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EXPERIMENTAL ARTICLES

An Organic Solvent-, Detergent-, and Thermostable Alkaline Protease from the Mesophilic, Organic Solvent-Tolerant *Bacillus licheniformis* 3C5[1]

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Abstract—*Bacillus licheniformis* 3C5, isolated as mesophilic bacterium, exhibited tolerance towards a wide range of non-polar and polar organic solvents at 45°C. It produced an extracellular organic solvent-stable protease with an apparent molecular mass of approximately 32 kDa. The inhibitory effect of PMSF and EDTA suggested it is likely to be an alkaline serine protease. The protease was active over a broad range of temperatures (45–70°C) and pH (8–10) range with an optimum activity at pH 10 and 65°C. It was comparatively stable in the presence of a relatively high concentration (35% (v/v)) of organic solvents and various types of detergents even at a relatively high temperature (45°C). The protease production by *B. licheniformis* 3C5 was growth-dependent. The optimization of carbon and nitrogen sources for cell growth and protease production revealed that yeast extract was an important medium component to support both cell growth and the protease production. The overall properties of the protease produced by *B. licheniformis* 3C5 suggested that this thermo-stable, solvent-stable, detergent-stable alkaline protease is a promising potential biocatalyst for industrial and environmental applications.

Key words: organic-solvent tolerant bacterium; *Bacillus licheniformis*; thermo-, organic-, solvent-, detergent-stable protease; alkaline protease

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INTRODUCTION

In recent years, important roles for organic solvent tolerant bacteria have been recognized in both industrial and environmental applications because the microorganisms, as well as their prominent enzymes, can actively function as biocatalysts in reactions where toxic organic solvents are present [1]. Numerous investigations have been conducted on not only the characterization of the organic-solvent tolerant bacteria, but also on the characteristics and the production of their enzymes. These microbial enzymes, which act in both biphasic aqueous-organic system and non-aqueous solvents [2], offer new possibilities and expand their potential use in chemical synthesis as well as in waste treatment applications.

Microbial proteases are one of the most important commercial hydrolytic enzymes and they are widely used in several industrial sectors, such as the food, pharmaceutical and detergent industries. In addition, solvent-stable proteases have been used as biocatalysts in non-aqueous media for various synthesis reactions. Due to the advantages of solvent-stable proteases, several attempts to improve regular enzymes for their activity and stability in the presence of organic solvents have been based on physical, chemical and protein engineering techniques. The stability of the modified enzymes in the organic media, however, has not yet been fully satisfied [2]. Therefore, an extensive search for naturally solvent-stable proteases has been carried out. Although Gram-positive bacteria, especially *Bacillus* sp., have been recognized as the predominant protease-producing bacteria, extensive studies on solvent-stable proteases have so far oly been carried out in Gram-negative, organic solvent-tolerant *Pseudomonas* sp. [3]. Only a few studies on solvent-stable enzymes from organic solvent-tolerant Gram-positive bacteria have been reported [4, 5].

In view of this, a group of mesophilic, organic solvent-tolerant Gram-positive bacteria, previously isolated in our laboratory, were expected to be prominent sources of organic solvent-stable proteases and so were screened. In this present study, an extracellular solvent-stable protease produced by the organic solvent-tolerant *B. licheniformis* 3C5 strain is reported. The enzyme characterization and the parameters affecting

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both bacterial growth and extracellular protease production are delineated.

MATERIALS AND METHODS

Bacterial type-strain and chemicals. The type strain of *B. licheniformis* ATCC 14580 was purchased from the BIOTEC culture collection, Thailand. All culture medium components were from Scharlau Chemie (Barcelona, Spain). Organic solvents were from Lab-Scan (Bangkok, Thailand). All chemicals used were of analytical grade.

Bacterial enrichment, isolation, identification and cultivation. The bacterial isolates were obtained from soil acclimatized with cyclohexane vapors in a sealed container at 45°C. The procedure of pure bacterial culture isolation was as described by Kongpol et al. [6] using either minimal salt basal medium (MSB) or MSB supplemented with 0.5% (w/v) yeast extract (MSBY) at pH 7.2. In addition to morphometric and biochemical classification (not shown) the likely species identity of the selected bacterial isolates were evaluated by comparison of the 16S rRNA sequence. Genomic DNA from each strain was extracted using a standard method [7], and the PCR mixture and the thermocycling conditions for amplification of a partial fragment of the 16S rRNA gene were carried out as previously described [6]. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, USA) and sequenced (Macrogen, Seoul, Korea). The partial 16S rRNA gene sequences were aligned and compared with the sequences placed in GenBank using BLASTN searches. As a result, a preliminary identification of the novel isolates was deduced by sequence similarity.

Determination of the organic solvent tolerance of the selected strain. The ability of the selected bacterial strain to survive in the presence of organic solvents was investigated by an overlay technique [6]. Briefly, the culture was streaked onto an agar slant and allowed to dry, and then the test solvent was added directly on top so that the solvent layer was approximately 5 mm-thick. The slant was incubated in a slanted position, so as to maintain the test solvent as an even 5 mm thick covering, at 45°C for 5 days. The colony formation on the slant surface overlaid with solvent was observed and used to indicate the tolerance when the growth was compared to that seen without the addition of any test solvent. The level of growth shown by the colony formation was expressed as – for no growth, +, ++ and +++ for enhanced growth after 3 days in comparison to that of bacterial colonies formed without organic solvent, and +/– for cell growth observed at a slower rate after 5 days.

Screening and selection of organic solvent-tolerant protease producers. The organic-solvent tolerant bacteria were examined for protease production. Cells were grown on skimmed milk agar at pH 7.0, 8.0 or 9.0, with and without cyclohexane overlay, and incubated at 45°C for 24 h. The diameter of each bacterial colony and the clear zone around the colony were measured and the ratio of the clear-zone diameter over the colony diameter was compared for at least four independent measurements. The bacterial strain that exhibited the highest ratio was then chosen for further studies.

