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Salicola sp. strain SBJ9: a novel extremely halophilic bacterium with an interesting protease activity

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Abstract:

A number of newly isolated halophilic microorganisms were screened for protease production. A bacterium designated as strain SBJ9 showed an important enzyme production at high salt concentrations and was then retained. The 16S DNA identification put this strain in the genus of *Salicola* with two reference species only. Protease production was higher at salinities ranging from 150 to 200 g/l (3.2 M) NaCl, when monitored at 35 °C and pH 7. The protease activity was optimal at 2.5 M NaCl, 40°C and pH 8, with high stability at wide ranges of salinity (1-5 M NaCl), temperatures (20- 70 °C) and pH values (5- 11). It was slightly improved by 5 mM $CaCl_2$ and totally inhibited by PMSF which indicated the dominance of serine proteases. Besides, it was perfectly stable in the presence of many detergent additives and organic solvents at high concentrations. These important features make *Salicola* sp. strain SBJ9 protease activity a good candidate for many industrial applications such as detergency and organic synthesis.

Keywords: extremely halophilic; *Salicola* sp.; protease; halo-thermostable; application.

1. Introduction

For many decades and even, proteases have been the first commercially available enzymes in the global enzyme market. In Fact, they are used in many industrial sectors as alternatives to chemicals to ameliorate the efficiency and the cost effectiveness [1]. As example, they are widely used in detergent, food and leather

industries [2-5]. Practically, proteases used in detergent formulations are facing many technical constraints that reduce their stability such as pH, ionic strength, salinity and the presence of surfactants. We noticed also the decrease of protease stability in liquid household detergents due to their high salt content. Thus, there are increasing studies on screening for new proteases that are active and stable under these harsh conditions.

In this context, we have focused on halophilic microorganisms (halophiles) which are known for the production of enzymes with high activity and stability at wide ranges of salinity and in law water content media [6]. For that, we have isolated over a hundred of halophilic microorganisms and we have screened them for extracellular proteases production. A bacterium showing an important protease production at higher salinities, strain SBJ9, was selected for its identification and the further study of its protease activity.

2. Results and Discussion

Over a hundred of halophilic and halotolerant microorganisms were screened from various saline and hypersaline biotopes. Strain SBJ9, isolated from the Salt lake Bou Djemal in Sfax (Tunisia), showed the most important protease production on agar plates containing 200 g/l NaCl (3.42 M) and was then retained. The 16S rDNA identification put the isolate in the genus *Salicola* which contain only two species (*S. salis* and *S. marasensis*). The study of the effect of salt on SBJ9 growth and protease production revealed that it is an extremely halophilic bacterium growing and producing protease activity optimally at 150- 200 g/l NaCl.

The biochemical characterization of *Salicola* sp. SBJ9 protease activity showed an optimal activity at 2.5 M NaCl, pH 8 and 40 °C with high stability at wide ranges of salinity (1.5 – 5M NaCl), pH (6- 10) and temperature (25- 65 °C) (**Figure 1**).

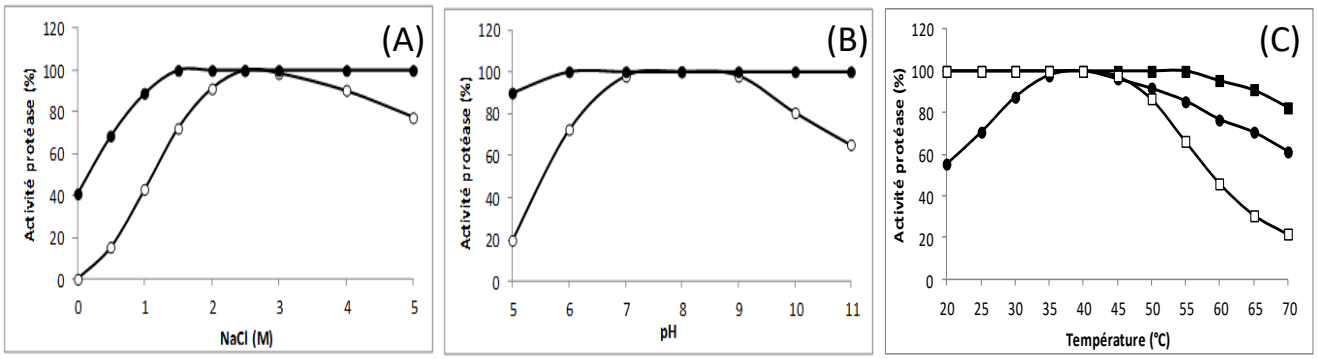

Figure 1. Effect of NaCl concentration (A), pH (B) and temperature (C) on protease activity and stability from *Salicola* sp. SBJ9.

