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Isolation and Application of Pathogenic and Biological Control Bacteria from Pupae of Golden Silkworm *Cricula trifenestrata* Helfer

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ABSTRACT

The decreasing productivity of silkworm cocoon is caused by attack of bacteria, fungi, protozoa and virus. The research was aimed to isolate of suspected pathogenic and biological control bacteria from damage and healthy pupae of golden silkworm (*Cricula trifenestrata* Helfer) and to do *in vivo* test of the bacteria toward 4th instar larvae of *C. trifenestrata* and mulberry silkworms, *Bombyx mori* Linn. The isolate UPC 60 was suspected as pathogenic bacteria due to cause the mortality of silkworms larvae. The bacteria UPC 40 and UPC 71 were selected as biological control bacteria which had capability to reduce the mortality of silkworms about 56.67 and 43.33%, respectively. Based on 16s RNA gene the bacteria UPC 40 had 97% identity with *Alcaligenes faecalis*, bacteria UPC 60 had 97% identity with *Aeromonas dhakensis* and bacteria UPC 71 had 98% identity with *Pseudomonas stutzuri*. The biological control bacteria showed antagonistic activity toward the pathogenic bacteria. The outcomes of the research that the biological control bacteria expected to be used to reduce pathogenic bacteria in the maintenance of silkworm so that the production and quality of silk yarn can be improved.

Key words: Pathogenic bacteria, biological control, golden silkworm

INTRODUCTION

Indonesia is a tropical country which has diversity of wild silkworms. One of which is *Cricula trifenestrata* known as Golden Silkworm. *Cricula trifenestrata* is a member of Saturniidae family which produces wild silk. Saturniidae consists of four groups, i.e., *Antheraea*, *Samia*, *Cricula* and *Attacus* (Triplehorn and Jhonson, 2005). *Cricula trifenestrta* habitats are plants with economic value, such as mango, avocado, *Anacardium*, cinnamon, *Spondias*, walnuts, cacao and kin trees (Amin *et al*., 2008). Such wide-ranged habitats are beneficial for wild silkworm cultivation.

Cricula trifenestrata produces unique cocoon which is perforated in shape and golden in colour. It is used as handicraft materials. However, the cocoon is still inadequately available throughout the year. Zhang *et al*. (2013) reported that besides predator, the decreasing productivity of cocoon can be caused by bacteria. According to Balavenkatasubbaiah *et al*. (2014) silkworm disease are among others *Grasserie, Flacherie*, *Pebrine* and *Muscardine*. They are caused by viruses, bacteria, protozoa and fungi (Nirupama, 2014), while the decreasing productivity of *Bombyx mori* silkworm is caused by *Nosema bombycis*, *Providencia rettgeri*, *Pantotea agglomerans*, *Klebsiella* sp., *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Erwinia* sp. and *Bacillus thuringiensis*

(Zhang *et al*., 2013). Therefore, this study aimed to isolate pathogenic bacteria that are responsible for the decreasing productivity of *C. trifenestrata* to isolate its biological control bacteria and to use the bacteria for application on growth of *B. mori* and *C. trifenestrata.*

MATERIALS AND METHODS

Isolation pathogenic and biological control bacteria silkworm pupae: Cocoons were collected from avocado tree at IPB Lecturer Residence, Soka Street, Darmaga, West Java, 06°33'145"S, 106°43'129"E and transferred to laboratory for isolation purpose. Cocoons were separated based on pupae condition. Healthy and unhealthy pupae were put in different plastics that had been sprayed with 70% ethanol. Macroscopic observation was conducted to differentiate healthy and unhealthy pupae, followed by sterilisation using 70% ethanol for 2 min and rinse using distilled water. A total of 3 g of each healthy and unhealthy pupae were homogenised in 27 mL Nutrient Broth (NB) media and diluted until 10^{-8} cell mL^{-1}. Then, 0.1 mL of each 10^{-6}, 10^{-7} and 10^{-8} cell mL^{-1} dilutions were taken and grown in Nutrient Agar (NA) media. The bacteria were incubated at 30°C for 2 days before being collected and stored as stocks for further examination (Demir *et al*., 2012).

