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CHARACTERIZATION AND STABILITY OF EXTRACELLULAR ALKALINE PROTEASES FROM HALOPHILIC AND ALKALIPHILIC BACTERIA ISOLATED FROM SALINE HABITAT OF COASTAL GUJARAT, INDIA

Mital S. Dodia[1]; Rupal H. Joshi[1]; Rajesh K. Patel[2]; Satya P. Singh[1]*

[1]Department of Biosciences, Saurashtra University, Rajkot, 360 005, Gujarat, India; [2]Department of Life Sciences, University of North Gujarat, Patan, India

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ABSTRACT

The present study deals with the isolation and characterization of the moderately halophilic-alkaliphilic bacteria from a saline habitat in western India. Eight different bacterial strains were isolated using enrichment techniques at 20% (w/v) NaCl and pH 10. The isolates exhibited diversity towards gram's reaction, colony and cell morphology. They were able to grow and produce alkaline protease over a broad range of NaCl, 5-20% (w/v) and pH, 8-10. None of the isolates could grow at pH 7, and one could not grow even at pH 8. Crude and partially purified proteases from strain S_5 were subjected to characterization with reference to pH, salt stability and protein folding. Optimum protease activity and stability was recorded at 10% salt and pH 9-9.5. Denaturation kinetics of S_5 alkaline protease along with a reference protease was studied at 8M urea followed by renaturation. The S_5 alkaline protease could be partially renatured up to 32% of the original activity. Despite of the fact that all the 8 isolates were from the same site, they displayed significant diversity with respect to their salt requirement for growth and enzyme secretion. While the effect of pH was less demarcated on growth, the protease production was significantly affected. Isolate S_5 produced substantial amount of halotolerant and alkaline protease. The activity and stability of the alkaline protease in a broader range of pH and salt would definitely make this enzyme an important candidate for various industrial applications.

Key words: halophiles, alkaliphiles, alkaline protease, protease stability, protein folding, extremophiles

INTRODUCTION

Extremophiles, the microbes dwelling in unusual habitats, can potentially serve in a verity of industrial applications. As a result of adaptation to extreme environments, extremophiles have evolved unique properties, which can be of biotechnological and commercial significance (15).

Extremophiles include halophiles, alkaliphiles, acidophiles, thermopiles and haloalkaliphiles. Hypersaline lakes are populated mostly with halophilic neutrophilic organisms, while the alkaline saline lakes (Soda lakes) are the habitats of Haloalkaliphilic species. Horikoshi (8) has described haloalkaliphiles as a subgroup of Alkaliphiles that require both an alkaline pH (>pH 9) and high salinity (up to 33% (w/v) NaCl). Tindall and his coworkers (25) have described haloalkaliphiles as moderate halophiles able to survive in alkaline saline habitats. The organisms living in such dual extreme environments possess special adaptation strategies that make them interesting not only for fundamental research but also towards exploration for applications (15).

During the past decades, the studies on ecology, physiology, and taxonomy of haloalkaliphiles have revealed an impressive diversity in highly saline and alkaline lakes (4,11,18). However, most of the studies related to them are based on phylogeny and only limited attempts have been made to explore their enzymatic potential (3,12). The quantity of proteases produced on a commercial scale worldwide is greater than any other enzymatic group of biotechnological relevance (17). Among them alkaline

*Corresponding Author. Mailing address: Microbiology & Head, Department of Biosciences, Saurashtra University, Rajkot, 360 005, Gujarat, India. Telefax: (+91 281) 258-6419. E-mail: satyapsingh@yahoo.com

proteases dominate the worldwide enzyme market, accounting for a two-thirds share of the detergent industry (6). As studies on the alkaline protease from Haloalkaliphilic group of bacteria are scarce in literature, it is of great importance to pursue such studies. In addition, gene cloning from extremophiles into mesophilic bacteria has gained considerable attention during the recent years. However, the over expressed proteins in host bacterium faced the problem of inclusion bodies. In order to overcome this problem we have demonstrated the denaturation and renaturation studies of alkaline protease from these groups of bacteria. Moreover, most of the haloalkaliphiles isolated until now are largely from Soda Lakes (1,24). In the present study, one natural saline habitat along the Gujarat Coast in Western India was selected for the isolation followed by the assessment of microbial diversity and enzymatic potential of haloalkaliphilic bacteria.

