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Effect of Medium Composition on Commercially Important Alkaline Protease Production by *Bacillus licheniformis* N-2

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Summary

Protease production by alkalophilic *B. licheniformis* N-2 was investigated in 50 mL of the growth medium consisting of (in g/L): glucose 10.0, soybean meal 10.0, K_2HPO_4 3.0, $MgSO_4 \cdot 7H_2O$ 0.5, NaCl 0.5 and $CaCl_2 \cdot 2H_2O$ 0.5 at pH=10. Different carbon and nitrogen sources in the form of fine powder of organic, inorganic and defatted meals were studied to select the suitable substrate for alkaline protease production. The highest level of alkaline protease (677.64 U/mL) was obtained in the medium containing glucose followed by soluble starch and wheat bran. Among various nitrogen sources, defatted soybean meal was found to be the best inducer of alkaline protease, while inorganic nitrogen sources in the form of ammonium salts repressed the enzyme activity up to 96 %. Thermostability studies showed that the enzyme in the presence of 10 mM Ca^{2+} ions retained its residual activity up to 80 % even after incubation at 40 °C for 12 h. The enzyme was found stable over a broad range of pH (8–11) and lost 52 % of its residual activity at pH=12. After the treatment with Tween 20, Tween 45, Tween 65, Triton X-405, H_2O_2 and sodium perborate, each at 1.0 % concentration, the enzyme showed residual activity of 105, 82, 116, 109, 135 and 126 %, respectively. The application of alkaline protease for removal of blood stains from cotton fabric also indicates its potential use in detergent formulations.

Key words: alkaline protease, surfactant and oxidant stability, *B. licheniformis* N-2

Introduction

Proteases execute a large variety of functions and have numerous applications in detergent, food, pharmaceutical and leather industries (*1*). The largest application of the proteases is in the laundry detergents, where they help in removing protein-based stains from clothing during washing. The enzymes to be used as detergent additives should be stable and active in the presence of typical detergent ingredients, such as surfactants, builders, bleaching agents, fillers, fabric softeners and various other formulations (*2*). Most of the alkaline proteases applied for the industrial purposes face some limitations due to low activity and stability towards surfactants and oxidants. *Bacillus* strains have the ability to secrete industrially significant proteases which are stable and compatible with various detergent components (*3,4*). *Bacillus licheniformis* has been extensively used in industry for alkaline protease production (*5*). However, the cost of alkaline protease is also a major issue in enzyme applications in different industries. About 30–40 % of the cost of industrial enzymes depends on the cost of the growth medium (*6*). The purification process of an enzyme also depends on the composition of the growth medium. Carbon and nitrogen sources, inorganic salts and other growth factors are important variables that affect the growth and products of microbes (*7,8*). The microorganisms isolated from natural habitats may also have some special

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potential for the utilization of local raw materials to produce valuable products like enzymes. Therefore, proper screenings of the medium ingredients are the basic need of any fermentation process to make it cost-effective and economically feasible at commercial scale.

Considering these facts, we attempted to screen some low-cost and easily available medium ingredients for the maximum yield of alkaline protease by locally isolated *Bacillus licheniformis* N-2. The enzyme produced by alkalophilic *Bacillus licheniformis* N-2 is found stable against oxidants and surfactants. It has also shown tremendous activity for the removal of blood stains from cotton cloth. These characteristics of the enzyme indicate its potential use as a detergent additive in laundry industry.

Materials and Methods

Microorganism

An alkalophilic *Bacillus licheniformis* N-2 was isolated from decaying organic soil in Microbiology Laboratory of Food and Biotechnology Research Center, PCSIR, Laboratories Complex, Lahore, Pakistan. The strain was identified with the methods recommended in Bergey's Manual of Determinative Bacteriology (*9*) and Bailey and Scott's Diagnostic Microbiology (*10*) and then selected for alkaline protease production after screening with comprehensive and discrete program (unpublished data). The culture was revived from nutrient agar slants (pH=10) at 37 °C after every month and maintained at 4 °C for further experimental study.

