Risk Assessment Summary Conducted Pursuant to the New Substances Notification Regulations of the *Canadian Environmental Protection Act* *Trichoderma longibrachiatum* RM4-100 NSN 6823

This document has been prepared to explain the regulatory decision made in September 1997 under the *Canadian Environmental Protection Act* (CEPA 1988)[1] regarding the manufacture or import of *Trichoderma longibrachiatum* RM4-100 by Iogen Corporation in a contained facility located in Ottawa.

Trichoderma longibrachiatum RM4-100 was notified pursuant to subsection 29.11(4) of CEPA New Substances Notification Regulations (NSNR).

The New Substances Branch of Environment Canada and the New Substances Assessment and Control Bureau of Health Canada have assessed the information submitted by Iogen Corporation and other available scientific information in order to determine whether *T. longibrachiatum* RM4-100 is *toxic*[2] or capable of becoming *toxic* as described in section 11 of CEPA 1988.

Regulatory Decision:

Based on the hazard and exposure considerations, the joint risk assessment conducted by Environment Canada and Health Canada concluded that *Trichoderma longibrachiatum* RM4-100 is not considered to be *toxic* to the Canadian environment or human health as described in section 11 of CEPA 1988.

Therefore, the manufacture in or import to a contained facility of *T. longibrachiatum* RM4-100 for use in the contained facility or for export only, may proceed after September 18, 1997.

This evaluation does not include an assessment of human health risk in the occupational environment nor does it include an assessment of the potential exposure and risk to humans associated with the use of the organism in or as an item that falls under the purview of the *Food and Drugs Act*.

---

[1] A new *Canadian Environmental Protection Act, 1999* was introduced in 1999.

[2] In accordance with section 11 of the CEPA 1988, a substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term effect on the environment; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health.

NSN Schedule: XVI (manufacture in or import into a contained facility of a micro-organism that is not for introduction outside a contained facility or is for export only)[3]

Organism Identity: *Trichoderma longibrachiatum* RM4-100

Notifier: Iogen Corporation, 310 Hunt Club Rd. East Ottawa, Ontario K1V 1C1 Canada

Date of decision: September 18, 1997

Proposed use: Commercial production, in a contained facility, of a novel thermophilic xylanase enzyme by the genetically engineered strain *Trichoderma longibrachiatum* RM4-100.

Strain History/Genetic Modification:

The notified strain RM4-100 was derived from an auxotrophic mutant of the parental strain M2C38[4] (ATCC 74252). The strain M2C38 was genetically modified in order to hyperproduce a genetically engineered, thermophilic xylanase enzyme through the ballistic introduction of the integrative transformation vector pC/XHML-TV. The expression cassette consists of the modified *T. reesei* xylanase II structural gene (*xln2*) under the control of *T. reesei* regulatory sequences. The selection cassette contains an antibiotic resistance marker gene under the control of *T. reesei* regulatory sequences. The parental strain M2C38 was a derivative of the *T. reesei* strain RUTC30 (ATCC 56765). RUTC30 is a mutagenic derivative of the wild-type *T. reesei* strain QM6a (ATCC 13631), which was isolated in the Solomon Islands from cotton canvas during World War II (Kuhls *et al.,* 1996).

Hazard considerations:

In addition to the information provided by the notifier, a review of in-house reference material and a comprehensive search of the scientific literature were conducted to gather information on potential harmful environmental and human health effects attributable to *T. reesei*.

Trichoderma species are common soil saprophytes and are metabolically versatile, aerobic, mesophilic, imperfect fungi (Nevalainen *et al*., 1994). The *Trichoderma* species are differentiated primarily by patterns of conidiophore branching and conidia morphology.

In general, large scale industrial manufacture of *T. reesei* enzyme preparations have a history of safe use in many industries including starch and animal feed processing, grain

---

[3] Provisions relating to organisms previously contained in Part II.1 of the NSNR are now contained within the NSNR (Organisms). These came into force on October 31, 2005, and included changes to Schedule numbering. Under the NSNR (Organisms), Schedule XVI is now referred to as Schedule 2.

[4]There was a discrepancy regarding the taxonomic designation of the organism. In the notification the parental strain was named *Trichoderma longibrachiatum* Rifai strain M2C38. Under synonyms, *Trichoderma reesei* was provided as the most commonly used name. The American Type Culture Collection classification of the parental strain was based on colony growth and morphological characteristics. After consulting with experts on fungal taxonomy to clarify the strain designation, there was an agreement in a meeting with the notifier, Environment Canada and Health Canada that the notified organism is a strain of *Trichoderma reesei.*

alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, in the pulp and paper industry, and in the textile industry (Hjortkjaer *et al*., 1986). Based on the criteria outlined in the Organisation for Economic Co-operation and Development guidelines entitled *Recombinant DNA Safety Considerations* (OECD, 1986) and the European Communities Council Directive 90/219/EEC on the contained use of genetically modified micro-organisms (ECC, 1990), *Trichoderma* species can be regarded as safe host organisms.

Trichoderma reesei has been shown to be non-pathogenic and non-toxic to healthy laboratory animals (Hjortkjaer *et al*., 1986). *Trichoderma reesei* is not reported to be a frank pathogen of plants or animals including humans. However, this species can act as an opportunistic pathogen to immunodepressed animals under extreme experimental conditions (Hjortkjaer *et al*., 1986). Some *Trichoderma* species have been cited as rare fungal pathogens (Munoz *et al.,* 1997; Jacobs *et al*., 1992; Seguin *et al.*, 1995).

