Jundishapur Journal of Microbiology
www.jjmicrobiol.com

Identification of a Bacterium Isolated From Soil of Ahvaz Contaminated by Oil and Determination of Its Protease Stability in Organic Solvents

Gholamhossein Ebrahimipour [1], Hossein Sadeghi [1*], Zahra Khosravi babadi [1], Soleiman Amiri [1]

[1] Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, IR Iran

* *Corresponding author:* Hossein Sadeghi, Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, IR Iran.Tel.: +98-2129902706, Fax: +98-2122431664, E-mail: Sadeghi.hsn88@gmail.com

A B S T R A C T

Background: Proteases are often used as catalysts for peptide synthesis. To shift the thermodynamic equilibrium in favor of the peptide synthesis, reaction media should contain organic solvent. However, known enzymes are usually inactivated by adding organic solvents to reaction media.

Objectives: In this study, we reported a bacterium isolated from soil of Ahvaz, Iran contaminated by crude oil producing an organic solvent-stable protease, and the effect of organic solvents on proteolytic activity was investigated.

Materials and Methods: Isolated bacterium was cultured in mineral salt medium containing glucose and peptone. After 48 hours incubation at 35 °C and 130 rpm, culture media were centrifuged and resulted supernatant filtered using 0.22 μm nitrocellulose membrane filter. Proteolytic activity of supernatant was determined by Keay and Wildi method (1970) by using casein as substrate. The effects of different concentrations of various organic solvents including acetone, ethanol, pentanol, cyclohexane, benzene, n-hexane, and n-decane and, also, the effects of temperature and pH on protease stability and activity were examined.

Results: According to 16SrDNA sequencing, strain ISA9 was identified as a new strain of Bacillus licheniformis. This strain was able to produce an extracellular organic solvent- tolerant protease. After 30 minutes incubation at 37 °C, caseinolytic activity of crude protease was increased in 25 and 50% of acetone, ethanol, benzene, cyclohexane, and hexane compared to non-solvent control. The enzyme was also activated 1.64, 1.23, and 1.17 times by 75% (v/v) of benzene, decane, and hexane, respectively. The protease was active in a broad range of pH (from 6 to 10) with the optimum pH 10. The optimum temperature for the activity of this protease was 70 °C and the enzyme remained active after incubation at 30-50 °C for 30 minutes.

Conclusions: In this study, we isolated *B. licheniformis* producing an organic solvent-stable protease from oil-contaminated soil. The protease was stable and active in various organic solvents. By purification, the protease could be used as a biocatalyst for organic solvent-based enzymatic synthesis.

Keywords: Soil; Bacillus licheniformis; Protease

Article type: Research Article; Received: 17 Apr 2012, Revised: 27 May 2012, Accepted: 07 Jun 2012; DOI: 10.5812/jjm.4575

Implication for health policy/practice/research/medical education:

Proteases are often used as catalysts for peptide syntheses. To shift the thermodynamic equilibrium in favor of the peptide synthesis, the reaction media should contain organic solvent. known enzymes are usually inactivated by the addition of organic solvents to the reaction medium. Several organic solvents stable enzymes producing bacteria have been isolated. This enzymes have intrinsic stability and high activity in organic solvents and can be used for synthesis of some commercially important organic products.

Please cite this paper as:
Ebrahimipour, G, Sadeghi, H., Khosravi babadi Z, Amiri Z. Identification of a Bacterium Isolated From Soil of Ahvaz Contaminated by Oil and Determination of Its Protease Stability in Organic Solvents. *Jundishapur J Microbiol.* 2013; **6**(2): 105-111. DOI: 10.5812/jjm.4575

1. Background

The vast majority of synthetic enzyme reactions are performed in organic media. Doing so in organic solvents or solvent/water mixtures has numerous advantages compared to traditional aqueous reaction system, including (i) increased solubility of nonpolar substrates and products; (ii) shift of thermodynamic equilibrium in favor of synthesis over hydrolysis, allowing reactions such as transesterification, thioesterification, and aminolysis that usually are not favored in aqueous solutions to occur; (iii) suppression of unwanted water-dependent side reactions, and (iv) elimination of microbial contamination in the reaction mixture (1, 2). However, the application of enzymes in organic media is restricted because most enzymes are less active and stable in the presence of organic solvents. Therefore, several physical and chemical methods have been employed to improve the activity and stability of proteases in the presence of organic solvents.

