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Studies on Alkaline Protease Produced by *Bacillus* sp. NG312

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Abstract

An alkalophilic hyperproducer of alkaline protease, *Bacillus* sp. NG312, was isolated, and the enzyme showed maximum activity at pH 11.0 and 60°C. The temperature optimum was increased by 10°C in presence of Ca^{2+}. The crude enzyme was found to have half-life of 11 d at 37°C and maximum stability at pH 9.0–10.0. It also exhibited very good stability in presence of detergent components and some locally available commercial detergent powders.

Index Entries: Alkaline protease; *Bacillus*; characterization; detergent compatibility.

Introduction

Proteases are an important group of industrial enzymes with applications in food, pharmaceutical, and detergent industries. This group of enzymes accounts for nearly 60% of total enzyme sales, of which detergent enzymes, mostly from *Bacillus* sp., contribute about 25% *(1)*. Apart from their industrial importance, proteases play an important role in many cellular functions, and are important tools in studying structure of proteins and peptides. Since the initial report on alkaline protease from *Bacillus (2)*, many studies have been carried out to explore proteases from various microbial sources *(3–10)*.

In recent years, the detergent industry has witnessed two major changes: One is the trend toward lower washing temperatures, and second is the change in detergent composition, with a shift away from

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phosphate-based detergents *(11)*, emphasizing the need for new alkaline proteases that are better suited for detergent applications. In the present study, an alkalophilic *Bacillus* sp. NG312, was isolated in this laboratory, and was found to produce alkaline extracellular protease. Its enzyme was assessed for suitability as a detergent additive in particular, and for other applications in general.

Materials and Methods

Bacterial Strain

The bacterial strain used in this study was isolated in this laboratory from alkaline soil samples from Leh and Ladakh, India. This bacterial strain was identified as belonging to genus *Bacillus* by Microbial Type Culture Collection Centre and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

Culture Conditions

Strain NG312 was cultivated at 37°C in an orbital shaker operating at 200 rpm in starch yeast extract biopeptone (SYB) medium *(12)* containing (per L) 10 g starch, 5 g yeast extract, 5 g biopeptone, 1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 10 g Na_2CO_3 (separately sterilized). The 12–16-h-old inoculum was used at 10% v/v level to inoculate the flasks containing 100 mL SYB medium for enzyme production. Each experiment was done in triplicate.

Samples were withdrawn at fixed time intervals, and culture supernatant, after centrifugation at 6000 *g* for 10 min, was used to estimate enzyme activity.

Enzyme Assay

The proteolytic activity was checked against azocasein (Sigma, St. Louis, MO), using the method of Samal et al. *(13)*. Reaction mixture containing 20 µL azocasein (5% w/v solution in assay buffer), 470 µL assay buffer (0.05 *M* glycine–NaOH buffer, pH 10.0), and 10 µL suitably diluted enzyme solution was incubated at 37°C for 30 min, followed by addition of 500 µL 10% (w/v) trichloroacetic acid to terminate the reaction, and samples were kept on ice for 15 min. After centrifugation, 800 µL supernatant was mixed with 200 µL 1.8 *N* NaOH, and the absorbance was measured at 420 nm.

Proteolytic activity in U/mL is defined as increase in absorbance caused by 1 mL enzyme/h under the given assay conditions.

Effect of Temperature and pH on Enzyme Activity

The enzyme assay was carried out at different temperatures, in the range of 30–80°C, to investigate the effect of temperature on enzyme activity.

The following assay buffers were used to study the effect of pH on enzyme activity: 0.05 *M* Tris-HCl buffer for pH 8.0–9.0 and 0.05 *M* glycine–

NaOH buffer for pH 10.0–12.0. The enzyme assay was performed at 50 and 60°C using the buffer of the given pH.

Thermal and pH Stability Studies

For thermal stability studies, 25 mL culture supernatant was incubated at different temperatures, and samples were withdrawn periodically to check for residual activity.

For pH stability studies, 1 mL enzyme solution was mixed with 5 mL buffer of given pH (0.05 *M* phosphate buffer was used for pH 7.0; the remaining buffers used were as described earlier). The mixture was incubated at 37°C for 24 h, then the residual activity was checked.

