ORIGINAL SUBMISSION

Mary Ditto, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740
U.S.A.

RECEIVED
FEB - 7 2012
Division of
Biotechnology and
GRAS Notice Review

January 31, 2012,

Re: GRAS Notice for functionally-enhanced baker's yeast that prevent acrylamide formation in foods and beverages

Dear Dr. Ditto,

In accordance with provisions of Sec. 170.30 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination], Functional Technologies Corp. hereby submits a notification that functionally-enhanced baker's yeast that prevent acrylamide formation in foods and beverages are GRAS and therefore are exempt from the premarket approval requirements of the Federal, Food, Drug and Cosmetic Act.

Enclosed are three copies of the GRAS report and one electronic copy.

Sincerely,

(b) (6)

Howard J. Louie

Executive Chairman
Functional Technologies Corp.

218-5511 West Boulevard, Vancouver, BC, V6M 4H3
(PH) 604-648-2200 (FX) 604-648-2201 (web) www.functionaltechcorp.com

Mary Ditto, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740
U.S.A.

January 31, 2012,

Re: GRAS Notice for functionally-enhanced baker's yeast that prevent acrylamide formation in foods and beverages

Dear Dr. Ditto,

In accordance with provisions of Sec. 170.30 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination], Functional Technologies Corp. hereby submits a notification that functionally-enhanced baker's yeast that prevent acrylamide formation in foods and beverages are GRAS and therefore are exempt from the premarket approval requirements of the Federal, Food, Drug and Cosmetic Act.

Enclosed are three copies of the GRAS report and one electronic copy.

Sincerely,

(b) (6)

Howard J. Louie

Executive Chairman
Functional Technologies Corp.

218-5511West Boulevard, Vancouver, BC, V6M 4H3
(PH) 604-648-2200 (FX) 604-648-2201 (web) www.functionaltechcorp.com

GRAS Notification

For the Use of a Functionally-Enhanced Yeast to Prevent Acrylamide in Heated Foods

Prepared by
Functional Technologies Corporation
Vancouver, British Columbia, Canada

_____

Howard Louie
Executive Chairman Chief Business Development Officer

Dated: January 31, 2012

Table of Contents

1. GRAS Exemption Claim 7

1.1 Name and address of the notifier 7

1.2 Common or usual name of the substance that is the subject of the GRAS exemption claim 8

1.3 Applicable conditions of use of the notified substance 8

1.3.1 Purposes for which the substance is used 8

1.3.2 Foods in which the substance is to be used 9

1.3.3 Levels of use of the Functional Technologies Acrylamide-Preventing Yeast 9

1.4 The basis for the GRAS determination 10

1.5 Statement that the data and information that are the basis for the notifiers GRAS determination are available for the Food and Drug Administration review and copying 10

2. Detailed information about the identity of the notified substance 11

2.1 Host microorganism 11

2.1.1 History of use 11

2.1.2 Characteristics 11

2.2 Donor organisms 12

2.2.1 Taxonomy 12

2.2.2 Genetic donor material 12

2.3 The modified microorganism 12

2.3.1 Final gene constructs used in the integration strategy 12

2.3.2 The transformation event 15

2.3.3 Genetic characterization of the modified microorganism 16

2.3.4 Absence of difference between genetic profiles of the transformed and the host strain 17

2.3.5 Method of manufacture of the Functional Technologies Acrylamide-Preventing Yeast. 17

3. Information on any self-limiting levels of use 18

4. Determination that the use of the active dry yeast obtained from the Functional Technologies Acrylamide-Preventing Yeast strain in food and beverage products is exempt from the pre-market approval requirements of the act because such use is GRAS 19

