

Final Report DACS Grant 2008-2009

PI: Dr. Dov Borovsky

Abstract:

Six synthetic genes tagged with (His₆) and code for Cry4Aa-TMOF, Cry11Aa-TMOF, Cyt1Aa-TMOF, GST-Cry4Aa-TMOF, GST-Cry11Aa-TMOF and GST-Cyt1Aa-TMOF were synthesized and cloned into pPICZB and pPIC3.5 plasmids (Invitrogen) using homologous recombination at the AOX1 and HIS loci on the yeast's chromosome. Recombinant yeast cells: AOX1:Cry4Aa-TMOF; AOX1:Cry11Aa-TMOF; AOX1: GST-Cry11Aa-TMOF and AOX1:GST-Cry11Aa-TMOF were fermented and fed to Aedes aegypti larvae to find the potency of the recombinant cells. First instar larvae that were fed whole yeast cells that were fermented for different periods and 3rd and 4th instars that were fed broken cells died within 1-6 days after the feeding. Cells that were fermented without zeocin were less effective than cells that were fermented in the presence of the antibiotic indicating that the recombinant genes were deleted by the yeast in the absence of zeocin. The fusion of GST (glutathione-S-transferase) to the recombinant proteins enhanced their solubility in the cell cytoplasm making the toxins more effective and heat stable.

Mass spectrometry analysis of the recombinant P. pastoris Cry11Aa-TMOF protein purified by Ni affinity chromatography showed that a full length Cry11Aa was synthesized by the transformed yeast cells.

The short duration of the grant period (12 months) did not allow us to ferment, test and evaluate all the genes that were cloned. Future funding, will allow us to biologically test all the genes that were cloned using small groups of larvae in the lab and larger groups outside in the Indian River Mosquito Control District.

Our results show for the first time, that yeast cells carrying Bti and TMOF genes can be successfully engineered producing Bti and TMOF at levels that are sufficiently high to kill mosquito larvae.

INTRODUCTION

Mosquitoes transmit many diseases that cause health and economical impact in Florida. Vector control in Florida is an important strategy in controlling and preventing vector borne diseases such as dengue and encephalitis including the West Nile encephalitis. Thus, new vector control products that are cost effective and target specific are urgently needed.

Trypsin Modulating Oostatic Factor (TMOF), a mosquito decapeptide, stops trypsin biosynthesis in the midgut of several species of female and larval mosquitoes by binding to a gut receptor. The hormone is an effective larvicide that causes larval starvation and death to *Aedes*, *Culex* and *Anopheles* larvae (Borovsky and Meola,

2004, Borovsky et al. 2006). Combining TMOF and *Bti* δ -endotoxins controls mosquito larvae because TMOF down regulates digestive enzymes and *Bti* causes an imbalance in the gut osmotic pressure with rapid death. The advantage of using this approach is:

- *Less Bti is needed because Bti is up to 10-fold more effective on starved 3rd instar larvae than on well fed larvae.*
- *A synergistic effect of Bti and TMOF can be utilized to produce an efficient and versatile mosquito larvicide.*
- *TMOF and Bti are natural biological moieties that do not cause harm to the environment.*
- *This approach will save money by reducing the amount of Bti that is currently needed to control larvae in Florida without causing harm to the environment.*
- *The new binary-larvicide will also be effective on all mosquito species which is not the case when Bti is used alone.*

BACKGROUND

General: Trypsin Modulating Oostatic Factor (TMOF) was originally found in the mosquito ovary (Borovsky 1985). The hormone is not species-specific, as injection of TMOF inhibits egg development and trypsin biosynthesis in *Culex quinquefasciatus*, *Culex nigripalpus* and *Anopheles albimanus* (Borovsky, 1988). TMOF has been purified, sequenced and characterized by means of mass spectroscopy as an unblocked decapeptide (YDPAPPPPPP) (Borovsky et al., 1990). Various synthetic peptide analogues (Barany and Merrifield, 1979) possess TMOF activity (Borovsky et al., 1990, 1991, 1993; Borovsky and Meola, 2004).

Synergistic effect of TMOF and *Bti* toxins: The effect of *Bti* on mosquito larvae (1st and 2nd instars) was tested and the LC₅₀ was determined (Table 1). We found out that

Table 1. Toxicity of *Bti* against *Ae. Aegypti* larvae

Larval Instar	LC ₅₀ (ng/ml)	
	Fed Larvae	Starved Larvae
First	55.6	1.6
Third	105.5	17.7

Mosquito larvae were fed *Bti* and assayed 24 h later for larval mortality. LC₅₀ was determined by Probit analysis (Borovsky, Zaritsky and Khasdan unpublished observations)

if mosquito larvae were starved prior to feeding them with *Bti* the mortality increased by 34.8 and 6-fold for first and third instar larvae, respectively (Table 1). Since TMOF blocks the biosynthesis of trypsin in the gut of larval mosquitoes and effectively starves

them to death, we fed TMOF and *Bti* toxin to *Ae. aegypti* larvae to assess synergism between TMOF and *Bti*.

Feeding *E. coli* (10^6 cells) transformed with plasmid pVE4-ADRC expressing the *Bti* toxins *cry4Aa*, *cry11Aa* and *cyt1Aa* to second instar larval *Ae. aegypti* caused 10% mortality in the presence of non-transformed yeast (10 mg/ml, 0.1% w/v). Feeding of *Pichia*-TMOF to second instar larvae (10 mg/ml yeast, 6 μ g TMOF) caused 95% mortality in 12 days. Larval mortality started at day 6 and ended at day 12. If *Pichia*-TMOF and *E. coli* transformed with pVE4-ADRC (plasmid that encodes for *Bti* toxins) were fed together larval mortality was 40% at day 2 and 90% at day 4 (Fig.1), indicating that TMOF enhances the activity of *Bti*.

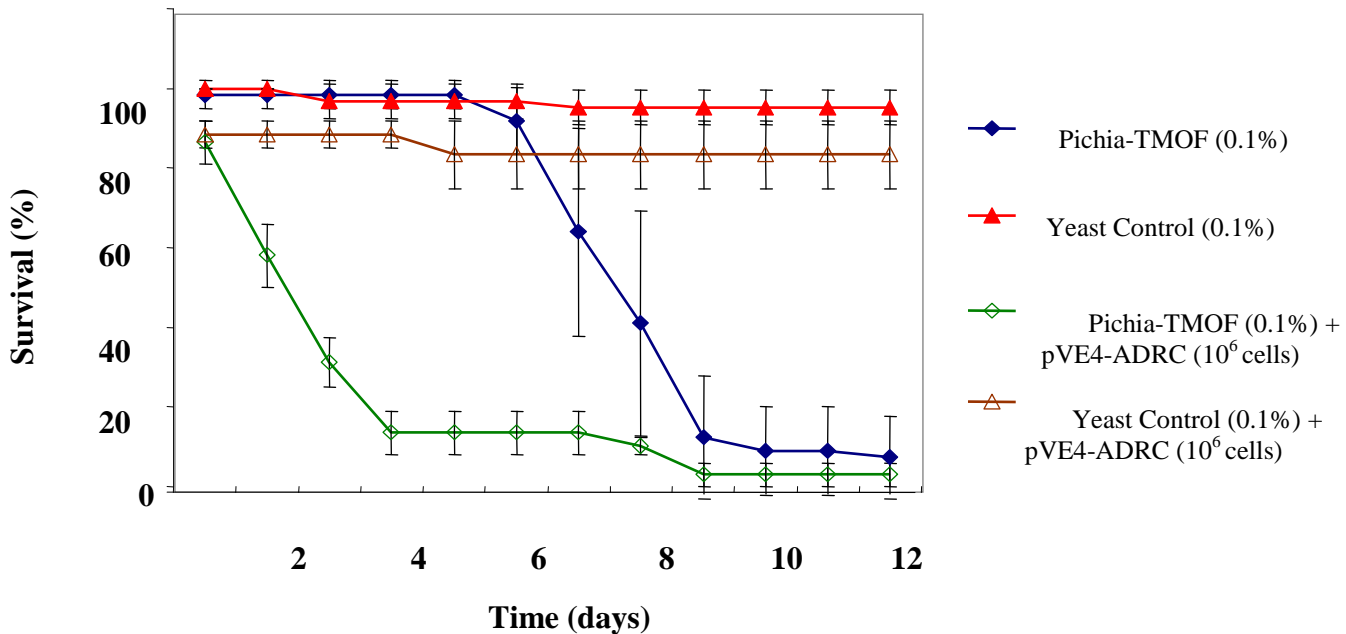


Fig. 1. Feeding of second instar larval *Ae. aegypti* *Pichia*-TMOF and *E. coli* transformed with plasmid pVE4-ADRC expressing *Bti* toxins (*cry4Aa*, *cry11Aa* and *cyt1Aa*) (Borovsky, Zaritsky and Khasdan, unpublished observations).

These preliminary results and the support that we have received from DACS, enabled us to clone and express several *Bti* toxins and TMOF in the methylotrophic *P.pastoris*. We report here for the first time, the cloning, fermentations and biological activity of several clones that were tested.

MATERIALS AND METHODS

***P. pastoris* constructs.** pPICZB (Invitrogen) and pPIC3.5, a Zeocin and Geneticin-selectable plasmids, were used for cloning and expression in *P. pastoris*. The plasmids contain an alcohol oxidase 1 promoter (*AOX1*) from *P. pastoris* fused to a multiple cloning site and an *AOX1* termination sequence. Fermentation in the presence of

methanol (0.5%) induces the AOX1 promoter to initiate transcription, translation and heterologous protein expression in the cytoplasm of the KM71H yeast cells (*Invitrogen*).