MATERIALS AND METHODS

Enrichment and isolation of moderately halophilic and alkaliphilic bacteria

The bacterial strains were isolated from salt enriched soil as sediment sample (one) collected from the natural hyper saline habitats from the Saurashtra region of the coastal Gujarat, India. The organisms were isolated by enrichment culture technique. The soil sample was added to a medium containing (g/liter): glucose, 10; peptone, 5; Yeast Extract, 5; KH_2PO_4, 5; NaCl, 200. The pH of the medium was adjusted to 10 by adding separately autoclaved Na_2CO_3 (20% w/v). They were incubated at 37ºC for 48 hours. After sufficient growth a loopful culture was streaked on the same media and based on colony characters different organisms were selected.

Characterization of organisms

The colony characteristics, cell morphology, gram reaction and nutritional susceptibility of the 8 isolates designated as S_1 to S_8 were studied. They were also screened for protease production using gelatin (g/liter; Gelatin, 30; Peptone, 10; NaCl, 100) and amylase using starch agar (g/liter: Starch, 2; Yeast extract, 3; and Peptone, 5; NaCl, 100). To study the effect of salt on the growth and protease secretion, NaCl in the range of 0 to 20% was included into the gelatin medium and the medium was inoculated with actively growing culture of all the 8 strains in the form of regular spots. After incubation at 37ºC, the colony diameter and the zone of gelatin hydrolysis was measured to study the effect of salt on the production of protease. Similarly, to examine the effect of pH in the range of 7 - 10, the NaCl concentration was maintained at 10%, w/v. The detection of protease was performed as described above.

Enzyme Assay

The enzyme was assayed by the Anson- Hagihara method (7) using casein as a substrate. One unit of alkaline protease activity (U) was taken as the amount of enzyme liberating 1 µg of tyrosine per min under the assay conditions. The estimations were based on a tyrosine calibration curve.

Partial purification and characterization of haloalkaline protease

The enzyme was partially purified by ammonium sulphate fractionation. The organism S_5 was grown as described earlier under shake flask conditions (100 rpm). After 23 h of growth ($A_{540,}$ 1.7), the cells were separated by centrifugation at 5,000rpm (3,000 X g); 4ºC for 15 min and the supernatant was used as crude preparation. Enzyme was fractionated by ammonium sulphate with 30, 50 and 90% saturation. Total protein content of the cell free extract was measured by Biuret method using 0-5 mg/mL BSA (Bovine serum albumin) as a standard. Partially purified and crude enzymes were characterized with reference to the effect of salt and pH on the stability and activity. To study the effect of NaCl on the catalysis of the protease, the substrate was prepared in the buffer contained different concentration of NaCl from 0 to 20%, w/v. After that the normal enzyme assay was performed and the activity was calculated. To study the effect of NaCl on the protease stability, the enzyme was incubated with 10 and 20% NaCl. The aliquots were withdrawn after definite time intervals for the enzyme assay. The protease activity before addition of the salt was considered as a 100% and the residual activity was measured accordingly.

Denaturation and renaturation of the enzyme

The effect of the denaturing agent Urea from concentrations 2-8M, on the crude protease and a reference enzyme was also studied. Various urea concentrations (2-8M) were used to denature enzyme. The enzyme was incubated with urea for 30min followed measurement to ascertain the loss of activity. For renaturation the completely denatured enzyme sample was dialyzed overnight against NaOH-Borex buffer (20 mM, pH 10) containing no urea.

RESULTS AND DISCUSSION

A group of 8 moderately halophilic and alkaliphilic bacteria were isolated. The isolates did not display much diversity as regards to their colony characteristics (Table 1a). However, the cell morphology and arrangement did vary significantly among these isolates from the same location (Table 1b). The gram-positive character dominated and isolates had limited variation in their colony pigmentation and texture (Table 1a).

All the isolates were able to grow over a wide range of salt (0-20%, w/v) except S_6 which could not grow in the absence of salt. Optimum growth required between 5-10% (w/v) NaCl. The isolates were found to be moderately halophilic and alkaliphilic in nature. Recently, a group of 122 moderately halophilic bacteria producing extracellular hydrolytic enzymes including proteases were isolated

Table 1(a): colony characteristics of Haloalkaliphilic bacteria isolated from saline habitats of India.