Determination of protease activity and protein concentration. Protease activity was determined as caseinolytic activity. The crude enzyme solution was mixed with a 1% (w/v) casein solution in 100 mM Tris-HCl buffer, pH 8.0, at an enzyme:substrate solution (v/v) ratio of either 1:9 or 1:4.5. The reaction volume was always adjusted to 550 μl using 100 mM Tris-HCl buffer, pH 8.0. This reaction mixture was then incubated at 45°C for 30 min, whereupon the reaction was stopped by immediately mixing with 450 μl of a 5% (w/v) TCA solution, incubating at 30°C for 15 min and removal of the precipitate by centrifugation at 6339 *g* for 15 min. The supernatant was harvested and assayed for absorbance at 280 nm, with the absorbance being used to estimate the amount of tyrosine liberated by comparison with a tyrosine standard curve produced from tyrosine concentrations in the range of 0–100 *g* ml^{-1}. Controls, one without casein substrate and another without enzyme, were always carried out and the amount of tyrosine generated by the controls, if any, was subtracted from the experimental value. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 *g* of tyrosine per min under the assay conditions. Protein concentration was determined by the method of Lowry [8]. Under some growth conditions, as later indicated, where a red pigment was formed, a slight modification of the assay protocol was performed [9] to avoid absorbance interference of the pigment at 242 and 280 nm. Briefly, after TCA addition, 350 μl of supernatant was mixed with 750 μl of a 500 mM Na_2CO_3 solution. The mixture was incubated in the dark at 37°C for 10 min, and then 150 μl Folin's reagent was added prior to incubation in the dark at 37°C for another 30 min. The mixture was then centrifuged at 12000 *g* for 5 min. An absorbance of the supernatant was then determined at 660 nm.

SDS-Polyacrylamide gel electrophoresis (PAGE) and zymogram analysis. Extracellular proteins in the cell supernatant were analyzed by SDS-PAGE followed by silver staining. The relative molecular mass of protein was estimated using a low-molecular weight (LMW) protein marker (GE Life Sciences, Singapore). For the zymogram analysis, the proteins were separated and the protease activity was analyzed simultaneously on a skimmed-milk SDS-PAGE, as previously described [10]. In brief, a 100 μg of the crude supernatant protein obtained from each stage of growth was loaded into the gel without heating. After gel electrophoresis, the gel was soaked in developer buffer (2.5% (v/v) Tritron X-100 in 50 mM Tris-HCl buffer, pH 8.0) to remove SDS and allow renaturation of proteins, and then the gel was incubated at 45°C for 1 h in the same

buffer to allow the reaction to occur, followed by staining and de-staining of the gel, respectively. The zone of proteolytic activity appeared as a cleared band on the gel. In separate gels run on aliquots from the same protein samples, phenylmethylsulphonyl fluoride (PMSF) (1 mM) was included in the develop buffer to investigate whether there was an inhibition of the enzyme activity by PMSF.

Parameters affecting protease activity and stability. The effect of organic solvents and detergents on the enzyme activity and stability were investigated as follows. The crude enzyme was mixed with either the test organic solvents at 35% (v/v) or detergents (0.5% or 1%, v/v or w/v as indicated). The control enzyme activity observed without solvent or detergent exposure under the same standard conditions was set as 100% activity and all other activities related to this as a relative %. The activity upon solvent or detergent mixing was used to represent the enzyme's sensitivity to the solvent, expressed as the percentage activity observed relative to that of the control. The stability of the enzyme to the test organic solvent or detergent was evaluated after incubation for 1 h and 6 h at 45°C in the presence of the organic solvent and for 6 h and 12 h at 45°C in the presence of the detergent. The residual activity was determined and expressed as a percentage relative to the activity of the enzyme without chemical addition as above.

The effect of metal ions (5 mM) and inhibitors (1 mM of PMSF and ethylene-diaminetetraacetic acid (EDTA)) on the protease activity were examined as follows. The crude enzyme was pre-incubated with the indicated metal ion or an inhibitor for 30 min at 45°C and the activity was then determined in comparison to that of the control without the pre-incubation.

An optimum pH for the crude protease was determined over the pH range of 4–12 using the following buffers (all at 0.1 M): acetate buffer (pH 4–6), phosphate buffer (pH 6–7), Tris buffer (pH 8–9), glycine-NaOH (pH 10–11), and carbonate buffer (pH 11–12). The pH stability of the enzyme was investigated by pre-incubating the enzyme in the appropriate buffer at 45°C and determining the residual enzyme activity after 12 h and 24 h under the standard assay conditions and expressed as a percentage relative to the activity of the enzyme without the pre-incubation, as above.

The optimum temperature of the crude enzyme was studied by monitoring the enzyme activity over temperature range of 25–80°C at pH 8.0 and pH 10. The temperature stability was determined by pre-incubating the enzyme for 12 h and 24 h at the various indicated temperatures and then determining the residual enzyme activity under standard conditions as a percentage relative to the activity of the enzyme without the pre-incubation, as above.

Parameters affecting cell growth and protease production. The effect of nutrient components on the growth and the protease production of the selected protease-producing strain were examined as follows. The selected strain was grown in Luria-Bertani (LB), MSB, MSBY medium or MSBY supplemented with one of the following carbon sources (10 g l^{-1}): glucose, fructose, lactose, xylose, sorbitol, soluble starch, sucrose, glycerol or crude glycerol (a by-product from the biodiesel production process). Then, the best carbon source among these in terms of yielding the greatest protease production was selected for further evaluation with the following nitrogen sources (10 g l^{-1}): peptone, tryptone, casein, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$ and urea. Cells were incubated at 45°C with shaking at 200 rpm. Cell growth was determined spectrophotometrically by measuring the absorbance at 600 nm (DU800, Beckman Coulter, Inc., USA) and the cell supernatant was assayed for the protease activity. The time course of the protease production by the selected strain was then examined by collecting cell culture at each stage of growth, removal of the bacteria by centrifugation and assaying the cell supernatant for the level of protease production, as above.

