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ISOLATION, CHARACTERIZATION AND ROLE OF GUT BACTERIA OF THREE DIFFERENT MILLIPEDE SPECIES

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ABSTRACT

Bacterial strains from the gut of three different millipedes, *Arthrosphaera magna*, *Alacobolus newtoni* and *Spinotarsus colosseus* were isolated and screened for their ability to produce different hydrolytic enzymes that are important in digesting the plant materials. In each millipede species, one predominant strain of amylase, cellulase, chitinase, xylanase and protease producer were isolated. Based on the clear zone formation, it is confirmed that 15 isolates such as *Alcaligenes faecalis*, *Citrobacter freundii*, *Bacillus pumilis*, *Corynebacterium sp, Corynebacterium kutcheri*, *Pseudomonas aeroginosa*, *Streptococcus mitis*, *Lactobacillus fermentum*, *Micrococcus roseus*, *Micrococcus varians*, *Corynebacterium xerosis*, *Lacobacillus casei*, *Enterobacter aerogenes*, *Bacillus cereus* and *Bacillus subtilis* have the ability to digest the plant carbohydrates and proteins.

KEY WORDS: *Arthrosphaera magna, Alacobolus newtoni, Spinotarsus colosseus, Xylanase*

Soil fauna play an important role in soil ecosystem through numerous interactions with microbes which can influence microbial species composition and faunal activities can indirectly affect decomposition rates and nutrient cycles. Soil macrofauna are known to contribute significantly to the breakdown of leaf litter by fragmentation in turn it favours the installation of bacteria and fungi by increasing the available surface for colonization and hence may accelerate decomposition (Kheirallah, 1990). It has been shown that macroarthropods, such as millipedes and isopods change the chemical composition of the leaf material during ingestion and favours the establishment of soil bacterial population (Tajovorsky et al., 1991).

Soil millipedes mainly feed on decomposing organic material and greatly affect decomposition processes both directly, through fragmentation of organic material and indirectly through the stimulation of microbial population and dissemination of their propagules (Lavelle and Spain, 2001). Leaf-litter feeding millipedes affect decomposer soil microorganisms, which enhance the activity of the latter and often increases biomass (Anderson and Bignell, 1980). Further, diplopods support the temporary microbial symbionts that destroy cellulose and pectin. Millipedes deprived of symbionts and own enzymes are largely characterized by low assimilation rates. Apparently to avoid this and get inoculated, these diplopods repeatedly eat their own faecal pellets (Striganova, 1980). Though, millipedes feed the polysaccharides like cellulose and xylan, but are not well equipped with specialized enzymes to enable them to digest the plant litter itself. It is suspected that microorganisms in the alimentary channel play a crucial role in the digestion of such food materials and indirectly influence the fluxes of nutrients (Maraun and Scheu, 1996) and converts the organic matter into humus (Dangerfield,1990). It is believed that the symbiotic gut microorganisms of millipedes split the cellulose components by their own enzymes. Taylor and Crawford (1982) reported that cellulose and hemicellulose degradation in a millipede gut is due to a rich aerobic microbial population. In the light of information presented above, the bacterial populations from the gut of three different millipede species such as *Arthrosphaera magna*, *Alacobolus newtoni* and *Spinotarsus colosseus* have been isolated, characterized and their role in hydrolyzing carbohydrates and proteins are also investigated in the present study.

MATERIALS AND METHODS

Adult millipede species, *A. magna*, *A. newtoni* and *S. colosseus* were collected from the reserve forest of

