

Review Article

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## Study on Melanized Shrimp Reveals *Bacillus* sp and *Acinetobacter* sp as Potential Sources for Bacterial Tyrosinase

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### ABSTRACT

Melanin is a biopolymer produced by phenol oxidases group of enzymes and plays an important role in radiational, thermal and biochemical stress tolerance. This pharmacologically active pigment has been reported to be produced by bacteria. However, there is a paucity of data for bacteria associated with melanisation in shrimp. In this context the present study was undertaken. The presence of bacteria with the extracellular phenol oxidase production from the melanized appendages of *Penaeus vannamei* is studied. Market drawn samples of white leg shrimp with melanization were checked for possible intervention of bacteria with phenol oxidase production. Out of 24 isolated checked, three bacterial isolates viz, TMA7, TMA9 and TMA10 shown potential phenol oxidase production by positive and negative screening with tyrosine. The sequencing analysis of 16S rRNA fragment confirmed that the TMA7, TMA9, TMA10 bacterial isolates were *Bacillus* sp, *Acinetobacter* sp, and *Bacillus megaterium* respectively. The study also suggests that *Bacillus* sp and *Acinetobacter* sp could be a potential source for tyrosinase which can be used in the fish processing industry.

#### Keywords

Tyrosinase, Phenol oxidase, Melanization, *Bacillus* sp

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### Introduction

Invertebrates such as shrimp lack advanced immune system and the immunity is dominated by innate immune responses. Prophenoloxidase system plays a major role in identification, sequestration and elimination of

invading pathogens. Melanization, which is performed by phenoloxidase (PO) and controlled by the prophenoloxidase (proPO) activation cascade, plays an important role in the invertebrate immune system in allowing a rapid response to pathogen infection (Amparyup *et al.*, 2013)

Tyrosinase is an enzyme that belongs to oxidoreductase group first discovered by Schoenben while studying the oxidation in mushrooms in 1856. Since then it is reported in most of the plant tissues, fungi, actinomyces and bacteria. Different bacteria are known to produce tyrosinase, such as *Streptomyces sp*, *Rhizobium*, *Pseudomonas putida*, *Bacillus thuringiensis*, *Marinomonas mediterranes*, *Ralstonia solanacearum*, *Cellulophaga thyrooxidans* (Whittager., 1995; Liu *et al.*, 2004; Mc Mahon *et al.*, 2007; Kahng *et al.*, 2009; Sanchez-amat *et al.*, 2010). Relationship of tyrosinases with the oxygen-binding and oxygen-transporting has been established (Dursewitz and Terwilliger., 1997; Decker and Terwilliger, 2000) by oxidizing monophenols to o-dihydroxyphenols and further to o-quinones. This enzyme belongs to the polyphenoloxidases family mainly known for the defense (Amparyup *et al.*, 2013), cuticle sclerotization and wound healing in crustaceans (Sugumaran, 1996, 1998).

Melanins are black or brown colored biopolymers biologically synthesized with the intermediate products viz., L-3, 4 dihydroxyphenyl alanine (L-Dopa), Dopachinone and dopamine by melanin forming enzymes such as tyrosinase and laccase. Melanin is synthesized as dopachrome and is converted to melanin by non-enzymatic reactions. Melanin has inherent properties like absorption of UV radiation, metals, sound and also have anti-oxidant and semi-conductor properties thus are used in the production of complex biopolymers (EMPA, 2010).

Melanosis, also called as black spot, the enzymatic browning of phenolic compounds in shrimp is considered as a challenge in processing industry. Melanosis in shrimp is due to innate immune response prophenoloxidase system and bacterial conversion of the phenolic compounds into

melanin with the help of phenoloxidase enzymes (Goncalves *et al.*, 2016). Shrimp melanosis due to innate immune response is well studied (Cerenius and Soderhall, 1998, 2004; Sritunyalucksana *et al.*, 1999). The shrimp pereopods were not reported to have PPO contrary to the carapace, cephalothorax pleopod and telson (Zamorano *et al.*, 2009). The bacterial associated melanosis studies were done in the context of spoilage microbes (Qian *et al.*, 2014; Charoensapsri *et al.*, 2014). However, there is a paucity in data pertaining to melanised appendages where the proPO activity is minimum and the bacterial associated melanosis other than spoilage bacteria. The pereopod melanisation in the market shrimp might be due to the action of microbes. Hence, this present study of screening bacteria as a source for tyrosinase enzyme was undertaken in shrimp melanised appendages.