Certain species of the genus *Trichoderma* can be used as biocontrol agents in agriculture since they are known antagonists to several plant pathogenic fungi. Some species of *Trichoderma* may also produce toxins under certain conditions; however, experience with *T. reesei* indicates that it is not likely to be toxigenic (Hjortkjaer *et al*., 1986). Tests conducted on commercial enzyme preparations confirm that neither antibiotics nor inhibitory substances are produced during the growth of industrial *T. reesei* strains (Hjortkjaer *et al*., 1986). A carbohydrase enzyme product manufactured by the notifier using the parental strain M2C38 was tested for aflatoxin with negative results.

The use of an antibiotic resistance marker gene in the construct is required to distinguish the strain RM4-100 from the parental strain M2C38. It does not confer resistance to clinically significant antibiotics and is unlikely to pose any health risk.

Trichoderma reesei is listed as ‘Biosafety Level 1’ organism by the American Type Culture Collection (ATCC). In addition, *T. reesei* has been designated as a ‘Risk Group 1’ organism by the Office of Laboratory Security of the Public Health Agency of Canada.

The DNA fragments used in the construction of the transformation vector are well characterized and do not contain any large undefined fragments. The ampicillin resistance gene used in the construction of the transformation vector is under the control of a bacterial promoter that will not function in *T. reesei*. In addition, the vector DNA has been shown to be stably integrated into the chromosome without loss or rearrangement of the sequence even after several generations on non-selective media. Therefore, the potential for lateral gene transfer from this organism to humans, animals or other microbes in the environment is extremely low.

Genetic modifications performed to develop the strain RM4-100 do not give rise to concerns of altered virulence or pathogenicity to humans, animals, plants or altered hazards to the environment. The inserted genetic material is well characterized and is not known to have any pathogenic or toxic capacity. The phenotypic changes resulting from the modification are well characterized and are not likely to influence the normal behavior of the strain.

Exposure considerations:

Trichoderma species, including *T. reesei* are common soil saprophytic fungal species found in all climate zones and are particularly prevalent in the litter of humid, mixed hardwood forests (Nevalainen *et al*., 1994).

The notified strain RM4-100 is manufactured solely as an intermediate in the production of a novel thermophilic xylanase II enzyme in a contained facility. The notifier indicated that the manufacturing process meets the standards for the *Good Large Scale Practice* (GLSP) level as defined in Appendix K of the *NIH Guidelines for Research Involving Recombinant DNA Molecules* (NIH, 1994). The notified strain is not intended for release outside the contained facility. Consequently, the potential exposure to the general population and the environment is expected to be low.

The notifier describes procedures which will limit potential worker exposure. These include the use of protective equipment, such as the National Institute for Occupational Safety and Health (NIOSH) approved respiratory masks with particulate filters, face shield, or safety goggles with side shields, rubber gloves, lab coats or overalls for workers who are chronically exposed to enzyme dusts or aerosols during such procedures as transfers of fermentation broths.

Precautions are in place and used by the notifier to ensure that exhaust and aerosols from the fermentor are decontaminated by UV irradiation and cyclone and scrubber system. The fermentor is equipped with an alarm to indicate high pressure, foam-over and low-level and is diked in the case of massive leakage.

When enzyme production is complete, the spent cell mass is chemically inactivated using a quaternary ammonium compound with 99.999% effectiveness prior to disposal in registered landfill or composting sites in accordance with provincial regulations. Given that the strain RM4-100 is not intrinsically hazardous, the likelihood of significant harm to the environment or human health resulting from this route of exposure is expected to be minimal.

References:

ECC. 1990. Council Directive 90/219/EEC on contained use of genetically modified micro-organisms. Official Journal L 117 , 08/05/1990 P. 0001 - 0014 http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/vol-1/dir_1990_219/dir_1990_219_en.pdf

Hjortkjaer, R.K., Bille-Hansen, V., Hazelden, K.P., McConville, M., McGregor, D.B., Cuthbert, J.A., Greenough, R.J., Chapman, E., Gardner, J.R. and Ashby, R. 1986. Safety evaluation of Celluclast®, an acid cellulase derived from *Trichoderma reesei*. Journal of Food and Chemical Toxicology. 24(1): 55-63.

Jacobs, F., Byl, B., Bourgeois, N., Coremans-Pelseneer, J., Florquin, S., Depre, G., Van de Stadt, J., Adler, M., Gelin, M. and Thys, J.P. 1992. *Trichoderma viride* infection in a liver transplant recipient. Mycoses. 35(11-12):301-3.

Kuhls, K., Lieckfeld, E., Samuels, G.J., Kovacs, W., Petrini, O., Gams, W., Borner, T. and Kubicek, C.P. 1996. Molecular Evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc. Nat. Acad. Sci. USA. 93: 7755-7760.

Munoz, F.M., Demmler, G.J., Travis, W. R., Ogden, A.K., Rossman, S.N. and Rinaldi, M.G. 1997. *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. Journal of Clinical Microbiology. 35(2): 499-503.

Nevalainen, H., Suominen, P. and Taimisto, K. 1994. Minireview on the safety of *Trichoderma reesei*. Journal of Biotechnology. 37: 193-200.

NIH. 1994. NIH Guidelines for Research Involving Recombinant DNA Molecules - Appendix K, US Department of Health and Human Services, in the Federal Register (United States), Vol. 59, No. 127.

Organisation for Economic Co-operation and Development (OECD). 1986. Recombinant DNA Safety Considerations: Safety Considerations for Industrial, Agricultural and Environmental Applications of Organisms Derived by Recombinant DNA Techniques. http://www.oecd.org/dataoecd/45/54/1943773.pdf

Seguin, P., Degeilh, B., Grulois, I., Gacouin, A., Maugendre, S., Dufour, T., Dupont, B. and Camus, C. 1995. Successful treatment of a brain abscess due to *Trichoderma longibrachiatum* after surgical resection. European Jo biology and Infectious Diseases. 14(5):445-8.