Journal of Aquatic Biology & Fisheries | 2020 | **8** | pp. 1-6

ISSN 2321–340X

Isolation of *Streptomyces* spp. with Bioprospecting Potential from Mangrove Regions of Ponnani, Kerala, India

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Abstract

Marine actinomycetes are increasingly becoming an important source in the production of medical and industrially important enzymes. Similarly, their role in the discovery of new antibiotics has paved the way for screening them from marine habitats that otherwise has not been explored. Under the above pretext, a study was envisaged with a focus on screening of actinomycetes with antibacterial properties from sediments of Ponnani mangrove region. The study was also aimed at determining the in-vitro antimicrobial activity of indigenous actinomycetes against selected pathogenic bacteria. A total of 126 isolates of actinomycetes were isolated from the sediment sample. The majority of the actinomycetes screened were identified as *Streptomyces* (73%) based on their colony morphology, and among them, two isolates (PP26 and PP59) showed very high antimicrobial activity against two Gram-negative bacteria (*Vibrio cholerae* and *Shigella* sp.). Among the 65 isolates that showed enzyme production, catalase activity was exhibited by all isolates while limited activities were seen for lipase (48 isolates), caseinase (42 isolates), gelatinase (39 isolates), amylase (26 isolates) and Urease (20 isolates). Molecular characterization through 16S rDNA amplification of four selected strains that showed high antimicrobial and better enzyme production revealed the species to be *Streptomyces exfoliates*, *S. rubrogriseus, S. sindenensis* and *S. spiroverticillatus*. The result elucidates that there is a rich consortium of many potent Streptomyces, which have bioprospecting potential. Further studies on the isolation and characterization of secondary bioactive metabolites from these groups will be beneficial for discovering novel biomolecules beneficial to humans. The study also shows that Ponnani mangroves are a potent source of actinomycetes useful for bioprospecting.

Keywords: *Streptomyces*, Mangrove, Antimicrobial, Bioactive compound, Hydrolytic enzyme

1. Introduction

Among the producers of commercially important secondary metabolites, bacteria have proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds. Among these, marine actinomycetes are one of the most efficient groups of secondary metabolite producers that show a range of biological activities and are different from those of terrestrial strains in producing different types of bioactive compounds including enzymes (Lekshmi *et al*., 2014). The demand for new antibiotics continues to grow due to the rapid emergence of multiple antibiotic-resistant pathogenic bacteria causing life-threatening infections, underscores the need to look for new antibiotics (Reyhanath and Kutty, 2014). Actinomycetes are the most promising group of bacteria able to produce a wide variety of bioactive metabolites (Baharum *et al*., 2010; Gulve and Dheshmukh, 2011). The terrestrial habitats have been explored at its most for various biologically active microbial products. On the contrary, marine habitats are relatively less scrutinized ones in this field. The marine ecosystem comprises different kinds of extreme environmental conditions and can be explored for the discovery of new bioactive metabolites.

Mangrove ecosystem is the intertidal region between marine and terrestrial environment are always confronted with continuous changes in various physico-chemical parameters. It is one of the most productive ecosystems (Shah and Sony, 2016). Mangrove sediments exhibit peculiar characteristics of high temperatures, high levels of salinity, high pH, and high levels of organic matter, low aeration and moisture which provide interesting substrate conditions conducive to the development of diverse microbial communities (Holguin, 2001). The bacterial community of mangrove sediments is an invaluable resource for bioprospecting and can serve as a renewable commercial resource for sustained human activity due to their unique environment (Bhagwat and Ingale, 2013). Mangrove actinomycetes have a diverse range of enzyme activities that are capable of catalyzing various biochemical reactions (Baskaran *et al.*, 2011). Various hydrolytic enzymes like amylases, chitinases, and proteases are produced by actinomycetes. They are also good decomposers of organic materials. Amylase is one of the commonly used enzymes in starch and textile industries. Proteases are important from an industrial perspective and cater 60% world enzyme market (Kalisz, 1998). Different microorganisms secrete urease enzyme and play an important role in the degradation of organic nitrogen. Lipase enzyme production by actinomycetes has been reported by many workers, and they are widely used in the food, pharmaceuticals, diagnostics and detergent industries (Schmid and Verger, 1998).

