Technical Opinion nº 2281/2010

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Applicant: Amyris Brasil S.A.
CNPJ: 09.379.224/0001-20
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Matter: Request for commercial release of genetically modified yeast (Saccharomyces cerevisiae) for production of strain Y1979 farnesene.
Previous summary: 2014/2009, published in the Federal Official Gazette nº 191, of October 06, 2009.
Decision: GRANTED.

CTNBio, following examination of an application for a Technical Opinion regarding biosafety of a class 1 biological risk genetically modified organism for the purpose of trade, industrial production of the chemical compound farnesene and any other activities related to this genetically modified organism and progenies thereof, decided favorably to the GRANTING of the request on the terms of this technical opinion. The Chairperson of Amyris S.A. Internal Biosafety Commission, Dr. Luciana Di Ciero requests CTNBio a technical opinion for commercial release of genetically modified yeast (Saccharomyces cerevisiae) for production of strain Y1979 farnesene. The organism to be commercially released is a yeast of the species Saccharomyces cerevisiae transformed by synthase farnesene of the non-pathogenic medicinal plant Artemisia annua L. for the purpose of commercialization, industrial production of the chemical compound farnesene and any other activities related to this GMO and progenies derived from it. CTNBio held as confidential the information so marked as such by applicant.
Under Article 14 of Law nº 11,105/05, as regulated by Decree nº 5,591/2005, CTNBio held that the information given by applicant verify the product biosafety and complies with CTNBio rules and applicable legislation in effect aimed at securing biosafety of the environment, agriculture, human and animal health.
Initial Description and General Considerations
The request relates to commercial release of a baker's yeast that was genetically modified to generate farnesene, an ubiquitous natural product, produced by innumerable plants yet not naturally synthesized by Saccharomyces cerevisiae yeast. The genetic construct involves several genes that are natural of the yeast, such as modified promoters, so that the mevalonate metabolic pathway becomes highly productive, coupled with just one gene of another organism, that codes for farnesene synthase, obtained from the medicinal plant Artemisia annua L. With this addition to the pathway, farnesyl pyrophosphate (FPP) of the yeast is converted into farnesene, a substrate for sustainable biodiesel production, since this yeast produces this fuel by fermenting sugarcane molasses.
Under Brazilian legislation, this request is being assessed by the sector in charge of safety related to human and animal health, though this yeast and its product are not targeted to human or animal alimentary consumption. The product generated is a "green" and renewable substitute for a fossil fuel undergoing foreseeable depletion in addition to being non-renewable within the time scale of human interest.

The company forwarded detailed documentation in five hundred and one (501) pages of text, fifty-six (56) figures, thirty (30) charts, table of contents, abbreviations, sequences of inserted DNA, thorough detailing of thirteen (13) methods used in generating the GMO and investigating possible adverse effects on environment organisms, monitoring plan and one hundred and thirty-six (126) bibliographic references. This opinion is a consolidation of an assessment conducted by CTNBio members Francisco C. Nóbrega (nine references) and José Luiz de Lima Filho (thirty-six references) and the opinion of an ad hoc specialist, Mário Henrique de Barros (eight references), in which all experts were favorable to granting the request made by Amyris. This genetic construct stems from a high socially responsible project that ended by originating the company, supported by the Bill & Melinda Gates Foundation in an attempt to produce artemisin for treatment of resistant malaria, which is limited by high costs and difficulty to obtain the necessary amount of the plant that produces the active principle (Artemisia sp.). The success of the project enabled production 90% cheaper than conventional extraction. The process, with no profitable purpose, was transferred to the company Sanofi-Aventis with the participation of the Institute for OneWorld Health, for the production of artemisinin. However, the idea of the sesquiterpene production pathway has many other commercial applications, with large potential to generate other highly valuable molecules such as complex chemical products, medicines, fuels and lubricants, creating goods of the so-called synthetic biology area. The company is therefore dedicated to development of products that are potential substitutes for hydrocarbon-derived, non-renewable materials, reducing the carbon footprint, generating chemical products by modifying microorganism metabolism.

Information on the Genetically Modified Organism

Saccharomyces cerevisiae yeast was the first organism to be used by man to process food and generate edible materials representing the most ancient technology. In the site of a Chinese neolithic village, vases were found containing a nine thousand years old beverage fermented from rice, honey and fruits, (McGovern et al., 2004). This organism is a classic representative of the GRAS (Generally Recognized As Safe) category, unrestricted for human consumption according to the FDA, present in the production of beverages and bread. Reliability in its safety also lead the National Institutes of Health (NIH) to release experiments with the yeast from most part of its restrictions, and the Environmental Protection Agency (EPA) to exempt Saccharomyces cerevisiae from most of the clauses envisaged by the Toxic Substances Control Act, the American legislation controlling introduction of new chemicals to the market.

