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ENDOSYMBIOTIC BACTERIA OF HONEY BEE IN BIOSYNTHESIS OF FUMARIC ACID

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ABSTRACT

Honey bees are a subset of bees of the genus *Apis*. Apitoxin is secreted by the workers and queen of honeybee. About 1 % of the world population is allergic to bee stings, but it has also shown therapeutic effects in the treatment of joint diseases and rheumatism due to its anti-inflammatory and anti-coagulant properties. The present study deals with the determination of endosymbiotic bacteria from the abdomen of honey bee and the involvement of these bacteria in the biosynthesis of fumaric acid. The bacterial culture was isolated from the abdomen of honey bee. The presence of fumaric acid and propionic acid was confirmed with GC-MS for the presence of fumaric acid in the bacterial broth and abdomen of *Apis mallifera*. Morphological, biochemical and molecular characterization showed the isolate VITPTS was closely related to *Serratia marsescens*. Hence forth the present study proves that the isolate obtained from the abdomen of honey bee plays a pivotal role in the biosynthesis of fumaric acid and propionic acid.

Keywords: Honey bee, endosymbiotic bacteria, fumaric acid, GC-MS.

INTRODUCTION

Endosymbiosis is a condition in which one organism survives inside other,there by behaving as a single organism. The endosymbiosis theory was first coined by Konstantin Mereschkowski of Russia in the year 1910. Although many plants and animals possess endosymbiotic microbes, the family *Insecta*scores maximum. The range of this association varies from obligate mutualism till facultative parasitism [1]. One of these *Insecta* families is honey bee.

Honey bee produces apitoxin which consists of components like apamin, melittin [2], phospholipase A2 [3], adlopin, hyaluronidase [4] etc. It also contains some small amounts of phospholipides, sugars, free amino acids and pheromonen [5]. Apitoxin is a colorless bitter liquid with its active site responsible for inflammation and anticoagulation. Its pH ranges from 4.5 to 5.5. Each of its component contributes to bee venom therapy in curing rheumatism, joint diseases etc. Recently a study in Washington, has revealed that the component melittin has the ability to poke holes on the envelope of HIV without hampering nearby cells thus preventing AIDS [6]. It has been reported that the major content of the toxin i.e. melittin named by Habermann consists of 26 amino acid residues [7]. Apamine, the second major content of the toxin was also first detected in Habermann's laboratory [8].

Toxins are predominantly produced by microorganism like bacteria and fungi, bacterial toxins can be either exotoxins or endotoxins. Exotoxins are actively secreted whereas endotoxins remain as a part of the bacterial outer membrane and it is not released until the microbe is not attacked by body immunogenic cell. This may result in inflammation and pain. The effect of honey bee toxin depends on the amount of toxin released or number of stings. For a person, not hypersensitive to it will have redness swelling itching along with pain but with its higher dose, it may lead to cramp, breathing problem or even paralysis.

This paper is concerned with the involvement of these endosymbiotic bacteria in the abdomen of honey bee in production of honey bee venom. Hence we have isolated microbes from the abdomen of honey bee and extracted the secondary metabolites from it by mass multiplication and solvent extraction. The extracted toxin from the bee abdomen was compared by GC-MS techniques. The isolate capable of producing compounds of apitoxin was characterized.

MATERIALS AND METHODS

Sample Collection

The honey bee samples were collected from the VIT campus. The head part was amputated and the abdomen of the honey bees were surface sterilized using 0.9% sodium hypochlorite, 70% ethanol and sterile distilled water ("Fig. 1"). Its dorsal and ventral surfaces were impregnated in the MPYGP solid medium (1% Muller-Hinton broth, 1.5%yeast extract, 0.3% K_2HPO_4, 0.2% glucose, 0.1% Sodium pyruvate, 2% agar, pH 7) [9], so as to confirm surface sterilization ("Fig. 2").