Received on: 01-01-2019; Revised on:10-04-2020; Accepted on: 13-04-2020

Streptomyces isolated from mangrove habitats is a potentially rich source for the discovery of anti-infection and anti-tumour compounds, and the agents for treating neurodegenerative diseases and diabetes (Hong *et al.*, 2009). There is an increasing awareness of the role of marine microorganisms in biogeochemical processes, in pollution, in disease and for biotechnology as relevant biochemical compounds, enzymes, single-cell protein and pharmaceutical compounds. Ponnani is a coastal town in Malappuram district of Northern Kerala, where the second longest river of the state, the Bharatapuzha, joins the Arabian Sea. The Ponnani estuary, which consists of a series of intricate brackishwater regions fringed with sparse mangrove vegetation, constitute a diverse environment for many aquatic species. Hence, a study was conducted to isolate, identify and characterize, actinomycetes with special reference to genus *Streptomyces* with a potential for producing various hydrolytic enzymes and antimicrobial activity against specific pathogenic bacteria from the mangrove region adjoining the Ponnani estuary.

2. Materials and Methods

2.1 ***Study area:*** The Ponnani backwater system located between 10° 46' and 10° 48' N and 75° 54' to 75° 56' E is part of an open estuary that is drained by a tributary of the Bharathapuzha River and which flows into the Arabian Sea. This region is flanked with a good patch of mangroves of the genus *Avicennia, Bruguiera, Kandelia, Rhizophora and Sonneratia* (Kaladharan and Ashokan, 2012). The estuarine systems are exposed to tides from the Arabian Sea, and hence water here is brackish almost throughout the year.

2.2 ***Sample collection*****:** Sediment samples were collected from three stations during pre-monsoon season (February to May) from Ponnani estuary that had healthy patches of mangroves, i.e., Veliyancode, Puduponnani and Biyyam. Samples were collected at a depth of 1-10 cm from the soil surface and placed in sterile polythene bags, sealed tightly and brought to the laboratory for analysis.

2.3 ***Pretreatment of the sample*****:** The collected sediment samples were air-dried at room temperature for a week and transferred to sterile petriplates and kept at 55°C for 10 minutes in order to retard the growth of slime forming bacteria.

2.4 ***Isolation of actinomycetes*****:** Actinomycetes from dried sediment samples were isolated by serial dilution method on SCA (Starch Casein Agar) medium supplemented with Nalidixic acid (25 mg/L) and Cycloheximide (50 mg/L) to minimize the growth of bacteria other than actinobacteria and fungi, respectively. All the plates were incubated at 28°C, and the colonies that appeared on petriplates were observed from 5th day onwards for one month.

2.5 ***Identification of actinomycetes*****:** Morphologically different colonies that appeared on Starch Casein Agar plates were selected and purified repeatedly. The features of morphology, physiology, and biochemistry of the strains were recorded as per International Streptomyces Project (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Williams *et al.*, 1989). The purified isolates were stored in -80°C for further analysis.

2.6 ***Screening of actinomycetes for enzyme production*****:** Based on the morphological study, 126 isolates were selected and inoculated on the suitable medium by streak inoculation method in order to check different enzymatic degradative activities viz., Amylase (Starch agar), Gelatinase (Gelatin agar), Caseinase (Skimmed milk agar), Lipase (Basal medium with egg yolk emulsion), Urease (Christenson's agar) and Catalase (using 3% hydrogen peroxide solution).

2.7 ***Screening of antibacterial activity*****:** All the 126 actinomycetes isolate obtained were tested for their antibacterial activity against seven Gram-negative and one Gram-positive pathogenic bacteria namely, *Salmonella typhi., Shigella sp., Escherichia coli*, *Vibrio cholera*, *V. parahaemolyticus, V. vulnificus* and *Staphylococcus aureus*. Screening for antibacterial activity of actinobacterial isolates was assessed by perpendicular streak method (*Egorov, 1985*) on SC Agar. A single streak of the actinomycetes was made on SCA and incubated at 37°C for 5 days. After observing a ribbon-like growth of the actinomycetes, the bacterial pathogens were streaked at a right angle to the first streak and were again incubated at 37°C for 24 to 48 hrs.

