RISK ASSESSMENT REPORT

OF THE GENETIC MODIFICATION ADVISORY COMMITTEE (GMAC)

FOR

AN APPROVAL APPLICATION TO RELEASE TMOF_Yeast (Technical Grade Active Ingredient, TGAI)

NBB REF NO: JBK (S) 602-1/1/5

APPLICANT: ENTOGENEX INDUSTRIES SDN BHD

DATE SUBMITTED: 19 April 2011

I - Summary of Assessment Process

The Genetic Modification Advisory Committee (GMAC, please refer to Appendix 1 for details of GMAC), under the purview of the National Biosafety Board was given the dossier by the Department of Biosafety on 6 May 2011 for release activities of TMOF _Yeast (Technical Grade Active Ingredient,TGAI). The application was filed by the Entogenex Industries Sdn Bhd (EntoGeneX, hereafter referred to as "the applicant").

Toxicology data and further information were sourced from the Environment Protection Agency (EPA) of the United States of America with reference to the registration of Trypsin Modulating Oostatic Factor (TMOF), EPA Registration number 74411-1.

A public consultation for this application was conducted from the 9 June 2011 to 8 July 2011 via advertisement in local newspapers. The Public Consultation for this application raised some technical and scientific issues regarding the release. GMAC considered these comments and addressed those that were not already included in the Risk Assessment.

GMAC had three meetings pertaining to this application and prepared the Risk Assessment Report and Risk Assessment Matrix along with its recommended decision, for consideration by the National Biosafety Board. GMAC also had a meeting on 21 March 2011 with representatives from other relevant agencies in the presence of the applicant to clarify a few issues which were very unclear throughout the assessment process. From this meeting, it was agreed that this assessment would be limited to the stock of TMOF_yeast that was supplied to the Ministry of Health, Malaysia in the form of two commercial larvicide products (Mousticide RH and Mousticide WP) and that was produced at the Chemical Engineering Pilot Plant (CEPP), Universiti Teknologi Malaysia.

A toxicity impact study was done by the Malaysian Palm Oil Board (MPOB) and was given as a supporting document by the applicant. GMAC invited Dr. Siti Ramlah Ahmad Ali from MPOB (Malaysian Palm Oil Board) to give a presentation on the toxicity study that was entitled "Acute Effect of TMOF (WP), TMOF (RH), MPOB Ecobac-1 (EC), against Oil Palm Pollinators, *Elaeidobius kamerunicus*" as a part of the assessment process (The contents of this presentation is not available as Dr. Siti has not submitted the document as agreed at the meeting). This report was further given full support (received full endorsement) by the Chairman of MPOB, who gave assurance in writing that the two products of the applicant had no toxic effect on the oil palm pollinating weevils.

GMAC invited an expert entomologist, as an independent evaluator, to review the assessment, especially with relevance to the impact of the two products on the oil palm pollinating weevil. Dr. A. Sivapragasam from Centre for Agricultural Bioscience International - Southeast and East Asia Regional Centre (CABI) was asked to review and give his opinion on the findings of MPOB and other supporting documents given by the applicant.

II - Background of Application

This application is for approval to release products of TMOF_yeast (genetically-modified *Pichia pastoris*) which were produced in Universiti Teknologi Malaysia, Johor. Specifically, these products are limited to the stock that have been supplied to the Ministry of Helath Malaysia. The TMOF_yeast products that are being assessed are Mousticide RH and Mousticide WP. The aim of the release is to use these products to control the *Aedes aegypti* larval population. These products, in the form of killed dried yeast cells containing TMOF peptide, are mosquito larvicides. They will be used to treat possible mosquito breeding habitats (water bodies) to kill mosquito larvae as a control measure to reduce the population of *Aedes* mosquitoes.

Information about TMOF_ yeast

The parent organism, *Pichia pastoris* is a yeast commonly used for the expression of biological pharmaceutical proteins with no known adverse health effects. It is a non-pathogenic microorganism with decades of safe utilization. Unmodified *P. pastoris* yeast has been approved by the United States Department of Agriculture (USDA) as a food additive in the livestock industry (Dossier of applicant).

