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July 29, 2011

**Via Hand Delivery**

Food and Drug Administration  
Division of Animal Feeds (HFV-224)  
Office of Surveillance and Compliance  
Center for Veterinary Medicine  
7519 Standish Place  
Rockville, Maryland 20855

**CONTAINS CONFIDENTIAL  
BUSINESS INFORMATION**

Re: **GRAS Notification by; Gevo; Our File No. GE14020.00002**

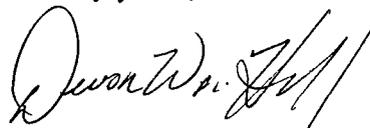
Dear Sir or Madam:

The purpose of this letter is to request that the enclosed Generally Recognized as Safe (GRAS) determination for the use of an inactivated modified *Saccharomyces cerevisiae* when present as a component in animal feed be accepted for review in the GRAS pilot program. 75 Fed. Reg. 31800 (June 4, 2010). This is a re-submission for part of the original GRAS Notification submitted on June 10, 2011 for isobutanol distillers grains (iDG). The Center for Veterinary Medicine requested that we submit separate GRAS Notifications for each substance in the original notification. This GRAS Notification is for the modified yeast. A separate notification for the distillers grains containing residual isobutanol will be submitted shortly.

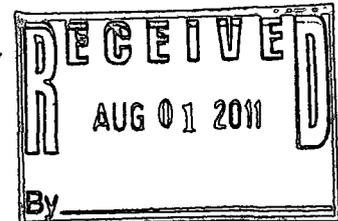
The submitter, Gevo, previously met with your office on December 17, 2009 to discuss a modified *S. cerevisiae* which will be inactivated and present in the distillers grains after isobutanol production.

This submission is provided in triplicate with one sanitized copy. We trust that this submission satisfies the Agency's needs, and will be deemed accepted and complete. Should any questions arise, please contact us, preferably by telephone or e-mail, so that we can promptly respond.

Sincerely yours,



Devon Wm. Hill



Enclosures

4836-5385-6778, v 1

**Generally Recognized as Safe (GRAS) Notification**

**for**

**an inactivated modified *Saccharomyces cerevisiae* when present as a  
component in animal feed**

Prepared for:

U.S. Food and Drug Administration  
Center for Veterinary Medicine  
Division of Animal Feeds (HFV-224)  
7519 Standish Place  
Rockville, MD 20855

Submitter:

Gevo  
345 Inverness Drive South  
Englewood, Colorado 80112

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## I. Introduction

This notification is submitted in support of the determination that the inactivated, modified *Saccharomyces cerevisiae* is Generally Recognized as Safe (GRAS) when present as a component of animal feed for target animal consumption. *S. cerevisiae* will be used in the fermentation and distillation of corn to produce isobutanol. Distiller's grain, a byproduct of the isobutanol distillation and which will contain the inactivated *S. cerevisiae*, will then be used as a component of feed for animals. As more fully explained below, the inactivated, modified *S. cerevisiae* is for all practical purposes identical to inactivated unmodified *S. cerevisiae*. Target animals include both food producing animals and pets and the amount of *S. cerevisiae* that could be present in the diet will be in accordance with good manufacturing practice and the typically feeding practice for distillers grains used in animal feed. Livestock for consideration include beef cattle, dairy cows, broiler chickens, egg-laying chickens, swine, and sheep. Pets for consideration include dogs, cats, rabbits, guinea pigs, and horses. The determination of the GRAS status is on the basis of scientific procedures and conforms to the guidance issued by the Food and Drug Administration (FDA) under *proposed* 21 CFR § 570.36, 62 Fed. Reg. 18938 (Apr. 17, 1997) and the FDA's Notice of Pilot Program; Substances Generally Recognized as Safe Added to Food for Animals, 75 Fed. Reg. 31806 (June 4, 2010). We submit information in the following areas:

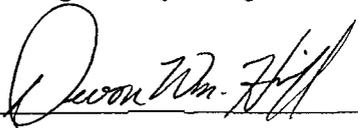
- Identity of the substance;
- A description of the method of manufacture;
- An estimation of daily intake for target animals;
- Safety data and safety evaluation; and
- GRAS determination, as determined by scientific procedures when inactivated, modified *S. cerevisiae* is present as a component of animal feed.

It is our expectation that FDA will concur that the information presented here fully supports the determination that the inactivated modified *S. cerevisiae* is GRAS when used as a nutritional component of animal feed.

**II. ADMINISTRATIVE INFORMATION**

**A. CLAIM OF GRAS STATUS**

The use of inactivated, modified *S. cerevisiae* as a component for animal food use has been determined to be exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 301 *et. seq.*)(the Act) because the Submitter has determined that such use is generally recognized as safe (GRAS).



7-29-11  
Date

**B. NAME AND ADDRESS OF THE SUBMITTER**

Submitter	Acknowledgement of Receipt of Notification and Inquiries to be Directed to:
Mr. Glenn Johnston, Vice President of Regulatory Affairs Gevo 345 Inverness Drive South Building C, Suite 310 Englewood, Colorado 80112	Keller and Heckman LLP 1001 G Street N.W. Suite 500 West Washington, DC 20001 ATTN: Martha Marrapese, Esq. and Devon Hill, Esq. <a href="mailto:marrapese@khlaw.com">marrapese@khlaw.com</a> <a href="mailto:hill@khlaw.com">hill@khlaw.com</a> 202-434-4123 (tel.)      202-434-4279 (tel.) 202-434-4646 (fax)      202-434-4646 (fax)

A letter authorizing Keller and Heckman to serve as agent for the submitter is provided as **Appendix 1.**

**C. COMMON OR USUAL NAME OF THE SUBJECT SUBSTANCE**

The subject of this notice is the inactivated, modified *S. cerevisiae* portion of distillers grains (DG). The inactivated, modified *S. cerevisiae* and the DG are obtained after the removal

of the alcohol by distillation after the yeast fermentation of a grain or grain mixture by methods employed in the grain distilling industry. The *S. cerevisiae* will remain part of the DG product and will not be marketed separately. The *S. cerevisiae* collectively includes lipids, proteins, amino acids, polysaccharides, etc., typically found in cells.

#### **D. INTENDED CONDITIONS OF USE AND TECHNICAL EFFECT**

The intended condition of use and technical effect of the inactivated modified *S. cerevisiae* is as a nutritional source, present in distillers grains at levels up to 20%, on a dry weight basis. This level is consistent with the yeast content of distillers grains produced from conventional ethanol distillation.<sup>1</sup>

The inactivated, modified *S. cerevisiae* can be a component of distillers dried grains, distillers dried grains with solubles, condensed distillers solubles, wet distillers grains, or wet distillers grains with solubles, depending on the processing after the removal of the alcohol by distillation after the yeast fermentation of a grain or grain mixture by methods employed in the grain distilling industry.

The inactivated, modified *S. cerevisiae* is to be a replacement for unmodified *S. cerevisiae* currently found in DG products. The inactivated, modified *S. cerevisiae*, present as part of the distiller's grain may be fed daily to target animals such as beef cattle, dairy cows, sheep, swine, egg-laying chickens, and broiler chickens, dogs, horses, rabbits, cats, and guinea pigs. The theoretical maximum feed rate levels of the inactivated, modified *S. cerevisiae* in the diet, based on levels of inactivated unmodified *S. cerevisiae* present in DG, are as follows:

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<sup>1</sup> Liu, K., "Chemical Composition of Distillers Grains, a Review," *Journal of Agricultural and Food Chemistry* 59:1508-1526 (2011).

**Table 1. Maximum feed rates of inactivated modified *S. cerevisiae* in target animal diets**

<b>Animal</b>	<b>% DG in Diet<sup>2</sup></b>	<b>% <i>S. cerevisiae</i> in the Diet<sup>3</sup></b>
<b>Beef Cattle</b>	60	12
<b>Dairy Cattle</b>	30	6
<b>Chicken (broiler)</b>	20	4
<b>Chicken (layer)</b>	15	3
<b>Sheep</b>	60	12
<b>Swine</b>	45	9
<b>Adult Dog</b>	25	5
<b>Horses</b>	20	4
<b>Rabbits</b>	20	4
<b>Adult Cat</b>	25	5
<b>Guinea Pig</b>	25	5

These feed rates are based on public literature for levels of *S. cerevisiae* in DG and feed rates for DG as identified in this notification.

#### **E. BASIS FOR THE GRAS DETERMINATION**

Pursuant to 21 C.F.R. 570.30(a)(1), scientific procedures were used to establish that the modifications to *S. cerevisiae* by the Submitter do not alter the characteristics of the modified *S.*

<sup>2</sup> The Submitter believes these elevated DG inclusion rates reported in the scientific literature are appropriate for worst-case dietary exposure. Although these inclusion rates are higher historic inclusion rates, these rates have been shown to be relatively safe for a short-term exposure. Based on the Submitter's experience, however, the higher inclusion rates are not generally used by animal producers because of negative impacts on animal health and performance. Animal producers use the historic levels because the additional ruminants and nutritional supplements that need to be added to the DG for overall animal health and performance have been established. Any changes to the inclusion levels would require establishing what the appropriate levels for each of the additives in order to maintain animal health and performance. Given batch variability in DG composition, each DG batch would need to be monitored to determine the appropriate level for the additives.

<sup>3</sup> The % *S. Cerevisiae* was calculated by multiplying the %DG in the target animal diet by 20%, which represents the worst-case amount of *S. cerevisiae* in the DG.

*cerevisiae* from those of the unmodified *S. cerevisiae*. Inactivated modified *S. cerevisiae* is substantially equivalent and indistinguishable from inactivated unmodified *S. cerevisiae*.

Therefore, it is our opinion that the inactivated modified *S. cerevisiae* is GRAS.

#### F. AVAILABILITY OF INFORMATION

The submitter will retain copies of the data and information that form the basis for the GRAS determination, which are available for FDA's review at reasonable times, and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Keller and Heckman LLP

1001 G Street, N.W.

Suite 500 West

Washington, DC 20001

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### III. DETAILED INFORMATION ABOUT THE IDENTITY OF THE NOTIFIED SUBSTANCE

#### A. *SACCHAROMYCES CEREVISIAE*

The yeast *S. cerevisiae* has an extensive history of safe use. It has been used for millennia in fermentation processes, such as bread leavening and wine or beer production. It is regarded as the yeast responsible for spontaneous fermentation of grape juice.<sup>4</sup> The Bureau of Alcohol, Tobacco and Firearms rates yeast or yeast cultures grown in juice of the same kind of fruit as a permitted material added in the production of natural wines. See 27 C.F.R. § 24.176. *S. cerevisiae* is ubiquitous in the environment and has been used in food production for several

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<sup>4</sup> Lodder J., Kreger-Van-Rij N.J.W. (Eds; 1967). The yeasts : a taxonomic study. 2nd ed. Amsterdam. North Holland Publishing Company.

thousands of years. *S. cerevisiae* is used as a model organism for molecular biology research and is generally regarded as non-pathogenic.

The specific *S. cerevisiae* strain used to produce isobutanol is a commercially available strain used in industrial fermentation in North America. The *S. cerevisiae* wild-type strain was selected for the addition of the isobutanol pathway because it was found to be more tolerant to high concentrations of isobutanol than other *S. cerevisiae* isolates that were available from public culture collections or from other commercial yeast vendors.