2.8 ***Molecular characterization of Streptomyces*****:** Isolates that showed good antibacterial and better production of hydrolytic enzymes were selected for molecular characterization. Total genomic DNA from the selected Streptomyces strain was isolated as per standard protocol (Sambrook *et al.*, 1989). Genomic DNA extraction and amplification was done using Actinobacteria specific primers (Monciardini *et al.*, 2006). The DNA samples were then dried under vacuum and dissolved in an appropriate volume of TE buffer (10 mM Tris; 1 mM EDTA, pH 7.5) and stored at –20°C. Agarose gel electrophoresis was done to check the purity. DNA sequencing of 16S rDNA of selected isolates was carried out according to Kumar *et al.* (2010). The 16S rDNA was amplified using 2X Taq master mix (Genaxy) and primers 8f (5'AGAGTTTGATCCTGGCTCAG 3') and 1492r (5'GGTTACCTTGTTACGACTT 3'). The conditions for thermal cycling were as follows: Denaturation of target DNA at 94°C for 2 minutes followed by 30 cycles at 94°C for 30 seconds and primer extension at 72°C for 1 minute, at the end of the cycling the reaction mixture was held at 72°C for 7 minutes and then cooled for 4 minutes. PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet fluorescence after ethidium bromide staining. PCR products from genomic DNAs were sequenced with 16S rDNA primers using ABI XL DNA analyzer, using the big dye Terminator kit (Applied Biosystems, USA) at SciGenom Cochin, India Ltd. The gene sequences obtained were compared by aligning the result with the sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) search program at the National Centre for Biotech Information (NCBI). A Maximum Likelihood (ML) tree was constructed using MEGA V. 7 (Kumar, Stecher, & Tamura, 2016), by including the 16S rDNA sequence of

Streptomyces sps., collected from Ponnani mangrove region, together with those retrieved from NCBI GenBank. *Bacillus subtilis* was used as an outgroup.

2.9 *Nucleotide sequence accession numbers*: Genomic sequences are available at NCBI RefSeq as follows: Accession No.: MT317111, MT317112, MT317113 and MT317114 respectively.

3. Results and Discussion

During the present study, a total of 126 actinomycetes isolates belonging to ten (10) Genus were identified from the sediment samples of Ponnani mangrove region. The actinomycetes included Genus *Actinomadura, Actinoplanes, Bifidobacterium, Micromonospora, Nocardia, Nocardiopsis, Prauserella, Rhodococcus, Streptomyces* and *Streptosporangium*. Among them, the highest representation was shown by Streptomyces (73%) (Fig. 1).

Streptomyces predominated in all the three stations; however, their abundance was considerably high in Puduponnani (9 cfu/g) compared to Veliyancode (5 cfu/g) and Biyyam (2 cfu/g). Of the 92 isolates of Genus *Streptomyces* identified only 35 isolates (38%) showed any kind of antimicrobial activity and among them, two isolates showed strong antimicrobial activity against two Gram-negative bacteria (*Vibrio cholerae* and *Shigella* spp.) (Fig. 2A & 2B). Table 1 depicts the colony characteristics of these antagonistic actinomycetes herewith referred to as Streptomyces PP26 and PP59.

Of the 92 isolates of Genus *Streptomyces*, 65 isolates (71%) showed enzyme production. Catalase activity was exhibited by all isolates while limited activities were seen for lipase (48 isolates), caseinase (42 isolates), gelatinase (39 isolates), amylase (26 isolates) and Urease (20 isolates) (Fig. 3). Among these 65 isolates, two strains (PP15 and PP56) showed very high production of all the six enzymes. Hence the molecular characterization of these four strains of Genus *Streptomyces* viz., PP15, PP26, PP56 and PP59, were carried out to ascertain the taxonomy of the Streptomyces.

Maximum Likelihood analysis based on 16S rDNA sequences (Fig. 4) revealed that *Streptomyces* isolated from Ponnani mangrove region formed a separate monophyletic clade with potential affinity to species isolated from the Arabian Sea. However, these four clades occur in separate lineages of Streptomyces. Results of molecular characterization showed that the four isolates belonged to four different species of Streptomyces namely *Streptomyces exfoliates* (PP15), *S. rubrogriseus* (PP26), *S. sindenensis* (PP56) and *S. spiroverticillatus* (PP59) respectively.