Fermentation methodology

Inoculum preparation

A 24-hour-old loopful culture was transferred into the sterilized inoculum medium consisting of (in g/L): glucose 5.0, soybean meal 10.0, K_2HPO_4 3.0, $MgSO_4{\cdot}7H_2O$ 0.5, NaCl 0.5 and $CaCl_2{\cdot}2H_2O$ 0.5. The inoculated broth was incubated in water bath shaker (Eyela, Japan) for 24 h at 37 °C for the propagation of bacteria up to 10^8–10^{10} cells/mL. Then, 1 % (by volume) of this inoculum medium was used to inoculate the growth medium for alkaline protease production.

Cultivation of growth medium

A volume of 50 mL of the growth medium comprising (in g/L): glucose 10.0, soybean meal 10.0, K_2HPO_4 3.0, $MgSO_4{\cdot}7H_2O$ 0.5, NaCl 0.5 and $CaCl_2{\cdot}2H_2O$ 0.5 in 250-mL Erlenmeyer flask was inoculated with 0.5 mL of the inoculum medium and then incubated in water bath shaker (Eyela, Japan) for 24 h at 37 °C with shaking speed of 140 rpm. After that, the supernatant was obtained by centrifugation at 9000×*g* for 10 min at 4 °C and the total protease activity was determined in the cell-free supernatant. The initial pH of the growth medium was adjusted at 10 with 1 M HCl or NaOH before sterilization (121 °C for 15 min).

Screening of carbon and nitrogen sources

Carbon sources

Different agroindustrial residues (wheat bran, wheat flour, rice bran, rice husk) and soluble carbon sources (glucose, soluble starch, sucrose, fructose, lactose, maltose, galactose and citric acid) were employed to find the suitable carbon source for alkaline protease production by *B. licheniformis* N-2. The agroindustrial residues were sieved through standard mesh sieve to get the fractions of fine particles of 0.25 mm. All these sources were studied at 1 % (by mass per volume) initial concentrations.

Nitrogen sources

Three categories, *viz.* organic nitrogen sources, inorganic nitrogen sources and different agricultural by-products in the form of defatted meals were employed. The growth medium was initially supplemented with different organic nitrogen sources, *i.e.* peptone, yeast extract, beef extract, corn steep liquor, malt extract, tryptone, casein, gelatin, Lab Lemco powder and urea, each at 1 % (by mass per volume). Among the inorganic nitrogen sources, $NaNO_3$, KNO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, NH_4NO_3, NH_4HCO_3 and NH_4Cl again at 1 % (by mass per volume) were tested. Different defatted meals such as sun flower meal, cotton seed meal, soybean meal, mustard seed meal, corn seed meal, canola seed meal and rape seed meal were collected from local market and processed through standard sieve to obtain fractions of fine particles (0.25 mm). These defatted meals were incorporated into the growth medium at 1 % (by mass per volume) to find the best inducer of proteolytic enzyme by *B. licheniformis* N-2.

Determination of proteolytic activity

Protease activity was determined by a slightly modified method of Yang and Huang (*11*). The reaction mixture containing 2 mL of 1 % casein solution in 0.05 M glycine-NaOH buffer (pH=11) and 1 mL of enzyme solution were incubated at 60 °C for 15 min and the reaction was then stopped with the addition of 3 mL of 10 % trichloroacetic acid. After 10 min the entire mixture was centrifuged at 9000×*g* for 10 min at 4 °C and absorbance of the liberated tyrosine was measured with respect to the blank at 280 nm. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 μg of tyrosine per minute, under assay conditions.

Determination of bacterial cell mass

The cell biomass was determined from a known amount of sample centrifuged at 9000×*g* for 10 min at 4 °C and the cell pellet was washed with sterilized normal saline three times to remove the suspended particles. The washed cell pellet was dried at 105 °C until constant mass and then cooled and weighed.

Protein assay

Protein content of the enzyme solution was measured by the method of Lowry *et al.* (*12*) using bovine serum albumin (BSA) as a standard.

Stability studies of alkaline protease

Effect of Ca^{2+} ions on the thermostability of alkaline protease

Thermostability of alkaline protease in the presence or absence of Ca^{2+} ions at different concentrations (5 and 10 mM) was studied by preincubating the enzyme in a water bath (Eyela, Japan) at different temperatures ranging from 30 to 80 °C for 1 h. After the treatment, the residual activity was measured as per described assay procedure.