Expression in *P. pastoris*. pUC57 plasmids carrying the synthetic genes: *Cry4Aa-TMOF*, *GST-Cry4Aa-TMOF*, *Cry11Aa-TMOF*, *GST-Cry11Aa-TMOF*, *Cyt1Aa-TMOF* and *GST-Cyt1Aa-TMOF* were cut with appropriate restriction enzymes and cloned into pPICZB and pPIC3.5 that were opened with the same restriction enzymes and cloned into *E. coli* INV α F cells (*Invitrogen*). Clones were selected in the presence of 25 μ g/mL zeocin and 250 μ g/mL Geneticin. Positive clones were grown in LB medium in the presence of Zeocin or Geneticin and plasmids were harvested, purified (Qiagen plasmid miniprep kit) and screened by PCR. Positive plasmids with inserts were linearized with *SacI* and the linearized plasmids were used to transform *P. pastoris* KM71H cells with the Easy Comp kit (*Invitrogen*). The transformed cells were plated on YPD plates containing sorbitol and 100 μ g/mL zeocin or Geneticin (0.25 mg/mL) (*Invitrogen*). Positive colonies were screened for zeocin resistance (100 to 3000 μ g/mL) or Geneticin resistance (0.25-4 mg/mL). Colonies that showed high resistance to zeocin (2000-3000 μ g/mL) (10 to 20, inserts per cell) were selected. Glycerol stock solutions were prepared and the cells were stored at -80^o C until used. At this stage, all the transformed cells are Mut^s (grow slow in the presence of methanol). For protein expression, single colonies were isolated after plating small aliquots from glycerol stock solutions on YPD plates containing zeocin (100 μ g/mL) or Geneticin (0.25 mg/mL). Colonies were grown in minimal glycerol medium (1%) containing yeast nitrogen base in the presence of 100 μ g/mL zeocin or Geneticin at 30^o C for 2-3 days. After incubation, the cells were centrifuged the supernatant removed and the cells induced with a fresh minimal medium (yeast nitrogen base with ammonium sulfate without amino acids) containing biotin and zeocin (100 μ g/mL) or Geneticin for 24-96 hours. Cells were broken with YPER or in the presence of 8M Urea and glass beads in a DNA FastPrep machine, centrifuged and supernatants removed and stored frozen at -20^o C until used.

Protein purification. Proteins obtained after fermentation and cell disruption using YPER or 8M urea were loaded on Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) and the column was washed with Phosphate buffer pH 8.0 and proteins were eluted with 20 mM and 250 mM imidazole. Samples from the eluted peaks were then removed and analyzed by SDS PAGE using 10% polyacrylamide slab gel. The gels were fixed and stained with coomassie brilliant blue and bands that migrated at the same molecular weight of the recombinant proteins were cut, digested with trypsin and analyzed by mass spectrometry at the University of Florida Biotech Center (<http://www.biotech.ufl.edu/about.html>).

Construction of synthetic genes. *Cry4Aa*, *Cry11Aa*, *Cyt1Aa*, *GST* and *TMOF* synthetic gene constructs were optimized using *Pichia* codons (www.kazusa.or.jp) and the synthetic genes were cloned into pUC57 and transferred into pPICZB and pPIC3.5K (Fig.2 and 3).

Transformation of *P. pastories* with pPICZB carrying Cry4Aa_TMOF and Cry11Aa_TMOF

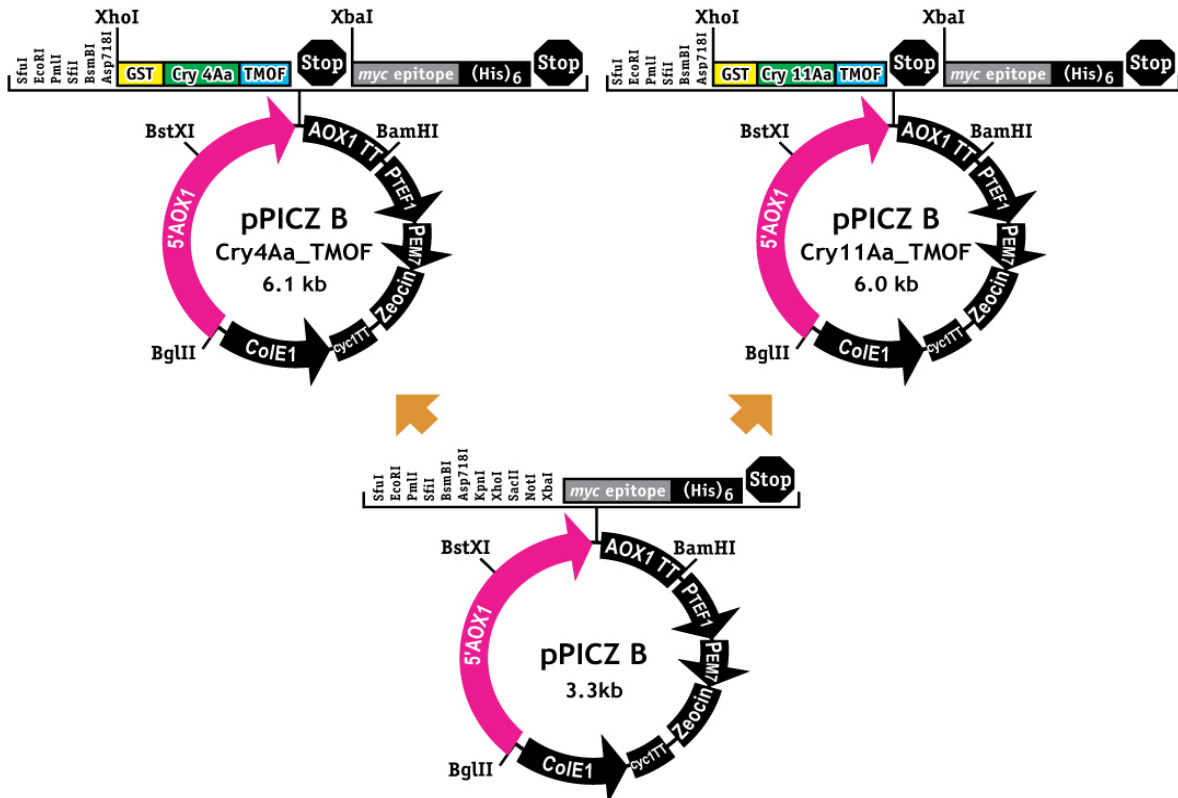


Fig. 2. Strategy for cloning of *GST-Cry4Aa-TMOF* and *GST-Cry11Aa-TMOF* into pPICZB multiple cloning site. Plasmid map shows the alcohol oxidase promoter (AOX1), terminator (AOX1 TT), the Zeocin resistant gene and the *E. Coli* origin of replication (CoIE1).

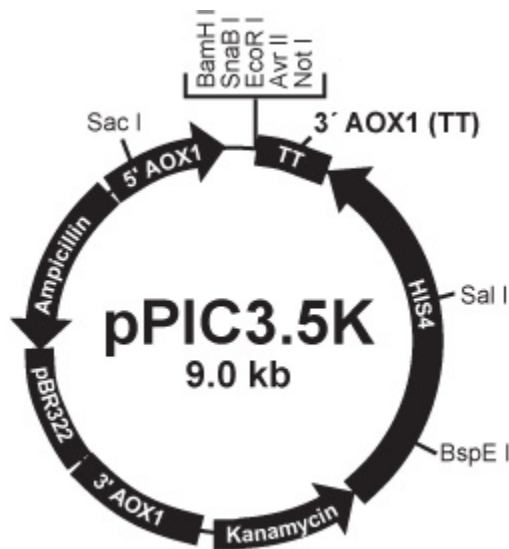


Fig. 3. pPIC3.5K map including the alcohol oxidase promoter (AOX1) and terminator (TT), the *HIS4* gene for cloning and the antibiotics resistant genes kanamycin, ampicillin and the multiple cloning site.

Synthetic genes with optimized codon for *P. pastoris* genes were synthesized and sequenced :

Cry4Aa-TMOF yeast codon optimized. Toxin sequence starts at ATG and end after TMOF sequence. The 5' end and 3' ends are used to clone into the yeast and bacterial plasmids.

```

ggggggGGATCCgggCTCGAGaaaaATGtctcatcaccatcaccatcacCCAATtGAGAACAGCCCCAAGCAGTTGT
TGCAGAGTACCAACTACAAGGACTGGtTGAACATGTGTCAACAGAACCAGCAGTACGGTGGTGACTT
CGAGACCTTCATCGACTCCGGTGAGTTGTCCGCTTACACCATCGTCGTCGGTACCGTCTTGACCGGT
TTCGGTTTTACCACCCCAATTGGGTTTGGCTTTGATCGGTTTTCGGTACCTTGATCCCAGTCTTGTTCC
AGCTCAGGACCAGTCCAACACCTGGTCCGACTTCATCACCCAGACCAAGAACATCATCAAGAAGGA
GATCGTTCCACCTACATCTCCAACGCTAACAAGATCTTGAACAGATCCTTCAACGTCATCTCCACCT
ACCACAACCATTGAAGACCTGGGAGAACAAcCCAACCCACAgAAcACcCAGGAcGTcAGaACcCAGa
TCCAGttgGTcCAcTACCacTTcCAGAAcGTCATcCCAGAGttgGTcAACTCcTGTCcCaAAcCCatccGAcT
GtGAcTACTAcAACATcttgGTcTTgTCctccTAcGcTcAgGcTcGcTAACTTgCActtgAcGcTcTTgAAcCAGGcTg
TCAAgtTcGAgGcTAcTTgAAgAAcAAcagaCAgTTCGAcTAcTTgGAGCCaTTGCCAACcGcTAcTcGAcTAc
TAcCCAGTcTTGACcAAgGCTATcGAgGAcTACAcCAcTAcTGTGTcACcACcTAcAAgAAgGGtTTgAAcTT
gATcAAgACcACcCAgActccAActtgGAcGGtAAcATcAACTGGAACAcCtTACAAcACcTAcagaACcAAgATG
ACcACcGCTGTcTTgGAcTgGTcGcTttgTTcCCaAAcTAcGAcGTcGGTAAgTAcCCAATcGGTGTCCAgTcC
GAgttgACcagaGAgATcTAcCAGGTccttgAACTTCGAgGAgTccCCaTAcAAgTAcTAcGACTTcCAGTAcCAGg
AGGAcTCcttgACcagaAGACCcAcTTgTTcACcTGGttgGAcTcCTTGAAcTTcTAcGAgAAgGcTcAgACcAG
cCCaAAcAAcTTcTTCACCTccCAcTAcAAcATGTTcCAcTACAcCctTgGAcAAcATcTCCCAgAAgTcctccGTcT
TcGGtAAcCACAAcGTcACcGAcAAgTTgAAgTCcttgGGTTTTGGCtACcAAcATcTAcATcTTcTTgTTgAAcGT
CATctccTTgGAcAAcAAgTAccttgAAcGAcTAcAAcAAcATctccAAgATGGAcTTcTTcATcACcAAcGGTACcAG
AttgTTGGAGAAgGAgttgACcGcTGGtTCCGGtCAgATcACcTAcGAcGTcAAcAAgAAcATcTTCGGfTTgCCA
ATcttgAAgagaAGAGAGAAcCAgGGtAACCCaACcttgTTcCCAACcTAcGAcAACTActccCAcATcTTgTCcTT
cATcAAgtccttgccATCCCaGcTAcCtAcAAgACcCAgGTcTAcACcTTcGCTTGGACcCACTCctccGTcGAcC
CaAAgAAcACcATcTAcACcCAcTTgACcACCCAgATcCCAGCTGTcAAgGcTAAcTcTgGGtACcGCTTcC
AAGGTcGTcCAGGGtCCaGGTCAcACcGGtGGtGAcTTgATcGAcTTCAAgGAcCAcTTCAAgATcACcTGTC
AgCACTCcaAAcTTcCAGCAgTcTAcTTcATcAGAATcagaTAcGCTTcCAcGGtccGcTAAcACcagaGCTGT
cATcAActtgccATCCAGGtGTcGcTgAgttgGGTATGGCttgAACCCaACcTTcTCCGGTACcGAcTAcACcA
AcTTgAAgTAcAAgGAcTTcCAGTACTTgGAgTTcTCCaACGAGGTcAAgTTcGCTCCcAAcCAgAACATcTC
cttgGTcTTcAAcCGaTcTAcGAcTcTAcACcAACACcACcGTctTgATcGAcAAgATTGAgTTctTGCCAATTACT
CGTTCcATcAGAGAGGATAGAGAGAAgCAgAAgTTgGAgACcGTcCAACAAatcgaggtagatagatccagctcct
ccacctccacctccaTAATCTAGAgggGAATTCgggggg

