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DIVERSITY OF BACTERIAL FLORA IN THE MID GUT OF FIFTH INSTAR LARVAE OF SILK WORM, *BOMBYX MORI* (L) (RACE: PM X CSR2)

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ABSTRACT
Silk worm, *Bombyx mori* (L.) (Lepidoptera: Boimbycidae) deserve significant feature with reference to silk synthesis and therefore it have been domesticated and widely used for silk production. Being a lepidopteran insect, it exhibits a typical life cycle, which includes the life stages like: egg, larval instars, pupa within a silky cocoon and adult moth. The larval instars feed voraciously on mulberry leaves, carbohydrate contents of which are composed of pectins, xylan, cellulose and starch. Some of the digestive enzymes that degrade these carbohydrates might be produced by mid gut bacteria. Eleven isolates were obtained from the digestive tract of multivoltine, cross breed silk worm, *Bombyx mori (L) (PM x CSR2).* The isolates include: the Gram positive *Bacillus circulans and Gram negative Proteus vulgaries, Klebsiella pneumonia, Escherichia coli, Cittrobacter freundii, Serratia liquefaciens, Enterobactor sp., Pseudomonas fluorescens, P. aeruginosa, Aeromonas sp. and Erwinia species. The three of these isolates, P. vulgaris, K. pneuomonae, C. freundii were found belonging to cellulolytic and xylanolytic group. P. fluorescens and Erwinia sp. were pectinolytic. The species K. pneuomniae was found degrading the starch. Aeromonas sp. was able to utilize the CMcellulose and xylan. S. liqquefaciens* was able to utilize three polysaccharides including CM cellulose, xylan and pectin. B. circulans was able to utilize all four polysaccharides with different efficacy. The mid gut of fifth instar larvae of silk worm, *Bombyx mori* (L) has an alkaline pH and of the isolated bacterial strains were found to grow and degrade polysaccharides at alkaline pH. The cellulolytic bacteria were found to increase with each instar.The nutritional contribution of mid gut microbiota and endosymbionts may be of several forms including: improved digestion efficiency; improved ability to live on suboptimal diets; acquisition digestive enzymes and the provision of vitamins.

KEYWORDS: silkworm, bacterial flora, cellulolytic bacteria, CM cellulose, xylan, pectin etc.

INTRODUCTION

Silk worm, *Bombyx mori* (L) is well known Lepidopteran (Family: Bombycidae), the larval instars of which feed on the leaves of mulberry, Morus (L). used for silk production. Being a lepidopteran insect, silk worm exhibit a typical life cycle, which include the life stages like egg, larval instars, pupa within the cocoon and adult moth. There are five larval instars, which feed voraciously on the mulberry leaves. The mature fifth instar larva spin the silky cocoon around its body and get changes into pupa. Within the silky cocoon, the pupa undergoes the metamorphic changes and gets converted into adult moth, which emerges out piercing the shell of cocoon. After mating, the female moth lays the eggs and life cycle continues. The larval instars deserve significant feature concerned with voracious feeding and utilizing the mulberry nutrients for spinning the silky cocoon. They consume 30,000 times its own weight, of mulberry leaves and grow rapidly (Fenemore and Prakash 1992). The first instar larvae feed particularly on young leaves which are rich in protein and water content. The mature instar larvage feed on mature leaves that are rich in carbohydrate with lower amount of protein and water content (Aruga, 1994).The foliage leaves are the most conspicuous organ of a plant. The structural component (primary and secondary cell wall) of leaf is composed of cellulose, xylan, pectic substance and lignin (Salisbury and Ross, 2001). Mulberry leaves are mainly composed of pectin, xylan, cellulose and starch. The cellulose compound is the main compound in the plant cell wall. The mulberry leaves (Dry Matter basis) consist of 121 g/Kg^{-1} cellulose and 107 g/Kg of hemicellulose (Kandylis *et al*., 2009). Cellulose is a biopolymer of glucose linked by β-1, 4 glycosidic linkages (Stryer, 1996). The β confirmation allows the cellulose to form a linear straight chain (Lynd *et al* , 1999). In most cases, cellulose fibers are embedded in a matrix of other structural biopolymer; primarily hemicellulose (xylan), pectin and lignin (Marchessault and Sundarajan, 1993). Xylans consist of backbone of β-1, 4 xylopyranose residues and it is less tightly associated than cellulose in plant cell wall (Warren, 1996). Pectin is a natural structural Polymer commonly found on middle lamella and in primary cell wall (Salisbury and Ross, 2001). Pectin is composed of poly (1-4)-α-D- polygactopyruanosyl uronic acid, in which neutral sugarss are covalently bound to the polymer (Cote, 1977). Starch is accumulated in chloroplast directly during photosynthesis, which is the major storage carbohydrate in plants (Jenner *et al.*,1982). It is composed of D-glucose connected by α-1-4 linkages and these linkages make starch chains to coil into helices (Setup *et al*., 1983).