RESULTS AND DISCUSSIONS

Selection of organic solvent-stable protease producing bacterial isolates. Twenty four strains of mesophilic organic-solvent tolerant bacteria, previously isolated at 45°C from cyclohexane enriched-soil in our laboratory, were examined for their ability to produce any extracellular caseinolytic protease. All of the strains tested were able to produce protease with different extent, yielding a clear zone around their colonies on the skim-milk agar plate. The result varied in extent between the different bacterial isolates (Fig. 1). The phylogenetic position of the studied strains, determined by 16S rRNA gene comparison, indicated that they are all affiliated with the genus *Bacillus*, which is well known for its protease producing representatives. Nevertheless, when the colony on plate was submerged with cyclohexane and incubated at 45°C for 24 h, the protease from only eight strains, i.e. strains 4/1, 5/1, 21, 24, 24/1, 45, 46 and 3C5, remained active and was able to hydrolyze the skimmed milk generating a significant clear zone. This result suggested that the enzymes produced by the organic solvent-tolerant bacteria does not necessarily exhibit natural resistance to organic solvents as previously presumed [5]. In this study, the protease from strain 3C5 remained highly active in the presence of cyclohexane; therefore, strain 3C5 was selected as the most potent protease producer for further study. The strain was identified from the 16S rRNA sequence analysis as *Bacillus licheniformis* 3C5.

In addition to the cell tolerance to cyclohexane, *B. licheniformis* 3C5 demonstrated tolerance ability towards other organic solvents with a broad range of log P_{ow} values, that is partition coefficients of the solvent from a water phase into an octanol phase, as shown in table. When a similar test was performed with

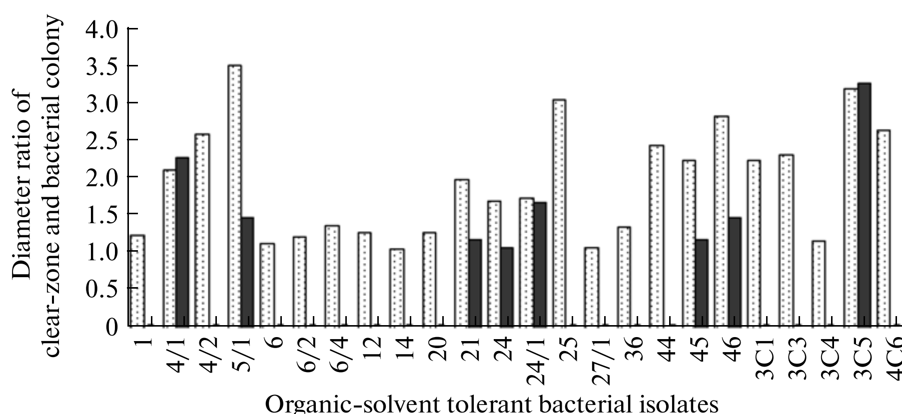

Fig. 1. Screening of organic-solvent tolerant bacterial strains for their ability to produce extracellular organic-solvent stableprotease. The ratio of the clear-zone diameter and the bacterial colony diameter on a skimmed milk agar plate in the absence (⊡) and in the presence (■) of cyclohexane overlaid on the plate. The diameters of clear zone and bacterial colony were evaluzted after incubation for 24 h at 45°C. A minus symbol (-) indicates no detectable clear zone on the plate in the presence of overlaid cyclohexane. Data are shown for each bacterial isolate (isolate code on the X axis) as the mean ± 1SD, derived from at least four independent experiments.

a type-strain *B. licheniformis* ATCC 14580, it tolerated only some of the organic solvents with a log P_{ow} greater than 4.66 which, theoretically, are non-toxic for microorganisms [1]. The result clearly indicated that the organic solvent tolerance was, in fact, strain-specific. However, it should be noted that the tolerance level derived from the growth on agar medium overlaid with the organic solvent could be different from that in a two-liquid phase fermentation system in which additional parameters, such as aeration, may be involved [1].

SDS-PAGE and zymogram analysis of the *B. licheniformis* 3C5 protease. Previous reports have shown that a bacterium can produce not only a number of proteases simultaneously, but their characteristics can also be largely varied. Molecular masses of previously reported proteases from *Bacillus* spp. vary from 28 kDa for *B. pumilus* MK6-5 [11] up to 55 kDa for that from *B. licheniformis* RSP-09-37 [5]. In this study, the zymogram analysis of the extracellular proteins of the cell supernatant using a skimmed-milk-SDS-PAGE revealed that *B. licheniformis* 3C5 produced only one type of active extracellular protease, as defined by SDS-PAGE estimated size and inhibitor sensitivity, under the growth and assay conditions provided (Figs. 2a and 2b). The protease appeared as a monomeric protein with an apparent molecular mass of approximately 32 kDa (Fig. 2b). Its activity was completely inhibited by 1 mM PMSF (Fig. 2c) suggesting a type of serine protease.

Effect of organic solvents and detergents on the protease activity and stability. Since our interest originated from the fact that *B. licheniformis* 3C5 protease could actively function in the presence of cyclohexane, the enzyme activity and stability towards various organic solvents was investigated prior to further characterization. Generally, organic solvents adversely affect enzyme activity because they interact directly with the essential water surrounding the enzyme molecule [2]. The enzymatic activity is generally high in a non-polar organic solvent with log $P_{ow} > 4$, moderate in solvents with log P_{ow} 2–4, and a low activity in a polar organic solvent with log $P_{ow} < 2$ [1]. Nevertheless, the protease activity of *B. licheniformis* 3C5 was increased by up to 20% when initially exposed to most of the organic solvents tested, although it was partially inactivated (by 4–20%) upon the exposure to diethyl ether, DMF, and

