Trends in Life Sciences
An International Peer-reviewed Journal
www.sciencejournal.in

CULTURE DEPENDENT STUDIES ON GUT BACTERIAL FLORA OF *COCCINELLA SEPTUMPUNCTATA* (L) WITH REFERENCE TO ISOLATION, IDENTIFICATION AND CHARACTERIZATION FROM NASHIK DISTRICT (M.S.), INDIA

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ABSTRACT

The ladybird beetle *Coccinella sepempunctata*, acts a biocontrol agent against the aphids, mealy bugs, whiteflies that is damaging pest's of assorted crops. The gut microbiota of ladybird beetle is responsible for digestion of intake of proteinous food material. The isolation, identification and characterization of gut microflora was allotted by cultural dependent technique and confirmed by Vitek-2 system that is machine-controlled microbic system utilizing growth based technology. The bacterial flora was analyzed and noted as *Kocuria rhizophila, Kocuria kristinae, Kocuria rosea, Lysinibacillus fusiformis, Cronobacter sakazaki* group and *Bacillus cereus*. All bacterial strains which were isolated from gut may be pathogenic. The strains were studied for enzymatic assay like catalase, oxidase, amylase, urease, protease, gelatinase, lipase, nitrate reduclase, phsphastase, chitinase, cellulose, and L-asparaginase. The presence of these digestive enzymes within the gut region indicates that the symbiotic microorganism from gut region have a functional role in digestion and survival of insect species and indicating the gut as a principal site for enzymes secretion. This study suggests that the gut microbiota may responsible for insect's evolution, reproduction, growth and development that rely on on digestive microbes and secretion of various types of enzymes from secretory organs of gut region may have prevailing conditions in insects gut such as redox potential and pH, that play essential role in digestion to enhance it's biocontrol activity for the harmful pest.

KEYWORDS: Gut bacteria**,** *Coccinella septempunctata*, Culture based isolation, Vitek system.

INTRODUCTION

The ladybird beetles, *Coccinella septempunctata* is one of the most economically valuable biocontrol agent, they are originated in Europe and Asia, spread to middle east, India and North America, United state, Canada (Gordon, 1985; Honek and Martinkova, 2005; Maredia *et al.,* 1992). Depending upon climatic condition and availability of food source the life span of beetle is about one-two years (Beverley *et al.,* 2012). The adult and larval stage feed nearly about 5,000 aphids in its life cycle (Honek and Martinkova, 2005).Other coccinellides are the natural predators of aphids and scales insects, which ultimately decrease the population of this pest from valuable agricultural crops likes wheat, sorghum, potato, bean etc (Abassi *et al.,* 2001; Peterson *et al.,* 2005; Riddick *et al.,* 2009). During the last five years various crops were damage by sucking and chewing types of insects, such as aphids (Solangi, 2008; Lohar *et al.,* 2005). Aphids are small hemipterans that eat phloem of various plants and also transferring diseases (Tatchell, 1989). Now a day's various types of synthetic chemicals were used for controlling the pest population which ultimately pollute the environment and leads to kill the natural biopredators to controlling the agricultural pest but in spite of its economic interest it has attracted sufficient attention towards the gut content as well as the enzymes profile. The feeding potential of beetle increased with age, so investigation of the bacterial gut flora of ladybird beetle for digestion and utilization of their food was under taken.

The present investigation concerned with isolation, identification of gut content for bacterial flora present in *Coccinella septumpuncta* with its morphological, biochemical and enzyme activity was performed by cultural dependent method to find out that isolated gut microflora is responsible to digest the ingested food material with survival of beetle. Because Insect guts represent restricted environments for microbial colonization, which potentially give many desirable and esteemed characteristics to host as Insect's evolution, reproduction, growth and development with survival depend upon digestive microbes as it can withstand in extreme pH, temperature and ionic compositions within the gut and multiplies very fast as compared to in-vitro (Cash and Hooper, 2005). The microbes which are present in to the gut having beneficial functions in nutrition as well as protection from pathogenic parasites in the form of immune responses to the insect's accepting the connection among gut microbes and insects which ultimately look up for biocontrol activity for pest control, as the main center of attention for gut microbial studies (Tang *et al.,* 2012).