Colony Characteristics	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
Size	Large	Large	Large	Large	Large	Large	Small	Large
Shape	Round	Round	Round	Round	Round	Round	Round	Round
Opacity	Opaque	Opaque	Translucent	Opaque	Opaque	Opaque	Opaque	Opaque
Margin	Regular	Regular	Regular	Regular	Irregular	Regular	Regular	Regular
Elevation	Raised	Raised	Raised	Slightly Raised	Raised	Flat	Raised	Raised
Texture	Smooth	Mucoid	Smooth	Smooth	Mucoid	Smooth	Smooth	Smooth
Pigmentation	Cream-White	Cream	Cream	Cream Dark Centered	Cream	Cream	Cream-White	Cream

Table 1(b): cell morphology and arrangement of Haloalkaliphilic bacteria isolated from saline habitat of India.

Cell Morphology	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
Size and Shape	Thin ong rods	Thin crystal like rods	Short rods	Short thin rods	Short thin rods	Very thin rods	Thin long rods	Short rods
Arrangement	Single	Single	Single	In chain	In chain	Single	Single	Single
Gram's reaction	-	-	-	+	+	+	+	+

from hypersaline environment in South Spain (23). Similar kind of results have also been found for the moderately halophilic and alkaliphilic coccus *Salinicoccus alkaliphilus* sp. Nov., isolated form Baer Soda Lake in Mongolia, which could grow over a range of 0-25% (w/v) NaCl with optimum at 10% (w/v) (30). In addition, some extremely Haloalkaliphilic sulfur-oxidizing bacteria have also been reported (24). There appears to be a great diversity among the isolates with respect to their ability to utilize carbon sources. Interestingly, for most of the isolates enzyme secretion was optimum with lactose (Data not shown).

All the 8 isolates were screened for protease and amylase secretion. All the 8 isolates secreted protease in haloalkaline medium, but none secreted amylase. The salt requirement for optimum enzyme secretion varied significantly among the isolates. The salt dependency was, however, relatively lower when compared to the extreme haloalkaliphilic archaean isolates from Soda Lake (27).

Optimum pH for growth and protease secretion from the isolates under study was 9 and 9-10 respectively. However, isolate S_8 grew optimally at pH 8. None of the isolates were able to grow at pH 7 and S_7 did not grow even at pH 8. This suggested strict alkaliphilic nature of these isolates (Table 2). There was hardly any variation in optimum pH for growth, although the isolates had significant variation in their salt requirement. Another trend which emerged was that requirement of pH and salt for the optimum growth and enzyme secretion varied significantly (Table 2). For instance, the optimum pH for growth was 9 for the majority of the isolates, while the variation in pH with regards to enzyme secretion varied between pH 8-10.

Among the isolates, S_5 was the most potent producer of alkaline protease. The level of protease production was maximum

Table 2. Effect of pH and salt concentration on growth and protease production by isolates obtained from saline habitat in India

Isolates	Optimum pH		Optimum salt (NaCl %, w/v)	
	Growth	Protease Production	Growth	Protease Production
S_1	9	10	10	15
S_2	9	10	10	5
S_3	9	9	5	10
S_4	9	10	5	10
S_5	9	10	10	15
S_6	9	8	5	15
S_7	9	10	5	5
S_8	8	9	15	5

at the late exponential phase after 24 h of growth. Results based on the shake flask cultures indicated maximum growth at 10% salt (w/v) whereas for maximum protease production 15% salt was required (Fig. 1). The trend in Fig. 1 indicated differential effect of salt on growth and protease secretion. While the growth was adversely affected with increasing salt from 5-15% (w/v), protease secretion had reverse relationship. This suggested higher salt requirement for the enzyme secretion. For a moderate halophile, *Pseudoalteromon,* maximal protease production was detected at the end of the exponential growth phase (23). These results clearly suggested the prominent role of extracellular proteases in ecological sustenance of these organisms.

The alkaline protease from S_5 isolate was partially purified by ammonium sulphate fractionation, where 2-fold purification was achieved in the 90% fraction. This enzyme has a specific need of NaCl because after dialysis the specific activity of the enzyme was decreased by 82%. The salt requirement for optimum activity was 10% in case of partially purified enzyme but crude enzyme displayed nearly the same enzyme activity over a broad range of salt concentrations (Fig. 2). The crude preparation had a higher activity as compare to partially purified protease, indicating the possible role of certain component/s present in crude preparation which support protease stability at high salt concentrations (20). The enzyme retained almost 80% activity when incubated at 10% salt (w/v) for 20 min, after which the activity sharply decreased. The stability, however, was adversely affected at salt concentrations above 10%. As reflected in Fig. 3, the enzyme stability gradually decreased with time when incubated with 20% (w/v) salt. A similar kind of high salt tolerance was also observed in a moderate halophile, *Pseudoalteromona* CP1 protease. The CP1 protease, showed high tolerance over a wide range of NaCl concentrations (0 to 4 M NaCl), the optimal activity being at 7.5% total salts (23). Generally, these halophilic proteins maintained their stability and activity by increased ion binding and glutamic acid content, both allowing the protein inventory to compete for water at high salt (9). Further, in moderate halophiles, compatible solutes such as glycine, betaine and hydroxyectoine are known to protect protein, at high salt concentration (13,14,17,21,22).

