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A protease stable in organic solvents from solvent tolerant strain of *Pseudomonas aeruginosa*

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Abstract

A solvent tolerant strain of *Pseudomonas aeruginosa* (PseA) was isolated from soil samples by cyclohexane enrichment in medium. The strain was able to sustain and grow in a wide range of organic solvents. The adaptation of *P. aeruginosa* cell towards solvents was seen at membrane level in transmission electron micrographs. It also secreted a novel protease, which exhibited remarkable solvent stability and retained most of the activity at least up to 10 days in the presence of hydrophobic organic solvents ($\log P \geqslant 2.0$) at 25% (v/v) concentrations. The protease was able to withstand as high as 75% concentration of solvents atleast up to 48 h.

P. aeruginosa strain and its protease, both seem promising for solvent bioremediation, wastewater treatment and carrying out biotransformation in non-aqueous medium.

Keywords: Bioremediation; Biotransformation; *Pseudomonas aeruginosa*; Solvent tolerant; Protease; Transmission electron micrographs

1. Introduction

Proteases find diverse applications in peptide synthesis, protein processing, food, pharmaceutical and detergent industries (Anwar and Saleemuddin, 1998; Gupta et al., 2002). They hydrolyze peptide bonds in aqueous environments and synthesize them in non-aqueous environments. To be used as biocatalysts for peptide synthesis, proteases need to be stable in the presence of organic solvents. However, enzymes in general are inactivated and give low rate of reactions under such conditions (Gupta, 1992). Several physical and chemical methods such as chemical modification, immobilization, entrapment, protein engineering and directed evolution have been employed for the stabilization of enzymes in the presence of organic solvents (Ogino and Ishikawa, 2001; Gupta and Roy, 2004). However, if enzymes are naturally stable and exhibit high activities in the presence of organic solvents, such stabilization of enzymes is not necessary.

In recent years a new class of solvent tolerant microbes having unique ability to sustain under non-aqueous system has drawn considerable attention. Such organisms are attractive for applications in solvent bioremediation and biotransformation in non-aqueous media (Isken and deBont, 1998; Pieper and Reineke, 2000; Sardessai and Bhosle, 2004). Solvent tolerant bacteria have been isolated from ecological niche such as soil or deep sea and identified to belong to genera *Pseudomonas* (Inoue and Horikoshi, 1989; Ramos et al., 1995; Ikura et al., 1997), *Bacillus* (Bustard et al., 2002), *Flavobacterium* (Moriya and Horikoshi, 1993) and *Rhodococcus* (Paje et al., 1997).

Some of these microbes are reported to be rich source of the solvent stable enzymes. Very few reports are available in literature concerning the screening of microorganisms, which produce organic solvent-stable proteases. Protease from solvent tolerant *Pseudomonas* sp. is novel in this regard (Ogino et al., 1999; Geok et al., 2003).

The present work describes the isolation of solvent tolerant microbes from soil by cyclohexane enrichment. One of the isolates, identified to be *Pseudomonas aeruginosa* PseA grew well at high concentrations of solvents. It produced an

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extracellular protease, stable in the presence of various organic solvents. Its behavior and stability in non-aqueous media was investigated.

2. Methods

2.1. Materials

The media components were purchased from Hi Media Laboratories (Mumbai, India). Casein (Hammarsten) was a product of SISCO Research Laboratories (Mumbai, India). All chemicals used were of analytical grade.

2.2. Microorganism

Soil samples were collected from the proximity of a solvent extraction unit in New Delhi, India. A known amount of soil was suspended in sterilized distilled water and 100 µl of suspension was spread on modified King's B medium agar (King et al., 1954) plate. The plates were overlaid with 7 ml of cyclohexane and incubated at 30 °C for 24 h. Microorganisms which formed colonies on the surface of plates covered with organic solvent were selected. Growing colonies were further purified by repeated streaking. The protease producers among purified colonies were detected by plating on skim-milk agar (Ghorbel et al., 2003). The microbes showing clear zones were identified as protease producers. The strain showing maximum zone diameter was selected as potent producer of the proteolytic enzyme and was maintained on nutrient agar slants at 4 °C for further studies. It was designated as strain PseA, and identified to be *Pseudomonas* sp. based on morphological and biochemical tests and the Microbial Type Culture Collection Facility, Institute of Microbial Technology (IMTECH), Chandigarh, India and *P. aeruginosa* based on further FAME analysis by the Microcheck Microbial Analysis Laboratory, Vermont, USA.