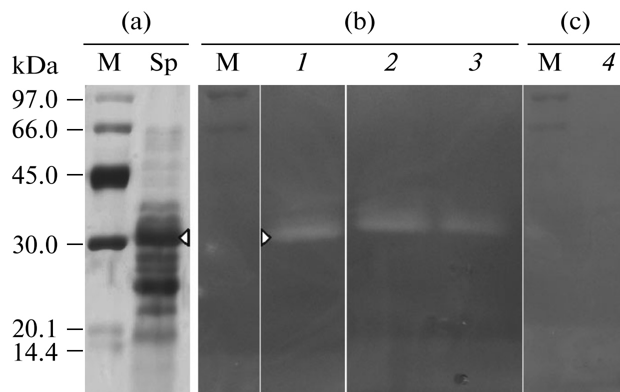

Fig. 2. Analysis of a protein profile (a) and the proteolytic activity (b) of the extracellular protease from *B. licheniformis* 3C5. (a) SDS-PAGE of 100 μg extracellular cell supernatant protein (Sp) next to 5 μl of a low-molecular weight (LMW) protein marker (M), and visualized by silver staining. (b) Skimmed-milk SDS-PAGE zymogram of 100 μg extracellular cell supernatant protein from 15-h (Lanes *1* & *4*), 18-h (Lane *2*), and 24-h (Lane *3*) cultures and developed in buffer (lane *1–3*) alone or with 1 mM PMSF (Lane *4*). The arrows represent the protein band with the proteolytic activity. Gels shown are representative of at least three independent repeats.

Organic solvent tolerance of *B. licheniformis* 3C5 and *B. licheniformis* ATCC 14580 type-strain

Organic solvent	[a] $\log P_{ow}$	[b]Bacterial growth	
		B. licheniformis 3C5	*B. licheniformis* ATCC 14580
None	–	++++	++++
n-Octane	5.18	++	++
n-Decane	5.01	+++	+++
n-Heptane	4.66	++	++
n-Decanol	4.57	+	–
Cyclohexane	3.44	+++	–
Ethylbenzene	3.15	+	–
n-Octanol	3.00	+/–	–
Toluene	2.73	+	+/–
n-Heptanol	2.62	–	+/–
Benzene	2.13	+	–
Trichloromethane (Chloroform)	1.97	+	–
Butyl acetate	1.78	++	–
Methyl butyl ketone	1.38	+	–
Dichloromethane	1.25	+	–
Diethyl ether	0.89	–	–
n-Butanol	0.88	+	–
Ethyl acetate	0.73	+/–	–
iso-Butanol	0.61	++	–
Methyl ethyl ketone	0.29	+/–	–
Acetone	–0.24	+/–	–
Acetonitrile	–0.34	–	–
Dimethylformamide	–1.01	–	–
Dimethylsulfoxide	–1.35	++	+

Notes: [a] $\log P_{ow}$ values were obtained from KOWWIN v.1.67 (2000) (US EPA, USA).

[b] *B. licheniformis* 3C5 and *B. licheniformis* ATCC 14580 type-strain were grown at 45°C and 37°C, respectively, on an MSBY agar slant overlaid with each organic solvent. The symbols, +++, ++, and + represent the relative cell growth to that without organic solvent exposure observed after 3 days of incubation, +/– represents a relative cell growth observed at a slower rate (at 5 days of incubation) and – means no relative cell growth.

DMSO (Fig. 3). Overall, the protease was well stable towards various types of non-polar and polar organic solvents at a relatively high temperature in that it retained more than 80% of its initial activity after solvent exposure for 1-h, while the activity was only slightly further decreased (20–25%) after incubation for 6 hours.

Given that bacterial proteases have their main biotechnology-based applications in the wastewater treatment and laundry detergent industries, the detergent-stability of the protease was determined. The three non-ionic surfactants, Triton X-100, Tween80, and Tween20, and the strong anionic surfactant, SDS, were selected for this study. The protease activity was slightly increased when either the non-ionic or anionic surfactant was initially added (Fig. 4). Its activity was stable and remained relatively high after a 6 h (≥95% of the original activity) and 12 h (~70–85% of the original activity) incubation period at 45°C, while the activity of the solvent-stable protease from *B. licheniformis* RSP-09-37 dropped by 20% and 30% when it was incubated for 1 h with 1% (v/v) Triton X-100 or 1% (w/v) SDS, respectively [5]. The relatively good stability of the protease produced by *B. licheniformis* 3C5 in the presence of surfactants or organic solvents over a certain period of time (6 h) at a relatively

