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Ministério da Ciência e Tecnologia

GOVERNO FEDERAL BRASIL PAÍS RICO É PAÍS SEM POBREZA

Ministry of Science Technology and Innovation – MCTI

National Biosafety Technical Commission – CTNBio

comissão técnica nacional CTNBio de biossegurança

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Technical Opinion no. 3877/2013

(Authors: Dr. Odir Antonio Dellagostin; Dr. Marcos Dornelas Ribeiro; Dr. Ricardo Vilela Abdelnoor; and Dr. Marcia Margis)

Proceedings:	01200.001454/2013-37
Applicant:	Bio Celere Agroindustrial Ltda.
CQB:	352/12
Próton:	11865/13
Matter:	Request for Commercial Release with confidential information
Previous Extract:	3609/13, published on 05.22.2013
Meeting:	168th CTNBio Regular Meeting, held on December 05, 2013
Decision:	**GRANTED**.

CTNBio, following examination of application for Technical Opinion on commercial release, reached a FAVORABLE conclusion, on the terms of this Technical Opinion.

Regarding the competences provided by Law no. 11105/2005 as regulated by Decree no. 5591/2005, the Commission found that the request complies with CTNBio rules and applicable

legislation aimed at securing environment, agriculture, human and animal health biosafety.

TECHNICAL OPINION

SUMMARY: The company Legal Representative and the Chairman of the Internal Biosafety Commission – CIBio requested CTNBio to analyze the matter and issue a Technical Opinion related to lineage RN1016 of yeast *Saccharomyces cerevisiae* for the production of ethanol. The organism to be commercially released is yeast of the species *Saccharomyces cerevisiae* genetically modified by introduction of the xylose isomerase coding gene coming from the non-pathogen fungus *Piromyces* sp., with increased expression of natural yeast genes *XKS1, TAL1, TKL1, RPE1* and *RKI1* and deletion of gene *GRE3*. The applicant request a Technical Opinion for the purposes of transportation, marketing, industrial production of ethanol, disposal and any other activities related to the purpose of this GMO and progenies derived therefrom.

Abstract:

The Chairman of the Internal Biosafety Commission of the company Biocelere Agroindustrial Ltda. Dr. Celso S. Fiori, requests CTNBio a technical opinion for the Biosafety Report of lineage RN1016 of the genetically modified yeast (*Saccharomyces cerevisiae*) for the production of ethanol. The organism to be commercially released is a yeast of *Saccharomyces cerevisiae* genetically modified with the introduction of the xylose isomerase coding gene (*Xy1A*) coming from the non-pathogen fungus *Piromyces* sp., increasing the expression of natural genes of the yeast, namely, genes *XKS1, TAL1, TKL1, RPE1* and *RKI1* and deletion of gene *GRE3*. CTNBio deemed to be confidential the information marked as such by the applicant. Regarding the competences provided by Law no. 11105/2005 as regulated by Decree no. 5591/2005, the authors find that the information contained in the proceedings fail to indicate evidence of risks for the environment and health provided the yeast is used under the contention regime submitted by applicant and complies with CTNBio rules and applicable legislation aimed at securing environment, agriculture, human and animal health biosafety.

1. General Information.

Yeast *Saccharomyces cerevisiae* is a ubiquitous eukaryote and unicellular organism. It was the first organism used by man to process food and generates edible products, representing the most ancient biotechnology. *Saccharomyces cerevisiae* is an organism classified as GRAS (Generally Recognized As Safe) and with no restriction to human consumption according to the US agency Food and Drug Administration (FDA) and, due to this classification, is widely used in basic and applied research. The absence of adaptive and reproductive advantages of genetically modified yeast was demonstrated in the production of wine in the United States and Canada when compared with its sylvan parental. The first eukaryote organism to have its genome sequenced was the laboratory standard yeast S188c, revealing a structure containing about 6000 genes distributed in 16 chromosomes (Goffeau *et al*., 1996).

The ability of this microorganism to produce alcoholic fermentation was described in 1860 by Louis Pasteur and man has been benefitting from the fermentative ability of yeasts in preserving and producing beverages for thousands of years. In recent years, with the development of new techniques, both in the fermenting process and in molecular biology enabled the yeast cells to assume the role of true bioreactors in the production of different products. Examples are: production of heterologous proteins, organic acids, glycerol, and the natural production of flavoring derivatives and protein, mineral and vitamin nutritional supplements and, most prominently, the production of bread, biomass and ethanol. The latter is the center of large world notoriety for its importance in the beverage industry (wine, sake, beer, brandy, among others), and most prominently, as a biofuel (ethanol) source. The advantage of using plant biomass, such as residues of the sugarcane and other industries (straw and bagasse) is proving to be economically feasible after many years of research. This technology became known as Cellulose Ethanol or Second Generation Ethanol (2G Ethanol).

