Production technology of entomopathogenic nematodes

Richou Han

Guangdong Entomological Institute
105 Xingang Road West, Guangzhou 510260, China

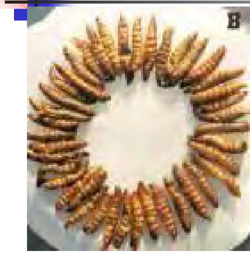
This talk…

- To introduce the production technology of entomopathogenic nematodes (EPNs) in China.

What are EPNs?

- Small worms in the genera of *Steinernema* and *Heterorhabditis.*
- Biological control agent.
- Not toxic to vertebrates.
- The second (after *Bacillus thuringiensis*) most highly traded group of bio-control products in the industrialized countries.

How to produce EPNs?



From Ralf Ehlers

In vivo *In vitro*

- *In vivo* vs *in vitro*
- Axenic vs monoxenic culture
- Solid vs liquid culture

Solid production

- Flasks and metal trays

Liquid production

- Fermentation system

Factors for the optimized production of EPNs

- Medium
- Bacterial status
- Gas supply
- Inoculum size
- Temperature
- Culture time

Improvement of medium ingredients

Commercial media with low cost and good character for down stream process, containing soy flour, wheat flour and egg yolk powder etc.

Table 4 Typical medium formulae developed in China

	Component	Concentration (%)	Nematode in cultures	Average yield or range ($\times 10^4$/g)	Reference
A.	corn flour	20.00	*S. carpocapsae*	47.4	Li *et al.* (1985)
	beef extract	0.25	Agriotos	(25.7-59.8)	
	peptone	0.50	*S. glaseri*	9.1 (7-11)	
	H_2O	74.25	*S. carpocapsae*	63.6	
	sponge	5.00	DD-136	(53.3-121.1)	
B.	peptone	1.00	*S. carpocapsae*	<60	Li *et al.* (1987)
	beef extract	1.00	Agriotos		
	egg	20.00			
	wheat flour	15.00			
	lard	5.00			
	H_2O	50.00			
	sponge	8.00			
C.	fish intestine	60.00	*S. glaseri*	8-12	Xu *et al.* (1985)
	H_2O	19.00			
	sponge	10.00			
D.	peptone	1.00	*S. carpocapsae*	38.5-77.2	Han *et al.* (1990)
	yeast extract	1.00	*Heterorhabditis* spp.	17.5-58.7	
	soy flour	15.00			
	egg	20.00			
	lard	5.00			
	H_2O	48.00			
	sponge	10.00			
E.	peptone	0.33	*S. glaseri* NC34	Not mentioned	Zhao *et al.* (1992)
	beef extract	0.33			
	egg	6.67			
	potato flour	20.00			
	wheat flour	5.00			
	H_2O	15.00			
F.	chicken offal	50.00	*Steinernema* sp. CRB	18.9-37.1	Han *et al.* (1992)
	corn flour	10.00			
	fish meal	10.00			
	lard	10.00			
	H_2O	10.00			
	sponge	10.00			
G.	duck offal	unknown	*S. carpocapsae*	52.7	Zhang *et al.* (1991)
	lard				
	deoiled silk worm pupae				
H.	pig offal	unknown	*S. carpocapsae*		Li *et al.* (1992)
	lard				

Phase Variation

- Phase variation of symbiotic bacteria can significantly influence the production of EPNs

Phase I and phase II *of Photorhabdus luminescens*

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Effect of inoculum sizes on the yield of *Steinernema carpocapsae* A24 in liquid medium flasks（Han *et al*., 1996)

