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Probiotic Characterization of Bacterial Isolates in the Gut of *Oreochromis mossambicus* (Peters, 1852)

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Abstract: In this study an attempt has been made to screen the gut microbes from freshwater fish *Orechromis mossambicus* for their probiotic effficiency. A total of 20 isolates from the gastrointestinal tract (GI tract) and skin of *O. mossambicus* were evaluated for their antimicrobial properties by agar well diffusion method against fish pathogens. Based on the better antibacterial features, 8 isolates from *O. mossambicus* were selected for further characterization of probiotic efficiency. Among the selected 8 isolates, two isolates from *O. mossambicus* showed higher tolerance to bile salt and moderate tolarence to low pH. These isolates exhibited proteolytic, amylolytic, lipolytic and cellulase activity and susceptibility to various antibiotics. 16S rRNA sequencing revealed that the isolates exhibited 99% sequence homology with *Bacillus cereus* of the database substantiating morphological and physiological characterization. Survival at low pH and bile salt authenticated their adaptability in the fish intestinal microenvironment. Absence of antibiotic susceptibility, presence of protease, amylase, lipase and cellulose activities and non-pathogenic ability of the above mentioned isolates rendered them as a potential probiotic microbes to use in aquaculture practice.

Keywords: *Enzymatic activity,* Probiotic bacteria, *Bacillus cereus, Bacillus thuringenesis*

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Introduction

Fishes receive bacteria in their digestive tract from the aquatic environment through water and food. Being rich in nutrient, the gastro-intestinal environment of fish confers conducive environment for colonization and further proliferation of microorganisms. The importance of intestinal bacteria in the nutrition and well–being of their hosts has been established for homeothermic species, such as birds and mammals. However, such information pertaining fish, the poikilothermic vertebrates is gaining momentous attention (Westerdhal *et al*., 1991).

In biological systems gut micro flora plays an important role in the digestive process, growth

and disease resistance of the host species. However, certain bacteria which possess the ability to tolerate the low pH in gastric juices, resist the action of bile acids, lysozyme secretion in intestine, elucid immune responses and adheres to the mucus or enteric wall surface and thereby persist for a relatively longer time and eventually make intestinal micro flora specific to each host animal (Westerdhal *et al*., 1991)

Furthermore, aerobic (Bairagi *et al*., 2002) and anaerobic (Ramirez and Dixon, 2003) intestinal bacteria isolated from the gut of diverse fish species have been shown to posses potential probiotic efficiency. During the last decade there has been an improved understanding on the importance of commensal intestinal microbiota in fish (Ringø *et al*., 2010). The gut microbiota may be categorized as either autochthonous (indigenous; adherent) or allochthonous (transient) depending upon its ability to adhere and colonize the mucus layer in the digestive tract (Ringø and Birkbeck, 1999; Ringø *et al*., 2003). It has been reported that Indian major carps advocated beneficial aspects of gut-associated microbiota in the host fish with regard to nutrition (Ghosh *et al*., 2002; Ray *et al*., 2010).

The gastro-intestinal tract of fish contains a number of beneficial bacteria called probiotics. The term probiotic means for life, as opposed to the term antibiotic which means against life (Tanasupawat *et al*., 2002). Fish bodies have a symbiotic relationship with probiotics. They involve diverse physiological and biochemical processes and help the host to digest food, kill harmful microorganisms and upright the immune functioning apart from synthesizing vital molecules (Sugita *et al*., 1985).

The present study aims at investigating information on enzymatic bacterial population of the cultivable freshwater fish Tilapia, *Orechromis mossambicus.*

Materials and Methods

Isolation of suspected bacterial isolates

Freshwater fishes *Oreochromis mossambicus* were collected from freshwater bodies and then transported to laboratory, with least disturbance. After transportation, the fishes were maintained in the laboratory under optimal environmental conditions (pH 7.8 and DO >5 mg/l) for 24 h. The fishes were then washed with sterile distilled water and dissected to remove the gastro-intestinal tract (GI tract) and skin under sterile condition. The GI tract and skin were homogenized in sterile distilled water and centrifuged. After centrifugation, the supernatant was taken and serially diluted with sterile distilled water in the test tubes to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions and were pour plated on nutrient agar plate. Then the pour plates were incubated for 24 h at 37 °C in incubator. Individual, well separated colonies were taken based on variation in physiological and morpho-logical characteristics and transferred to subculture. Further on subsequent quadrant streak, pure cultures representing well isolated single colonies were obtained. Selected colonies were then characterized and identified following Bergey's Manual of Systematic Bacteriology (Holt *et al*., 2009) through their colony cell morphology, gram staining, biochemical and physiological tests.