It has also been widely used in animal foods as a source of proteins and other nutrients. In human food, the yeast has been used in the form of derivatives, such as flavoring, to enhance taste and as a nutritive complement for its high quality protein, richness in lysine, B-complex vitamins and important minerals, including selenium and zinc, in addition to fibers, represented by cell wall mananes and glicanes. Even genetically modified yeasts were already approved for human consumption in wine production (United States and Canada) and in the United Kingdom, for beer and bread production. (Aldhous, 1990; Dequin, 2001), considerations that comply with Ruling Resolution nº 5, Annex III, section 1. Because of its easy cultivation and genetic manipulation, both for nuclear and for mitochondrial genes, yeast Saccharomyces cerevisiae became the most scientifically studied and characterized organism among all eukaryotes, being the first to have its genome entirely sequenced (Goffeau, 1996) and considered a model unicellular eukaryote for research. For over ten years, a collection of Saccharomyces cerevisiae strains is available, each containing a specific mutation in one of its six thousand genes, enabling highly interesting studies of gene function. (Brachmann,

1998).
Its pathogenicity was held null until scarce cases of Saccharomyces cerevisiae presence were described in isolated clinical cases of individuals affected by severe immune deficiency. There are also rare reports of allergy, mainly in bakery workers. In addition to its role in producing food, this yeast is always around us, present in fruit peels and grain surfaces. Its safety and industrial importance is evident, since it is responsible for the five main industrial products derived from fermentation: beer, wine, cell protein, baker's yeast and citric acid. Its effect as probiotic in human and animal food was evidenced in several studies (Gaioto, 2005, Caballero-Cordoba and Sgarberi, 2000). The demands of CTNBio Ruling Resolution nº 5, Annex 3, related to sections 1 and 2 do not apply, since the yeast or the biodiesel resulting from industrial fermentation is not targeted for human or animal consumption.
Strain Y1979 is derived from lineage PE-2, coming from Usina da Pedra, isolated and identified in 1994. It is used in the Brazilian production of ethanol from sugarcane in about thirty percent of the ethanol plants in the country, therefore responsible for over ten percent of the ethanol currently produced in the world. The economic importance of the lineage encouraged its full generic characterization, and its genome was recently sequenced (Argueso et al., 2009).
Summary of Molecular Manipulations Involved
Strain Y1979 is a diploid, a state that blocks the natural crossing between the haploid forms *f*Ñ and a. Additionally, genes STE5 and IME1 were inactivated in this strain, hindering production of haploid ascospores, reducing to insignificant levels the likelihood that the yeast enters in genetic combination with other yeasts present in nature, including laboratory lineages. The change, coupled with the alterations of the mevalonate pathway, make this strain a laboratory organism, highly dependent on specific conditions for proliferation and maintenance, in an analogy with plants domesticated by humankind along thousands of years, warranting the extreme difficulty for the organism in colonizing the environment by invasion or competition with the natural microbiota.
The company scientists developed an efficient framework for modular construction of integration cassettes, including a library of plasmids containing genetic elements flanked by ligand segments that are propagated in Escherichia coli. The genetic elements are promoters, terminators, interest genes, target loci, selection markers, etc. (Confidential Information).
Stability of Strain Constructed and Competiveness
This is a diploid strain with two identical copies of each chromosome. Adverse pleiotropic and epistatic effects from the several overexpressed genes and from heterologous gene FS were not recorded in the strain. Stability of FS gene was molecularly tested for over forty generations and proved immutable. Inactivation of STE5 and IME1 genes makes the strain incompetent for meiosis and mating. A competitiveness test of the Y1979 strain in continuous growth with six usual ethanol-producing strains, such as PE-2, was started with ninety percent of Y1979 and ten percent of competitors. In all cases, an inversion of percentages took place after three days, and 90% of competitors and 10% of Y1979 were recorded.
Assessment of Risk to Human and Animal Health
Although the yeast is targeted solely for biodiesel production, the company conducted a series of experiments to examine possible adverse effects, given the likely accidental exposure of workers to the organism. CanTox, a Canadian company was hired to study the genes expressed in the yeast and the heterologous gene, tracing a possible resemblance with known allergens and toxins. Data bases consulted were MedLine