Effect of Ca^{2+} ions on pH stability of alkaline protease

The effect of Ca^{2+} ions on pH stability of alkaline protease was determined by preincubating the enzyme without substrate in water bath (Eyela, Japan) at different pH values (6–12) for 12 h at 40 °C. The pH was adjusted using the following buffers (0.05 M): sodium phosphate (pH=6–7), Tris-HCl (pH=8–9) and glycine--NaOH (pH=10–12) in the presence or absence of 5 and 10 mM Ca^{2+} ions. The residual activity was then measured according to the standard assay procedure.

Effect of surfactants and oxidants on the stability of alkaline protease

The effect of different surfactants and oxidizing agents on alkaline protease stability was studied by measuring the enzyme activity in the presence of these agents. The alkaline protease was incubated with different concentrations of surfactants like Tween 20 (0.5 and 1.0 %), Tween 45 (0.5 and 1.0 %), Tween 65 (0.5 and 1.0 %), Triton X-405 (0.5 and 1.0 %), sodium dodecyl sulphate (SDS) (0.5, 1.0 and 5.0 %) and oxidizing agents like H_2O_2 (0.5, 1.0 and 5.0 %) and sodium perborate (0.5, 1.0 and 5.0 %) for 1 h at 40 °C. The residual activity was then measured according to the standard assay conditions (pH=11 and temperature of 60 °C).

Application of alkaline protease in removing the blood stains

The application of alkaline protease in removing the blood stains was observed according to the method of Najafi *et al.* (2) after slight modification. A clean piece of pure white cotton cloth was soaked in animal blood for 15 min and then allowed to dry at 80 °C for 5 min in hot air oven. The dried cloth was cut into equal sizes (4×4 cm^2) and incubated with crude enzyme at 40 °C for different incubation periods (10, 20, 30, 40 and 50 min). After a given incubation, the cloth was rinsed with tap water for 2 min without scrubbing and then dried in open air. The same procedure was done with the control without the enzyme exposure.

Chemicals used and statistical analysis applied

The chemicals used for all of the experiments were of analytical grade. All the experiments were carried out independently in triplicates and the data represented here are the mean value±SD. Significance was presented with Duncan's multiple range test in the form of probability ($p \leq 0.05$) values using CoStat software.

Results and Discussion

The most significant aspect of the present study is the production of alkaline protease from *B. licheniformis* N-2 by using cheaper and easily available substrates. In a preliminary study, the effect of different carbon sources on the growth and yield of the alkaline protease was observed in the basal medium (Table 1). Results indicate that different carbon sources have different impact on growth and production of extracellular alkaline protease by *B. licheniformis* N-2. It was observed that the production of alkaline protease was greatly enhanced by the addition of glucose (677.64 U/mL), followed by soluble starch (595.82 U/mL), as compared to the control (320.46 U/mL). These findings indicate that glucose is the best carbon source for alkaline protease production by *B. licheniformis* N-2. Similar effect of glucose on protease production by *Bacillus* sp. has been observed in earlier investigations (*13*,*14*). It was found that protease production increased as the concentration of glucose increased. Some other researchers also found a considerable increase in alkaline protease production by *Bacillus* sp. with glucose as a carbon source compared to control (without external carbon source) (*15*,*16*). However, the presence of citric acid and trisodium citrate in the fermented medium repressed the growth and yield of alkaline protease by *Bacillus licheniformis* N-2. This indicates that *B. licheniformis* N-2 does not have the ability to tolerate citric acid, although in an earlier investigation (*17*)

Table 1. Effect of different carbon sources (each at 1 % except control) on growth profile and alkaline protease production by *Bacillus licheniformis* N-2 after a 24-hour incubation at 37 °C and initial pH=10