```

Cry11Aa codon optimized to use for *Pichia* cloning. Sequence starts at ATG and end after TMOF sequence. The 5' and 3' upstream sequences before the start and stop signals are used for cloning into bacterial and yeast plasmids.

```

GGGGGGGGATCCGGGCTCGAGAAAAATGGCTCATCACCATCACCATCACGAAGATAGTTCTTTGGA
TACTCTAAGTATTGTTAACGAAACTGACTTCCCATTGTACAACAACACTGAACCTACTATCGCTCC
AGCCTTGATTGCTGTTGCTCCCATCGCTCAGTACCTTGCCACTGCTATCGGGAAGTGGGCTGCTAAG
GCTGCCTTCTCTAAGGTCCTGTCCCTGATCTTCCAGGTTCTCAACCAGCTACTATGGAGAAGGTTCC
GTACCGAAGTCGAGACCCTCATCAATCAAAAGTTGAGCCAAAGATCGAGTCAACATCTTGAACGCCGA
GTACAGAGGGATCATTGAGGTTAGTGTCTTGTGATGCCTACATCAAGCAACCAGGTTTCACTCCCT
GCTACTGCTAAGGGTTACTTCTCAACCTGAGTGGTGTATCATCCAACGATTGCCTCAATTTGAGGT
TCAAACATACGAAGGAGTCTCTATCGCCCTGTTCACTCAAATGTGTACACTTCATTTGACTTTTGTGA
AAGACGGAATCCTAGCAGGTAGTGCATGGGGATTTACTCAAGCTGATGTTGATTCTTCATCAAGTT
GTTCAATCAAAAAGTCTTCGATTACAGGACTAGATTGATGAGAATGTACTACTGAAGAGTTCCGGAAGAT
TGTGTAAGTCAAGTCTTAAAGATGGATTGACGTTCCGGAATATGTGTAATTTGTATGTGTTTCCATTTG
CTGAAGCCTGGTCTTTGATGAGATATGAAGGATTGAAGTTACAAAGCTCTCTATCATTGTGGGATTAT

```

GTTGGTGTCTCTATTCTGTCAACTACAACGAATGGGGAGGACTAGTCTACAAGTTGTTAATGGGTG
AAGTTAATCAAAGATTGACAACTGTTAAGTTCAATTACTCCTTCACTAATGAACCAGCTGATATACCAG
CAAGAGAAAATATTCGTGGTGTCCATCCTATCTACGATCCTAGTTCTGGTCTTACTGGATGGATCGGA
AACGGAAGAACTAACAACCTCAACTTCGCTGATAACAATGGCAATGAAATTATGGAAGTTAGAACTCA
AACTTTCTACCAAAATCCAACAACGAGCCTATCGCTCCTAGAGATATCATCAACCAAATTTTGACTG
CCCCAGCTCCAGCTGATCTATTCTTCAAGAATGCTGATATCAACGTTAAGTTCACTCAGTGGTTTCAA
TCTACTCTATACGGTTGGAACATTAAGCTTGGTACACAACTGTCTTGAGTAGTAGAACCGGAACAAT
TCCACCAAATTACTTGGCCTACGATGGATACTACATTCGTGCTATTTTCACTTGGCCAAAGAGGAGTCT
CCCTTGCATACAATCACGATCTTACTACTCTAACATACAATAGAATTGAGTACGATTCACCTACTACTG
AAAATATTATTGTCGGGTTTGTCTCCAGATAATACTAAGGACTTCTACTCTAAGAAGTCTCACTACTTAA
GTGAAACCAACGATAGTTACGTCATTCCTGCTCTGCAATTCGCTGAAGTTTCAGATAGATCATTCTTG
GAAGATACTCCAGATCAAGCTACTGACGGTAGTATTAAGTTTCGCTCGTACTTTTATTAGTAATGAAGC
TAAGTACTCTATTAGACTAAACACTGGGTTCAATACCGCTACTAGATACAAGTTGATTATCAGAGTTA
GAGTTCCATATCGCTTGCCTGCTGTTTCCGTTACAATCTCAGAACTCAGGAAATAATAGAATGCTA
GGCAGTTTCACTGCAAAATGCTAATCCAGAATGGGTGGATTTTCGTCAGTGCCTTCACTTCAACCTCAACG
ACTTGGGTATTACAACCTCAAGTACAACACTGTTTTGTTTCAGTATCTTTCAGATAGCTTGAACCTCTGGA
GAAGAGTGGTACTTGTCCCAGTTGTTCTTGGTCAAGGAATCCGCCTTCACTACGCAAATTAACCCATT
GCTAAAGATCGAAGGTAGATACGATCCAGCTCCTCCACCTCCACCTCCATAATCTAGAGGGGAATTC
GGGGGG

Cyt1Aa yeast codon optimized with TMOF gene. The 5' and 3' ends are used for cloning into yeast and bacterial plasmids like the other genes.

GGGGGGGGATCCGGGCTCGAGAAAATGGCTCATCACCATCACCATCACGAAAACCTGAATCATTG
TCCATTGGAAGATATCAAGGTCAACCCATGGAAAACCTCAATCTACTGCTAGAGTTATTACCTTGC
GTGTTGAAGATCCAAATGAAATCAACAACCTTCTTTCTATTAACGAAATTGATAATCCAAATTACATCT
TGCAAGCCATTATGTTGGCTAACGCCTTCCAAAACGCATTGGTTCCAACCTTCTACTGATTTCCGGTGAT
GCTCTTCGCTTCAGTATGCCAAAAGGTTTGGAAATTGCTAACACTTACTCCAATGGGTGCTGTTGT
CAGTTACGTTGATCAAAACGTTACTCAAACCTAACACCAAGTTAGTGTATGATTAACAAGGTCTTGG
AAGTCTTGAAGACTGTCTTGGGAGTTGCCTTGAGTGGATCTGTCATTGATCAATTGACTGCTGCTGTT
ACCAACACCTTCACTAACTTGAACACTCAAAGAATGAAGCTTGGATCTTCTGGGGTAAGGAAACTG
CTAATCAAACCTAACTACACCTACAACGTTCTGTTTGAATCCAAAACGCTCAAACCTGGTGGTGTATG
TACTGTGTTCCAGTTGGTTTTGAAATTAAGGTCTCTGCTGTTAAGGAACAAGTCTTGTCTTCACTATT
CAAGATTCTGCTAGTTACAACGTTAACATTCAATCTTTGAAGTTTGCCTCAACCTTGGTTAGCTCTAGT
CAATACCCAATTGCTGATCTTACTAGTGTCTATTAATGAAGTCTCATCGAAGGTAGATACGATCCAGC
TCTCCACCTCCACCTCCATAATCTAGAGGGGAATTCGGGGGG

GST-Cry4A-TMOF optimized sequence with restriction enzymes: 2782 bp

ggggggGGATCCgggCTCGAGaaaaATGtctcatcaccatcaccatcacTCCCCTATtCTAGGTTATTGGAAGATTAA
GGGtCTTGTGCAACCaaACTCGACTTCTTTTGGAAATATCTGAAGAgAAgTATGAAGAGCATTGTATGA
GCGtGATGAAGGTGATAAgTGGCGAAACAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTT
ATTAcATTGATGGTGATGTcAAgTTgAcTCAaTCTATGGCtATCATAcGTTAcATcGCTGACAAGCACAAc
ATGTTGGGTGTTGTCCAAAAGAGCGTGCtGAGATTTCAATGCTTGAAGGAGCtGTTTTGGATATTAG
ATACGGTGTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCT
ACCTGAAATGCTGAAAATGTTGGAAGATCGTTTATGTCATAAAACATAcTTgAATGGTGATCATGTAACt
CATCCTGACTTCATGTTGTATGACGCTCTTGTGTTTgTACATGGACCCAATGTGtCTGGATGCc
TTCCAAAAGTTgGTTTGTTCAAgAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAATCCA
GCAAGTATATcGCATGGCCTTTGCAaGGtGGCAAGCtACGTTTGGTGGTGGCGACCATCCTCCAAA
TcTgATCTtGTTCCaCGTCCAATtGAGAACAGCCCCAAGCAGTTGTTGCAGAGTACCAACTACAAGGAC
TGGtGAACATGTGTCAACAGAACCAGCAGTACGGTGGTACTTCGAGACCTTCATCGACTCCGGTG
AGTTGTCCGCTTACACCATCGTCGTCGGTACCGTCTTGACCGTTTTCGTTTTACCACCCCATTGGG
TTTTGGCTTTGATCGTTTTCGGTACCTTGATCCCAGTCTTGTCCAGCTCAGGACCAGTCCAACACC
TGGTCCGACTTCATCACCCAGACCAAGAACATCATCAAGAAGGAGATCGCTTCCACCTACATCTCCA
ACGCTAACAAGATCTTGAACAGATCCTTCAACGTCTCTCCACCTACCACAACCACTTGAAGACCTG

