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ORIGINAL PAPER

Organic solvent tolerance of an alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1

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Abstract A salt-tolerant alkaliphilic actinomycete, Mit-1 was isolated from Mithapur, coastal region of Gujarat, India. The strain was identified as *Streptomyces clavuligerus* and based on 16S rRNA gene sequence (EU146061) homology; it was related to *Streptomyces* sp. (AY641538.1). The organism could grow with up to 15% salt and pH 11, optimally at 5% and pH 9. It was able to tolerate and secrete alkaline protease in the presence of a number of organic solvents including xylene, ethanol, acetone, butanol, benzene and chloroform. Besides, it could also utilize these solvents as the sole source of carbon with significant enzyme production. However, the organism produced spongy cell mass with all solvents and an orange brown soluble pigment was evident with benzene and xylene. Further, the enzyme secretion increased by 50-fold in the presence of butanol. With acetone and ethanol; the enzyme was highly active at 60–80°C and displayed optimum activity at 70°C. The protease was significantly stable and catalyzed the reaction in the presence of xylene, acetone and butanol. However, ethanol and benzene affected the catalysis of the enzyme adversely. Crude enzyme preparation was more stable at 37°C in solvents as compared to partially purified and purified enzymes. The study holds significance as only few salt-tolerant alkaliphilic actinomycetes are explored and information on their enzymatic potential is still scares. To the best of our knowledge this is the first report on organic solvent tolerant protease from salt-tolerant alkaliphilic actinomycetes.

Keywords Alkaline protease · *Streptomyces* sp. · *Streptomyces clavuligerus* organic solvent tolerance · Non-aqueous catalysis

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Introduction

Microbial life can be found in the most diverse conditions, including extremes of temperature, pressure, salinity, pH, nutrient concentrations, water availability and presence of high levels of radiations, harmful heavy metals and toxic compounds such as organic solvents and hydrocarbons. Organic solvents can be extremely toxic to cells by virtue of their ability to partition into and disrupt the normal functioning of biological membranes. They accumulate in the bacterial cell membrane changing structural and functional integrity followed by cell lysis [9, 16, 27]. Organic solvent tolerant bacteria are a relatively novel group of extremophilic microorganisms with novel tolerance mechanisms, which enable them to overcome the toxic and destructive effects of organic solvents in solvent-saturated environments [8, 10, 24, 30, 33].

Solvent tolerance is a strain-specific property and the toxicity of a given solvent correlates with the logarithm of its partition coefficient in n-octanol and water (log P_{ow}). Organic solvents with a log P_{ow} between 1.5 and 4 are extremely toxic for microorganisms and other living cells because they partition preferentially in the cytoplasmic membrane, disorganizing its structure and impairing vital functions. Every micro-organism has a limiting log P_{ow} value below which it is unable to grow. In addition, the

solvent toxicity also depends on the intrinsic tolerance of the organisms; for instance certain strains of *Escherichia coli* are resistant to cyclohexane (log P_{ow} 3.44), while others are sensitive [23].

Solvent tolerant organisms are ecologically significant and hold immense potential in non-aqueous bio-catalysis [3, 14] involving biphasic organic aqueous fermentation systems, effluent treatment and bioremediation in hydrocarbon-saturated environments. Solvent-tolerant proteases, lipases, and cyclodextrin glucanotransferase were isolated from *Pseudomonas aeruginosa* PST-01, LST-03 and *Paenibacillus illinoisensis*, respectively [4, 5, 18, 19]. However, there are only few reports on the organic solvent-tolerant enzymes from halophilic and alkaliphilic organisms. Fukushima et al. [6] reported an organic solvent-tolerant amylase from a newly isolated *Haloarcula* sp. strain S-1.

While majority of the studies related to organic solvent tolerance have focused on rod shaped bacteria [2, 3], the capacity of actinomycetes in this context is yet to be explored and understood [33]. Among the actinomycetes, salt-tolerant alkaliphilic groups are rarely explored for their enzymatic potential and organic solvent tolerance [12, 13, 28, 29, 32]. Therefore, studies on the solvent tolerance of haloalkaliphilic actinomycetes may appear as an important tool to get insight into the molecular adaptation of such organisms under non-aqueous conditions. These organisms would possess enzymes that are not only active and stable at high temperatures in the presence of salt, but may also display solvent tolerance. In the light of above realization, the present report highlights on the organic solvent tolerance and non-aqueous catalysis of alkaline protease from a salt tolerant alkaliphilic actinomycete.

