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comissão técnica nacional CTNBio de biossegurança

Ministry of Science, Technology and Innovation–MCTI

National Biosafety Technical Commission – CTNBio

Ministério da Ciência e Tecnologia

GOVERNO FEDERAL BRASIL PAÍS RICO É PAÍS SEM POBREZA

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Technical Opinion no. 4526/2015

Proceedings:	01200.004293/2014-37
Applicant:	BioCelere Agroindustrial Ltda.
CQB:	352-12
Proton:	41217/2014 and 41223/2014
Matter:	Application for Commercial Release, **with confidential information**
Previous Extract:	4348/2014, published on 11.20.2014.
Meeting:	179th CTNBio Regular Meeting held on February 09, 2015
Decision:	**GRANTED**

CTNBio, following examination of the proceeding requesting a Technical Opinion on biosafety of a product for commercial release, reached a conclusion favorable to the granting of such commercial according to this Technical Opinion.

Within the range of its competences established under Law no. 11105/2005, as regulated by Decree 5591/2005, the Commission reached a conclusion that the within request complies with CTNBio rules and related legislation aimed at securing biosafety of the environment, agriculture, human and animal health.

CONSOLIDATED TECHNICAL OPINION

Applicant, BioCelere Agroindustrial Ltda., according to provisions of CTNBio Ruling Resolution no. 05, requests analysis and issuance of a technical report related to commercial release of lineage Celere-2L of yeast *Saccharomyces cerevisiae* for the production of ethanol. The genetically modified organism, a yeast of the species *Saccharomyce cerevisiae* lineage Celere-2L, is an organism genetically modified through insertion of a homologue recombination of gene *xylA* coding for enzyme Xylose isomerase, where the only exogenous sequence introduced, by homologue recombination, is the gene coding for one enzyme, coming from a non-pathogen microorganism. Other genetic modifications produced permanent deletion of one gene and increased expression of endogenous genes, with no introduction of exogenous DNA sequences. All additional modifications are related to the metabolism of this sugar. Applicant intends to use this GMO for the purposes of transportation, marketing, industrial production of ethanol, discarding and any other activities related to the purpose of this GMO, its progenies and derivatives.

Initial considerations

Yeast *Saccharomyce cerevisiae* lineage Celere-2L is an organism genetically modified by homologous recombination of enzyme Xylose isomerase gene *xylA*. On the other hand, Xylose isomerase is one of the most used enzymes for industrial purposes because of its ability in sugar isomerization. The main example of such use is in large scale production of corn syrup

since the sixties, a market that currently has an important role in the food industry. For this reason, the use of this enzyme for several years in the food industry verifies its safety for human and animal health.

Yeasts of the species *Saccharomyce cerevisiae* are risk class I organisms, widely used for hundreds of years in the production of food and beverages, with no consistent record of unwanted effects on human and animal health and the environment. The use of this microorganism in the production of food and beverages, as well as its natural occurrence in fruits, has promoted its direct and frequent contact with humans and animals for thousands of years along the evolution of species. For the above reasons, regulatory agencies around the world consider this microorganism safe for human and animal health and for the environment, as exemplified by the American Food and Drug Administration (FDA), which lists *Saccharomyce cerevisiae* in a group known as “GRAS” (generally recognized as safe).

According to the detailed information supplied by Applicant, this yeast will be used in closed fermentation systems, having just lignin and vinasse as byproducts. Both spinoffs are generated after the distillation process, where the content of the fermentation vat is submitted to temperatures around 85^{o}C for about 10 to 15 minutes. Experiments presented by Applicant showed that thermal treatments for times and temperatures considerably lower than that are sufficient to completely inactivate the microorganism. Despite the sufficiency of the distillation process to fully inactivate the GMO, the industrial process includes specific treatments for both byproducts. The lignin obtained after filtering is fully burned in a tank within the industrial facility to produce electrical power. Vinasse, in turn, may be directed to an evaporating system by concentration, where temperatures range from 67^{o} to 103^{o}C for long periods of time.