Natural enzymes with organic solvent-tolerance are useful to apply in the organic solvents as reaction media without any modification to stabilize the enzymes. Microorganisms are the most important source of enzyme. It was postulated that extracellular enzymes produced by organic solvent-tolerant bacteria showed the stability in the presence of organic solvents. Thus, the solvent-tolerant bacteria were isolated and screened for production of organic solvent-tolerant enzymes (1, 3). Among these enzymes, hydrolases, especially carboxylesterases, lipases, and proteases, were mostly studied. Useful reactions performed by hydrolases in the presence of organic solvent include resolution of racemic mixtures by transesterification, enantio- and regioselective hydrolysis, and synthesis of natural and non-natural pro-drugs, detergents, polyesters, peptides, and additives (3).

Proteases are among the most important hydrolytic enzymes and account for approximately 60% of total enzyme sales in the world. These enzymes are used in detergent, food, pharmaceutical, leather, and protein processing industries and also used for peptide synthesis (4). Microbial proteases have attracted considerable attention due to commercial application of peptide and ester synthesis in media containing organic solvents. The first screening report of an organic solvent-tolerant protease from Pseudomonas aeruginosa PST-01is related to Ogino *et al*. (5). Since then, various organic solvent-tolerant enzymes have been reported.

The Bacillus and Pseudomonas species have been found to produce solvent-stable proteases with potential industrial applications (3, 5-8). Geok *et al*. isolated P. aeruginosa strain K, a benzene-toluene-xylene-ethylbenzene (BTEX) tolerant bacterium that produced organic solvent-tolerant protease from contaminated soils of a wood factory (9). Li *et al*. reported a solvent-stable alkaline protease produced by *Bacillus licheniformis* YP1A isolated from oil-contaminated soil. This extracellular protease YP1A was active in a broad range of pH (8.0–12.0) (6). Sareen *et al*. used a protease secreted by a mutant strain, *B. licheniformis* RSP-09-37, in aqueous-acetonitrile media in order to synthesize kyotorphin precursor (10).

Organic solvent-tolerant bacteria found in oil-contaminated area and halophilic bacteria producing halotolerant enzyme are considered to be valuable tools as biocatalysts in aqueous–organic media (1, 3).

2. Objectives

The aim of this study was investigation on the ability of a native organic solvent-tolerant bacterium isolated from oil-contaminated soil around Ahvaz, Iran to produce valuable organic solvent-stable protease.

3. Materials and Methods

3.1. Methods

RD1 and FD1 primers were purchased from Gen-fanavaran, Iran. 1 kb PCR marker was obtained from Fermentase, Lithuania, high pure PCR kit from Roch, Germany, peptone from Difco, USA; and other material used in this study from Merck, Germany.

3.2. Bacterium Strain

The bacterium used in this study, strain ISA9, was isolated from oil-contaminated soil around Ahvaz, Iran. The early purpose was the isolation of a bacterium with ability to desulfurize gas oil. Because of high tolerance of this strain in organic solvents, it was screened for production of solvent-stable extracellular protease.

3.3. Identification of Bacterium

Isolated bacterium was identified based on biochemical and morphological tests as given by Bergey's Manual of Systematic Bacteriology (11). The results of the biochemical tests were further confirmed by molecular testing. DNA of strain ISA9 was extracted using high pure PCR product kit. PCR amplifications were performed on a Primus 25 PCR system (PEQLab, Germany). After 5 minutes of denaturation at 94 °C, thermal profile of PCR was 30 cycles consisting of 94 °C for 40 seconds, primer annealing at 56 °C for 40 seconds, and extension at 72 °C for 1 minute. PCR was followed by a final extension at 72 °C for 10 minutes. Universal primers used in this process included forward: 5'-AGAGTTTGATCCTGGCTC-3´and reverse: 5'-GCCTAAGGAGGTGATCCA-3´. The amplified product was purified by agarose gel electrophoresis. Nucleotide sequencing analysis was performed with dideoxy chain termination method (SEQLAB, Germany). The 16SrRNA gene sequence of strain ISA9 was compared with simi-

lar sequences of reference organisms by BLAST algorithm (www.ncbi.nlm.nich.-gov/BLAST/).