Materials and methods

The organism

A salt-tolerant and alkaliphilic actinomycete, Mit-1, was isolated from saline soil of Mithapur, Gujarat, India. The saline soil (10 g) was incubated at 45°C with $CaCl_2$ (1 g) for one week. The treated soil was enriched at 30°C under shake flask conditions in Actinomyces broth (Hi Media Ltd.) containing NaCl (5%, w/v). The pH of the medium was earlier adjusted to 9 by adding separately sterilized Na_2CO_3 (20%, w/v). The enriched culture was streaked on the Actinomyces agar (5% w/v NaCl, pH 9). After the incubation for 6 days at 30°C, a typical chalky white colony was picked up and re-streaked to ensure the purity of the colony. The culture was maintained at 4°C on Actinomyces agar slants (5% w/v NaCl and pH 9).

Organic solvent tolerance of Mit-1

Various organic solvents; xylene (log P_{ow}, 3), ethanol (log P_{ow} 2.92), benzene (log P_{ow}, 2.1), chloroform (log P_{ow}, 1.9), acetone (log P_{ow} −0.24) and butanol (log P_{ow}, 0.8) were added at 1%, v/v, in gelatin broth (g/l): gelatin, 10; peptone, 5; yeast extract, 5; NaCl, 50 and pH 9. An actively growing culture of Mit-1 was inoculated (10%) in the medium and the flasks were incubated at 30°C under shaking conditions for 8 days followed by the measurement of growth and protease activity. Because the organisms formed spongy bead like culture morphology, the growth was measured in terms of dry weight. The crude enzyme was harvested by filtration on cellulose filter and the protease activity was assayed. The growth and enzyme activity in gelatin broth devoid of any solvent was considered as control.

Enzyme assay

The enzyme was assayed at 70°C by the Anson-Hagihara method [7] using casein as a substrate. One unit of alkaline protease activity (U) was described as the enzyme liberating 1 μg of tyrosine per min under the assay conditions. The estimations were based on a tyrosine calibration curve.

Protease production with organic solvents as the sole source of carbon

Minimal medium (5% NaCl, pH 9) containing leucine as the sole source of nitrogen and devoid of glucose was used to assess the ability of the strain to grow and produce protease in the presence of organic solvent. The solvents viz. ethanol, benzene, xylene, butanol and acetone were included into the minimal medium at 0–0.5%, v/v, final concentrations. The culture media after inoculation were incubated at 30°C under shake flask conditions for 8 days followed by the measurement of growth and protease production.

Purification of Mit-1 alkaline protease

The partial purification of Mit-1 protease was carried out by precipitation of the crude enzyme with ammonium sulphate between 30 and 60% saturation. The partially purified enzyme was desalted by dialysis against 20 mM NaOH-Borax buffer (pH 10) at 4°C and was further purified by ion exchange chromatography on DEAE cellulose [28].

Catalysis and stability of alkaline protease in the presence of organic solvents

Catalysis of the partially purified and purified Mit-1 alkaline protease was performed in the presence xylene, ethanol,

butanol, benzene and acetone. The reaction mixture was supplemented with varying concentrations of solvents (0.25–2%, v/v). The activity was calculated after the enzyme assay and related to control. The stability of the enzyme was investigated by incubating it at 37°C with solvents (0.25–2%, v/v); butanol, xylene, acetone, ethanol and benzene. Enzyme aliquots withdrawn at 20, 40, 60, 120 and 1,440 min were assayed for the residual activity.

Results

The organism Mit-1, isolated from coastal region of Gujarat (India), was gram-positive having filamentous structure. It was identified as *Streptomyces clavuligerus* based on the morphological, physiological and biochemical characteristics, including cell wall constituents. The culture was assigned MTCC 7037 as accession/strain number by Microbial Type Culture Collection and *Gene Bank,* IMTECH, Chandigarh (India). However, Mit-1 (EU146061) was phylogenetically related to *Streptomyces* sp. (AY641538.1) based on 16S rRNA gene sequence homology.