## **Materials and Methods**

### **Sample collection**

*Penaeus vannamei* with melanized appendages were procured from retail fish market of Ukkadam, Coimbatore, Tamil Nadu. The sampled shrimp were brought to the laboratory in chilled condition in an insulated box and processed for the isolation within four hours of the sampling.

### **Isolation of tyrosinase producing bacteria**

The shrimp surface was sterilized with 70% alcohol and chitin shell was removed from the shrimp. Melanized appendages sampled from the shrimp were macerated and transferred to sterile tyrosinated saline (0.1% tyrosine supplemented with normal physiological saline) used for isolation of tyrosinase producing bacteria. Composition of the media used for isolation for a litre of media includes tryptone 1%, L-tyrosine 0.1%, magnesium

sulphate 0.025%, potassium phosphate dibasic 0.05%, NaCl 0.85%, Agar 1.5%). In the autoclave sterilized media, macerated melanized appendages were inoculated. Plates were incubated at room temperature for 48 h. The colonies producing dark pigmentation were taken for further identification by biochemical tests (Leifson, 1963). Confirmation of potential tyrosinase producing ability was once again checked in positive and negative selection method.

### **DNA isolation and 16S rRNA identification**

Potential tyrosinase producing isolates *viz*; TMA 7, TMA9, and TMA10 were selected for further identification. The total DNA was extracted using phenol chloroform method (Sambrook and Russel, 2006). DNA (~100ng/µl) was used as template for amplification of 16S rDNA region. The forward and reverse primers used were 27F AGAGTTTGATCCTGGCTCAG and 1492R ACGGYTACCTTGTTACGACTT respectively. The PCR master mix includes 1X Taq buffer, 2.5 mM MgCl<sub>2</sub>, 0.5 µM of forward and reverse primers each, 0.25 mM dNTP mixture and 1 U of Taq polymerase (Fermentas, USA). The PCR conditions used were as follows: 95°C for 5 min (initial denaturation); 35 cycles of 95 °C for 1 min (denaturation), 55°C for 1 min (annealing) and 72 °C for 1 min (extension) followed by final extension for 72 °C for 10 min. The amplicons of 1500 bp were visualized in gel documentation system (Biorad, Germany). Gel extraction kits were used for purification of amplicons (Sigma, USA) and outsourced for sequencing (Scigenome Pvt ltd, Cochin, India) and the data was analyzed with NCBI blast program. The 16S rRNA gene sequences were aligned using CLUSTAL W software (Thompson *et al.*, 1994). The phylogram was constructed using Mega 6 software (Kumar *et al.*, 2018). Evolutionary tree was constructed using neighborhood joining method (Tamura and Nei, 1993)

### **Results and Discussion**

The prophenoloxidase mediated melanosis distribution is organ specific *viz.*, more in the cephalothorax and very less or nil in the pereopods (Zamorano *et al.*, 2009). The melanized pereopods might be a result of bacteria mediated melanosis. The melanosis caused by spoilage bacteria such as *Carnobacterium maltaromaticum*, *Shewanella purefaciens*, *Aeromonas salmonicida* and *Fusarium solanii* were studied (Quin *et al.*, 2012; Charoensapsri *et al.*, 2014). But the bacterial associated melanosis is not elucidated in the tyrosinase producing bacterial context.

### **Screening for tyrosinase producing bacteria**

From tyrosine agar total twenty-four colonies of distinct morphology and color formation were streaked on tyrosine agar. Among them three isolates *i.e.* TMA7, 9, and TMA 10 were shown zone size more than 5 mm with maximum production of tyrosinase enzyme with positive and negative screening (Fig. 1). The three isolates were purified and biochemically characterized (Table 1). Screening of extracellular tyrosinase was first carried out by Nurudeen and Ahearn in 1979 and recognized the importance of brown pigments in the screening of tyrosinase. Plate method for screening *Streptomyces* sp was established (Lemos *et al.*, 1985) and for tyrosinase (Hagerman *et al.*, 1985). Zone of catalysis, the positive and negative screening method (Shivaveerakumar *et al.*, 2014) with and without tyrosine was used in the present study. The colonies namely TMA7, TMA9 and TMA 10, with brown coloration in the positive plates and without brown coloration in the negative plates were taken for biochemical characterization (Leifson, 1963). All the three colonies were non- oxidase but catalase producers. TMA7 and 10 were motile whereas TMA9 was non-motile. TMA7 was not having casein hydrolysis ability where the