Lemaitre and Hoppman, (2007) suggest a number of insects become a functional model for laboratory experimental work on microbial communities. The symbiotic associations between insects and gut bacteria have been studied widely in a number of systems, mainly in termites and aphids (Breznak, 1982; Chen and Purcell, 1997; Lilburn et al., 1999; Ohkuma, 2003). The most important step up during the evolution is that the gut represents active food absorption system and availability of food determines the species diversity, in support to digestion of ingested food compounds eaten by animals are particularly able to digest by microbial fauna of midgut (Natarajan, 2012). The isolation and characterization of the gut bacteria *Klebsiella* and *Citrobacter* species from *Aspidomorpha milliaris* (tortoise beetles) are herbivores, feed on *Ipomoea carnea* (morning glory spp.), which is poisonous to cattle and human beings these beetles feed and live on this plant, and species of bacteria have capacity be helping in digestion of the toxic leaf by converting it into harmless form before it is getting absorbed by the gut of the beetle (Sharavati *et al.,* 2012).The communication within insect and bacteria is responsible for nutrition as well as behavior of insects illustrate by example that aim that the correlation within gut microflora with its host determined by the niche (Cordero *et al.,* 2012). In *Cansiliella servadeii* (Coleoptera: Leptodirini) which is an common troglobite, having a pool of bacterial communities which is colonized with live microbes like *γ-Proteobacteria, actinobacteria, firmicutes* (Paoletti, *et al.,* 2013).

In search of new genera insects are successful animals of the world in species richness, various microbes are providing some novel compounds in the form of enzymes. Various functioning microbial enzymes are they act as a biomolecules. These enzymes produced by the microbes were used on commercial level in the form of secondary metabolites. They also help in breakdown, mineralization and cycling of organic compound which is present in their food material. Most of the enzymes are derived from the insects gut sample microbiota act as principle site for enzyme production. Oluwakemi, *et al.,* (2014) studied the presence of digestive enzymes, α-amylase, β-amylase, γ-amylase, proteinase and lipase to in the fore, mid- and hindgut regions of adult male and female, *Periplaneta americana* (L.). The digestive tract of the insects harbors amylase, urease, protease, gelatinase, lipase, nitrate reduclase, phsphastase, chitinase, cellulose, L-aspanginase. These enzymes were utilized for various purposes which finally increased the economy of industrialization (N. Sangetha Kamatshi *et al.,* 2012). The consequences on culture-based method play important role in extraction of hydrolytic enzymes from the gut, which is also helpful in finding exo-enzyme produced by micro-organisms (Heo *et al.,* 2006; Kwak *et al.,* 2007; Park *et al.,* 2007). So growing interest in developing environmentally safe pest control, present studies focuses on isolation, identification and characterization of bacterial gut flora from ladybird beetles, *Coccinella septempunctata* with its morphological, biochemical and enzyme activity by cultural dependent method and identification was done by Vitek system to find out that isolated gut microflora is responsible to digest the ingested food material with survival of beetle.

MATERIALS AND METHODS

Collection and rearing of beetles: The adults, *Coccinella septempunctata* were collected by hand picking method from Tapovan, Agriculture fields of panchavati Nasik, Maharashtra, India (The area location is from Latitude 19.9942 ^{0} N, Longititude 73.7972 ^{0} E) in early morning and in evening during march 2014 to october 2014 from grassland, agricultural crops and vegetables infested with aphids, mealybugs, whiteflies. The collected beetles were brought and placed in the insects rearing cages and acclimatized at laboratory condition (temp=21±1, RH=60-65%). While rearing beetles were fed with live aphids, mealybugs, whiteflies as food.

Dissection: The collected *Coccinella septumpunctata* beetle were surface sterilized with 70% ethanol for 1 min, the dissecting plate also sterilized with ethanol and further placed in laminar air flow cabinet for 2 hours in aseptic condition All the material for the dissection were previously autoclaved at 15 lbs for 15 min, The dissection were performed under laminar air flow hood, (Lemeke *et al.,* 2003). The specimens were fixed in wax plate and dissected using sterile phosphate buffer saline (pH 6.8). The dissected gut sample of *Coccinella septumpunctata* was placed in microfuge tube with 50µl sterile phosphate buffer saline solution, vortexed at 250 rpm for 1 minute to separate microbial cells from gut.