2.3. Growth of P. aeruginosa

For bacterial growth, the inoculum was prepared by inoculating a loopful of *P. aeruginosa* cells from slant into 5 ml of modified King's B medium followed by incubation at 30 °C and 140 rpm. One millilitre of this overnight grown culture was used to inoculate 100 ml of modified King's B medium (overlaid with 50 ml cyclohexane). The incubation was carried out at 30 °C with constant shaking at 140 rpm in an orbital shaker. To prevent the evaporation of solvent, flasks were sealed with butyl rubber stoppers. *P. aeruginosa* was also grown in the absence of solvent as a control under similar conditions and same culture medium without solvent. Growth was followed by recording absorbance at 660 nm and by dry cell mass estimation.

2.4. Dry cell mass estimation

For dry cell mass measurement, 3.0 ml culture broth was centrifuged at 11,000*g* and 4 °C for 10 min to pellet down the cell mass. The pellet was washed twice with distilled water and dried under vacuum till constant mass was achieved.

2.5. Organic solvent tolerance of P. aeruginosa

The solvent tolerance of the microorganism was checked both on solidified medium and in liquid medium. In the first set of experiments, the cells were spread on King's B agar medium. The surface was overlaid with 7.0 ml of different organic solvents and the plates were incubated at 30 °C for 24 h. In the second set of experiments, it was cultured in 500 ml Erlenmeyer flasks containing 100 ml of the medium and 50 ml of organic solvents at 30 °C with shaking at 140 rpm. All flasks were plugged with butyl-rubber stoppers to prevent the evaporation of solvent. The cell growth was monitored by measuring the dry cell weight.

2.6. Culture conditions for protease production

Inoculum was prepared by transferring loopful of *P. aeruginosa* stock culture in pre-culture medium ($g l^{-1}$): peptone, 5.0; yeast extract, 5.0; NaCl, 0.5 (pH 7.0). The cultivation was performed at 30 °C with shaking at 140 rpm. 1.25 ml of above inoculum having 17×10^9 cfu was seeded into 50 ml (in 250 ml Erlenmeyer flask) of protease production medium consisted of ($g l^{-1}$): K_2HPO_4, 7.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; casein, 4.0; yeast extract, 6.0; NaCl, 0.5; glycerol, 7.0; $CaCl_2$ (0.6 mM), pH 7.0. Incubation was carried out at 250 rpm in an orbital shaker maintained at 30 °C. The culture was centrifuged at 11,000*g* for 10 min at 4 °C. The supernatant was assayed for protease activity.

2.7. Effect of organic solvents on protease production

To check the effect of organic solvents on protease production from *P. aeruginosa,* the bacterium was grown in 100 ml of protease production media in the presence of different organic solvents (50 ml). The flasks were sealed with butyl rubber stoppers and incubated at 30 °C and 140 rpm for 72 h. The samples were withdrawn from the aqueous phase at different time intervals. These were centrifuged (11,000*g* for 10 min at 4 °C) and the supernatants were assayed for proteolytic activity.

2.8. Protease assay

Protease activity was determined as described by Shimogaki et al. (1991), using casein as the substrate. Enzyme solution (0.5 ml) was added to 3.0 ml of substrate solution (0.6% casein in 0.1 M Tris–HCl buffer, pH 8.0) and the mixture was incubated at 30 °C for 20 min. The reaction was stopped by addition of 3.2 ml of TCA mixture (containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) and kept at room temperature for 30 min followed by filtration through Whatman filter paper No. 1. The absorbance of the filtrate was measured at

280 nm. One unit of protease activity is defined as the amount of enzyme required to produce 1 μg of tyrosine per minute under the conditions described above.

2.9. Transmission electron microscopy (TEM)

P. aeruginosa cells, grown for 24 h in King's B medium in the absence and presence of cyclohexane (33%, v/v) were harvested and quickly washed with 0.1 M phosphate buffer (pH 7.4). These were fixed in modified Karnovsky's fluid and processed as per the procedure of David et al. (1973). Transmission Electron Micrographs were recorded in TEM Philips, CM-10 model (AIIMS, New Delhi facility, India).

2.10. Effect of solvents on protease stability

P. aeruginosa was grown in protease production medium (as described previously) and the resultant culture was centrifuged at 11,000*g* and 4 °C for 10 min. The supernatant was filtered with cellulose acetate membrane filter (pore size 0.2 μm). One ml of organic solvent was added to 3.0 ml of the cell-free supernatant in screw-capped tubes and incubated at 30 °C, 140 rpm for 10 days.