(1950-2009 Feb.), ToxFile (1965-2009 Feb.), Biosis Previews (1926-2009 Feb.), Agricola (1970-2009 Feb.), and Embase (1974-2009 Feb.). No record was found in the literature that may suggest association between the expression of the mevalonate and FS pathway genes and increased pathogenicity and/or virulence or allergenicity of the noticeably innocuous yeast (complies with CTNBio Ruling Resolution nº 5, Annex III, section 10, though not intended for food). The plant origin of the farnesene synthase is also long used by large human populations for centuries in treating fevers and malaria. Farnesene sesquiterpene itself belongs to a class of products that is widely disseminated in nature. It may be toxic in high doses. MB Research, an independent laboratory, was hired to assess safety aspects related to the farnesene resulting from fermentation by strain Y1979. Acute oral toxicity, acute dermal toxicity, acute eye irritation, lymph node sensitization and mutagenicity were assayed. Results in all cases suggested that farnesene is well tolerated and not mutagenic. Farnesene DL50 in rats was determined as being above 5000 mg/kg of weight. The dose was applied to the skin of rabbits and there was no dermal reaction in twenty-four hours, light reaction in the seventh day and an even lighter reaction after fifteen days. Therefore, regarding the pure substance farnesene and foreseeing any likely accidental exposure, aspects related to sections 7 (immunologic and histological analysis), 8 (possible toxic effect) and 9 (toxicologic analysis) were assessed. Compounds of this chemical group may act as alarm pheromones for termites and other insects and constitute a substantial part of odorant substances such as the ones present in the gardenia perfume. Plants attacked by parasites issue volatile compounds including farnesene (Kannaste et al., 2009). The compounds have an inhibiting effect in bacterial proliferation. The fruits we consume, such as apples and oranges, produce farnesene and the compound is present in essential oils of sandal, basil, bergamotta, lemon, carrot seed, gardenia, ginger, grape and many others. Regarding differences in chemical and nutritional composition between the food coming from the genetically modified plant and from the non-modified plant, either in natura or processed, and existence of substantial equivalence between the GMO and its parental organism, as well as investigation on digestibility of the yeast and its fractions, the establishment of comparisons is meaningless, since the yeast will not be used as human food nor incorporated to animal ration. Studies conducted by CanTox demonstrated that there is no biological basis for effects in the immunological system or toxicity, in addition to not being the organism targeted for consumption as food. Therefore, recommendations of CTNBio Ruling Resolution nº 4, Annex III, regarding section 4 (nutritional performance), 5 (digestibility and stability of expressed proteins) and 6 (teratogenic potential) are not applicable to the request submitted by the company. To cause inactivation after the fermentation process, the company decided to use superheated vapor, easily generated in its facilities, that effectively kills the yeast without leaving environmentally aggressive residuals. The company reports that a temperature of 62 ºC for 90 minutes inactivates completely the strain under the different conditions studied (water and vinasses). In producing the yeast, Amyris shall use higher temperatures (120 ºC), securing full and safe inactivation.

Assessment of Environmental Impact of the GM strain

Specific environmental biosafety aspects of strain Y1979.

Page 11 to page 163 of the proceeding present a host of experiments tailored and conducted to assess indicative aspects involving the recombinant yeast environmental safety.

Risk Level of the Recipient Organism

There are many evidences that the baker’s yeast is safe, the main one being the yeast wide use, for centuries, in producing bread, beer, wine and a large number of other