3.4. Protease Production Test

The strain ISA9 was cultured in skim milk agar (skim milk powder, 100 g, and agar, 15 g in 1000 ml distilled water) and incubated at 37 °C for 24 hours. Proteolytic activity of bacterium was investigated by appearance of clear zone around the colonies on skim milk agar plate (6, 12).

3.5. Protease Production Medium

The inoculum was prepared by transferring a loopful of fresh cultured strain ISA9 into nutrient broth medium followed by incubation at 37°C and 130 rpm for 24 hours. Then, the culture was used to inoculate 250 ml of the protease producing medium in 1000 ml Erlenmeyer flasks. The composition of the medium was as follows: (g/L): glucose, 10; peptone, 10; K2HPO4, 0.5; NH4Cl, 1; MgSO4.7H2O, 0.2; FeSO4.7H2O, 0.01; CaCl2.2H2O, 0.01; and 1 ml trace elements (ZnCl2, 70 mg; MnCl2.4H2O, 100 mg; CoCl2.6H2O, 200 mg; NiCl2.6H2O, 100 mg; CuCl2.2H2O, 20 mg; NaMoO4.2H2O, 50 mg; Na2SeO3.5H2O, 26 mg; NaVO3.H2O, 10 mg; and Na2WO4.2H2O, 30 mg in 1000 ml distilled water) (13). Flasks were incubated at 37 °C and 130 rpm for 48 hours. Afterward, to remove bacterium cells, the culture media were centrifuged at 13000 × g for 10 minutes. Resulted supernatant was filtrated using 0.22 μm nitrocellulose membrane filters and stored in 1000 ml sterile bottles in 4 °C as crude protease solution.

3.6. Assay of Protease Activity

Caseinolytic activity of supernatant was measured by a slight modification of Keay and Wildi method (14). The supernatant was diluted by water (enzyme/water, 1:3) and pre-incubated at 37 °C for 5 minutes. The reaction was started by adding 1 ml of casein 2.0% (w/v) at pH 7. The reaction mixture was then incubated in the incubator at 37 °C for 10 minutes and terminated by adding 2 ml of trichloroacetic acid (TCA), 0.4 molar. A vortex mixer was used to ensure complete mixing at various stages of these assay procedures. This mixture was further incubated at 37 °C for 20 minutes, followed by centrifugation at 13000 × g for 10 minutes. The supernatant was harvested. Five ml of Na2CO3 (0.4 molar) and 1 ml of folin ciocalteau reagent diluted by water (1:3, v/v) were added to 1 ml of resulted supernatant to yield a blue color. The colored mixture was incubated in an incubator at 37 °C for 20 minutes. Then, the absorbance was read at 660 nm. A blank was prepared by the same procedure, the trichloroacetic acid being added at zero time and the casein after the 10 minutes incubation. One unit (U) of protease was equivalent to 0.5 μg tyrosine liberated by 1.0 ml enzyme solution under the assay conditions. The amount of tyrosine was determined using tyrosine standard curve.

3.7. Effects of Organic Solvents on the Stability of Protease

The stability of the crude protease against several organic solvents was tested. The solvents used for this study were: acetone, ethanol, 1-pentanol, cyclohexane, benzene, hexane, and n-decane. The stability of protease at different concentrations (0, 25, 50, 75%, v/v) of organic solvents was examined. The cell-free supernatants with different percentages of organic solvents were shaken at 150 rpm and 37 °C for 30 minutes in the sterile capped tubes (1.6 cm in diameter). The stability of crude protease was also examined by incubation of 1 ml of organic solvent with 3 ml of cell-free supernatant in sterile capped tube at 37 °C and 150 rpm for 14 days. The remaining proteolytic activities were measured by Keay and Wildi method. Stability was expressed as the ratio of remaining proteolytic activity to non-solvent-containing control (0%, v/v). Each experiment was performed in triplicates.

3.8. Effect of Temperature on Protease Stability and Activity

For determination of temperature stability, aliquots of 1 ml of crude protease were diluted by 3 ml distilled water and incubated for 30 minutes at various temperatures, i.e.:30, 37, 40, 50, 60, 70, and 80 °C in screw capped tubes in water bath (Memmert, Germany). Then, samples were removed and cooled rapidly in an ice bath and pre-incubated at 37 °C, and protease activity was determined by aforementioned method. The activity at 37 °C was considered as control. To study the effect of temperature on protease activity, 1 ml of diluted supernatant (1:3 with water) was mixed with 1 ml of 2% casein and incubated at screw capped tubes for 10 minutes at above mentioned temperature in water bath. Afterward, the samples were removed and cooled rapidly in an ice bath (4 °C) and pre-incubated at 37 °C, and protease activity was determined. All experiments were performed in triplicates.