The effect of solvent concentrations on protease stability was examined following the method as described by Geok et al. (2003). The cell-free supernatants with different percentages (0%, 25%, 50%, 75%, v/v) of organic solvents were shaken at 140 rpm, 30 °C for 48 h. The residual proteolytic activities were measured in all the samples. Stability is expressed as the remaining proteolytic activity relative to control without any solvent (100%).

3. Results and discussion

Tolerance to grow in the presence of solvents is often observed among the microbes inhabitating the soil exposed to solvents. Their enzymes function under non-aqueous/low water environment, hence naturally stable towards solvents. In the present work, soil samples from the sites near to the solvent extraction unit were screened for solvent tolerant microbes. Cyclohexane was enriched in the medium right at the beginning of soil inoculation so that only solvent tolerant microbes were able to grow. Protease producers among these were selected on the basis of zone formation on skim milk agar plate. The isolate showing maximum zone diameter was selected for further studies. It was found to be aerobic, gram-negative, motile rod with very simple nutritional requirements that grow best at neutral pH and temperatures in the mesophilic range. Based on biochemical tests by the Microbial Type Culture Collection Facility, Institute of Microbial Technology (IMTECH), Chandigarh, India and FAME analysis by the Microcheck Microbial Analysis Laboratory, Vermont, USA, it was identified as *Pseudomonas aeruginosa* and designated as PseA.

The growth curve of *P. aeruginosa* in the absence and presence of cyclohexane (33%, v/v) is shown in Fig. 1. The growth decreased in the presence of cyclohexane. The dry

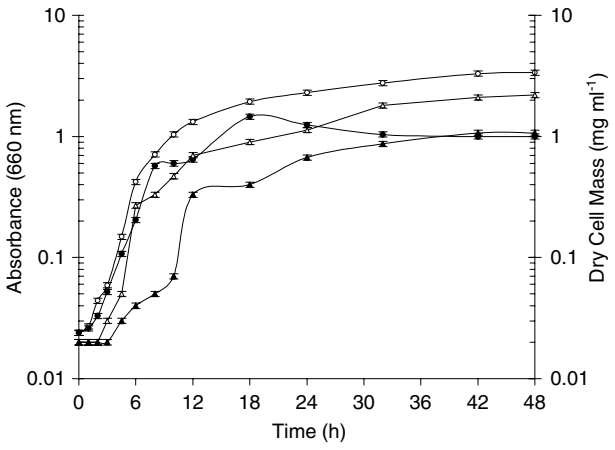

Fig. 1. Growth of *P. aeruginosa* in the absence and presence of cyclohexane. The *Pseudomonas* was grown in culture media in the absence and presence of cyclohexane (33% v/v). The growth (absorbance at 660 nm and dry cell masses, mg ml^{-1}) was recorded in asceptically-withdrawn samples at various time intervals. In absence of cyclohexane: OD_{660nm} (○) dry cell mass (△) and in presence of cyclohexane: OD_{660nm}; (●) dry cell mass (▲). The experiment was carried out in triplicates and the difference in the individual results was less than 5%.

cell mass of the culture in the presence of cyclohexane was about one-half of that in its absence. In the presence of cyclohexane, the specific growth rate, determined during the exponential growth phase showed a decrease from 0.35 h^{-1} (for the cells grown in the absence of cyclohexane) to 0.17 h^{-1}. Since butyl rubber covered flasks were used in both the cases, availability of oxygen may not be responsible for lesser growth in the presence of solvent. It may be caused by the direct effect of cyclohexane on cells. Similar growth behavior was observed by Ogino et al. (1995) in the case of *P. aeruginosa* PST-01 while 23% cyclohexane was incorporated in the media.

Since the isolation was done by cyclohexane enrichment, it was worthwhile to check behavior of the *P. aeruginosa* isolate towards other solvents. Its growth was checked on the medium plates overlaid with solvents of varying log *P* values (logarithm of the partition coefficient of a particular solvent between *n*-octanol and water). The results are summarized in Table 1. Bacterium grew well on the medium plates overlaid with isooctane, *n*-octane, *n*-heptane, cyclohexane, hexane but did not grow in the presence of toluene, chloroform, benzene, isopropanol, 1-butanol and ethanol, thus indicating its tolerance for hydrophobic solvents rather than hydrophilic.