Feeding the inoculum in the propagation system is made with reduced amounts of yeast, transported in purpose-appropriate robust packages, minimizing any risk of leak or accidentally contact with the operator or with the external environment. The reduced inoculum amount, coupled with the closed fermentation system that ends up with the distillation and complete elimination of the GMO reduce to negligible levels all likelihood of exposure of the yeast to humans, animals and the environment.

GMO Characteristics

Event Celere-2L is a diploid yeast of the species *Saccharomyce cerevisiae*, developed from the wild lineage Pedra-2 (PE2). PE2 is one of the first lineages introduced by the Brazilian ethanol industry, being also an important model for research with molecular biology of yeasts, widely known by the Brazilian and international scientific community.

The GMO has a single exogenous gene, namely gene *xylA*, derived from a non-pathogenic anaerobic fungus. Both microorganisms, host and donor, belong to risk class 1.

Transgene donor organism

This information is confidential. Up to this moment there are no records of any adverse or pathogenic effect caused by the donor microorganism to human beings, animals or the environment, therefore placing this microorganism in Risk Class 1.

Environment risk assessment

The series of results and experimental data submitted by Applicant enabled a complete risk assessment of the GMO as against its unmodified parental.

In order to show the effect of genetic manipulations in to the GMO survival, Celere-2L growth in different carbon sources was assessed in comparison with its parental. Both lineages display

equivalent growth in sucrose, glucose and fructose. The only recorded difference was during cultivation in a medium containing xylose as a carbon source. Being this the aim of the genetic modifications, it became clear that the yeast Celere-2L has the ability to ferment xylose sugar, contrary to its non-GM parental. The results verify that the generic modification of lineage Celere-2L is restricted to the process of converting xylose into ethanol. This data strengthen the evidence of absence of adverse effects caused by genic, pleiotropic or epistatic interactions.

The ability to resist desiccation was assessed in comparing the GMO and the parental to each other. Cells of both lineages, collected during the exponential and stationary phases of growth, were submitted to desiccation for 12 and 24 hours. After the treatment, the cells were rehydrated and plated for the counting or viable cells. No significant differences were recorded between GMO and its parental in both the sampled points. Both lineages are significantly affected by the stress condition, reducing the cell viability to less than 20% after 12 hours of treatment. After 24 hours, no viable cells were recorded.

Attempting to observe the effect of sterilizing and antimicrobial agents, GMO and parental were submitted to five independent sanitizing treatments including sodium hypochlorite, ethanol, antibiotics hygromycin and geneticin, and temperature. Agents were applied in different treatments of concentration and/or exposure time. The results were presented in dose-response charts, correlating exposure time and concentration. All tested agents were equally effective, promoting complete inactivation of the GM and parental treatments in lethal doses.

In the essay with *Daphnia similis*, the percentage of static test-organisms in the control condition was less than 5%, validating the essay. According to Fisher's Exact Test the recorded

immobility rate was not significant, even in the highest concentration employed. The recorded Immobility Coefficient (CE(I)50; 48H) of both lineages is above 2% of cells in total volume of the aqueous matrix. The result suggests that both GM and parental lineages fail to display toxicity.

Possible changes in chemical and physical properties of the water were also assessed. Parameters such as pH and oxygen content were assessed for the different concentration of GM and parental yeasts. Despite some small changes recorded in the comparison between them, the values resulting from the GMO and parental treatments are not different. The results demonstrate, again, that the genetic modification of yeast Celere-2L fails to alter the microorganism physiology beyond what is required.

In essays conducted with *Damio rerio*, toxicity is established by the concentration of the sample sufficient to cause lethality of 50% of the organisms in essay conditions after 96 hours [LC(I)50; 96h]. During this period, observations were made on the conditions of the organisms each 24 hours recording mortality, abnormal natation movements and any other unexpected reaction. The results show that both treatments (GMO and parental) fail to cause any significant toxic effects, according to the Fisher's Exact Test, even in the highest concentration tested (2%). Therefore, the lethal median concentration [LC(I)50; 96h] for either case was considered to be higher than 2%, an amount extremely high of cells in the aqueous medium.