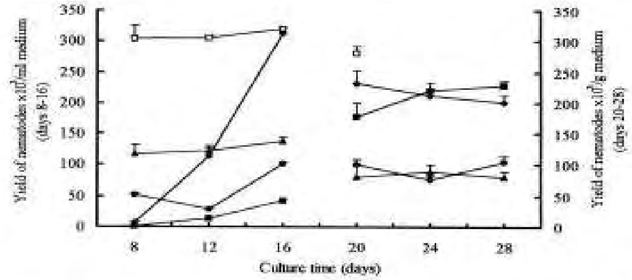

Fig. 1. Average number of *Steinernema carpocapsae* A24 in 100 g liquid medium flasks with different nematode inoculum sizes. Inoculum (infectives/g medium): 80 000 ●—●; 8000 □—□; 800 ▲—▲; 80 ◆—◆; 8 ■—■. For the 8000 inoculum, the culture after 20 days contained only infectives and counting was discontinued.

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Effect of inoculum sizes on the yield of *Heterorhabditis bacteriophora* H06 in liquid medium flask（Han *et al*., 1996)

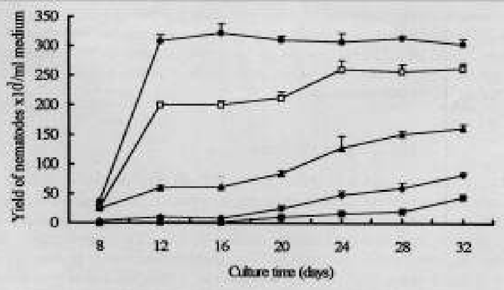

Fig. 2. Average number of *Heterorhabditis bacteriophora* H06 in 100 g liquid medium flasks with different nematode inoculum sizes. Inoculum (infectives/g medium): 56 000 ●—●; 5600 □—□; 560 ▲—▲; 56 ◆—◆; 5.6 ■—■

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Symbiotic relations of the EPNs and their symbionts in nematode production

- Food signals
- Nutrients
- Trans-specific toxins
- Bacterial colonization of IJs

Symbiotic bacteria: nematodes

- **Food signals:** The signals from the symbiotic bacteria for triggering the recovery of the infective juveniles during reproduction.
- **Nutrients:** The bacteria provide nutrients for nematode production.
- **Trans-specific toxins:** Symbiotic bacteria may produce toxic effect on the non-indigenous nematodes.
- **Bacterial colonization of nematodes:** Significant differences in the ability of the nematodes to retain the bacterial cells in the intestines of the IJs.

Known parameters influencing the IJ recovery

Parameters	Effect on IJ recovery		References
	S	H	
Insect haemolymph	+	+	Strauch & Ehlers,1998; Ciche & Ensign, 2003
Insect tissue culture of			
Drosophila melanogaster DL-1	N	+	Ciche & Ensign, 2003
Spodoptera litura SL-zsu-1	+	-	Han *et al.*, 2000
Symbiont culture in stationary phase	+	+	Strauch & Ehlers,1998
Compounds of artificial media	-	-	Strauch & Ehlers,1998 Ciche & Ensign, 2003
Fresh human blood	N	-	Ciche & Ensign, 2003
Increase of CO_2 concentration	N	+	Strauch & Ehlers,1998 Jessen *et al.*, 2000

Note: S: *Steinernema*; H: *Heterorhabditis*; "+",effect; "-", no effect; N, data not available.

Known parameters influencing the IJ recovery

Parameters	Effect on IJ recovery		References
	S	H	
Incompatible symbionts of *Photorhabdus*	-?	+	Han & Ehlers, 1998
Incompatible symbionts of *Xenorhabdus*	+	-?	Han & Ehlers, 1998
Other microorganisms *Escherichia coli*	+	-	Strauch & Ehlers, 1998
Photobacterium phosphoreum	+	-	Han *et al.*, 2001
Insect hemolymph			Ciche & Ensign,2003
Centrifuged, filter sterilized	N	+	
Heat treatment (80 ℃,30min)	N	+	
Dialyzed (10kDa)	N	-	
Centricon (10kDa) filtrate	N	+	
Through anion-exchange column (Sepharose Q)	N	+	
Through caton-exchange column (Sepharose S)	N	-	
Pronase (20ug/ml, 50 ℃,2h)	N	+	

