Research Article

International Journal of Bioassays
ISSN: 2278-778X
www.ijbio.com

ORGANIC SOLVENT TOLERANT ACTINOMYCETE WHICH SECRETES ORGANIC SOLVENT STABLE ENZYMES

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Received for publication: May 11, 2013; **Accepted:** June 24, 2013

Abstract: A solvent tolerant actinomycete was isolated from Balaghat ranges of Maharashtra, India. The actinomycete was identified as *Streptomyces* and based on 16S rRNA phylogenesis it was identified as *Streptomyces hygroscopicus*. The organism could grow with up to pH 9.0 at 35°C and optimally at pH 7.0 at 45°C within 24 h. The organism could utilize most sugars and reduced nitrates. It shows caseinase, amylase, catalase, lecithinase, cellulose and lipase activities. It was able to tolerate and secrete the enzymes in the presence of a number of organic solvents including methanol, acetone, butanol, benzene and toluene. However, the organism produced a granular cell mass with all solvents and a brown soluble melanin pigment which was evident in the absence of solvents disappeared in the presence of solvents. The study holds significance as only few solvent tolerant actinomycetes from soil are explored and information on their enzymatic potential is still scarce. To the best of our knowledge this is the first report on organic solvent tolerant *Streptomyces hygroscopicus* from soil.

Keyword: Soil, *Streptomyces*, Enzymes, Solvent Tolerance, Organic Solvent.

INTRODUCTION

In recent years a new class of solvent tolerant microbes having a unique ability to sustain under non aqueous system has drawn considerable attention. Such organisms are attractive for applications in bioremediation and biotransformation in non-aqueous media [1]. Several bacteria have been screened but there are relatively very few reports on isolation of solvent tolerant actinomycetes. There are very few reports on screening of solvent tolerant proteases, lipases and tyrosinases and further the enzymes from actinomycetes. Actinomycetes are considered as major source for antibiotics and hence it seems very little attention has been directed towards enzyme production. There are a few reports on isolation of organic solvent tolerant actinomycetes secreting solvent tolerant proteases and lipases [2,3,4]. Further studies need to be carried out to check the presence of novel isolates, producing extracellular enzymes, stable in solvent atmosphere. The study also holds significance as only few salt tolerant alkaliphilic actinomycetes are explored and information on their enzymatic potential is still scarce. Actinomycetes usually are slow growers. Hence it would be interesting to find and collect the fast growers and check their solvent tolerance. Earlier reports though very few, the sites of isolation have been the halophilic / alkaliphilic marine regions [2,3]. Other ecological niches need to be explored. Here we report the isolation from the deep soil sites.

Uses of organic solvents provide advantages to improve the solubility of substrates and ease of product recovery in organic phase. It is known that most of enzymes easily lose their activities in organic solvent[7]. When enzymes are naturally stable and active in hostile environments, they would be excellent biocatalyst for application [5]. The isolated actinobacteria could grow in a medium containing organic solvents and secrete several enzymes [2,3,4]. Such solvent tolerant enzymes which show enhanced activity in the presence of solvents can be used in biodegradation in the coastal environments thus enhancing eco restoration. It was observed that lower concentrations were more favorable for retaining and enhancing the enzyme activities.

Organic solvents or organic-aqueous two phase media are favorable for some reactions, especially the prospective fields as pharmaceutical industry and biodiesel production. Besides the use of organic solvents instead of water promises many advantages: (i) the relative high solubility of hydrophobic substrates, (ii) the relative ease of recovery of products in organic phase, (iii) the possibility of reducing the degree of undesirable substrate and/or product inhibition[4, 5]. Nevertheless, as we know that enzymes are generally very labile catalysts and easy to lose their activities in organic solvent, several techniques such as medium engineering, substrate engineering, and protein engineering have been employed so far to improve the stability of