TABLE 1: Chemical composition of polysaccharide substance in mulberry leaves and the enzymes required for digestion.

Polysaccharide	Plant cell wall (in %)[a]	Content in mulberry leaves	Enzymes involves digestion	Mechanism
Cellulose	9-25 (primary Cell wall)	19-25% [b]	Cellobiohydrolase (FPcellulase;EC 3.2.1.91)[d]	Cellobiohydrolase acts on the reducing or non-reducing ends of the cellulose generating either glucose or cellobiose.
	41-45 (Secondary cell wall)		Endo-beta-1.4- glucanase; EC 3.2.1.4)[d]	Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating cellobiose.
			Beta- glucosidase (cellobiase; EC 3.2.1.21)[d]	B glucosidases hydrolyze soluble cellobiose to glucose
Xylan	25-50 (Primary cell wall)	10-40% [b]	Endo-beta-1.4-xylanase (1.4-β-D-xylanase xylanohydrolase;EC 3.2.1.8)[d]	Digest xylan into oilgomers
	30 (Secondary cell wall)		Beta- xylanohydrolase (1.4-β-Dxylan sylohydrokase; EC3.2.1.37)[d]	Hydrolyze β-1.4 - xylosidic bonds of xylan resulting in xylose
Pectin	10-35 (primary cell wall)	4.6 gram% [b]	Pectin methylestrease (EC 3.1.1.11)[e]	Acts as a de-methoxylating enzylating enzyme i.e removal of methyl group from pectin resulting in demethylated pectin
			Polygalactouranase (EC 3.2.1.15)[e]	Hydrolyse polyglacturonic chain of demethoxylated pectin
			Pectin lyase [e]	Cleaves pectin in exo action pattern generating oilgomers
Starch	--	16.77 grams% [b]	α-amylase (EC 3.2.1.10)	Hydrolyze starch granules into glucose.
			β-amylase	Digest the product produced by α-amylase
			Starch phosphorylase	

[a]Salisbury and Ross 2001; [b]Lohan 1980, Sing and Makkar 2002;[c]Ghosh et al2003: [d] Warren 1996, Rexova- Benkova et al.19769; Setup *et al.*,1983

The composition of cellulose, xylan, pectin and starch in mulberry leaves, and the enzymes required for digestion of the above substrates along with their mechanism are summarized in Table 1.The significant feature of the mid gut of fifth instar larvae of silk worm, *Bombyx mori* (L) is absence of any specialized structures like diverticula. It is has been assumed that microorganisms play little part in nutrition and digestion (Appel, 1994; Bignell and Eggleton, 1995 and Vitthalrao B. Khyade, 2004). More recently, evidence has been presented that mid guts of Lepidopteran insects contain bacteria that produce digestive enzymes that help digestion of mulberry leaf constituents such as cellulose, xylan, pectin and starch (Dillon and Dillon, 2004). Here the hypothesis is tested that the digestive tract of *B. mori* contains bacteria that produce enzymes that digest polysaccharides including cellulose, xylan, pectin and starch that are normally difficult to digest. Further, it has been hypothesized that, the nutritional contributions of mid gut microbiota and endosymbionts may be of several forms concerned specially with: improved digestion efficiency; improved ability to live on suboptimal diets; acquisition of digestive enzymes and provision of vitamins. With this supposition, the study has been planned.