In order to ensure that the starting cells were *S. cerevisiae*, the submitter sequenced the *RDN25-1* region, which corresponds to the 25S ribosomal RNA gene, and compared it to the published genome sequence of the laboratory strain *S. cerevisiae* strain S288c. A sequence alignment demonstrated the sequence in the parental cell is 100% identical to the S288c sequence. Further, a BLAST analysis using the non-redundant nucleotide database at the National Center for Biotechnology Information (NCBI) using the Fungi subset to generate "Distance Tree of Results," as shown in **Figure 5 of Attachment 7**, shows that the sequence is from *S. cerevisiae*. The commercially obtained parental strain is verified in this manner as *S. cerevisiae*. The modifications to the strain were verified to not alter its species identity. The modified organism is substantially equivalent to the wild type organism based on the amount of heterologous DNA inserted. The yeast genome contains approximately 12,156,677 base pairs (bp). The genetic modifications made in the commercially available strain results in the addition of a net +31,026 bp. The fraction of the genome reflected by the additional base pairs is:

$$31,026 \text{ bp} / (12,156,677 + 31,026) = 0.0025, \text{ or } 0.25\%.$$

Thus, the 0.25% difference in the modified *S. cerevisiae* does not significantly change the genome from that of the unmodified *S. cerevisiae* parent cell.

## B. COMPOSITION

The inactivated modified *S. cerevisiae* may consist up to 20% of the DG, on a dry weight basis, as sourced from the public literature on the typical composition of DG.<sup>5</sup> The inactivated, modified *S. cerevisiae* collectively includes lipids, proteins, amino acids, polysaccharides, etc., typically found in cells.

## C. MANUFACTURE

Based on published literature, it is generally recognized that yeasts are inactivated at temperatures of 63°C (145°F) for 30 minutes. Specifically, based on pasteurization temperatures taken from U.S. Food and Drug administration, as shown in the Table reproduced below and in **Appendix 3**, pasteurization is dependent on the time of exposure at the temperature shown. Yeast cells also have been identified as being inactivated (non-viable) in DG products such as thin stillage, condensed distillers solubles (CDS), and dried distillers solubles as described in **Appendix 4**, page 12.

Table 1. Milk pasteurisation temperature-time relationships.

Temperature	Time
63°C (145°F)	30 minutes
72°C (161°F)	15 seconds
89°C (191°F)	1.0 second
90°C (194°F)	0.5 seconds
94°C (201°F)	0.1 seconds
96°C (204°F)	0.05 seconds
100°C (212°F)	0.01 seconds

US Food and Drug Administration (2003).

### 1. Distillation Temperatures

(b) (4)

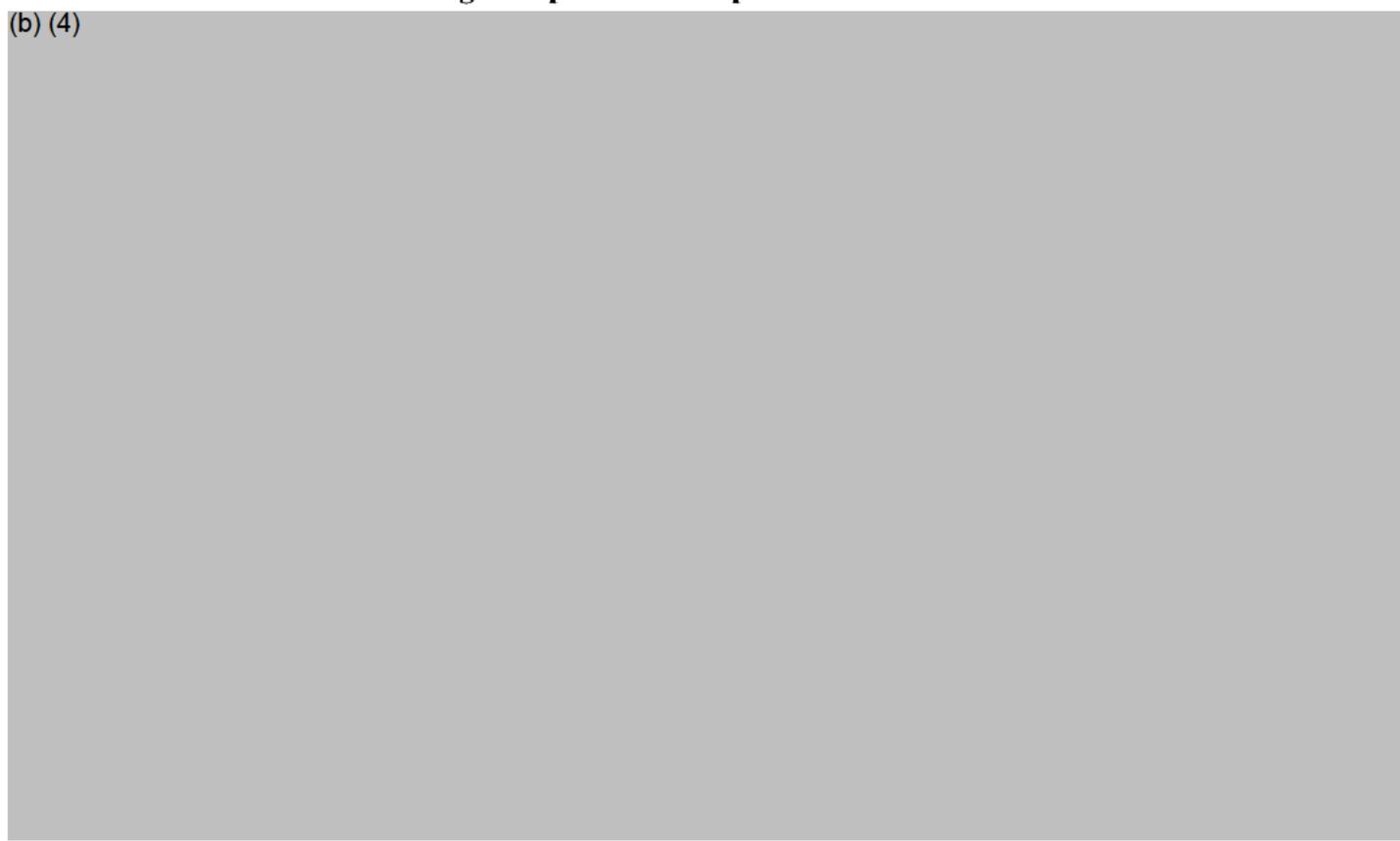
<sup>5</sup> Liu, K., "Chemical Composition of Distillers Grains, a Review," *Journal of Agricultural and Food Chemistry* 59:1508-1526 (2011).

(b) (4)



**2. Thin Stillage Evaporation Temperatures**

(b) (4)



### 3. Grain Processing Temperatures

(b) (4)

Data, in which dry DG with solubles containing the inactivated modified *S. cerevisiae* were analyzed using the accepted protocol for DDGS, the DDGS containing the inactivated, modified *S. cerevisiae* to be nutritionally identical to DDGS containing unmodified *S. cerevisiae*. **Appendix 6** contains the DG analysis and the protocol used.

#### D. INFORMATION ON ANY SELF-LIMITING LEVELS OF USE

The level of the inactivated modified *S. cerevisiae* in DG is limited by the amount of yeast that can be supported in the fermentor during isobutanol production. However, we do not expect that the amount of inactivated, modified *S. cerevisiae* in DG will be different than the amount of inactivated, unmodified *S. cerevisiae* found in DG that is a byproduct of conventional ethanol distillation, which may be up to 20%. See **Appendix 4**. Our conclusion is based upon analysis of DG containing the inactivated modified *S. cerevisiae*, **Appendix 6**, which demonstrates that the nutritional content of DG with the inactivated, modified *S. cerevisiae* is the same as DDGS with inactivated, unmodified *S. cerevisiae*.

The amount of DG containing the inactivated, modified *S. cerevisiae* used in the animal diet will be dependent upon the nutritional needs for the specific target animal and is based on historical use levels. For example, broiler chickens can have inclusion rates up to 20% but higher inclusion rates, such as 30%, decreases body weight and requires additional amino acids and enzymes to be provided to the chickens.<sup>6</sup> Also, for egg layers, inclusion rates are 15%

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<sup>6</sup> Wang, Z. *et al.*, "Use of Constant or Increasing Levels of Distillers Dried Grains with Solubles (DDGS) in Broiler Diets," *International Journal of Poultry Science* 6: 501-507 (2007).

because inclusion rates at 20% and higher results in smaller eggs.<sup>7</sup> Therefore, since the nutritional content of DG containing the inactivated modified *S. cerevisiae* is the same as DG containing inactivated unmodified *S. cerevisiae*, the dietary limitations of DG containing inactivated unmodified *S. cerevisiae* are applicable to DG containing inactivated modified *S. cerevisiae*.

#### E. SAFETY CONSIDERATIONS DUE TO THE NATURE OF MODIFICATIONS TO THE YEAST

The original strain was diploid, and therefore possessed two copies of every gene. In order to expedite strain engineering, it was advantageous to engineer a haploid progeny to simplify the modification process. To reduce the number of chromosomes from diploid to haploid, the beginning strain was sporulated and the haploid cells isolated to generate the haploid descendents. Sporulation in diploid yeast is the biological process where the strain undergoes meiosis to form haploid progeny, analogous to the production of germ cells by mammals. Following sporulation, a single haploid cell was selected for further strain engineering.

The genes inserted into the modified yeast come from organisms commonly used to produce enzymes that are used in the food industry. **Table 2** provides a list of clearances and GRAS Notifications using the organisms as sources for food-grade enzyme preparations. We conclude that the GRAS Notifications (and the data that support them) indicate that the organisms, the genes corresponding to the products listed, and the resulting gene products are GRAS when used consistent with good manufacturing practices to produce food and food ingredients that result in exposures substantially equivalent to those described in the GRAS Notifications and public literature.

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<sup>7</sup> “How Much DDGS Will Benefit Layers?” found at [http://www.wattagnet.com/How\\_much\\_DDGS\\_will\\_benefit\\_layers\\_.html](http://www.wattagnet.com/How_much_DDGS_will_benefit_layers_.html) (last accessed June 6, 2011).

**Table 2. Gene source organisms**

Organism	Reference
<i>Bacillus subtilis</i>	21 C.F.R. § 184.1148 “Bacterially-derived carbohydrase enzyme preparation.” 21 C.F.R. § 184.1150 “Bacterially-derived protease enzyme preparation.” GRN: 20, 114, 205, 274
<i>Escherichia coli</i>	21 C.F.R. § 184.1685 “Rennet (animal-derived) and chymosin preparation (fermentation-derived)” GRN: 289, 299
<i>Lactococcus lactis</i>	21 C.F.R. § 184.1985 “Aminopeptidase enzyme preparation derived from <i>Lactococcus Lactis</i> ”

*Bacillus subtilis* is a ubiquitous, saprophytic, soil bacterium which is thought to contribute to nutrient cycling due to its ability to produce a wide variety of enzymes. This latter feature of the microorganism has been commercially exploited for over a decade. *B. subtilis* has been used for industrial production of proteases, amylases, antibiotics, and specialty chemicals. U.S. EPA’s *Bacillus subtilis* Final Risk Assessment (February 1997). In addition to use in producing industrial substances, *B. subtilis* has been used for the production of food-grade enzymes, as shown in **Table 2**. Since *B. subtilis* has been used and modified to produce food-grade products and still been considered safe, genes extracted from *B. subtilis* may also be considered safe.

