Molecular diversity and enzymatic potential of salt-tolerant alkaliphilic actinomycetes

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Salt-tolerant alkaliphilic actinomycetes are difficult to isolate as their natural environments are much more different and hostile than laboratory conditions. Due to these limitations, majority of the microbial population remain uncultivated. During the recent years, development and application of molecular techniques, such as; DGGE, TGGE, RFLP and DNA-DNA hybridization have proved quite valuable in judging the distribution and diversity of these organisms. While majority of the studies related to enzymes are confined to halophilic/alkaliphilic bacteria, similar account with respect to halo-tolerant alkaliphilic actinomycete is nearly unattended. Salt-tolerant alkaliphilic actinomycetes produce alkaline proteases, amylases, cellulases and lipases that are functional under extreme conditions. Consequently, having unique properties, these biocatalysts would attract several novel applications in industrial processes. Recent studies have revealed vast spectrum of enzymes; alkaline proteases, amylases and cellulases in salt-tolerant alkaliphilic actinomycetes from the saline habitats along the coast of Gujarat in India.

Key words: Salt-tolerant alkaliphilic actinomycetes, enzymes from actinomycetes, molecular diversity, alkaline proteases, saline habitats

Introduction

Microbial communities are found in most diverse conditions, including extremes of temperature, pressure, salinity and pH. In order to survive under such conditions, these organisms have developed adaptive features to function under extreme conditions. These microorganisms, referred as extremophiles, grow optimally under one or more environmental extreme, while polyextremophiles grow optimally under multiple extremes. The extremophiles can grow optimally in some of the earth's most hostile environments of temperature (-2ºC to 15ºC; Psychrophiles; and 60ºC to 115ºC; Thermophiles), salinity (2-5M NaCl; Halophiles), pH (<4 Acidophiles and >9; Alkaliphiles), anaerobicity (Methanogens), and/or pressure (Barophiles).

New developments for cultivation of microbes in general and extremophiles, in particular, along with the cloning and expression of their genes in heterologous hosts will add to the possibilities of enzyme-driven reactions in chemical, food, pharmaceutical and other industrial applications[1-3]. Extremophiles belong to all three domains of life. Those living in salt are called Halophiles and can thrive in concentrated brine, having salinity several times more than that of sea water. Alkaliphiles grow from pH 9 to 12.

The natural and man-made environments may harbor a large population of halophilic and alkaliphilic actinomycetes. Actinomycetes are Gram-positive bacteria which comprise a group of branching unicellular microorganisms, exhibiting a wide range of morphological forms from coccoid through fragmenting hypha to permanent and highly differentiated branched mycelium [4]. They have only recently focused attention from the point of view of their diversity, phylogeny and biotechnological potential [5]. Members of the actinomycetes, which live in marine environment, are poorly understood and only few reports are available pertaining to actinomycetes from saline and alkaline habitats. Recent findings from culture-dependent and culture-independent methods have demonstrated that there is tremendous diversity and novelty among the halotolerant and alkaliphilic actinomycetes present in saline and alkaline environments. *Nesterenkonia alba* sp. nov., an alkaliphilic actinobacterium was recently reported to grow optimally at pH 9-10 [6]. Similarly, Chen et al. reported a halophilic marine actinomycete, *Nocardiopsis litoralis* sp. nov., isolated from a sea anemone [7].

Actinomycetes are physiologically diverse group, as evident by their production of numerous extra cellular enzymes and by the thousands of metabolic products they produce. Actinomycetes are the major antibiotic producers in the pharmaceutical industry [8, 9]. Although they have provided many important bioactive compounds of high commercial value, exploration of their biocatalytic potential is relatively a new phenomenon [10]. The salt-tolerant alkaliphilic actinomycetes from coastal Gujarat (Western India) indicated significant morphological, biochemical and molecular diversity. In addition, secretion of high level of extracellular enzymes, displaying high stability under unconventional conditions of pH, temperature and chemical denaturants has generated considerable attention [11-13].