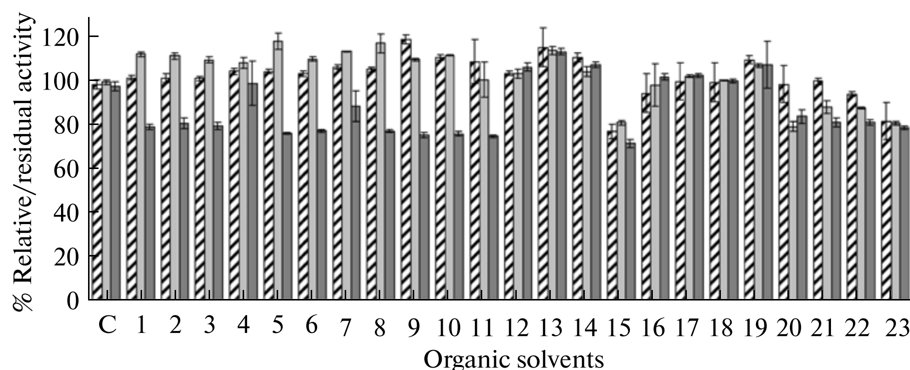

Fig. 3. Effect of organic solvents on the activity and stability of the *B. licheniformis*3C5 extracellular protease. Each organic solvent was mixed with the crude enzyme solution to 35% (v/v) and incubated at 45°C. The relative enzyme activity was determined after mixing (▨), and the residual activity was measured after 1-h (▒) and 6-h (■) incubation and compared to that (100%) of a non-solvent containing control (C). The organic solvents, in order of Log P_{ow} (in parenthesis), were; 1, *n*-octane (5.18); 2, *n*-decane (5.01); 3, *n*-heptane (4.66); 4, *n*-decanol (4.57); 5, cyclohexane (3.44); 6, ethylbenzene (3.15); 7, *n*-octanol (3.00); 8, toluene (2.73); 9, *n*-heptanol (2.62); 10, benzene (2.13); 11, chloroform (1.97); 12, butyl acetate (1.78); 13, methyl butyl ketone (1.38); 14, dichloromethane (1.25); 15, diethyl ether (0.89); 16, *n*-butanol (0.88); 17, ethyl acetate (0.73); 18, isobutanol (0.61); 19, methyl ethyl ketone (0.29); 20, acetone (–0.24); 21, acetonitrile (–0.34); 22, dimethyl formamide (–1.01); 23, demethyl sulfoxide (–1.35). Data are shown as the mean ± 1SD and are derived from at least three experiments. Log P_{ow} values were obtained from KOWWIN v.1.67 (2000) (US Environmental Protection Agency, USA).

high temperature suggests its potential application in the detergent industries, as well as in municipal and industrial wastewater treatment systems, where toxic organic solvents and/or surfactants are accumulated [12].

Effect of metal ions and inhibitors on protease activity. The addition of Ca^{2+} or Mg^{2+} resulted in an increase in the enzyme activity of up to 131 ± 12% and 108 ± 7% of the initial activity, respectively. On the other hand, the addition of Cu^{2+}, Mn^{2+} or Ni^{2+} decreased the activity almost two-fold to 58 ± 10 %, 45 ± 6%, and 48 ± 5%, respectively. These results are in agreement with a previous report which indicated a role for Ca in the stabilization of an enzyme in its active conformation [13]. Subsequently, the addition of EDTA (1 mM), a potent inhibitor of metalloprotease, caused a drop in the enzyme activity by 15% as a result of Ca^{2+} chelation, while the protease activity was completely inhibited by PMSF (1 mM). These results support that the protease of *B. licheniformis* 3C5 is an alkaline serine protease, where Ca^{2+} plays a role in enzyme stabilization leading to the slight increase in the overall activity under the conditions used.

Effect of pH and temperature on the protease activity and stability. The protease appeared to maintain its activity across the broad pH range of 5–12 with an optimum pH at pH 10 and minimum with this tested range at pH 4.0 (Fig. 5a). Likewise, it exhibited a relatively high activity within the temperature range of 30–70°C, when tested at pH 8.0 and pH 10.0, increasing in activity with increasing temperature up to a maximum at 65–70°C, and then the enzyme activity declines at temperatures higher than 70°C (Fig. 5b). When the enzyme stability towards a range of pH values was determined, the residual activity was found to still be higher than 75% after the incubation for 12 h and 24 h within the pH range of 5–11 (Fig. 5c). As to the enzyme's thermo-stability, when determined at pH 8.0, the remaining activity was higher than 75% after incubation at 30–45°C, while it gradually decreased with incubation at temperatures higher than

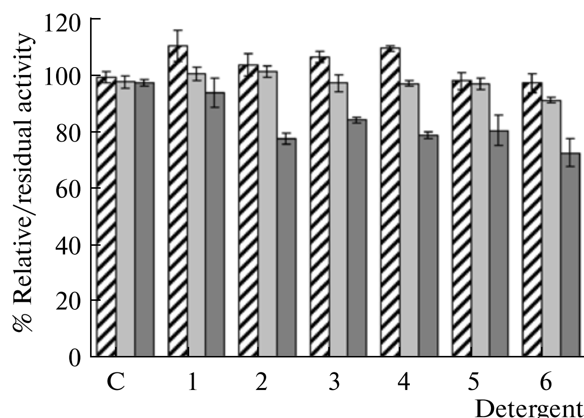

Fig. 4. Effect of detergents on the activity and stability of the *B. licheniformis* 3C5 extracellular protease. The detergent was mixed with the crude enzyme solution and incubated at 45°C. The relative activity was determined after mixing (▨), and the residual activity was measured after 6-h (▒) and 12-h (■) incubation and compared to that (100%) of a non-solvent containing control (C). The detergents evaluated were the non-ionic detergents (1) 1% (v/v) Triton X-100, (2) 1% (v/v) Tween 80, (3) 1% (v/v) Tween 20 and (4) 1% (v/v) Brij 35; as well as the anionic detergent (5) 0.2% (w/v) SDS and (6) 1% (w/v) SDS. Data are shown as the mean ± 1SD and are derived from at least three experiments.