The effect of various chemical reagents on SBJ9 protease activity was also studied. **Table 1** showed the effect of different metal ions and protease inhibitors on the enzymatic activity. It revealed a slight amelioration by Ca^{2+} ions and a total inhibition by Co^{2+}, Fe^{2+} and PMSF which indicated that most of proteases exhibiting this activity are serine proteases. In addition, the protease activity was very stable in the presence of several organic solvents at 50% (v/v) and detergent additives. As shown in **Table 2**, it is unaffected by acetonitrile and DMSO and presented more than 71.5% of residual activity with ethanol, isopropanol and butanol. Besides, it is perfectly stable in the presence of SDS (1%), CTAB (25 mM), Triton X-100 (10%), Tween 20, 40 and 80 (10%) and Na_2CO_3 (100 mM), exceeding 77.9% of residual activity. Then, *Salicola* sp. SBJ9 protease activity is considered as a good candidate for detergent industry and organic biosynthesis.

Table 2. effect of metal ions and protease inhibitors on protease activity from *Salicola* sp. SBJ9

Chemical agent	Protease activity (%)
None	100
Metallic ions (5 mM)	
Cu^{2+}	85.5
Mn^{2+}	54.4
Mg^{2+}	100
Ba^{2+}	110.3
Zn^{2+}	52.7
Ca^{2+}	150.6
Co^{2+}	24.2
Fe^{2+}	12.6
Protease inhibitors (5 mM if not indicated)	
Pepstatin A (10 µg/ml)	100
NEM	96.2
TPCK	94.5
EDTA	86.2
Iodoacetamide	57.8
PMSF	2

Table 1. effect of organic solvents and detergent additives on protease activity from *Salicola* sp. SBJ9

Chemical reagent	Protease activity (%)
None	100
Detergent additives	
H_2O_2 (1% (v/v))	60.4
SDS (1% (w/v))	92.4
CTAB (25 mM)	80.6
Tween 20 (10% (v/v))	77.9
Tween 40 (10% (v/v))	92.5
Tween 80 (10% (v/v))	98.8
Triton X-100 (10% (v/v))	90.7
Na_2CO_3 (100 mM)	100
Organic solvents (50%)	
Methanol	66
Ethanol	71.5
Isopropanol	80.6
Butanol	89.4
Acetonitrile	100
DMSO	100

3. Materials and Methods

The screening of new halophilic microorganisms was monitored from various saline and hypersaline biotopes, on agar plates containing increasing concentrations of NaCl (50, 100, 150 and 200 g/l NaCl). Protease production was detected by the presence of halo of degradation around the clone, in medium supplemented with 20% of skimmed milk. Molecular identification of the isolate SBJ9 was performed by the amplification of the 16S rDNA gene, using the universal primers S73 (5'-AGAGTTTGATCCTGGCTCAG) and S74 (5'-AAGGAGGTGATCCAGCC) as direct and reverse primers, respectively. The PCR product (~ 1.5 Kb) was purified, cloned in the pGEM-T Easy vector (Promega, USA) and sequenced in both directions.

Protease activity was assayed by the method of Kembhavi et al. [7] using Hammerstein casein (Merck, Germany) as substrate. One unit (U) of protease activity was defined as the amount of enzyme which liberated 1 µg of tyrosine per minute under the experimental conditions. Protease activity represents the means of, at least, two determinations performed in duplicate. The difference between values did not exceed 5%. The effect of NaCl concentration, pH and temperature on protease activity and stability was studied by incubating the crude enzyme at 0 to 5 M NaCl, 5 to 11 and 20 to 70 °C, respectively, and measuring relative and residual activities at standard assay conditions.

The effect of the different organic solvents and detergent additives on protease stability was examined by incubating the crude enzyme with each solvent at 50% (v/v) and each additive at the appropriate concentration, indicated in table 2, for 1 h at 30 °C, under kind shaking. Residual activities were carried under standard assay conditions. Enzyme activity without any additive was taken as control (100 %).

4. Conclusions

As a conclusion, *Salicola* sp. SBJ9 is an extremely halophilic bacterium producing an interesting protease activity which is distinguished by a good activity at high salt concentrations, neutral to alkaline pH values and room temperatures, conditions that are very required by many industrial applications. Besides, it is halo-alkalo-stable, thermo-solvent stable and compatible with different detergent chemicals. These important properties make strain SBJ9 protease activity as an effective candidate for many industrial and

biotechnological applications at harsh conditions such as detergent industry, organic biosynthesis and bioremediation of saline environments. This report is the first one presenting a biochemical characterization of a protease activity from a *Salicola* sp. strain. Besides, SBJ9 Protease activity was about 170 U/ml which is much higher than the sole reported uncharacterized protease from *Salicola* sp. IC10 which is 0.1 U/ml [8]. Due to the importance of the protease activity from *Salicola* sp. SBJ9, and probably of its other hydrolytic enzymes, the genome of this bacterium was sequenced in our laboratory, for the first time, and the corresponding genes were isolated to be then expressed.

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Conflicts of Interest
The authors declare no conflict of interest.

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