Food signals: Effect of bacterial strains on the recovery of *H. bacteriophora* H06

（Han and Ehlers, 1999)

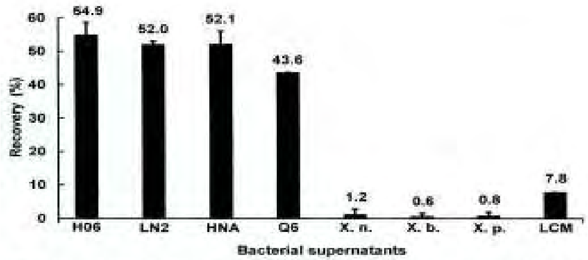


Fig. 1. Percent recovery of axenic dauer juveniles of *Heterorhabditis bacteriophora* H06 in LCM culture supernatant of *Photorhabdus luminescens* isolated from *H. bacteriophora* H06, *H. indica* LN2, *H. megidis* HNA and *Heterorhabditis* sp. Q6 and in supernatants of *X. nematophilus*, *X. bovienii* and *X. poinarii* (vertical bars represent standard deviation).

Nutritional significance for nematode production

- Insects
- Artificial media
- Symbiotic bacteria
  - Strains
  - Phase variation
  - Crystalline inclusion proteins

Nutrient suppliers and nematode reproduction

Nutrient suppliers	Nematode		
	Steinernema spp.	*Heterorhabditis* spp.	*Panagrellus redivivus*
Axenic *Galleria mellonella* larvae	+ (Han & Ehlers,2000)	- (Han & Ehlers,2000)	Not tested
Cell culture of *Spodoptera litura* SL-zsu	+ (Han *et al*., 2000)	- (Han *et al*., 2000)	Not tested
Artificial media with high nutrient factors	+ (Friedman 1990; Ehlers *et al*. 1990; Lunau *et al*., 1993)	- (Friedman 1990; Ehlers *et al*. 1990; Lunau *et al*., 1993)	Not tested
Xenorhabdus bacteria on lipid agar plates	(Han & Ehlers, 2000)	(Han & Ehlers, 2000)	
Phase I	+++ (indigenous) or — (some non-indigenous)	-	+
Phase II	+	-	Not tested

Nutrient suppliers and nematode reproduction (continued)

Nutrient suppliers	Nematode		
	Steinernema spp.	*Heterorhabditis* spp.	*Panagrellus redivivus*
Photorhabdus bacteria			
Phase I	-	+++ (indigenous) or — (some non indigenous)	–
Phase II	+	+	–
Other bacteria, such as *E. coli* etc.	+ (Boemare, 1983; Ehlers *et al*., 1990; Aguillera *et al*., 1993; Grewal *et al*., 1997)	- (Ehlers *et al*.,1990; Han *et al*.,2000)	+
P. asymbiotica	N	+ (Yoshiga *et al*.,2005)	Not tested

Symbiosis between nematodes and bacteria

- No nematode strains can use all bacterial isolates.
- Not every bacterial isolates can be used by all nematode strains.

Specificity between nematodes and bacteria (Han *et al.*, 1990)

Table 2

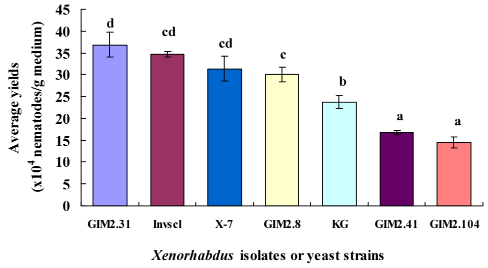
Monoxenic *in vitro* cultures of various combinations of *Heterorhabditis* spp. and *Xenorhabdus luminescens*

Nematode	Combinations maintained in lipid agar							
	Bacterium							
	X1HNA	X1H06	X1G12	X1H3	X1HNZ	X1Q6	X1V16	None
H. megidis HNA	+	–	–	–	+	+	+	–
Heterorhabditis								
sp. H06	+	+	–	–	+	+	+	–
sp. G12	+	+	+	+	–	–	–	–
sp. H3	+	+	+	+	–	–	–	–
H. heliothidis HNZ	+	+	–	–	+	+	+	–
Heterorhabditis								
sp. Q6	+	+	–	–	+	+	+	–
sp. V16	+	+	–	–	+	+	+	–

\+ = Nematode developed in this combination.
– = Nematode did not develop in this combination.