MATERIALS AND METHODS

The study has been planned through the materials and methods, which was consisted of : A. Rearing of larvae of silk worm; B. Isolation and characterization of bacterial flora with reference to the property of utilization of cellulose, xylan, pectin and starch from the mid gut of larval instars of silk worm, *Bombyx mori* (L); C. Enumeration of cultivable total bacteria and cellulolytic bacteria from the first to the fifth instar larvae of silk worm, *Bombyx mori* (L); D. Screening and identification of bacteria; E. Preparation of culture medium; F.Bioassy of enzyme (Cellulase, Xylanase, Pectinase and amylase) activity; and G. Subjecting the data for statistical-analysis.

A). **Rearing of Larvae of Silk worm, *Bombyx mori* (L)**

The disease free laying (DFLs) of multivoltine , crossbreed race (PM x CSR2) were procured from the sericulture unit of Agriculture Development Trust Malegaon (Baramati). They were processed for incubation (black boxing); transfer of hatched larvae to the rearing bed made up of mulberry leaves (brushing); and rearing the larvae from first to fifth instar in sterile cages at laboratory conditions (Temperature: 32 ± 1^0c and humidity: 8 2—90%). The method followed for the rearing the larvae belongs to Krishnaswami, *et al.* (1978) with modifications (Upadhyayay and Miishra (2002) and suggestions (Vitthalrao B. Khyade, 2004). Larvae were fed mulberry leaves that had been sterilized by exposure to UV light. The sterilization was done in precaution to reduce external bacterial contamination. No antibiotics were used in the precaution, and none were used by the breeder. The feeding schedule was followed for appropriate quqlity and quantity of leaves of mulberry, *Morus alba* (L) (M- 5 Variety). The rearing was repeated for three times through the use of DFLs from same source.

B). Isolation and characterization of bacterial flora with reference to the property of utilization of cellulose, xylan, pectin and starch From the mid gut of larval instars of silk worm, *Bombyx mori* (L)

The fifth instar larvae were used for the study. At 120 hours after the fourth moult, the fifth instared larvae (50 +50) were selected randomly and weighed individually on electronic balance. One group of larvae was used as stock group (of 5o larvae) and another group (of 50 larvae) was for actual study. The larvae of study group were anesthetized with chloroform soaked cotton pads and dissected in 0.9 percent saline solution. The entire alimentary canal of individual larva was aseptically separated and isolated in a UV laminar flow hood. The isolated digestive tract was washed with sterile ice-cold saline (0.9 percent NaCl) solution, chopped with a sterile blade, homogenized and incubated for 30 minutes at 37^0C. The supernatant was taken and serially diluted 1000 to 10,000 times. The pour plate method was used to estimate total bacterial count on lysogenic broth (Bertani, 2003) agar plates and on Berg's agar plates (Bergs, *et al,*1972) containing different substrates. The ability of the bacteria to degrade a substrate was checked using 0.1% carboxy methyl cellulose (CMC); 1% citrus pectin; 1% oat spelt xylan (or 1% starch), as respective substrates. Anaerobic cultures were made to screen obligative anaerobic bacteria on these substrates. The total viable count was expressed as the number of colony forming units (CFU) in 1ml of sample from substrate agar plates and lysogenic broth agar plates. Cellulolytic activity of bacteria (Cellulose degrading) in CMC medium was assyed using degradation of Whatmann No.1 filter paper in Berg's broth. As a control, a single agar plate from each batch was opened in the UV laminar flow hood for 15 minutes. This was done to check the contamination from within the hood.

C). Enumeration of cultivatable total bacteria and cellulolytic bacteria from the first to the fifth to instar larvae of silk worm, Bombyx mori (L):

Before setting the larvae for each moult (First , Second, third, fourth and fifth, the larvae (50 + 50) were selected randomly; anaesthetized; weighed and dissected in insect saline (0.9percent). And the entire alimentary canal from individual larva was separated and isolated. The isolation procedure was carried out as given described in B. section. The cellulose degrading bacteria were enumerated by serial dilution in Berg's agar plates containing CMC (Teather and Wood, 1982), while the total bacteria were enumerated on lysogenic broth agar plates. The total viable count of cultivatable total bacteria and celluloytic bacteria were expressed as the number of CFU in 1ml of sample. The experiments were repeated with different batches of larvae reared through the use of DFLs procured from original source.