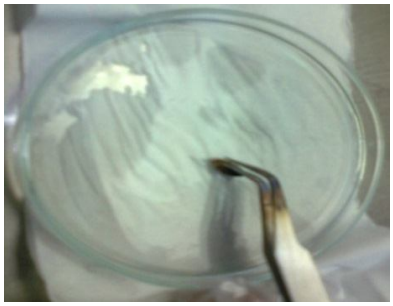

Fig. 1 Surface sterilization

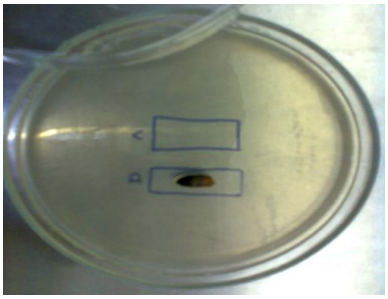

Fig. 2 Impregnation

Isolation of endosymbiotic bacteria

The surface sterilized abdomens were dissected and the semi fluid material from the internal abdominal part was taken with the help of a loop and was streaked on MPYGP solid medium. Some of freshly surface sterilized abdominal sample was homogenized with 2 % saline with the help of a mortar and pestle. About 1 ml of the crushed solution was spread on the same MPYGP solid medium. Plates were kept for incubation for 24 h at 37°C. The bacterial colony obtained from the dissection and crushed parts were named as VITPTS.

Growth kinetics

In order to determine the synthesis of secondary metabolites the growth kinetics was performed. About 2 ml of the culture was taken at 0.5 OD and was inoculated in a side-arm flask containing Luria Bertani broth. Same was repeated with the other isolate. The optical density was taken at an interval of 2 h at 600 nm. A growth curve was plotted and the stationary phase was determined [10].

Mass multiplication of the isolates

The isolate VITPTS was inoculated in a 250 ml Erlenmeyer flask, containing 100 ml of LB broth. It was kept for incubation till the O.D reached 0.5. Further 2% of the culture was taken from the flask and was inoculated in a 1 L conical flask, containing 800ml of LB broth. The flask was incubated for 7-8 days.

Extraction of solvent

About 500 ml of mass multiplied broth was taken from the flask and was centrifuged at 10,000 rpm for 8 min. The supernatant of the flask was taken in a beaker and the supernatant was evaporated by concentration technique. The sample was prepared by scraping 0.2 g of

the left over residues from the beaker in 2 ml eppendorf tube and it was mixed with 0.5 ml of polar solvent methanol.

GC-MS analysis of the isolates

For the detection of various components present in the venom and their production by our isolate the sample was given for GC-MS analysis along with the controls i.e. only the LB media and the crushed abdomen. The solvent extract was subjected to GC-MS analysis. GC-MS analysis was performed using Perkin Elmer GC model (30 m × 0.25 mm ×0.25 µm)Clarus 680 (Mass spectrometer Clarus 600 EI). The Clarus 680 GC used purified helium as the carrier gas, at a constant flow rate of 1 mL/min. One microliter of samples were injected and oven temperature was programmed from 60°C to 300°C for 2 mins at the rate of 10°C/min and then isothermally held for 6 min until the analysis was completed.

Morphological and biochemical characterization

In order to identify the isolate morphological studies were performed which includes Gram's staining, hanging drop motility and endospore's staining. Biochemical tests like indole, methyl red, Voges Proskauer, citrate was also performed for the identification of culture [11].

Identification of bacterium by 16S rRNA technique.

Bacterial strains were characterized using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). DNA was extracted from cells and the 16S rRNA sequence was determined by fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on a ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic analyzer, Tokyo, Japan). The aligned sequences were computed using ClustalW software and sequence homologies were determined using BLASTn search to create an evolutionary distance matrix [12].

RESULTS AND DISCUSSION

Isolation of endosymbiotic bacteria

Bacterial isolate was identified on the basis of its morphological characteristics. The impregnated surface sterilized honey bee samples on the MPYGP solid medium showed no colonies, whereas visible growth was observed in the plates inoculated from dissected region.

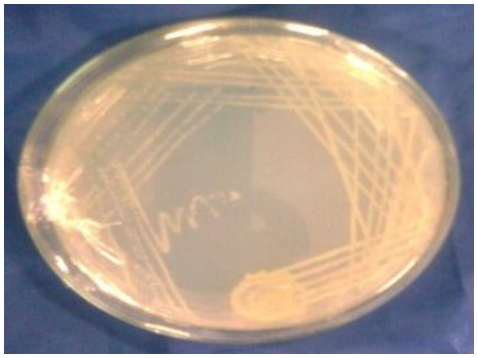

Fig. 3 VITPTS isolated in LB agar medium

Morphological and biochemical characterization

The colony morphology of VITPTS isolate showed mucoidal, round, small and white colonies (Table. 1) and biochemical characterization showed Gram negative rod, motile and non-endospore forming bacteria (Table. 2). From the result of carbohydrate fermentation it was detected that VITPTS was able to ferment glucose, sucrose and mannose but failed to ferment lactose.