Xenorhabdus bacteria for non-EPNs

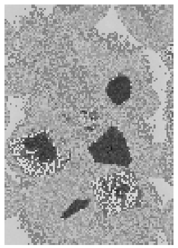
Nutrient potential of *Xenorhabdus* bacteria for a free living *Panagrellus redivivus* nematode (a fish food for the aquaculture) (Cao *et al.*, 2008)

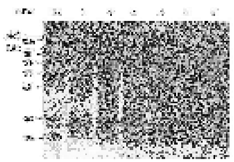


***Panagrellus redivivus* nematode**

Nutrients: Crystalline inclusion proteins

- *Photorhabdus* CipA or CipB can promote nematode development and IJ formation（You *et al.*, 2006)



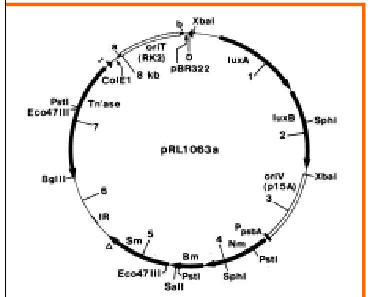


Development-related genes of EPNs

- A full-length cDNA library from recovered IJs of *S. carpocapsae* A24 was constructed using RLM-RACE method (Xia *et al.*, 2008).
- A set of candidate genes associated with the development of the infective juveniles were obtained (Xia *et al.*, 2008).

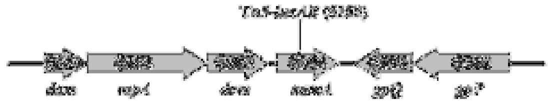
Trans-specific toxins

Identification and characterization of a novel gene involved in the trans-specific nematicidal activity of *P. luminescens* (Qiu *et al.*, 2009)



A novel *namA* gene encoding a protein of 364 amino acids

Trans-specific toxins



9586 bp sequence flanking the Tn*5-luxAB*
Accession number: EU850396.

Six ORFs

- ORF1, ORF2, ORF3, ORF5, and ORF6 similar to genes of *dam*, *repA*, *dcm*, *gpQ*, and *gpP*, respectively.
- One ORF: 1095 bp, was inserted by the Tn*5* transposon and designated *namA* (nematicidal activity mutant A)

Specificity: Bacterial colonization of nematodes (Han et al., 1990)

Table 3

The percentage of the infectives with bacteria in *in vivo* culture of various combinations of *Heterorhabditis* spp. and *Xenorhabdus luminescens*

Nematode strain	Bacterium X1HNA	X1H06	X1G12	X1H3	X1HNZ
H. megidis	94 (3.9)[a]	—[b]	—	—	41 (3.4)
Heterorhabditis					
sp. H06	0	98 (1.4)	—	—	83 (4.0)
sp. G12	11 (1.1)	0	92 (1.3)	98 (0.1)	—
sp. H3	27 (1.8)	n[c]	94 (3.9)	94 (0.9)	—
H. heliothidis					
HNZ	1 (0.01)	88 (2.3)	—	—	93 (2.3)

[a] Percentage with (SE).
[b] Not successful combination. See Table 2.
[c] n = No infectives available.