Actinomycetes are characterized by the ability to produce a large variety of metabolites, such as vitamins, enzymes and antibiotic. Actinomycetes enzymes have an array of biological, industrial and environmental applications, like polymer hydrolysis, synthesis of chemicals, soil decontamination, biological control of diseases, and decomposition of organic matter (Minotto *et al.*, 2014). There were investigations by a number of research workers about the diversity, enzymatic and antibacterial activity from marine and freshwater environment. However, only a few reports are available pertaining to actinomycetes diversity in mangrove soil of India (Gulve and Deshmukh, 2011). Also, little is known about the activities of actinomycetes in mangrove waters and sediments (Bhagwat and Ingale, 2013). The present study hence was perceived to screen actinomycetes of genus *Streptomyces* from the mangrove ecosystems of Ponnani estuary that had the potential for producing hydrolytic enzymes and antimicrobial compounds. Results showed the region to be rich in both diversity and abundance of actinomycetes and among them two strains of Streptomyces viz., *Streptomyces rubrogriseus* and *S. spiroverticillatus* were identified as a potential source for antimicrobial compounds. Similarly, the production of hydrolytic enzymes of industrial importance was high in two strains of Streptomyces viz., *Streptomyces exfoliates* and *S. sindenensis*. The results also showed that Streptomyces is an important group of actinomycetes capable of producing novel antimicrobial and enzymes that have significance to humans. Several workers have investigated the antibacterial and antifungal activity of actinomycetes isolates along with the enzymatic activity. Of these, Malisorn and Nikhome (2014) reported that actinomycetes strains *Streptomyces, Microbispora*, and *Microtetraspora* exhibited antagonistic reaction against *Fusarium* species. Similar findings were recorded from India by Rathnakala and Chandrika (1999) and Dharmaraj (2010) who isolated actinobacterial cultures from mangrove environment that showed antagonism against most of the pathogens present in fishes and the aquatic environment. Apart from these, actinomycetes have also proven to be producing many antiviral and antifungal antibiotics (Kumar *et al*., 2006; Pindi *et al*., 2012; Mohanraj and Sekar, 2013), most of which have been isolated from mangrove sediments. *Streptomyces rubrogriseus*, the strain of Streptomyces that showed good antimicrobial property in the present study is known to produce an antibiotic Streptomycin (Kusaka *et al*., 1968). Similarly, *S. spiroverticillatus* has been reported to produce antibiotic Tautomycin (Li *et al*., 2008). Hence the present study is in consonance to the earlier reports of mangrove swamps being a good source of actinomycetes with antimicrobial properties.

In the present study, an attempt to isolate and screen the actinomycetes from mangrove sediment of Ponnani estuary showed many strains of Streptomyces with good enzymatic production. Majority of the actinomycetes isolated (71%) produced hydrolytic enzymes and among them two strains *Streptomyces exfoliates*, and *S. sindenensis* produced all the major hydrolytic enzymes. In a similar study, Lekshmi *et al.*, (2014) have reported the isolation of actinomycetes strains capable of protease, cellulase and lipase activity from sediment samples. Similarly, Gulve and Deshmukh (2011) have revealed the difference in enzymatic activity for protease, gelatinase, amylase, lecithinase, cellulose and Urease. Cellulases are important industrial enzymes sustainable for production of biofuel as they convert the cellulose into fermentable sugars (Mukhtar *et al.*, 2018). Cellulases used as a supplement in detergents, textile, animal additives and paper and pulp industry have also

