Isolation of Moderately Halophilic *Pseudoalteromonas* Producing Extracellular Hydrolytic Enzymes from Persian Gulf

M. Roayaie Ardakani · A. Poshtkouhian ·
M. A. Amoozegar · H. Zolgharnein

Received: 12 November 2010 / Accepted: 1 December 2011 / Published online: 17 December 2011
© Association of Microbiologists of India 2011

Abstract Extracellular hydrolytic enzymes such as amylases, proteases, lipases and DNases have quite diverse potential usages in different areas such as food industry, biomedical sciences and chemical industries, also it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations and temperature. Halophiles are the most likely source of such enzymes, because not only their enzymes are salt-tolerant, but many are also thermotolerant. The purpose of this study was isolation of hydrolytic extracellular enzyme producing halophilic bacteria from water and sediment of the Persian Gulf. Isolated bacteria from water and sediment were inoculated in media with concentration of 0–20% NaCl to determine the optimum salt concentration for growth, isolates were also inoculated in 4 types of solid medium containing substrates of 3 extracellular hydrolytic enzymes including amylase, Protease and Lipase, to determine the quantitative detection of enzyme production, selected strains after more accurate physiological and biochemical studies were identified regarding phylogeny and molecular characteristics using 16S rRNA technique. Isolated enzyme producing bacteria belong to *Pseudoalteromonas* genera.

Keywords Moderate halophilic · *Pseudoalteromonas* · Hydrolytic enzymes · Isolation and screening · Persian Gulf

Introduction

Pseudoalteromonas comprise one of the most abundant taxonomic groups of the proteobacteria widely distributed in the marine environment [1–4]. Extracellular hydrolytic enzymes such as amylases, proteases, lipases and DNases, have quite diverse potential usages in different areas such as food industry, feed additive, biomedical sciences and chemical industries [5–8]. Industrial processes are carried out under specific physical and chemical conditions which cannot always be adjusted to the optimal values required for the activity of the available enzymes. For that reason, it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations and temperature. Halophiles are the most likely source of such enzymes, because not only their enzymes are salt-tolerant, but many are also thermotolerant. The isolation of moderate halophiles which are able to produce extracellular enzymes will provide the possibility to have optimal activities at different salt concentrations. Moreover, the application of halophilic microorganisms for purification of polymer containing wastes has obvious advantages. Moderately halophilic bacteria are a group of halophilic microorganisms able to grow optimally in media containing a wide range of NaCl concentrations (3–15% NaCl) [9]. A screening programme, performed on saline habitats such as sea water and sediment, for the isolation of extracellular enzyme producers will provide insight on the possibility to use different moderate halophiles as a source of extremophilic enzymes in biotechnological processes. In this study we describe the screening for extracellular

M. R. Ardakani (✉) · A. Poshtkouhian
Department of Biology, Faculty of Science, Shahid Chamran
University, Ahwaz, Iran
e-mail: roayaei_m@yahoo.com

M. A. Amoozegar
Department of Microbiology, Faculty of Biology, University
of Tehran, Tehran, Iran

H. Zolgharnein
Khoramshahr University of Marine Science and Technology,
Khoramshahr, Iran

hydrolytic enzymes produced by moderately halophilic *Pseudoalteromonas* isolated from the Persian Gulf.

Materials and Methods

Sampling

Some sediment and Water samples were collected from different depths, of the Persian Gulf seawater at 4 different sites. Water pH, salinity and temperature were measured.

Isolation of Halophilic Microorganisms

Collected samples were incubated in marine broth, at 30°C for 3 days, then were transferred to Moderate Halophilic Medium (NaCl, 81.0 (g/l); MgSO₄·7H₂O, 9.6 (g/l); MgCl₂·6H₂O, 7.0 (g/l); KCl, 2.0 (g/l); CaCl₂·2H₂O, 0.36 (g/l); NaHCO₃, 0.06 (g/l); NaBr 0.026 (g/l); Yeast extract, 10.0 (g/l) Proteose peptone No. 3, 5.0 (g/l); Glucose, 1.0 (g/l); Agar, 20.0 (g/l); distilled Water, 1000 ml) so that the colonies appear [10]. After subculturing of the colonies, isolation was achieved. In order to detect the production of extracellular hydrolases such as protease, lipase and amylase, enzymatic agar plate assays, detailed below, were performed. Optimal concentration of NaCl for growth was determined by inoculating the isolates into nutrient broth with 9 different concentrations of NaCl (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20% (w/v)) and reading the absorbance at 600 nm every 2 h from the time of inoculation until 48 h later.