Table 1. Morphological and biochemical characterization of the sample

Colony features	**VITPTS**
Shape	Circular
Margin	Undulate
Elevation	Convex
Size	Small
Texture	Mucoid
Appearance	Smooth
Pigmentation	Non- pigmented (cream)
Optical property	Translucent

Table 2. Morphological staining

Staining	**VSTPTS**
Gram's staining	-ve
Hanging drop motility	+ve
Endospore staining	-ve

Table 3. Carbohydrate fermentation

Carbohydrates	VSTPTS
Glucose	+ve
Sucrose	+ve
Lactose	-ve
Mannose	+ve

Table 4. Biochemical Characterization

Biochemical Test	VITPTS
Indole	-ve
Methyl Red	+ve
Vogesproskauer	-ve
Simmon citrate	+ve
Catalase	+ve
Oxidase	+ve

Growth kinetics of the isolate

From the growth curve it was observed that the isolate VITPTS exhibited its stationary phase at the 10th h, after inoculation ("Fig.4"). Further the isolates were incubated for 8 d for mass multiplication.

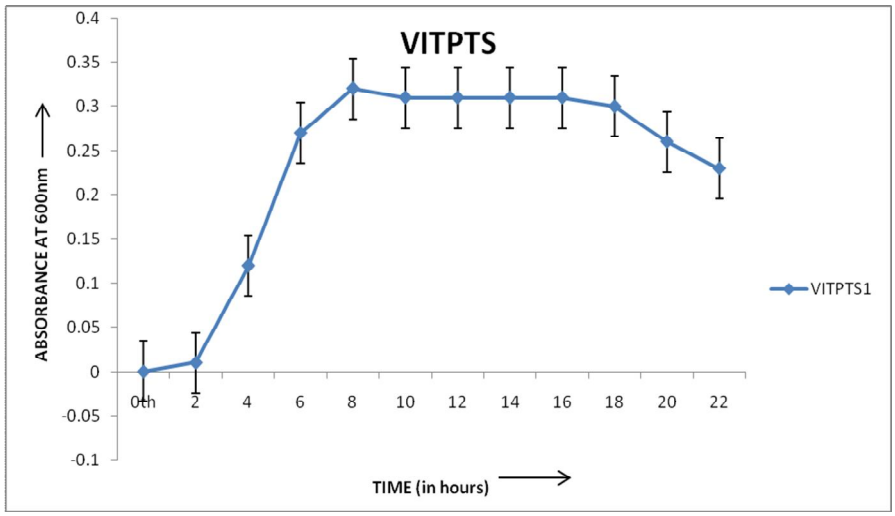

Fig. 4 Growth curve of VITPTS

GC-MS analysis of the isolate

From the GC-MS results it was concluded that fumaric acid was synthesized by the isolate VITPTS. Fumaric acid was detected in the abdomen extract and also in the bacterial broth of VITPTS. In the previous studies it has reported that fumaric acid is found in increased concentration in honey dew honey [13]. Our study proves that the bacteria VITPTS obtained from abdomen of honey bee is capable of producing fumaric acid.

Table 5. Endosymbiotic bacterial secondary metabolites

Serial no.	Compound	Molecular weight	Molecular formula
1	Fumaric acid	116.07 g/mol	$C_4H_4O_4$
2	Propionic acid	74.07854 g/mol	$C_3H_6O_2$