P. pastoris KM71H strain was modified with pPICZ B plasmid containing gene sequences for expression of the TMOF peptide, which was originally isolated from ovaries of the female *Aedes aegypti* mosquito. The mode of action of TMOF is by hormonal distruption of transcription and translation of trypsin, a critical enzyme needed by the adult and larval digestive system for the digestion of their protein diet. The lack of free amino acids liberated from the blood meal in adult females causes inhibition of egg development and lack of free amino acids in the larval gutcauses anorexia leading to starvation and death of larval mosquitoes.

The active ingredient, TMOF, is a small protein containing 10 amino acids. The genes for making TMOF have been inserted into *P. pastoris* so that the yeast cells can make large amounts of the protein. The yeast cells are then killed by exposure to extremely high temperatures. For this application, killed yeast cells have been formulated to produce Mousticide WP and Mousticide RH. These products are for application onto bodies of water to control mosquito larvae. Once ingested by mosquito larvae, TMOF interferes with the production of trypsin.. Exposed larvae are therefore unable to digest food and starve to death.

III - Risk Assessment and Risk Management Plan

GMAC evaluated the application with reference to the following documents:

(i) Roadmap for Risk Assessment of Living Modified Organisms, (according to Annex III of the Cartagena Protocol on Biosafety produced by the *Ad Hoc* Technical Expert

Group (AHTEG) on Risk Assessment and Risk Management of the Convention on Biological Diversity).

(ii) the risk assessment and risk management plan submitted by the applicant.

(iii) United States Environmental Protection Agency Office of Pesticide Programs Biopesticides Registration Action Document for Trypsin Modulating Oostatic Factor (TMOF). This report states that the EPA assessment of TMOF was conducted **for manufacturing use only** and **any end use product using TMOF should be evaluated individually** to determine any risk or labeling issues. This report specifies that in any evaluation of end use product using TMOF, additional information must be provided such as data on food clearances/tolerance, toxicity of end product on non-target organisms, efficacy of product, impact on endangered aquatic invertebrate species.

GMAC took cognizance of the following as suggested within the AHTEG guidelines:

(i) That the risk assessment exercise should be specific to the details of this particular application

(ii) That the risk assessment exercise should be specific to the receiving environment in question, and

(iii) That any risk identified should be compared against that posed by the unmodified organism.

A Risk Matrix was prepared based on an assessment mechanism developed by Office of the Gene Technology Regulator, Australia (OGTR, 2005). In applying this matrix, GMAC identified potential hazards, and then added a value/rank for the likelihood of each hazard as well as its consequences. The likelihood of each hazard occurring was evaluated qualitatively on a scale of 1 to 4, with 1 for „highly unlikely", and 4 for „highly likely". The consequences of each hazard, if it were to occur, were then evaluated on a scale of 1 to 4, with 1 for „negligible" and 4 to denote a „severe consequence". A value was finally assigned for the overall risk from the identified potential hazard. The general formula: Overall Risk = Likelihood x Consequence was employed. GMAC also proposed risk management strategies for some of the potential hazards. This methodology of assessment follows the procedure of Risk Assessment in Annex III of the Cartagena Protocol on Biosafety.

The Risk Assessment was conducted over a series of three meetings. To start with, the possible pathways to risk/hazard arising from release of the products were identified and listed. The potential hazards identified arose from three main areas:

(i) **Effects on the ecology of the receiving environment**

Issues pertaining to animal health, pollination, food chains and toxicity of the gene product/s to non vector mosquito species and other non-target organisms.

(ii) **Effects on human health**

Issues pertaining to toxicity and allergenicity through exposure to the products

(iii) **Effects of the transgene**

Issues pertaining to horizontal gene transfer, changes in expression, interaction and persistence in the environment.