Carbon source	γ(cell biomass) g/L	Enzyme activity U/mL
Control	$(2.75\pm0.06)^{ef}$	$(320.46\pm7.34)^{i}$
Xylose	$(2.81\pm0.08)^{ef}$	$(335.67\pm9.04)^{h}$
Rice bran	$(3.35\pm0.13)^{d}$	$(407.12\pm10.78)^{g}$
Rice husk	$(3.90\pm0.15)^{bc}$	$(515.35\pm10.59)^{d}$
Wheat bran	$(3.48\pm0.09)^{d}$	$(541.23\pm12.22)^{c}$
Glucose	$(3.72\pm0.11)^{c}$	$(677.64\pm8.25)^{a}$
Soluble starch	$(4.12\pm0.08)^{a}$	$(595.82\pm9.31)^{b}$
Waste bread powder	$(3.95\pm0.10)^{ab}$	$(488.60\pm8.24)^{e}$
Wheat flour	$(4.10\pm0.15)^{ab}$	$(462.55\pm7.11)^{f}$
Galactose	$(3.90\pm0.11)^{bc}$	$(320.27\pm9.20)^{i}$
Lactose	$(2.87\pm0.12)^{e}$	$(288.05\pm6.26)^{j}$
Mannose	$(3.02\pm0.10)^{e}$	$(255.11\pm5.22)^{k}$
Arabinose	$(2.98\pm0.08)^{e}$	$(260.34\pm9.81)^{k}$
Maltose	$(3.45\pm0.13)^{d}$	$(200.90\pm7.40)^{l}$
Sucrose	$(1.54\pm0.08)^{h}$	$(165.13\pm3.33)^{m}$
Fructose	$(1.88\pm0.10)^{g}$	$(107.22\pm4.11)^{n}$
Citric acid	$(0.08\pm0.00)^{i}$	0.00°
Trisodium citrate	$(0.09\pm0.01)^{i}$	0.00°

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates. Values followed by different letters differ significantly from each other at $p \leq 0.05$ using CoStat software

citric acid was used as a sole carbon source for alkaline protease production by *B. licheniformis*. On the other hand, the variations in our findings might be due to the difference in physiological characteristics and behaviour of *B. licheniformis* N-2 in the presence of citric acid. Some researchers also reported that citric acid inhibited the microbial growth by chelating divalent ions from the medium, resulting in ion depletion in the growth medium (*18*,*19*). Magnesium is an essential cofactor for many of the glycolytic enzymes and depletion of magnesium in the culture broth inhibits glycolysis (*20*). These findings also suggest that the inhibition of the growth of *B. licheniformis* N-2 in the medium containing citric acid might happen due to the magnesium-chelating activity of carbon sources.

Complex nitrogen sources are usually needed for alkaline protease production, but the requirement for specific nitrogen sources differs from organism to organism, or even among the same species isolated from different sources (*21*). Considering these facts, the effect of different nitrogen sources in the form of organic, inorganic or different defatted meals on alkaline protease production and growth of *Bacillus licheniformis* N-2 was studied to investigate the suitable nitrogen source. Organic nitrogen sources at initial concentration of 1 % (by mass per volume) showed significant effect on alkaline protease yield (Table 2). However, the addition of urea abruptly repressed the enzyme biosynthesis as well as the growth of *B. licheniformis* N-2. Among various complex nitrogen sources, yeast extract and casamino acid were also found the most suitable sources for alkaline protease production in earlier investigations (*16*,*22*). Inorganic nitrogen sources, especially ammonium salts, inhibited the growth and protease production by *B. licheniformis* N-2 whenever they were added into the growth medium. Minimum growth (0.25, 0.53 and 0.81 g/L) and protease activity (19.57, 60.77 and 90.14 U/mL) were obtained in the presence of ammonium chloride, ammonium sulphate and ammonium nitrate, respectively, each employed at 1 % (by mass per volume) concentration (Table 3). The repression of growth and protease biosynthesis might be attributed to the fast release of ammonia from these inorganic nitrogen sources. Comparable results for thermostable alkaline protease produced in the presence of organic and inorganic nitrogen sources through *B. stearothermophilus* F1 was reported by Rahman *et al*. (*23*). Many other researchers have also reported that organic nitrogen sources are better for enzyme production than inorganic ones (*7*,*24*,*25*). In contrast to both sources, maximum yield of protease (680.61 U/mL) was obtained in the presence of defatted soybean meal, followed by sunflower meal, as shown in Table 4. This indicates that defatted soybean meal is a potent inducer for alkaline

Table 2. Effect of different organic nitrogen sources (each at 1 % except control) on growth profile and alkaline protease production by *Bacillus licheniformis* N-2 after 24-hour incubation at 37 °C and initial pH=10