Genes involved in the bacterial colonization of IJs

- **Genes involved in the bacterial colonization of *Steinernema* nematodes:**
  - **Four putative regulators required for nematodecolonization: *RpoS, RpoE, Lrp and NilD RNA* (Vivas *et al.*, 2001; Heungens *et al.*, 2002)**
  - **Nutrient transport proteins: Nil proteins (Cowles *et al.*, 2004)**
  - **Metabolism related: *iscRSUA-hscBA-fdx* (Martens *et al.*, 2003)**
  - **Nutrient exchange: *serC-aroA* (Martens *et al.*, 2005; Orchard *et al.*, 2005)**
  - ***mrx*A encoding the structural subunit of type 1 fimbriae (Chandra *et al.*, 2008)**

Genes involved in the bacterial colonization of IJs

- Genes involved in the bacterial colonization of *Heterorhabditis* nematodes: *pbgE1* (Bennett *et al*., 2005)

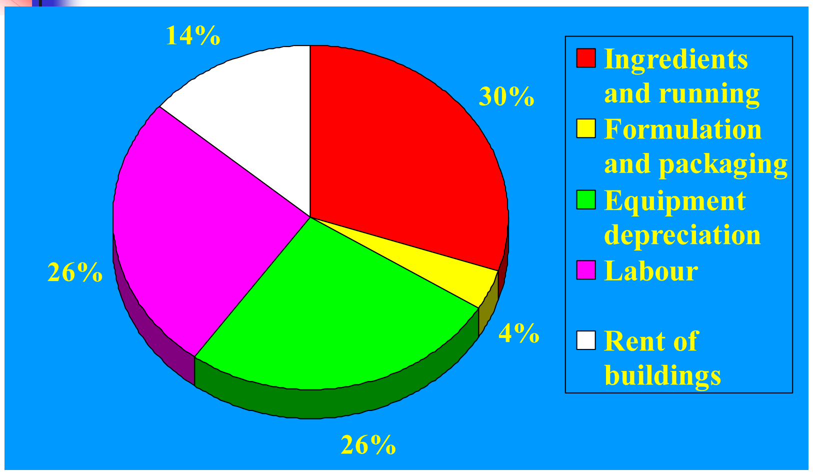
Commercialization of EPNs in China

- 2000: Biogreen Company was established to produce EPNs

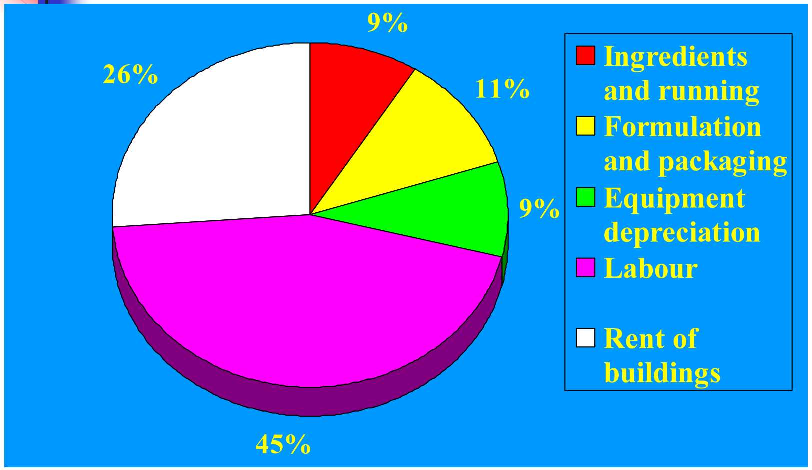
Nematode-based products

- *S. carpocapsae* All
- *S. feltiae*
- *S. longicaudum* X7
- *S. glaseri*
- *H. bacteriophora* H06
- *H. megidis*
- *H. indica*

Production cost breakdown for *Steinernema carpocapsae* All in liquid fermentation system



Production cost breakdown for *Steinernema carpocapsae* All in solid culture system



Future perspectives

- Biocontrol agents show high potentials for plant protection
- EPNs play a major role in IPM
- Great progress can be expected from further R&D in EPN biotechnology

Many thanks to my collegues and students