foods and nutritional supplements and, more recently, its use in the industrial production of ethanol.
The US National Institutes of Health holds this microorganism so safe that releases certain procedures for risk assessment, as detailed in Appendix C-III. Saccharomyces Host-Vector Systems (see http://oba.od.nih.gov/oba.rac.guidelines_02/NIH_Guidelines_Apr_02.htm).
Detection
Detection of recombinant yeast by PCR is simple and accurate. Native yeasts have just one band of 270 pb, while genetically modified yeasts may be easily distinguished by the presence of two bands, one of 279 pb and another of 417 pb.
Stability of Construct
The gene for FS is integrated to the Saccharomyces cerevisiae genome and strain Y1979 fails to contain plasmids, therefore one expects maintenance of gene stability. However, in order to access an unlikely instability, the company developed an essay to detect presence of FS gene. Samples were isolated from cultivations in different periods and submitted to the colony PCR technique in an attempt to detect the presence of FS gene. Initiators were specifically designed for flanking regions, outside gene FS, to show that, in fact, the gene remained intact during experimentation. Results showed that the FS gene was present in all phases and that no genetic instability was detected in strain Y1979 for over 72h of continuous cultivation (about forty generations); each of the 24 colonies tested after 24h, 48h and 72h of continuous cultivation contained all five genetic integrations. The essay therefore suggests that genetic stability of the construct is maintained in Y1979 strain.
Variation of Gene Expression
There is no theoretical reason to expect a change in gene expression regarding those genes unrelated to the mevalonate production pathway. Experimental results submitted fail to show any indication of such change. On the other hand, gene expression, assessed by farnesene production, was always within the expected range.
Destination of Biomass, Gases and Effluents
As mentioned in the proceeding, fermentation shall be conducted in a closed reactor system with the purpose of minimizing environmental risk. The biomass produced during the process shall be inactivated before being discarded. Amrys Brazil will produce farnesene in its own facilities and intends in the future to license the technology to third parties.
During the industrial fermentation process, the risks of the GM yeast escaping to the environment shall be taken into consideration. Cells multiply vegetatively in fermenters and the company intends to use a protocol in which the GM lineage is inactivated by heat generated by high temperature steam, after fermentation. The purpose is minimizing the release of living cells into the industrial environment.
The productive process, starting by feeding the pre-fermenter (the tank for multiplication and preparation of the innoculant), following by feeding the fermentation vats and ending at the separation centrifuges, is conducted within an absolutely closed system, with flows confined to pipes and feeding pumps. Yeasts coming from the fermentation process are recycled and feedback the vats. Exceeding cells and the effluent from fermentation (known as vinasses) shall be inactivated by heat. Data presented in the proceeding on pages 112 to 114 show that Y1979 stops multiplying when heated to 66 ºC for 120 s, both when suspended in water and in vinasses. Since the temperature of the industrial process is around 120 ºC, this in practice ensures death of one hundred percent of cells.
Vertical gene flow

Strain Y1979 was incapacitated for sexual reproduction and sporulation at any detectable level, by knocking-out genes STE5 and IME1. STE5 is needed for mating in Saccharomyces cerevisiae. Strains with absence of STE5 display reduction of sexual reproduction in over six orders of magnitude (Elion, 2001). Gene STE5 codes for a protein necessary for Saccharomyces cerevisiae sexual reproduction and comprises at least other three necessary components for a well-succeeded reproduction. In the absence of STE5, yeast cells become unable to detect the presence of sexual partners in the environment. On its turn, gene IME1 is the main meiosis transcriptional regulator and mutants that have this gene deleted fail to sporulate (Kassir et al.). Additional precautionary measures to reduce an unlikely event of gene transfer from the GM strain to other yeasts were taken. It is a known fact that diploid yeasts do not mate and the Y1979 strain yeast is a diploid.

Horizontal Gene Flow

The likelihood of gene flow from yeasts to organisms belonging to other phylogenic families is low. For the Y1979 strain, which has no plasmids, this likelihood is even lower. Frequency of horizontal gene transfer between species is estimated to be about 2.0 x 10-17, as inferred from evolutionary data (Schlüter et al., 1995). Besides, strain Y1979 is significantly less robust than PE-2 and other naturally occurring yeasts and is quickly overcame by them in nature. However, the most important element in this risk analysis is that the yeast will be dead by heat before being released into the environment. Therefore, horizontal transfer would have to be passive, from cell remnants, which is even more unlikely.

Competiveness with Native Yeasts

Under conditions of controlled fermentation, the recombinant strain apparently fails to compete with the most common Brazilian commercial lineages. Results are very clear and are adequately presented in the proceeding. The experiment in vases containing soil of sugarcane farming area also indicate that survival of Y1979 strain in soil does not exceed one hundred and twenty days and that this strain is less competitive than native ones.

Survival and thermal resistance

Strain Y1979 is noticeably more sensitive to heat in sterilization processes than strain PE-02, featuring survival of 3 x 10-5% after sixty seconds at 66 ºC, two magnitude orders lower than PE-02 strain. Both strains are killed within not less than ninety seconds at 90 ºC. Results are clearly recorded on page 113 of the proceeding. The results are ratified by another experiment described on page 114 of the proceeding and together they show that sterilization of vinasses and vent (above the vat) containing the yeast is viable.

Impact on Water Collections

When added to water, strains PE-02 and Y1979 affected several physicochemical parameters in an essentially equivalent way (pages 115-116 of the proceeding).

Impact on Soil

When cell pellets of the two strains or respective vinasses were added to vases containing earth with sugarcane plants, there was no significant difference due to application of vinasses of strains Y1979 and PE-2 for almost all nutrients assessed. However, after addition of pellets higher levels or potassium and calcium were found in treatments with PE-2 when compared to that of Y1979. Addition of yeast pellets to sugarcane culture, however, is not a routine procedure, not even exceptionally, being just an experimentation device. Differences recorded have not, therefore, negative implications in environment safety. Results are clearly shown in Tables VII.6 and VII.7 on pages 117 and 118 of the proceeding.