3.9. Effect of pH on Protease Stability and Activity

The effect of pH on protease activity and stability was measured at pH: 4, 5, 6, 7, 8, 9, 10, 11, and 12 using casein as the substrate at 37 °C. For pH stability studies, 1 ml of supernatant was mixed with 3 ml of 50 mM buffer of specific pH. The mixtures were incubated at 37 °C for 30 minutes and 24 hours. Then, the pH of samples was adjusted at pH 7 by using 1 M NaOH and 1 M HCl and the residual activity was determined using Keay and Wildi

method. The effect of pH on protease activity was studied in pH 4-11 using casein as substrate. One ml of crude protease (supernatant) mixed with 3 ml of buffer at specific pH. Then, 1 ml of this solution was added to 1 ml of 2% casein. The mixtures were incubated for 10 minutes in 40 °C and protease activity was measured by aforementioned method. Buffer systems used were sodium dihydrogen phosphate-NaOH (pH 4-8) and disodium hydrogen phosphate-NaOH (pH 9-12).

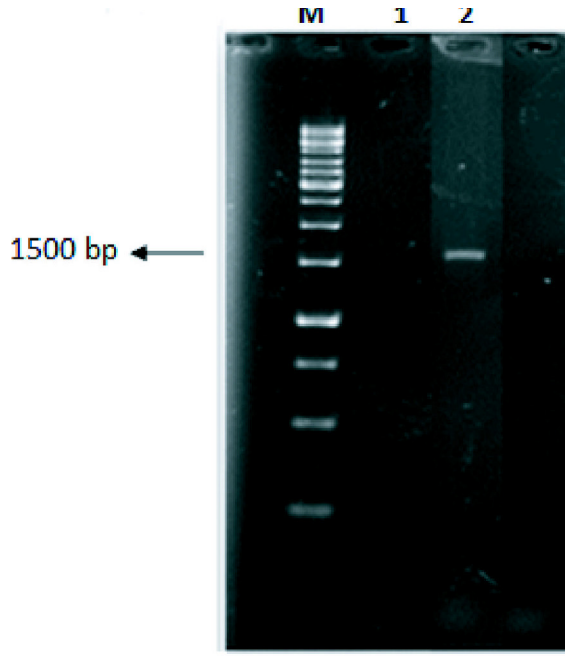
4. Results

4.1. Identification of Bacterium

The sequence of 16SrDNA was obtained after DNA extraction and PCR amplification result in 1500 bp (*Figure 1*). The sequence was reversed, aligned, and compared with similar database sequences using the Bioedit software (Ibis Biosciences, USA) . BLAST analysis demonstrated 99% similarities with *Bacillus licheniformis* and *Bacillus* sp. Morphological and biochemical properties of the isolated bacterium were investigated by Bergey's Manual of Systematic Bacteriology (11).

Summarized results of biochemical and morphological tests were shown in Table 1. According to Table 1, physiological-biochemical characteristics of strain ISA9 were similar to *Bacillus licheniformis* that was confirmed by phylogenetic analysis of the 16SrRNA gene.

Figure 1. Separation of PCR Product of 16SrDNA on 1% Agarose Gel



M: 1kb, DNA ladder; 1, negative control; 2, Strain ISA9

4.2. Proteolytic Activity of Strain ISA9

The strain ISA9 was cultured in skim milk agar plates. After incubated at 37 °C for 24 hours, clear zone around the bacterium appeared as a result of casein hydrolysis (*Figure 2)*. Caseinolytic activity of its supernatant measured by Keay and Wildi method was 346 U/mL at 37 °C and pH 7.