GGAGAACAACCCAAACCCACAgAAcACcCAGGAcGTcAGaACcCAgATCCAGttgGTcCAcTACCAcTTcCAgAaAcGTCATcCCAGAGttgGTcAACTCcTGTCcCaCAaAcCCatccGAcTGtGAcTACTAcAACATcttgGTcTTgTCctccTAcGcTcAgGcTcAACTTgCActtgACcGTcTTgAAcCAgGcTGTCAAgtTcGAgGcTAcTTgAAgAAcAAcagaCAgTTCGAcTAcTTgGAGCCaTTGCCAACcGcTAcGAcTAcTAcCCAGTcTTGACcAAgGCTATcGAgGAcTACACcAAcTAcTGTGTcACcACcTAcAAgAAgGGTTgAAcTTgATcAAgACcACcCCaGActccAActtgGAcGGtAAcATcAACTGGAACACcTACAACcTAcagaACcAAgATGACcACcGCTGTcTTgGAcTgGTcGCTttgTTcCAaAcTAcGAcGTcGGTAAgTAcCCAATcGGTGTCCAgTcGAgttgACcagaGAgATcTAcCAGGTcttgAACTTCGAgGAggtccCCaTAcAAgTAcTAcGACTTcCAgTAcCAgGAGGAcTccttgACcagaAGACCAcAcTTgTTcAcCtGGttgGAcTcTTGAaAcTTcTAcGAgAAgGcTcAgACcCAaAcAAcTTcTTCACctccCAcTAcAAcATGTTcCAcTACAcctTgGAcAAcATcTCCCAgAAgTcctccGTcTTcGGtAAcCACAaAcGTcACcGAcAAgTTgAAgTccttgGGTTTGGcTAcCAaAcATcTAcATcTTcTTgTgAAcGTCATctccTTgGAcAAcAAgTAcctgAcGAcTAcAAcAAcATctccAAgATGGAcTTcTTcATcAcCAcAAcGGTAcCAgAttgTTGGAGAAgGAgttgACcGcTGGtTcCGcTcAgATcAcTAcGAcGTcAAcAAgAAcATcTTCGGtTgCCAATcttgAAgagaAGAGAGAAcCAgGGtAAcCCaACcTtgTTcCCAACcTAcGAcAACTActccCAcATcTTgTcTcATcAAgtccttgccATCCcAGcTAcCcTAcAAgACcCAgGTcTAcACcTTcGCTTGGACcCACTCctccGTcGAcCCaAAgAAcACcATcTAcACcCAcTgAcACCCAgATcCCAGCTGTcAAgGcTAAcTcTgGGtAcCgCTTcCAAGGTcGtCAGGGtCCaGGTCAcAcGGtGGtGAcTTgATcGAcTTCAAgGAcCAcTTCAAgATcACcTGTCAgCACTCcAAcTTcCAGCAgTcTAcTTcATcAGAATcagaTAcGCTTCCaAcGGttccGcTAAcACcagaGCTGTcATcAActgtccATCCcAGGtGTcGcTgAgttgGGTATGGCttgAACCCaAcTTcTcGGTAcGAcTAcACcAAcTTgAAgTAcAAgGAcTTcCAGTACTTgGAgTTcTcAACGAGGTcAAgTTcGCTCCcAAcCAgAACATcTccttgGTcTTcAAcCGaTcTgAcGTcTAcCcAACACcACcGTctTgATcGAcAAgATTGAgTTctTGCCAATTACTCGTTCcATcAGAGAGGATAGAGAGAAgCAgAAgTTgGAgACcGTcCAACAaAtcgaggtagatatacgcagctcctccacctccacctccaTAATCTAGAgggGAATTCgggggg

GCT-Cry11Aa-TMOF with restriction enzymes optimized sequence:2710 bp

GGGGGGGATCCGGGCTCGAGAAAAATGGCTCATCACCATCACCATCACTCCCCTATcTAGGTTAT TGGAAGATTAAGGGtCTTGTGCAACCaACTCGACTTCTTTTGAATATCTTGAAGAgAAgTATGAAGAG CATTGTATGAGCGtGATGAAGGTGATAAgTGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCC CAATCTTCTTATTAcATTGATGGTGTGtAAgTTgAcTcAaTCTATGGcTATCATACGTTAcATcGCTGA CAAGCACAAcATGTTGGGTGGTTGTCCAAAAGAGCGTGTGAGATTTCAATGCTTGAAGGAGcTgTTT TGGATATTAGATACGGTGTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTC TTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAAACATAcTTgAATGGTG ATCATGTAACtCATCCTGACTTCATGTTGTATGACGCTCTTGTGTTTTgTACATGGACCCAATGT GtCTGGATGCcTTCCAAAgTTgGTTTGTTCAAgAAACGTATTGAAGCTATCCCACAAATTGATAAGTA CTTGAAATCCAGCAAGTATAcGCATGGCCTTTGCAaGGtTGGCAAGcTACGTTTGGTGGTGGCGACC ATCCTCCAAAATcTgATCTtGTTCCaCGTGAAGATAGTTCTTTGGATACTCTAAGTATTGTTAACGAAc TGACTTCCCATTGTACAACAActACACTGAACCTACTATCGCTCCAGCCTTGATTGCTGTTGCTCCCA TCGCTCAGTACCTTGCCACTGCTATCGGGAAGTGGGCTGCTAAGGCTGCCTTCTAAGGTCTGTc CCTGATCTTCCAGGTTCTCAACCAGCTACTATGGAGAAGGTTcGTACCGAAGTCGAGACCCTCATC AATCAAAGTTGAGCCAAGATCGAGTCAACATCTTGAACGCCGAGTACAGAGGGATCATTGAGGTTA GTGATGTCTTCGATGCCTACATCAAGCAACCAGGTTTCACTCCTGCTACTGCTAAGGGTTACTTCTC AACCTGAGTGGTGTATCATCCAACGATTGCCTCAATTTGAGGTTCAAACATACGAAGGAGTCTCTAT CGCCCTGTTCACTCAAATGTGTACACTTCATTTGACTTTGTTGAAAGACGGAATCCTAGCAGGTAGTG CATGGGGATTTACTCAAGCTGATGTTGATTCTTCATCAAGTTGTTCAATCAAAGTCTTCGATTACA GGAActAGATTGATGAGAATGTACACTGAAGAGTTCCGAAGATTGTGTAAAGTCAGTCTTAAAGATGG ATTGACGTTCCGGAATATGTGTAATTTGTATGTGTTCCATTTGCTGAAGCCTGGTCTTTGATGAGAT ATGAAGGATTGAAGTTACAAAGCTCTATCATTGTGGGATTATGTTGGTGTCTcTATTCTGTCAAC TACAACGAATGGGGAGGACTAGTCTACAAGTTGTTAATGGGTGAAGTTAATCAAAGATTGACAActGT TAAGTTCAATTACTCCTTCACTAATGAACCAGCTGATATACCAGCAAGAGAAAATATTCTGTTGTCC ATCCTATCTACGATCCTAGTTCTGGTCTTACTGGATGGATCGGAAACGGAAGAActAAcAACTTCAAC TTCGCTGATAACAATGGCAATGAAATTATGGAAGTTAGAActCAAActTCTACCAAAATCCAAACAA CGAGCCTATCGCTCCTAGAGATATCATCAACCAAAATTTGACTGCCcAGCTCCAGCTGATCTATTCT TCAAGAATGCTGATATCAACGTTAAGTTCACTCAGTGGTTTCAATCTACTCTATACGCTTGAACATTA AGCTTGGTACACAActGTCTTgAGTAGTAGAACCGGAACAATTCCACCAAAATTACTTGGCCTACGAT

GGATACTACATTTCGTGCTATTTTCAGCTTGCCCAAGAGGAGTCTCCCTTGCATACAATCACGATCTTAC
 TACTCTAACATACAATAGAATTGAGTACGATTACCTACTACTGAAAATATTATTGTCGGGTTTGCTCC
 AGATAACTAAGGACTTCTACTCTAAGAAGTCTCACTACTTAAGTGAAACCAACGATAGTTACGTCA
 TTCCTGCTCTGCAATTCGCTGAAGTTTCAGATAGATCATTCTTGAAGATACTCCAGATCAAGCTACT
 GACGGTAGTATTAAGTTTCGCTCGTACTTTTCATTAGTAATGAAGCTAAGTACTCTATTAGACTAAACACT
 GGGTTCAATACCGCTACTAGATACAAGTTGATTATCAGAGTTAGAGTTCCATATCGCTTGCCTGCTGG
 TATTCGGGTACAATCTCAGAACTCAGGAAATAATAGAATGCTAGGCAGTTTCACTGCAAAATGCTAATC
 CAGAATGGGTGGATTCGTCACTGATGCCTTCACTTCAACGACTTGGGTATTACAACCTCAAGTACA
 AATGCTTTGTTGAGTATCTCTTCAGATAGCTTGAACCTGGAGAAGAGTGGTACTTGTCCAGTTGTT
 CTTGGTCAAGGAATCCGCCTTCACTACGCAAATTAACCCATTGCTAAAGATCGAAGGTAGATACGAT
 CCAGCTCCTCCACCTCCACCTCCATAATCTAGAGGGGAATTCGGGGGG