Same conditions were used to assess the possible impact in the physical and chemical properties of water in tests with *Damio rerio*. pH and oxygen content were assessed. In all observations it became clear that when the GMO and the parental are compered between them, variations are equivalent and statistically non-significant, both in the beginning and the end of the essay.

As the absence of both adverse effects on indicator organisms and significant changes in the

physical and chemical properties of the water was demonstrated, the effects of the GMO in the water microbiota were assessed. For this purpose, metagenomics was used, enabling identifying and quantifying the organisms in the ecosystem. Samples of water were collected from natural environments were collected and received treatment with different concentrations of the GMO parental. Samples were taken in 1, 1.5, and 30 days after application, total DNA was extracted and regions 16S and ITS were sequenced.

In analyzing region 16S, seven bacterial classes were identified (*Alpha Proteobacterium*, *Chloroflexia*, *Beta Proteobacterium*, *Planctomycetacia, Cyanobacterium, Gamma Proteobacterium* and *Caldilineae*). The results obtained from sequencing and analysis of the ITS region resulted in the identification of five genera of microalgae, all representative of the class Chlorophyceae (*Desmodesmus*, *Lobochlamys*, *Monoraphidium*, *Chlamydomonas* and *Scenedesmus*). Comparisons conducted with the GMO and parental in each point sampled show absence of significant differences in bacteria and microalgae populations, demonstrating that the GM yeast has no effect on the aquatic microbiota when contrasted to the non-GM parental organism.

As a whole, the results obtained by the three approaches (essay with *Daphnia*, essay with *Danio rerio* and metagenomic) demonstrate that the genetic modifications used in the construction of this GMO fails to add to the lineage characteristics that could establish a difference regarding its parental from the viewpoint of environmental safety.

The same approach was used to assess the implications of the GMO in water quality was used to assess the effects of the lineage in soil quality. The ecotoxicity test conducted was construed with three independent experiments using complementary approaches: i) acute toxicity essays with indicator organism; ii) analyses of soil physical and chemical properties; and iii) analysis of

the microbial community after application of the GMO.

For the acute ecotoxicity test, earthworms of the species *Eisenia fetida*. The experiment was conducted by rigorously following the rule ABNT NBR 15537:2007, the protocol of which is appended to this Technical Opinion. Viability shown in all treatments was always above 92% and no significant differences were recorded between treatments with the GMO and the non-GM parental. Therefore, treatments were held to be equivalent and, in the concentrations used, toxicity is discarded.

To assess the effect to physical and chemical properties of the soil, a high concentration of cells (3000mg/dm^3) was applied to new soil samples. In this experiment, physical and chemical properties of the soil were analyzed after the treatments. Untreated soil was used as experimental control. About 300g of soil was collected 15 and 30 days after GMO and parental application.

In analyzing physical properties (or granulometry) clay, silt, sand and gravel were the parameters assessed. No significant change was recorded after treatments with the GMO and parental in every time of the sample collection.

Analysis of chemical properties included as main parameters: macro- and micronutrients, organic matter, cation exchange capacity (CEC) . Contrary to granulometry, small changes are recorded after application of the yeasts. However, all result equivalent when the GMO and the parental are compared. Absence of significant differences between GMO and parental shows again that genetic changes fail to incorporate undesired characteristics to the GMO.

To demonstrate the effects of the GMO on soil microbiota, a metagenomic analysis was conducted after application of the treatments. Examining the results obtained by sequencing

the region 16S, eight bacterial classes were identified (*Anareo Linear, Gamma Proteobacterium, Alpha Proteobacterium, Beta Proteobacterium, Actinobacterium, Sphingobacterium, Delta Proteobacterium, and Acidobacterium*) as being present in all treatments/points analyzed. Results obtained by sequencing region ITS, include identification of four fungi (*Sordariomycetes, Pezizomycetes, Leotiomycetes, Saccharomycetes and Agaricomycetes*).