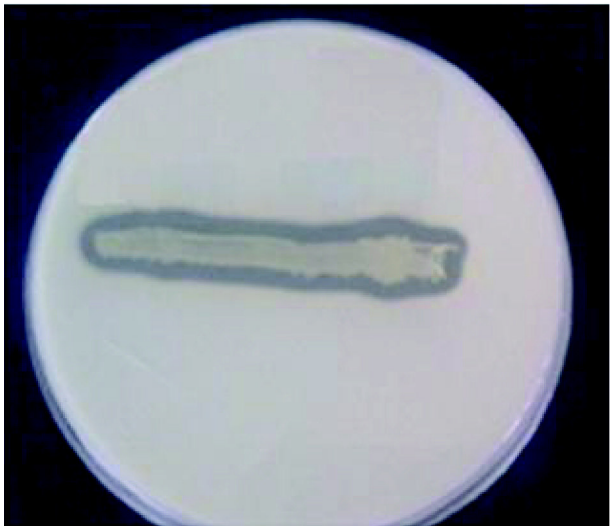


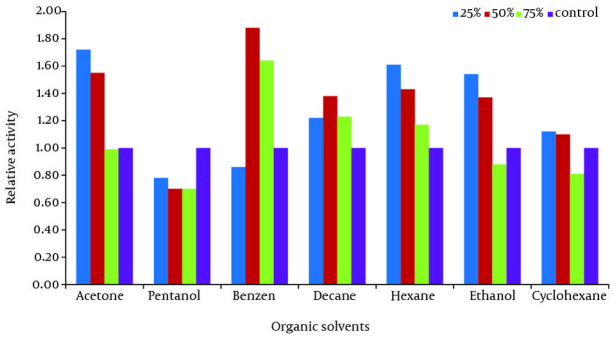
Figure 2. Clear Zone around Bacterium Colonies in Skim Milk Agar After 24 hours Incubated at 37 °C

4.3. Effects of Organic Solvents on Protease Stability

The effects of various organic solvents including acetone, ethanol, 1-pentanol, cyclohexane, benzene, hexane and decane in concentrations of 0, 25, 50, and 75% on the stability of crude protease were examined. *Figure 3* showed relative activity of protease after 30 minutes incubation in 37 °C and 150 rpm. Caseinolytic activity of crude protease increased in acetone, ethanol, benzene, and hexane compared to control (non-solvent sample). In concentration of 50%, acetone caseinolytic activity of supernatant was 1.8 times greater than that of control. Except of 1-pentanol, crude protease was stable and active in concentrations of 25 and 50% (v/v %) of all tested solvents. In 1-pentanol with log Po/w 1.3, the stability of protease decreased in all tested concentrations. Stability of crude protease in various organic solvents was also tested for 14 days (Table 2).

No clear relationship observed between solvent polarity and protease activity. Acetone and ethanol caused 1.36 and 1.2 times increasing in caseinolytic activity of supernatant. Compared to the control, 1-pentanol and cyclohexane proteases lost 12% and 19% of their activities, respectively. In other solvents, no changes were observed in protease activity compared to the control.

Figure 3. Effects of Different Percentages of Organic Solvents on the Stability of Protease

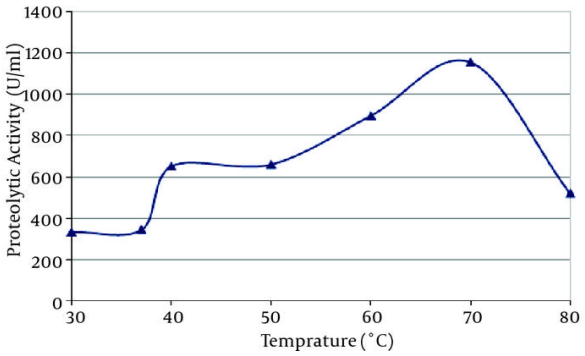


The cell-free Supernatant was incubated at 37 °C and 150 rpm in the presence of 0% (v/v) (without organic solvents), 25% (v/v), 50% (v/v), and 75% (v/v) of organic solvents for 30 minutes. Each experiment was performed in triplicates.