*Escherichia coli* is a normal inhabitant of the gastro-intestinal tract where it produces vitamin K for the host.<sup>8</sup> *E. coli* also has a history of use in producing food-grade products, antibiotics, and hormones, such as human insulin.<sup>9</sup> Since *E. coli* is found as a normal constituent

<sup>8</sup> Bentley R and Meganathan R, “Biosynthesis of vitamin K (menaquinone) in bacteria,” *Microbiological Review* 46: 241–80 (1982).

<sup>9</sup> See EPA’s *Escherichia coli* Final Risk Assessment at [http://epa.gov/biotech\\_rule/pubs/pdf/fra004.pdf](http://epa.gov/biotech_rule/pubs/pdf/fra004.pdf) (last accessed June 8, 2011).

of the gastro-intestinal tract, has been used and modified to produce food-grade and drug products and still been considered safe, genes extracted from *E. coli* may also be considered safe.

*Lactococcus lactis*<sup>10</sup> is used extensively in the production of cheeses, buttermilk and vaccines.<sup>11</sup> Since this organism is safe for use in producing food, genes extracted from *L. lactis* may also be concluded to be safe.

#### F. ALLERGENICITY

Regarding *S. cerevisiae* and allergenicity, a study by Baldo and Baker<sup>12</sup> examined the results of skin prick tests and radioallergosorbent tests (RASTs) and found positive reactions to protein extracts from *S. cerevisiae* and purified enolase from *S. cerevisiae* in people with inhalant allergies to airborne fungi. The study emphasized that although the results demonstrate a high incidence of positive skin tests and RAST reactions in those subjects, it does not mean that if the subjects were exposed to the proteins, an allergic response would occur. The tests merely demonstrate that the subjects have antibodies against the proteins, but presence of an antibody does not equate to an allergic response. No further studies were identified that indicates the potential for *S. cerevisiae* to cause an allergic response, nor were any studies located examining the sensitivity of allergic responses to *S. cerevisiae*.

To address the potential allergenicity of the inserted genes, a 6 or 8 sliding amino acid window, based on the complete protein sequence, was used to compare the amino acid sequence

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<sup>10</sup> Wisconsin has recently named *L. lactis* as its state microbe because of its use in cheese making. Davey, Monica, "And Now, a State Microbe," New York Times (April 15, 2010) found at <http://www.nytimes.com/2010/04/16/us/16microbe.html> (last accessed June 8, 2011).

<sup>11</sup> See Todars' Online Textbook of Bacteriology for *L. lactis* use in various cheeses and buttermilk production at [http://www.textbookofbacteriology.net/featured\\_microbe.html](http://www.textbookofbacteriology.net/featured_microbe.html) (last accessed June 8, 2011).

<sup>12</sup> Baldo, B.A. and Baker, R.S., "Inhalant Allergies to Fungi: Reactions to Bakers' Yeast (*Saccharomyces cerevisiae*) and Identification of Bakers' Yeast Enolase as an Important Allergen," International Archives Allergy Applied Immunology 86: 201-208 (1988).

of the inserted protein against public databases of known allergens. AllergenOnline, a website run by the University of Nebraska-Lincoln, provides access to a peer reviewed allergen list and a searchable database of allergen sequences to identify potential allergenic cross-reactivity and uses an 8 amino acid window. AllerMatch is a website that allows a comparison of a protein sequence to a database of allergenic proteins based on the bioinformatics approaches recommended by the Codex Alimentarius Commission and FAO/WHO Expert Consultation on Foods Derived from Biotechnology and uses a 6 amino acid window. AllerMatch is maintained by RIKILT - Institute of Food Safety and Plant Research International, which are both part of Wageningen University and Research Center in Wageningen, The Netherlands. Based on the search results, which are provided in **Appendix 7**, no allergenic response based on the inserted proteins are expected.

#### G. ANTIBIOTIC RESISTANCE

The Submitter represents that although *phleo*<sup>13</sup> and *hph*<sup>14</sup> antibiotic resistance genes were used during the creation of the modified *S. cerevisiae*, no antibiotic resistance genes remain in the genome of the notified *S. cerevisiae* strain. Loss of gene expression is demonstrated by phenotype analysis on phleomycin and hygromycin containing plates, shown in Figures 10 and 11 of **Appendix 8**. As shown in the figures, the cells are sensitive to the antibiotics, thereby demonstrating loss of *phleo* and *hph* resistance.

The *URA3* gene was also used as a selection marker. *URA3* is the gene involved in uracil synthesis and expression will allow cells that are defective in the ability to synthesize uracil to survive in a uracil free environment. In order to be able to re-use the *URA3* gene for selection, the gene was flanked by *loxP* sites. *Cre* recombinase was then used to “looped-out” the *loxP* site leaving one *loxP* site behind. The final strain does not contain the *URA3* gene either, as demonstrated by phenotype analysis on plates lacking uracil in Figure 12 of **Appendix 8**. As

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<sup>13</sup> *Phleo* confers resistance to phleomycin.

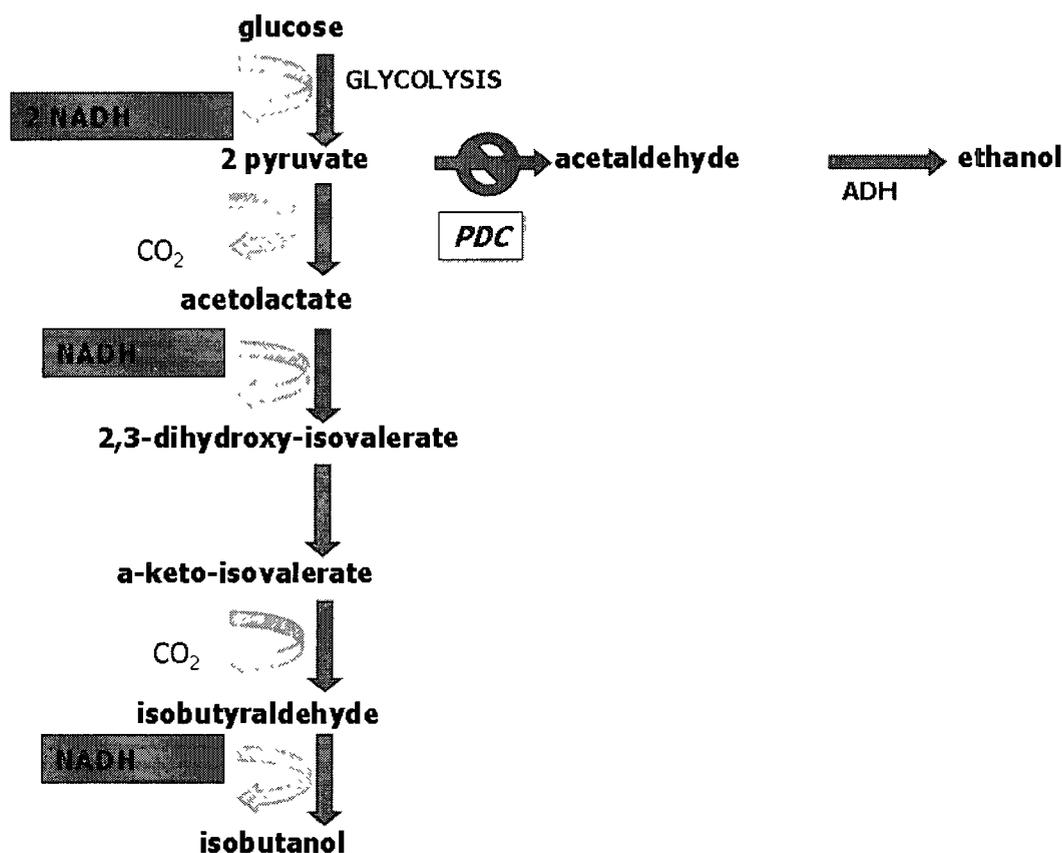
<sup>14</sup> *Hph* confers resistance to hygromycin.

shown in the figure, the cells are unable to grow on plates lacking uracil, thereby demonstrating loss of *URA3*.

#### H. ANTICIPATED METABOLIC PATHWAY

The Submitter would like to note that the insertion of the metabolic pathway will result in the presence of residual isobutanol in the DG, but since this is a separate substance, it will be addressed in a separate notification. This notification only applies to the inactivated modified *S. cerevisiae*.

**Figure 3.** The isobutanol biosynthetic pathway.



The purpose of the inserted traits are: (1) for inactivation of the ethanol production pathway; and (2) replacement of the ethanol production pathway with the isobutanol production pathway as shown in **Figure 3**. All insertions were integrated directly into specific gene loci

within the chromosomal DNA as confirmed by PCR analysis reviewed by Keller and Heckman LLP scientific personnel qualified as experts in the field.

The intermediates in the inserted metabolic pathway, used to achieve production of isobutanol from glucose, also occur in wild type *S. cerevisiae* fermentation (**Attachment 9**). For example, in wild type *S. cerevisiae*, isobutyraldehyde is rapidly converted to isobutyrate.<sup>15</sup>

**Table 3** provides citations documenting the occurrence of the expected individual metabolic intermediates from the isobutanol pathway in the wild type yeast.

**Table 3. Isobutanol intermediates in wild-type yeast**

Metabolic Intermediate	Reference Documenting Occurrence
Acetolactate	Falco, S.C., et al. (1985). Nucleotide sequence of the yeast ILV2 gene which encodes acetolactate synthase. <i>Nucleic Acids Res.</i> Vol. 13(11):4011-27.
2,3-Dihydroxyisovalerate	Ryan, E.D. and Kohlhaw, G.B. (1974). Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast. <i>J. Bacteriol.</i> Vol. 120(2):631-7.
alpha-Ketoisovalerate	Dickinson, J.R., et al. (2000). An investigation of the metabolism of isoleucine to active Amyl alcohol in <i>S. cerevisiae</i> . <i>J. Biol. Chem.</i> Vol. 275(15): 10937-42.
Isobutyraldehyde→Isobutyrate	Lucie A. Hazelwood, L.A., et al. (2008). Minireview: The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on <i>Saccharomyces cerevisiae</i> Metabolism. <i>Appl. Environ. Microbiol.</i> , Vol. 74(8): 2259–2266.
Isobutanol	Dickinson, J.R., et al. (1998). An Investigation of the Metabolism of Valine to Isobutyl Alcohol in <i>Saccharomyces cerevisiae</i> . <i>J. Biol. Chem.</i> 273(40), 25751–25756.

These published scientific references permit the Submitter to conclude that all of the anticipated metabolic products of the isobutanol pathway have been documented to occur in a variety of fermentation food products employing *S. cerevisiae* and, therefore, support the substantial equivalence of the modified organism and the wild type organism, and their equivalent GRAS status. These metabolic intermediates are not expected to be produced at

<sup>15</sup> Lucie A. Hazelwood, L.A., et al. (2008). Minireview: The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on *Saccharomyces cerevisiae* Metabolism. *Appl. Environ. Microbiol.*, Vol. 74(8): 2259–2266.

levels higher than previously reported in the literature because the efficiency of the pathway has been designed to maximize the output of isobutanol and thus the flow of carbon through the pathway will be directed to the end product, rather than to the production of these metabolic intermediates.