GST-Cyt1Aa-TMOF with restriction enzymes optimized sequence: 1528 bp

GGGGGGGGATCCGGGCTCGAGAAAAATGGCTCATCACCATCACCATCACTCCCCTATCTAGGTTAT
 TGGAAGATTAAGGGTCTTGTGCAACCcACTCGACTTCTTTTGAATATCTTGAAGAgAAgTATGAAGAG
 CATTGTATGAGCGtGATGAAGGTGATAAgTGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCC
 CAATCTTCCTTATTAcATTGATGGTGATGTcAAgTTgAcTcAaTCTATGGCtATCATACGTTAcATcGCTGA
 CAAGCACAAcATGTTGGGTGGTTGTCCAAAAGAGCGTGCtGAGATTTCAATGCTTGAAGGAGCtGTTT
 TGGATATTAGATACGGTGTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTC
 TTAGCAAGCTACCTGAAATGCTGAAAATGTTTCGAAGATCGTTTATGTCATAAAACATAcTTgAATGGTG
 ATCATGTAACtCATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTgTACATGGACCCAATGT
 GtCTGGATGCcTTCCCAAAGTTgGTTTGTTCcAAgAAACGTATTGAAGCTATCCCACAAATTGATAAGTA
 CTTGAAATCCAGCAAGTATATcGCATGGCCTTTGCAaGGtTGGCAAGCtACGTTTGGTGGCGACC
 ATCCTCCAAAATcTATCTtGTTCCaCGTGAAAACCTTGAATCATTGTCCATTGGAAGATATCAAGGTCAA
 CCCATGGAAAACCTCAATCTACTGCTAGAGTTATTACCTTGGCTGTTGAAGATCCAAATGAAATCA
 ACAACCTTCTTTCTATTAACGAAATTGATAATCCAAATTACATCTTGCAAGCCATTATGTTGGCTAACG
 CCTTCCAAAACGCATTGGTTCCAACCTTCTACTGATTTCCGGTATGCTCTTCCGTTTCAAGTATGCCAAAA
 GGTTTGGAAATTGCTAACACTATTACTCCAATGGGTGCTGTTGTCAGTTACGTTGATCAAAAACGTTAC
 TCAAACATAACAACCAAGTTAGTGTTATGATTAACAAGGTCTTGGAAAGTCTTGAAGACTGTCTTGGGAG
 TTGCCTTGAGTGGATCTGTCATTGATCAATTGACTGCTGCTGTTACCAACACCTTCACTAACTTGAAC
 ACTCAAAGAATGAAGCTTGGATCTTCTGGGGTAAGGAAACTGCTAATCAAACATAACTACACCTACAA
 CGTTCTGTTTGCAATCCAAAACGCTCAAACCTGGTGGTGTATGTACTGTGTTCCAGTTGGTTTTGAAA
 TTAAGGTCTCTGCTGTTAAGGAACAAGTCTTGTCTTCACTATTCAAGATTCTGCTAGTTACAACGTTA
 ACATTCAATCTTTGAAGTTTGTCAACCATTGGTTAGCTCTAGTCAATACCCAATTGCTGATCTTACTA
 GTGCTATTAATGGAACCTCATCGAAGGTAGATACGATCCAGCTCCTCCACCTCCACCTCCATAATCT
 AGAGGGGAATTCGGGGGG

RESULTS AND DISCUSSION

Cloning of Bti toxins and TMOF into *Pichia pastoris*.

Our approach was to clone and express Bti toxins and TMOF in the methylotrophic yeast *Pichia pastoris*. To achieve this goal we initially cloned and expressed single toxins in *Pichia pastoris* and checked their biological activity against mosquito larvae. In our initial progress reports we have reported that expressing Cry4Aa and Cry11Aa fused with TMOF caused rapid death and starvation to *Aede aegypti* larvae. However, we have noticed that not all the toxins were soluble in the cell cytoplasm because cells tend to precipitate heterologous proteins to get rid of them. To overcome this problem, we have constructed and cloned new genes that were fused to GST (Glutathione- S-Transferase) to keep the heterologous proteins soluble in the cells cytoplasm. The synthetic genes were lifted from pUC57 with *XbaI* and *XhoI* for cloning into pPICZB and

with *BamHI* and *EcoRI* for cloning into pPIC3.5K. *P. pastoris* KM71H cells were transformed and glycerol stock solutions were prepared. Table 2 lists all the recombinant yeast cells that were transformed using our optimized synthetic genes.

Table 2. List of synthetic genes that were cloned into *P. pastoris* KM71H cells at the *AOX1* and *HIS* loci.

Transformed Cell	Location of Transformation	LT ₅₀ (Days)
<i>AOX1:Cry4Aa-TMOF</i>	<i>AOX1</i>	8
<i>AOX1:Cry11Aa-TMOF</i>	<i>AOX1</i>	2-4
<i>AOX1:GST-Cry4Aa-TMOF</i>	<i>AOX1</i>	3-5
<i>AOX1:GST-Cry11Aa-TMOF</i>	<i>AOX1</i>	3
<i>AOX1:GST-Cry11Aa-TMOF</i> (cell-extract)	<i>AOX1</i>	>1
<i>AOX1:TMOF; HIS:Cry4Aa-TMOF</i>	<i>AOX1,HIS</i>	N.D
<i>AOX1:TMOF; HIS:Cry11Aa-TMOF</i>	<i>AOX1,HIS</i>	N.D
<i>AOX1:GFP-TMOF; HIS:Cry4Aa-TMOF</i>	<i>AOX1,HIS</i>	N.D
<i>AOX1:GFP-TMOF;HIS:Cry11Aa-TMOF</i>	<i>AOX1,HIS</i>	N.D

Pichia pastoris cells 10⁸ cells/mL were fed in 24 well plates to 6 groups of mosquito larvae (8 larvae per group) containing one *Ae.aegypti* larva per well in 1.0 mL sterile water. Larval survival was followed daily. LT=Time required to kill 50% of the tested larvae. N.D= Not determined. GFP=Green Fluorescent Protein gene. TMOF=Trypsin Modulating Oostatic Factor. *Ae.aegypti* 4th instar larvae, were fed cell extracts of broken *P.pastoris* cells at different times after fermentation, and larval survival was checked as above.

Although we proposed to show activity of 3 toxin-genes cloned simultaneously into P.pastoris cells the paucity in time (12 months) did not allow for ample time to incorporate all the genes into the yeast chromosome. Thus, a more realistic approach was undertaken in which individual genes were cloned and expressed in the yeast cells. We also managed to finish the simultaneous cloning of TMOF and GFP-TMOF on the AOX1 loci and Cry4Aa-TMOF and Cry11Aa-TMOF on the HIS loci, however, we did not check the activity of these 4 construct yet (Table 2). Cyt1Aa-TMOF was not cloned into P.pastoris KM71H cells because it enhances the activity of the other genes but by itself is not very toxic.

Fermentation and biological activity of the recombinant cells.

Following the cloning and selection of multiple inserts in KM71H cells, several clones were fermented and tested against *Ae. aegypti* larvae. Each clone was fermented for 24, 48, 72 and 96 hours in the presence of methanol (0.5%; materials and methods). After fermentation, cells were washed by centrifugation in sterile distilled water and aliquots removed and heat inactivated (50⁰ C for 3 hours) and compared with the potency of cells that were not heat-treated. Aliquots (10⁸ cells/mL) were fed to individual larvae in 24 well plates. Larval survival was followed at 24 hours intervals.

When transformed *P. pastoris Cry4Aa-TMOF* were initially fed to mosquito larvae we observed that cells that were fermented for short intervals (24 and 48 h) were more efficient in killing the larvae (72% mortality in 10-12 days), whereas cells that were fermented for longer intervals (72 and 96 h) were not as efficient (24% and 50% mortality, respectively; Fig.4). There are two explanations for these observations:
a. Cry4A-TMOF precipitates in the cell cytoplasm and, thus, is not active against the

larvae or, b. The transformed yeast cells excised the cloned genes during the fermentation because these genes are toxic.

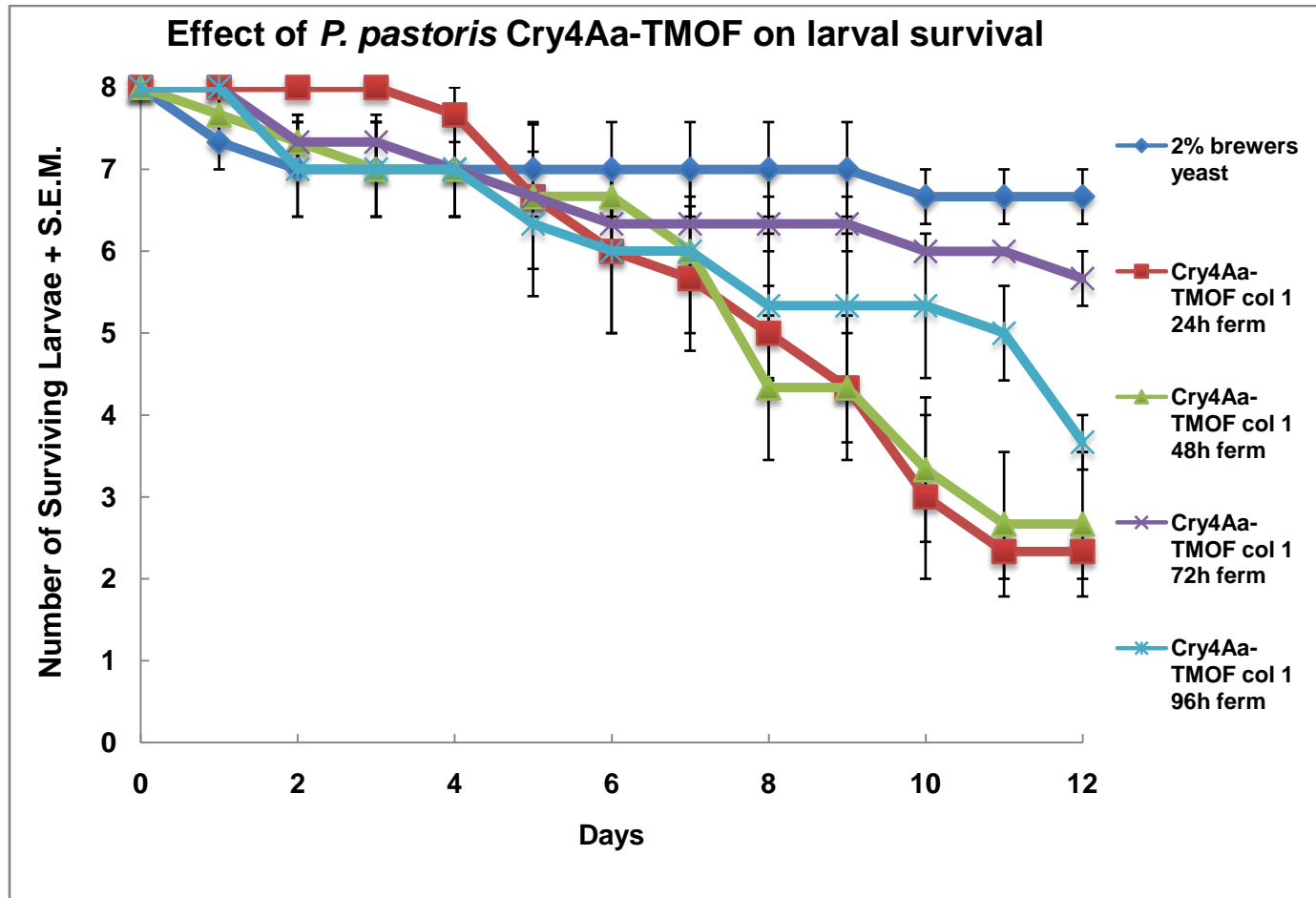


Fig. 4. Effect of *P. pastoris* *Cry4Aa-TMOF* cells on *Ae. aegypti*. Cells were fermented for 24, 48, 72 and 96 hours and fed to first instar larvae in 24 well plate. Larval survival was recorded and expressed as mean Survival \pm S.E.M. Brewer's yeast was used as non-transformed cell control.

