

Symbiotic Flagellated Protozoa Isolated From Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier), (Coleoptera: Curculionidae)

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Abstract: Cultures of flagellated protozoa FP-007 isolated from the hindgut of the red palm weevil, *Rhynchophorus ferrugineus*. Results showed that the isolated protozoa required NaHCO₃ and fetal bovine serum for good growth; the presence of yeast extract is stimulatory. Under these conditions, H₂ was a major protozoan fermentation product. Hydrogen production was closely paralleled to cell yields. Flagellated protozoa FP-007 used powdered cellulose, corncob and cereal leaves as fermentation energy sources, on the other hand chitin showed no growth. Under these conditions H₂ was a major protozoan fermentation product. The improved growth of the Flagellated protozoa FP-007 in vitro should facilitate further studies on the cell biology and biochemistry of these symbiotic, anaerobic protozoa.

Key words: Flagellated protozoa, red palm weevil, *Rhynchophorus ferrugineus*, Hydrogen production

INTRODUCTION

Red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) is the most dangerous and deadly pest of date, coconut, sago and other palms (Cox, 1993).

Normally, the red palm weevil prefers to infest palms below the age 20 years, where the stem of the young palm is soft, juicy and easily penetrated. The weevils are destructive pests to palms.

The larvae are responsible for damaging the palm, and once they have gained access, the death of the palm generally ensues. The larva never comes to the surface, since it begins its life inside the palm chewing fibers and cellulose.

Red palm weevil insects are among the most important lingo- cellulose digesting insects and possess a variety of symbiotic microorganisms in their hind guts, including protozoa.

In the digestive tracts of *R. ferrugineus* seems to be synergistically degraded by flagellated protozoa.

This is the first report on the presence of anaerobic protozoa in the red palm weevil guts. Further research is needed to better understand the ecology of these microbes.

MATERIALS AND METHODS

Organism: flagellated protozoa FP-007 was isolated from the hind gut of red palm weevil *R. ferrugineus* at Pests and Plant Protection Dept. NRC, Egypt.

Media and Cultivation Conditions:

Strict anaerobic techniques Holdeman *et al.* (1975) were employed for the preparation of media and for the cultivation and manipulation of cells.

The culture medium was slightly modified from that of Yamin (1978) and contained (in millimolar) K₂HPO₄ (10.8), KH₂PO₄ (6.9); KCl (21.5); NaCl (24.5); CaCl₂ (0.5); MgSO₄ (5.3); Pfennig metal solution McInerney *et al.* (1979) (0.1% V/V); Cellulose powder 0.1% (W/V), glutathione (reduced form) (3.2); NaHCO₃ (9.5); Yeast extract (0.3 W/V); heat inactivated fetal bovine serum (2.5% V/V).

To prepare this medium, a basal solution containing the inorganic salts, cellulose powder and glutathione was placed in tubes under N₂ in 9.25ml amounts and heat sterilized. The pH of the medium before inoculation was 6.7 to 6.8, and inoculated tubes were incubated vertically and unshaken at 24 to 26°C.

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Stock cultures of flagellated protozoa FP-007 were maintained by transferring 5% (V/V) inoculums to fresh medium every 20 to 30 days.

Culture purity was periodically verified by microscopic examination of wet mount preparations and by inoculation of cultures into brain heart infusion broth, supplemented with 0.3% (W/V) glucose and 0.05% cysteine hydrochloride, 0.3% (W/V) cellobiose, under which conditions flagellated protozoa does not grow.

Nutrition and Growth Studies:

Nutritional and growth characteristics of flagellated protozoa FP-007 were evaluated by determining their growth rate and cell yield, as well as gas production, in response to changes in medium composition or incubation conditions. Three to four replicates were done for each culture condition tested. Cell densities were determined by direct microscopic counts on samples drawn into 50mm long rectangular glass capillary tubes.

Analysis of Metabolic Products:

H₂ in the headspace of flagellated protozoa FP-007 cultures were analyzed by gas chromatography, for H₂, a column of Molecular sieve 5A was used with thermal conductivity detection (Uffen, 1976).

Substrate and Chemicals:

Cereal leaves, chitin, and corncob were further dry ball milled for 24-72h at ambient temperature to produce a fine powder.