Organic solvent tolerance of Mit-1

Among various solvents (1% w/v), the growth of Mit-1 was optimum with acetone, ethanol and benzene followed by xylene and butanol (Fig. 1). However, the organism could not grow at all in the presence of chloroform. Mit-1 produced spongy, spiky bead type of growth in the presence of solvents with large variation in their size. Beads were larger with butanol followed by benzene and xylene. With acetone and ethanol; however, the normal filamentous cotton like growth was evident (Fig. 2). The solvents also induced the production of pigments in the broth which otherwise was absent. Intense orange brown and soluble pigment was evident with benzene and slightly lighter in color intensity

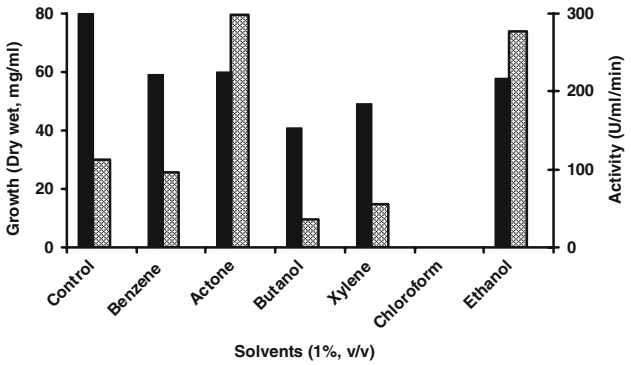

Fig. 1 Effect of various organic solvents on growth (*filled rectangle*) and protease production (*shaded rectangle*)

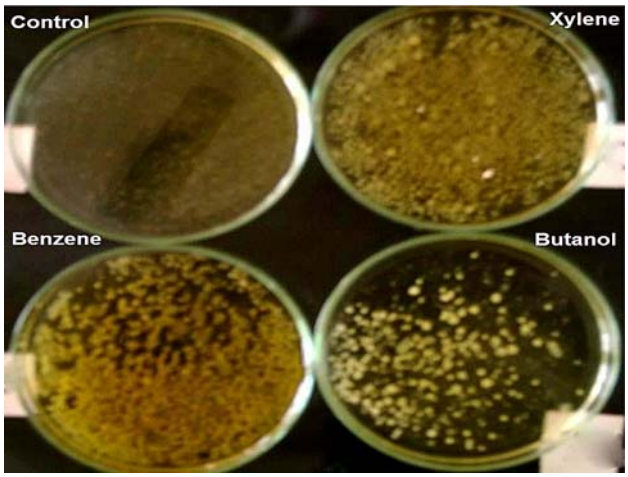

Fig. 2 Growth pattern of Mit-1 in the presence of organic solvents. The organism was grown with 1%, v/v of xylene, benzene and butanol in gelatin broth having 5% w/v NaCl and pH 9. The control represented the growth in the absence of solvent

with xylene, while the pigment was absent with ethanol and butanol (data not shown).

Solvents also affected protease production significantly when applied at the concentration of 1% w/v. Acetone and ethanol favored the protease production with 300 and 277 U/ml activities, respectively. The enzyme activity was, thus, nearly 3 times greater than that in normal (113 U/ml) gelatin broth. Although the growth was optimum with xylene and benzene, protease production significantly retarded (Fig. 1).

Protease production with organic solvents as the sole source of carbon

Mit-1 grew with almost all the solvents when employed as the sole source of carbon, in the presence of leucine as nitrogen source in minimal medium. Interestingly, Mit-1 gave regular cottony growth in the presence of solvent in minimal medium compared to spongy, spiky beads as observed in complex medium containing solvents. Xylene, acetone and butanol supported protease production to a greater extent, although the growth was quite limited in comparison to complex media. Poor enzyme production was evident with alcohol and benzene.

Xylene, in the range of 0.1–0.3%, favored the growth, with slight decrease at 0.5%, v/v. Protease production was sharply enhanced at 0.1% v/v xylene followed by sharp decline at higher concentrations (Fig. 3a). The enzyme production was substantially higher (250 U/ml) at 0.3% of acetone, while the growth was limited.