In the second approach, various solvents were added to the medium broth. The growth of *P. aeruginosa* was monitored in the presence of each solvent. Fig. 2 shows the effect of different solvents on dry cell mass. The results are in agreement with those obtained with solvent overlaid over medium agar plates. Here again solvent with higher log *P* (i.e. hydrophobic) were tolerated better.

Transmission electron micrographs of the *Pseudomonas* cells growing in the absence and presence of cyclohexane are shown in Fig. 3a and b. Convoluted and disorganized

Table 1
Effect of organic solvents on growth of *P. aeruginosa*

Solvent	log *P*	Growth
None	–	+[a]
Isooctane	4.5	+
n-Octane	4.5	+
n-Heptane	4.0	+
n-Hexane	3.5	+
Cyclohexane	3.2	+
Toluene	2.5	–[b]
Benzene	2.0	–
Chloroform	2.0	–
1-Butanol	0.8	–
2-Propanol	0.28	–
Ethanol	−0.24	–

The isolate was grown on King's B medium agar plates overlaid with 7.0 ml of organic solvents of varying log *P* values as described in the text.
[a] Indicates bacterial growth on plates after 24 h of incubation.
[b] Indicates no bacterial growth on plates after 24 h of incubation.

membrane structure leading to electron transparent regions and accumulation of solvent is clearly seen in the presence of solvents. Similar changes have been reported by Cruden et al. (1992) and Gupta et al. (2005) for *Pseudomonas* sp. cells grown in *p*-xylene and *Enterobacter* sp. grown in the presence of cyclohexane respectively. Solvents are reported to damage the integrity of cell membrane structure. This causes loss of permeability regulations. In extreme cases leakage of cell RNA, phospholipid and protein also take place (Sikkema et al., 1995). Solvent tolerant cells adapt by making changes in fatty acid composition and protein/lipid ratio in cell membrane to restore the fluidity (Isken and deBont, 1998). Structural changes seen in TEM of *Pseudomonas* cells confirmed the effect of solvent at membrane level.

Thus it was established that the isolated *P. aeruginosa* PseA strain was solvent tolerant and able to grow well in the presence of range of hydrophobic solvents. The adaptation was at membrane level.

As the strain was protease producer, the effect of the incorporation of various solvents on proteolytic enzyme secretion by *P. aeruginosa* PseA was studied (Fig. 4). Protease production decreased in the presence of solvents as compared to control (without solvent). The highest protease activity (1142 U/ml) was observed with isooctane in culture medium followed by *n*-decane and *n*-dodecane, while very little amount of protease was produced in the presence of heptane, hexane, cyclohexane and xylene.

Since interest was, in obtaining a solvent stable protease for applications in non-aqueous systems, the protease secreted by *P. aeruginosa* strain was incubated with 25% of various organic solvents at 30 °C with constant shaking. Table 2 shows the residual protease activities after 10 days of incubation. This protease showed remarkable stability in the presence of solvents. In the presence of solvents having log *P* values above 2.0 (except hexane), the protease activity was comparable to that without solvent up to 10 days. This level of stability towards hydrophobic solvents is unique.

The protease was less stable (75–80%) in the presence of hydrophilic solvents viz., *N*,*N*-dimethylformamide (log *P* = −1.0) and 1-butanol (log *P* = 0.8) as compared to hydrophobic solvents viz., isooctane, *n*-decane, *n*-dodecane etc.