On the other hand, cells that were transformed with *Cry11Aa-TMOF* were more effective. After 96 hours of fermentation, larval mortality was 62.5% at day 2 and about 87.5% at day 8 (Fig. 5). Cells that fermented for longer periods (72 and 96 h) contained more toxins. These cells were more effective than cells that were fermented for shorter time (24 and 48 h, respectively, Fig. 5). Cells that were fermented for shorter times caused 50% mortality at days 6 and 7, respectively and 81% mortalities at day 12 (Fig. 5). Therefore, it appears that *Cry11Aa-TMOF* gene is more stable during the fermentation, and the toxin does not readily precipitate in the cell cytoplasm, or being excised from the chromosome.

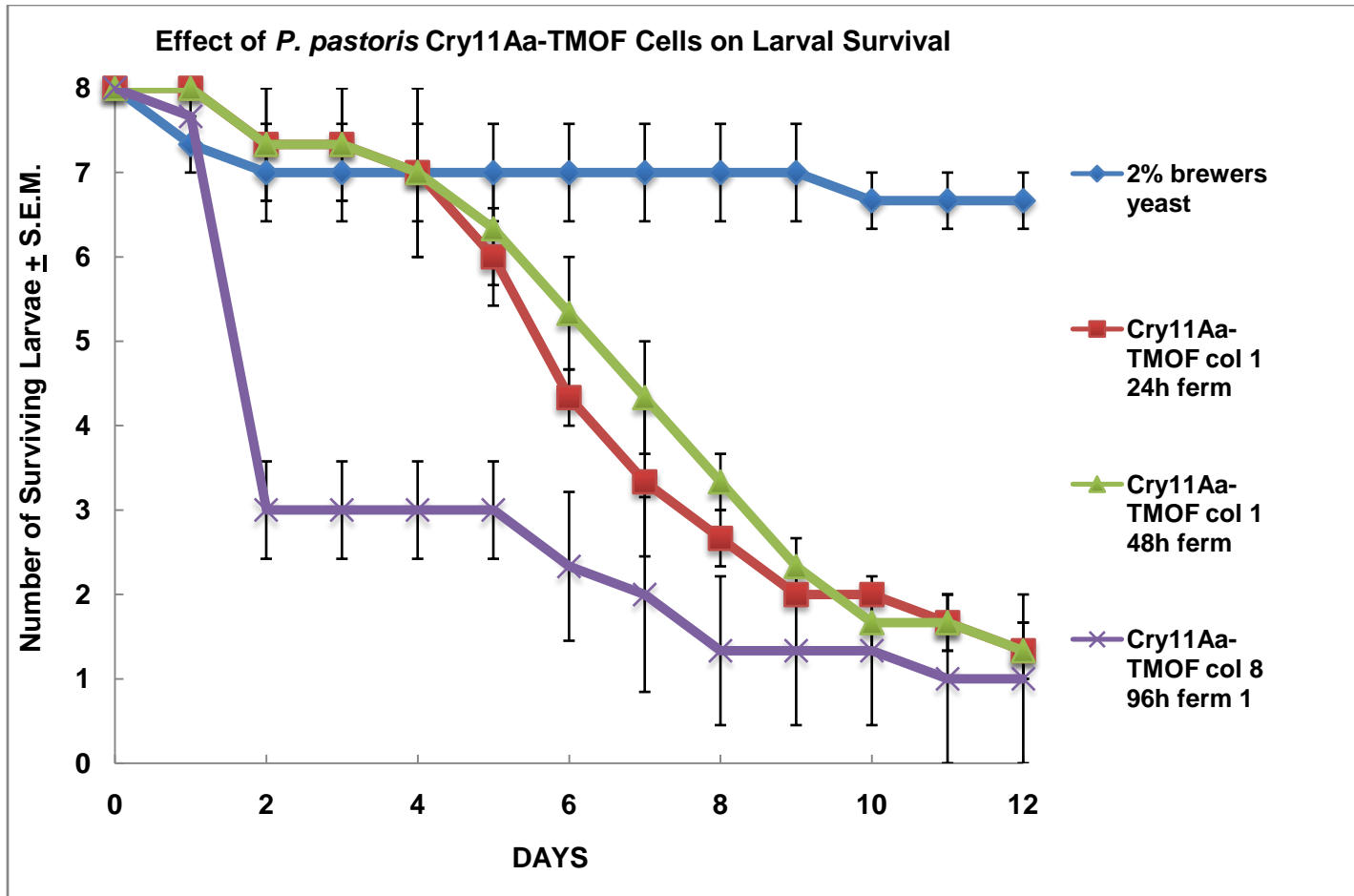


Fig. 5. Effect of *P. pastoris* Cry11Aa-TMOF on *Ae. aegypti* larval survival. Cells were fermented for 24, 48 and 96 hours and fed to first instar larvae in 24 well plate. Larval survival was recorded and expressed as mean Survival \pm S.E.M. Brewer's yeast was used as non-transformed cell control.

To find out if we can increase the activity of the recombinant *P. pastoris* cells by increasing the solubility of our heterologous proteins during fermentation, GST (glutathione-S-transferase) was fused with *Cry4Aa-TMOF* and *Cry11Aa-TMOF*. Cells were fermented at different intervals and the biological activity of these cells was followed. After *P.pastoris* cells were transformed with *GST-Cry11Aa-TMOF*, the biological activity of these cells increased several fold (Fig. 6). About 87.5% of the tested larvae died at 5 days (Fig. 6) and shorter fermentation periods produced cells that did not have as much toxin as compared with cells that were fermented for 96 h. These results indicate that GST-Cry11Aa-TMOF does not precipitate in the cell cytoplasm during the fermentation. The biological activity of cells that were heat inactivated at 50⁰ C for 3 hours was also checked to ensure that recombinant cells that will be used in the field are all dead. Because high temperatures denature the conformations of native proteins, we were interested to find out if GST-Cry11Aa-TMOF in yeast cells that were heat inactivated (50⁰C for 3 h) retains its biological activity.

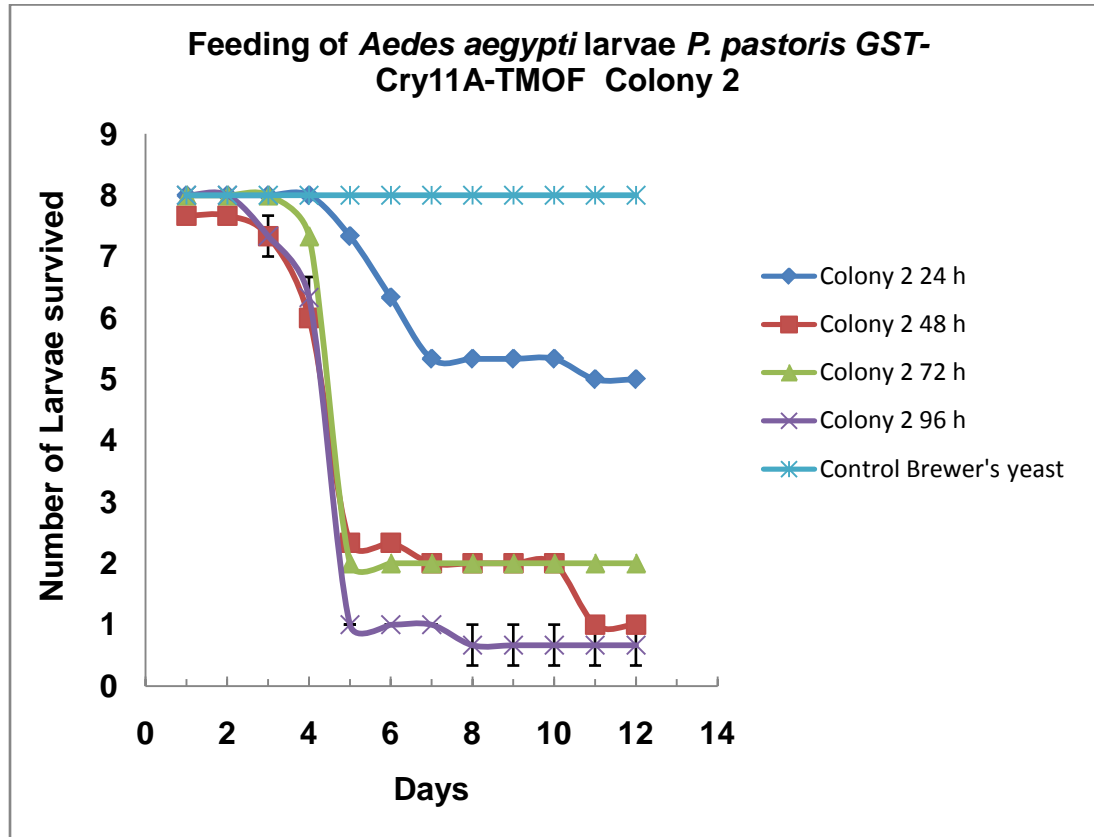


Fig. 6. Effect of *P. pastoris* *Cry11Aa-TMOF* on *Ae. aegypti* larval survival. Cells were fermented for 24, 48, 72 and 96 hours and fed to first instar larvae in 24 well plate. Larval survival was recorded and expressed as mean Survival \pm S.E.M. Brewer's yeast was used as non-transformed cell control.

To find out the heat stability of the recombinant protein, *P. pastoris* cells were transformed with *GST-Cry11Aa-TMOF* and fermented in the presence of methanol (0.5%) for 96 h. After fermentation, the cells were heat inactivated (50^o C for 3 h) and fed to mosquito larvae. Our results show that these cells were *almost as effective* as cells that were not treated (Fig.7) indicating that GST protects the recombinant protein from heat denaturation while the yeast cells are killed during this process. Thus, inactivated yeast cells can be used in the future to treat mosquito larvae in the field.