The metabolic efficiency of the modified *S. cerevisiae* to metabolize glucose into isobutanol is demonstrated in **Table 4**. **Table 4** data shows a carbon loop that identifies the metabolites and the amounts being produced from glucose metabolism. As shown in the GEVO6215 column, the bulk of the glucose generates isobutanol (49%) and carbon dioxide (34%). Similar results were obtained for GEVO06143. GEVO6143 and GEVO6215 are two different isobutanol-producing cell isolates that are related to the notified strain.

**Table 4: Carbon yields for the production phase of the HR3 fermentation. Shown are only compounds with a carbon rate of at least 0.01 C-mmol/(L\*h).**

Product	GEVO6143		GEVO6215	
	$\frac{\text{C-mmol}_{\text{Product}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}}{\text{C-mmol}_{\text{Glucose}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}}$			
Isobutanol	0.42	±0.01	0.49	±0.004
CO <sub>2</sub>	0.38	±0.03	0.34	±0.02
Isobutyrate	0.13	±0.004	0.07	±0.001
EtOH	0.03	±0.003	0.03	±0.001
CDW	0.00	±0.01	0.02	±0.000
Meso-2,3 butanediol	0.03	±0.004	0.02	±0.003
Acetoin	-0.02	±0.003	-0.01	±0.004
Diacetyl	-0.01	±0.001	-0.01	±0.001
Acetate	0.01	±0.00	0.01	±0.000
Total	1.0	±0.03	0.99	±0.02

#### I. INFORMATION INCONSISTENT WITH GRAS DETERMINATION

There are reports that *S. cerevisiae* is an opportunistic pathogen. A 2006 chapter by McCusker provides a list of *S. cerevisiae* infections described in the literature. The list includes infections in patients with AIDS; it does not identify which of the other patients were otherwise immuno-compromised. A 2005 report by Muñoz *et al.* (2009) described three (3) ICU patients

that had *S. cerevisiae* fungemia at Hospital General Universitario. As part of the report, the authors searched MEDLINE for reports of *S. cerevisiae* fungemia since 1966. Their search returned fifty seven (57) additional reported cases. Since *S. cerevisiae* is commonly used in the biotechnology industry, Murphy and Kavanagh (1999) examined the potential pathogenicity of *S. cerevisiae*. They concluded that *S. cerevisiae* can be regarded as an opportunistic pathogen for the immuno-compromised, but one of low virulence. Copies of these papers are provided in **Appendix 10.**

As the U.S. Environmental Protection Agency (EPA) recognized in its “Final Risk Assessment of *Saccharomyces cerevisiae*” (February 1997) (p. 9), “[m]any scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen.” The agency concluded that *S. cerevisiae* has an extensive history in food processing and neither it nor other closely related species “has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment” (p.2). With respect to human exposure, EPA concluded on p. 3 of the Final Risk Assessment that:

*There are individuals who may ingest large quantities of S. cerevisiae every day, for example, people who take the yeast as part of a “health food” regimen. Therefore, studies were conducted to ascertain whether the ingestion of large numbers of these yeasts might result in either colonization, or colonization and secondary spread to other organs of the body. It was found that the installation of very large numbers of S. cerevisiae into the colons of animals would result in both colonization and passage of the yeasts to draining lymph nodes. It required up to  $10^{10}$  S. cerevisiae in a single oral treatment to rats to achieve a detectable passage from the intestine to the lymph nodes (Wolochow et al., 1961). The concentrations of S. cerevisiae required were well beyond those that would be encountered through normal human daily exposure.*

EPA further concluded that: “*Saccharomyces*, as a genus, present low risk to human health or the environment.” The habitat of *S. cerevisiae* is diverse such that it is geographically distributed throughout the world. EPA described the geographic distribution of *S. cerevisiae* as ubiquitous,

as has Environment Canada (EC). Liti *et al.* (2006) report on the reproductive isolation of *S. cerevisiae*. Copies of the EPA, EC and Liti reports are provided in **Appendix 11**. Liti *et al.* were able to isolate *S. cerevisiae* from each continent. In terms of source, “*S. cerevisiae* is a normal inhabitant of soils and is widespread in nature.”<sup>16</sup> *S. cerevisiae* is known to be “ubiquitous in nature, being present in fruits and vegetables.”<sup>17</sup> Wild strains have been isolated from mushroom fruiting bodies as well as oak tree-associated soils and fluxes.<sup>18</sup>

In addition, Environment Canada concluded in its *Risk Assessment Summary Conducted Pursuant to the New Substances Notification Regulations (Organisms) (NSNR[o]) of the Canadian Environmental Protection Act, 1999, EAU-288: Saccharomyces cerevisiae strain ECMo01 (August 23, 2006)* that “despite its ubiquitous nature and wide use in the food and wine industries, reports of *S. cerevisiae* pathogenicity to insects, birds, fish, animals, and plants in the available scientific literature are exceedingly rare.” The Environment Canada risk assessment did note one reported case associating *S. cerevisiae* with chronic diarrhea in a dog.<sup>19</sup>

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<sup>16</sup> *Id.* at p. 4.

<sup>17</sup> “Final Risk Assessment of *Saccharomyces Cerevisiae*,” U.S. Environmental Protection Agency (February 1997) [last updated Sept. 24, 2007].

<sup>18</sup> Capriotti A (1954) Yeasts in some Netherlands soils. *Antonie van Leeuwenhoek* 21: 145–156; Capriotti A (1967) Yeasts from U.S.A. soils. *Archiv für Mikrobiologie* 57: 406–413; Naumov GI, Naumova ES, Korhola M (1992) Genetic identification of natural *Saccharomyces sensu stricto* yeasts from Finland, Holland and Slovakia. *Antonie van Leeuwenhoek* 61: 237–243; Sniegowski PD, Dombrowski PG, Fingerman E (2002) *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. *FEMS Yeast Research* 1: 299–306; Naumov GI, Naumova ES, Sniegowski PD (1998) *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* are associated with exudates of North American oaks. *Canadian Journal of Microbiology* 44: 1045–1050; Goddard MR, Burt A (1999) Recurrent invasion and extinction of a selfish gene. *Proceedings of the National Academy of Sciences* 96: 13880–13885.

<sup>19</sup> Milner R.J. *et al.*, “Chronic episodic diarrhoea associated with apparent intestinal colonization by the yeasts *Saccharomyces cerevisiae* and *Candida famata* in a German shepherd dog,” *Journal of South African Veterinary Association* 68:147-9 (1997).

The characterization of *S. cerevisiae* as an opportunistic pathogen applies to living *S. cerevisiae*. As discussed above, the modified *S. cerevisiae* present in the DG will be inactivated. We did not locate any published reports of adverse effects related to inactivated *S. cerevisiae* consumption or exposure. This allows us to conclude that these data on the unmodified *S. cerevisiae* is GRAS, and since the modifications to the *S. cerevisiae* does not change the characteristics, it is likewise GRAS.

#### IV. DETAILED SUMMARY OF THE BASIS FOR THE DETERMINATION FOR SUBMITTER'S GRAS DETERMINATION

##### A. TARGET ANIMAL SAFETY

###### 1. *S. cerevisiae*

*S. cerevisiae* is a common food component whose long history of safe use for many food products intended for both human and animal consumption is well-documented in the public literature. The Associate of American Feed Control Officials (AAFCO) list includes active dry yeast, brewers dried yeast, grain distillers dried yeast, brewers liquid yeast, yeast extract, and hydrolyzed yeast for use as animal feed ingredients. Yeast is a common and important nutritional component of DG for vitamin and protein content. According to AAFCO, "grain distillers yeast" must contain no less than 40% crude protein (**Appendix 12**).

The yeast species *S. cerevisiae* is also known as baker's yeast and brewer's yeast through its use in the baking, brewing, and winemaking industry. Its genome has entirely been sequenced which has confirmed that this yeast is free of known pathogenicity traits.<sup>20</sup> No published studies were identified to indicate that the strain contains known pathogenic genes based on the use of the search phrase "*Saccharomyces cerevisiae*" and pathogen\* in public scientific databases, such as PubMed and Toxnet, as of April 27, 2011. The following organizations have evaluated *S. cerevisiae* and concluded it is safe and well-characterized:

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<sup>20</sup> *Saccharomyces* Genome Database. <http://www.yeastgenome.org>.

- The Scientific Committee for Human Food of the European Community in its 27th report indicates that this yeast has a safe history of use in food and belongs to a species which is known not to produce toxins.<sup>21</sup>
- The U.S. EPA has included *Saccharomyces cerevisiae* as a recipient microorganism for exemptions from EPA review and expedited EPA review (40 C.F.R. § 725.420). This decision was based on the fact that *Saccharomyces cerevisiae* is found to have little potential for adverse effects and that the introduction of genetic material will not increase the potential for adverse effects (provided that the genetic material is limited in size, well characterized, free of certain sequences and poorly mobilizable).<sup>22</sup>

The American Type Culture Collection (ATCC) has classified the recipient strain *S. cerevisiae* as a Biosafety Level (BL) 1 organism based upon the fact that the organism is not known to cause disease in healthy humans.

## 2. Consumption in the Animal Gut of Heterologous DNA and Proteins

As consumption of DNA (and RNA) by target animals from modified yeast are broken down into nucleic acids by digestive enzymes, it was concluded that “DNA from GMOs is equivalent to DNA from existing food organisms that has always been consumed with human diets” and that “any risks associated with the consumption of DNA will remain, irrespective of its origin, because the body handles all DNA in the same way” (**Appendix 13**). The breakdown of DNA into individual nucleic acids decreases the potential of any transfer of genes into microorganisms into the gut flora of the target animals. In addition, the final production strain

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<sup>21</sup> SCF (1996). Opinion on Invertase from *Saccharomyces cerevisiae*. Expressed on 23 September 1994. Reports of the Scientific Committee for Food (SCF); 35th Series. European Commission.

<sup>22</sup> EPA (1997). Final Risk Assessment of *Saccharomyces Cerevisiae*. U.S. Environmental Protection Agency. Updated Sept. 24, 2007. Also see: *Saccharomyces cerevisiae* TSCA Section 5(h)(4) Exemption: Final Decision Document. [http://epa.gov/biotech\\_rule/pubs/fra/fd002.htm](http://epa.gov/biotech_rule/pubs/fra/fd002.htm).

has no antibiotic resistance genes as discussed under the section “Safety Assessment of the Modification.”

Although excess dietary consumption of RNA and to lesser extent DNA have been implicated in the disease gout, genetic modification of food will not increase the overall oral consumption of DNA (**Appendix 13**).

None of the donor organisms or protein products resulting from expression of the inserted exogenous genes are associated with known toxicity issues. Furthermore, we anticipate that the inserted proteins will be degraded and denatured (non-functional) after exposure to high distillation and DG processing temperature and broken down into amino acids, as described in **Appendix 4** during target animal consumption.

Therefore consumption of the modified DNA and proteins expressed in the modified yeast are safe for target animal consumption and no different than from consumption of unmodified yeast.

### **3. Target Animal Exposure Calculations for Modified *S. cerevisiae***

The inactivated, modified *S. cerevisiae* is to be a replacement for the inactivated, unmodified *S. cerevisiae* currently found in DG. The Submitter’s safety analysis is based upon the current DG intake levels reported in the scientific literature and the amount of *S. cerevisiae* in the DG. The DG containing the inactivated modified *S. cerevisiae* will be fed as a portion of daily feed to target animals such as beef cattle, dairy cows, sheep, swine, egg-laying chickens, and broiler chickens. In addition, dogs, horses, rabbits, cats, and guinea pigs, are addressed as well.