Once more, it became clear that eventual changes from time to time are equivalent between parental and GMO when treatments are compared to each other, showing absence of unwanted effects caused by the genetic modification. On a whole, results demonstrate an absence of adverse effects of yeast Celere-2L on physical, chemical and biological features of the soil.

There are no evidences that yeasts of the species *Saccharomyce cerevisiae* (or other species of the genus) may influence in air contamination. Biologic activities of these organisms involve production of Volatile Organic Compounds (VOC) that are formed during DNA, amino acids and fat acid syntheses.

In order to determine emission of CO_2 and VOC from glucose and xylose fermentation, three replicas of the GMO and parental were grown in media containing the two sugars as the only carbon source. Summarizing, total gas collection was made through fermentation in airtight closed bottles coupled with appropriate bags (Gas Sampling Bags – Sigma-Aldrich®. At the end of the growth experiment, sugar consumption and CO_2 and VOC production were quantified.

Bags containing gases collected in the GMO and parental/glucose and GMO/xylose conditions were sent to the TASQA company, where presence of VOCs was assessed by chromatography and mass spectrometry (CC-MS). At the beginning, 53 VOCs analyzed in air quality tests were

quantified and none of them was identified. Additionally, a quantitative method analyzing the remaining 64 VOCs was conducted. In total, among all 117 (53+64) compounds analyzed, ethanol was the only identified VOC, an expected outcome due to the fermentative activity of yeasts. Results showed that the yeast biologic activity, either GMO or parental, fails to produce even detectable amounts of volatile organic compounds associated to deviations in air quality.

Finally, in order to assess survival of both lineages in water and soil, an experiment was established applying the GMO and parental treatments to natural samples of water and soil. Survival of both linages was determined from time to time by real-time quantitative PCR (qPCR) using the method of standard-curve based absolute quantification. The analysis was conducted by the staff of the Soil Microbiology Laboratory of the University of São Paulo (Luiz de Queiroz – ESALQ campus). After 30 days, both treatments, GMO and parental, displayed a behavior equivalent in what concerns survival. For the water sample, no living cells were found after 30 days for both treatments, while for the soil sample an uniform population decay was recorded for both, GMO and parental. Results show that genetic modification produced in yeast Celere-2L fail to ensure additional survival ability and dispersion in water and soil.

Risk Assessment for human and animal health

Experiments presented and discussed in the report show that yeast cells are completely inactivated by thermal treatments for a time and temperature below the level that is already part of the industrial process of cellulose ethanol production.

Experiments also showed that the yeast fails to present potential risk and is not different from its conventional equivalent for different relevant assessments as far as the environmental and human and animal health viewpoints are concerned. Taking into consideration the approach of the opinion on issues related to human and animal health, it shall be noted that yeast

Celere-2L was approved by thorough exam in what regards (1) toxic effects on indicator organisms in water and soil, (2) impact on water microbiota, and (3) resistance to physical and chemical sterilizers.

Experiments demonstrated that application of living cells failed to cause changes in physical and chemical properties and microbial diversity of water and soil when compared with its conventional (parental) equivalent. Besides, yeast Celere-2L was found to be equally sensitive to different sterilizing and antimicrobial agents when compared to its conventional equivalent.

Regarding provisions of Annex III of Ruling Instruction no. 5, of March 12, 2008 (Assessment of risks to human animal health) specific information shall be included on: "(A) organisms that were consumed as food, or (B) microorganisms used as vaccines".

Lineage Celere-2L is not a vaccine and under no circumstances shall be used for direct consumption or additive to human or animal food. For these reasons, the topic is not applicable to the purposes of this GMO.

According to Applicant (section 1.1, pg. 20), the intended use of lineage Celere-2L is restricted to industrial production of ethanol from sugars derived from lignocellulosic materials, such as plant biomass. Celere-2L shall be used in closed vats and pipes and the microorganism is not discarded in the environment in its viable form.