4.1 Safety assessment of the host strain 19

4.2 Safety assessment of the genetic material used to construct the modified organism 20

4.2.1 Foreign genetic material source and product 20

4.2.2 Native genetic material source and product: *ASP3* sequences 20

4.2.3 DNA integration cassettes 20

4.2.4 Transformation of the host strain with the integration cassette ... 20

4.3 Safety assessment of the modified organism 21

4.3.1 Characterization of the transformation event 21

4.3.2 L-Asparaginase II 22

4.3.3 Consequence of the genetic modification on the physiology of the FT Acrylamide-Preventing Yeast strain 22

4.4 Safety assessment of the product derived from the modified organism 23

4.4.1 Changes processing procedures as a consequence of the Functional Technologies Acrylamide-Preventing Yeast fermentation 23

Reduction of aspragine in bread dough 23

5. References 57

Figures

Figure 1: Acrylamide formation in the Maillard Reaction. 26

Figure 2: The catalytic activity of the periplasmic enzyme asparaginase II in yeast. 27

Figure 3: LEU2-ASP3 cassette 28

Figure 4: A schematic of the plasmid ASP3-pUC57. 29

Figure 5: A schematic of the plasmid vector URA3-pMA. 30

Figure 6: URA3-ASP3 cassette 31

Figure 7: TRP1-ASP3 cassette 32

Figure 8: Transformation events of ASP3 cassette into host strain. 33

Figure 9: Confirmation of the loss of the KanMX marker that conveys resistance to the antibiotic G418. 34

Figure 10: Southern blot analysis of the KanMX gene that encodes for Geneticin®G418 resistance. 35

Figure 11: PCR of genetic patterns of the host baker's yeast and Functional Technologies Acrylamide-Preventing Yeast strains. 36

Figure 12: Growth curve of Functional Technologies Acrylamide-Preventing Yeast strain versus the parent strain. 37

Figure 13: Flow diagram describing the manufacture of active dry yeast. 38

Tables

Table 1: PCR primers used for the ASP3 integration cassette construction..... 40

Table 2: Plasmids used for the construction and integration of the integration cassette..... 41

Appendices

Appendix 1. Genomic sequence of the *ASP3* gene (1089 nucleotides) in FASTA format. 43

Appendix 2. Protein sequence of the *ASP3* gene in FASTA format. 44

Appendix 3: Nucleotide sequence of the linear LEU2-KanMX-ASP3 cassette in FASTA format. 45

Appendix 4: Nucleotide sequence of the linear LEU2-ASP3 cassette in FASTA format. 47

Appendix 5: Nucleotide sequence of the linear URA3-KanMX-ASP3 cassette in FASTA format. 49

Appendix 6: Nucleotide sequence of the linear URA3-ASP3 cassette in FASTA format. 51

Appendix 7: Nucleotide sequence of the linear TRP1-KanMX-ASP3 cassette in FASTA format. 53

Appendix 8: Nucleotide sequence of the linear TRP1-ASP3 cassette in FASTA format. 55

GRAS Notice

1. GRAS Exemption Claim

1.1 Name and address of the notifier

Functional Technologies Corporation
Suite 1500 - 885 West Georgia Street
Vancouver, British Columbia, V6C 3E8
Canada

Telephone: (604) 648-2200
Facsimile: (604) 648-2201

Scientific Manager: Dr. John Husnik
jihusnik.functionaltechcorp□gmail.com

Regulatory: Dr. John Husnik
jihusnik.functionaltechcorp□gmail.com

The research and development of an Acrylamide-Preventing yeast strain was completed in the research laboratory of Functional Technologies Corp., Charlottetown, PEI, Canada. The technology is based on the *ASP3* gene that encodes for the enzyme L-asparaginase II which is involved in asparagine catabolism.

1.2 Common or usual name of the substance that is the subject of the GRAS exemption claim

The subject of this notice is a *Saccharomyces cerevisiae* strain obtained from a commercial baker's yeast strain. This commercial strain carries extra copies of the native *S. cerevisiae ASP3* gene. There is no foreign DNA involved in this self-cloned organism. This novel strain is called **Functional Technologies Acrylamide-Preventing Yeast**.