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enzymes[10,11]. However, if enzymes were naturally stable and active in hostile environments, they would be excellent biocatalyst for application. Thus, the search for natural enzyme that shows high stability and organic solvent tolerance is more straightforward. These days, the ability of an enzyme to retain its activity in the presence of organic solvents is an attractive property, as many reaction media for enzymatic reactions involve the use of organic solvents. Organic solvents are basically known to be toxic to most bacteria as it compromises the structural and functional integrity of the cell [12]. Organic solvent tolerant bacteria exhibit certain adaptations to circumvent the toxic effect, such as, by having solvent efflux pump, rapid membrane repair, decreased cell surface hydrophobicity, lower cell membrane permeability and increased membrane rigidity. Since an organic medium is a hostile environment for living cells, microbial contamination is negligible. This is particularly important for reactions on an industrial scale, where maintaining sterility may be a serious problem. Immobilization of enzymes is often unnecessary because they may be recovered by simple filtration after the reaction due to their insolubility in organic solvents [13]. The enzymes produced by these organic solvent tolerant microbes are logically stable in a solvent-rich environment [9]. Enzymes of these microbes are attuned to work under solvent rich environment, thus exhibiting solvent stability and some of them have already been commercialized [6,9,14].

MATERIALS AND METHODS

Collection of soil sample:

Soil samples were collected in 4 x 6 cm sterile polythene bags from Balaghat ranges of Maharashtra, India, by separating outer layer of soil by digging up to 1 feet under the soil surface. The sample was labeled and brought to the laboratory in an ice box and stored in refrigerator until further use.

Isolation and Identification:

The actinomycetes were isolated by serial dilution technique using 0.1ml of 10^{-5} dilution on glycerol asparagin agar (GAA) [15] supplemented with antifungal antibiotic griseofulvin at 50µg/ml concentration. Plates were incubated at room temperature for 4 days. At the end of incubation, plates were observed for actinomycetal growth. Typical actinomycetal colonies were transferred on the Glycerol aspargine agar slants. Slants were labeled, incubated at room temperature for four to five days. The growth on the slants was further used for study. The slants were preserved in the refrigerator.

Biochemical tests were carried out using the Bergey's Manual of Determinative Bacteriology [16]. The actinomycetes grow with whitish tough colonies with white spore mass and is Gram positive and spore forming. The purified isolate was identified using the 16S rRNA phylogenic analysis at National Center for Cell Sciences, Pune. The media components were obtained from Hi Media Laboratories (Mumbai, India). All other chemicals used in this study are of analytical grade .All the solvents used in the present study were membrane filtered.

Maintenance of cultures:

Stock cultures were maintained by periodic transfer on Glycerol aspargine agar medium supplemented with antifungal griseofulvin, by two months intervals.

Effect of temperature and pH on the growth of actinomycete:

Optimum temperature and pH for the maximum growth was evaluated by incubating the inoculated media at various temperatures and pH ranges. For 48 h at 120 rpm in an orbital shaker. Growth was studied by centrifugation and the size of pellet formation.

Organic solvents tolerance of the *Actinomycetes sps:*

Solvent tolerance was determined by plate overlay solvent tolerance method as described by Ogino et al. [7]. The Bennet's agar medium was prepared and sterilized by autoclaving at 121°C at 15 lbs for 15 minutes. To such cooled, sterile medium was added the organic solvent, the concentration of which was increased in a stepwise manner. All the organic solvents were filter sterilized and used. Loopful of an overnight culture was spotted on an Bennet's agar medium plate and then solvents like butanol, acetone, toluene, methanol and benzene (3ml) were poured onto the plates. The plates were sealed with parafilm to avoid evaporation of solvents. Colonies were examined for solvent tolerance after incubation at 35°C for 48 h. The experiments were later repeated in liquid medium.

Enzyme profile of *Streptomyces hygroscopicus:*

A number of research workers in earlier investigation have been reported that soil and water bodies possess high number of enzymatically active actinomycetes [17]. Kulkarni and Deshmukh [18] have detected presence of protease activity among isolated actinomycetes. Shejul and Kapadnis have reported the gelatinase activity actinomycetes [19]. Amylase activity in actinomycetes has been reported by Stamford *et al.* and Nawani *et al.* [20,21]. Cellulase activity in actinomycetes has been reported by George [22].

Qualitative Enzyme assays:

The qualitative enzyme assays were carried out both in the presence and absence of solvents. Whenever the tests were carried out in the presence of solvents the inoculated plates were overlaid with three ml of the solvent concentrations and the plates were

sealed with parafilms so as to avoid evaporation of the solvents.