Screening of extra cellular enzymatic activity and Probiotic characterization

(i) *Protease activity*

The selected isolates were screened for the production of extra-cellular protease. In brief, cultures were streaked as single streak on skim milk agar plates and incubated at 37^{0}C for 24- 48 h. Presence of zone of clearance surrounding the culture streak was considered as a measure of protease production.

(ii) *Amylase activity*

For the screening of extracellular amylase production, cultures were streaked individually on starch agar plates and incubated at 37°C for 24-48 h. The culture plates were then flooded with 1% lugol iodine solution to identify amylase activity by formation of transparent zone surrounding the

colony (Jacob and Gerstein, 1960).

(iii) *Lipase and cellulase activity*

A loop full of isolated culture was streaked on Spirit blue agar plates and CMC plates. The plates were then incubated at 24-48 h at 37°C and formation of zone around the colony indicates the lipid synthesis and Cellulase synthesis observed by using congo red stain (Basavaraj *et al*., 2014).

(iv) *Antibiotic susceptibility test* (*AST*)

AST was performed for isolated fourteen bacterial strains against three antibiotics such as Ampicilin, Amoxycillin and Kanamycin (HiMedia – India). The single isolated bacterial culture grows in broth culture was used for AST. Kirby Bauer Disc Diffusion method was performed by using a cotton swab in which the bacterial suspension was spread on Mueller Hinton agar plate. Antibiotic discs were prepared at a concentration of 25 µg/ml, placed on the prepared MHA medium plate and incubated at 37°C for 24 h. The zone of inhibition of each disc was measured (ZOI) and expressed in mm.

(v) *Antimicrobial activity test*

Antimicrobial activity of the isolated bacterial strains was conducted on selected fish pathogens such as, *Vibrio vulinifucus*, *Vibrio parahemolytis*, *Proteus mirabilis* and *Vibrio harveyi*. Then fish pathogens and isolated bacterial strains were inoculated in nutrient broth independently and incubated for 12 h at 37 °C. The freshly prepared nutrient agar plates were swabbed with selected fish pathogens and wells were made according to the number of bacterial samples. The bacterial broth cultures were separately centrifuged at 6000 rpm for 10 min. The clear 20 ml supernatant obtained from each sample is used to fill the wells. Each 80 µl supernatant of sample was used to inoculate the respective wells on the agar plates. The inoculated plates were kept in an incubator for 24 h at 37°C. The diameter of the bacterial inhibition zone was measured by a normal millimeter scale.

(vi) *pH tolerance test*

Nutrient broth with varying pH such as 2, 4, 6, and 7 were prepared using 1 N hydrochloric acid (1N HCl) and 1 N sodium hydroxide (1N NaOH) in sterile labelled bottles. The nutrient broth media along with control were sterilized at 15 lbs pressure for 30 min and then inoculated with 12 h culture of the test bacterial samples. After two hours of incubation, optical density (OD) were measured by spectrophotometer at 600 nm, as an index for bacterial growth.

(vii) *Bile salt tolerance test*

The nutrient broths with 0.0%, 0.05%, 0.1% Oxgall bile salt were prepared in sterile labelled bottles. The nutrient broth media along with control were sterilized at 15 lbs pressure for 30 min. Nutrient broth bottles containing different concentrations of bile salts were inoculated with 30 µl of the cultured sample and incubated for 24 h at 37 °C. The growth was assessed by measuring the optical density (OD) by using spectro-photometer at 600 nm after 24 h.