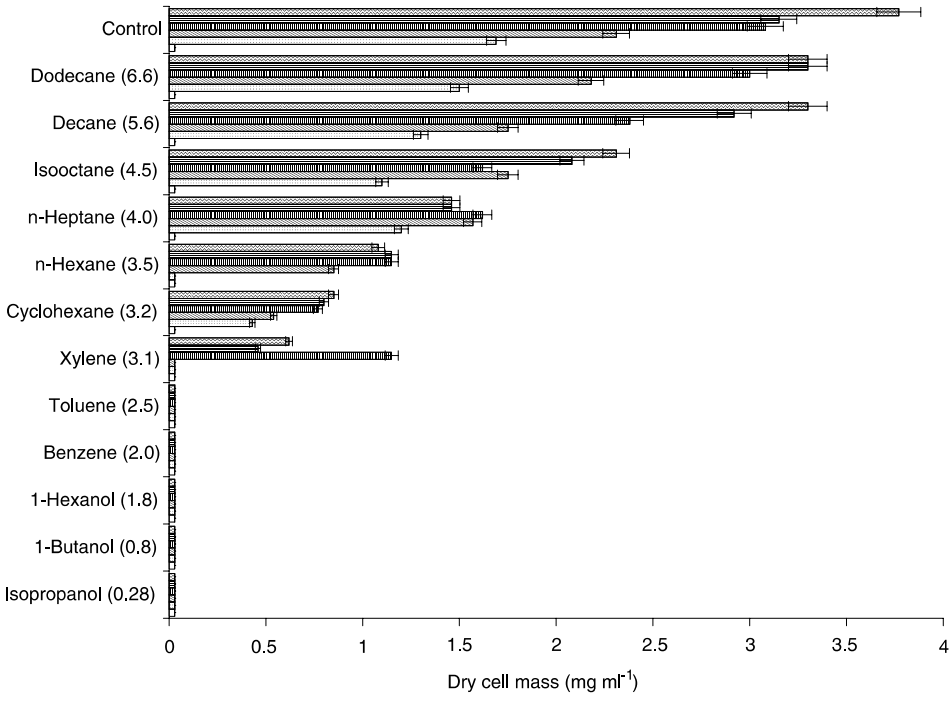

Fig. 2. Growth of *P. aeruginosa* in various organic solvents. The isolate was grown in 100 ml of liquid media in the presence of different organic solvents (50 ml) of varying log *P* values as discussed in the text. Dry cell masses (mg ml^{-1}) were recorded after 0 h (□) 12 h (⚄), 24 h (▨), 36 h (▥), 48 h (▤) and 60 h (🞕) of growth. Each experiment was done in duplicate and the difference between two sets of experiments was less than 4%.

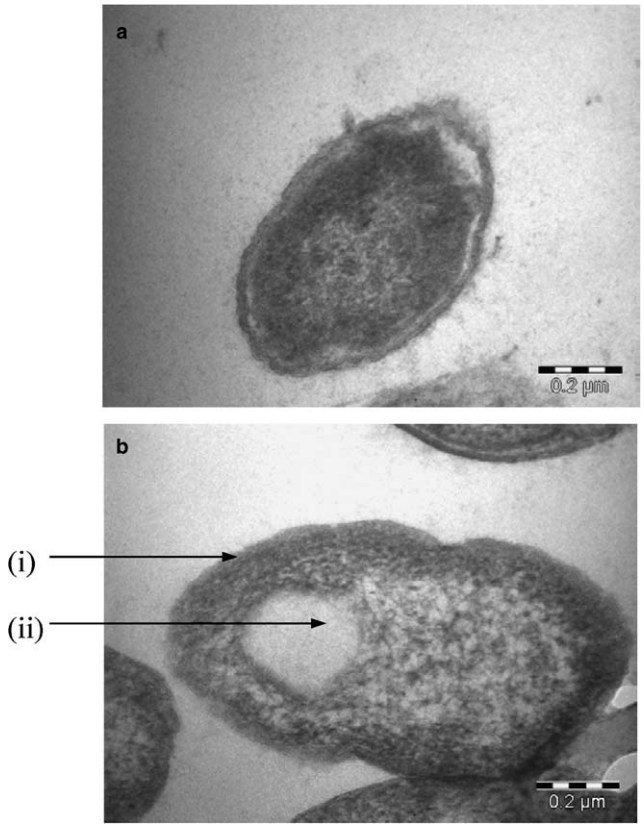

Fig. 3. (a) Transmission electron micrographs (bar 0.2 μm) of the *Pseudomonas* cells grown in the absence of cyclohexane. (b) Transmission electron micrographs (bar 0.2 μm) of the *Pseudomonas* cells grown in the presence of cyclohexane (33%, v/v). (i) Convoluted and disorganized cell membrane. (ii) Accumulation of solvent.