1.3 Applicable conditions of use of the notified substance

1.3.1 Purposes for which the substance is used

In 2002 a group of Swedish researchers attracted world-wide attention when they announced that commonly consumed heat-treated carbohydrate-rich foods contain significant levels of acrylamide (Swedisch National Food Administration, 2002; Tareke *et al*., 2002). Acrylamide is a known neurotoxin, a carcinogen in animals, and considered probably carcinogenic to humans (IARC, 1994; Lingnert *et al*., 2002; Friedman, 2002). In animal studies, mice and rats given purified acrylamide developed tumors in the thyroid gland, testes, mammary gland, lung, clitoral gland, and brain (Rice, 2005). In humans, long-term studies of high acrylamide food is linked with a slightly higher occurrence of human ovarian, endometrial, and kidney cancer (Hogervorst et al., 2008; Olesen et al., 2008; Wilson et al., 2010).

Acrylamide occurs naturally during the cooking or processing of certain foods, particularly in foods high in carbohydrates and low in proteins. Although acrylamide in food is formed through several routes, it is predominately formed from free amino acids, mainly asparagine, and reducing sugars via the complex Maillard reaction (Hogervorst *et al*., 2002, Mottram *et al*., 2002, Stradler *et al*., 2002, Yaylayan *et al*., 2003, Zyzak *et al*., 2003) (Figure 1).

Based on the levels of acrylamide in foods and consumption patterns of these foods, the dietary intake of acrylamide in the US is estimated to be 0.4 ug/kg of body weight/day and the international range is 0.2-0.4 ug/kg-body weight/day (FDA, 2009).

Many strategies have been used to reduce acrylamide formation in food. These include changes to the raw material, formulation, baking temperature and time, process, and technology. However, these changes may have potential negative consequences on the flavor, texture, and appearance of the final product.

Functional Technologies Corporation proposes the use of a self-cloned *S. cerevisiae* strain named Functional Technologies Acrylamide-Preventing Yeast to prevent acrylamide formation in food and beverage products. Through modern DNA technology, the strain carries three additional copies of the *S. cerevisiae ASP3* gene that are over expressed. The *ASP3* gene encodes for the periplasmic enzyme asparaginase II, which is capable of hydrolyzing both L-asparagine and D-aspragine into aspartate and ammonia (Figure 2). The modified strain has increased expression of the *S. cerevisiae* L-asparaginase which enhances its ability to catalyze asparagine. By reducing asparagine in the food matrix, acrylamide formation is significantly reduced

when heated. The FT Acrylamide-Preventing Yeast strain's enhanced ability to rapidly catalyze asparagine means that no modification is required to the production process.

Since all DNA fragments are obtained from *S. cerevisiae*, Functional Technologies Acrylamide-Preventing Yeast is non-transgenic.

1.3.2 Foods in which the substance is to be used

The FT Acrylamide-Preventing Yeast is intended for use in food and beverage products. The following food and beverage products are known to have acrylamide formation occur during the heating process and may benefit by using the FT Acrylamide-Preventing Yeast strain.

- Grain-Based Foods
  - Bread (rye, whole wheat, white, etc.)
  - Toasted breads
  - Cereals
  - Cookies
  - Tortillas
  - Baguettes
  - Doughnuts
  - Bagels
  - Biscuits
  - Pizza crust
  - Crackers
  - Wheat-based snacks
  - Extruded snacks and other snack foods
- Vegetable-based Foods
  - French-fried potatoes
  - Potato chips
  - Formed potato products (eg. Hashbrowns)
  - Potato-based snack foods
- Beverages
  - Coffee
  - Coffee substitutes

1.3.3 Levels of use of the Functional Technologies Acrylamide-Preventing Yeast

To prevent acrylamide formation in the variety of food and beverage products that the strain can be applied to, we recommend an approximate range of 0.5 to 5% as an appropriate level of use for FT Acrylamide-Preventing Yeast.

1.4 The basis for the GRAS determination

Through scientific analysis, Functional Technologies Corp. has determined that the Acrylamide-Preventing Yeast strain is "Generally Recognized as Safe" (GRAS) for use in food and beverage product. Functional Technologies Corp. also asserts that the Acrylamide-Preventing Yeast strain is substantially equivalent to the host strain from which it was derived, except for the enhanced asparagine catabolic activity. Specifically, the recombinant *ASP3* gene inserts that encode for asparaginase II, which catalyzes the hydrolysis of asparagine to aspartic acid, and is derived from *S. cerevisiae*.