RESULTS AND DISCUSSION

With the culture conditions employed (static, vertical, culture tubes), flagellated protozoa FP-007 settled to the bottom and grow on and amongst a soft pellet of cellulose particles. The incubation of cultures horizontally, was detrimental to growth.

General growth characteristics and nutrition of flagellated protozoa isolated from red palm weevil larvae. Flagellated protozoa FP-007 grow in modified Yamin medium achieved final yields of 8400 to 10370 cells/ml after 30 days of incubation.

Growth of cells was accompanied by the production of H₂ and acetate. No other organic acids or ethanol was found.

Evaluation of Soluble Medium Constituents:

FP-007 required fetal bovine serum and NaHCO₃ for good growth Table (1). Cell yields was 8400±1650 in the control (no omission), while when omitted yeast extract, NaHCO₃ or fetal serum, all yields were 4050±2100, 620±305 or < 20, respectively. Hydrogen production was parallel to cell yields, where it was 700±35, 374±53, 141±57 for the control (no omission), omission of yeast extract or NaHCO₃, respectively. Showed that cell yields in the absence of yeast extract were significantly lower than that of the control at P< 0.1 level (t test). Therefore, yeast extract was judged to be stimulatory. Hydrogen production by flagellated protozoa EG-007 closely paralleled cell yields.

Table 1: Effect of medium components on growth of flagellated protozoa FP-007

Components omitted from medium ^(a)	Yield as ^(b)	
	No. of cells/ml	m mol. of H ₂ /ml of culture
No Omission (control)	8400±1650	700±35
Yeast extract	4050±2100	374±153
NaHCO ₂	620±305	141±57
Fetal bovine serum	< 20	-

a-Growth medium was modified Yamin medium. The initial pH of all media was 6.7±0.2.

b-Determined 30 days after inoculation into the medium values are the means ± standard error, of the mean (n=4).

Evaluation of Insoluble Polysaccharides:

Insoluble polysaccharides were tested for their ability to support growth of flagellated protozoa FP-007, cellulose powder supported the best growth and hydrogen production. Powder cereal leaves and corn cob, also supported growth, but to a lesser extent, chitin showed little or no growth of protozoa.

Table (2) showed that cell yield of the growth, of flagellated protozoa EG-007 on insoluble polysaccharides, was 1020±578, 1190±170, 5700±1530, 2890±170 and 10370±2720 for the control (no substrate), chitin, corncob, cereal leaves and cellulose, respectively.

Table. 2: Growth of flagellated protozoa FP-007 on insoluble polysaccharides

Polysaccharide tested ^(a)	Yield	
	No. of cells /ml	m mol. of H ₂ /ml of culture
Cellulose	10370±2720	1021±36
Cereal leaves	2890±170	354±124
Corncob	5700±1530	746±68
Chitin	1190±340	141±85
No substrate	1020±578	117±73

a- Polysaccharides were incorporated into the medium at a final concentration of 0.1% (W/V).

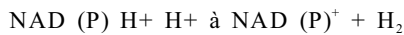
Hydrogen production was parallel to cell production. Where it was 117±73, 141±85, 746±68, 354±124 and 1021±36 for the control (no substrate), chitin, corn cob, cereal leaves and cellulose, respectively.

Discussion:

In vitro growth of flagellated protozoa in modified Yamin medium was markedly improved when added fetal bovine serum, and Na HCO₃, also yeast extract achieved good growth.

Results confirm and extend observations of Odelson & Breznak, (1985) that a particulate source of cellulose is required as a fermentable energy source by flagellated protozoa.

Results showed that hydrogen was the major or sole reduced and product of the flagellated protozoa. This observation suggested that some of the hydrogen produced by flagellated protozoa was derived from reduced pyridine nucleotide via.



A reaction that is thermodynamically at $p\text{H}_2 > 10^{-3}$ atom (Wolin, 1982).

Under these conditions, H₂ was produced, this is the first in vitro demonstration of H₂ during the anaerobic decomposition of cellulose in the hindgut of red palm weevil.

It is difficult to predict whether flagellated protozoa isolated from *R. ferrugineus* might ultimately prove to be useful in anaerobic bioconversion schemes designed to produce fuel, food, or chemical feeding stocks from lignocelluloses substrates.

Nevertheless, the improved growth of flagellated protozoa isolated from *R. ferrugineus* larvae as described herein, should facilitate further studies of cellulose and other hydrolytic enzyme activities of this symbiotic protozoan.

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