Effect of Metal Ions and Protease Inhibitors

$SrCl_2$, $BaCl_2$, $ZnCl_2$, $MgCl_2$, $CaCl_2$, and $CuCl_2$ were added to assay buffer at the 5 m*M* level. Assay buffer without any metal ion was used as a control. Appropriate blanks were maintained in each case.

10 µL enzyme and 470 µL assay buffer containing 10 m*M* ethylenediaminetetraacetic acid (EDTA), or 5 m*M* phenylmethylsulfonyl fluoride (PMSF), or 10 m*M* iodoacetic acid, or 10 m*M* 1–10, phenanthrolein (all from Sigma, St. Louis, MO) were mixed separately. The solutions were incubated at 37°C for 2 h, followed by enzyme assay to check residual enzyme activity.

Effect of Detergent Components

Equal volumes of enzyme solution and 0.2% sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO), or 1.0% sodium perborate (SPB) (Fluka Chemie Switzerland), or 0.2% dodecyl benzene sulfonate (DBS) (Sigma), were mixed and incubated at 37°C for 1 h. Assay buffer was used instead of solution of detergent component as control. The residual enzyme activity was estimated in each case.

Effect of Commercial Detergents

Commercial detergent powders, namely Nirma (Nirma Chemicals, Ahmadabad, India), Tatachem (Tata Chemicals, Bombay, India), Super Wheel (Hindustan Lever, Bombay, India) and Surf Ultra (Hindustan Lever), were dissolved in tap water at 5 g/L. Five hundred µL enzyme (culture supernatant) was added to 4 mL detergent solution. The residual enzyme activity was determined after 1 h of incubation at 37°C. Four mL tap water was used instead of detergent solution as control.

Results and Discussion

Bacillus sp. NG312 produced extracellular alkaline proteases mostly during the stationary phase in SYB medium. Maximum proteolytic activity

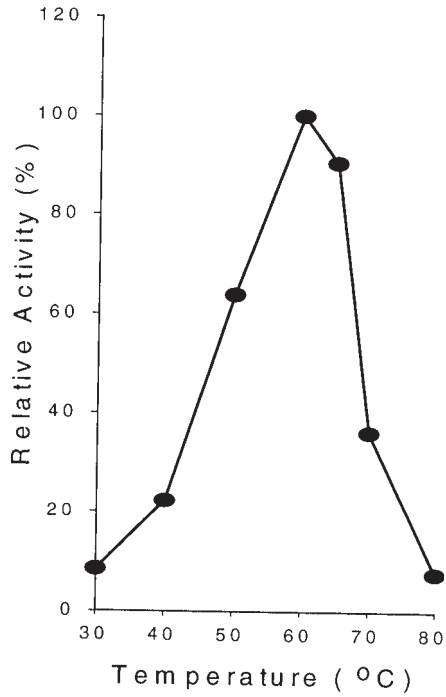

Fig. 1. Effect of temperature on enzyme activity.

of 486 U/mL in culture supernatant was observed at 72 h. The major part of proteolytic activity appeared after depletion of starch.

The desired properties in a detergent enzyme have been reported to be good temperature stability, stability at pH 9.0–10.5, and relative nondependence on metal ions, along with compatibility with detergent components, such as surface active reagents, builders, and bleaching agents *(1,11)*. Studies of the effect of temperature on protease activity (Fig. 1) revealed that the enzyme's optimum temperature for activity is 60°C. However, addition of 5 m*M* Ca^{2+} leads to an increase of 10°C in optimum temperature. This enzyme was active over a wide range of pH (from 8.0–11.0), with optimum at 11.0, when assayed at 60°C using azocasein as a substrate. However, at 50°C, it showed fairly good activity, even at pH 12.0 (Fig. 2). These characteristics make NG312 protease quite suitable for detergent applications, with the current trend toward lower washing temperatures, because of obvious energy savings *(11)*. Changes in detergent composition, with a shift away from phosphate based detergents, has also led to an increase in pH of suds *(11)*, and the highly alkalophilic nature of NG312 protease may be useful for this purpose. This may also be useful for other applications, such as in the leather industry, and also in the photographic film industry, for recovery of silver from used photographic films.