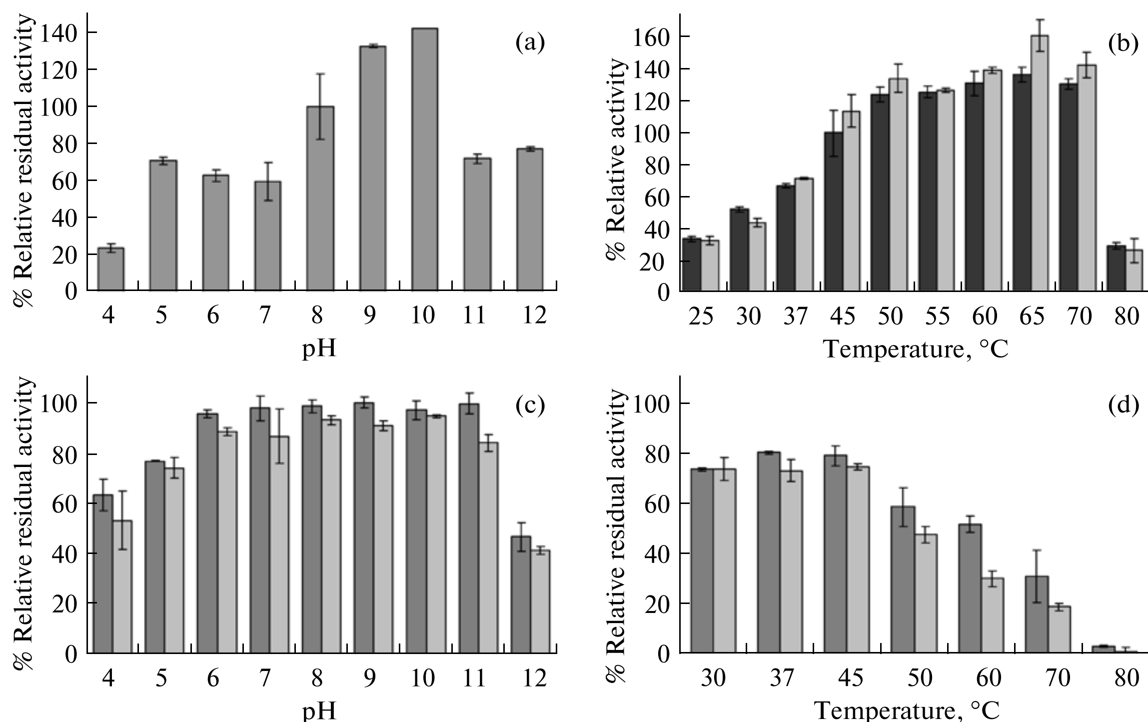

Fig. 5. Effect of pH (a), (c) and temperature (b), (d) on the activity (a), (b) and stability (c), (d) of the *B. licheniformis* 3C5 extracellular protease. The protease activity was assayed at 45°C for (A) different pH values using the following buffers (all 0.1 M): sodium acetate (pH 4-6), phosphate buffer (pH 6–7), Tris buffer (pH 8–9), glycine-NaOH (pH 10-11), and carbonate buffer (pH 11-12) and for (b) different temperature at either pH 8.0 (■) or pH 10.0 (□) The pH and temperature stability of the protease was determined by pre-incubating the enzyme in the buffer with (c) different pH values or (d) with different temperatures at pH 8.0 for either 12 h (■) or 24 h (□), and then evaluating the residual enzyme activity according to the standard assay conditions. Data are shown as the mean ± 1SD and are derived from at least three experiments.

that and was inactivated at 80°C (Fig. 5d). The apparent pH optimum of 10 for the protease produced by *B. licheniformis* 3C5 is similar to that of other previously reported alkaline proteases [5, 13]. However, it has a comparatively higher optimal temperature (65°C) than other alkaline proteases produced by *Bacillus* species, including the two solvent-stable proteases from *B. licheniformis* strains RSP-09-37 [5] and YP1A [14].

Effect of carbon and nitrogen sources on cell growth and protease production. The protease produced by *B. licheniformis* 3C5 is active and stable under harsh conditions, suggesting its potential use not only in chemical industries, but also in wastewater treatment. However, such applications would require a plentiful and cheap supply of enzyme. Therefore, the optimization of growth conditions by way of increasing active extracellular protease yield was carried out. *B. licheniformis* 3C5 grown on MSBY medium generally exhibited a specific growth rate of 0.126 ± 0.012 h^{-1} and yielded the highest protease activity of 5.6 ± 1.2 U ml^{-1}. Variation of carbon and nitrogen sources clearly influenced both the bacterial growth rate and the protease production. Since the addition of yeast extract was shown to be necessary to support cell growth, the effect of an additional carbon source supplemented into the MSBY medium was evaluated in terms of both the cell growth rate and active protease production (Figs. 6a and 6b). Medium containing different sugars (10 g l^{-1}) showed an enhancement of cell growth, ranging from 14–80%, while those supplemented with glycerol or crude glycerol reduced the cell growth by approximately 25%. Contrary to the cell growth enhancement, the addition of a supplement carbon source not only caused the pH to drop by 1–2 units, but also substantially reduced the protease yield, probably due to the presence of a readily utilizable carbon source. Although a similar phenomenon has been reported by several other groups of researcher [15–18], it can not be taken for granted as in a few cases the addition of a readily utilizable carbon source was reported to actually enhance the protease production [19–21]. The addition of sugar also caused the formation of an extracellular, water-insoluble red pigment which was clearly observed upon the addition of most of the carbon sources, whereas the formation of such was at negligibly low level when cells were grown in MSBY, MSBY-lactose and MSBY-glycerol (data not shown). Further investigations indicated that this red