4.4. Effect of Temperature on Protease Activity and Stability

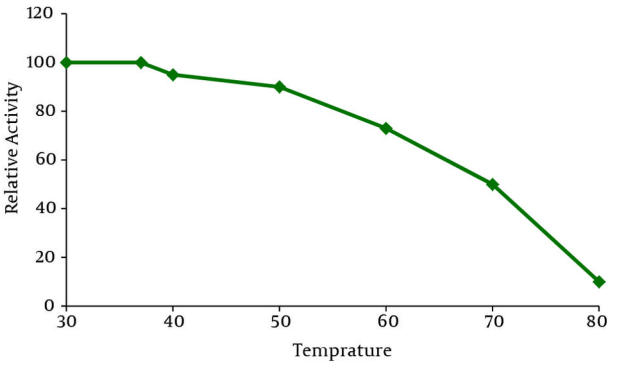
The effect of temperature on proteolytic activity of supernatant was measured at 30-80 °C and pH 7 by using casein as substrate. Maximum caseinolytic activity of supernatant was observed at 70 °C by 10 minutes incubation. The optimum temperature studies indicated that the strain ISA9 protease was active over a wide range of temperature from 30 -70 °C (*Figure 4*). Thermal stability of protease at 30-80 °C for 30 minutes at pH 7 showed that crude protease was stable at 30-50 °C (*Figure 5*). Incubation at 60 °C caused the loss of 27% of protease activity after 30 minutes incubation. Proteolytic activity decreased dramatically when temperature raised to above 70°C. At 70 °C and 80 °C, only 50% and 10% of activity observed in temperature 37 °C (control), were retained, respectively.

Figure 4. Effect of Temperature on Protease Activity



Protease activity was determined by using casein as the substrate incubated at the various temperatures, i.e.: 30, 37, 40, 50, 60, 70, and 80 °C

Figure 5. Effect of Temperature on Protease Stability

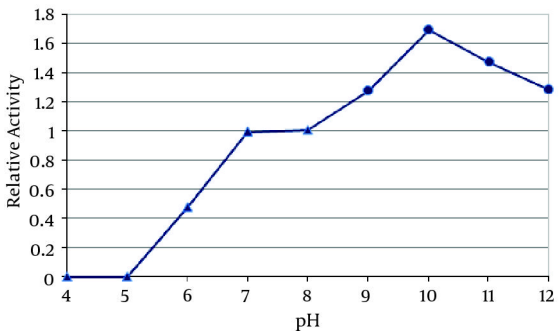


The crude protease was pre-incubated at temperatures: 37, 40, 50, 60, 70, and 80 °C for 30 minutes. The enzyme solution was cooled to 4 °C and the residual activity was measured under standard condition. The activity measured at 37 °C was taken as control (100% of relative activity).

4.5. Effect of pH on Protease Activity and Stability

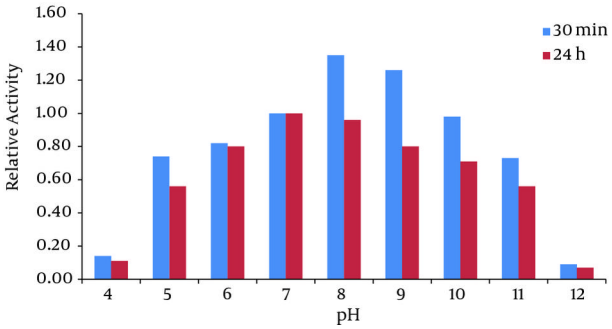
The relative protease activities, measured at various pH levels, were shown in *Figure 6* . The enzyme was active in pH range of 7–12 (up to 50%) and shown an optimum pH of 10 at 37 °C. Strain ISA9 protease retained 75.8 % of its maximal activity at pH 12, and 7% of its optimal activity at pH 4.0, 39% at pH 5, and 43% at pH 6. Study of pH stability demonstrated that when strain ISA9 protease was incubated at 37°C for 30 minutes, it was very stable between pH 6 and 11 (more than 70% of initial activity at pH 7). However, incubation of the supernatant at 37 °C for 24 hours showed that it was stable at pH range of 6 -10. Stability at pH 5 and 11 after 24 hours incubation was 56% (*Figure 7*).

Figure 6. Effect of pH on Protease Activity



Protease activity of the supernatant was measured at various pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0) at 37 °C using casein as the substrate. Buffer systems used were sodium dihydrogen phosphate-NaOH (pH 4.0-8.0) (▲), and disodium hydrogen phosphate -NaOH (pH 9.0-12.0) (●). The activity at pH 7 (346 U/ml) was taken as 1 and activity at other pH was compared with this pH.

Figure 7. Effect of pH on Protease Stability



One ml of supernatant was mixed with 3 ml, 50 mM Buffer of specific pH. The mixture was incubated at 37 °C for 30 minutes and 24 hours and the residual activity was determined at pH 7. Buffer systems used were sodium dihydrogen phosphate-NaOH (pH 4.0-8.0), and disodium hydrogen phosphate -NaOH (pH 9.0-12.0). The activity at pH 7 was taken as 1 and stability at other pH was compared with this pH.