(viii) *Pathogenicity test*

The pathogenicity of the experimental isolates, were tested against the fingerlings of Indian major carp, Rohu (*Labeo rohita*, Hamilton 1822) weighing 4.0–5.0 g obtained from commercial fish farms in the Tirunelveli district. Twelve 100 L plastic culture tanks, each with 10 fishes were maintained for four sets of experiment in triplicates. The fishes were acclimatized for a period of ten days and during its period they were maintained in dechlorinated water and fed with commercial diet @ 10% body weight twice daily. Each group was injected intraperitoneallywith 0.5 ml of fresh culture of either TS4 and TS7 in triplicates. Mortality was daily recorded in the groups of control and treated fish for 10 days.

(ix) *Molecular identification of test bacterial isolates: 16s rRNA*

DNA Isolation

To begin with overnight culture of test isolates were centrifuged and pellet was collected individually. The pellet was then suspended in

extraction buffer, kept in water bath for 20 min at 60 °C and re-centrifuged. The supernatant was collected and mixed with equal volume of isoproponal, centrifuged again and DNA was collected, dissolved in TE buffer for further analysis. Quality was analyzed both spectrophotometrically (Sambrook *et al*.,1989) and by running agarose gel, stored in TE buffer.

PCR amplification of 16s rRNA gene

Extracted DNA was used to amplify the 16s r RNA gene. Universal primers for 16s r RNA encoding genes were used to carry out the reaction. The primers used for amplification are 16SF5´-AGAGTTTGATC CTGGCTCAG-3´ and 16SR5´-GGTTA CCTT GTTACGACTT-3´ (Lie *et al*., 2014).

PCR reaction mixtures

The PCR cycle used was 95°C for 5 min, 95°C for 30 sec, 55°C for 30 sec, 72°C for 10 min and 10°C hold. The 1 µl of forward primer, 1 µl of reverse primer, 30 µl of PCR master mix and 2 µl of Isolated DNA was taken to run 30 cycles of PCR. The PCR parameters are in house developed and optimized for isolated DNA sample.

Sequencing the 16s rRNA gene

The product thus amplified is gel which was eluted and the product was then sequenced on ABI 3730xl Sequencer by Sangers method using internal primers specific for whole 16S region. The sequence of the insert was determined using the automated DNA sequencing service provided at Bioserve Laboratory (Pvt.) Ltd., Hyderabad, India.

Results

(i) *Screening of bacterial isolates for extra cellular enzymatic activity*

The isolated bacterial colonies from the foregut, midgut, hindgut regions and skin of freshwater fish *O. mossambicus* was qualitatively screened for extra-cellular enzyme production, such as protease, amylase, lipase and cellulase. The zone of clearance surrounding the colonies was recorded as positive result. In the fore gut, mid gut, hind gut and skin regions of *O. mossambicus*, the occurrence of amylase positive colonies was; 100%, 99.99%, 100% and 100%, respectively. Likewise, the protease positive colonies registered were; 66.66%, 40.0%, 75.0% and 80.0%, respectively from fore gut, mid gut, hind gut and skin. The lipase positive colonies represented from fore gut, mid gut, hind gut and skin were; 66.66%, 100%, 100% and 60%, respectively. In the tested regions of gut and skin, the percentage occurrence of cellulase positive colonies registerd were: 83%, 60%, 50% and 60%. Irrespective of the gut regions of *O. mossambicus*, the amylolytic enzymatic bacterial population was high when compared to other enzyme producers (Fig. 1).

(ii) *Screening and Identification of bacterial isolates*

Twenty isolates obtained from the GI tract and skin of healthy *O. mossambicus* was screened through gram staining, biochemical tests and antibacterial activity against the target fish pathogens. The results inferred that eight isolates (TS1 to TS8) out of twenty exhibited notable antimicrobial activity against the target fish pathogens. Hence, these eight isolates from *O. mossambicus* were selected for further probiotic potential screening. However, remaining tweleve bacterial isolates showed very low (<1 mm)/nil antagonistic activity against the target fish pathogens.