other two bacteria shown casein hydrolysis which evidences the TMA 7 is having tyrosine oxidizing capacity not the hydrolysis like *Cellulophaga thyrosinoydans* isolated from seawater of eastern coast of Jeju island, Korea (Kahng *et al.*, 2009).

**Molecular identification of the tyrosinase producing bacteria by 16s rRNA sequencing analysis**

16S rRNA sequencing analysis revealed that the isolates TMA7, TMA9, and TMA10 had similarity to *Bacillus* sp (NCBI access no MN336256), *Acinetobacter* sp (NCBI access no MN337281), *Bacillus megaterium* (NCBI access no MN337271) respectively.

The sequence of TMA 7 was compared with NCBI available sequences with the help of blast program. Blast analysis has shown 97% identity to *Bacillus niabensis*. The bootstrap

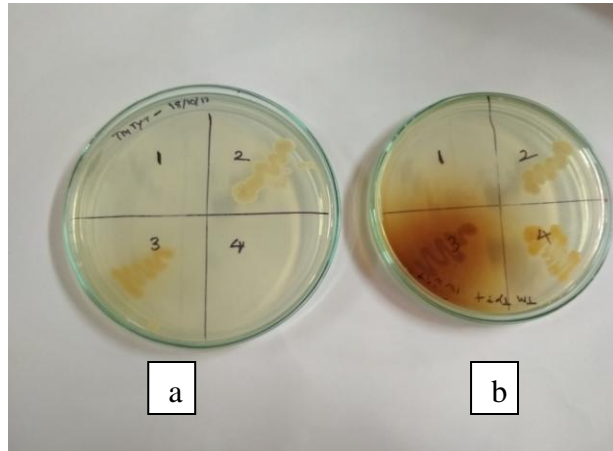
analysis with neighbor joining method was performed based on 1456 resampling. The closest relative strain was identified as *Bacillus niabensis* Marseille- p strain. Identification to species level was only 97% similarity with the closely related bacterial species in the NCBI gene bank database (Fig. 1). The next closest relative is *Bacillus niabensis* G3-1-20 KC4 (Jonathan *et al.*, 2014).

Blast analysis of sequenced TMA9 16s rRNA product revealed that 96% identity was shown to *Acinetobacter* sp. The closest strain identified was *Acinetobacter jhonsonii* MN307299 and *Acinetobacter bouvetii* strain 173 with only 96% similarity. Evolutionary tree was constructed using neighbor joining method and the phylogram was constructed using mega 6 software (Fig. 2) (Jonathen *et al.*, 2014).

**Table.1** Biochemical characteristic of tyrosinase producing bacteria

Biochemical Test	Bacterial isolates		
	TMA7	TMA9	TMA10
Oxidase	Negative	Negative	Negative
Catalase	Positive	Positive	Positive
Gram staining	Gram positive rod with spores	Gram negative diplococcic	Gram positive rod with spores
Casein hydrolysis	Negative	Positive	Positive
Tyrosine	Positive	Positive	Positive
Glucose	Positive	Positive	Positive
Mannitol	Positive	Positive	Positive
Motility	Motile	Non-motile	Motile

**Fig.1** Positive and negative screening method for isolation of tyrosinase producers



a). No discoloration of media as negative.

b). Dark brown discoloration of the media around the colonies of tyrosinase producing bacteria.