As reflected from Fig. 3b, acetone supported the growth at 0.1%, v/v. The enzyme production was substantially higher (250 U/ml) at 0.3% of the solvent, while the growth

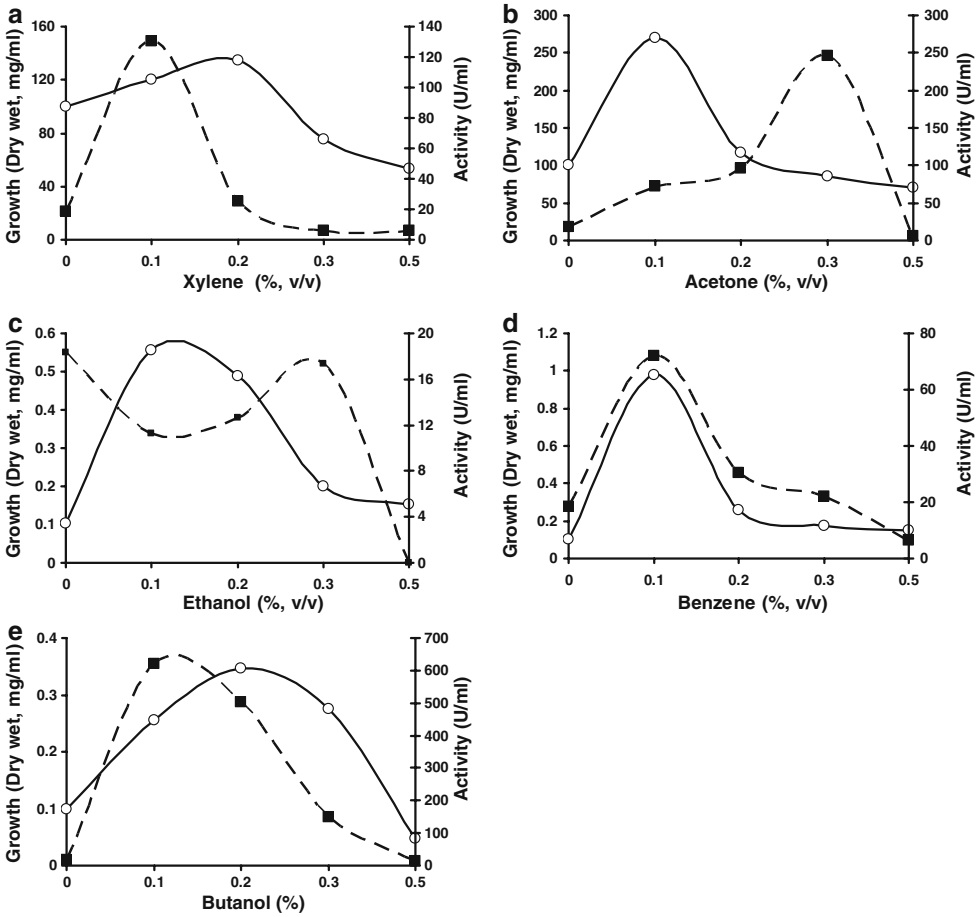

Fig. 3 Effect of xylene (**a**), acetone (**b**), ethanol (**c**), benzene (**d**) and butanol (**e**) on growth (*open circle*) and protease production (*filled square*) when applied as the sole source of carbon

was limited. On the other hand, only marginal protease production (16 U/ml) was detected with ethanol (Fig. 3c) although the growth was relatively significant. While growth was optimum with 0.2% v/v ethanol, the protease production was comparable in the range of 0.1–0.3%.

Although the growth was the highest with benzene among all solvents, protease production was only moderate. Similarly, substantial growth was evident with butanol in the range of 0–0.3%, v/v. It also emerged as the best carbon source for protease production among the organic solvents employed in the study (Fig. 3d). In fact, the enzyme production (620 U/ml) recorded with 0.1% butanol was higher than those obtained in any complex medium (Fig. 3e). Thus, benzene and butanol proved as the best carbon sources for growth and protease production, respectively (Table 1).

Mit-1 alkaline protease was earlier purified by the combination of ammonium sulphate fractionation and ion exchange chromatography with DEAE-Cellulose leading to 141-fold purification with the specific activity of 6,800 U/mg of protein. The apparent molecular mass of purified protease was estimated as 49–50 kDa and displayed optimum activity at pH 10 and 70°C temperature [28].