The activity of S_5 protease gradually increased with increase in the pH of catalysis from 7 to 9 and maximum activity was attained between pH 9-10 (Fig. 4). The S_5 protease was highly stable when exposed to pH 9.5 for 30 min at 37ºC. But at pH 7.5,

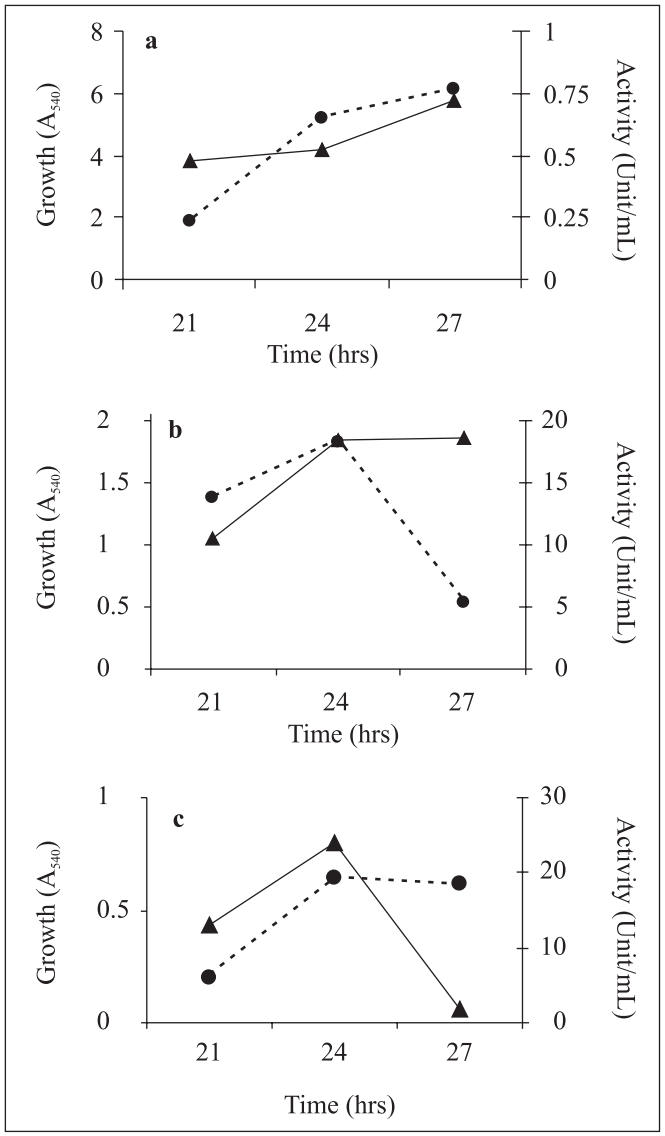

Figure 1. Effect of NaCl (%, w/v) on growth and protease production by strain S_5. Samples were taken at three different time intervals for determination of cell growth (OD_{540}) (●) and protease activity (▲). Figure 1(a), (b) and (c) represent the data with 5, 10 and15% NaCl (%, w/v) respectively.

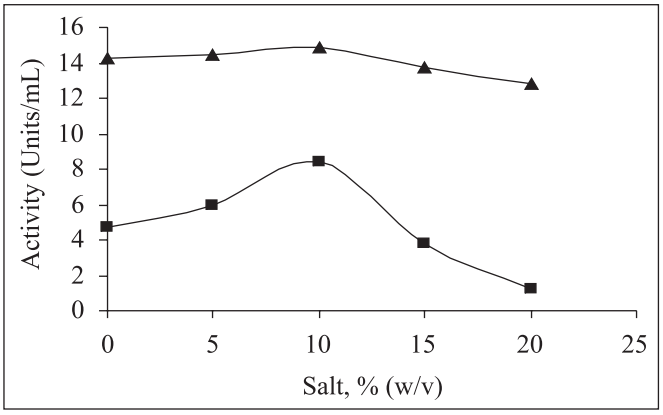

Figure 2. Effect of different NaCl concentrations (%, w/v) on protease activity; crude (▲) and partially purified protease (●).