D). Screening and identification of bacteria:

Colonies showing degradation capacity was assayed by plate screening using the Congo red overlay method and the

iodine method for each substrate (Wood, 1980; Hols, *et al* , 1994; Ruijssenaars and Hartsmans, 2000). Selected isolates were plated on respective agar plates for subsequent work and maintained as pure cultures. The selected colonies with degradation capacity were identified using the Congo red overlay method and the iodine method according to Bergey's Manual of Systemic Bacteriology (Sneath, et al , 1984). For the Congo red method, plates were flooded with 0.1% aqueous Congo red for 10 minutes and then washed with 1M NaCl solution . Congo red interacts with (1-4)-β-D-glucans, (1-4)-β-D-xylan and (1-4)-α-D- polygalacto-pyronosyl uronic acid. A clearing zone around the colony indicates the hydrolysis of polysaccharides namely CMC, xylan and pectin respectively (Wood 1980). For the iodine method starch plates were flooded with iodine solution resulting in dark blue plates with uncoloured zone where the starch had been degraded (Holes *et al.* , 1994).

E). Preparation of culture medium

Lysogenic broth agar was prepared using 10 g peptone, 5 gm yeast extract; 5 gm NaCl and 2% agar per liter. The pH was adjusted to 7.0 with NaOH, before adding agar to the medium and autoclaving. Isolated bacteria on plates were screened for ability to degrade various carbohydrates, using standard dyes: Congo red for cellulolytic, xylanolytic (Ruijssenaars and Hartsmans, 2000) and pectinolytic activity, and iodine for amyloytic activity (Holes et al , 1994). The following ingredients were used for the preparation of Berg's agar (Berg's et al , 1972): minimal medium without changing its composition (in g/100ml) of 0.2 gm $NaNO_3$; 0.05 gm $MgSO_4$; 0.005 gm $K2HPO_4$; 1mg $FeSO_4$; 2 mg $CaCl_2$; 0.2 mg $MnSO_4$ and 2% agar. Berg's agar with 0.1% CMC, 1% oat spelt xylan, 1% citrus pectin and 0.1% starch on respective plates as carbohydrate substrates. Except agar, all other requirements of Berg's agar minimal medium were added in the preparation of Berg's broth.

F). Bioassay for activities of enzymes (Cellulase; Xylanase; Pectinase and Amylase)

Enzyme activity for cellulase (1, 4-β endoglucanase and FP cellulase); xylanase (1, 4-β xylanase; pectinase (pectin methyl esterase and polygactouranase) and α- amylase were assayed by measuring the amount of reducing sugar liberated from the respective substrate dissolved in appropriate buffer. The reducing sugar was measured by Dinitrosalicylic acis (DNS; Miller, 1959).

For bioassay Cellulase

The substrate used for measuring 1,4-β endoglucanase (EC 3.2.1.4) and FP cellulase (EC 3.2.1.91) was one percent CMC and Whatman filter paper No.1 respectively, in 0.05M sodium phosphate buffer (pH 7.0) respectively. The enzyme action was arrested using DNS. The absorbance measured at 540 nm. One enzyme unit was defined as the enzyme amount which releases 1 µM of glucose equivalent from substrate per minute.

For bioassay of Xylanase

The substrate used for measuring 1, 4 - Beta endoxylanase (EC 3.2.8) was one percent oat spelt xylan in 0.05M potassium phosphate buffer (pH = 6.0). The enzyme action was arrested using DNS and absorbance measured at 540 nm. One unit of enzyme activity was defined as the enzyme amount that released one micro moles of xylose equivalent from oat spelt xylan per minute.