The author of the Application stresses that the GMO examined is a wild lineage of *Saccharomyce cerevisiae* (receptor organism), an ubiquitous microorganism, class risk 1, widely used in the food and beverage industry, held to be safe and with no alimentary restrictions or allergenicity record. The organism received a single exogenous gene derived from an anaerobic non-pathogenic fungus. The expressed enzyme is widely used in the food

industry with no record of allergenicity or any adverse effect to human and animal health.

Final Opinion

Based on a large set of information contained in the report forwarded by Applicant, in the relevant literature and in forerunner experience related to the industrial use of yeasts in the process of ethanol production;

Whereas

- *Saccharomyce cerevisiae* is the most known yeast species by the scientific community, with its genome sequenced, safe record of use in different industrial branches, such as fuels, beverages and food;
- The yeast is intended for the production of second-generation ethanol, which has no significant differences from the traditional industrial ethanol production and that the distillation phase, an important part of the process, is enough to cause complete inactivation of the microorganism;
- The gene donor has no record or even related occurrences of pathogeneicity to human beings or animals, and that the protein coded by the inserted gene has a fully known role in the food industry, failing to grant to the recombinant protein any characteristics additional to the intended ones.
- Industrial byproducts are not a source of GMO exposure, since it fails to contain viable forms of the GMO, and may receive further thermal treatments, as the case may be;
- The production industrial unit operates in a closed system, since inoculation of the yeast to the complete inactivation on the distillation process;

- Ethanol is the final product of the fermentation process, a pure molecule chemically defined and identical to the one traditionally produced by Brazilian industrial plants;
- The experiments reported in the Application show that the yeast cells are completely inactivated by thermal treatments for a time and under a temperature significantly lower than the time and temperature that are already part of the industrial process of cellulosic ethanol production;
- The experiments also show that the yeas has no potential risk and is not different from its conventional equivalent for several assessments, such as
    - Survival and dispersion in water and soil;
    - Toxic effects on indicator organisms in water and soil;
    - Impact on water and soil physical and chemical characteristics;
    - Impact on the water and soil microbiota; and
    - Tolerance to physical and chemical sterilizing agents.
- The genetic modification was the result of a stable integration of the transgene and, for this reason, there is no possibility of transmission of characteristics via plasmids. The transformation was made through the traditional system of homologue recombination, where the insert is specifically directed to the intended locus in the genome.
- In experiments of acute toxicity, in high concentrations of up to 300 mg/l of pure cells, the yeasts of lineage Celere-2L, as well as their parental, will not cause toxic effects on water indicator organisms (fish and micro-crustaceans). Similarly, the tests in soil with concentrations of 23 g/cm^3 of pure cells failed to show any toxicity for indicators

species of soil quality (earthworms). Besides, application of living cells failed to cause changes in physical, chemical properties and in water and soil microbial diversity when compared with its conventional equivalent (parental).

- Survival and potential impact assessment of viable yeasts of lineage Celere-2L in natural soil and water samples demonstrated that survival in water is equivalent and reduced to both lineages. In soil, the equivalence was also noticed so that the ability to consume xylose failed to grant toe GMO any adaptive advantages in the natural environment when contrasted to its non-GMO parental.

Now therefore, considering the wide and extended historic of use of the *Saccharomyce cerevisiae* species in different processes for the production of food, beverages and fuels, with no record of undesirable effects to human and animal health and to the environment, we understand that the application is in full compliance with the provisions of CTNBio Ruling Directive no. 05/2008, and proved the safety of event Celere-2L, we reach a conclusion favorable to the granting of the request for commercial release.

Therefore, this commission is favorable to the granting of the application made by BioCelere.

Dr. Edivaldo Domingues Velini

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, June 27, 2016.

Fees according to

Official Gazette of 04/15/2011 Marco Antônio Rochadel

Page 73 Public Translator