(iii) *Antibacterial activity test*

Antibacterial activity of the experimental strains against the test pathogens such as, *Vibrio vulinifucus*, *Vibrio parahemolytis*, *Proteus mirabilis* and *Vibrio harveyi*, indicated that among the tested 20 isolates eight isolates (TS1 to TS8) showed growth inhibition as the result of antagonistic activity. Further among the eight isolates, two isolates (TS4 and TS7) showed the highest zone inhibition in particular against both the *Vibrio vulnificus* and *Proteus mirabilis.*

(iv) *Antibiotic susceptibility test*

The susceptibility and resistance pattern obtained with the eight bacterial isolates against tested antibiotics are shown in Table 1. The interpretations of inhibition zone were determined according to zone size of chart of Kirby-Bauer test

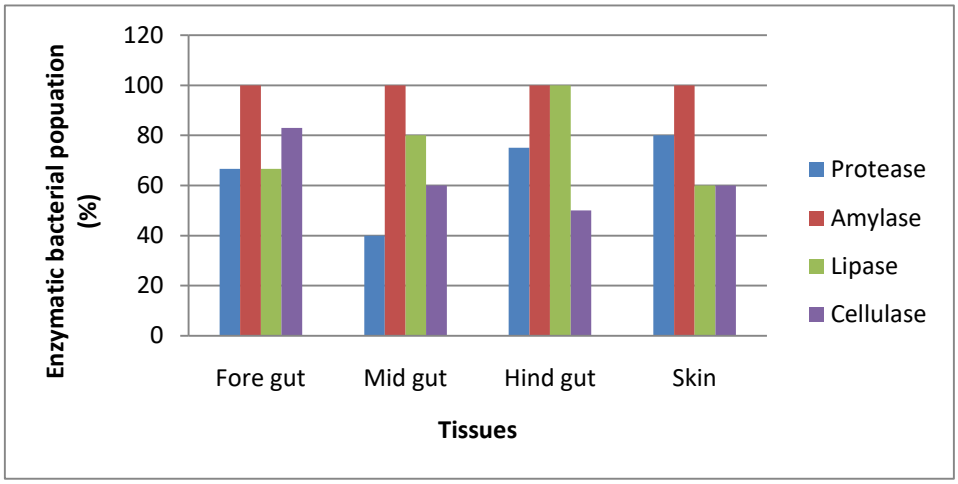

Fig. 1: Diversity of enzymatic bacterial population of freshwater fish *O. mossambicus.*

Table 1: Antibiotic sensitivity test for the suspected bacterial isolates of *O. mossambicus*

Antibiotics	**Suspected bacterial isolates**	
	TS4	**TS7**
Control	-	-
Amoxyclin	++	-
Ampicilin	+++	+
Kanamycin	++	++

- No activity; + > 5mm; ++ < 5mm; +++ <10mm

results (Bauer *et al*., 1966). The results indicated that the isolates such as TS4, TS7 were less susceptible to most of the antibiotics and developed resistance against amoxyclin and ampicilin. This antagonistic character of TS4 and TS7 isolates can be considered as a potent trait to be employed as probiotics.

(v) pH tolerance test

The tested pH ranges (pH 2, 4, 6, and 7) obviously affected the viability and growth performance of the isolated bacterial samples. Most of the bacterial samples showed viability and high growth at pH 7. Quit interestingly, two bacterial isolates such as TS4 and TS7 showed high viability and growth at pH 2 (Fig. 2). This result indicated that, these two isolated can tolerate and grow well at the lowest pH (2.0) and it's characteristic is considered as one of the most important criteria a bacterium should possess to use as probiotic organisms.

(vi) *Bile salt tolerance test*

Bile tolerance is the second important feature attributed by a probiotic strain. Bile salts play a very important role in lipid digestion and absorption. Bile salts were considered as natural bacterial growth inhibitors, hence the strain used as a probiotic supplement should exhibits tolerant to bile salts. In the present study, the bile salt tolerance for the isolated strains were tested at 0.5% and 1% bile salt. The bacterial isolates such