Yeasts are a standard component of DG and recognized as an important nutritional component (**Appendix 4**). Typically, inactivated yeast is thought to comprise about 5% of DG on a dry weight basis. A recent reference conservatively estimates as much as 20% of the

condensed solubles<sup>23</sup> can be comprised of yeast on a dry weight basis. Although the 20% dry weight composition relates to the amount of yeast in condensed solubles only, the Submitter used it as an overestimation of the inactivated modified *S. cerevisiae* component of DG as a worst-case scenario for calculating animal safety and exposure.

Historically, DG have comprised up to 30% of the diet for beef cattle, dairy cows, sheep, and swine, 15% of the diet for broiler chickens, and 25% of the for domestic animals, all on a dry weight basis.<sup>24</sup> More recently, due to market considerations related to corn and grain prices, it is thought that these historical levels could be higher today. A review of the current scientific literature and consultation with AAFCO and the Distillers Grains Technology Council have identified university studies that fed higher distillers grains levels to some target animal species:

- Beef Cattle: 60% based upon an article on the Iowa Beef Center website,<sup>25</sup> which provides a theoretical inclusion rate of 60% in the diet of beef cattle.<sup>26</sup>
- Dairy Cattle: 30% based upon a publication from South Dakota State University entitled “Distillers Grains for Dairy Cattle.”<sup>27</sup> The article recommends a diet consisting of no more than 20% DDGS, but the inclusion rate may go up to 30% if the forages are mostly corn silage.

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<sup>23</sup> Liu, K., “Chemical Composition of Distillers Grains, a Review”, *Journal of Agricultural and Food Chemistry* 59:1508-1526 (2011).

<sup>24</sup> SAX’S Dangerous Properties of Industrial Materials. Ninth Edition (1996). Table 2. Van Nostrand Reinhold Company. New York.

<sup>25</sup> [www.iowabeefcenter.org](http://www.iowabeefcenter.org). Last accessed June 6, 2011.

<sup>26</sup> “How Much Distillers Grains Can I Include In My Feedlot Diet?”, IBC 46 January 2011 found at <http://www.iowabeefcenter.org/sulfur/IBC46.pdf> (last accessed June 6, 2011).

<sup>27</sup> Article can be found at [http://pubstorage.sdstate.edu/AgBio\\_Publications/articles/ExEx4022.pdf](http://pubstorage.sdstate.edu/AgBio_Publications/articles/ExEx4022.pdf) (last accessed June 6, 2011).

- Broilers: 20%<sup>28</sup>
- Layers: 15%<sup>29</sup>
- Swine: 45%<sup>30</sup>
- Lamb: 60%<sup>31</sup>

Again, the DG feed levels above are thought to be higher than the inclusion rates typical in target animal diets.

Initially, the feeding data for animals which the Distillers Grain Technology Council has indicated that DG can be used in daily feed, which are presented in **Table 5** below were used, but the levels for the target animals discussed above were changed to mimic the levels of DG found in the current literature.

Because we did not locate published scientific data on DG levels in cat and guinea pig feed, we based our estimate of cat and guinea pig feed on the highest percentage for domestic animal feed, which is 25% of DG based on dog DG feeding studies.<sup>32</sup>

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<sup>28</sup> Bregendahl, K., "Use of Distillers Co-Products in Diets Fed to Poultry", Chapter 5, pages 99-132 in Using Distillers Grains in the U.S. and International Livestock and Poultry Industries, edited by Bruce A. Babcock, Dermot J. Hayes, John D. Lawrence, published by the Midwest Agribusiness Trade Research Center at the Center for Agricultural and Rural Development, Iowa State University (2008).

<sup>29</sup> "How Much DDGS Will Benefit Layers?" found at [http://www.wattagnet.com/How\\_much\\_DDGS\\_will\\_benefit\\_layers\\_.html](http://www.wattagnet.com/How_much_DDGS_will_benefit_layers_.html) (last accessed June 6, 2011).

<sup>30</sup> Cromwell, G.L., "Corn Distillers Dried Grains with Solubles in Diets for Growing-Finishing Pigs: A Cooperative Study," *Journal of Animal Science*, published online March 31, 2011 and available at <http://jas.fass.org/content/early/2011/03/31/jas.2010-3704> (last accessed June 4, 2011).

<sup>31</sup> Schauer, C.S. *et al.*, "Feeding of DDGS in Lamb Rations: Feeding Dried Distillers Grains with Solubles as 60 Percent of Lamb Finishing Rations Results in Acceptable Performance and Carcass Quality," *Sheep & Goat Research Journal* 23:15-19 (2008).

Weights and intakes of feed are nominal, meaning that they are representative of populations of animals generally, and may not be specific to particular categories of food animals raised under specific conditions.<sup>33</sup> The quantity of food consumed per day per animal may not be representative of food intakes for a specific period of time during growth, but rather reflect an average that approximates intakes over an expected lifetime.

**Table 5: Feeding data for DG in target animals\***

Animal	Weight*	Feed Consumed*	DG (dry weight basis) Consumed per Day		
			(%)	(g/day)	(g/kg bw)/day
<b>Beef Cattle</b>	500	10,000	60%	6,000	12.0
<b>Dairy Cattle</b>	500	10,000	30%	3,000	6.0
<b>Chicken (broiler)</b>	2 <sup>34</sup>	350	20%	70	35.0
<b>Chicken (layer)</b>	2	350	15%	53	26.3
<b>Sheep</b>	60	2,400	60%	1,440	24.0
<b>Swine</b>	60	2,400	45%	1,080	18.0
<b>Adult Dog</b>	10	250	25%	62.5	6.3
<b>Horses</b>	500	10,000	20%	2,000	4.0
<b>Rabbits</b>	2	60	20%	12	6.0
<b>Adult Cat</b>	2	100	25%	25	12.5
<b>Guinea Pig</b>	0.5	30	25%	7.5	15.0

<sup>32</sup> Shurson, J., "Effect of Feeding DDGS to Companion Animals: A Literature Review," Dept. of Animal Science, University of Minnesota, St. Paul located at <http://www.ddgs.umn.edu/articles-companion/2006-Shurson-%20Effect%20of%20feeding%20DDGS%20to%20horses%20and%20companion%20animals.pdf> (last accessed June 9, 2011); Allen, S.E. *et al.*, "Evaluation of byproduct feedstuffs as dietary ingredients for dogs," *Journal of Animal Science* 53:1538-1544 (1981) located at <http://www.ddgs.umn.edu/articles-companion/1981-Allen-%20Evaluation%20of%20byproduct%20feedstuffs-.pdf> (last accessed June 9, 2011).

<sup>33</sup> SAX'S Dangerous Properties of Industrial Materials. Ninth Edition (1996). Table 2. Van Nostrand Reinhold Company. New York.

<sup>34</sup> Poultry Fact Sheet No. 20. Cooperative Extension. University of California, Riverside. June 1995. Chicken Meat Production in California. Other information found on the internet is consistent with the current average weight of broiler chickens of approximately 5 pounds (2 kg).

\* Based on: SAX'S Dangerous Properties of Industrial Materials, 1996.

### Calculations for Table 5:

#### 1) Examples of calculations for g/day of DG for the following animals

Cattle (beef/dairy)

$$(10,000\text{g/day}) \times (0.60\text{g-distillers grains/g-food}) = 6,000\text{ g/day}$$

Chicken (broilers)

$$(350\text{ g/day}) \times (0.20\text{ g-distillers grains/g-food}) = 70\text{ g/day}$$

#### 2) Examples of calculations for (g/kg bw)/day of DG for the following animals:

cattle (beef/dairy):

$$(10,000\text{ g-food}/500\text{ kg bw/day}) \times (0.60\text{ g-distillers grains/g-food}) = 12\text{ g-distillers grains/kg bw/day}$$

chickens (broilers) :

$$(140\text{ g-food}/0.8\text{ kg bw/day}) \times (0.205\text{ g-distillers grains/g-food}) = 35\text{ g-distillers grains/kg bw/day}$$

The dietary intake of the DG by other animals is similarly calculated.

In order to determine the amount of inactivated modified *S. cerevisiae* consumed by the target animals, we have taken the data from **Table 5** and used it to calculate modified *S. cerevisiae* exposure. The maximum distillers grains consumed by beef cattle, on a dry weight basis, is 6 g/kg bw/day. With a maximum *S. cerevisiae* residual level of 20% in distillers grains on a dry weight basis, a maximum dietary intake for beef cattle in **Table 6**, normalized to body weight, is calculated as follows:

$$12\text{ g-distillers grain/kg bw/day}(\text{Table 4}) \times (0.2) = 2.4\text{ g/kg bw/d}$$

The dietary intake for beef cattle in **Table 6**, normalized to body weight, is calculated as follows:

$$6,000 \text{ g/day (Table 4)} \times (0.20) = 1,200 \text{ g/day}$$

The dietary intake of inactivated modified yeast by other animals in **Table 6** are similarly calculated.

**TABLE 6: Estimated daily intake for modified yeast in target animals normalized to body weight**

Animal	Yeast g/kg bw/day	Yeast g/day
Beef Cattle	2.4	1200
Dairy Cow	1.2	600
Chicken(broiler)	7.0	14
Chicken (layer)	5.3	10.6
Sheep	4.8	288
Swine	3.6	216
Adult Dog	1.25	12.5
Horse	0.8	400
Rabbit	1.2	2.4
Adult Cat	2.5	5.0
Guinea pig	3.0	1.5

As discussed above, dietary exposure to DG containing the inactivated modified *S. cerevisiae* were calculated using the DG containing inactivated unmodified *S. cerevisiae* inclusion levels considered GRAS and the inactivated unmodified *S. cerevisiae* component level in DG considered GRAS. In addition, nutritional analysis has shown DG containing the inactivated modified *S. cerevisiae* is identical to DG containing inactivated unmodified *S. cerevisiae* (**Appendix 6**). Based on all the information, the Submitter concludes that the dietary exposure to the inactivated modified *S. cerevisiae* is GRAS.

## V. HUMAN CONSUMPTION AND SAFETY

No EDI, carcinogenic, teratogenic, allergenic potential is required for human consumption of animal products and tissue from animals that have consumed the inactivated modified *S. cerevisiae*. Since the inactivated modified *S. cerevisiae* will be metabolized during animal digestion into endogenous essential compounds (such as lipids, proteins, carbohydrates, nucleic acids) just as any other ingested nutritional substance, the essential compounds derived from the inactivated modified *S. cerevisiae* will be indistinguishable from the essential compounds derived from other sources.

## VI. CONCLUSION

The Submitter has determined that the inactivated, modified *S. cerevisiae* is equivalent to inactivated, unmodified *S. cerevisiae*. The Submitter concludes that publicly available scientific information supports the general recognition of the safety of the gene sources used in the modified strain and the metabolic products produced from all modifications made to the organism. The public literature on *S. cerevisiae* establishes that there is a consensus among experts qualified by scientific training and experience to evaluate the safety of substances added to food that there is reasonable certainty that the inactivated modified *S. cerevisiae* is not harmful under the intended condition of use.