Isolation of bacteria: The collected 50µl sample was immediately inoculated on both oligotropic media (nutrient deficient media) and complete media (nutrient rich media) The plates were incubated at room temperature 28 ^{0}C for 24-48 hours to allow the growth. The bacterial colonies were observed on both the plates. On the bases of color and morphology of the colony the single colony were picked and inoculated separately on sterile nutrient agar plates. The incubated colonies were purified by subculturing on plates & stored in refrigerator (4^{0}C) for further studies, (Doo-sang park *et al.,* 2007). The culture was identified by varieties of tests like morphological characterization (colony morphology, gram staining, motility), enzymatic assay and identification of organisms was carried out by VITEK-2 system at Bac Test Lab's, Following the Standard protocol.

OBSERVATION AND RESULTS

Morphological characteristics: Total 17 different bacterial strains were isolated from the gut samples of *Coccinella septempuntata* and coded as LB-A to LB-P to study the characteristics of the bacteria like morphological characterization according to Bergey's manual of Determinative Bacteriology.

Table 1: Results of morphological characteristics of bacterial isolates grown on nutrient agar plates.

Sample	size	shape	color	margin	elevation	consistenc	opacity	Gram	motility
LB-A	2	Circular	Cream	Entire	Convex	mucoid	opaque	+ ve rods	motile
LB-B	1	Circular	Orange	Entire	Convex	mucoid	opaque	+ ve cocci	nonmotile
LB-C	1	Circular	Orange	Entire	Convex	mucoid	opaque	-ve rods	motile
LB-D	1	Oval	White	Entire	Convex	mucoid	opaque	+ ve cocci	nonmotile
LB-E	Pinpoint	Circular	Orange	Entire	Convex	mucoid	opaque	+ ve cocci	nonmotile
LB-F	Pinpoint	Circular	Yellow	Entire	Convex	mucoid	opaque	-ve cocci	nonmotile
LB-G	2	Circular	White	Irregular	Convex	mucoid	opaque	+ ve rods	nonmotile
LB-H	1	Circular	White	Irregular	Convex	mucoid	opaque	- ve rods	Motile
LB-I	1	Oval	cream	Irregular	Convex	mucoid	opaque	+ ve rods	nonmotile
LB-J	1	Circular	White	Irregular	Convex	mucoid	opaque	- ve rods	motile
LB-K	2	Circular	yellow	Irregular	Convex	mucoid	opaque	+ ve rods	nonmotile
LB-L	3	Circular	Cream	Irregular	Flat	mucoid	opaque	+ ve rods	nonmotile
LB-M	1	Circular	White	Irregular	Convex	mucoid	opaque	+ ve rod	motile
LB-N	Pinpoint	Circular	White	Irregular	Convex	mucoid	opaque	+ ve rods	motile
LB-O*	2	Circular	White	Irregular	Convex	mucoid	opaque	+ ve rods	motile
LB-O^D	1	Circular	White	Entire	Convex	mucoid	opaque	+ ve rod	nonmotile
LB-P	1	Circular	White	Irregular	Convex	mucoid	opaque	+ ve rod	motile

Table -2- All bacterial samples obtained in pure culture form from the gut of *Coccinella septempunctata* were studied for enzyme profile.

	LB-A	LB-B	LB-C	LB-D	LB-E	LB-F	LB-G	LB-H	LB-I	LB-J	LB-K	LB-L	LB-M	LB-N	LB-O*	LB-O^D	LB-p
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Amylase	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Protease	+	+	-	-	-	-	+	+	+	+	+	+	+	-	+	+	-
Gelatinase	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
Lipase	-	-	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-
Nitrate reductase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphatase	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Chitinase	-	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+
Cellulase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LAsparginase	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Identification of organisms was carried out by VITEK system at Bac Test Laboratory, Nashik Following the Standard protocol.

Table 3-Shows the identified bacterial strain

Sample	Identified Microorganisms
LB-A	*Bacillus cereus*
LB-B	*Kocuria rhizophila*
LB-C	*Cronobacter sakazaki group*
LB-D	*Kocuriakristinae*
LB-E	*Kocuria rosea*
LB-F	*Kocuria rhizophila*
LB-G	*Bacillus cereus*
LB-H	*Bacillus cereus*
LB-I	*Bacillus cereus*
LB-J	*Bacillus cereus*
LB-K	*Bacillus cereus*
LB-L	*Bacillus cereus*
LB-M	*Lysinibacillus fusiformis*
LB-N	*Lysinibacillus fusiformis*
LB-O*	*Bacillus cereus*
LB-Od	*Bacillus cereus*
LB-P	*Bacillus cereus*