Table 1 Comparison of specific enzyme production with complex medium and with organic solvent as the sole source of carbon (0.1%)

Medium	Specific enzyme production (enzyme activity/growth)	Comparative fold
Complex medium (gelatin broth)	49	1.0
MM + butanol	2,400	48.9
MM + xylene	1,083	22.1
MM + acetone	268	5.5
MM + benzene	73.8	1.5
MM + ethanol	20.21	0.412

MM minimal medium

Catalysis of alkaline protease in the presence of organic solvents

The crude, partially purified and purified alkaline protease preparations were studied with respect to their activity in the presence of various organic solvents. Crude enzyme was active up to 1% v/v xylene with slight decline at 2%. On the other hand, the activity slightly increased in the

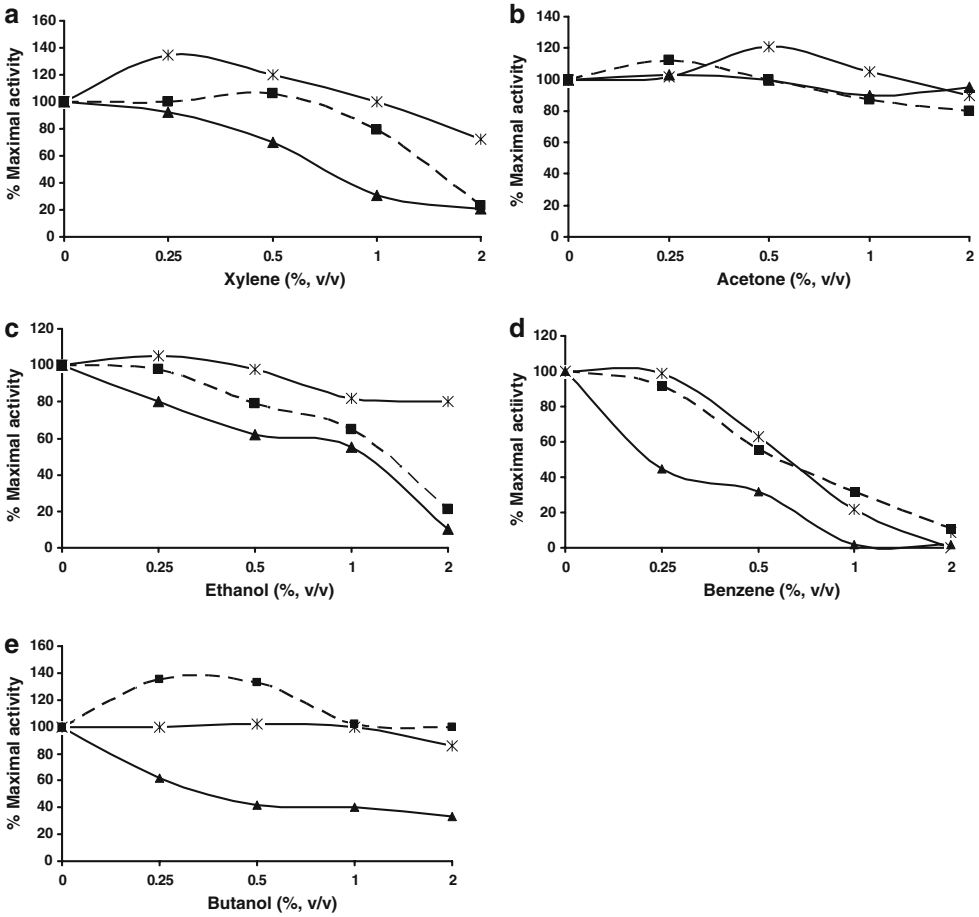

Fig. 4 Effect of xylene (**a**), acetone (**b**), ethanol (**c**), benzene (**d**) and butanol (**e**) on the activity of crude (*asterisks*) and partially purified (*filled square*) and purified (*filled triangle*) alkaline protease

presence of 0.25% xylene. Compared to crude, the partially purified and homogenous preparations were much sensitive to xylene. However, the sensitivity was more pronounced at higher concentrations; for instance at 2% v/v xylene, 80% of the original activity of the purified enzyme was lost (Fig. 4a).