Extracellular protease production

Proteolytic activity of the cultures was screened qualitatively in a saline medium containing skim milk (10%) plus

10% (w/v) total salts [11]. Formation of clear zone around the colonies over the next 48 h was considered as indication of protease production (Fig. 1).

Extracellular Amylase Production

The presence of amylolytic activity on plates was routinely determined following the method described by Coronado [12], using HM medium supplemented with 0.5% (w/v) soluble starch. After incubation at 32°C for 3 days, the plates were flooded with 0.3% I₂—0.6% KI solution, a clear zone around the growth indicated hydrolysis of starch (Fig. 2).

Extracellular Lipolytic Production

Lipolytic activity of the isolates was detected by screening for zones of hydrolysis around colonies growing on HM plates containing 1% Tween-80, turbid around the growth indicated hydrolysis of Tween-80 [13] (Fig. 3).

Amylase Assay

For amylase production, the bacteria (1.5×10^8 CFU/ml) were inoculated in 250 ml Erlenmeyer flasks containing 50 ml medium (Beef extract, 3 (g/l); soluble starch, 10 (g/l); MgSO₄·7H₂O, 9.6 (g/l); MgCl₂·6H₂O, 7.0 (g/l); KCl, 2.0 (g/l); CaCl₂·2H₂O, 0.36 (g/l); NaHCO₃, 0.06 (g/l); NaBr 0.026 (g/l); distilled water, 1000 ml, (NaCl was added according to optimal amount needed for each isolate's growth). The flasks were incubated on a rotary shaker (200 rpm) at 30°C for 48 or 72 h [14]. Every 24 h after inoculation cells and insoluble materials were removed by centrifugation at 10,000×g for 10 min at 4°C and the cell-free supernatant was used as the source of crude enzyme. Amylase activity was assayed by using soluble starch as a substrate by the modified method of Bernfeld [15]. 0.5 ml of 1% soluble starch in sodium phosphate buffer (pH 7.0) was

Fig. 1 Screening of proteolytic strains (A proteolytic isolate, B non proteolytic isolate)

Fig. 2 Screening of amylolytic strains

Fig. 3 Screening of lypolytic strains

mixed with 0.5 ml of enzyme solution. After incubation at 40°C for 30 min, 1 ml of the dinitrosalicylic acid color reagent was added to the mixture. Tubes were incubated in a boiling water bath for 5 min. Cooled to room temperature and then 10 ml of distilled water was added to dilute the mixture in order to measure the Absorbance at 540 nm in a spectrophotometer versus blank. Micromoles maltose released was determined from maltose standard curve which was prepared as followed: In numbered tubes, 10 maltose dilutions ranging from 0.3 to 5 micromoles/ml was prepared. Two blank tubes with reagent grade water only were included. 1 ml of each dilution of maltose was pipetted into a series of corresponding numbered tubes. 1 ml of dinitrosalicylic acid color reagent was added to the mixtures then were incubated in boiling water bath for 5 min and cooled to room temperature. 10 ml of reagent grade water was added to each tube and mixed well. The absorbance for both the test and blank was read by spectrophotometer at 280 nm. Hydrolysis of 1 μ mol of soluble starch in 1 min was defined as 1 U of enzyme activity.

Lipase Assay

For lipase production, the bacteria (1.5×10^8 CFU/ml) were inoculated in 250 ml Erlenmeyer flasks containing 50 ml medium (Olive oil, 10.0 (g/l); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 9.6 (g/l); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 7.0 (g/l); KCl, 2.0 (g/l); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.36 (g/l); NaHCO_3 , 0.06 (g/l); NaBr 0.026 (g/l); Yeast extract, 5.0 (g/l); distilled water, 1000 ml (NaCl was added according to optimal amount for each isolate's growth). The flasks were incubated on a rotary shaker (200 rpm) at 30°C for 48, 72 or 96 h. Every 24 h after inoculation cells and insoluble materials were removed by centrifugation at $10,000 \times g$ for 10 min at 4°C and the cell-free supernatant was used as the source of crude enzyme. Lipase activity was measured by a modification of the method used by Kanlayakrit and Boonpan [16]. Olive oil (10%) was used as a substrate and treated with 7% solution of Arabic gum.