Based on the above, a final list of 18 potential hazards were identified. Most of these hazards were rated as having an Overall Risk of 1 or "effectively zero".

GMAC also took extra caution and further discussed pre-emptive mitigation procedures for hazards where the Overall Risk was estimated to be above the minimal, and also for a few hazards that required further evaluation and data acquisition. Some of these risks are expected to be managed effectively with the risk management strategies proposed (please refer to section IV of this document). Taking into consideration the limited amount of product to be released (limited to the amount in the possession of the Ministry of Health) and short term use, some of the identified hazards were found to be low. However, these issues should be thoroughly reassessed for the accumulative impact from long term usage of this product or if TMOF is used for the formulation of another type of end product.

One pertinent potential hazard where the Overall Risk was found to be 3 is highlighted below along with the appropriate management strategies. In addition, risks that may have significant consequences to biodiversity through the accumulative effect of long term usage of this product are also described here:

a) Possibility of toxic effects on the oil palm pollinating weevil

TMOF may have toxic effects on important agricultural pollinators, specifically the oil palm pollinating weevil. EPA reports on the negative effects of TMOF on cotton boll weevil, citrus weevil as well as on minor effects on other insect species. The weevils are of the same family as the oil palm pollinating weevil. Any detrimental impact on the oil palm pollinating weevil will have devastating effects on the oil palm industry. The consequence of this risk is unacceptable unless mitigation is highly effective and feasible, and this is a strong basis to request for a

toxicity report from the applicant. Although the study that was done by MPOB demonstrated low toxicity to the oil palm weevil, GMAC strongly recommends that more comprehensive experiments be conducted, consistent with the independent evaluator's recommendations. GMAC lacks confidence in the scientific rigour of the study done, but was willing to accept the findings of the study on condition that it was endorsed in writing by MPOB as sufficient to prove the TMOF products to be harmless to the oil palm pollinating weevil. GMAC is of the opinion that the study could be re-designed for better scientific rigour, a view shared by the independent evaluator.

The independent expert evaluator, Dr. A. Sivapragasam gave the following input:

(i) MPOB studies - Reason for doing non target species effect with pollinator weevil – beneficial to know based on impact on the oil palm industry. Results from MPOB (for Bangi strain only) suggested no serious problems on pollinator weevil (<30% mortality considered harmless or slightly harmful based on WHO Toxicity Class – see below). However, the mortality numbers are high enough to discount them due to natural causes. Thus, there is need to: (a) Examine the egg development and oviposition of treated adults (*vis a vis* untreated check) since there is suggested implication on egg development (lack of amino acids from feed meal in mosquitoes) and (b) examine the conditions of the midgut peritropic membrane of weevils which survived but may be impaired in terms of subsequent development.

(ii) Missing link in UKM study - Effect of non-targets (besides the target pest species- *Aedes aegypti)* in the endemic aquatic environments not clear. The generic insecticidal effects on proteases across a wide target (in aquatic environments) spectrum is known. Therefore, important to check on effects on other aquatic non targets.

(iii) All studies done with financial (?) support of registrant Company (question of non-partiality of trials)

b) <u>Accidental release of live genetically- modifed yeast.</u>

Upon review of the heat-kill protocol provided by the applicant, it was found that the heat-kill protocol may not have been effective enough. Viable yeast cells may still be present and survive in the final preparation. This will result in unintentional release of LMO into the environment. *P. pastoris* may be able to thrive in suitable ecological niches e.g. decaying fruits. Therefore, this risk may require mitigation beyond normal practices. GMAC recommends that the applicant has to implement stricter quality control measures that ensure that the yeast is 100% non viable for all future applications.

c) <u>Synergistic effect of TMOF with other biopesticides</u>

TMOF may act synergistically with other biopesticides when combined in the environment. TMOF has been demonstrated to act synergistically with *Bacillus thuringiensis israelensis* (Bti) which is able to enhance the effect of TMOF. This will result in undesirable widespread effects on other non target organisms due to possible increased target spectrum and toxicity. This risk requires mitigation actions that need to be demonstrated as effective. Therefore, any other combination of TMOF with biopesticides should be evaluated separately and on a case-by-case basis.