5. Discussion

Some commercially important synthetic organic products and some substrates used in these enzymatic reactions are insoluble or unstable in water, and the presence of organic solvent in reaction media is necessary to carry out reactions (10). In the presence of organic solvent, hydrolytic enzyme can also catalyze synthetic reactions. By adding organic solvent to enzyme reaction media, molar fraction of water reduces and shifting the reaction equilibrium towards synthetic route occurs. Enzymes with stability in organic solvents are produced by novel extremophilic microorganisms. Organic solvent-stable proteases were identified in Pseudomonas and Bacillus species (4). Some of these proteases showed stability in hydrophobic and others in hydrophilic solvents. In this study, an organic solvent-tolerant bacterium was isolated from oil-contaminated soil around Ahvaz, Iran. According to 16SrDNA sequencing and biochemical and morphological features, strain ISA9 was identified as *Bacillus licheniformis*. Several solvent-tolerant Bacillus species have been reported for the production of organic solvent-stable protease including Bacillus pumilus 115b (15), Bacillus cereus BG1 (16), and *Bacillus licheniformis* (5, 7, 8, 10, 17, 18). Li *et al*. reported a novel solvent-stable alkaline protease produced by solvent-tolerant *Bacillus licheniformis* YP1A isolated from oil-contaminated soil (6). This extracellular protease YP1A retained more than 95% of its initial activity in the presence of 50% (v/v) of DMSO, DMF, and cyclohexane. Rachadech *et al*. isolated *Bacillus licheniformis* 3C5, as mesophilic bacterium, from cyclohexane-enriched soil that exhibited tolerance over a wide range of nonpolar and polar organic solvents (7). The optimal temperature for proteolytic activity of crude ISA9 protease was 70 °C and it was active over a wide range of temperature from 30 -70 °C. The organic solvent-stable protease produced by *Bacillus licheniformis* 3C5 was active over a broad range of temperature, i.e. 45–70 °C, with an optimum activity at 65 °C (7). Sareen *et al*. reported the optimum temperature of organic solvent-tolerant protease from *Bacillus licheniformis* strains RSP-09-37 at 50 °C (10).

Strain ISA9 protease had a higher optimal temperature compared to those produced by other strains of *Bacillus licheniformis*. Most proteases from Bacillus sp. showed an optimum pH values ranged from 8-10 (8). Optimal pH for proteolytic activity of crude ISA9 protease was observed at pH 10. Optimum pH for casein hydrolysis of organic solvent-stable protease for *Bacillus licheniformis* 3C5 (7) and *Bacillus licheniformis* RSP-09–37 (10) was 10 and for *Bacillus licheniformis* YP1A (6) was 9.5. Hence, the optimal pH of strain ISA9 protease was similar to other known alkaline proteases produced by Bacillus species. Strain ISA9 protease was stable in organic solvents with log P < 2 such as ethanol, acetone, and butanol. In the presence of water soluble organic solvents or alcohols, the activity of crude strain ISA9 protease was much higher than that seen in the absence of organic solvent. This phenomenon might be due to conformational changes of enzyme that increased its catalytic activity (KCat) or its affinity to substrate (Km). The precise determination of these parameters needs to purification of the enzyme and structural studies. Several publications reported that the solvent-stable proteases are quite stable in the organic solvents with the log P values equal to or more than 3.0 at 25% concentration, but only a few of them were stable in organic solvents with the log P values below 2.0 (5, 8, 9, 17). Rachadech *et al*. reported that the activity of organic solvent-tolerant protease from *Bacillus licheniformis* 3C5 raised up to 20% when it was initially exposed to 35% of butanol, acetone , and benzene, and the protease retained more than 80% of its initial activity after solvent exposure for an hour (7). The stability of Pseudomonas aeruginosa strain PT121 solvent-stable protease in the presence of organic solvents with the log P < 2.0 (acetone, butanol, and ethanol) was below 0.7, compared to the control when incubated for 14 days in a 25% organic solvents (19).

The protease from the strain K (9) was activated in the presence of organic solvents with the log P values over 4.0 compared to the control, but the stability of protease in the presence of 25% organic solvents with the log P values below 4.0 was not more than 0.65 after 14 days of incubation. The results in this study showed that the protease from ISA9 strain was not only quite stable in the presence of various organic solvents with the log P values equal to or more than 3.0, but also in the presence of several organic solvents with the log P values below 2.0. These results indicated that the protease may be a novel solvent-stable protease.