For bioassay of Pectinase

The substrate used for measuring pectin methyl esterase (EC 3.1.1.11) and polygalactouranase (EC 3.2.1.15) was 1% citrus pectin in 0.05M Sodiun phosphate buffer (pH = 7.0). The polygalactouranase was measured by stopping the reaction with DNS and reading the absorbance at 540 nm. One enzyme unit was defined as the enzyme amount which releases one micro moles of equivalent galactouronic acid per minute. Pectin methyl esterase was analyzed by the release of methanol with the help of alcohol oxidasee. The absorbance was measured at 412 nm. One enzyme unit was defined as the enzyme amount which releases one micro moles of methanol per minute,

TABLE 2: Characteristics of the bacteria isolated from the digestive tract of *Bombyx mori*

Organism	Source	Oxygen tolerance	LB agar[a]	Cellulose[a]	Xylan[a]	Pectin[a]	Starch[a]
Bombyx mori	Entire digestive tract	Faculative anaerobe	6 080 ± $3.08X10^{11}$	4. 056 ± $0.13 X 10^5$	3. 96 ± $0.15X 10^5$	3.78± $0.25X 10^2$	6.12 ± $0.14 X 10^5$
		Obligative anaerobe	2 70 ± $0.21 X10^6$				

[a]CFU/ml (Mean± SD)

For bioassay of amylase

The substance used for studying α-amylase (EC 3.2.1.10) was 1% starch. The reaction was arrested using DNS and absorbance measured at 540 nm. One enzyme unit was defined as the enzyme amount which releases one micro moles of maltose per minute from the substrate.

Enzyme activity at different pH

The isolated bacterial strains were subjected to grow on different pH ranging from pH 4.0-10 in lysogenic broth to check its growth in alkaline pH. Selected cultivatable bacterial strains were subjected to different pH ranging from pH 4.0-10.0 and analyzed for FP cellulase; 1,4-β endoglucanase; 1,4-β endoxylanase; pectin methyl esterase; polygalactouranase and amylase activity. The substrates used in the study were as described above. The bacterial strains used in the attempt were include: *S. liquefaciens* for FP cellulase and 1,4-β endoglucanase , *B. circulans* for 1,4-β

endoxylanse and α-amylase, and *Erwinia sp*., for pectin methyl esterase and polygalactouranase.

G). Subjecting the data for Statistical Analysis

All the attempts were repeated for three times for consistency in the results. The data collected was subjected for analyzing through statistical methods (Norman and Baily, 1955).Results are expressed in terms of Mean; ± SD of three replicates and sign.

RESULTS

The results on bacterial flora in the mid gut of larval instars of silk worm, *Bombyx mori* (L) are presented in the sequence as in materials and methods, which include : Isolation of bacteria from the mid gut of larval instars of silk worm, *Bombyx mori* (L) and their Identification ; Isolates of bacteria utilizing polysaccharides from the mid gut of larval instars of silk worm, *Bombyx mori* (L) ;

1). Isolation of Bacteria from the mid gut of larval instars of silk worm, *Bombyx mori* (L) (PM x CSR2) and their identification

The results on cultivatable bacteria in the mid gut of larval instars of silk worm, *Bombyx mori* (L) (PM x CSR2) are presented in table - 2. The total cultivatable bacterial count of entire mid gut was found to be 6.080 (± 3.08) x $I0^{11}$ CFU/mI of larval instars of silk worm, *Bombyx mori* (L) mid gut suspension for cultivatable facultative anaerobic bacteria and 2.7 (± 0.21) x 10^6 CFU/ml for cultivatable obligatory anaerobic bacteria (Table 2). The ANOVA studies shows exhibit statistical significance between each instar of silk worm, *Bombyx mori* (L) (PM x CSR2) and cultivatable cellulose facultative anaerobic bacteria (P>0.O5). Eleven isolates were selected from the facultative bacteria and characterized biochemically. These colonies were found to be *BacilIus circulans* (L) ; *Proteus vulgaris* (L) ; *Klebsiella pneumonia* (L) ; *Escherichia coli* (L) ; *Citrobacter freundii* (L) ; *Serratia liquefacien* (L) ; Enterobacter sp. *; Pseudomonas fluroscens* (L) ; *Proteus aeruginosa* (L) ; Aeromonas sp. ; and Erwinia sp. The bacterial species *Proteus aeruginosa* (L) and E. coli were found not to utilize any of the polysaccharide substrates used: cellulose, xylan, pectin and starch. Given its omnipresent nature, *E. coli* might have been a contaminant. No obligatory anaerobic bacteria were isolated from the mid gut of larval instars of silk worm, *Bombyx mori* (L) (PM x CSR2) with the property to degrade cellulose, xylan, pectin or starch. The reason might be that those bacteria may not be cultivatable with the available methods. No fungal colonies were observed during the experiments. There were no colonies growing control plates. This clearly indicates that, there was minimal contamination from the outside.