Organic nitrogen source	γ(cell biomass) g/L	Enzyme activity U/mL
Control	(2.45±0.13)^{e}	(245.90±5.56)^{d}
Corn steep liquor	(3.81±0.28)^{a}	(253.22±9.11)^{d}
Malt extract	(2.97±0.11)^{d}	(333.12±11.67)^{c}
Casein	(3.78±0.17)^{a}	(464.11±8.90)^{b}
Lab Lemco	(3.78±0.19)^{a}	(413.78±7.51)^{b}
Yeast extract	(3.65±0.10)^{ab}	(551.37±10.25)^{a}
Peptone	(3.30±0.27)^{c}	(470.31±10.08)^{b}
Tryptone	(3.51±0.13)^{b}	(453.56±9.02)^{b}
Beef extract	(2.49±0.10)^{e}	(445.21±11.10)^{b}
Gelatin	(2.98±0.05)^{d}	(229.31±5.88)^{d}
Urea	(0.02±0.00)^{f}	0.00^{e}

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates. Values followed by different letters differ significantly from each other at p≤0.05 using CoStat software

Table 3. Effect of different inorganic nitrogen sources (each at 1 % except control) on growth profile and alkaline protease production by *Bacillus licheniformis* N-2 after 24-hour incubation at 37 °C and initial pH=10

Inorganic nitrogen source	γ(cell biomass) g/L	Enzyme activity U/mL
Control	(2.51±0.13)^{a}	(251.45±7.04)^{a}
$NaNO_3$	(1.23±0.08)^{c}	(197.96±5.89)^{c}
KNO_3	(1.56±0.09)^{b}	(245.41±5.66)^{ab}
$NH_4H_2PO_4$	(1.10±0.11)^{c}	(241.49±7.81)^{b}
$(NH_4)_2HPO_4$	(1.13±0.10)^{c}	(175.11±6.08)^{d}
$(NH_4)_2SO_4$	(0.53±0.03)^{e}	(60.77±2.11)^{g}
NH_4NO_3	(0.81±0.05)^{d}	(90.14±4.40)^{f}
NH_4HCO_3	(1.45±0.07)^{b}	(106.54±4.08)^{e}
NH_4Cl	(0.25±0.02)^{f}	(19.57±1.20)^{h}

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates. Values followed by different letters differ significantly from each other at p≤0.05 using CoStat software

Table 4. Effect of different defatted meals (each at 1 % except control) on growth profile and alkaline protease production by *Bacillus licheniformis* N-2 after 24-hour incubation at 37 °C and initial pH=10

Defatted meals	γ(cell biomass) g/L	Enzyme activity U/mL
Control	(2.81±0.08)^{d}	(257.50±5.25)^{e}
Sunflower meal	(3.07±0.10)^{c}	(562.06±11.50)^{b}
Cotton seed meal	(4.05±0.17)^{a}	(456.23±9.26)^{c}
Soybean meal	(3.74±0.20)^{b}	(680.61±10.89)^{a}
Mustard seed meal	(2.51±0.13)^{e}	(145.76±4.06)^{f}
Corn seed meal	(3.11±0.11)^{c}	(367.89±8.88)^{d}
Canola seed meal	(2.11±0.18)^{f}	(105.21±7.23)^{h}
Rape seed meal	(2.05±0.09)^{f}	(123.12±3.32)^{g}

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates. Values followed by different letters differ significantly from each other at p≤0.05 using CoStat software

protease biosynthesis by *B. licheniformis* N-2, as compared to the other nitrogen sources. The highest protease production (3050 U/mL) was reported with soybean meal and glucose by recombinant *Bacillus subtilis* in the absence of inorganic nitrogen source (*26*). Some other researchers also reported that the presence of soybean meal in the growth medium substantially improved the production of alkaline protease (*6,27,28*). All these findings suggest that the level of protease production by *B. licheniformis* N-2 is highly responsive to the nature of nitrogen sources.

Stability studies of alkaline protease

Effect of Ca^{2+} ions on the thermostability of alkaline protease

Thermostability of alkaline protease was examined by measuring the residual activity at 60 °C after incubation of the enzyme without substrate for 1 h at various temperatures ranging from 30 to 80 °C (Fig. 1). The enzyme was found stable up to 50 °C and above this temperature its activity decreased. However, the alkaline protease retained 100 % of its original activity at 50 °C, and 61 % at 60 °C in the presence of 5 mM Ca^{2+} ions. In the presence of 10 mM Ca^{2+} ions, 92 % of the original activity was retained at 60 °C. This finding indicates that Ca^{2+} ions may stabilize the structure of the enzyme, which consequently increases its thermal stability. Ca^{2+} ions have been described to keep 78 % of residual activity of thermostable enzymes after incubation at 80 °C for 1 h in the presence of 10 mM Ca^{2+} ions (*29*).