Gelatinase: The gelatinase activity was tested with gelatin medium (Hi media). The bacterial cultures were inoculated into sterile medium in test tubes, and looked for liquification of the medium after 24, 36 and 48 h. Liquification indicated positive test.

Caseinase: The qualitative test for proteolytic activity was done using 10% milk agar medium. The bacteria were spot inoculated on the sterile medium in petriplates, incubated for 24-36 h. The zone of clearance around the colony indicated positive proteolysis.

Amylase: The isolated bacteria were tested for their amylolytic activity by inoculating them onto petriplates containing sterile starch agar (Hi Media) medium for 24 h. The zone of clearance seen around the culture after the addition of Iodine solution indicated a positive test.

Cellulase: The isolated culture was tested for their cellulolytic activity by inoculating them onto petriplate containing sterile Czepeck mineral salt agar (Hi Media) medium for 48 h. The zone of clearance seen around the culture indicated a positive test.

Lecithinase: The isolated culture was tested for their cellulolytic activity by inoculating them onto petriplate containing sterile Egg yolk agar (Hi Media) medium for 48 h. The opaque (cloudy) zone seen around the culture indicated a positive test.

Urease: The isolated culture was tested for their urease activity by inoculating them onto petriplate containing sterile Christensen's agar (Hi Media) medium for 48 h. The development of pink color indicated a positive test.

Catalase: The test was performed by mixing loopful of culture with a drop of Hydrogen peroxide on a clear slide. Effervescence indicated positive test.

Lipase: The test was performed for their lipolytic activity in Luria-Bertani (LB) agar plate containing 1.0% tributyrin at 35°C for two days. After two days incubation the plates were kept at 4°C to brighten the zone of clearence on the plate.

RESULTS AND DISCUSSION

Morphological and Biochemical characteristics:

The purified isolate identified as *Streptomyces hygroscopicus* subsp. *angustmyceticus* strain AS 4.207 using the 16S rRNA phylogenic analysis. Typical morphology of spiral spore chain arrangement also confirmed that isolated actinomycete belongs to *Streptomyces hygroscopicus*.

The typical Morphological and biochemical characteristics of *Streptomyces hygroscopicus* are shown in Table.1and Table.2.

Fig.1: Growth of *Streptomyces hygroscopicus* on glycerol Aspargin agar (GAA)

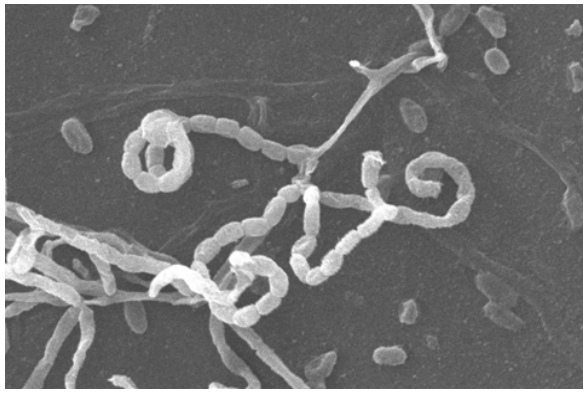

Fig.2: SEM image of *Streptomyces hygroscopicus* showing spiral spore chain morphology.

Effect of temperature and pH on growth of actinomycetal isolate:

The culture grows within 24 h in a wide pH range from pH 5.0 to pH 10.0 and in temperature range of 25°C. to 45°C but the actinomycete had luxuriant growth at pH 7.0 between temperature range of 35° C and 45°C.

Organic solvent tolerance of *Streptomyces hygroscopicus:*

An enrichment culture technique, as used by Akira Inoue [23] for screening of the solvent tolerance of the actinomycete under study. The culture was suspended in 10ml of sterile water and 0.2ml of this suspension was inoculated into 5 ml of the media in screw capped tubes. The culture was incubated at 35°C for one week in a test tube shaker. The rotary incubator shaker was adjusted to 100 rpm. The experiment was repeated in 250mL Erlenmeyer flasks with fresh sterile medium, where the concentration of the solvent was raised at each successive step from 2%, 5%, 10%, 15%, 20%, 25%, up

to 30% v/v. At every stepwise rise in solvent concentration in liquid medium, the acclimated culture was directly streaked on agar plates containing the same nutrients as given in the medium.