## VII. ENVIRONMENTAL ASSESSMENT

Neither an Environmental Assessment (EA) nor an Environmental Impact Statement (EIS) are required for this submission. *Proposed* 21 CFR § 570.36, 62 Fed. Reg. 18938 (Apr. 17, 1997) and the FDA's Notice of Pilot Program; Substances Generally Recognized as Safe Added to Food for Animals, 75 Fed. Reg. 31806 (June 4, 2010) do not list an EA or EIS as one of the criteria to be addressed for eligibility as GRAS.

More specifically, under 21 C.F.R. § 25.15(a) “[a]ll applications or petitions requesting agency action require the submission of an [Environmental Assessment] EA.” This Notification does not request “agency action” by FDA in this context. As FDA states in April 17, 1997 proposed rule

under the notification procedure a notifier explicitly accepts full responsibility for the GRAS determination by signing a GRAS exemption claim (under proposed § 170.36(c)(1)). In contrast, under the petition process a petitioner requests that FDA attest to a GRAS determination.

62 Fed. Reg. at 18953. The Submitter is accepting full responsibility for the GRAS determination by signing the GRAS exemption claim. FDA has made it clear that it does not take any action based on a Submitter's GRAS conclusion, as supported in the letter FDA provides to submitters if there are no questions. In such a letter, FDA typically will state that "[t]he agency has not, however, made its own determination regarding the GRAS status of..." indicating that FDA has not taken an action that rises to the need for an EA. Furthermore, in the 1997 proposed rule, FDA states that "[a] response that does not advise that the agency has identified a problem with the notice would not be equivalent to an affirmation of GRAS status by the agency."<sup>35</sup> The agency further stated that "although FDA would maintain a readily accessible inventory of notices received and the agency's response to them, this inventory would be neither codified nor referenced in the agency's regulations."<sup>36</sup> Collectively, these statements are admissions which support the conclusion that FDA is not taking any action that requires an EA or an EIS.

This legal conclusion is additionally supported by a review of the available GRAS notification submissions on the GRAS Notice Inventory database administered the Center for Food Safety and Applied Nutrition (CFSAN). Of the current 378 GRAS submissions that are posted on FDA's GRAS Inventory website at <http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing>, none contain either an EA or an EIS. Although we understand that the listings are reviews conducted by CFSAN and not CVM, we believe that there should be consistency throughout the agency and with the requirements of the law.

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<sup>35</sup> 62 Fed. Reg. at 18956.

<sup>36</sup> *Id.*



May 31, 2011

Feed Safety Team  
HFV-222  
Food and Drug Administration  
Center for Veterinary Medicine  
7519 Standish Place  
Rockville, Maryland 20855

Re: Authorization to Act as Agent for Gevo

Dear Sir or Madam:

This is to advise that the law firm of Keller and Heckman LLP, its employees, associates, and agents, specifically including, but not limited to Devon W. Hill, Scott A. Krygier and Martha E. Marrapese are hereby authorized to act as agents on behalf of Gevo with regard to submissions to the U.S. Food and Drug Administration by Gevo.

This letter is our authorization to you to permit said firm to undertake appropriate communications relevant to making submissions or inquiring as to the status of any and all submissions filed or to be filed by or on behalf of Gevo, including examination of all relevant information including confidential business, proprietary, and trade secret information submitted or developed under the Federal Food, Drug, and Cosmetic Act.

Best Regards

A handwritten signature in black ink that reads "Glenn Johnston". The signature is written in a cursive, flowing style.

Glenn Johnston  
Vice President of Regulatory Affairs  
Gevo Inc.

c.c. File

345 Inverness Drive South  
Building C, Suite 310  
Englewood, CO 80112

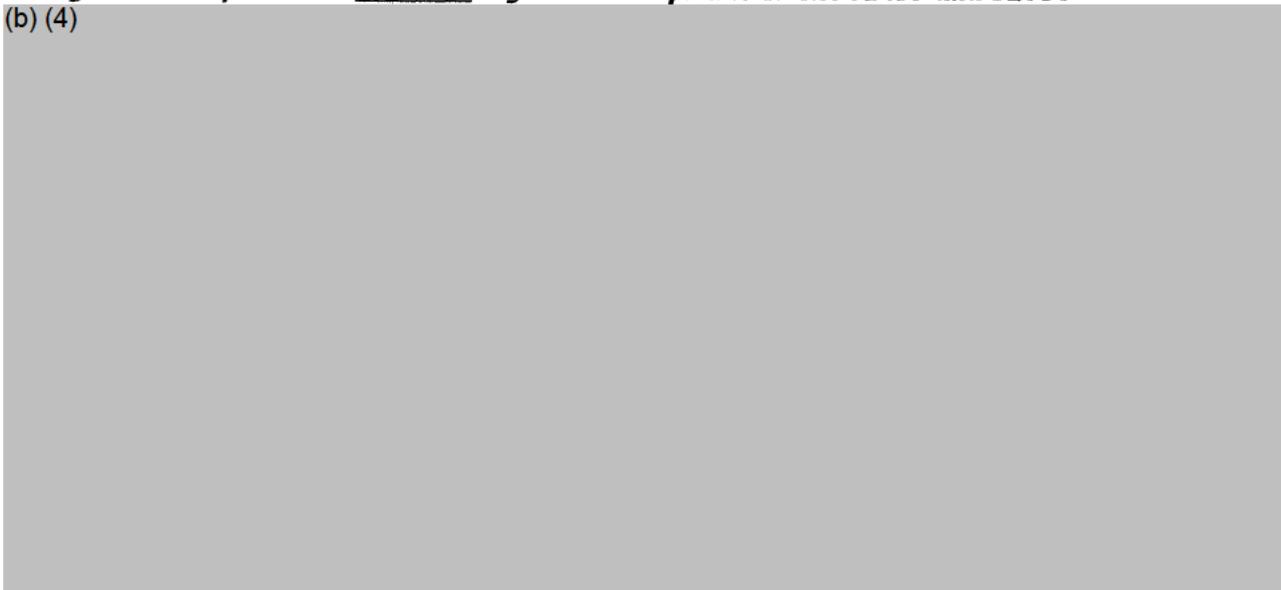
PHONE 303-858-8358  
FAX 303-858-8431

gevo.com



**Figure 4 Comparison of RDN25-1 region DNA sequence of GEVO1437 and S288c**

(b) (4)



(b) (4)





**Figure 5 NCBI Distance Tree Results using GEVO1473 RDN25-1 nucleotide sequence (Error! Reference source not found.) as query.**

(b) (4)



Pages 37-47 (Appendix 3) have been removed in accordance with copyright laws. Please see table of contents in document for list of references of copyrighted information.

Pages 48-66 (Appendix 4) have been removed in accordance with copyright laws. Please see table of contents in document for list of references of copyrighted information.