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Alagarmalai Hills ($10^0 0'$-$10^0 30'$ N and $75^0 55'$-$78^0 20'$E) and maintained on sterile food in laboratory at room temperature (28 ± 1^0C) for one week. These millipede species were surface sterilized with 1% $Hgcl_2$ in ethanol for 2 minutes and washed with sterile water. The alimentary canal of the test animals were dissected out and washed with sterile Ringer's solution. For isolation of THB (Total heterotrophic bacteria), 1g of gut sample was suspended in 99ml sterile distilled water, agitated for few minutes in a shaker and the sample was serially diluted on $10^{-4,}$ 10^{-5}, 10^{-6} and10^{-7} dilutions. In each dilution, 0.1 ml of sample was spread on petri plates containing nutrient agar medium and incubated at 37^0C for 24hrs and the colony density was observed and expressed as CFU g^{-1} dry weight of millipede gut. Representative colonies were selected, sub cultured and stored at 4^0C. The selected screening media were used to isolate the bacterial strains for the following enzymes production namely, amylase, protease, cellulose, chitinase and xylanase by using standard methods. The utilization of the substances, carbohydrate and protein were determined by observing the clear zone formed around the colonies. The screening experiments were performed in triplicates. The selected bacterial strains were identified using standard biochemical tests according to Bergey's Manual of Systemic Bacteriology.

RESULTS

The average wet weight of the gut was 0.33± 0.02, 1.02± 0.01, and 3.29± 0.10 g for *A. magna*, *A. newtoni* and *S. colosseus* respectively. The p^H of the gut content of *A. magna* (6.4), *A. newtoni* (6.7) and *S. colosseus* (6.3) was also recorded. The total bacterial count in the gut was remarkably higher in *S. colosseus* ($4.7x10^{11}$ CFU g^{-1} dry wt.) than *A. magna* ($3.8x10^{11}$ CFU g^{-1} dry wt.) and *A. nwetoni* ($3.1x10^{11}$ CFU g^{-1} dry wt.). The amylase, protease, cellulose, chitinase and xylanse producing bacterial count vary from species to species. Similarly, maximum density of amylase (2.6×10^{11} CFU g^{-1}) and protease ($1.4x10^8$ CFU g^{-1}) producers were observed in *S. colosseus* and it was minimum in *A. newtoni* (amylase: $1.0x10^9$ CFU g^{-1}, protease: $1.0x10^6$ CFU g^{-1}). But very high density of cellulase ($4.3x10^{10}$ C CFU g^{-1}) and xylanse ($5.7x10^8$ CFU g^{-1}) producers were observed in *A. newtoni* and low cellulase ($1.7x10^{10}$ CFU g^{-1}) and xylanase ($4.0x10^9$ CFU g^{-1}) producers were observed in *S. colosseus* and *A. magna* respectively. Density of chitinase ($3.7x10^{10}$CFU g^{-1}) producers was higher in *A. magna* and lower ($1.9x10^{10}$CFU g^{-1}) in *A. newtoni* (Table, 1). Through the screening medium, fifteen predominant strains of hydrolytic and proteolytic bacteria were isolated from three species of millipedes (Table, 2). The clear zones formed as a result, indicates that 15 strains were capable of producing hydrolases and proteolases enzymes. Based on biochemical characterization, they were identified through different tests such as staining, morphology, motility and ability to produce acids from carbohydrates, utilized citrate, indole production, pyruvic acid and metabolism, oxidasese synthesis and also utilization of different substrate like Arabinose, Xylose, Lactose, Sucrose, Raffinose, Galactose, Maltose and Manitol etc. were presented in table.,3,3a &3b

DISCUSSION

Millipedes are associated with many other organisms that also inhabit soil surface and subterranean environments. These include, but are not limited to bacteria, fungi, nematodes, nematomorphs, annelids, insects and mites. The actual decomposition of complex molecules from fragmented leaf litter and wood is accomplished almost exclusively by microorganisms that reside in the soil system, specifically bacteria, which are the predominant organisms possessing the enzymes capable of breaking down the complex compounds produced by plants. They produce many enzymes for the decomposition of plant compounds such as cellulases, hemicellulases and other cellulolytic enzymes. The conditions of the millipedes gut (high humidity and low oxygen) are such that it creates a suitable habitat for anaerobic bacteria. The roles of the microorganisms associated with millipedes and the role of the millipedes themselves is complicated by the practice of coprophagy, the ingestion of faces of one's own or of another. Communities of microbes present on faeces can be a source of nutrition for millipedes and / or a source of various enzymes which the millipedes cannot produce