Table.1: Morphological and biochemical characteristics of *Streptomyces hygroscopicus*

Morphological characteristics				Hydrolysis of						Utilization of							
Spore morphology	Colour of AM	Colour of SM	Spore mass	Gelatin	Starch	Lipid	Casein	Urea	Mannitol	Glucose	Galactose	Fructose	Maltose	Dextrose	Rhamonose	Arabinose	Raffinose
Spiral	Dark gray	Coffee brown	Dark gray	+ +	+++	+	++	+	+	++++	+	+	+++	++	-	+	+

AM= aerial mycelium, SM =substrate mycelium, Excellent ++++ , Good +++ , Fair ++ , Poor + Nil – .

Table.2: The growth of *Streptomyces hygroscopicus* in presence of various nitrogen sources

Nitrogen source utilization													
D-alanine	L-arginine	Potassium nitrate	L-phenylalanine	L-tyrosine	L-Aspergine	L-Citrulline	L-Histidine	Glysine	L-Proline	Valine	Serine	Methinine	L-Arginine	Melanin Pigmentation
+	++	-	+	++++	+++	-	+	+	-	+	+	+	+	+++

Excellent ++++, Good +++, Fair ++, Poor + Nil –.

Table.3: Enzymatic activity of *Streptomyces hygroscopicus*

Enzyme	Activity in the absence of solvent	Activity in the presence of solvent											
		Acetone [logP -0.24]		Benzene [logP 2.13]		Butanol [logP 0.8]		Toluene 2.69]	[logP	Methanol [logP 0.76]	
		10%	20%	10%	20%	10%	20%	10%	20%	10%	20%
Caseinase	+	+	-	+	+	+	++	+	+	+	-
Amylase	+	+	+	+	+	+	++	+	++	+	+
Cellulase	+	+	+	+	++	+	++	+	++	+	+
Lecithinase	+	+	-	+	+	+	+	+	+	+	-
Catalase	+	+	+	+		+	+	+	+	+	+
Lipase	+	+	+	+	++	+	++	+	++	+	+

Effect of temperature and pH on growth of actinomycetal isolate:

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When solid medium was used, the sterile medium in the petriplates were inoculated by streaking and the medium was then overlaid with solvents. The petriplates were sealed using cellophane tape to avoid the loss of the volatile solvents. The plates were incubated at 35°C for four days. The colony characters were noted. The actinomycete tolerated highest concentration i.e., 20% v/v concentration of the solvents were reinoculated on the same enrichment medium, without the overlay of solvent to get the pure cultures for further study. The growth of the cultures, in 20% v/v solvent concentrations, though was very less, the cells were still viable after more than one month.

The growth of the organisms in the liquid medium containing 0%, to 30%, solvent concentrations was checked. The effect of the absence and presence of solvents on the growth of organisms after 48 hours cultivation on a rotary shaker at 100 rpm at specific temperatures was noted for the cultures (Plate 1). The increase in turbidity in the sterile medium with aseptically inoculated culture after 48 hours of incubation was taken as the measure of growth, keeping an uninoculated sterile medium sample as control. As observed maximum growth was seen in the absence of solvent and the growth decreased with increasing solvent concentration.

It is observed that the actinomycete *Streptomyces hygroscopicus* produces the melanin pigment (Table 2). It is important to note that the pigment was not produced in the presence of any of the solvents used and it could be observed only in the control Erlenmeyer flasks.

Plate.1: The growth of *Streptomyces hygroscopicus* in presence of various concentration of methanol

Qualitative enzymatic Analysis:

Inoue and Horikoshi made the first discovery of organic solvent stable bacterium, a strain of *Pseudomonas putida* IH-2000 which could actively grow and multiply in the presence of 50% (v/v) toluene [23]. The enzymes produced by these organic solvent tolerant microbes are logically stable in a solvent-rich environment [23]. The earliest study on such an organic solvent tolerant lipase was reported by Ogino *et al.*, [7] wherein the lipolytic activity of *P. aeroginosa* LST-03 increased in the presence of toluene, cyclohexane, ethanol and acetone [13]. Since then, many organic solvent tolerant lipases were isolated; mainly from *Pseudomonas* and *Bacillus*[14]. Solvent tolerant cholesterol biotransforming bacillus has been reported by the author earlier [1]. There are a few recent reports on solvent tolerant actinomycetes and their enzymes. Singh and Thumar report an organic solvent tolerant alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1 [4]. Isolation and characterization of a novel thermophilic-organic solvent stable lipase from *Acinetobacter baylyi* is reported by Uttatree *et al.*,[2].