2). Isolates of bacteria utilizing polysaccharides from the mid gut of larval instars of silk worm, *Bombyx mori* (L) (PM x CSR2)

The bacterial count concerned with the total cultivatable cellulose degrading was found to be 4.056 (+0.13) x 10^5 CFU/ml mid gut suspension of larval instars of silk worm, *Bombyx mori* (L).

TABLE - 3: Bacteria isolated from the digestive tract of *Bombyx mori*

Characteristic feature	Isolate I
Grams stain	+
Morphology	Rod
Motility	+
Cellulose utilization	+
Xylan utilization	+
Pectin utilization	+
Starch utilization	+
Catalase	+
Indole	-
Dihydroxyacetone	-
VP[a]	-
Citrate	+
Acid from	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
Gas from glucose	-
Identified as	*Bacillus circulans*

TABLE 4: Gram-negative bacteria of Enterobacteriaceae family isolated from the mid gut of larval instars of silk wom, Bombyx mori (L).

Characteristic feature	Isolate 2	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 9	Isolate 10
Gram stain	-	-	-	-	-	-	-
Morphology	R	R	R	R	R	R	R
Celllulose utilization	+	+	+	+	+	-	-
Xylan utilization	+	+	+	+	-	-	-
Pectin utilization	-	-	-	-	-	+	-
Starch utilization	-	+	-	+	-	-	-
Motility	+	+	+	-	+	+	+
Indole production	-	(-)	(+)	-	-	+	+
Methyl red test	(+)	(+)	(+)	+	-	-	(+)
VP	(-)	(+)	(-)	-	(+)	+	(-)
Cirtate utilization	(+)	(+)	(-)	+	(+)	-	(-)
H_2S production	+	(-)	(+)	(-)	(-)	+	(-)
Oxidase	(-)	-	(+)	(+)	+	(+)	(+)
Catalase	(+)	+	(+)	(+)	+	(+)	+
Nitrate reduction	+	+	+	(+)	+	(+)	+
Ornithine decarboxylase	-	+	-	(-)	(+)	(-)	+
Urease	+	(-)	+	-	-	-	(-)
Phenylalanine deaminase	-	-	+	-	-	-	-
Urease	+	(-)	+	-	-	-	()-
Phenylanine deaminase	-	-	+	-	-	-	-
Gelatin liquefaction	-	(+)	+	-	-	+	-
KCN, growth in	+	+	+	+	+	+	-
Gas form glucose	+	(+)	(+)	+	+	+	+
Acid form							
Glucose	+	+	+	+	+	+	+
Lactose	+	-	-	+	+	+	+
Sucrose	+	+	+	(-)	+	(+)	+
Sorbitol	+	+	-	+	+	+	+
Manitol	+	+	-	+	+	(+)	+
Identified as	Cirtobacter freundii	Serratia liquefaciens	Proteus vulgaris	Klebsiella pneumonia	Enterobacter sp.	Erwina sp.	E.coli

R, Rod;VP, Voges Proskauer

(+/-)Main test for identification of this species.

TABLE 5: Characteristic features of Aeromonas sp., isolated from the mid gut of larval instars of silk worm, *Bombyx mori* (L).

Characteristic feature		Isolate 3
Gram stain		Negative
Morphology		Rod
Motility		+
Cellulose utilization		+
Xylan utilization		+
Pectin utilization		-
Starch utilization		-
Oxidase		(+)
Catalase		(+)
Nitrate reduction		+
Ornithine decarboxylase		(-)
Indole production		+
Methyl red test		+
VP		-
Cirtate utilization		+
H_2S production		-
Urease		(-)
Phenylalanine deaminase		-
Gelatin hrdrolysis		(+)
KCN, growth in		+
Gas form glucose		-
Acid form		
	Glucose	+
	Lactose	-
	Sorbet	+
	Manito	+
Identified as		*Aeromonas sp.*

VP, Voges Proskauer, (+/-)Main test for identification of this species.