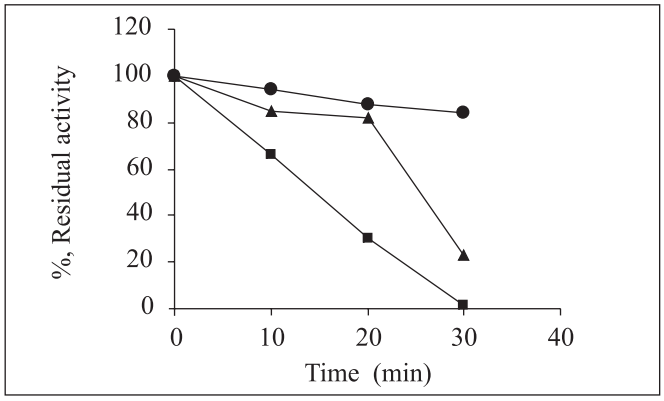

Figure 3. Effect of NaCl concentrations (%, w/v) on protease stability. Enzyme was incubated with corresponding amount of salt and samples were withdrawn at different time intervals for protease assay; 0 (●), 10 (▲) and 20% (■).

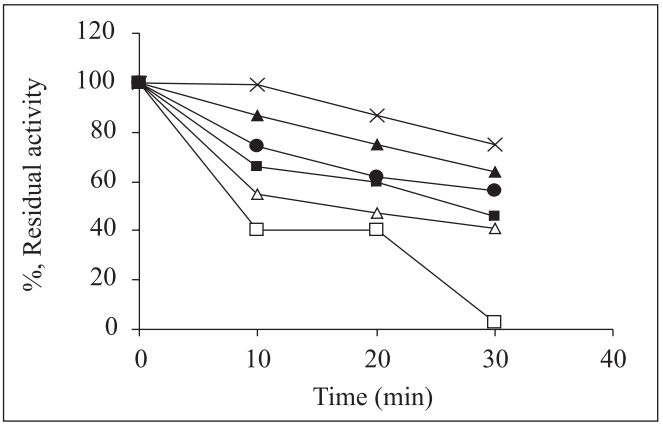

Figure 5. Effect of pH on Protease stability. Enzyme was incubated with respected buffer and samples were withdrawn at different time interval for protease assay; pH 7.5(△), 8.0 (□), 8.5 (●), 9.0 (×), 9.5 (▲) and 10 (■).

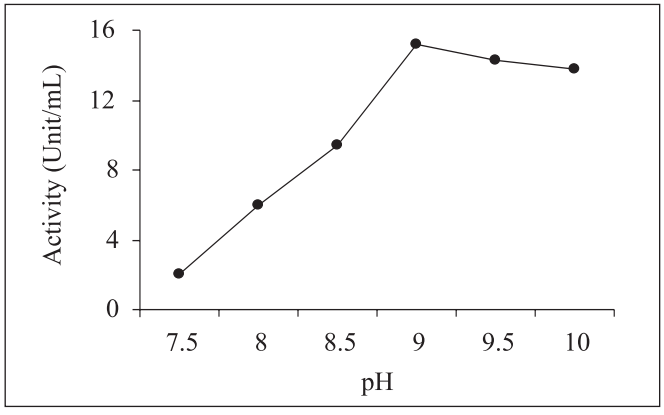

Figure 4. Effect of pH on Protease activity. Buffer used were Sodium Phosphate buffer (pH 7.5), Tris-HCl buffer (pH 8, 8.5, 9), NaOH-Borex buffer (pH 9.5, 10).

the enzyme was quite unstable (Fig. 5). The stability gradually decreased with lowering of the pH towards 7 indicating the alkaline nature of this protease. Similar results were also reported for *Bacillus* sp. in which enzyme was highly active and stable at pH 9-10 (10). Two alkaline protease producing alkaliphilic bacteria, designated as AL-20 and AL-89, were isolated from a naturally occurring alkaline habitat (5). Protease AL-20 was active in a broad pH range displaying over 90% of its maximum activity between pH 7.5 and 11.5, the peak being at pH 10. An extracellular protease purified from a psychrophilic *Pseudomonas* sp., displayed optimal activity at 40ºC and pH 10 (29). A novel extracellular serine protease designated Pernisine was purified from the archaeon *Aeropyrum pernix* K1 and was active over broad range of pH (5.0-12.0) with maximal activity between pH 8.0 and 9.0 (2). However, our enzyme was maximally active over a relatively narrow alkaline pH range (9-10).