Impact on biota

Soil is the main ecosystem to receive an unwanted accidental release of Y1979. Results on page 133 of the proceeding (Table VII.12) show that yeast Y1979 had a toxic effect equal or less than that of PE-2 on Daphnia similis.

Page 136 of the table show that strain Y1979 is no more toxic for Dugesia tigrina (a freshwater planarian) individuals than the strain of the control yeast PE-2. Mortality of Danio rerio associated to strain Y1979 was also equivalent to that of the control yeast, PE-2, as shown on Table VII.2, page 138 of the proceeding.

The impact of the yeast and its vinasses was assessed on two indicator species internationally used in soil toxicity tests; the collembola species, Folsomia candida, used as a representative of the mesofauna, and the earthworm species, Eisenia Andrei, used as a representative of the edaphic macrofauna. There was no difference between strain Y1979 and its non-modified equivalent PE-2.

Regarding the microfauna, different approaches were used. The abundance of bacteria in vases kept in nurseries where Y1979 cells and vinasses were applied was assessed through soil plating in growth medium. The diversity of bacteria, fungi and yeasts was assessed by the Denaturing Gradient Gel Electrophoresis (DGGE) technique on soils extracted from these same vases. Compatibility tests were conducted in laboratory using fungi and bacteria representative of important functional groups such as entomopathogens, decomposers and phytopathogen antagonists. Besides, analysis was conducted of an important soil ecological process, namely decomposition of organic matter, a process that is developed by a diversity of organisms of the soil fauna. This is an indirect way of assess changes in the soil fauna.

No statistically significant differences were found between strain Y1979 and yeast PE-2 in what relates to change in microbial diversity or in abundance of *f*Ñ-proteobacteria, actinobacteria or fungi in soil during a period of sixty days.

Strain Y1979 also failed to show significant impact in the soil microorganism model indicating soil health conditions, such as Metarhizium anisopliae (an entomopathogen), Trichoderma harzianum (a phytopathogen antagonist fungus), Baccillus CL16 (a cellulose decomposing fungus) and Pleurotus sajor-caju (a lignin decomposing fungus). Once more, we stress that the live yeast will not be released into the environment, except on an accident scenario.

Strain Y2979 also failed to show significant impact in sugarcane cultures and in soil decomposition rates or its chemical properties when compared to the native yeast PE-2. The survey was simulated in experiments where vinasses of Y1979 strain was employed in irrigation of sugarcane, which is currently done with PE-2 and other yeast vinasses in Brazilian ethanol plants.

Results of tests to assess potential effects of farnesene in insect communities in general, given their activities with pheromones. In the course of a five-month study no impacts on the local insect community were identified when the traps containing farnesene were compared to control traps containing no pheromones (herbivores, parasitoids and predators).

Presence of farnesene in the vicinity is, eventually, an additional concern. Indeed, farnesene accumulates both in the liquid part of the fermented mass and in the inside of the yeast. Most part of the fermentation farnesene is withdrawn before discarding the sterile vinasses. In the leaven mass, concentration may be considerably higher. However, the product will not be used in nutrition, unless new studies so recommend. Discarding over the soil is the most likely destination, in the short run, of this byproduct.

Monitoring Plan

The first purpose of the plan shall be following up efficiency of the genetically modified microorganism (GMM) inactivation in the industrial effluent. Any escape shall be monitored to assess persistence of the organism in the environment. A farm worker will not enter in contact with the GMM. However, a record of events shall be kept to monitor workers' health standards in order to detect any adverse effect. Data obtained shall be presented in annual reports for five years.

Colony Polymerase Chain Reaction (PCR) technique shall be routinely used in yeast samples collected in the industrial plant environment to monitor leaks. The monitoring molecular methodology to monitor the environment is described in the proceeding.

Conclusion

Considering that there are no adverse effects resulting from undesirable interactions between genes expressed in yeasts and the inserted transgene, or that its action may result in behavior changes (virulence, pathogenicity, competitiveness) of this yeast to the environment or to humans and animals; that the lineage of origin is commonly used and safe; that the fermentation product is a substance abundantly present in nature, significantly toxic solely for insects and that its production will take place in a closed system and the effluent inactivation methods are efficient and the proposed monitoring is adequate, we reach the conclusion that the risks to human and animal health are practically inexistent and that this industrial production is safe, complies with the Brazilian biosafety legislation aimed at protecting the environment, agriculture, human and animal health.

CTNBio reached the decision by eighteen votes for, one vote against and two abstentions for the granting of the commercial release to yeast Y1979 for the purpose of farnesene production.

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