d) Impact of TMOF to human health

There might be an impact on human health through the exposure to insect proteins via inhalation or skin contact during the spraying of wettable powder in an aquatic environments. This is likely if personal protective equipment (PPE) is not used during spraying and other necessary precautions are not taken. Exposure will cause allergic reactions in hypersensitive individuals. The EPA report includes dermal toxicity data which showed allergic reactions in rabbits: slight to moderate erythema, edema, focal eschar and desquamation. These reactions persisted in some animals for more than 7 days. This risk requires actions of mitigation that need to be demonstrated as effective. Product handling and safety instructions should be provided with the product. These instructions should include the need for PPE and proper usage procedure including notification; and follow-up medical monitoring of staff handling the product.

e) Effect on other non target organism

EPA describes significant effects (growth retardation and limited mortality) on some spp of Lepidoptera, Hemiptera, Coleoptera and Diptera. The current application does not provide any data on local species. Any such effect on local species will result in disruption of the food chain and the ecosystem. This risk requires actions of mitigation that need to be demonstrated as effective. GMAC has recommended that the applicant conduct a population study on local key indicator species in representative areas where TMOF will be applied.

f) Persistence of the transgene in the environment

The transgene construct does not provide any selective advantage in the wild. However, there is a likelihood that the heat kill protocol may not be 100% effective. GMAC considers the volume of samples taken during the validation test as too small and not adequate to completely rule out the presence of viable cells in the final product. Accidentally released GM *P. pastoris* may mate with wild strains and perpetuate as ascospores. Genetic contamination of environment is more likely with long term continuous use arising from accumulative effects. This risk requires actions of mitigation that need to be demonstrated as effective. The applicant has to ensure that the product being considered is 100% non viable. GMAC recommends that stratified random sampling and testing for the presence of viable yeast cells is to be performed prior to release of the stock for field use.

IV - Proposed Terms and Conditions for Certificate of Approval

Based on the 18 potential hazards identified and assessed, GMAC has drawn up the following terms and conditions for the certificate of approval for the release of this product:

a) Final TMOF product should not be applied to waterways such as finished, treated drinking water sources.

b) For long term use of TMOF_yeast in the formulation of another type of end product a reassessment of this product is required. Therefore, this approval is restricted only to the limited amount already supplied by the applicant to the Ministry of Health in the form of the two existing products – Mousticide WP and Mousticide RH.

c) The requirement for proper labeling is imposed; product handling and safety instructions should be provided with the product. These instructions should include the need for PPE and proper usage procedures including notification and follow up medical monitoring of staff handling the product.

V - Other Regulatory Considerations

No other regulatory considerations are recommended.

VI - Identification of issues to be addressed for any subsequent release of this product

Some additional issues have been identified that would be important during the assessment of an application for long term usage of this product. These include:

a) Risk: Heat kill protocol may not be effective

The heat kill protocol used may not be 100% effective. The post-heat kill quality control test uses samples of 0.3 ml, which may not be sufficient to detect the presence of viable GM *P. pastoris* cells at low levels.

The LMO under evaluation here is considered as a "GMO with no known survival advantages when released into the wild" and the required killing efficiency is at least a 6-log kill (in line with the US EPA"s requirement). The sampling procedure should thus be adequate to contain at least 10^7 cells per sample in order to detect viability at this level. There was no data on the initial number of cells in the fermenter prior to the heat kill process, and it was thus difficult to judge if the heat kill process has been 100% efficient.