Selection and antagonistic test of pathogenic bacteria from golden silkworm *C. trifenestrata* pupae: Collected bacteria from healthy and unhealthy pupae were tested by making dots on blood agar to determine potentially pathogenic bacteria. Suspected of pathogenic bacteria are characterised by their ability to haemolyse blood agar. Suspected pathogenic bacteria were grown in NB media and incubated until 10^{-8} cell mL^{-1}. Culture of suspected pathogenic bacteria was taken 0.1 mL and plated on NA media. The collected bacteria were tested by making dots on NA media prior to incubation. Clear zone-forming bacteria are the potential suspected of biological control bacteria. Suspected of pathogenic and biological control bacteria were run through antagonistic test and both cultured in liquid medium until 10^{-8} cell mL^{--1}. A total of 0.1 mL of each suspected pathogenic bacteria was spread on NA media, while 30 µL of each suspected biological control bacteria was dropped on paper disc and placed on NA media, prior to incubation for 24 h and clear zone measurement (Shaekh *et al*., 2013).

Pathogenicity test of suspected pathogenic bacteria against silkworm: Healthy 4th instar larvae *B. mori* were run through pathogenicity test by feeding the larvae with leaves that have been given 4 mL of liquid culture of pathogenic bacterial candidate. Observation was carried out by counting the mortality of larvae after application for 4 days. The percentage of mortality was corrected following Abbott formula (Abbott, 1925):

$$\text{Mortality} = \frac{X - Y}{100 - Y} \times 100\%$$

Where:

X = Percentage of larvae mortality because of treatment
Y = Percentage mortality of larvae on control

Effectiveness test of biological control bacteria against pathogenic bacteria in *B. mori* and *C. trifenestrata* silkworm: Healthy larvae of 4th instar were run through test by feeding them with leaves that have been given 4 mL of liquid culture of biological control bacteria for

5 days, followed by liquid culture of pathogenic bacteria on 6th day while at the same time the mortality was observed until 10th day. Pathogenic and biological control bacteria capable of showing lowest percentage of mortality were singly-applied against silkworms and used for further tests.

Growth curve and antagonists activities of the selected bacteria: A total of 1 loop of each pathogenic and biological control bacteria liquid culture were inoculated into 50 mL NB media and incubated in shaker incubator until 10^{-8} cell mL^{-1}. One milliliter of each result culture were taken, grown in 100 mL NB media and incubated in shaker incubator with agitation speed of 100 rpm at 37°C for 48 h. The culture were measured once every 6 h on the density of liquid culture at 620 nm, in addition to be tested for their antagonistic activity.

Identification of selected bacteria: Morphology identification was carried out using gram staining and cell shape identification (Zhang *et al*., 2013) while molecular identification consisted of bacteria genomic DNA isolation, Polymerase Chain Reaction (PCR) and DNA sequencing. The DNA was isolated from both pathogenic and biological control bacteria. Bacteria colonies were inoculated in NB media and incubated using shaker incubator at 37°C for 18 h (overnight). A total of 1.5 mL culture bacteria was put into microtube and centrifuged at 13.000 rpm for 2 min to obtain pellets. The step was repeated twice to throw the component of bacterial cell away (Demir *et al*., 2012). Genome isolation was carried out following Geneid protocol that has been modified for its prelysis phase. The purity and concentration of DNA were measured using Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, De, USA). 16S rRNA gene was amplified using 63F(5'-CAGGCC-TAA-CACATGCAAGTC-3') and 1387R (5'-GGGCGGCGTGTACAAGGC-3') primers (Marchesi *et al*., 1998). Total PCR reaction volume was 50 μL, consisted of 25 μL Taq GoGreen Master Mix 2X, 0.5 mL (100 pmol) of each primer, 0.625 mL (250 ng) template and 23.4 mL nuclease free water. The PCR conditions used were predenaturation (94°C for 5 min), denaturation (94°C for 1 min), annealing (55°C for 1 min), elongation (72°C for 1 min) and post-elongation (72°C for 7 min) in 30 cycles. The result of which was visualized using electrophoresis gel. The PCR products were sequenced and analyzed using BLAST. The data obtained were compared to database in NCBI GenBank.

RESULTS

Isolation and selection pathogenic and biological control bacteria: Cocoons with healthy pupae *Cricula* (HPC) (Fig. 1a) are brownish yellow in colour and odourless. Cocoons with unhealthy pupae *Cricula* (UPC) (Fig. 1b) are blackish brown in colour, foul odour and produce black liquid. There were a total of 104 bacteria from healthy pupae and 101 bacteria from unhealthy pupae obtained.