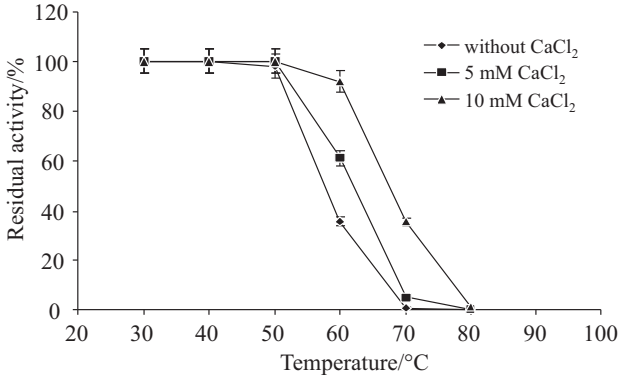

Fig. 1. Effect of Ca^{2+} ions on thermostability of alkaline protease produced by *B. licheniformis* N-2. The stability was expressed in percentage of residual activity

Effect of Ca^{2+} ions on pH stability of alkaline protease

The effect of Ca^{2+} ions on pH stability of protease was determined by preincubation of the enzyme at 40 °C for 12 h with different buffers at various pH values ranging from 6 to 12 in the presence or absence of 5 and 10 mM Ca^{2+} ions. The enzyme was found stable over a broad range of pH (8–11) and lost 52 % of its residual activity at pH=12 (Fig. 2). The function of an enzyme is absolutely dependent on its three-dimensional structure. The pH level is one of the factors that affect the structure of not only enzymes but all proteins. The pH values beyond the range of 8–11 could alter the three-dimensional structure of alkaline protease by disturbing the electrostatic interactions among the charged amino acids, resulting in loss of enzyme activity. However, in the presence of 5 and 10 mM Ca^{2+} ions, the enzyme retained its residual activity up to 71 and 80 %, respectively. Similar results were reported by Sookkheo *et al.* (*30*), who found 60 % proteolytic retention at pH=10 in the presence of 5 mM Ca^{2+} ions. All these investigations indicate that thermal and pH stabilities of the enzyme depend on the available concentration of Ca^{2+} ions in the enzyme solution.

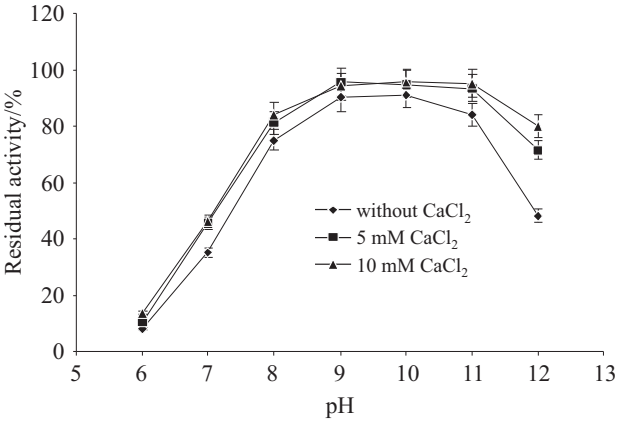

Fig. 2. Effect of Ca^{2+} ions on pH stability of alkaline protease produced by *B. licheniformis* N-2. The stability was expressed in percentage of residual activity