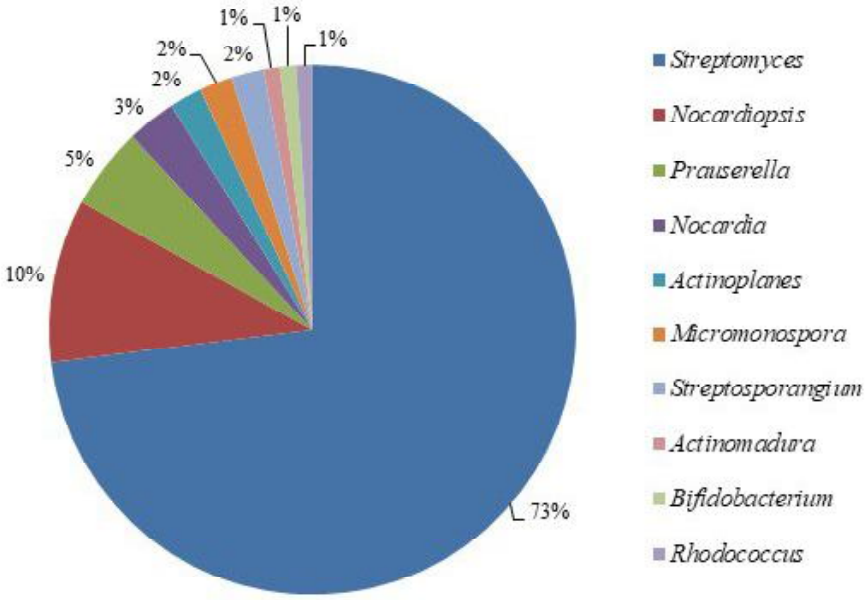

Fig. 1. Percentage representation of different Actinomycetes from Ponnani estuary

Table 1. Colony characteristics of antagonistic Streptomyces isolates

Colony Characteristics	Streptomyces isolates	
	Streptomyces PP26	Streptomyces PP59
Form	Filamentous	Filamentous
Elevation	Flat	Flat
Margin	Filiform	Filiform
Surface	Wrinkled	Smooth, granular
Edges	Lobate	Lobate
Colour	White	Creamy white
Opacity	Opaque	Translucent
Gram's staining	+ve	+ve
Microscopical structure	Filamentous	Long rods

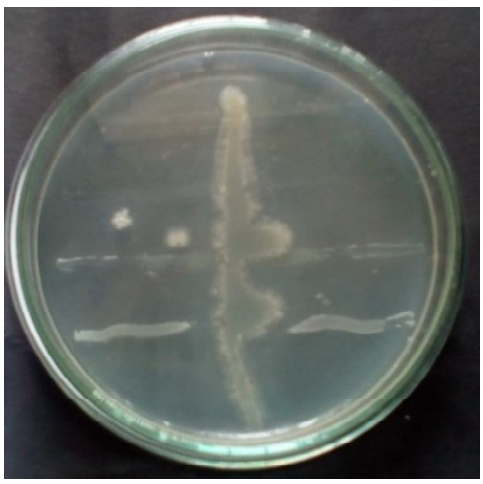

Fig. 2A. Antimicrobial activity of Streptomyces PP26 against *Vibrio cholera*

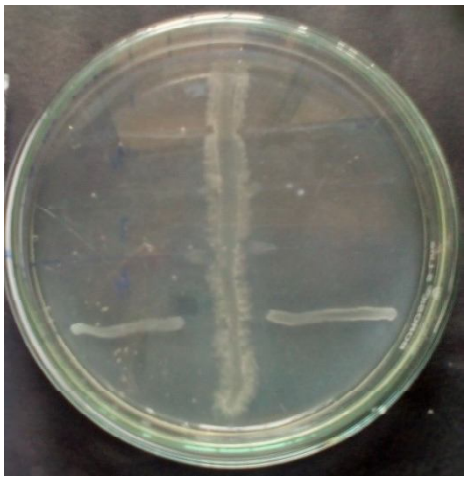

Fig. 2B. Antimicrobial activity of Streptomyces PP59 against *Shigella* spp.

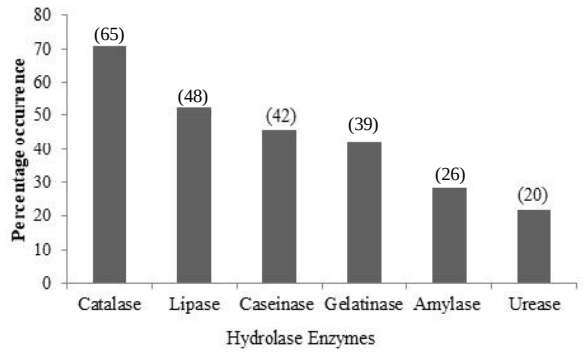

Fig. 3. Percentage representation of hydrolytic enzyme-producing isolates of Streptomyces