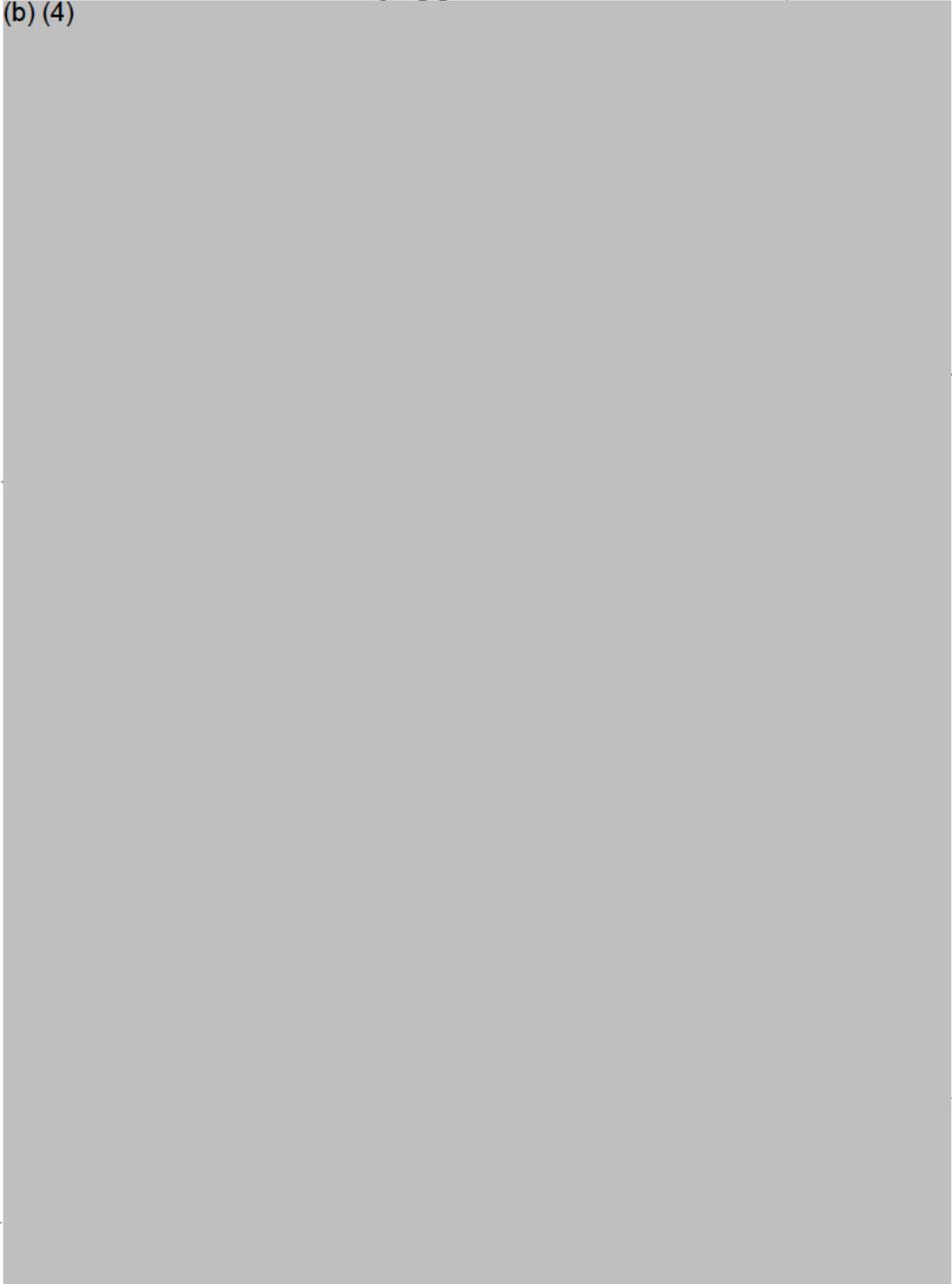


**Appendix C | Heat Sensivity Study of GEVO6397**

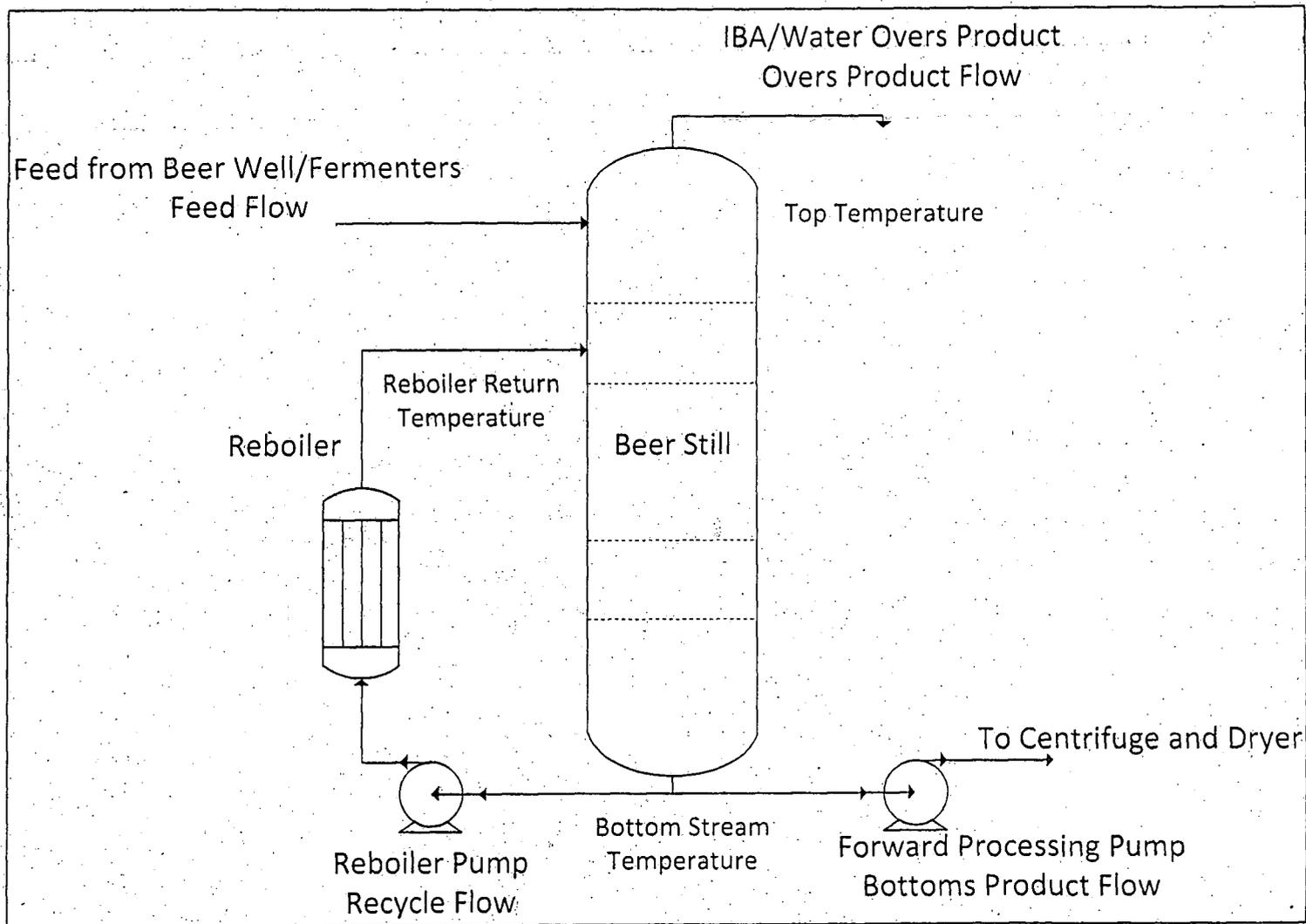
(b) (4)

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(b) (4)



Luverne Beer Still Temperature and Residence Time  
Based on current EtOH production and Aspen modeled iBuOH production.



Process Parameters	Units	EtOH	iBuOH	Assumptions
Feed Flow	GPM	245	452	
Overs Product Flow	GPM	35	118	
Bottoms Product Flow	GPM	210	334	
Reboiler Recycle Flow	GPM	Unknown	1600	
Reboiler Return Temperature	F	Unknown	182	
Top Temperature	F	157	161	
Bottom Stream Temperature	F	196	177	
Reboiler Volume (Process)	Gallons	882	882	
Beer Still Sump Operating Volume	Gallons	805	805	Assumes continued operation at current 35% level.
Residence time in sump and reboiler ((Sump Operating Volume + Reboiler Volume)/ Bottoms Product Flow)	Minutes	8.0	5.1	

(b) (4)

GEVO  
GLENN JOHNSTON  
345 INVERNESS DR S BG C SE 310  
ENGLEWOOD CO 80112

HIGH PROTEIN ANIMAL FEED

FEED NUTRIENT ANALYSIS

Date Sampled	Received	Reported	Lab #
	02/18/11	02/22/11	9697976

Sample ID: (b) (4)

Feedstuff: FINISHED FEEDS

ANALYSIS RESULTS

Component	As Sent	Dry Wt.
Moisture (%)	9.38	//////
Dry Matter (%)	90.62	//////
Crude Protein (%)	33.1	36.5
Crude Fat (%)	8.82	9.73
acid Detergent Fiber (%)	11.6	12.8
Cellulose (%)	11.1	12.2
Total digestible nutrients (%)	72.0	79.4
Net energy-lactation (Mcal/lb)	0.75	0.83
Net energy-maint. (Mcal/lb)	0.78	0.86
Net energy-gain (Mcal/lb)	0.52	0.57
Digestible energy (Mcal/lb)	1.44	1.59
Metabolizable energy (Mcal/lb)	1.28	1.41

COMMENTS

- (b) (4) is certified by the National Forage Testing Association (NFTA) for wet chemistry methods and mineral analysis.
- Analysis for:  
(27326) GEVO  
Phone: (303) 858-8358

(b) (4)

(b) (4)

GEVO  
GLENN JOHNSTON  
345 INVERNESS DR S BG C SE 310  
ENGLEWOOD CO 80112

HIGH PROTEIN ANIMAL FEED

FEED NUTRIENT ANALYSIS

Date Sampled	Received	Reported	Lab #
	02/18/11	02/22/11	9697977

Sample ID: (b) (4)

Feedstuff: FINISHED FEEDS

ANALYSIS RESULTS

Component	As Sent	Dry Wt.
Moisture (%)	9.73	//////
Dry Matter (%)	90.27	//////
Crude Protein (%)	33.5	37.1
Crude Fat (%)	8.66	9.60
Acid Detergent Fiber (%)	9.86	10.9
ash (%)	10.2	11.3
Total digestible nutrients (%)	72.8	80.6
Net energy-lactation (Mcal/lb)	0.76	0.84
Net energy-maint. (Mcal/lb)	0.79	0.87
Net energy-gain (Mcal/lb)	0.52	0.58
Digestible energy (Mcal/lb)	1.45	1.61
Metabolizable energy (Mcal/lb)	1.29	1.43

COMMENTS

- (b) (4) is certified by the National Forage Testing Association (NFTA) for wet chemistry methods and mineral analysis.
- Analysis for:  
(27326) GEVO  
Phone: (303) 858-8358

(b) (4)

(b) (4)

DDGS

(b) (4)

FEED NUTRIENT ANALYSIS

Date Sampled	Received	Reported	Lab #
	12/13/10	12/15/10	9672089

Sample ID: (b) (4)

Feedstuff: DISTILLER DRIED GRAIN

ANALYSIS RESULTS

Component	As Sent	Dry Wt.
Moisture, Distillers Grains (%)	12.59	//////
Dry Matter (%)	87.41	//////
Crude Protein (%)	25.1	28.7
Crude Fat (%)	9.75	11.2
acid Detergent Fiber (%)	13.6	15.6
neutral Detergent Fiber (%)	28.2	32.2
Total digestible nutrients (%)	67.3	77.0
Net energy-lactation (Mcal/lb)	0.70	0.80
Net energy-maint. (Mcal/lb)	0.69	0.79
Net energy-gain (Mcal/lb)	0.47	0.54
Relative Feed Value		222

COMMENTS

1. Relative Feed Value (RFV) is calculated using National Forage Testing Association (NFTA) guidelines.
2. (b) (4) is certified by the National Forage Testing Association (NFTA) for wet chemistry methods and mineral analysis.
3. Moisture determined using 3hr@105 Deg. C Method.
4. Analysis for:  
 (b) (4) (b)  
 (b) (4) )

(b) (4)

<b>MODIFIED DISTILLERS GRAINS</b> with solubles		
Moisture	max	43.00%
Crude Protein	min	15.00%
Crude Fiber	max	12.00%
Crude Fat	min	5.00%
Contents		Bulk
Manufactured By		
(b) (4)		
This product was made in a feed manufacturing facility that does not handle or store products containing animal proteins prohibited in ruminant feed.		

<b>CORN DISTILLERS DRY GRAIN</b> with solubles		
Moisture	max	13.00%
Crude Protein	min	25.00%
Crude Fiber	max	12.00%
Crude Fat	min	8.00%
Calcium	min .01%	max .40%
Phosphorus	min	.35%
Sulfur	max	.85%
Contents		Bulk
Manufactured By		
(b) (4)		
This product was made in a feed manufacturing facility that does not handle or store products containing animal proteins prohibited in ruminant feed.		

Analytical Methods For The Analysis  
Of The Ethanol Industries Co-Products  
September 11, 2006

Presented by:  
John Torpy & Dr. Jerome King

(b) (4)

**Analytical Methods for the Analysis of the Ethanol Industry's Co-Products**  
**PROXIMATES**

***Moisture (Dry Matter)***

**Dried Distillers Grains (DDGS)**

**Modified** AOAC Official Method 935.29 - "Moisture in Malt" Gravimetric Method.  
3 hours in forced air convection oven at 105°C.

**Syrups**

AOAC 44.2.02 - "Moisture in Molasses."  
Four (4) hours under 20-25 psi; vacuum at 70°C

**Wet Distillers Grains**

AOAC 930.15- "Loss on drying (moisture) for feeds"  
Forced air oven overnight at 60 deg c for sample preparation.  
Residual moisture at 135 deg c for two hours.  
Moisture values combined for total moisture.

**Crude Protein**

AOAC 990.03 - Protein (Crude) in Animal Feed.  
Dried distillers grains and wet distillers grains which have been dried and ground.  
Combustion method LECO - TruSpec™

**Syrups**

AOAC 988.05 - Protein (Crude) in Animal Feed and Pet Food  
Kjeldahl method using TiO<sub>2</sub> - CuSO<sub>4</sub> catalyst.

**CRUDE FAT**

**Dried Distillers Grains and Wet Grains that are dried and ground**

AOAC 945.16 - "Oil in Cereal Adjuncts."  
Petroleum Ether extraction Method using a Tecator soxtec 1043 extractor.

**Syrups**

AOAC 954.02 - "Gravimetric Method."  
Acid hydrolysis method manual extraction.

## FIBER

### Crude Fiber, Acid Detergent Fiber, Neutral Detergent Fiber in Distiller Dried Grains (DDGS), Wet Grains & Syrups

All fiber analyses are performed using Ankom Filter bag procedures.  
Wet grains are dried and ground for analysis.

## ASH

### Distiller Dried Grains (DDGS), Wet Distillers Grains & Syrups

AOAC 942.05 – Ash of Animal Feed  
Muffle furnace at 600° for two (2) hours.

## MINERALS

### Distiller Dried Grains (DDGS), Wet Distillers Grains (Dried & Ground), Syrups and Fermentation Samples

Wet digestion using nitric acid mixture in 50 mL tubes in a hot block digester.  
Sulfur, phosphorus, potassium, calcium, magnesium, iron, copper and zinc.  
AOAC 985.01 “Metals and Other Elements.”  
Inductively Couple Plasma Spectroscopic Method.