**Fig.2** Phylogram of *Bacillus* sp TMA7]

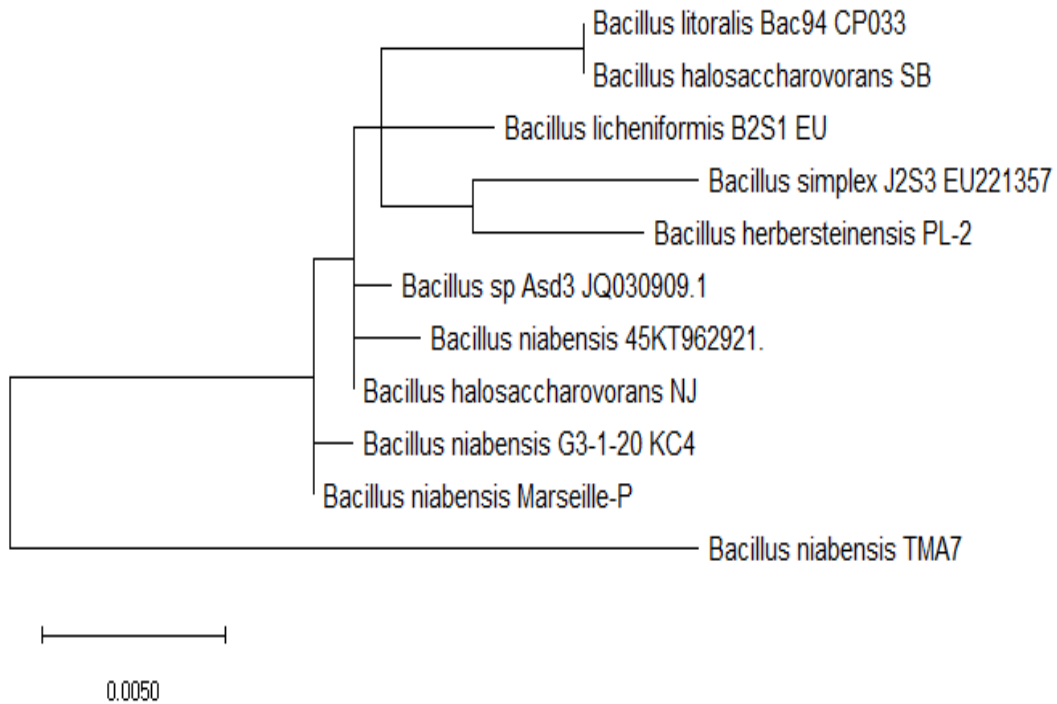


Fig.3 Phylogram of *Acinetobacter* sp TMA 9

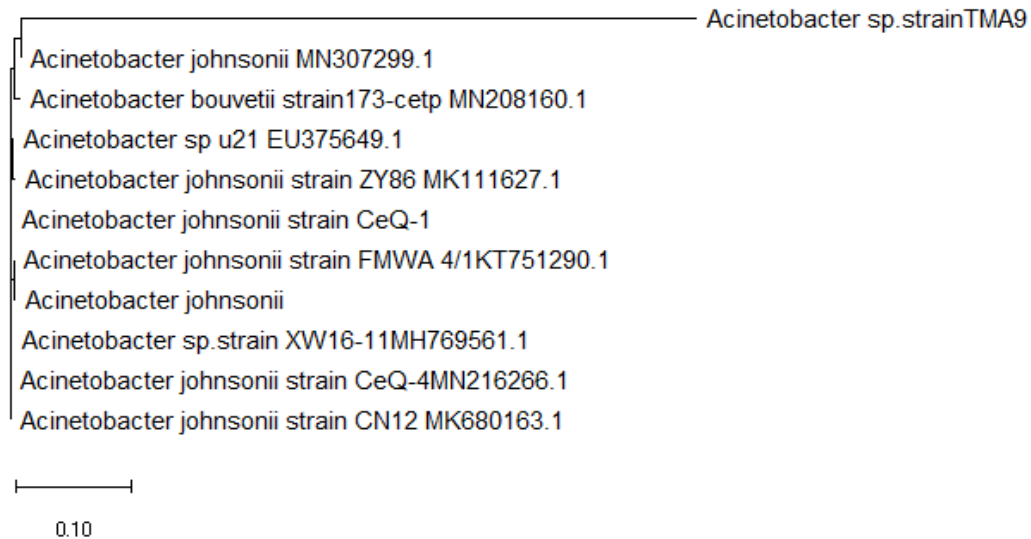
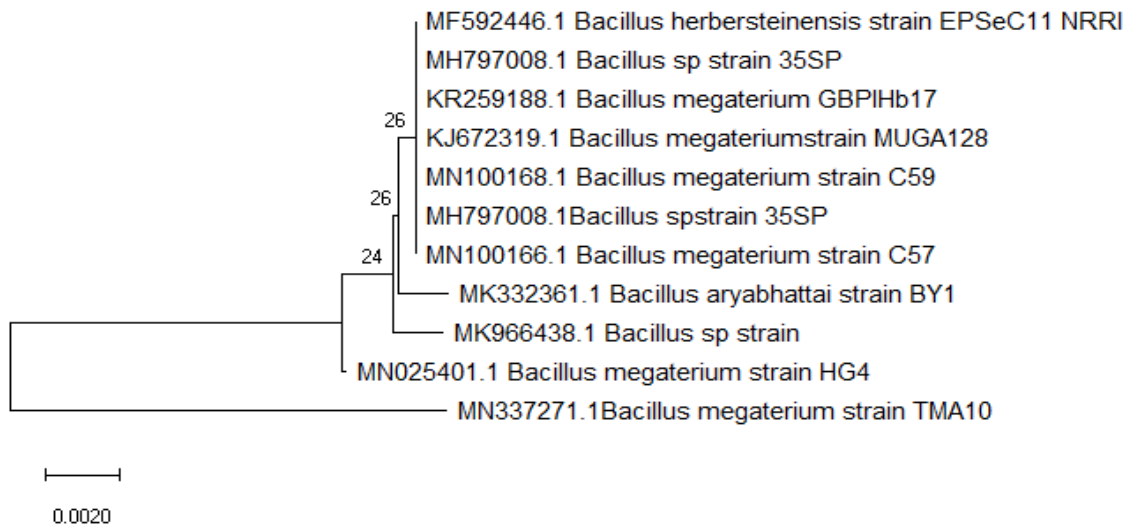