been isolated from Streptomyces spp., such as *S. ruber*, *S. lividans* and *S. rutgersensis* (Jang and Chang, 2005). Actinomycetes *Streptomyces erumpens* have shown to produce amylases for extracellular digestion and has been used in baking, pharmaceutical, paper and pulp industry (El-Sersy *et al.*, 2010). While *Streptomyces exfoliates* that produce lipases to hydrolyze the ester bonds in triglycerides to glycerol and fatty acids, have the potential to be used in the processing of oils and fat, cosmetics, diagnosis and detergents (Aly *et al.*, 2012). Similarly *Streptomyces thermoviolaceus* are known as chitinases producers. Chitinase has applications in biomedical and food industry (Bhattacharya *et al.*, 2007). The presence of a rich source of nutrients makes the mangrove environment highly productive and a homeland of microbes. It is a potent source for the isolation of microorganisms and the bacterial community of this environment is an invaluable resource for bioprospecting of enzymes of industrial needs, especially from marine actinomycetes. These enzymes have well-known applications as biocatalysts in several areas of industry, such as biotechnology, agriculture and pharmaceuticals, etc. Hence the present study clearly identifies Ponnani estuarine sediment as a good source of enzyme-producing actinomycetes. All the enzymes studied in the present study have great industrial importance, and hence the biochemical activity of these selected strains to produce various enzymes such as amylase, gelatinase, caseinase, lipase, Urease and catalase enable them to be a potential source of further investigation. The present study is the primary attempt to screen the actinomycetes with bioprospecting potential from an unexplored habitat of Ponnani mangrove regions. The study emphasizes the need to study in detail the actinomycetes diversity, especially Streptomyces that can provide a rich source of bioactive compounds which could effectively be used in large scale production for industrial and pharmaceutical applications.

Acknowledgements

The authors thank the Principal, MES Ponnani College, for providing the necessary facilities. Reyhanath is supported by the Maulana Azad National Fellowship for Minority Students [MANF-2013-14-MUS-KER-24858] of the University Grants Commission, Government of India. Ranjeet thank the help rendered by Syam Krishna, NBFGR, Arya Sidharthan and Liju Thomas, KUFOS during molecular analysis.

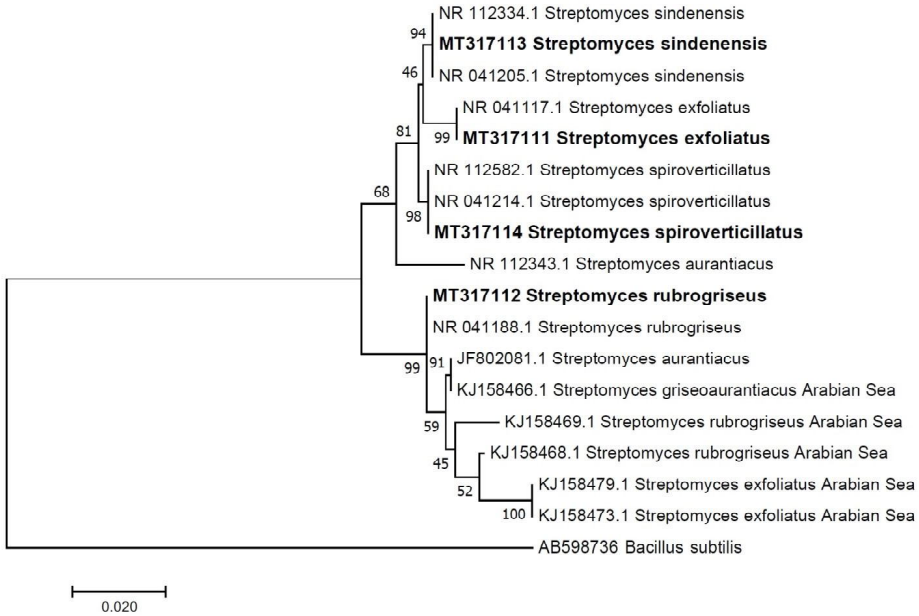

Fig. 4. Maximum-likelihood tree based on 16S rDNA gene of four different *Streptomyces* spp. isolated from Ponnani mangrove region

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