Effect of surfactants and oxidants on the stability of alkaline protease

Effects of various surfactants and oxidizing agents at different concentrations on protease activity after preincubation at 40 °C for 1 h were examined and then the stability of enzyme in terms of residual activity was determined (Table 5). The enzyme produced by *B. licheniformis* N-2 showed 123, 102 and 118 % activity in the presence of Tween 20, Tween 45 and Tween 65 respectively, each at 0.5 % (by volume). The enzyme was also found to express >100 % activity in the presence of Tween 20 and Tween 65, each at 1 % concentration. However, the enzyme lost its activity in the presence of SDS and Triton X-405. Hydrogen peroxide is known to be a strong oxidizing agent, and it mediates oxidative inactivation of proteins. Met has been identified as a primary site for oxidative inactivation of the enzyme, and most of the subtilisins containing Met residue next to the catalytic Ser residue undergo oxidative inactivation after the treatment with oxidizing agents such as H_2O_2 (*31*). Therefore, the enzymes showing extreme stability towards oxidizing agents are of immense commercial significance for detergent industry because peroxides and perborates are common ingredients of modern bleach-based detergent formulations. H_2O_2 and sodium perborate, each at 1 % concentration, stimulated the residual alkaline protease activity up to 135 and 126 %, respectively, whereas 124 and 108 % increase in residual activity was observed at 5 % concentration of H_2O_2 and sodium perborate, respectively. Previous reports on stability of alkaline protease indicated that *Bacillus clausii* I-52 protease exhibited residual activity up to 114 % after treatment with 1 %

Table 5. Effect of different oxidizing agents and surfactants on alkaline protease activity produced by *B. licheniformis* N-2

Surfactants/ oxidizing agents	*w*/%	Residual activity/%
Control	–	100.00
Tween 20	0.5	123.45
	1.0	105.50
Tween 45	0.5	102.95
	1.0	82.36
Tween 65	0.5	118.05
	1.0	116.78
Triton X-405	0.5	98.93
	1.0	109.85
	5.0	7.42
SDS	0.5	33.54
	1.0	27.12
	5.0	15.17
H_2O_2	0.5	118.43
	1.0	135.36
	5.0	124.07
Sodium perborate	0.5	115.35
	1.0	126.61
	5.0	108.23

Residual activity was expressed as a percentage of the activity level in the absence of surfactants and oxidizing agents. Separate blank was prepared for each agent

H_2O_2 (*6*), while an alkaline protease from *Vibrio fluvialis* VM10 strain showed 132 % activity after incubation with 4 % H_2O_2 (*32*). From the viewpoint of stability of oxidants, the alkaline protease from *B. licheniformis* N-2 is an excellent candidate for industrial applications, especially in detergent industry.

Application of enzyme in removing the blood stains

The blood stain was removed from a white cotton cloth by simply incubating the cloth in enzyme broth for different time intervals (Fig. 3). It was seen that alkaline protease produced by *Bacillus licheniformis* N-2 had high capability of removing the blood stain, which indicates its potential in detergent industries. In earlier reports, proteases from alkaliphilic bacteria and *Pseudomonas aeruginosa* PD100 were used to remove the blood stain from the cotton cloth in the absence of detergents (*2*,*33*).

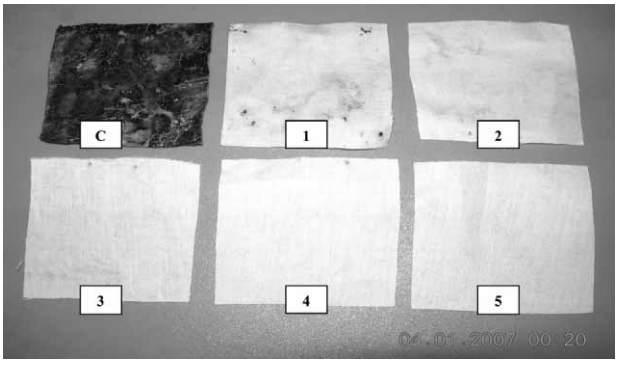

Fig. 3. Removal of blood stains from cotton cloth pieces (4×4 cm^2) by alkaline protease produced by *B. licheniformis* N-2. The blood-stained cotton cloth pieces were incubated with alkaline protease broth ((680.61±10.89) U/mL) at 40 °C for different time intervals. C=control (without treatment), 1=10 min, 2=20 min, 3=30 min, 4=40 min and 5=50 min

Conclusions

From the results, it has been concluded that the screening of suitable medium ingredients plays an important role in the production of proteolytic enzymes by *B. licheniformis* N-2. The presence of glucose and soybean meal in the growth medium has inducible effect on enzyme production, whereas ammonium salts repress the production of enzymes. Stability studies indicate that the enzyme is stable over a broad range of pH and temperature in the presence of Ca^{2+} ions. The enzyme was also found very effective in removing the blood stains from a cotton cloth and is compatible with most of the surfactants and oxidizing agents. These properties indicate that alkaline protease produced by *B. licheniformis* N-2 is an excellent candidate for use as detergent additive in laundry industry.

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