Selection and antagonistic test of pathogenic bacteria from golden silkworm *C. trifenestrata* pupae: There were 5 bacteria which were capable of haemolysing blood agar. The suspected pathogenic bacteria consisted of UPC 12, UPC 51, UPC 60, UPC 80a and UPC 80b. The collected bacteria from healthy and unhealthy pupae *Cricula* were used for *invitro* antagonistic test against pathogenic bacteria. The suspected biological control bacteria were characterised by the formation of clear zone (Fig. 2). There were a total of 4 suspected biological control bacteria, i.e., UPC 40, UPC 70, UPC 71 and UPC 72 (Table 1).

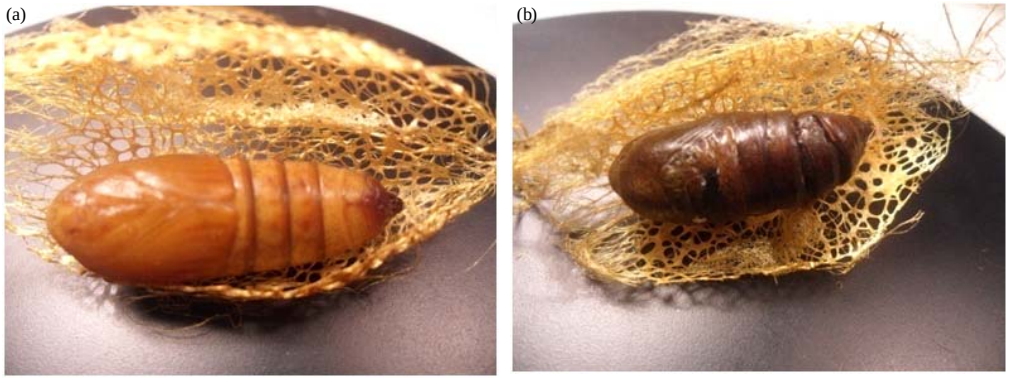

Fig. 1(a-b): (a) Healthy Pupae *Cricula* and (b) Unhealthy Pupae *Cricula*

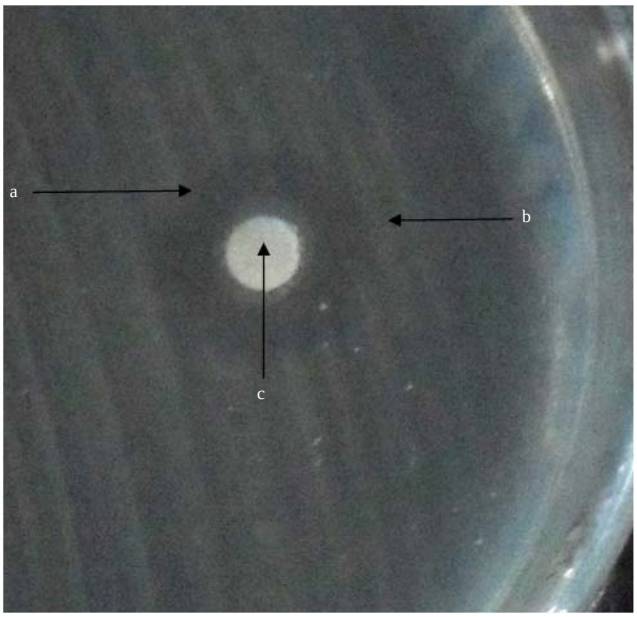

Fig. 2: *In vitro* antagonist test biological control bacteria against pathogenic bacteria, a: Clear zone, b: Pathogenic bacteria and c: Biological control bacteria

Table 1: Inhibition zone of biological control bacteria against pathogenic bacteria

Pathogenic bacteria code	Biological control bacteria code	Diameter of inhibition zone (mm)
UPC 12	UPC 70	8
	UPC 72	2
UPC 51	UPC 71	1
	UPC 72	1
UPC 60	UPC 40	7
	UPC 71	8
UPC 80a	UPC 70	10
	UPC 72	3
UPC 80b	UPC 40	10
	UPC 70	4

Pathogenicity test pathogenic bacteria against silkworm *B. mori*: Pathogenicity test of pathogenic bacteria UPC 60 against *B. mori* 4th instar larvae revealed to have the highest mortality percentage 56.82% (Fig. 3).