Our earlier results (Fig. 4) indicate that *Cry4Aa-TMOF* may precipitate in the cell cytoplasm. To overcome this problem we cloned and express *GST-Cry4Aa-TMOF* in *P. pastoris* cells with the hope of stabilizing the toxin and preventing precipitation. The new recombinant cells were fermented to find out if the new clone will be more soluble and effective against mosquito larvae. Our results, however, indicate that *GST-Cry4Aa-TMOF* rapidly loses its activity in the cells after 96 h fermentation (Fig. 8). The recombinant cells were effective for only one day killing 50% of the larvae. These results indicate that during the fermentation most of the cloned genes were deleted. We also noted that heat treatment inactivated the protein indicating that fusion with GST did

not protect the protein against heat denaturation (Fig. 8). This is probably due to deletion of the *GST-Cry4Aa-TMOF* gene from the yeast chromosome during the prolonged fermentation. To overcome this problem, we plan to express GST-Cry4A-TMOF in the presence of Zeocin, thus, preventing the cells from deleting the gene. Apparently, this is a common phenomenon that is known to occur during prolonged fermentation in recombinant *P. pastoris* cells (*Invitrogen* personal communications).

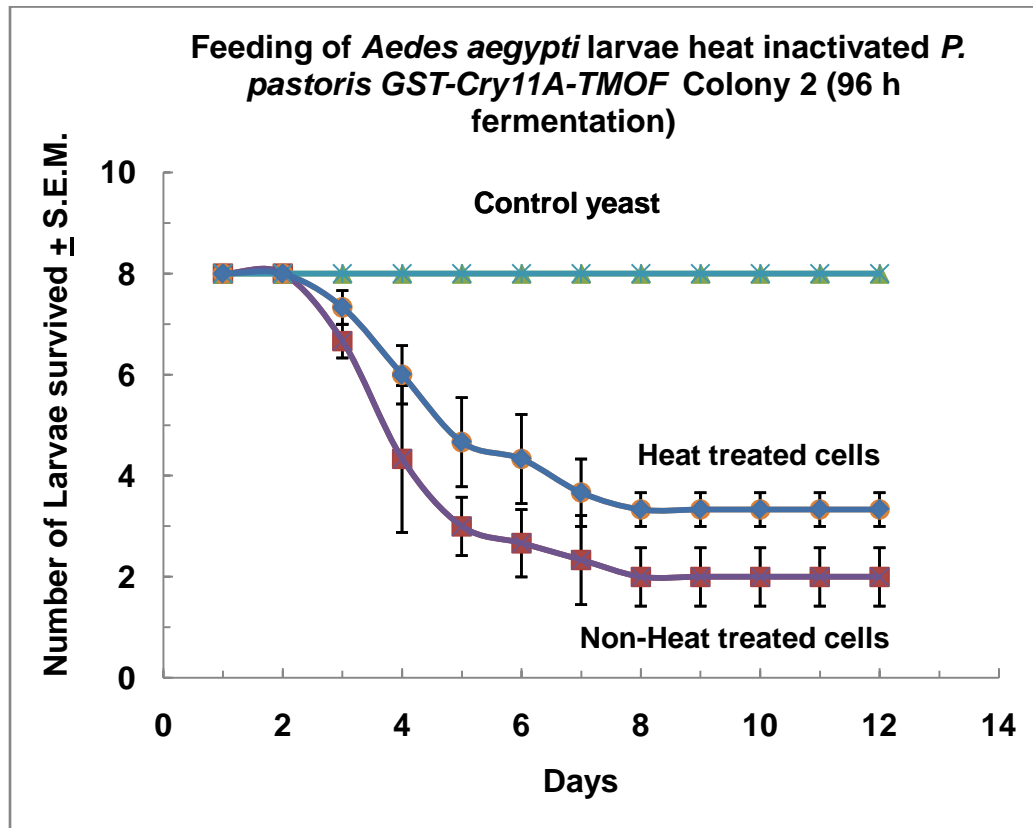


Fig. 7. Feeding *Ae. aegypti* larvae heat inactivated *P. pastoris* GST-Cry11Aa-TMOF. Cells were fermented for 96h, half the cells were heat inactivated (50^o C for 3 h) and the other half was kept at room temperature. Heat inactivated and native cells were fed to first instar mosquito larvae for 12 days, and larval survival was daily recorded. Results are expressed as mean Survival \pm S.E.M. *P. pastoris* cells transformed with empty plasmids were used as a control.

Biological activity of cell extracts

We have shown that whole cells of *P. pastoris* GST-Cry11Aa-TMOF cause mortality to first instar larvae. To find out if *Pichia* GST-Cry4Aa-TMOF cell extracts are effective on 4th instar larvae. Cells of colony 1 and colony 2 after 24, 48, 72 and 96 h fermentations were broken with YPER (Pierce) and the extracts (10 μ L) were fed to two groups of sixteen, 4th instar *Ae. aegypti* larvae. Twenty-four hours later, larval mortality was recorded. Cells from colony 2 were more efficient in killing the 4th instar larvae.

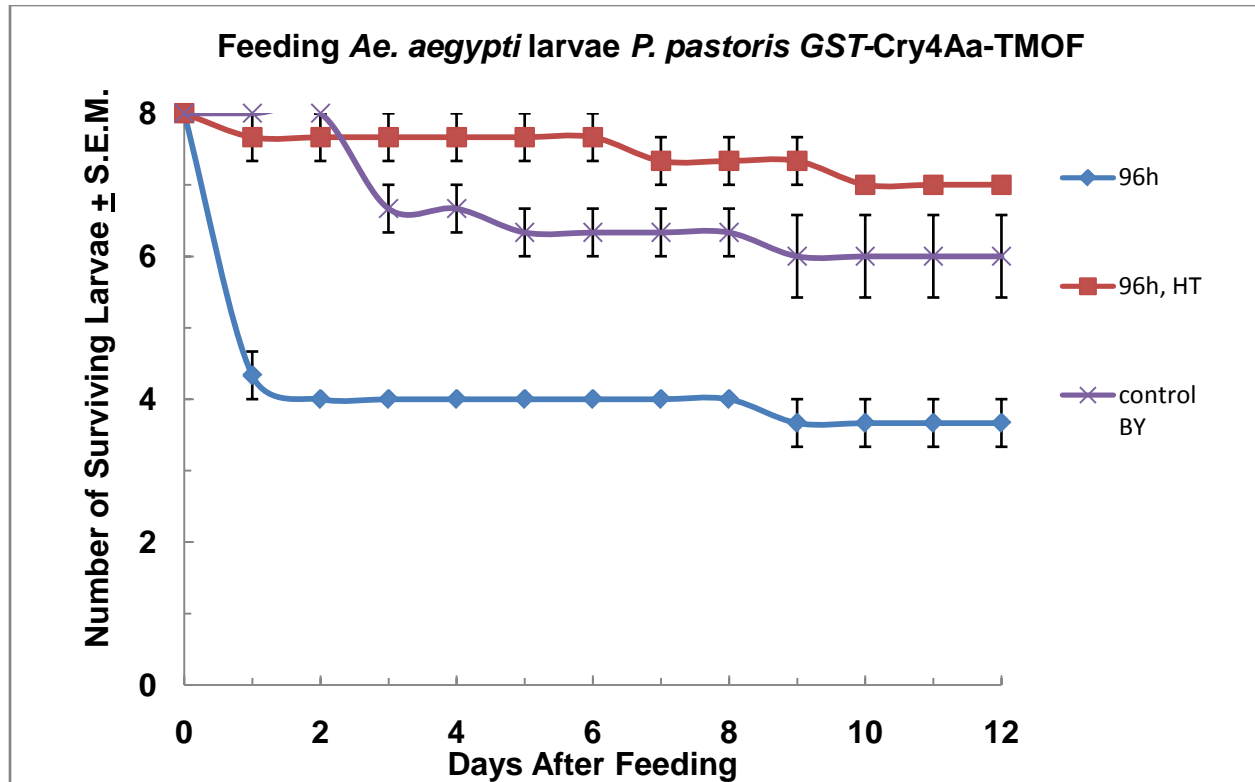


Fig. 8. Feeding *Ae. aegypti* larvae heat inactivated *P. pastoris GST-Cry4Aa-TMOF* cells. Cells were fermented for 96h, half the cells were inactivated at 50^o C for 3 h and the other half kept at room temperature. Heat inactivated and native cells were fed to first instar mosquito larvae for up to 12 days and larval survival was daily recorded. Results are expressed as mean Survival ± S.E.M. Non-recombinant Brewer's yeast cells were used as a control

At 48 and 96 h larval mortality reached 62.5%, whereas colony 1 reached maximum mortality of 44% at 72 h (Fig. 9). Controls that were fed YPER that was used to extract the cells, caused 6% mortality at 24 h. These results show that the *GST-Cry11Aa-TMOF* cell extracts are effective against early 4th instar larvae

Protein characterization and mass spectrometry analysis.