The enzyme catalysis of partially purified protease was not significantly affected by acetone. Crude enzyme was highly active with acetone in the range of 0.25–2% with slight increase in activity at 0.5%, v/v. Similar trend was also evident with pure enzyme (Fig. 4b).

There was varied effect of butanol on the catalysis of different enzyme preparations. Crude and partially purified enzymes were highly active in the presence of butanol up to 2%, v/v. Activity of the partially purified protease was substantially increased by butanol at 0.25 and 0.5%. In comparison, the pure enzyme lost almost more than 50% of its original activity at higher concentrations (Fig. 4c).

Benzene exerted drastic effect on the catalysis of all enzyme preparations. As the concentration increased, protease activity gradually decreased and the enzyme lost 50% of the activity at 0.5% benzene. A complete loss of enzyme activity was evident with 2% v/v, benzene (Fig. 4d). Therefore, it appears that in comparison to other solvents, enzyme catalysis was more sensitive to benzene.

Ethanol, a well known organic solvent was studied for its effect on protease catalysis and stability. Interestingly, in its presence, the catalysis of crude protease was significantly enhanced followed by a marginal decrease at 2%, v/v. On the contrary, the activity of partially purified and purified protease gradually decreased with increase in ethanol concentrations; at 2% ethanol, about 80% activity was lost (Fig. 4e). The results indicated that the presence of ethanol was favorable for the catalysis of crude enzyme, but on purification, the enzyme got sensitive.

Effect of organic solvents on the stability of Mit-1 protease

The stability of partially purified and homogenous enzyme was determined in the presence of solvents in the range of 0–2%, v/v (Table 2). As the concentration of the solvent increased, the stability of enzymes decreased significantly after the incubation of 120 min. The partially purified and purified protease displayed significant stability with acetone and xylene followed by butanol and ethanol. However, the stability of both preparations was drastically affected

Table 2 Stability of Mit-1 alkaline protease with different organic solvents

Solvent	% Residual activity after 120 min (a/b)				
	0%	0.5%	1%	1.5%	2%
Xylene	100	82/75	66/75	69/70	65/71
Acetone	100	99/100	89/77	66/52	79/39
Butanol	100	93/90	81/64	87/39	55/05
Ethanol	100	89/78	62/59	53/40	24/16
Benzene	100	86/66	50/60	22/14	17/07

Activity at 0 min was considered as 100% to determine the residual activity

[a] Partially purified Mit-1 protease

[b] Purified Mit-1 protease

with benzene resulting in a loss of about 85% of the original activity. The partially purified enzyme was relatively more stable with all the solvents (Table 2).

Discussion

Most bacteria and their enzymes are destroyed or inactivated in the presence of organic solvents. Organic solvent tolerant bacteria are a relatively novel group of extremophilic microorganisms that combat these destructive effects and thrive in the presence of high concentrations of organic solvents due to various adaptive strategies. The degradation or transformation of a range of organic solvents as pollutants and the production of alternative energy are some of the recent and important fields of extremophiles. The biodegradation (transformation or mineralization) of a wide range of hydrocarbons, including aliphatic, aromatic, halogenated and nitrated compounds, has been shown to occur by some halophilic and haloalkaliphilic microorganisms [1, 14]. Therefore, studies on organic solvent tolerance in the presence of high salt concentrations at alkaline pH appear quite attractive for the bioremediation of oil- polluted salt marshes and industrial wastewaters, contaminated with aromatic or chlorinated hydrocarbons [30].

While extensive work has been reported on the organic solvent tolerance mechanisms in Gram-negative bacteria; particularly *E. coli* and *P. aeruginosa*, only limited information are available on this aspect in Gram-positive bacteria including actinomycetes. Mit-1, a salt-tolerant and alkaliphilic actinomycete used in the present study, exhibited large variation in the patterns of organic solvent tolerance. The isolate displayed growth in the presence of butanol, acetone and ethanol (included in the complex medium) which was quite comparable to that in normal medium. Interestingly, the organism formed swollen and spongy bead like mass, a pattern quite uncommon in actinomycetes. The cells might have aggregated to protect the population against stress conditions, resembling with a phenomenon of quoram sensing, where bacteria co-ordinate their behavior in response to stressful conditions. Nikolaev et al. [16] and Vakhitov and Petrov [31] recently described the reversible adhesion of cells in submerged bacterial cultures under stress conditions and the processes of cell reactivation in the post-stress period in *E. coli*, *Vibrio* and few other organisms. Similarly, Khmel [10] reported that this communication was achieved by the production of small molecules (termed as autoinducers), which, at sufficient concentrations, trigger a variety of cellular responses.