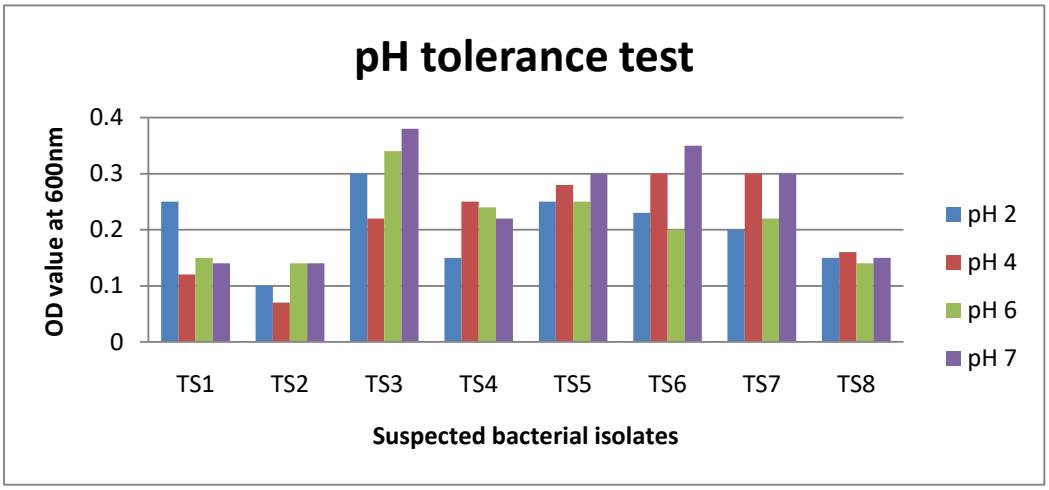

Fig. 2: pH tolerance test for the suspected isolates of *O. mossambicus.*

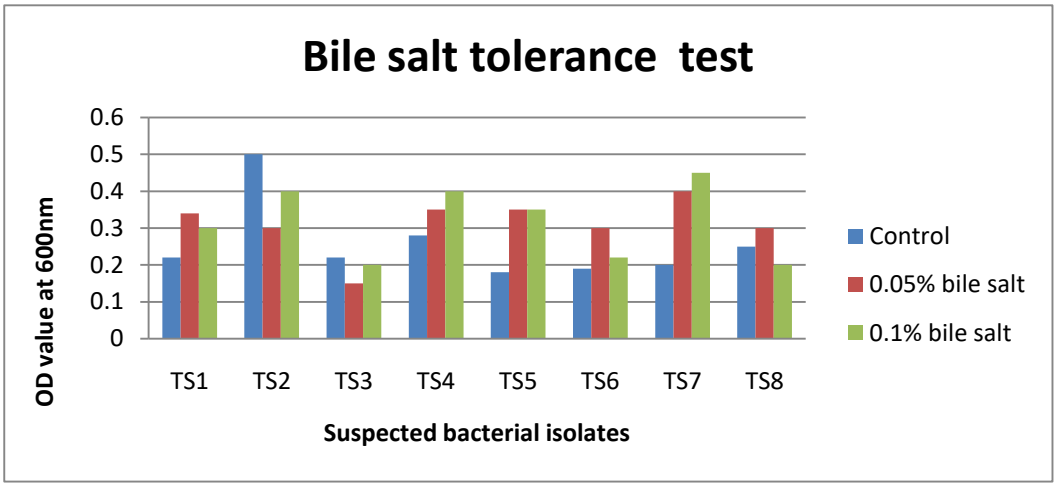

Fig. 3: Bile salt tolerance testfor the suspected isolates of *O. mossambicus.*

as TS4, TS7, CS3 and CS5 showed better bile salt tolerance when compared to the remaining ten tested isolates (Fig. 3).

(vii) *Pathogenicity Test*

Non-pathogenicity is an unique probiotic character and hence this property was tested for TS4 and TS7 bacterial isolates against the experimental fish, *L. rohita*. The result indicated that experimental fishes displayed better growth and it was even more when compared with control group. It clearly indicated the non-pathogenitic nature of these tested bacterial isolates.