Cellulose or Second Generation Ethanol is produced from lignocellulose material. This includes agricultural, forest and urban plant residues, energy cultures (sugarcane, sorghum) and

forestry cultures. Conversion of lignocellulose biomass in ethanol takes place in three main phases: pre-treatment, hydrolysis and fermentation. The pre-treatment phase has the purpose of increasing the accessibility of plant cell wall polymers to the components that will later perform the process of hydrolysis. Hydrolysis, either acid or enzymatic, is responsible for making available the cellulose and hemicellulose sugars that may be converted into ethanol through fermentation by specialized microorganisms. Recent analyses show that the use of cellulose ethanol as fuel has the great advantage of reducing fossil fuels dependence, stabilizing fuel prices, strengthening rural infrastructure and, in addition, reducing greenhouse effect (Bracmort, 2012). Besides, this biofuel will not compete with the production of foodstuffs, since the residues of first generation plants will be used in the production process.

Pathogenicity of *Saccharomyces cerevisiae* was held as basically insignificant until very rare cases of the yeast presence were described in isolated cases of severely immunocompromised individuals. There are also rare reports of allergy, mainly workers of the bakery industry, in this case due mainly to protein fractions of wheat. In addition to its role in food production, this yeast is always around us, on fruit peels and surface of grains. Its safety and industrial importance is patent, since it is responsible for the top five industrial products derived from fermentation: beer, wine, cell protein, bakery yeast, and citric acid. Its effect as a probiotic in human and animal food was verified in several studies (Gaiotto, 2005, and Caballero-Cordoba and Sgarbieri, 2000).

2. Description of GMO

Yeast *Saccharomyces cerevisiae* lineage RN1016, subject of this plea, is an organism genetically modified through introduction of homologous recombination of gene *Xy1A* that codes for the xylose isomerase enzyme, coming from the non-pathogenic fungus *Piromyces* sp., displaying in addition an increased expression of the yeast natural genes *XKS1*, *TAL1*, *TKL1*, *RPE1*, and *RKI1* and deletion of gene *GRE3*. This enzyme grants the yeast the ability to ferment xylose, a five-carbon sugar, enabling it to produce ethanol from biomass that is processed as a carbon

source. Other changes produced increased expression of the yeast endogenous genes as well as deletion of one gene, without introducing DNA exogenous sequences for increasing efficiency of the fermentation process.

Lineage RN1016 does not produce sporulation due to its genetic constitution type MATa/MATa, and therefore has no ability to persist for large periods in soil or any other environmental matrices and does not resist to desiccation, as experimentally shown. The absence of sporulation also limits the mating of RN1016 with other yeasts, restricting its reproduction to the vegetative (non-sexual) cycle. On the other hand, insertion in the chromosome secures stability of the transgenic construct.

Therefore, the genetically modified yeast, event RN1016 (or simply event) is clearly an organism featuring risk class 1.

Regarding this plea, the use of this yeast shall be restricted to closed fermentation systems having as sole byproducts the lignin obtained from filtration of the fermented hydrolyzed material, which is entirely burned in the interior of the industry and the distilled vinasse that is typically obtained in the ethanol producing process, which is submitted to a sterilizing treatment with ultraviolet light and may in addition be submitted to a cooking process for concentration, as required.

Considering that the event involves a well-known host that is riskless to human and animal health, that here are no records of health harms related to consumption or exposure to proteins expressed by the transgene, that the bioinformatics analysis supports the hypothesis of allergenic pathogen absence for a the protein, that the event and its byproducts are not targeted for human or animal consumption and that the industry workers will be minimally exposed to the yeast, one may conclude that the event fails to pose risks that are different from the ones posed by the non-transgenic yeast for the intended purposes and we are favorable to the granting of the request for commercial release of this yeast.

3. Biosafety of the Product

Analysis of the GM microorganism pursuant to Ruling Resolution no. 05, of March 12, 2008, Annex III

3.a. Intended Use of the Microorganism

The microorganism subject of this application was genetically modified to be able to use 5-carbon sugars in the process of alcoholic fermentation. Therefore, this yeast has the ability to consume not only saccharose but also sugars derived from lignocellulosic biomass made available through specific treatments.