1.1 Taxonomy and diversity of Halo-tolerant and alkaliphilic actinomycetes

The halophilic and alkaliphilic actinomycetes are difficult to isolate from the saline and marine environment. Although their ecological role in the marine ecosystem has been largely neglected, recent advancements in marine microbial

ecology employing molecular tools and metagenomics are significant steps. Cultivation methods and molecular techniques may lead to greater insights into marine actinobacterial biodiversity and biogeography. Recently, the actinobacterial community in a historic lake sediment core of Ardley Island, Antarctica, spanning approximately 1,600 kms, was investigated by molecular approaches targeting the 16S rRNA gene fragments [14]. Tang and coworkers [15] investigated the biological characteristics of 43 actinomycete from saline and alkaline soils in Xinjiang, Hebei and Qinghai (China) and representative strains of four other genera under differing conditions of pH and varying concentrations of the mineral salts Na+, K+, Mg2+ and Ca2+. Similarly, 38 halophilic and halo-tolerant actinomycetes were isolated from Challenger deep sediment from the Mariana Trench [16]. The phylogenetic analysis based on 16S rRNA gene sequencing showed that the isolates belonged to the genera *Streptomyces, Dermacoccus, Kocuria, Micromonospora, Tsukamurella and Williamsia.* Diversity of halophilic and alkaliphilic actinomycetes is reflected in table 1a [17-26] and 1b [27-35]**.**

Table 1a Diversity of halophilic actinomycetes with respect to salt-tolerance

Organism	**Site of Isolation**	**NaCl, w/v (Range)**	**Reference**
Nocardiopsis litoralis sp. nov	Sea anemone South China	01-15%	[17]
Streptomonospora amylolytica sp. nov. and *Streptomonospora flavalba sp. nov.*	Salt lake in the north-west of China	05-15%	[18]
Saccharopolyspora qijiaojingensis sp. nov.	Salt lake in the north-west of China	06-22%	[19]
Amycolatopsis marina sp. nov.	Ocean Sediment of South China Sea	08-15%	[20]
Gracilibacillus halophilus sp. nov	saline soil in the Qaidam Basin, China	07-30%	[21]
Thermobifida halotolerans sp. nov.	salt mine, China South-West China	0- 15%	[22]
Nocardiopsis aegyptia sp. nov.	Marine sediments of Abu Qir Bay, Egypt	05-30%	[23]
Nocardiopsis Halotolerans sp. nov.	Kuwait salt marsh soil	0-15%	[24]
Saccharomonospora halophila sp. nov.	Marsh soil of Kuwait	10-25%	[25]
Nocardiopsis kunsanensis sp. nov.	Saltern in Kunsan Republic of Korea	10-20%	[26]
Streptomyces clavuligerous Strain Mit-1	Saline soil of Mithapur Gujarat, India	0-15%	[13]

Table 1b Diversity of alkaliphilic actinomycetes, site of isolation and pH -tolerance

Organism	Site of Isolation	pH Range (Growth)	Reference
Nitriliruptor alkaliphilus gen. nov., sp. nov	soda lake sediments	8.4-10.6	[27]
Nocardiopsis valliformis sp. nov.	alkali lake soil in China	8.0-14.0	[28]
Streptomyces deccanensis sp. nov.	Gulbarga, Karnataka Province, India	8.0-10.5	[29]
Kucuria aegyptia sp. nov.	Saline, alkaline Egypt	9.0-10.0	[31]
Streptomyces sodiiphillus sp.nov	Chaka salt lake, China	9.0-10.0	[32]
Nocardiopsis alkaliphila sp.nov.	Eastern desert of Egypt	9.5-10.0	[33]
Nocardiopsis metallicus sp.nov.	Alkaline slag dump, Germany.	8.5-10.5	[34]
Dietzia natronolimnaios sp. nov.	East African soda lake	9.0-13.0	[35]
Streptomyces sannanensis Strain RJT-1	Gujarat Province, India	7.0-10.0	[8]