TABLE 6: Characteristics features of Pseudomonas species isolated from the digestive tract of *Bombyx mori*

Characteristic feature		Isolate 8	Isolate II
Gram stain		-	-
Morphology		R	R
Motility		+	+
Cellulose utilization		-	-
Xylan utilization		-	-
Pectin utilization		+	-
Starch utilization		-	-
Growth at 4^0c		+	-
Diffusible nonfluorescent		(-)	(+)
Pigment production		(Fluorescent green)	(Blue- green)
Indole production		+	+
Methyl red test		-	-
VP		+	+
Cirtate utilization		+	+
H_2S production		-	-
Urease		(-)	(+)
Gelatin hrdrolysis		+	+
Acid form			
Glucose		+	+
	Lactose	-	-
	Sucrose	-	-
Identified as		Pseudomonas fluorescens	Pseudomonas aeruginosa

From that , seven isolates were selected with cellulolytic activity. Among the seven isolated bacterial colonies, one isolate belongs to Gram-positive group and other six isolates were found to be Gram-negative group. The Gram-positive bacteria found to *Bacillus circulans* (L). The Gram-negative bacterial isolates were *Proteus vulgaris* (L); *Klebsiella pneumonae* (L); Enterobacter sp. ; *Citrobacter freundii* (L); *Serratia liquefaciens* (L) and Aeromonas sp. Except the Aeromonas sp., other bacterial isolates utilizing CMC were found to utilize Whatmann No.1 filter paper in the Berg's broth. This confirm the Cellulolytic nature of Isolates of bacteria in the mid gut of larval instars of silk worm, *Bombyx mori* (L)(PM x CSR2) .The total cultivable xylanolytic bacterial colonies were found to be 3.96 ($\pm$ 0.15) x 10^5 CFU/ml of the suspension of mid gut of larval instars of silk worm, *Bombyx mori* (L) (PM x CSR2). The isolates utilizing xylans were found to *Bacillus circulans* (L); *Citrbacter freundii* (L); *Klebsiella pneumoninae* (L); *Proteus vulgaris* (L); *Serratia liquefaciens* (L) and Aeromonas sp. Total cultivatable pectinolytic bacterial colonies were about 3.78 ($\pm$ 0.25) x10^3 CFU/ml of the suspension of mid gut of larval instars of silk worm, *Bombyx mori* (L) (PM x CSR2). And the isolates found to belong : *Bacillus circulans* (L); *Pseudomonas fluorescens* (L) and Erwinia sp. The total cultivatable starch degrading bacterial colonies were about (6.12$\pm$ 0.14) x 10^5 CFU/ml of the suspension of mid gut of larval instars of silk worm, *Bombyx mori* (L) . And these isolates belongs to : *Bacillus circulans* (L); *Serratia liquefaciens* (L) and *Klebsiella pneumonia* (L).

3). Identification of isolates of Bacteria in the mid gut of larval instars of silk worm, *Bombyx mori* (L) :

The bacteria mentioned in the Table - 3 are the Gram-positive strain bacteria found in the mid gut suspension and they confirmed as *Bacillus circulans* (L).The isolated strains of Gram-negative bacteria were rod shaped. Upon biochemical classification (Bergey's Manual of Systematic Bacteriology), these isolates were confirmed to belong to the Family Enterobacteriaceae (summarized in Table - 4). The isolate with morphology of straight rod was confirmed to be *Aeromonas s*pecies (Table - 5). Members of the genus Pseudomonas was identified by their positive result for motility, indole utilization, VP, citrate utilization, glucose fermentation, oxidize reaction and nitrate reduction and negative results for methyl red and H_2S production (Table - 6).

4). Cellulolytic bacteria in the mid gut of larval instars of silk worm, *Bombyx mori* (L)

Enumeration cultivatable bacteria from the mid gut of larval instars of silk worm, *Bombyx mori* (L) show that, there was a gradual decrease in the total number. In contrast, there was a sharp increase in the total cellulolytic bacterial count. Both trends were found to be

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