Mit-1 failed to grow in the presence of chloroform (log P_{ow} 2). A halotolerant *Streptomyces* sp, isolated from an oil field in Russia was earlier reported to degrade crude petroleum [15]. Similarly, Ogino et al. [20] had reported on the organic solvent tolerance in *Pseudomonas aeruginosa* PST-01. It did not grow in the presence of organic solvent with log P_{ow} values equal or less than 2.5. However, the tolerance of *Pseudomonas aeruginosa* PST-01 was a little less than that of earlier described strain *Pseudomonas aeruginosa* LST-03 [21].

Similar to growth, protease production by Mit-1 was also affected in the presence of solvents; particularly benzene, xylene and butanol. Enzyme production was greatly enhanced by acetone and ethanol with delayed production. Ogino and coworkers [20] reported the delayed production of proteolytic enzymes in the presence of cyclohexane in *Peudomonas aeruginosa* PST-01, where delayed enzyme production was due to direct effect of cyclohexane on the bacterium.

Mit-1 was highly versatile for its nutritional requirements, since it utilized the solvents as sole carbon source. *Stenotrophomonas maltophilia* T3-c, a halophilic bacterium, isolated from a biofilter for the removal of benzene, toluene, ethyl benzene, and xylene (BTEX), could grow in a mineral salt medium (MEM) containing toluene, benzene or ethyl benzene as a sole source of carbon [11]. Recently, Segura and coworkers [26] reported on the marine strains of bacteria that were able to grow with toluene, as the sole source of carbon.

The study of stability and activity of Mit-1 protease in the presence of organic solvents reflected some interesting features. The crude enzyme preparation, as compared to partially purified and homogenous enzyme, was more stable and active in almost all the solvents used. It appears that certain other proteins/peptides and organic matters present in the crude preparation might be protecting the enzyme from the adverse effect of the solvent. The enzyme was highly active in the presence of butanol (log P_{ow} 0.8), where as greater stability was evident with ethanol (log P_{ow} −0.24) in comparison to other solvents. It indicated that solvents with log P_{ow} less than 1 provided better stability

and catalysis. Fukushima et al. [6] studied the organic solvent tolerance of halophilic protease and amylase secreted by *Halobacter mediterranei.* The enzymes displayed greater stability in the presence of chloroform and other organic solvents. More recently, Rahman et al. [22] demonstrated a protease from *Bacillus pumilus* 115b, which was stable in 25% (v/v) benzene and toluene and activated 1.7 and 2.5- fold by *n*-dodecane and *n*-tetradecane, respectively. Similarly, the protease from organic solvent –tolerant *Psuedomonas aeruginosa* PST-01 catalyzed peptide synthesis with higher yields and higher reactions rates in the presence of organic solvents such as dimethyl sulfoxide, *N*, *N*-dimethyl formamide and methanol than in the absence of organic solvents [17]. PST-01 protease displayed enhanced conformational stabilities in the presence of methanol. Furthermore, it was suggested that the organic solvent stability of enzymes closely related to the secondary structure, as monitored by the conformational transitions of polyamino acids in the presence and absence of methanol. However, the enzyme stability was only marginally affected by the solvents having log P_{ow} values less than 3.2. In comparison, Mit-1 protease was highly stable with the solvents having the log P_{ow} less than even 1.

To emphasize again, the extracellular halophilic enzymes with solvent tolerance can be used in several processes where high salt concentrations and hydrophobic organic solvents are present. The Mit-1 protease may also prove valuable for peptide synthesis under non-aqueous conditions, which otherwise may be thermodynamically unfavorable in water. This property could be exploited to carry out bioremediation and biocatalysis in organic phase [25]. It is now known that enzymes display striking novel properties in the presence of organic solvents. The role of solvent-stable enzymes in nonaqueous biocatalysis needs to be explored and could result in novel applications. Further, the study as a whole appears quite interesting as haloalkaliphilic organisms, in general and actinomycetes in particular are rarely explored for their tolerance under organic solvents.

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