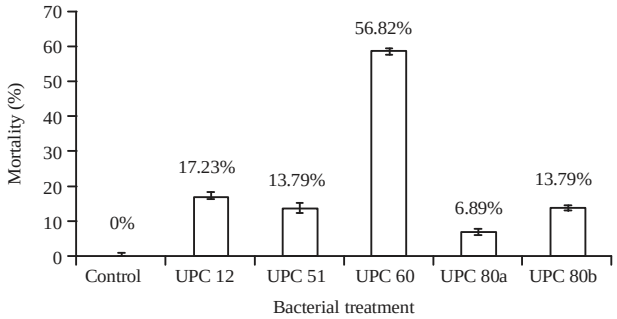

Fig. 3: Pathogenicity of 24 h suspected pathogenic bacteria against 4th instar larvae of *B. mori* for 4 days

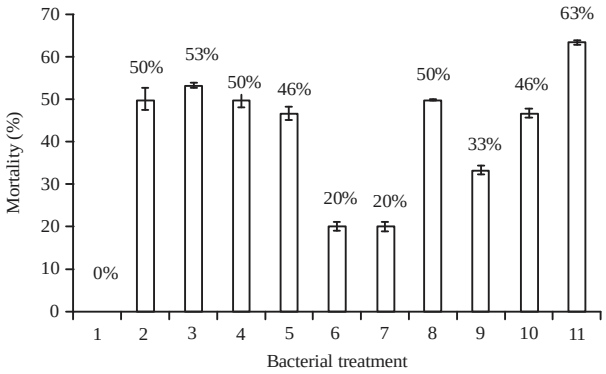

Fig. 4: Effectiveness test of biological control bacteria against pathogenic bacteria 4th instar larvae of *B. mori* for 10 days, 1: Control, 2: Bacteria UPC12+UPC70, 3: Bacteria UPC12+UPC72, 4: Bacteria UPC51+UPC71, 5: Bacteria UPC51+UPC72, 6: Bacteria UPC60+UPC40, 7: Bacteria UPC60+UPC71, 8: Bacteria UPC80a+UPC70, 9: Bacteria UPC80a+UPC71, 10: Bacteria UPC 80b+UPC 40 and 11: Bacteria UPC80b+UPC70

Effectiveness test of biological control bacteria against pathogenic bacteria in *B. mori* and *C. trifenestrata* silkworm: The lowest mortality was seen between biological control bacteria UPC 40 and UPC 70 against pathogenic bacteria UPC 60, i.e., 20% (Fig. 4). Control without any treatments did not show any mortality until 10th observation day.

Pathogenic and biological control bacteria were tested in *B. mori* for 10 days. The result revealed that pathogenic bacteria UPC 60 caused the highest mortality (96%). Biological control bacteria UPC 40 mixed with pathogenic bacteria UPC 60 was capable of decreasing the percentage of mortality to 36.67%, while biological control bacteria UPC 71 mixed with pathogenic bacteria UPC 60 to 23.33% (Fig. 5).

The result of *in vivo* test of biological control bacteria UPC 40 and UPC 71 against pathogenic bacteria UPC 60 in golden silkworm. *Cricula trifenestrata* 4th instar larvae for 7 days revealed that pathogenic bacteria UPC 60 caused 100% mortality. *In vivo* test of biological control bacteria UPC 40 against pathogenic bacteria UPC 60 reduced the mortality percentage to 43.33%, while biological control bacteria UPC 71 against pathogenic bacteria UPC 60 to 56.67% (Fig. 6).

Growth curve and antagonistic activities of the selected bacteria: Bacterial growth can be observed through the increasing cell number against time. Biological control bacteria UPC 40 were

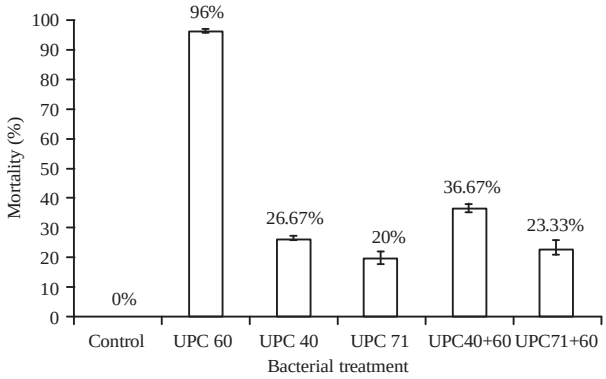

Fig. 5: *In vivo* test of biological control bacteria UPC 40 and UPC 71 against pathogenic bacteria UPC 60 to 4th instar larvae of *B. mori* for 10 days