1.2 Molecular approaches to assess the diversity of halo – alkaliphilic actinomycetes

Molecular approaches based on 16S rRNA sequence analysis allow direct investigation of the community structure, diversity, and phylogeny of microorganisms [36]. There are about 65000 bacterial 16S rDNA sequences in EMBL database and 16,000 in the Ribosomal Database Project (RDP) [37, 38]. Stackebrandt et al. [39] and Mehling et al. [40] used 16S rDNA sequencing to determine those regions suitable for detection of streptomycetes, and proposed a genus-specific probe and primers targeting the 16S rRNA gene. Huddleston et al. [41] described a method for the molecular detection of streptomycin-producing streptomycetes in soil with DNA probes targeting the streptomycin biosynthetic genes. In addition, the existing databases [42] can be used to compare the DNA-sequences of unknown microorganisms and allow a phylogenetic identification.

The 16S rRNA from majority of our isolates from Coastal Gujarat was amplified with Universal primers, while some of the isolates were amplified by specific primer sets [43]. The amplification with different primers at different annealing temperatures revealed that the amplification was highly temperature specific. Many reports in literature have highlighted amplification of 16S rRNA gene of actinomycetes with U1 and U2 primer sets [44, 45]. Similarly, reports are also indicative of the amplification by StrepB/StrepE, StrepB/StrepF and NF/R specific primer sets [46, 47].

1.2.1 Denaturing gradient gel electrophoresis

For the assessment of diversity, DGGE is applied as a fingerprinting tool; where separation is based on changes in electrophoresis mobility of DNA fragments [48]. In DGGE, DNA fragments of the same length but with different

sequences can be separated on the basis of electrophoretic mobility of the single stranded DNA [49]. The basic need for DGGE necessitates the DNA fragments having at least two melting domains. The mobility of these branched fragments in the polyacrylamide gel is abruptly retarded. Since, Tm for a given domain is determined by its sequence and base composition, two DNA fragments differing by a single base change (and thus in Tm) in the lowest melting domain, will be separated from each other at the end of the run in DGGE [50] as well as in TGGE [51].
Different hyper variable (V) regions of the archaeal 16S rRNA gene (rrs) were compared systematically to establish a preferred V region(s) for use in Archaea-specific PCR-denaturing gradient gel electrophoresis (DGGE) [52]. Using this approach, Sabine et al., [53], members or close relatives of the genera *Halomonas, Clostridium,* and *Frankia* were identified. Further, Olajire and Mathew et al., [54] proposed *C. circinans* as a distinct species from *C. coccodes*. PCR amplification of 16S ribosomal DNA fragments from the co-culture, analyzed by denaturing gradient gel electrophoresis, resulted in two distinct 16S ribosomal DNA bands, indicating two different bacterial components. Sequencing showed that the bands were derived from a *Desulfovibrio* strain and an *Arcobacter* strain [55]. The band patterns of endocytic bacteria and free-living marine bacteria were different, indicating the development of a specific bacterial population within *N. scintillans* [56]. DGGE was carried out with five different primer sets targeting 16S rRNA gene of salt-tolerant alkaliphilic actinomycetes isolated from coastal Gujarat (Western India) [22]. The amplicon size of the isolates amplified with the same primer was quite similar, while it differed in size with other primer sets. It's quite apparent from these findings that DGGE generated valuable information on the group specificity and phylogenetic relatedness of salt-tolerant actinomycetes.