Recently, renaturation of the over expressed but wrongly folded proteins have gained considerable attention (19,26). We attempted *in vitro* protein folding of S_5 protease and a commercial protease. About 97% denaturation of S_5 enzyme occurred at 8M urea, while the commercial enzyme lost about 87% activity under similar circumstances (Fig. 6). Dialysis based renaturation yielded 7.25 % and 32% renaturation of commercial and S_5 enzyme respectively (Table 3). This decreased

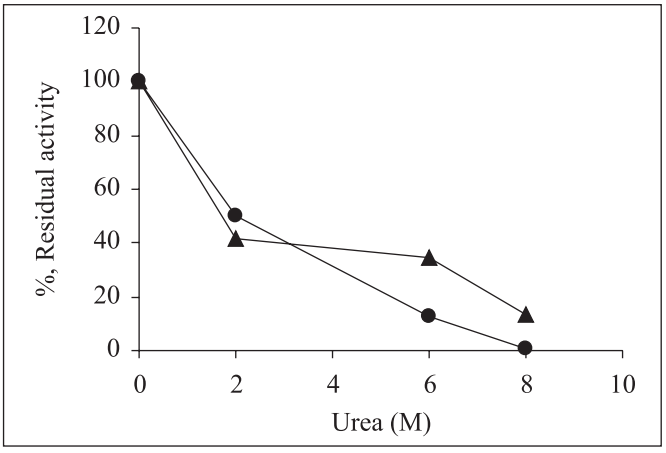

Figure 6. Denaturation kinetics of S_5 protease and reference protease. Both enzymes were incubated with different concentrations of urea and incubated for 30min at 37ºC, after which protease activity was measured. S_5 (●) and Reference protease (▲).

Table 3. Denaturation by urea and renaturation of S_5 and reference protease.

Enzyme Sample	Activity (Units/mL/min)			
	Original Activity	After Denaturation (8 M)	After Renaturation (By dialysis)	% Recovery
Reference Protease	40	5.4	2.9	7.25
S_5	10.96	0.47	3.5	32

renaturation yield may be due to the kinetic competition of folding and "Wrong aggregation", because we do not know the optimum concentrations of the denaturant for the effective renaturation of a denatured protein. Previously the slow dialysis method yielded encouraging results for lysozyme (28). Thus, gradual and slow removal of urea could be an effective and simple means to renature the protein under *in vitro* conditions. However, renaturation of S_5 protease would need to be investigated in further details for various factors affecting the *in vitro* folding of this enzyme. Nevertheless, this approach of protein folding has immense biotechnological potential and could contribute significantly in protein engineering.

RESUMO

Caracterização e estabilidade de proteases alcalinas extracelulares de bactérias halofílicas e alcalifílicas isoladas de habitat salino de Gujarat, Índia

O presente estudo relata o isolamento e caracterização de bactérias moderadamente halofilicas e alcalífilicas de um habitat salino no oeste da Índia. Oito cepas diferentes de bactérias foram isoladas empregando técnicas de enriquecimento em NaCl a 20% (p/v) e pH 10. As cepas apresentaram diversidade em relação à coloração de Gram e à morfologia das colônias e células. As cepas foram capazes de multiplicar e produzir protease alcalina em uma ampla faixa de concentração de NaCl (5 a 20%) e pH (8 a 10). Nenhuma das cepas foi capaz de se multiplicar em pH 7, e uma não se multiplicou nem em pH 8.0. Proteases naturais e parcialmente purificadas da cepa S_5 foram submetidas à caracterização com relação ao pH, estabilidade salina, e estrutura protéica. Atividade e estabilidade ótimas da protease foram obtidas com 10% de sal e pH 9-9,5. A cinética de denaturação da protease de S_5, juntamente com uma protease de referencia, foi avaliada com uréia 8M seguida de renaturação. A protease alcalina de S_5 foi renaturada a 32% da atividade original. Apesar de provenientes do mesmo local, as oito cepas mostraram grande diversidade em relação à exigência de sal para multiplicação e secreção enzimática. Enquanto o efeito do pH na multiplicação foi menos marcante, o efeito na produção de protease foi significativamente afetada. A cepa S_5 produziu uma quantidade substancial de protease alcalina e halotolerante. A atividade e estabilidade da protease alcalina em uma faixa mais ampla de pH e sal tornam essa enzima uma importante candidata para diversas aplicações industriais.

Palavras-chave: halófilos, alcalifilicos, extremofilicos, protease alcalina, estabilidade de protease

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