene, ethylene glycol, butanol and DMSO (Table. 4). This result is similar to one (Ghorbel et al., 2003) that showed *B.cereus* was not sensitive to DMSO and isoproponal. The mechanism suggested is the increase of hydrophobic interactions within proteins caused by the organic solvents which in turn

improves the active site substrate interaction (Hadj-Ali et al., 2007). Hexane, Ethanol, methanol and octane showed slight inhibitory effect on enzyme activity, similar to literature (Wang and Yeh., 2006).

Reference

Adinarayana, K., Bapi Raju, K.V.V.S.N., Ellaiah, P., 2004. Investigations on alkaline protease production with *Bacillus subtilis* PE-11 immobilized in calcium alginate gel beads., *Proces Biochem.,* **39**: 1331–1339.

Adinarayana, K., Jyothi, B., Ellaiah, P., 2006. Production of Alkaline Protease with Immobilized Cells of *Bacillus subtilis* PE-11 in Various Matrices by Entrapment Technique., *AAPS PharmSciTech.* **6**: 3.

Anwar, A., Saleemuddin, M., 1997. Alkaline-pH- acting digestive enzymes of the polyphagous insect pest *Spilosoma obliqua*: stability and potential as detergent additives, *Biotechnol Appl Biochem*, **25**: 43-46.

Anwar, A., Saleemuddin, M., 1998. Alkaline proteases. A Review, *Bioresour Tech*, **6**: 175-183.

Anwar, A., Saleemuddin., M., 2000 Alkaline protease from *Spilosoma obliqua*: potential applications in bioformulation., *Biotechnol Appl Biochem*, **31**: 85-89.

Banerjee, U.C., Sani, R.K., Azmi, W., Soni, R., 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive, *Proces Biochem*., **35**:213-219.

Elibol, M., Moreira, A. R., 2003 Production of extracellular alkaline protease by immobilization of the marine bacterium *Teredinobacter turnirae*., *Proces Biochem*., **38:** 1445-1450.

Ghorbel, B., Kamoun, A. S., Nasri, M., 2003. Stability studies of protease from *Bacillus cereus* BG1., *Enzym Microb Technol.,* **32**:513.

Gupta, R., Beg, Q. K.., Lorenz, P., 2002. Bacterial alkaline proteases: Molecular approaches and Industrial Applications., *Appl Microbiol Biotechnol*, **59:** 15-32.

Haddar, A., Kamoun, A. S., Zouari, N.F., Hmidet, N., Nasri, M., 2010. Characterization of detergent stable and feather degrading serine proteases from *Bacillus mojavensis* A21., *Biochem Eng J*., **51:** 53–63.

Hadj-Ali, N. E., Rym, A., Basma, G. F., Alya, S. K., Safia, K., Moncef, N., 2007. Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1., *Enzym Microb Technol*, **40:** 515–523.

Hemachander, C., Bose, N., Puvanakrishnan, R., 2001.Whole cell immobilization of Ralstonia pickettii for lipase production., *Proces Biochem*., **36:** 629–633

Ishikawa, H., Ishimi, K., Sugiura, M., Sowa, A., Fujiwara, N., 1993. Kinetics and mechanism of enzymatic hydrolysis of gelatin layers of X-ray film and release of silver particles., *J Ferm Bioeng*, **76**: 300-305.

Joo, H. S., Kumar, C. G., Park, G. C, Paik, S. R., Chang, C. S., 2003. Oxidant and SDS-stable alkaline protease from *Bacillus clausii*-52: production and some properties., *J Appl Microb*, **95:** 267.

Kumar, C. G., Takagi, H., 1999. Research review paper: microbial alkaline proteases: from a bioindustrial viewpoint., *Biotechnol Adv*, **17:** 561–94.

Li, T., Li, S., Wang, N., Tain, L., 2008. Immobilization and stabilization of

pectinase by multipoint attachment onto an activated agar-gel support., *Food Chem*., **109**: 703–708

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Rondal, R. L., 1951. Protein measurement with the folin phenol reagent, *J. Biol Chem*., **193:** 265–73

Masui, A., Fujiwara, N., Takagi, M., Imanaka, T., 1999. Feasibility study for decomposition of gelatin layers on X-ray films by thermostable alkaline protease from alkaliphilic *Bacillus sp*., *Biotechnol Tech*., **13**, 813-815.

Mei, C., Jiang, X., 2005. A novel surfactant and oxidation-stable alkaline protease from *Vibrio metschnikovii* DL 33–51., *Proces Biochem*., **40:** 2167–2172.

Nam, G.W., Lee, D. W., Lee, H. S., Kim, B. C., Choe, E. A., Hwang, J. K., Suhartono, M. T., Pyun, Y. R., 2002. Native-feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase-producing thermophilic anaerobe., *Arch Microbiol*., **178**:538–547.

Najafi, M. F., Deobagkar, D., Deobagkar, D., 2005. Potential application of protease isolated from *Pseudomonas aeruginosa* PD100, *Electr J Biotechnol*, **8**: 2.

Shankar, S., More, S. V. Seeta Laxman, R., 2010. Recovery of silver from waste x-ray film by alkaline protease from *conidiobolus coronatus*., *Kathmandu Univ J Sci Eng Technol*., **6**: 60-69

Sjodahl, J., Emmer, A., Vincent, J., Roeraade, J., 2002. Characterization of proteinases from Antarctic krill (*Euphausia superba*). *Protein Exp Puri*, **26**:153-161.

Suntornsuk, W., Suntornsuk, L., 2003. Feather degradation by *Bacillus sp*. FK 46 in submerged cultivation., *Bioresour Technol*., **86**:239–243.

Turner, K., Serantoni, M., Boyce, A., Walsh, G., 2005. The use of proteases to remove protein-based residues from solid surfaces., *Proces Biochem*., **40**:3377–3382

Vijayalakshmi, S., Venkatkumar, S., Thankamani, V., 2010. Optimization and cultural characterization of *Bacillus* RV.B2.90 producing alkalophilic thermophilic protease *(Communicated).*

Vijayalakshmi, S., Venkatkumar, S., Thankamani, V., 2010. Purification and characterization of thermophilic alkaline protease from B*acillus cereus* RV.B2.90 *(Communicated).*

Wang, S. L., Yeh, P. Y., 2006. Production of a surfactant- and solvent-stable alkaliphilic protease by bioconversion of shrimp shell wastes fermented by *Bacillus subtilis* TKU007., *Proces Biochem*., **41:**1545–1552

Xu, J., Jiang , M., Sun, H., He, B., An organic solvent-stable protease from organic solvent-tolerant Bacillus cereus WQ9-2: Purification, biochemical properties, and potential application in peptide synthesis., *Bioresour Technol*., **101**:7991–7994