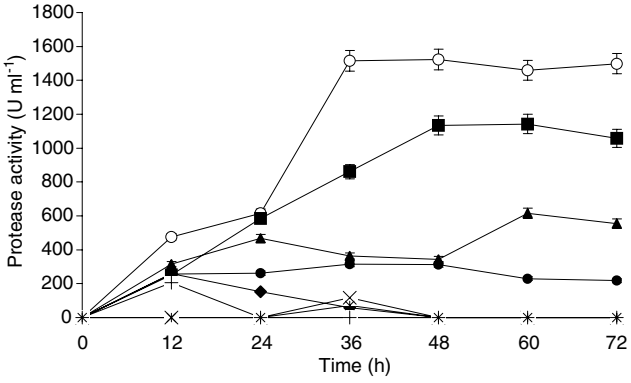

Fig. 4. Effect of organic solvents on protease production from *P. aeruginosa*. The bacterium was grown in the protease production media in the absence and presence of different solvents (33%, v/v) under the conditions described in text. The samples were withdrawn from the aqueous phase at different time intervals and supernatants were assayed for protease activity. Protease activity in the absence of solvent (control) (○), in the presence of *n*-dodecane (●), *n*-decane (▲), isooctane (■), heptane (◆), hexane (×), cyclohexane (+), xylene (∗). The experiment was done in duplicates. Error bars show the percent error.

wherein more than 90% protease activity was retained. Ethanol ($\log P = -0.24$) was exception among hydrophilic solvents which caused no loss in activity. Considering ethanol and hexane as exception in case of hydrophilic and hydrophobic solvents respectively, no clear correlation between stability of protease and solvent polarity ($\log P$ value) could be established. Almost similar stability trends for proteases in the presence of various organic solvents have been reported by others (Ogino et al., 1995; Gupta et al., 2006).

In order to see whether stability was retained if higher concentrations of solvents were used, the effect of different percentages of benzene, *n*-decane and *n*-dodecane on *P. aeruginosa* PseA protease was studied (Fig. 5). It was found to be stable in the presence of these solvents up to concentration as high as 75% atleast for 48 h except a marginal decrease in the case of *n*-dodecane.

Table 2
Effect of organic solvents on crude protease stability

Organic solvent	log *P*	Stability (%)
None		100
N,*N*-Dimethylformamide	−1.0	77
Ethanol	−0.24	112
1-Butanol	0.8	75
Benzene	2.0	98
Toluene	2.5	92
Xylene	3.1	97
Cyclohexane	3.2	94
Hexane	3.5	75
Heptane	4.0	90
Isooctane	4.5	96
n-Decane	5.6	102
n-Dodecane	6.6	104

One milliliter of organic solvent was added to 3 ml of the cell free supernatant and incubated at 30 °C, 140 rpm for 10 days. The remaining proteolytic activity was measured. The protease activity of the non-solvent containing control has been taken as 100%. Each experiment was carried out three times and the difference in the individual results in each set of experiments was less than 5%.

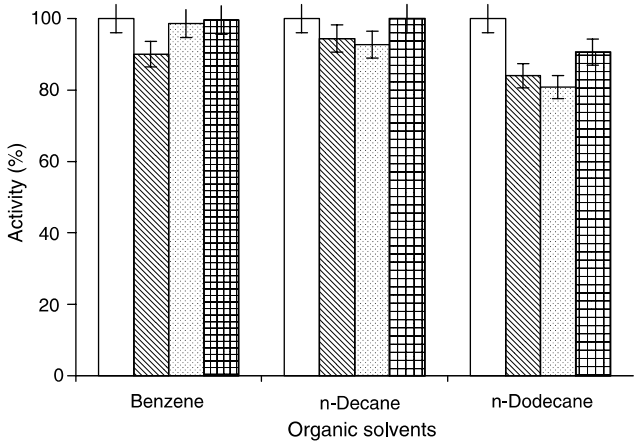

Fig. 5. Effect of organic solvent concentrations on crude protease stability. The cell-free supernatant was incubated at 30 °C with constant shaking in the absence (□) or presence of 25% (v/v) (▨), 50% (v/v) (⊡) and 75% (v/v) (▦) organic solvents for 48 h. The remaining proteolytic activity was determined in aqueous phase under standard assay conditions. The experiment was carried out in triplicate and the difference between individual set of readings was less than 4%.

Although *P. aeruginosa* protease was stable in the presence of hydrophobic solvents, it showed lesser stability in hydrophilic solvents. Solvent polarity is one of the factors reported for determining the stability of enzymes (Laane et al., 1987; Geok et al., 2003).

Nonetheless the extracellular protease from *P. aeruginosa* isolate (PseA) exhibits significant stability with range of hydrophobic organic solvents even at high concentrations. This property makes them attractive for synthesis in non-aqueous media especially in two-phase systems. The *P. aeruginosa* strain itself is able to grow effectively in the presence of various solvents, a trait which can be exploited for bioremediation of solvent rich-wastes.

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