Some extrapolation could be done from the OD_{600} reading taken at 0 hours for the 48 h-YPD pre-heat kill experiment (OD_{600} = 0.690 = ~3.5 x 10^7 cells/ml; assuming 1 OD = ~ 5 x 10^7 cells/ml). Taking into account the dilution factor of 60/0.3 = 200, this gives an initial cell concentration of ~7.0 x 10^9 cells/ml. However, this remains guesswork without the proper data. Furthermore, the OD readings after 48 hours of culture in YPD = 1.238 (eq to ~6.2 x 10^7 cell/ml). This seems to indicate that only one doubling of cells has occurred. The expected doubling time for *P. pastoris* in YPD is ~2 hours, thus in the 48 hour culture period, at least 12 generations of cell growth should have occurred, even after factoring in a lag phase, growth saturation and possible inhibitory effects of the zeocin. While *P. pastoris* has been known to grow to high densities under optimum conditions, there is again no indication of the fermentation parameters to judge this. Thus, GMAC is of the opinion that there is a lack of robust data on the effectiveness of the heat kill procedure at this juncture.

GMAC recommends that proper quality control and data keeping to be practiced by the applicant. The quality control procedure should be properly validated or modified to ensure that the heat kill process is sufficiently effective for a 6-log reduction in the number of viable cells.

For testing of efficacy of the heat kill protocol used, the applicant should thus conduct batch-by-batch testing (Mousticide WP and Mousticide RH) containing the GM *P. pastoris* prior to field release. At least 2% of each release batch should be tested (e.g. if 100 packages is to be used for one batch, two should be tested). The current protocol for post-heat kill validation is acceptable but the volume of sample to be taken for analysis should contain at least 10^6 *P. pastoris* cells (dead or alive) per sample. The addition of zeocin and the use of a selective media should suppress growth of most other organisms.

b) <u>Risk: Detrimental effect of TMOF on local fauna</u>

The effect of TMOF on the local fauna is unknown. While the EPA report provided reassuring data on the limited toxicity on TMOF to a variety of non-target organisms, it also contains evidence of possible toxic effects to certain insect families. Of particular concern is the possibility of toxicity to important pollinator species such as the oil palm pollinating weevil. The applicant is thus required to conduct studies to establish the toxicology of TMOF on (i) local insect species that were not covered in the EPA report and (ii) on key indicator species for the local ecology, especially aquatic organisms. Ideally, a population study should be carried out on the effect of Mousticide (WP/RH) on key indicator species *in situ* at the several selected sites where the products have been used.

The result of these studies should be communicated to GMAC within 24 months of the date of approval for this particular batch of TMOF. This study will form an important basis for the further approval of open use of TMOF and products containing TMOF.

c) Risk: Synergistic effect of TMOF in combination with other biopesticide

The data provided indicate that TMOF when combined with Bti exhibits synergistic toxicity. There is thus the possibility that combining TMOF with Bti or other biopesticides may lead to an increased spectrum of target organisms, or an increased toxicity level in mildly-affected organisms. There is no data addressing this aspect. If TMOF is to be mixed with other biopesticides and used in such a formulation, similar studies as detailed in item (b) of this Section must be carried out to determine possible synergistic toxicity effects.

The results of such studies should be communicated to GMAC within 24 months from the date of this approval, and will form an important basis for the further approval of open use of TMOF and products containing TMOF.

VII – Conclusion and Recommendation

GMAC has conducted a thorough evaluation of the application for the release activities of TMOF _Yeast (Technical Grade Active Ingredient, TGAI) product and has determined that the release of this product in limited amount does not endanger biological diversity or human, animal and plant health (as per the amounts currently with the MOH). This applies to the use of Mousticide WP and Mousticide RH (produced from CEPP, UTM). However, assessment should be done again if the TMOF contains a different formulation other than the Mousticide WP and Mousticide RH as well as if these products (Mousticide WP and Mousticide RH) are produced again in another facility as there may be variables in the effectiveness of the protocols used as well as variations in the risk exposure pathways.

GMAC recommends that the proposed application for release be **APPROVED WITH TERMS AND CONDITIONS** as listed in section IV - Proposed Terms and Conditions for Certificate of Approval.