Fig.4 Phylogram of *Bacillus megaterium*



TMA 10 16S rDNA segment were amplified, sequenced using Sanger sequencing and blast analysis revealed that sequence has shown 97% similarity to *Bacillus megaterium*. The closest strain identified was *Bacillus megaterium* strain HG4. The next closest relative is *Bacillus* sp MK966438.

Evolutionary tree was constructed using neighborhood joining method. The phylogram was constructed using mega 6 software (Fig. 3 and 4).

The production of microbial enzymes is mainly based on the environment. Valipour

and Arikian (2016) reported that *Bacillus megaterium* produces enzymes as per the environment requirement. Kwon *et al.*, (2007) isolated *Bacillus niabensis marseille* strain from cotton waste for mushroom cultivation also reported to be tyrosine degrading. *Bacillus* sp isolated from Iranian soil is reported to produce cresolase, catecholase, tyrosinase and laccase (Dalford *et al.*, 2006). The substrates used were Tyrosine, Syringaldazine and 2, 6 dimethoxy phenol. *Bacillus subtilis* NA2 from potato field soil of Egypt also proved to produce tyrosinase (Elsayed *et al.*, 2018). When *Bacillus* sp is inoculated to shrimp sample it has reduced the tyrosine level (Cobb *et al.*, 1971) which shows the *Bacillus* sp can be a potential source for tyrosine enzyme production.

The *Acinetobacter* sp also found to degrade various pollutants (Sun *et al.*, 2012; Ziang *et al.*, 2013) and phenoloxidase is also one of the characteristics in *Acinetobacter calcoaceticus* PFEA-2 which was isolated from phenol polluted waters of China, utilizes phenol as a sole source of carbon energy (Xu *et al.*, 2003). Even though few studies are being carried out in the various environment for the isolation of tyrosinase producing bacteria, to the best of our knowledge this is the first reports on the association of tyrosinase producing bacteria (*Bacillus* sp and *Acinetobacter* sp) in melanized appendages of shrimp. So, these strains TMA7, TMA9 & TMA10 belonged to (*Bacillus* sp and *Acinetobacter* sp) may be a potential candidate for bioremediation in fish processing industry in future.

The study concludes that melanized appendage of shrimp may be a good source of tyrosinase producing bacteria. *Bacillus* sp and *Acinetobacter* sp are the potential tyrosinase producer identified. Further studies are needed to utilize these bacteria for bioremedial applications

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