(viii) *16s r RNA Gene sequencing*

Based on the probiotic traits, two out of eight isolates exhibited similar characterization. These two isolates (TS4 and TS7) were further characterized and identified through molecular techniques (16s rRNA) as *Bacilus cereus.*

Discussion

Bacteria are abundant in the environment in which fish live and it is quite possible for them being a constituent of fish diet. As opined by Strom and Olafsen (1990), the microbial community of water plays an important role in the colonization of microflora within the gastro-intestinal (GI) tract

of fish. While reaching the GI tract through food or water, the microorganisms adhere and colonize the host's gut epithelial surface or escape after some time without colonizing, depending on which the gut microbiota of fish has been classified as either autochthonous or allochthonous, respectively (Ringo and Birkbeck, 1999; Merrifield *et al*., 2010; Ray *et al*., 2012). In the present study, occurrence of considerable amount of microbial flora was detected in the skin and gastro-intestinal tract of *O. mossambicus*. Two isolates from *O. mossambicus* skin and gut were characterized morphologically and biochemically. The fish intestine is a favourable ecological niche for microorganisms, which reach must higher numbers than surrounding water (Austin,1987). Probiotic properties of the two isolates were also analysed in the present study. The antagonistic activity was caused by the release of chemical substance with bactericidal or bacteriostatic effects. The reduction of pathogen growth and cell density indicate that extracellular bacteriolytic products produced from the probiotic bacteria were responsible for this inhibition.

Enzymatic analysis of the suspected bacterial isolates (TS4 and TS7) indicated that the bacterial isolates possess the ability to degrade proteins, lipids, starch and cellulose. Amylase activity was exhibited by a majority of the isolates reflecting that the bacterial flora associated with the intestinal tract of *O. mossambicus* are capable of digesting foods rich in carbohydrates. Earlier studies have also established that bacteria in the digestive tract of fish demonstrated proteolytic, lipolytic, amylolytic and cellulolytic activities (Ghosh *et al*., 2002; Saha *et al*., 2006; Ray *et al*., 2010; Sumathi *et al*., 2011; Ariole and Kanu, 2013) and it was found to be influenced by the feeding behaviour.

The bacterial flora of the gastrointestinal tract with diversified enzymatic potential plays a vital role in major part of the metabolism of the host animal (Clements, 1997). The digestive enzymes present in fish digestive tract can elucidate some aspects of their nutritive physiology and thus be supportive to develop nutritional strategies for fish feeding and diet formulation (Alexander *et al*., 2002; Ghosh *et al*., 2002; Nibeta and Ghosh, 2008; Ray *et al*., 2010). Therefore, the indigenous multiple enzyme-producing bacteria can be effectively exploited for use as probiotics while formulating cost-effective aquafeeds of the host fish species.

Being resistant to low pH is one of the major selection criteria for probiotic strains (Cakur, 2003). So as to reach the small intestine the probiotic microbes have to pass through from the stressful conditions of stomach. Although in the stomach, pH can be as low as 1.0, in most *in vitro* assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad *et al*., 1998). The strains, resistant to low pH, were screened for their ability to tolerate the bile salt. In the present study, the two isolates such as TS4 and TS7 were screened for their ability to tolerate low pH and bile salt. Although the bile concentration of the human gastro-intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h. Tolerance to bile is important for the probiotic strains to grow and survive in the digestive tract. Salminen *et al.* (2004) reported that bile salt tolerance is required for probiotic bacterial strains to grow and survive in fish intestine. Cebeci and Gurakan (2003) determined that *L. plantarum* as a probiotic could survive in 0.3% of bile salt. The probiotics that exhibit low pH tolerance and bile salt tolerance means they not only can transit through stomach and be active in intestine; but also are able to be alive and survive in stress conditions (Cebeci and Gurakan, 2003).

Furthermore, the characterized and identified probiotic bacterium *Bacillus cereus* is a Gram-positive, rod-shaped, facultatively anaerobic, motile, beta hemolytic, spore forming bacterium commonly found in soil and food. The specific name, *cereus*, meaning "waxy" in Latin, refers to the appearance of colonies grown on blood agar.

Some strains are harmful to humans and cause food borne illness, while other strains can be beneficial as probiotics for animals. The bacteria are classically contracted from fried rice dishes that have been sitting at room temperature for hours. *B.cereus* bacteria are facultativeanaerobes, and like other members of the genus *Bacillus,* can produce protective endospores.

Conclusion

The present study revealed that the isolated *Bacillus cereus* meet out the required criteria for probiotic such asnon-pathogenicity, tolerance to harsh conditions such as low pH and high bile salt concentration. These isolates were emerged as potential probiotics.

Conflict of Interest

The authors declare no conflicts of interest.

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