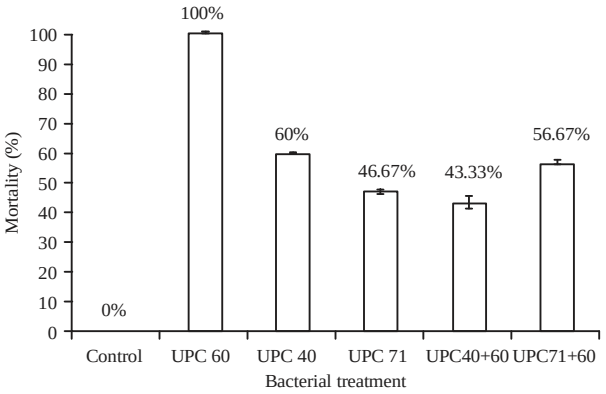

Fig. 6: *In vivo* test of biological control bacteria UPC 40 and UPC 71 against pathogenic bacteria UPC 60 to 4th instar larvae of golden silkworm *C. trifenestrata* for 7 days

in exponential phase until 24th h, followed with stationary phase until 42th h and decreased slowly (Fig. 7). Biological control bacteria UPC 71 were in exponential phase until 30th h, followed with stationary phase until 36th h and decreased at 48th h. While pathogenic bacteria UPC 60 were in exponential phase until 12th h, followed with stationary phase at 24th h until 36th h.

Test of inhibition activity of biological control bacteria UPC 40 against pathogenic bacteria UPC 60 produced 1 mm clear zone during logarithmic phase (12th-24th h) and the diameter increased to 2 mm during stationary phase (30th-36th h). Biological control bacteria UPC 71 produced 1 mm clear zone during logarithmic phase (18th-24th h) and the diameter increased to 2 mm during stationary phase (36th-48th h).

Identification of the selected bacteria: Based on the ability of biological control bacteria of inhibiting pathogenic bacteria in vitro and in vivo against *B. mori* and *C. trifenestrata* larvae been two biological control bacteria there were UPC 40 and UPC 71 and UPC 60 as pathogenic bacteria. Third isolates were identified becoming based on morphology and molecular identification. Biological control bacteria UPC 40 and UPC 71 and pathogenic bacteria UPC 60 belong to Gram negative. Isolated DNA genome of the three selected bacteria were of around 1.9 in quality at A_{260}/A_{280}. 16S rRNA gene amplification of the three selected bacteria using 63F and 1387R primers (Marchesi *et al*., 1998) generate done amplicon of around 1300 bp in size (Fig. 8).

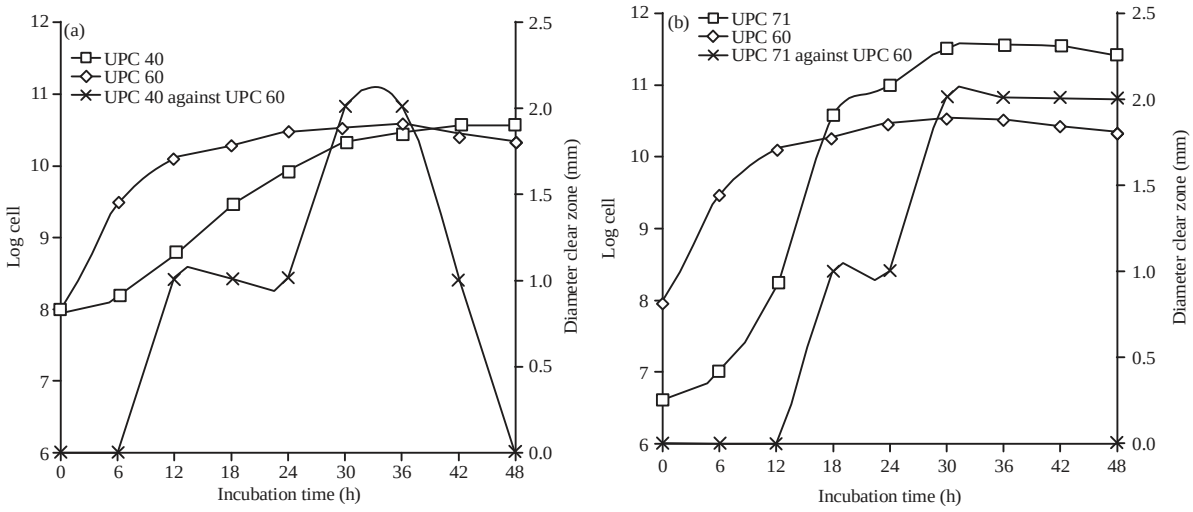

Fig. 7(a-b): (a) Growth curve of biological control bacteria, pathogenic bacteria and antagonist activity of biological control bacteria UPC 40 against pathogenic bacteria and (b) Growth curve of biological control bacteria, pathogenic bacteria and antagonist activity of biological control bacteria UPC 71 against pathogenic bacteria