Molecular Characterization

Analysis of 16S rRNA and phylogenetic tree construction

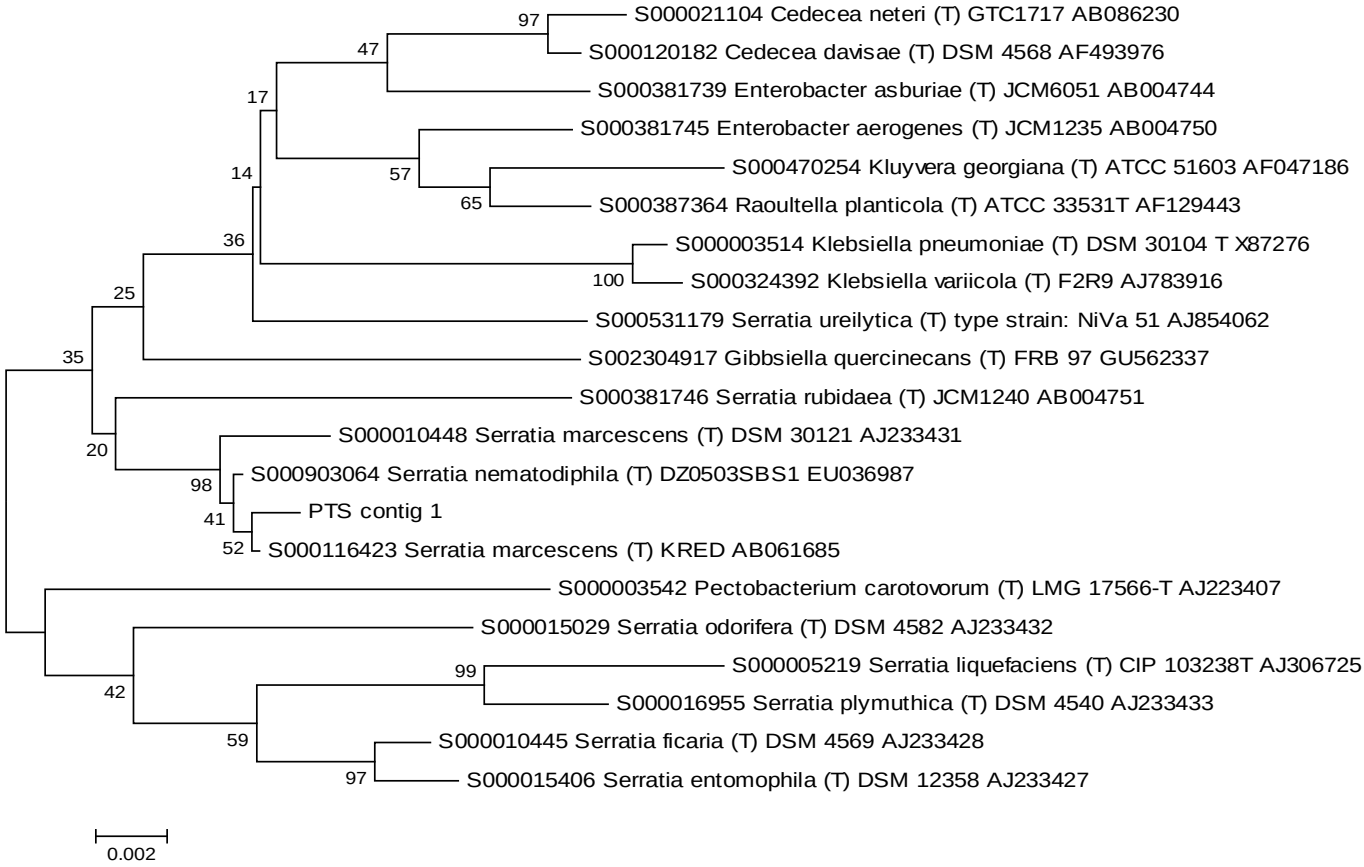

Fig.5 Phylogenetic tree of effective isolate VITPTS

Identification of 16S rRNA gene sequencing analysis ascertained the bacteria belong to the family *Enterobacteriaceae* and it has a closer relationship with *Serratia*. A phenogram reflecting the relationship among the strains and candidate sequences of related strains obtained from the NCBI database. The result obtained by 16S rRNA sequencing clearly showed that the bacterial isolate VITPTS isolated from the abdomen of honey bee is the nearest neighbor of *Serratia* with the percentage similarity of 99%. This present study is the first report of the presence of *Serratia marcescens* as endosymbiotic bacteria in the abdomen of honey bee species. Recently Endo and Salminen 2013 have reported fructophilic lactic acid bacteria from the honeybees, larvae, fresh honey and bee pollen [14].

From the above phylogenetic tree ("Fig. 5"), it was concluded that the isolate VITPTS is 99% similar to *Serratia marcescens.*

CONCLUSION

The present study concludes that the fumaric acid was synthesized by the isolate VITPTS which was also isolated from the abdomen of honey bee.

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