The whole process shall be conducted in vats located in alcohol producing plants. The yeast will not be released alive in the environment. Neither the yeast nor its products will be consumed in the form of food or ration, supplement or other form of ingestion.

3.b. Modified Organism and its Biology

Event RN1016 was obtained through transformation of bread yeast *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* is the most known and studied eukaryote organism in the world, being an important model of cell study, subject matter of the first program of gene sequencing. It is also widely employed in the food and beverages industry since the beginning of human civilizations. For this and other reasons, several Regulating Agencies all over the world have exempted this organism from regulatory restrictions. The Food and Drug Administration, for instance, includes *Saccharomyces cerevisiae* in the group known as GRAS (Generally Recognized as Safe) due to the abovementioned characteristics. There are no doubts that the parental organism is innocuous from the viewpoints of human and animal and alimentary health.

3.c. Transgene donor Organism

Event RN1016 has just one new gene, due to the anaerobic ruminal fungus *Piromyces*.

Strict anaerobic fungi have an essential ruminal role in enzyme-breaking the cell plant wall polysaccharides, supplying nutrients to the host and guaranteeing its survival. High specific activities detected for cellulases and hemicellulases that are present in the rumen of herbivores have attracted great attention for the discovery of new potentially industry-application enzymes (Qiu *et al.*, 2000; Harhangi *et al.* 2003; Steenbakkers *et al.*, 2008).

The xylose isomerase found in RN1016 was obtained from the anaerobic E2 *Piromyces* sp. fungus (ATCC 76762). The choice of this organism was due to its high ability to degrade cellulose and hemicellulose, suggesting the presence of genes related also to the xylose metabolism (Harhangi, 2002; Harhangi *et al.*, 2003). This microorganism is a symbiont of its host and has no pathogenic characteristics as against the ruminant and other organisms. Indeed, there are no reports of pathogenicity in organisms of the *Neocallimasticaceae* family. Gene *Xy1A* isolated from *Piromyces* sp. is related solely to hydrocarbon metabolism and is not related to pathogenicity mechanisms elsewhere described. The organism *Piromyces* sp. E2 (ATCC 76762) has no history of undesired effects to human and animal health and to the environment. A search conducted by the authors on the Pulmed databank failed to return any reference that may indicate suspicion, or even suggestion, of pathogenicity.

3d. Introduced Gene and its Specific Function

The sole exogenous gene introduced in yeast *Saccharomyces cerevisiae* to obtain event RN1016 was gene *Xy1A*, coming from the non-pathogen *Piromyces* sp., coding for the xylose isomerase enzyme. Other genetic changes made so as to increase the expression of the yeast endogenous genes in order to maximize the efficiency of the fermentation process. However, the changes and introduction of new genetic elements were conducted in the chromosome, granting their stability along generations and classic Mendelian inheritance.

Xylose isomerase, also known as glucose isomerase (XI, D-xylose isomerase EC 5.3.1.5), is the product of the transgene and it catalyzes the reversible reaction of D-xylose into D-xylulose. It

is one of the most industrially used enzymes, largely used in the production of high-fructose corn syrup (HFCS) (van Maris *et al* 2007). Facing an increasingly higher demand, and driven by the booming market for HFCS, the production of this enzyme currently commands a large part of the food industry (Bhosale *et al.*, 1996). This safe record of use of the enzyme in the food industry significantly reduces the uncertainties of alimentary safety that may remain based on possible homologies with other sequences or any other reasons not directly linked to food.

3.e. Production Targets

Taking into account the intended use and the scope of the risk assessment, it is clear that all aspects of human health shall be restricted to contact with the transgene yeast during routine operations in the industry and, in the case of vinasse for agricultural purposes, to contact with the derived product, containing yeasts that were inactivated by heat or other adequate procedure.

Therefore, we understand that the protection target derived from general aims established by the country laws is the plant laborer's health and, to a lesser extent, the farmer's health in case the vinasse is foreseen to be used for farm purposes. There is no reason to extend this protection target to the general public or to animals, since they will neither get in contact with the transgenic yeast nor with any of the yeast's byproducts.