DISCUSSION

In finding better productive and secure biological control factor against aphids, mealy bugs, whiteflies etc. which is the destructive pests of vegetables and agriculture crops, studies has been performed on the determination of bacterial flora from the gut of ladybird beetle *Coccinella septempuntata*, The gut bacteria may have a symbiotic relationship to digest the ingested food by secretion of various enzymes. This study provides new information on intestinal microbiota. The isolation, identification and characterization of microbial gut flora performed on the basis of it's morphological, biochemical and enzyme activities like catalase, oxidase, amylase, urease, protease, gelatinase, lipase, nitrate reduclase, phsphastase, chitinase, cellulose, and L-aspanginase. According to preliminary observation and tests, Total 6 bacterial colonies were isolated from the gut of *Coccinella septempuntata*.

All the isolated strains were identified as gut bacterial microbiota judged by Vitek System of Microbiology which is computer programmed base method. The system is accessible in 3 formats (VITEK a pair of compact, VITEK 2, and VITEK a pair of XL) that dissent in increasing levels of capability and automation, all 3 systems accommodate constant quantitative analysis chemical agent cards that are incubated and taken mechanically. According to that, we confirmeded gut microbiota which includes the *Kocuria rhizophila, Kocuria kristinae, Kocuria rosea, Lysinibacillus fusiformis Cronobacter sakazaki group* and *Bacillus cereus* strains.

In our finding, the bacterial communities within the gut of ladybird beetle *Coccinella septempuntata* were investigated by culture dependent method in which the gut content was homogenized in phosphate buffered saline (pH 6.8) and plated on both oligotropic media and complete media, by transferring colonies to fresh agar, Total of 6 bacterial strains were isolated, like *Bacillus cereus* and *Enterobacter* spp. This finding is supported by Doo-Sang Park (2007) within the guts of several longicorn beetles, this work is also supported by Vasanthakumar A., (2006). Moore (1972) isolated *Enterobacter aerogenes, Pseudomonas fluorescens, Serratia marcescens*, and *Bacillus* spp. from the alimentary canal of the southern pine beetle, *Dendroctonus frontails* however, in our finding, we observed many genera of bacteria such as *Bacillus cereus, Enterobacter spp*. of *Cronobacter sakazaki group, Kocuria rhizophila, Kocuriakristinae, Kocuria rosea, Lysinibacillus fusiformis.*

The bacterial species *Lysinibacillus fusiformis* reported in our findings was also reported by R. Michael Lehman (2008), by using the different method like tRFLP (terminal restriction fragment analyses) on wild predatory ground beetle *P. chalcites,* which contribute to the control of insect and weed pests. This finding is supported by the report for the intestinal gut community from honeybees (Babendreier, 2007), aphids (Haynes, 2003), ant lions (Dunn, 2005), and gypsy moth larvae (Broderick, 2004). These researches confirms that the insects gut bacterial communities have *Lysinibacillus fusiformis* bacterial species

The bacterial gut content of omnivorous carabid beetles *Harpalus pensylvanicus*, *Rhynchophorus ferrugineus*, *Helicoverpa armigera* harbor a gut microbial community belonging to *Bacillus* and *Enterobacteriaceae* community. (Jonathan, 2010; Marcello, 2014; Natarajan, 2012). According to previously mentioned results the *Bacillus* and *Enterobacteriaceae* species may occur at high frequencies in insects gut microbiota. *B. cereus* originate in the rhizosphere of some plants which deriving nutrients from its host, and also give microflora of insects (Vilain, 2006). It is associate degree timeserving human infectious agent and isn't connected with infections inflicting periodontic diseases and different a lot of serious infections (Hoffmaster, 2006). Upset patients are in danger to bacteriemia, carditis, meningitis, pneumonia, and endophthalmitis (Wijnands, L. 2006). It's potential to cause general infections publically health and medical specialty considerations. Thus, the ordering sequence of *Bacillus cereus* is vital so as to determine genetic background data for coming investigations like ordering sequencing that finally facilitate in development of antimicrobial medicine. *Bacillus cereus* will metabolize a spread of compounds as well as carbohydrates, proteins, peptides and amino acids for growth and energy. In present finding *Bacillus cereus* moreover gives positive results in nitrite reductase, catalase, amylase, protease, gelatinase, phsphastase, chitinase, cellulose, L-aspanginase assay. Davender Kumar (2012) shows the positive results in Screening, isolation and production of lipase/esterase producing *Bacillus sp*. strain DVL2 which have potential in estimation of esterification and resolution reactions, we got positive results in *Kocuria kristinae*, *Kocuria rosea*, *Lysinibacillus fusiformis* and *Cronobacter sakazaki group.*