In this paper we described an organic solvent-stable protease from native *Bacillus licheniformis* strain ISA9 isolated from oil-contaminated soil around Ahvaz, Iran. Our study

showed that the protease is stable in various organic solvents, and in some cases the activity of crude protease increased by adding solvents. By purification, this protease could be used in organic solvent-based synthesis.

Acknowledgements

The authors wish to thank Dr. Dariush Minaei for his guidance and Dr. Mohammad Yaghubi and Aazam Moradi for their assistance with the experiments.

Financial Disclosure

None declared.

Funding/Support

None declared.

Authors' Contribution

None declared.

References

1. Doukyu N, Ogino H. Organic solvent-tolerant enzymes *Biochem Eng J*. 2010;**48**(3):270-82.
2. Ogino H. *Protein Adaptationin Extremophiles*. 2008; p. 193-236
3. Torres S, Pandey A, Castro GR. Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials *Biotechnol Adv*. 2011;**29**(4):442-52.
4. Tang XY, Wu B, Ying HJ, He BF. Biochemical properties and potential applications of a solvent-stable protease from the high-yield protease producer Pseudomonas aeruginosa PT121 *Appl Biochem Biotechnol*. 2010;**160**(4):1017-31.
5. Ogino H, Yasui K, Shiotani T, Ishihara T, Ishikawa H. Organic solvent-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme *Appl Environ Microbiol*. 1995;**61**(12):4258-62.
6. Li S, He B, Bai Z, Ouyang P. A novel organic solvent-stable alkaline protease from organic solvent-tolerant *Bacillus licheniformis* YP1A *J Mol Catal B: Enzym*. 2009;**56**((2-3)):85-8.
7. Rachadech W, Navacharoen A, Ruangsit W, Pongtharangkul T, Vangnai A. An organic solvent-, detergent-, and thermostable alkaline protease from the mesophilic, organic solvent-tolerant *Bacillus licheniformis* 3C5 *Microbiology*. 2010;**79**(5):620-9.
8. Rahman RNZRA, Geok LP, Basri M, Salleh AB. An organic solvent-stable alkaline protease from Pseudomonas aeruginosa strain K: Enzyme purification and characterization *Enzyme Microb Tech*. 2006;**39**(7):1484-91.
9. Geok LP, Razak CNA, Abd Rahman RNZ, Basri M, Salleh AB. Isolation and screening of an extracellular organic solvent-tolerant protease producer *Biochem Eng Jl*. 2003;**13**(1):73-7.
10. Sareen R, Mishra P. Purification and characterization of organic solvent stable protease from *Bacillus licheniformis* RSP-09-37 *Appl Microbiol Biotechnol*. 2008;**79**(3):399-405.
11. Sneath P, Mair N, Sharpe M, Holt J. 1986.
12. Brown A. *Benson's Microbiological Applications: Laboratory Manual in General Microbiology, Short Version*. 2009.
13. Schlegel H. *General Microbiology*. 1993.
14. Keay L, Wildi BS. Proteases of the genus Bacillus. I. Neutral proteases *Biotechnol Bioeng*. 1970;**12**(2):179-212.
15. Rahman RN, Mahamad S, Salleh AB, Basri M. A new organic solvent tolerant protease from Bacillus pumilus 115b *J Ind Microbiol Biotechnol*. 2007;**34**(7):509-17.
16. Ghorbel B, Sellami-Kamoun A, Nasri M. Stability studies of protease from Bacillus cereus BG1 *Enzyme Microb Tech*. 2003;**32**(5):513-8.
17. Ogino H, Watanabe F, Yamada M, Nakagawa S, Hirose T, Noguchi A. Purification and characterization of organic solvent-stable protease from organic solvent-tolerant Pseudomonas aeruginosa PST-01 *J Biosci Bioeng*. 1999;**87**(1):61-8.
18. Gao Y, Dai J, Peng H, Liu Y, Xu T. Isolation and characterization of a novel organic solvent-tolerant Anoxybacillus sp. PGDY12, a thermophilic Gram-positive bacterium *J Appl Microbiol*. 2011;**110**(2):472-8.
19. Tang X, Pan Y, Li S, He B. Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease *Bioresource Technol*. 2008;**99**(15):7388-92.