Our biological results (Figs. 4-8) strongly suggest that Cry11Aa-TMOF and Cry4Aa-TMOF were synthesized *de novo* by the recombinant yeast cells. Our success in fermenting and keeping Cry11Aa-TMOF in solution prompted us to try to extract the recombinant protein, purify it by Ni affinity chromatography, separate it on SDS PAGE and sequence the purified protein band by mass spectrometry. *P. pastoris-Cry11Aa-TMOF* cells were broken with glass beads and extracted with 8M urea and adsorb onto a Ni column (0.5 x 10 Cm). The column was washed with phosphate buffer, followed with 20 mM imidazole and the recombinant protein was eluted with 250 mM imidazole (Fig. 10), concentrated by centrifugation and dried by Speed Vac. The dried protein

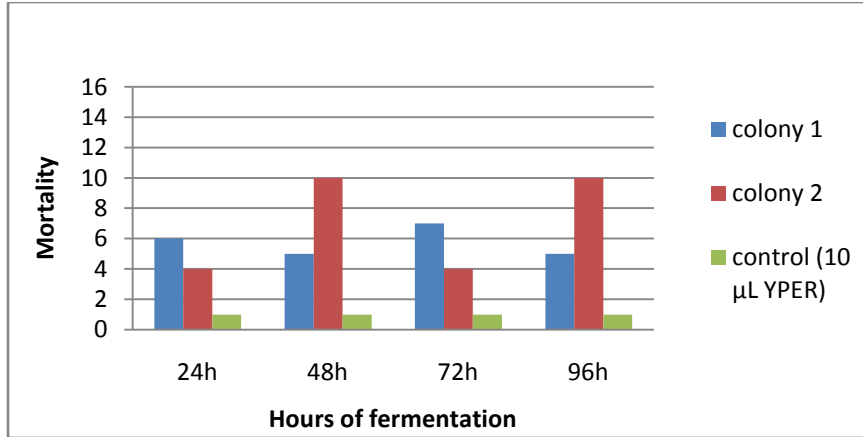


Fig. 9. Feeding of early 4th instar *Ae.aegypti* larvae with YPER extract of recombinant *P. pastoris* GST-Cry11Aa-TMOF. Two groups of 16 early 4th instar larvae were fed cell extracts from colony 1 and colony 2 of recombinant *P. pastoris* cells, and larval mortality was checked 24 h after the feeding. Results are expressed as averages of two determinations.

was taken up with a Tris- buffer containing 4% SDS and 4% mercaptoethanol, heated at 95^o C for 10 min and size-separated by SDS- PAGE. A protein band of M_r 72 kDa was identified (Fig. 11), cut from the gel, digested with trypsin and analyzed by mass

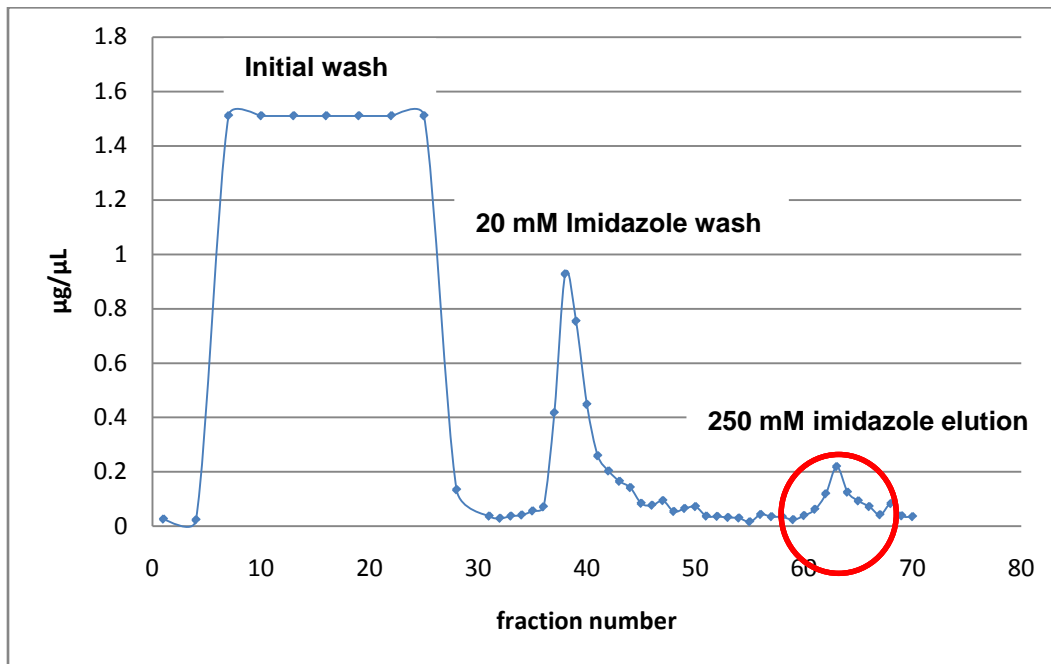


Fig. 10. Elution of Cry11Aa-TMOF by Ni Chromatography. *P. pastoris* cells KM71H *Cry4Aa-TMOF* (OD₆₀₀=400) were broken by glass beads and extracted in 8M urea. The protein was adsorb onto Ni column (Quiagen) and the column was washed in phospahte buffer pH 8.0 without imidazole, in the presence of 20 mM imidazole, and Cry11Aa-TMOF eluted with 250 mM imidazole. Fractions 61-65 (235 μg protein) were collected, concentrated and dried by Speed Vac

spectrometry at the University of Florida Biotechnological Center (materials and

methods). After mass spectral analysis, several peptides from the tryptic digestion spanning 14% of the entire of Cry11Aa were identified (Fig. 12). These results prove (probability of 95-100%) that Cry11Aa was synthesized by the recombinant yeast cells.

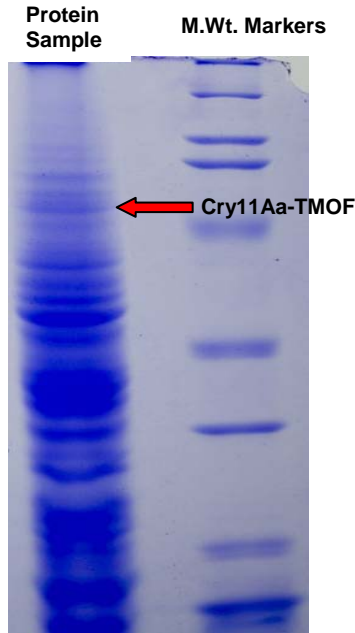


Fig. 11. SDS-PAGE of the 250 mM imidazole elution peak from the Ni affinity chromatography. A putative protein band that ran at the same molecular weight of Cry11Aa-TMOF was identified (red arrow), cut and digested with trypsin and analyzed by mass spectrometry.

gi|117327 (100%), 72,352.1 Da

Pesticidal crystal protein cry11Aa (Insecticidal delta-endotoxin CryXIA(a)) (Crystalline entomocidal protoxin) (72 kD
8 unique peptides, 8 unique spectra, 9 total spectra, 87/643 amino acids (14% coverage)

MEDSSLD T L S	I V N E T D F P L Y	N N Y T E P T I A P	A L I A V A P I A Q
Y L A T A I G K W A	A K A A F S K V L S	L I F P G S Q P A T	M E K V R T E V E T
L I N Q K L S Q D R	V N I L N A E Y R G	I I E V S D V F D A	Y I K Q P G F T P A
T A K G Y F L N L S	G A I I Q R L P Q F	E V Q T Y E G V S I	A L F T Q M C T L H
L T L L K D G I L A	G S A W G F T Q A D	V D S F I K L F N Q	K V L D Y R T R L M
R M Y T E E F G R L	C K V S L K D G L T	F R N M C N L Y V F	P F A E A W S L M R
Y E G L K L Q S S L	S L W D Y V G V S I	P V N Y N E W G G L	V Y K L L M G E V N
Q R L T T V K F N Y	S F T N E P A D I P	A R E N I R G V H P	I Y D P S S G L T G
W I G N G R T N N F	N F A D N N G N E I	M E V R T Q T F Y Q	N P N N E P I A P R
D I I N Q I L T A P	A P A D L F F K N A	D I N V K F T Q W F	Q S T L Y G W N I K
L G T Q T V L S S R	T G T I P P N Y L A	Y D G Y Y I R A I S	A C P R G V S L A Y
N H D L T T L T Y N	R I E Y D S P T T E	N I I V G F A P D N	T K D F Y S K K S H
Y L S E T N D S Y V	I P A L Q F A E V S	D R S F L E D T P D	Q A T D G S I K F A
R T F I S N E A K Y	S I R L N T G F N T	A T R Y K L I I R V	R V P Y R L P A G I
R V Q S Q N S G N N	R M L G S F T A N A	N P E W V D F V T D	A F T F N D L G I T
T S S T N A L F S I	S S D S L N S G E E	W Y L S Q L F L V K	E S A F T T Q I N P
L L K			

Fig. 12. Mass spectral analysis of the trypsin digested protein band after SDS PAGE. Nine distinct peptides (colored yellow) were identified, throughout the protein sequence, identifying the protein with 95-100% probability as Cry11Aa.

Summary and Future Work.

This report shows for the first time that it is possible to express in *P. pastoris* fusion proteins of Cry4Aa-TMOF, Cry11Aa-TMOF, GST-Cry4Aa-TMOF and GST-Cry11Aa-TMOF that retain their biological activity and efficiently control mosquito larvae. The addition of GST to the fusion proteins increased solubility of the recombinant proteins in the cell cytoplasm, conferred heat stability and activity during the heat inactivation step necessary to inactivate the yeast cells in future field applications. The submitted research proposal suggested two goals which were only partially achieved due to the complexity of the project, and to the enormous time that was spent on synthesizing and cloning of the synthetic genes. We have been able to clone and express 4 single genes and combinations of 2 genes (Table 2). We successfully tested the biological activities of the single genes in the laboratory before time ran out. Because of the short research period (12 months), we were not able to perform large scale tests outside the laboratory in the Indian River Mosquito Control District.

We would like to continue this promising research project, and apply in the near future for additional support funds from DACS to be able to develop a Bti-TMOF larvicide for field applications in Florida and beyond.

LITERATURE CITED

- Barany, G. and R. B. Merrifield. 1979. In the Peptides, Vol. 2, E. Gross and J. Meienhofer, eds, Academic Press, New York. pp. 1-284.
- Borovsky, D. 1985. Isolation and characterization of highly purified mosquito oostatic hormone. Arch. Insect Biochem. Physiol. 2: 333-349.
- Borovsky, D. 1988. Oostatic hormone inhibits biosynthesis of midgut proteolytic enzymes and egg development in mosquitoes. Arch. Insect Biochem. Physiol. 7: 187-210.
- Borovsky, D., Carlson, D. A., Griffin, P. R., Shabanowitz, J. and Hunt, D. F. 1990. Mosquito oostatic factor a novel decapeptide modulating trypsin-like enzyme biosynthesis in the midgut. FASEB J. 4: 3015-3020.
- Borovsky, D., Carlson, D. A. and D. F. Hunt. 1991. Mosquito oostatic hormone a trypsin modulating oostatic factor. In Insect Neuropeptides Chemistry, Biology and Action, edited by J. J. Menn, T. J. Kelly and E. P. Masler, Chapter 13, pp. 133-142. ACS Symposium Series 453.
- Borovsky, D., Carlson, D. A., Griffin, P. R., Shabanowitz, J. and Hunt, D. F. 1993. Sequence analysis, synthesis and characterization of *Aedes aegypti* trypsin modulating oostatic factor (TMOF) and its analogs. Insect Biochem. Mol. Biol. 23: 703-712.
- Borovsky, D., and Meola, S. M. 2004. Biochemical and cytoimmunological evidence for the control of *Aedes aegypti* larval trypsin with *Aea*-TMOF. Arch. Insect Biochem. Physiol. 55: 124-139.