In the our finding insect's gut contain *Kocuria rhizophila* that is that the members of the genus *Kocuria*, is additionally according from an outsized sort of natural sources with class skin, soil, rhizosphere, hard foods, clinical specimens, H_2O and marine sediments, suggesting that every *Kocuria* species is very custom-made to individual niche. The bacterium *Cronobacter sakazaki* isolated from foods, water, hospitals and houses. It is an emerging opportunistic pathogen, which is responsible neonatal infections of intestinal track. Kloos,W.E (1974),reported positive results for protease test but we got negative results for protease analysis.

Kocuria kristinae found in the gut of *Coccinella septumpunctata* is also reported in skin, oral cavity and urinary track of patient (Sneath, 1986). Michael Boudewijns (2005) identified *Kocuria kristinae* by Vitek 2 Automated System Basaglia, (2002) reported that it has been recorded once in an ovarian cancer patient with catheter-related bacteremia. Stackebrandt, (1995) shows positive catalase and negative nitrate reduction test, in our finding shows the positive test for catalase, nitrate reduction, amylase, urease, gelatinase, lipase, cellulase and L-asparginase.

Kocuria rosea, species are all over gift within the setting and compose a standard flora of human's skin and mucosa, and in different mammals, (Stackebrandt, 1995). These are uncommon human pathogens and principally infect disorder persons (Dunn, 2011). Recently, this organism has been involved in brain symptom, acute rubor, infective carditis and different tube connected bacteriaemia (Tsai, 2010; Moissenet, 2012). S. Allen, *et al.,* (2006) reported that *Kocuria rosea* are catalase positive

Lysinibacillus fusiform is naturally occurring bacterium of factory wastewater and farming soil.its strains are responsible for causing disease connected to respiratory infections and ulcer formation. Suppiah (2013), isolated *Lysinibacillus fusiformis* from the gut of an estuarine fish which produce alkaline protease.

In the present study all the bacterial strains which are isolated from gut may be pathogenic and nonspore-forming bacterial pathogen multiply in the gut, but they can be recognized themselves in the haemocoel, if they have sufficient time to pass through the wall and go into susceptible cells (Coppel and Martins, 1977). The occurrence of these enzymes like catalase, oxidase, amylase, urease, protease, gelatinase, lipase, nitrate reduclase, phsphastase, chitinase, cellulose, L-aspanginase in the digestive tract of *coccinella septumpunctata* shows that the species may have ominivorus feeding habit. In present finding the presence of enzymes in the digestive track confirms that the isolated gut bacteria may be helping in the insect's survival.

The absolute results are positive the catalase, nitrate reduclase, which indicates the high presence of enzymes activity, as well as amylase gelatinase, L-aspanginase phsphastase, chitinase, protease are present approximately 70-80% in insects gut flora, which may indicate that gut because the principal site for accelerator secretion and food digestion in insects. Klowden (2007) and Nation (2008) rumored that this might result to the actual fact that these enzymes secreted by the secretion glands that square measure set in conjunction with the gut. The humour organs have existing conditions in numerous regions of insect gut like oxidation-reduction potential and hydrogen ion concentration may additionally have differential assets on organic process enzymes (Vinokurov *et al.*, 2007). A minor quantity of enzyme, oxidase, enzyme indicates that they need alternative perform like replica. this findings supported by Beenakkers (1981), within which he conclude that lipids square measure the foremost necessary sources of energy for the developing embryo. Ziegler and Van port, (2006), complete that just about all the lipids should be foreign, significantly from eaten food. Consequently, it was determined that presences of identified bacterial microbiota in the gut of *Coccinella septumpunctata* with its enzymatic activity have great effort in digestion as natural enemies, to improve its biocontrol activity.

Form the present studies it may be concluded that all the microbiota found in the gut of *Coccinella septumpunctata*, which is secreting various type of enzymes in the gut region have the symbiotic microorganism which have a functional role in digestion and survival of insect species and indicating the gut as a principal site for enzymes secretion.

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