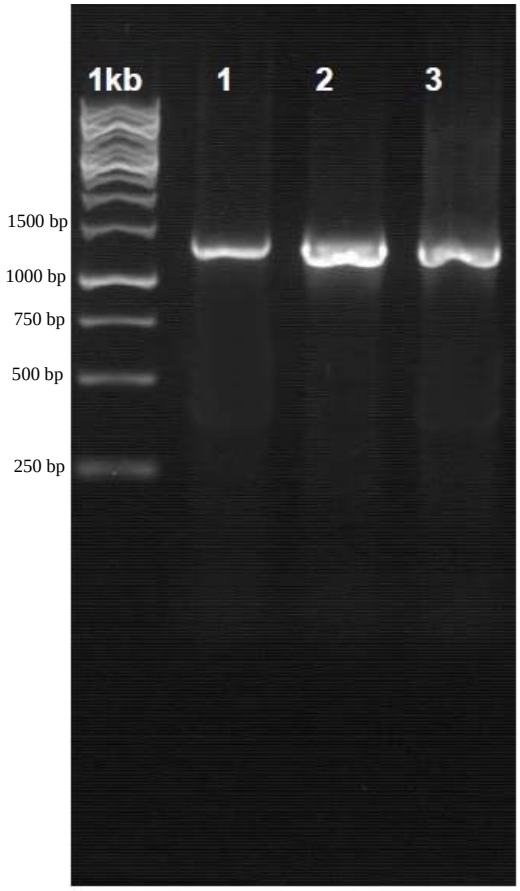

Fig. 8: Electrophoresis amplification result of three selected bacteria based on 16S rRNA gene M: 1 kb, well1, 1: UPC 40, 2: UPC 60 and 3: UPC 71

Table 2: Result of sequences analysis 16S rRNA genes of three selected bacteria at NCBI database

Bacteria description	Query cover	Identity (%)	E-value	Accession No.
UPC 40				
Alcaligenes faecalis strain NBRC 13111	100	97	0.0	NR_113606.1
Alcaligenes faecalis subsp. *parafeacalis* strain G	100	97	0.0	NR_025357.1
Alcaligenes faecalis strain IAM 12369	100	97	0.0	NR_043445.1
Alcaligenes aquatilis strain LMG 22996	100	97	0.0	NR_104977.1
UPC 71				
Pseudomonas stutzeri A1501	100	98	0.0	NR_074829.1
Pseudomonas stutzeri strain ATCC 17588	100	98	0.0	NR_041715.1
Pseudomonas stutzeri strain DSM 5190	100	98	0.0	NR_114751.1
Pseudomonas stutzeri strain CCUG 11256	100	98	0.0	NR_118798.1
UPC 60				
Aeromonas dhakensis strain P21	100	97	0.0	NR_042155.1
Aeromonas hydrophila strain DSM 30187	100	97	0.0	NR_119190.1
Aeromonas caviae strain ATCC 15468	100	97	0.0	NR_029252.1
Aeromonas enteropelogenes strain CECT 4487	100	97	0.0	NR_116026.1

Phylogenetic tree analysis showed biological control bacteria UPC 40 had 97% identity with *Alcaligenes faecalis*. Biological control bacteria UPC 71 have 98% identity with *Pseudomonas stutzeri*. Pathogenic bacteria UPC 60 have 97% identity with *Aeromonas dhakensis* (Table 2).

DISCUSSION

The usual description of bacteria life cycle comprises four phases: lag, logarithmic, stationary and death phase (Bacun-Druzina *et al*., 2011). The stationary phase is the third phase of bacteria growth, the number of bacteria has been constant between the number of dead and the number of living because of the availability of nutrients is getting a bit (Monod, 1949). Bacteria generally produce secondary metabolites such as antibiotics against other bacteria (Chellaram *et al*., 2012). The metabolic activity of bacterial cell modifies the composition of the medium in which they growth. Depending on the initial condition and on the properties of the strains one or another. The factors commonly found to be limiting be classified in one of following groups (a) exhaustion of nutrients, (b) accumulation of toxic metabolic products and (c) changes in ion equilibrium especially pH (Monod, 1949).