GMAC also recommends that section VI – “Identification of Issues to be addressed for subsequent release of this product” be communicated at the same time to the applicant.

VIII - Bibliography

1) Ad Hoc Technical Expert Group (AHTEG). *Final Report of the Ad Hoc Technical Expert Group on Risk Assessment and Risk Management under the Cartagena Protocol on Biosafety*. UNEP/CBD/BS/AHTEG-RA&RM/2/5 document, 5 May 2010

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6) OGTR. 2005. Risk Analysis Framework.

7) Ramlah Ali *et al*. "Acute Effect of TMOF (WP), TMOF (RH), MPOB Ecobac-1(EC), Against Oil Palm Pollinators, *Elaeidobius kamerunicus." Unpublished.*

8) United States Environmental Protection Agency Office of Pesticide Programs. *Biopesticide Registration Action Document Trypsin Modulating Oostatic Factor (TMOF).* PC Code 105403

9) United States Environmental Protection Agency Trypsin Modulating Oostatic Factor (105403) Fact Sheet (http://www.epa.gov/opp00001/biopesticides/ingredients/factsheets/factsheet_105403.htm)

Appendix I

GENETIC MODIFICATION ADVISORY COMMITTEE (GMAC) MEMBERS INVOLVED IN SPECIFIC RISK ASSESSMENT AREAS FOR THE APPROVAL FOR RELEASE OF PRODUCTS OF TC1507 CORN FOR SUPPLY OR OFFER TO SUPPLY

Genetic Modification Advisory Committee (GMAC) members divided the task of looking up more information for the Risk Assessment matrix based on three broad categories. The scope of research aspects for each group is as listed below. Each sub-committee had a nominated leader to coordinate the work and report back to the main GMAC. The respective leader contacted the sub-committee members and discussed the work process with their members. The groupings of GMAC sub-committee members and their assigned tasks are as below:

1. **ENVIRONMENT**

   Effect on ecology of receiving environment due to unintentional release and planting (e.g. weediness, gene transfer to bacteria, accumulation of the PAT protein in the environment, cross pollination and toxic effects on non-target organisms)

   - **Assoc. Prof. Dr. Mohd. Faiz Foong bin Abdullah (Universiti Teknologi MARA) (Leader)**
   - Dr. Sim Soon Liang (Sarawak Biodiversity Centre)
   - Dr. Martin Abraham (Malaysian Society of Marine Sciences)
   - Madam Atikah binti Abdul Kadir Jailani (Department of Agriculture)
   - Dr. Wan Abdul Rahaman (Malaysian Biotechnology Corporatiom Sdn bhd.)

2. **HUMAN HEALTH**

   Effect on human health (e.g. acute toxicity of the novel protein, potential allergenicity, mutagenic/tetragenic/carcinogenic effects, reproductive toxicity, potential transfer of antibiotic resistance genes in the digestive tract, the pathogenic potential of donor microorganisms and nutritional equivalence)

   - **Madam T.S. Saraswathy (Institute of Medical Research)(Leader)**
   - Prod. Dr. Son Radu (Universiti Putra Malaysia)
   - Dr. Chow Keng See (Malaysian Rubber Board)
   - Dr. S. Ravigadevi (Malaysian Palm Oil Board)
   - Madam Shamsinar binti Abdul Talib (Ministry of Health)

3. ANIMAL HEALTH

Effect on animal health (e.g. allergenicity, toxicity, anti-nutritional properties, compromised nutritional content, metabolic breakdown of products, survivability, horizontal gene transfer and animal product contamination)

- **Prof. Dr Jothi Malar Panandam (Universiti Putra Malaysia) (Leader)**
- Dr. Ahmad Parveez bin Hj Ghulam Kadir (Malaysian Palm Oil Board)
- Dr. Tan Swee Lian (Academy of Science Malaysia)-plant breeding
- Prof. Dr. Helen Nair (Academy of Science Malaysia)
- Dr. Tan Chon Seng (Malaysian Agricultural Research and Development Institute)