The olive oil emulsion (15.0 ml) was hydrolyzed with 1.0 ml enzyme solution in 4.0 ml of 0.2 M phosphate buffer (pH 7.0) at 37°C, 250 rpm for 1 h and the reaction was stopped by addition of 15 ml of a mixture solution containing acetone and 95% ethanol in the ratio of 1:1. The liberated oleic acid was calculated by titration with 0.05 N NaOH. One unit of lipase activity was defined as the amount of enzyme required to release 1 μ mol of oleic acid/ml/hour.

Protease Assay

For protease production, the bacteria (1.5×10^8 CFU/ml) were inoculated in 250 ml Erlenmeyer flasks containing 50 ml medium (Peptone, 5 (g/l); Glucose, 5 (g/l); Yeast extract, 5 (g/l); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 9.6 (g/l); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 7.0 (g/l); KCl, 2.0 (g/l); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.36 (g/l); NaHCO_3 , 0.06 (g/l); NaBr 0.026 (g/l); distilled water, 1000 ml, (NaCl was added according to optimal amount for each isolate's growth). The flasks were incubated on a rotary shaker (200 rpm) at 30°C for 48, 72 or 96 h. Cells and insoluble materials were removed by centrifugation at $10,000 \times g$ for 10 min at 4°C and the cell-free supernatant was filtered through a 0.45- μ m pore-size membrane filter and was used as the source of crude enzyme. Proteolytic activity was assayed by a modified method of Abdelnasser [17]. Samples containing 400 μ l of 0.5% (w/v) casein in 50 mM Tris–HCl buffer (pH 7) with 100 μ l enzyme sample were incubated in a water bath at 50°C for 20 min. The enzyme reaction was terminated by addition of 500 μ l of 10% (w/v) trichloroacetic acid and was kept at room temperature for 10 min. The reaction mixture was centrifuged at $10,000 \times g$ for 10 min at 4°C and the absorbance was measured against a blank (non-incubated sample) at 280 nm. One unit of proteases activity was defined as the amount of the enzyme releasing the equivalent of 1 μ mol of tyrosine/minute under the defined assay conditions. Standard curve of tyrosine was drawn using 10, 20, 30, 40, 50, 60, 70, 80 and 100 mg/ml tyrosine in 50 mM Tris–HCl buffer (pH 7).

Identification of Bacterial Strains

Initial Identification

The characters of the organisms were studied following the standard microbiological methods. Morphology, vegetative cells and spore characters were observed. The physiological and biochemical characters such as: catalase and oxidase test, starch hydrolysis, production of acid from glucose, manitol, xylose, arabinose, citrate utilization, casein hydrolysis, nitrate reduction and gelatin hydrolysis were studied.

Molecular Analysis by 16srRNA Sequencing

Molecular identification of the selected isolates was performed by the amplification of 16srRNA with eubacterial universal primers [18], (F: 5'CCGAATTCGTCGACAA CA3' and R: 5'CCCGGGATCCAAGCTATC3') Phylogenetic tree were drawn by Mega4 software (Fig. 4).

Results

Isolation of halophilic microorganisms: according to Molecular analysis by 16srRNA sequencing, 5 isolates belonged to *pseudoalteromonas* genus (Fig. 4). These isolates were able to produce extracellular enzymes (Table 1).

Enzyme Assay

Maximum enzymatic activity of all isolates was observed in the logarithmic phase of growth. Maximum amylase activity for T1 was observed 12 h after inoculation in assay medium explained earlier, for isolate T4, 18 h, for isolates T2 and T15, 32 h and for isolate T14, 48 h after inoculation (Fig. 5). Maximum lipase activity of isolates T1, T14 and T15 was observed in the 5 day of the lipase assay (Fig. 6). Also maximum protease activity of isolates T1 and T14 was observed in the 4th day and for isolate T15 in the 3rd day of protease assay (Fig. 7).

Fig. 4 Phylogenetic tree of strains T1, T2, T4, T14, T15 (Neighbor joining phylogenetic method)

Table 1 Moderate halophilic *pseudoalteromonas* isolates producing extracellular hydrolytic enzymes

Enzyme	Lipase	Protease	Amylase
Isolate			
T1	+	+	+
T2	-	-	+
T4	-	-	+
T14	+	+	+
T15	+	+	+