Thermal stability studies indicated that crude alkaline protease of NG 312 has a half-life of approx 11 d, 24 h, and 40 min at 37°, 50°, and 60°C respectively, in absence of any stabilizer. Studies on pH stability of the

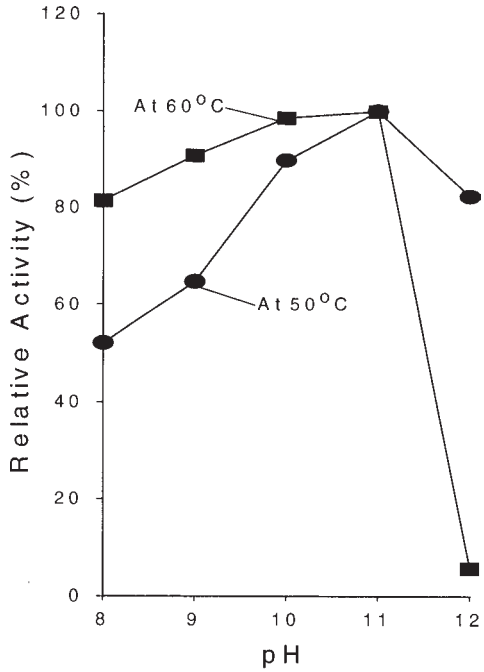

Fig. 2. Effect of pH on enzyme activity.

enzyme revealed that this enzyme is fairly stable at a wide range of pH values from 7.0–11.0, with maximum stability being exhibited at pH 9.0–10.0. This is the pH range of most detergent solutions.

There are reports on thermostable proteases with half-life of 1 h at 50°C *(8)*, or at 25 min at 60°C *(14)*. In present-day laundering processes, proteases are generally used for 10 min at 60°C *(10)*. Hence, better stability characteristics of NG312 protease (half-life of 24 h at 50°C and 40 min at 60°C) make it a potential candidate for use in laundry detergents.

Of all divalent cations studied, presence of both Cu^{2+} and Zn^{2+} led to decreased proteolytic activity, but Mg^{2+} increased it marginally (Table 1).

Among the various protease inhibitors tested for their effect on enzyme activity, only PMSF, which is a class-specific inhibitor of serine proteases, resulted in almost 90% inhibition at 5 m*M* level, which clearly indicated that this enzyme belonged to the serine class of proteases.

This enzyme retained more than 80% activity after exposure to SDS, SPB, and DBS (Table 2), which are commonly used detergent components. Presence of surface-active components or bleaching agents seemed to have little effect on NG312 protease.

This enzyme was almost 100% stable in presence of 5 g/L solution of Nirma, Tatachem, and Super Wheel detergents. However, it was relatively less stable in presence of Surf Ultra (Table 1). All the above characteristics of NG312 proteases make it a promising candidate for varied industrial applications.

Table 1
Effect of Metal Ions and Protease Inhibitors

Additive	Relative activity (%)[a]
Metal ions	
Sr^{2+}	101
Ba^{2+}	99
Zn^{2+}	70
Mg^{2+}	108
Ca^{2+}	99
Cu^{2+}	77
Protease inhibitors	
EDTA	84
PMSF	9
Iodoacetic acid	100
1,10-Phenanthrolein	92

[a]Relative activity (%) in the presence of additives was calculated as % of the activity in the control (without any additive).

Table 2
Effect of Detergent Components
and Locally Available Commercial Detergent Powders

	Concentration g/L	Relative residual activity (%)[a]
Detergent component		
SDS	1.0	83.5
SPB	5.0	86.3
DBS	1.0	93.3
Commercial detergent powders		
Nirma	5.0	100.0
Tatachem	5.0	97.0
Super Wheel	5.0	100.0
Surf Ultra	5.0	37.0

[a]Relative residual activity (%) in the presence of additives was calculated as % of the residual activity in control (without any additive).

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