1.3 The Enzymatic Potential of Salt-Tolerant Alkaliphilic Actinomycetes

During the last several years, there has been increasing interest in biocatalysts with novel features. Microorganisms are the first choice as the source of enzymes because of their rapid growth, broad biochemical diversity and ease of genetic manipulation. Despite the fact that till date more than 3000 different enzymes are identified and some are used in biotechnological and industrial applications, the available enzymatic range and their properties are still not sufficient to meet the ever inceasing demand. During the last few years, some extracellular enzymes from halophilic and alkaliphilic actinomycetes have been studied [57]. However, it is evident from the literature that the exploration of the enzymatic potential of these microbes is just the beginning and till date only few enzymes are investigated. Salt-tolerant alkaliphilic actinomycetes from the saline habitats along the Coastal Gujarat (India) secrete a range of extracellular enzymes including proteases, amylases, cellulases and lipases. Overall, above 60% of these isolates produced extracellular alkaline proteases, amylases and cellulases at higher salt and alkaline pH [13]. The salt tolerance of these isolates was comparable to those of *Haloactinospora alba* gen. nov., sp. nov. [58] and *Saccharopolyspora halophila* sp. nov. [59]. However, it was relatively higher than that of *Haloglycomyces albus* gen. nov., sp. nov., a halophilic, filamentous actinomycete of the family *Glycomycetaceae* [60] and *Paraliobacillus quinghaiensis* sp. nov. [61].

1.3.1 Production of extracellular alkaline protease by salt-tolerant alkaliphilic actinomycetes

Proteases represent one of the three largest groups of industrial enzymes and account for 60% of the total worldwide sale of enzymes. Alkaline proteases of microbial origin are widely used as detergent additives for household laundry, industrial and institutional cleaning. Ideally, proteases used in a detergent formulation should have a high level of activity over a broad range of pH and temperatures. The enzymes from alkaliphiles have stability in alkaline range but usually are thermally sensitive. Thus, it is desirable to search for new proteases with novel properties from as different sources.

The study of enzymes usually involves a search for optimal media for their production. This is achieved by a systematic study on the suitability of large number of carbon and nitrogen sources. Further, it is important to relate various cultural, nutritional and environmental factors to the production of the desired product. The actinomycetes from Gujarat Coast (India) produced enzymes over a broad range of salt and pH [13]. An alkaliphilic actinomycete, *Nocardiopsis* sp. TOA-1, has been reported to produce alkaline protease optimally at pH 9-10 [62].

1.3.2 Purification of alkaline proteases produced by halo-tolerant alkaliphilic actinomycetes

Purification of extremozymes is significant to study enzymatic characteristics and understand cellular metabolism and regulatory pathways. It is also significant for commercial production of several industrially and pharmaceutically important enzymes. Since enzymes require specific strategy for purification, it's important to develop novel strategies for the purification of individual extremozyme. Similarly, the characterization of the enzymes is important towards understanding the biochemical reactions and maintenance of stability under extreme conditions [63].

A serine alkaline protease from the *Streptomyces clavuligerus* Mit-1 was active and stable over a range of pH [12] and high temperature optimum for enzyme activity was a unique feature. In addition, enzyme stability in the presence of various surfactants and oxidizing-reducing agents would add to its commercial value. Extreme resistance of alkaline proteases from Mit-1 against chemical denaturation by urea was another unique feature [12]. Higher temperature optima for catalysis and significantly enhanced thermal stability of the alkaline proteases were novel features of the salt tolerant

alkaliphilic actinomycetes from saline habitats. Since only few enzymes are purified and characterized from extremophilic actinomycetes, the work on the enzymatic characteristics and stability assumes significance [12, 13,]. A novel thermostable serine protease was purified and charactreized from the extreme halophilic archaeon, *Halogeometricum borinquense* strain TSS101 [64].The enzyme stability under multitude of extremities; salt, pH, temperature and resistance against chemical denaturation offers unique avenues for biotechnological applications and provides an opportunity to investigate stability of macromolecules.

The above accounts present the current status of research on the biology and biotechnology of salt-tolerant and alkaliphilic actinomycetes. The trends are quite interesting in view of the diversity, novelty and distribution of these actinomycetes. The fairly enhanced salt and pH tolerance of these actinomycetes, along with their capacity to secrete commercially valuable primary and secondary metabolites, appears as attractive features of these organisms. The characterization of proteases from different organisms indicated that although synthesized under similar conditions of growth, the enzymes may display greater variation in their properties. However, the structural elucidation of these enzymes may provide some important clues responsible for varied chemical sensitivity. Further, the search and development of cloning and expression systems can be used for the production of enzyme in large quantity and would certainly add to the possibilities of altering the enzyme for desired traits employing protein engineering and random mutagenesis.

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