The isolated actinomycete was tested for their different enzymatic activities, both in the presence and absence of solvents. The results have been summarized in the Table 3.

Organic solvent stability of enzyme:

Enzyme activity in the presence and absence of the solvents was observed after 48 hrs of incubation. The plates were inoculated by point inoculation, The cells got dispersed in the presence of the three mL layer of the solvent on the medium. Hence there were multiple colonies with zones of clearance. The plates were sealed with cellophane tapes when being incubated to avoid evaporation of the solvents. The activity was observed with both organic and inorganic solvents. The activity of most enzymes was found to be stable in the presence of the solvents. Most enzymes show stable and better activity in the presence of organic solvents like benzene, butanol and toluene than in the presence of inorganic solvents like acetone and methanol whose logP values are less than Zero. Caseinase and lecithinase did not show any activity in the presence of increase in concentrations of these solvents by 10%, though they retained their activity in the presence of 10% of the acetone and methanol. The maximum activity of caseinase and lipase is found to be with butanol exposure for 48 hours. The lipase, amylase and cellulose enzymes from the *Streptomyces hygroscopicus* retained their activity throughout in the presence of all the solvents but in contrast has shown the most efficient activity in the presence of the butanol whose log Po/w value is 0.8.

These results revealed that these enzymes are not only stable in the presence of water miscible and water immiscible solvents tested but also most of the solvents tested could even enhance the enzyme activity, Although there is a general belief that polar water miscible solvents are more destabilizing than water immiscible solvents [24,25]. As a conclusion, there was no clear correlation between the log *P* value of an organic solvent and the stability of the enzyme in its presence. It can be suggested that the water miscibility is not the only critical factor of solvents affecting enzyme stability. Other factors such as the solvents molecular structures and their functional groups as well as enzyme structure and the type of surface amino acids may also play their roles [24,25]. Review of the literature reveals that microbial lipases are generally stable in organic solvents but they possess different sensitivity to the solvents.

CONCLUSIONS

In this study we report organic solvent tolerant actinomycetes, *Streptomyces hygroscopicus* strain which is found to display extreme stability in the presence of three hydrophobic and two hydrophilic organic solvents. Thus organic solvent tolerant actinomycete grows at moderate temperature, neutral pH and stable activity in the presence of 20% concentration of solvents. The results look promising for the usage of the isolated enzymes both in industries like the detergent industry and in containing the oil spills and environmental conservation, Actinomycetes are considered as major source for antibiotics and hence it seems very little attention has been directed towards enzyme production. There are a few reports on isolation of organic solvent tolerant actinomycetes secreting solvent tolerant proteases and lipases. Further studies need to be carried out to check the presence of novel isolates, producing extracellular enzymes, stable in solvent atmosphere. The study also holds significance as only few salt tolerant alkaliphilic actinomycetes are explored and information on their enzymatic potential is still scarce.

The effect of parameters like solvent concentrations, pH, temperature, NaCl concentrations, nitrogen and carbon sources, presence of surfactants, can be studied to get the superbug, which would be a novel and unique actinomycetes. Based on the merits of the strains, they could be highly efficient with potential applications in industries. Once isolated and identified their electron microscopy after exposure to the extreme solvent environment would be interesting.

Further the studies using HPLC on what exactly do these actinomycete enzymes breakdown the substrates to would be enlightening, specially looking at actinomycetes being the important antibiotic producers and hence proving medicinally important it would be interesting to know if they can play a role in steroid biotransformation. The studies on whether the actinomycetes are able to use solvents as their sole source of carbon may highlight their role in xenobiotic /solvent bioremediation too.

ACKNOWLEDGEMENT

The authors are grateful to Yogesh Souche of NCCS, Pune, India, for the culture identification. The project was partly funded by DST, Govt. of India, under the Woman Scientist Scheme. The authors are also thankful to Nishigandha Mestry and Yashada Bhagwat for their help.

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Source of support: Nil
Conflict of interest: None Declared