themselves. The results of Bignell (1989) experiments in which bacteria were stripped from litter in digestion through the gut of the millipedes led him to conclude that coprophagy is potentially important to millipedes. Interestingly, the passage through the gut tends to repopulate the faeces with bacteria. Most of the investigations revealed that ingestion by millipedes results in significant alteration of bacterial communities. Generally millipedes are attracted to the products of decomposition which are the results of the presence of microbes which can change the chemical and physical characteristics of the soil or litter making it more appetizing for millipedes. By utilizing cellulolytic enzymes bacterial colonies decompose the litter surface and the resulting sugars are what may attract millipedes. Millipedes cannot degrade plant material to the stage where simple sugars are generated. When feeding on soil, the millipede tended to put their anterior body into the soil and at the same time, faecal egestion on the soil surface was observed. These faecal pellets formed a macro-aggregate, and bacterial activity in the aggregate is known to be enhanced by passage through the animal gut.

A comparison of the elemental composition of the leaf litter and the millipede feaces quite clearly shows that whilst the more labile, metabolically useful carbon results in loss of organic matter it is only a minor loss. The majority of organic matter (non-hydrolysable macro-molecules, lignin, etc) the remains in a more intractable, condensed state following passage through the millipede gut (Rawlins et al., 2007). This strongly supports the conclusion that such faecal material constitutes a highly stabilized and recalcitrant form of organic matter in the terrestrial environment with the changes in biochemical composition brought about the gut passage being a significant factor in stabilization (Rawlins et al., 2007). Szabo et al., (1990) had isolated over 100 species of bacteria from millipedes guts.

Consumption of leaf litter will provide the pill millipede with the bulk of its amino acid requirements. However, consuming leaf litter would also mean the consumption of bacteria and fungi growing on the leaf litter, which are stimulated by passage through the gut of the pill millipede (Anderson and Bignell, 1980). The presence of gut bacteria in the millipede is also a possible source of the amino acids detected in the faecal pellets due to the excretion of the gut lining and associated symbiotic microorganisms. The degradation of glucose, predominantly sourced from cellulose suggests the presence of appreciable cellulolytic activity in the gut of the millipede, associated with either endogenous cellulases or symbiotic gut bacteria capable of cellulose degradation. The findings of the present study suggest that symbiotic bacteria of three millipede species are involved in metabolic process, such as synthesis of essential enzymes for digestion of plant litter for example cellulose, chitin, xylan and some proteins. As the millipede species is essential and important component for forest ecosystem, the gut bacteria of millipedes can able to synthesize many enzyme for digestion of plant material, some essential nutrient are derived from these materials after the action of gut bacteria of millipedes. The raw materials of plants cannot directly utilize the plants, hence it is complex and unassimilable forms. The gut bacteria of millipedes act on complex plant substance and converted into simple assimilable nutrient form. Moreover, millipede is a predominant organism for enrich the fertility of soil and recycling the plant material in ecosystem with the association relationship of gut bacteria. This observation corroborated the results of Gilbert and Hazlewood (1993), in which bacteria produced cellulases and xylanases. Similarly, the presence of bacterial species belonging to *Klebsiella, Sarcina, Bacillus* and *Corynebacteriaum* were also reported in the gut of millipede *Schizophyllum sabulosum* (Baleux and Virares,1974). Hence, the present study confirms that 15 different bacterial strains are present in these different species of millipedes play a crucial role in the degradation and digestion of complex plant materials..

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Table1: **Colony density of different enzyme producing bacteria isolated from three millipede species**

Enzyme	Density (CFU/g^{-1} dry wt.)		
	A. magna	*A. newtoni*	*S. colosseus*
THB	$3.8x10^{11}$	$3.1x10^{11}$	$4.7x10^{11}$
Amylase	$1.6x10^{10}$	$1.0x10^{9}$	$2.6x10^{6}$
Protease	$1.1x10^{10}$	$1.0x10^{6}$	$1.4x10^{8}$
Cellulase	$2.0x10^{9}$	$4.3x10^{10}$	$1.7x10^{10}$
Chitinase	$3.7x10^{10}$	$1.9x10^{10}$	$2.9x10^{10}$
Xylanase	$4.0x10^{9}$	$5.7x10^{8}$	$5.0x10^{8}$