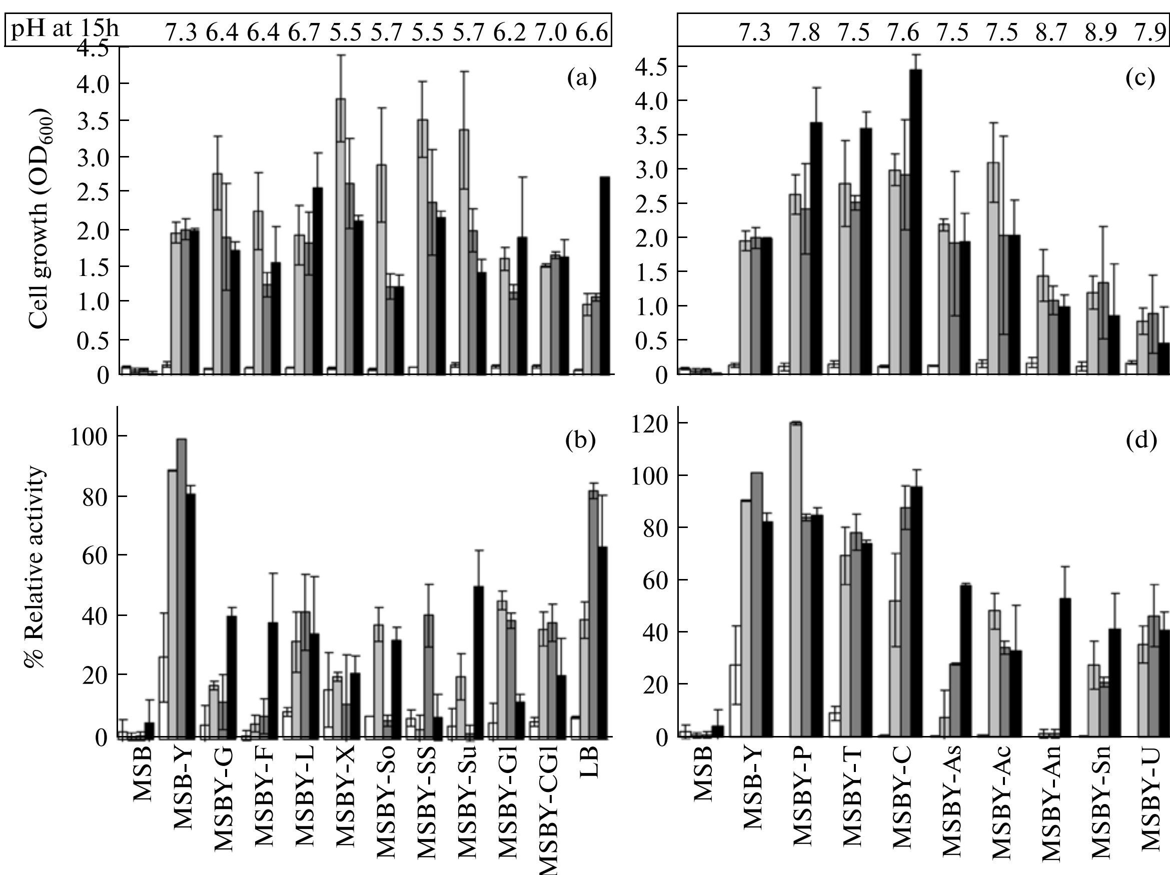

Fig. 6. Effect of carbon and nitrogen sources on the (a), (c) cell growth, and (b), (d) protease production, of *B. licheniformis* strain 3C5. *B. licheniformis* strain 3C5 were grown at 45°C in Luria-Bertani medium (LB) or minimal salt basal medium (MSB) as base references, and compared to that when grown in MSB supplemented with yeast extract (5 g.L^{-1}) (MSBY) with or without various carbon source (10 g.L^{-1}) and nitrogen source (10 g.L^{-1}) sources, before (a), (c) the cell growth and (b), (d) extracellular protease activity levels were determined at 0 h (□), 15 h (▥), 18 h (▥), and 24 h (■) culture. Supplemented carbon sources (a), (b) were: glucose (G), fructose (F), lactose (L), xylose (X), sorbitol (So), soluble starch (SS), sucrose (Su), glycerol (Gl), crude glycerol (CGl). Supplemented nitrogen sources (c), (d) were: peptone (P), tryptone (T), casein (C), ammonium sulfate (As), ammonium chloride (Ac), ammonium nitrate (An), sodium nitrate (Sn), urea (U). The pH of the medium after 15 h of culture is shown above. Data are shown as the mean ± 1SD and are derived from at least three experiments.

pigment was pulcherrimin [22] and its formation was significant only when the additional sugars were provided (data not shown).

B. licheniformis 3C5 grew to a much lower extent in the absence of yeast extract, even if yeast extract was replaced with another organic nitrogen source (data not shown). Nevertheless, when the organic nitrogen (except urea) was individually combined to MSBY medium, cell growth was significantly enhanced (Fig. 6c). On the other hand, the addition of inorganic nitrogen, apart from $(NH_4)_2SO_4$ and NH_4Cl, was observed to adversely affect cell growth. As has been observed in the production of several other proteases [17, 20, 23, 24], protease production by *B. subtilis* 3C5 was stimulated in the presence of complex organic nitrogen sources (Fig. 6d). In this study, the highest protease production was obtained when using MSB-Y supplemented with peptone. The addition of inorganic nitrogen sources, such as $(NH_4)_2SO_4$ and NH_4Cl, provided readily utilizable nitrogen for the microorganism and thus resulted in a lower protease production. That a higher cell growth was observed when using complex organic nitrogen sources (Fig. 6c) might be a result of the additional carbons that are also supplied by organic nitrogen sources.

The highest protease productivity was obtained from cells grown in MSBY supplemented with peptone (Fig. 6d). Therefore, a time course study was performed with cells grown in MSBY-peptone medium to investigate the correlation between cell growth and extracellular protease production. During the cultivation, the pH of culture medium continuously increased until it reached a final pH of 7.8 ± 0.5. The protease production reached a maximum yield of 8.0 ± 2.4 U ml^{-1} in the mid-late exponential growth phase (~15 h) and then dramatically decreased thereafter

with *ca*. 70% of the protease activity remaining in the medium after 24 h. During this period, no spore formation was observed. The dramatic decrease in the enzyme activity when cells enter the stationary phase has been widely reported and is perhaps was caused by an enzymatic degradation [21].

In conclusion, *B. licheniformis* 3C5 was isolated as a mesophilic, organic-solvent tolerant bacterium. It has the indigenous property of being tolerant to a wide range of non-polar and polar organic solvents at a relatively high temperature (45°C) and produced an extracellular thermo-stable, solvent-stable alkaline serine protease with a molecular mass of approximately 32 kDa. The protease was active over a broad temperature (45–70°C) and pH (8–10) range with an optimum activity at pH 10 and 65°C. Its activity was comparatively stable in the presence of a high concentration (35% (v/v)) of organic solvents, and both nonionic and ionic detergents (1%) (v/v) or (w/v)), at a relatively high temperature (45°C). The growth and the protease production by this bacterium required only minimal medium components which is practical for commercial practice. The overall properties of the protease produced by *B. licheniformis* 3C5 suggest that this thermo-stable, solvent-stable, detergent stable alkaline protease is potentially a promising biocatalysts for industrial and environmental applications.