Fig. 5 T1, T2, T4, T14 and T15 amylase activity

Fig. 6 T1, T14 and T15 lipase activity

Fig. 7 T1, T14 and T15 protease activity

Discussion

Although moderately halophilic bacteria have a great biotechnological potential, only few studies have been carried out concerning their production of extracellular enzymes [19]. In this study most environmental isolates that were able to produce hydrolytic enzymes belonged to the gram-negative genus *Pseudoalteromonas*, a genus that is widely distributed in marine environments. It is interesting that combined hydrolytic activities have been detected in few strains. Only two *Pseudoalteromonas* isolates presenting the four hydrolytic activities tested have been isolated. These isolates could have an application for biotechnological purposes. Also enzymes from halophiles are expected to show optimal activities in extreme conditions, thus the possibility to have a wide variety of moderate halophiles producing extremozymes will be of invaluable help for biotechnological applications. All six investigated strains had hydrolytic activity at 1.5 M NaCl. At 3.5 M NaCl, many strains appeared to lose their hydrolytic properties and the predominant activity at 3 M NaCl was the lipase hydrolyzing Tween-80 or olive oil, suggesting that strains having this activity can be studied for further investigations regarding lipid degradation in saline conditions or some oil degradation in seawater and wastewater environments. In the case of isolates which produced multiple hydrolytic enzymes amylase was the first enzyme produced by them. Protease was produced a few hours later and lipase production lasted longer than other hydrolyses.

Acknowledgment The authors would like to acknowledge the Research Vice Chancellor of Shahid Chamran University of Ahwaz for offering a grant for this research.

References

- Gauthier G, Gauthier M, Christen R (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *Int J Syst Bacteriol* 45:755–761
- Baumann P, Baumann L (1981) The marine gram-negative eubacteria; genera *Photobacterium*, *Beneckea*, *Alteromonas*, *Pseudomonas*, and *Alcaligenes*. In: Starr MP, Stolp H, Truper HG (eds) *The prokaryotes: a handbook on habitats isolation, and identification of bacteria*. Springer Verlag, Berlin, pp 1302–1330
- Baumann P, Gauthier MJ, Baumann L (1984) Genus *Alteromonas* Baumann, Baumann, Mandel and Allen 1972. In: Kreig R, Holt JG *Bergey's Manual of Systematic Bacteriology*, vol 1. Williams & Wilkins, Baltimore pp 343–352
- Gauthier MJ, and Breittmayer VA (1992) The genera *Alteromonas* and *Marinomonas*. In: Balows A, Triper HG *The Prokaryotes*. Springer Verlag, New York pp 3046–3070
- Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. *FEMS Microbiol Rev* 23:411–456
- Niehaus F, Bertoldo C, Kahler M, Antranikian G (1999) Extremophiles as a source of novel enzymes for industrial application. *Appl Microb Biotechnol* 51:711–729
- Pandey A, Benjamin S, Socol CR, Nigam P, Krieger N, Socol VT (1999) The realm of microbial lipases in biotechnology. *Biotechnol Appl Biochem* 29:119–131
- Pandey A, Nigam P, Socol CR, Socol VT, Singh D, Mohan R (2000) Advances in microbial amylases. *Biotechnol Appl Biochem* 31:135–152
- Ventosa A, Nieto JJ, Ore A (1998) Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* 62:504–544
- Atlas RM (2005) *Handbook of media for environmental microbiology*. CRC Press, New York, pp 84–256
- Ventosa A, Quesada E, Rodríguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A (1982) Numerical taxonomy of moderately halophilic gram-negative rods. *J Gen Microbiol* 128:1959–1968
- Coronado MJ, Vargas C, Hofmeister J, Ventosa A, Nieto JJ (2000) Production and biochemical characterization of an alpha-amylase from the moderate halophile *Halomonas meridiana*. *FEMS Microbiol Lett* 183:67–71
- Hasan F, Ali Shah A, Hameed A (2009) Methods for detection and characterization of lipases: a comprehensive review. *Biotechnol Adv* 27:782–798
- Rohban R, Amoozegar AM, Ventosa A (2009) Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *J Ind Microbiol Biotechnol* 36: 333–340
- Worthington Biochemical Corporation manual, Beta Amylase Assay. 2010
- Kanlayakrit W, Boonpan A (2007) Screening of halophilic lipase-producing bacteria and characterization of enzyme for fish sauce quality improvement. *Kasetstar J. (Nat.Sci.)* 41:576–585
- Abdelnasser SS, Ibrahim SS, El-Shayeb Nefisa MA, Sohair S Mabrouk (2007) Isolation and identification of alkaline protease producing alkaliphilic bacteria from an Egyptian Soda Lake. *J Appl Sci Res* 3(11):1363–1368
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173:697–703
- Ventosa A (1988) Taxonomy of moderately halophilic heterotrophic eubacteria. In: Rodríguez-Valera F, Boca Raton FL (eds) *Halophilic bacteria*. CRC Press, USA, pp 71–84