Bacteria infected insects particularly during in larval stage. The infected larvae are seen darker in skin colour and their body parts become softer. Internal tissue and organs are damaged and release foul odour. The high number of bacteria causes mortality (Tanada and Kaya, 1993).

Phylogenetic tree analysis (Fig. 9) indicated that biological control bacteria UPC 40 was 97% identity to *Alcaligenes faecalis*. *Alcaligenes faecalis* is a gram negative oxidase positive rod with peritrichous flagella which exists in soil and water (Kahveci *et al*., 2011). The presence of *A. faecalis* in soil and water enhances its chance of contaminating wounds and initiating opportunistic infections in hospitals and in individuals who are immunocompromised (Mordi *et al*., 2013). *Alcaligenes faecalis* produces antibiotic compounds capable of inhibiting the growth of Gram-positive and Gram-negative bacteria (Zahir *et al*., 2013). Recombinant of *A. faecalis* with *Escherichia coli* capable of producing penicillin G in high quantities (Cheng *et al*., 2007). Biological control bacteria UPC 71 was 98% identity *Pseudomonas stutzeri*. *Pseudomonas stutzeri* is known as the cause of pathogenicity in humans (Bisharat *et al*., 2012).

Pathogenic bacteria UPC 60 was 97% identity to *Aeromonas dhakensis*. *Aeromonas dhakensis* is pathogenic in fish (Soto Rodriguez *et al*., 2013) and human (Rogo *et al*., 2009). *Aeromonas* are responsible for ulcerative disease outbreaks in Mtera Dam (Shayo *et al*., 2012). *Aeromonas* species are ubiquitous bacteria in terrestrial and aquatic milieus. They are becoming renowned as enteric

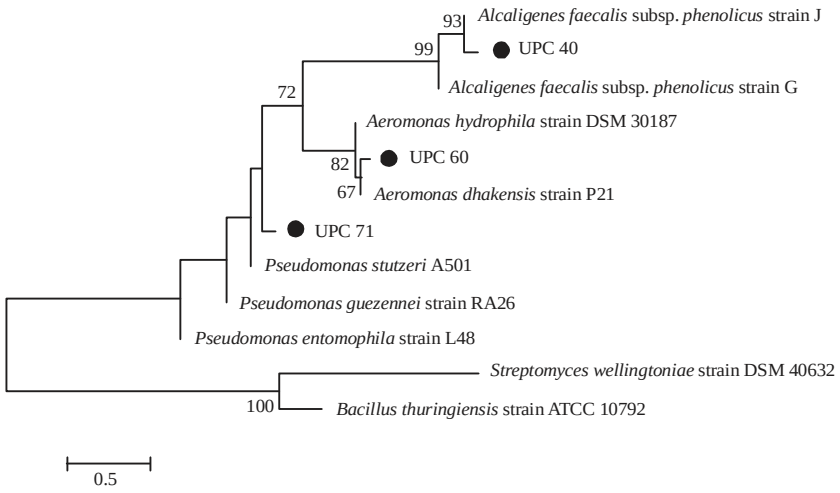

Fig. 9: Construction of a phylogenetic tree of the three selected bacteria based on amplification of gene 16S rRNA

pathogens of serious public health concern as they acquire a number of virulence determinants that are linked with human diseases, such as gastroenteritis, soft-tissue, muscle infections, septicemia and skin diseases (Igbinosa *et al*., 2012).

CONCLUSION

This research had been isolated 104 HPC bacteria from healthy pupae *Cricula trifenestrata* and 101 UPC bacteria from unhealthy pupae *C. trifenestrata* which is produce 5 pathogenic bacteria and 4 biological control bacteria. Pathogenic bacteria UPC 60 is a bacteria that causes highest mortality. Biological control bacteria, UPC 40 and UPC 71 could inhibit pathogenic bacteria UPC 60 as *in vitro* and *invivo*. Molecular identification 16S rRNA showed biological control bacteria UPC 40 have 97% identity with *Alcaligenes faecalis*, biological control bacteria UPC 71 have 98% identity with *Pseudomonas stutzuri* and pathogenic bacteria UPC 60 had a 97% identity with *Aeromonas dhakensis*.

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