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REFERENCES

1. Sardessai, Y.N.. and Bhosle, S., Industrial Potential of Organic Solvent Tolerant Bacteria, *Biotechnol. Prog.*, 2004, vol. 20, pp. 655–660.
2. Ogino, H. and Ishikawa, H., Enzymes Which Are Stable in the Presence of Organic Solvents, *J. Biosci. Bioeng.*, 2001, vol. 91, pp. 109–116.
3. Isken, S. and de Bont, J.A., Bacteria Tolerant to Organic Solvents, *Extremophiles*, 1998, vol. 2, pp. 229–238.
4. Fang, Y., Liu, S., Wang, S., and Lv, M., Isolation and Screening of a Novel Extracellular Organic Solvent-Stable Protease Producer, *Biochem. Eng. J.*, 2009, vol. 43, pp. 212–215.
5. Sareen, R. and Mishra, P., Purification and Characterization of Organic Solvent Stable Protease from *Bacillus licheniformis* RSP-09-37, *Appl. Microbiol. Biotechnol.*, 2008, vol. 79, pp. 399–405.
6. Kongpol, A., Kato, J., and Vangnai, A.S., Isolation and Characterization of *Deinococcus geothermalis* T27, a Slightly Thermophilic and Organic Solvent-Tolerant Bacterium Able to Survive in the Presence of High Concentrations of Ethyl Acetate, *FEMS Microbiol. Lett.*, 2008, vol. 286, pp. 227–235.
7. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., *Current Protocols in Molecular Biology*, 1994, New York: John Wiley.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, pp. 265–275.
9. Cupp-Enyard, C., *Sigma'S Non-Specific Protease Activity Assay-Casein as a Substrate, J. Vis. Exp.*, 2008, DOI: doi: 10.3791/899.
10. Lee, S.O., Kato, J., Nakashima, K., Kuroda, A., Ikeda, T., Takiguchi, N., and Ohtake, H., Cloning and Characterization of Extracellular Metal Protease Gene of the Algicidal Marine Bacterium *Pseudoalteromonas* sp. strain, p. A28, *Biosci. Biotechnol. Biochem.*, 2002, vol. 66, pp. 1366–1369.
11. Kumar, C.G., Purification and Characterization of a Thermostable Alkaline Protease from Alkalophilic *Bacillus pumilus, Lett. Appl. Microbiol.*, 2002, vol. 34, pp. 13–17.
12. Gupta, A. and Khare, S.K., A Protease Stable in Organic Solvents from Solvent Tolerant Strain of *Pseudomonas aeruginosa, Bioresour. Technol.*, 2006, vol. 97, pp. 1788–1793.
13. Strongin, A.Y., Izotova, L.S., Abramov, Z.T., Gorodetsky, D.I., Ermakova, L.M., Baratova, L.A., Belyanova, L.P., and Stepanov, V.M., Intracellular Serine Protease of *Bacillus subtilis*: Sequence Homology with Extracellular Subtilisins, *J. Bacteriol.,* 1978, vol. 133, pp. 1401–1411.
14. Li, S., He, B., Bai, Z., and Ouyang, P., A Novel Organic Solvent-Stable Alkaline Protease from Organic Solvent-Tolerant *Bacillus licheniformis* YP1A, *J, Mol. Cat. B: Enzyme*, 2009, vol. 56, pp. 85–88.
15. Fairbairn, D.J. and Law, B.A., The Effect of Nitrogen and Carbon Sources on Proteinase Production by *Pseudomonas fluorescens, J. Appl. Bacteriol.*, 1987, vol. 62, pp. 105–113.
16. Ferrero, M.A., Castro, G.R., Abate, C.M., Baigor, M.D., and Sieriz, F., Thermostable Alkaline Proteases of *Bacillus licheniformis* MIR29: Isolation, Production and Characterization, *Appl. Microbiol. Biotechnol.*, 1996, vol. 45, pp. 327–332.
17. Mehtaa, V.J., Thumara, J.T., and Singh, S.P., Production of Alkaline Protease from an Alkaliphilic Actinomycete, *Bioresour. Technol.*, 2006, vol. 97, pp. 1650–1654.
18. Shivananda, P. and Jayaraman, G., Production of Extracellular Protease from Halotolerant Bacterium, *Bacillus aquimaris* strain VITP4 Isolated from Kumta Coast, *Process Biochem.*, 2009, vol. 44, pp. 1088–1094.

19. Kaur, S., Vohra, R.M., Kapoor, M., Beg, Q.K., and Hoondal, G.S., Enhanced Production and Characterization of a Highly Thermostable Alkaline Protease from *Bacillus* sp. P-2, *World J. Microbiol. Biotechnol.*, 2001, vol. 17, pp. 125–129.
20. Nadeem, M., Qazi, J.I., Baig, S., and Syed, Q., Effect of Medium Composition on Commercially Important Alkaline Protease Production by *Bacillus licheniformis* N-2, *Food Technol. Biotechnol.,* 2008, vol. 46, pp. 388–394.
21. Rahman, R.N., Geok, L.P., Basri, M., and Salleh, A.B., Physical Factors Affecting the Production of Organic Solvent-Tolerant Protease by *Pseudomonas aeruginosa* strain K, *Bioresour. Technol.*, 2005, vol. 96, pp. 429–436.
22. Canale-Parola, E., A Red Pigment Produced by Aerobic Spore-Forming Bacteria, *J. Arch. Microbiol.*, 1963, vol. 46, pp. 414–427.
23. Joshi, G.K., Kumar, S., and Sharma, V., Production of Moderately Halotolerant, SDS Stable Alkaline Protease from *Bacillus cereus* MTCC 6840 Isolated from Lake Nainital, Uttaranchal State, India, *Brazilian J. Microbiol.*, 2007, vol. 38, pp. 773–779.
24. Shafee, N., Aris, S.N., Rahman, R.N.Z.R.A., Basri, M., and Salleh, A.B., Optimization of Environmental and Nutritional Conditions for the Production of Alkaline Protease by a Newly Isolated Bacterium *Bacillus cereus* Strain 146, *J. Appl. Sci. Res.*, 2005, vol. 1, pp. 1–8.