## TOTAL STARCH

### Distiller Dried Grains (DDGS), Wet Distillers Grains (Dried & Ground), Syrups and Fermentation Samples

AOAC 996.11, AACC 76-11 YSI Application Number 319  
Sample is treated with specific enzyme that hydrolyzes starch to glucose. Glucose measured using YSI 2700 Select Glucose Analyzer. Total Starch results include all hydrolysable carbohydrates, sugars and starches.

## CAROTENES and XANTHOPHYLLS

### Dried Distiller Grains (DDGS), Wet Distiller Grains (Dried and Ground), Syrups

AOAC 970.64 – “Spectrophotometric Method.”  
Carotenes and Xanthophylls are extracted and separated on a column and quantitated by color absorption on a spectrophotometer.

## **Mycotoxin Analysis**

### **Not an AOAC Referenced Method**

Mycotoxins are toxic chemicals produced by molds and are tested at Midwest Laboratories, Inc. using LC-MS (Liquid Chromatography with Mass Selective Detectors). This technology has not been validated by AOAC; however, many government agencies (USDA and FDA), universities, and research scientists use LC-MS to analyze mycotoxins. Mycotoxins comprise six (6) different types of chemicals namely:

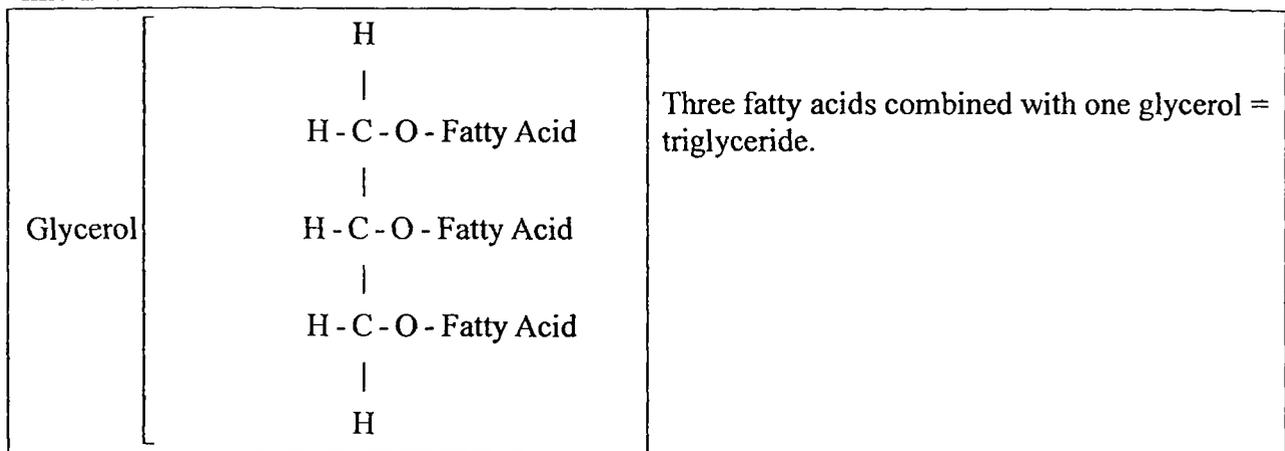
- Aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>)
- Fumonisin (B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>)
- Ochratoxin
- T-2
- Zearalenone
- Deoxynivalenol (DON) or Vomitoxin

The analysis of mycotoxins historically has been carried out using Thin Layer Chromatography (TLC), gas chromatography, or liquid chromatography. Using the current AOAC methods, each mycotoxin must be analyzed using a single method, but use of LC-MS allows analysis of multiple mycotoxins on a single instrument run and the time required for analysis is also quite short; less than ten (10) minutes. These advantages, plus the selectivity of the method makes LC-MS an excellent analytical tool for mycotoxins.

The process of analysis is the initial extraction of the mycotoxins from the sample. A sample amount of twenty-five (25) grams is generally used, but sample amount could vary. To extract the sample, a combination of methanol and water is used with the mycotoxins removed in the methanol/water combination. The extract is "cleaned" up to remove oils or other materials that could interfere with the test. After the sample is "cleaned" up, a volume of extract is passed through an affinity column. The affinity column contains antibodies that are specific for types of mycotoxins, so as the liquid passes through the column, mycotoxins that are present combine with the specific antibody and are "held" as the rest of the liquid passes through. After the liquid has been drawn through, another chemical is added that releases the mycotoxins from their specific antibodies and collected in a vial. The material in the vial is injected into the HPLC (High Pressure Liquid Chromatography) and the column separates the individual mycotoxins. As each mycotoxin comes out of the column, they are analyzed by a Mass Selective Detector (MS). The MS monitors the molecular weight of chemicals, thus the combination of separation by Liquid Chromatography and specific analysis of a chemical by its unique mass; it is possible to quantitate all mycotoxins at very low levels.

**FATTY ACID PROFILE**  
**AOAC 996.06**

Fatty acids are organic chemicals that are associated with all oils (e.g. corn oil) and fats, and along with glycerine made up what we call “oils or fats.” The chemical composition would look like this:



The role of a fatty acid profile is to determine what type and how many fatty acids are in a sample. The AOAC reference for Fatty acid Profiles is AOAC 996.06 “*total, saturated, and unsaturated fatty acids in foods.*”

The initial step of sample preparation involves the removal of the fat from the rest of the sample using either ether extraction, pet ether extraction, acid hydrolysis fat, or acid hydrolysis fat depending on the material. The next step in the process of analysis involves separation of the fatty acid from the glycerol and the reaction of the fatty acids with a chemical called Boron Trifluoride with the formation of chemicals called “fatty acid methyl ester” (FAMES). The FAMES are analyzed by Gas Chromatography with a flame ionization detector (GC/FID).

As the sample passes through the column, the various fatty acids are separated and leave the column at different times. Use of known fatty acids are used as controls to know when the fatty acids come off the column and also helps to determine how much of a certain fatty acid is present.

The fatty acids can also be divided into four major categories: saturated, mono-unsaturated, poly-unsaturated and trans-fatty acids. This method does provide a break down of each class of fatty acid, and also can provide levels of the omega-3, omega-6, and omega-9 fatty acids.

## **Amino Acid Profile**

### **AOAC 988.12, AOAC 988.15 & AOAC 985.28**

The analysis of Amino Acids is based on the traditional method of sample hydrolysis and the analysis of the individual amino acids using HPLC (High Performance Liquid Chromatography) with Post Column Derivatization. The method is based off of AOAC 994.12. To determine the total amino acids, three (3) methods are required, including the 994.12 (acid hydrolysis), but in addition, analyses of tryptophan requires base hydrolysis (AOAC 988.15), and the sulfur-containing amino acids (methionine and cystine) require a pre-oxidation step (Modified AOAC 985.28).

The process of sample preparation requires use of a relatively small amount of sample, generally less than 0.50 grams, thus the sample used for analyses must be well ground and representative of the submitted sample. A small amount of a non-homogenous sample will alter the final results. To hydrolyze the sample, the sample is placed in a container with acid and then the acid heated for twenty-four (24) hours to break down the protein into the constituent amino acids. A second sample is required for analysis of tryptophan, but instead of using an acid to break down (hydrolyze) the protein, a base (alkalai) is used. The preparation of methionine and cystine requires a pre-oxidation step that needs a twenty-four (24) hour process and then the acid hydrolysis step, so the minimum time of sample preparation for methionine and cystine is forty-eight (48) hours.

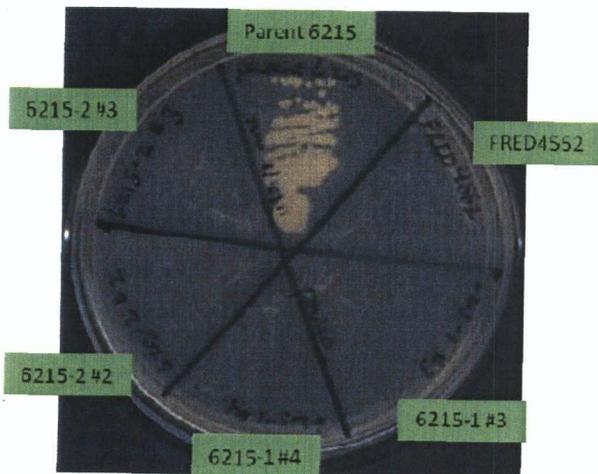
After the samples are hydrolyzed, the extracts are filtered to remove any remaining particulates, and then analyzed by HPLC. The HPLC columns obtain separation of the various individual amino acids. When the amino acids leave the HPLC column, they react with a special chemical (Ninhydrin) that allows the detector to see the amino acids. To quantitate the amount of amino acid present, a known amount of amino acid (a standard) is analyzed at the same time. By calculating the instrument response to the amount found with the sample, it is possible to quantitate the individual amino acids.

In doing Quality Control, it is important to compare the amount of amino acid to the total protein because the amino acid (natural) comprises the protein. If nitrates or non-protein nitrogen is present, the amount of protein (if using a nitrogen analyses) will be higher than the calculated amount from the amino acid profile.

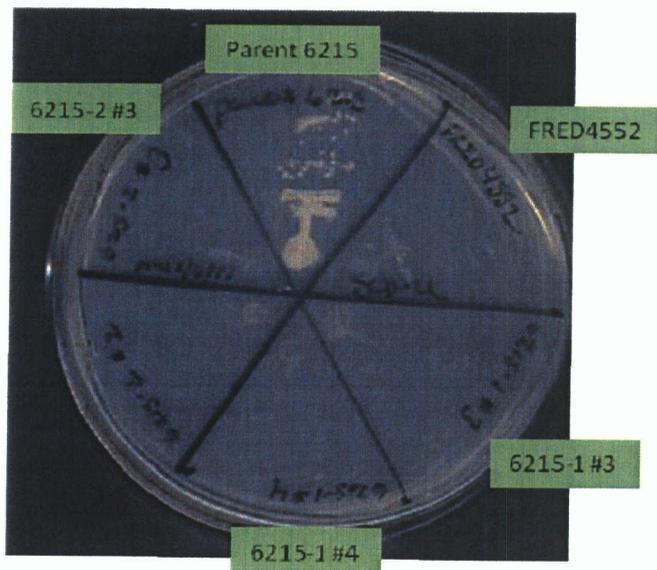
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**Figure 10. Phenotype confirmed by streaking for singles onto 0.1 g/L hygromycin, starting at the top (clockwise): Positive control parent FRED6215, deletion strains FRED6215-1 #3 (GEVO6397), FRED6215-1 #4, FRED6215-2 #2, FRED6215-2 #3 and negative control FRED4552 (lacking antibiotic markers and the *URA3* gene).**



**Figure 11. Phenotype confirmed by streaking for singles onto 40 mg/L phleomycin, starting at the top (clockwise): Positive control parent FRED6215, negative control FRED4552 (lacking antibiotic markers and the *URA3* gene), deletion strains FRED6215-1 #3 (GEVO6397), FRED6215-1 #4, FRED6215-2 #2, and FRED6215-2 #3.**



**Figure 12. Phenotype confirmed by streaking for singles onto SCD-U, starting at the top (clockwise): Positive control parent 6215, negative control FRED4552 (lacking antibiotic markers and the *URA3* gene), deletion strains FRED6215-1 #3 (GEVO6397), FRED6215-1 #4, FRED6215-2 #2, and FRED6215-2 #3.**

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