Considering the information brought by the base document and those available in the literature, we understand that:

1. There is no evidence that aspiration or contact with the event may cause light or moderate allergies in a significant part of workers, since xylose isomerase has no record of allergenicity or sequences of amino acids that may suggest such potential harm;
2. No evidence was found that the expression of this new gene and/or the superexpression of the remaining exogenous genes have modified, even to a

minimum extent, the behavior of this transgenic yeast, under any scientifically well-based hypothesis;

4. Environmental Safety

The intended use of this GMO shall be solely for closed-system fermentation, where the only byproducts are lignin, resulting from filtering the fermented hydrolyzed matter and vinasse, a typical product of the ethanol industry, which in this particular case may be treated by exposure to ultraviolet light to guarantee sterilization or by a heating process to boost concentration and consequent inactivation of all yeasts, as the case may be. The resulting lignin may be used in heat generation in the plant.

Therefore, the limited capacity of dispersion into the environment, the intended use in a closed industrial system and the processing characteristics are the main issues to be taken into consideration to assess environmental impacts. Compared with conventional alcohol production, from sugarcane, there is no glimpse of significant processing differences, except for the fact that it represents a second-generation ethanol production system.

4.1 Identification of protected targets for potential environment damages and harm caused to the agrosystem represented by an accidental release of yeast RN1016.

Applicant has correctly identified potential damages to protection targets specified in Brazilian legislation, as well as their hypothetical causal mechanisms:

(1) survival and dispersion in water and soil;

(2) impact on biodiversity and soil and water indicator organisms;

(3) impact on physicochemical characteristics of water and soil;

(4) impact on water and soil micro-biodiversity;

(5) tolerance to physical and chemical sterilizing agents; and

(6) allergenic potential.

Based on these risk elements, the applicant carried out a wide bibliographic review and conducted an extended set of experiments to assess potential risks associated to the yeast management, inactivation systems and possible accidental release. Experimental and bibliographical details may be found as follows:

- **Survival and dispersion;**
- **Impact on physic-chemical characteristics of water and soil;**
- **Impact on soil and water biodiversity and indicator organisms;**
- **Tolerance to physical and chemical sterilizing agents;**
- **Allergenic potential.**

The complete methodology and discussion of results are supported by an extended and complete bibliography.

5. CTNBio Final Opinion

Based on the full and complete information contained in the dossier forwarded by applicant, on the related literature and on previous experience on industrial use of yeasts in the process or ethanol production, considering that the event involves a well-known host, *Saccharomyces cerevisiae*, with its genome already sequenced, featuring a safe history of use by the industry for multiple uses and with no risks to human and animal health; taking into consideration in addition that there are no reports of damages to health related to consumption or exposure to the protein expressed by the transgene; considering that the bioinformatics analysis supports the hypothesis of no allergenic potential for the protein; that the intended use of this yeast for industrial production of second-generation ethanol has no significant differences of the common industrial process of garapa fermentation, which contains closed and later inactivation system; that the gene donor is neither a human nor an animal pathogen and that *Xy1A* participates solely in the catalysis of the reversible relation of D-xylose and D-xylulose, a

process widely known by the food industry; that the transformed organism is deemed to be safe and widely used in the industry and even in artisanal fermentative processes; that the xylose isomerase enzyme, the only new protein produced by the related yeast, is extensively employed in the food industry; that the expression of a new gene, as well as the change in the expression levels of other genes related to the modified metabolic pathway of RN1016 fails to grant the recombinant yeast any modified characteristics of competitiveness in the environment; that RN1016 has reduced survival abilities due to absence of sporulation ability that drastically limits its dispersion in nature and restricts its growth to industrial vats, under optimized conditions that are non-competitive with natural yeasts or other microorganisms; that industrial residues are not a source of environmental exposure to the GMO, given the biologic characteristics of the RN1016 yeast, which facilitate its inactivation within the conventional ethanol production processes; and the low competitiveness of the yeast in natural environments, even in case it is accidentally released alive; that the additional procedures to secure full inactivation of modified yeasts are foreseen in the industrial plant and will be incorporated to the productive process when necessary; and, above all considering that the event and its byproducts are not targeted for human and animal consumption and that the industry workers will be minimally exposed to the yeast, our conclusion is that the event fails to pose risks to protection targets different from the non-transgenic yeast to the intended purposes and we are favorable to granting the applicant's request.

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DR. FLÁVIO FINARDI FILHO

CTNBio President

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In Witness Whereof, I have hereunto set my hand and seal in this City of Brasília, Federal District, Brazil, this Monday, April 27, 2015.

Fees according to

Official Gazette of 04/15/2011 Marco Antônio Rochadel

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