Table 2: Selected bacterial strains isolated from the gut of different species of millipede

Enzyme	Millipede species		
	A. magna	*A. newtoni*	*S. colosseus*
Amylase	*Alcaligenes faecalis*	*Citrobacter freundii*	*Bacillus pumilis*
Cellulase	*Corynebacterium sp*	*Corynebacterium kutcheri*	*Pseudomonas aeroginosa*
Chitinase	*Streptococcus mitis*	*Lactobacillus fermentum*	*Micrococcus roseus*
Xylanase	*Micrococcus varians*	*Corynebacterium xerosis*	*Lacobacillus casei*
Protease	*Enterobacter aerogenes*	*Bacillus cereus*	*Bacillus subtillus*

Table 3: Characteristics of gut bacteria of *A. magna*, *A. newtoni* and *S. Colosseus*

Biochemical test	*B. subtilis*	*B. cereus*	*E. aerogenes*	*B. pumilis*	*C. freundii*
Gram´s staining	+	+	–	+	–
Morphology	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Catalase	+	+	+	+	+
Indole	–	–	–	–	–
Methyl red	–	–	–	–	–
Voges proskauer	+	+	+	+	+
Citrate utilization	+	+	+	–	+
Starch hydrolysis	+	+	+	–	–
Gelatin hydrolysis	+	+	+	+	+
Spore test	+	+	–	+	+
Glucose	+	+	+	+	+
Arabinose	+	+	–	+	+
Xylose	–	+	+	–	–
Lactose	+	+	+	+	+
Sucrose	+	+	+	+	+
Raffinose	–	+		–	–
Galactose	–	–	+	–	–
Maltose	–	+	+	–	–
Manitol	+	–	–	+	+
Oxidase	+	+	–	–	–

Table 3a:Characteristics of gut bacteria of *A. magna*, *A. newtoni* and *S. Colosseus*

Biochemical test	***M. roseus***	***L. fermentum***	***S. mitis***	***L. casei***	***C. xerosis***	***M. varians***
Gram´s staining	+	+	+	+	+	+
Morphology	Cocci	Rod	Cocci	Rod	Rod	Cocci
Motility	_	-	+	-	+	+
Catalase	+	-	-	-	+	+
Indole	_	+	+	+	+	+
Methyl red	_	-	+	-	+	+
Voges proskauer	_	-	_	-	_	_
Citrate utilization	+	+	+	+	+	+
Spore test	_	-	_	-	_	_
Glucose	+	+	+	+	+	+
Arabinose	_	+	_	+	+	_
Xylose	_	_	_	_	_	_
Lactose	-	+	-	+	_	_
Sucrose	+	+	+	+	_	+
Raffinose	-	-	-	_	_	_
Galactose	-	-	-	_	_	_
Maltose	-	-	-	_	_	_
Manitol	-	+	+	+	+	_
Oxidase	+	+	+	+	+	+

Table 3b: Characteristics of gut bacteria of *A. magna*, *A. newtoni* and *S. Colosseus*

Biochemical test	***A. faecalis***	***P. aeroginosa***	***C. kutcheri***	***Corynebacterium* sp.**
Gram´s staining	–	-	+	+
Morphology	Rod	Rod	Rod	Rod
Motility	+	+	–	–
Catalase	+	+	+	+
Indole	–	–	–	–
Methyl red	–	–	–	–
Voges proskauer	–	–	–	–
Citrate utilization	+	+	–	–
Starch hydrolysis	–	+	+	+
Gelatin hydrolysis	–	+	–	–
Spore test	–	–	–	–
Glucose	+	+	+	+
Arabinose	–	+	–	+
Xylose	–	–	–	–
Lactose	–	+	–	–
Sucrose	+	+	–	–
Raffinose	–	+	–	+
Galactose	–	–	–	+
Maltose	–	+	+	–
Manitol	–	–	–	–
Oxidase	+	+	–	–

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