1.5 Statement that the data and information that are the basis for the notifiers' GRAS determination are available for the Food and Drug Administration review and copying

Functional Technologies Corp. undertakes that all data and information used in this GRAS determination for use of the Acrylamide-Preventing Yeast strain in food and beverage products will be made available for the FDA to review and copy at the following address:

Functional Technologies Corporation
Suite 1500 - 885 West Georgia Street
Vancouver, British Columbia, V6C 3E8
Canada
Attention: Dr. John Husnik

Data will be sent to the FDA upon request.

2. Detailed information about the identity of the notified substance

2.1 Host microorganism

The host yeast strain is an industrial bread yeast strain of the domesticated *Saccharomyces cerevisiae* which is widely used in the baking industry.

2.1.1 History of use

The yeast *Saccharomyces cerevisiae* is found as a commensal organism on our bodies. It is in the air that we breathe and it grows naturally on many foods that we eat regularly. This yeast strain has been used in food production for over five thousand years. Ancient Egyptians, Hebrews and Greeks utilized yeast to produce fermented foods and beverages such as baked goods, wine and beer. Over 2.5 million tons of yeast are commercially produced each year worldwide, the largest volume of any microorganism.

S. cerevisiae serves as a model organism for scientific research for numerous reasons. Yeast cells are structurally and genetically similar to human cells. They each contain about 6,000 genes, many of which are identical or similar to human genes. Over the last 20 years, researchers have found that yeast genes can be manipulated with relative ease and efficacy. As a result, most of what we know about the cell cycle and human cancer was discovered from research on yeast. Throughout time, the classification of *S. cerevisiae* has undergone many changes (a historical survey of the species is found in Lodder and Kreger-van-Rij, 1967; Lodder, 1970; Kregar-van Rij, 1984; Kurtzman and Fell, 1998).

Yeast are very convenient microorganisms to work with in small and large volumes. Yeast needs are very basic, their growth rates are high and predictable, and their proliferation can be controlled and scaled. The primary reason that yeast has become one of the most widely-studied and understood organism, is that *S. cerevisiae* are known to be generally non-pathogenic and safe.

2.1.2 Characteristics

The commercial baker's yeast is commonly used in the baking and snack food industry. The production of baker's yeast vegetative cells during manufacturing occurs asexually through budding.

Baker's yeast is used for its ability to ferment dough thereby leavening and conditioning the dough. In addition, the yeast contributes to the flavor and structure of the final baked product.

The commercial baker's yeast strain is normally grown on cane and beet molasses under highly aerobic conditions.

2.2 Donor organisms

2.2.1 Taxonomy

Only *Saccharomyces cerevisiae* sequences were used in the modification of the Functional Technologies Acrylamide-Preventing Yeast, and therefore this enhanced yeast strain is 100 *Saccharomyces cerevisiae*. The history of use of this species is given in Section 2.1.1.

2.2.2 Genetic donor material

The three ASP3 integration gene cassettes all contain the *ASP3* ORF and the *KanMX* ORF but have different flanking sequences. The first integration cassette contains homologous flanking sequences for targeted integration into the *LEU2* locus (Figure 3). The second integration cassette contains homologous flanking sequences for targeted integration into the *URA3* locus (Figure 6). The third integration cassette contains homologous flanking sequences for targeted integration into the *TRP1* locus. After the integration of each of the gene cassette, the transformed strain was sub-cultured to lose the *KanMX* ORF via homologous recombination. The recombinant genetic insert in the FT Acrylamide-Preventing Yeast strains contains only the *S. cerevisiae ASP3* gene and the *S. cerevisiae PGK1* promoter and *PGK1* terminator. The *PGK1* promoter and terminator allows for the constitutive expression of the *ASP3* gene.

2.2.2.1 The ASP3 gene

The *ASP3* gene was derived from the *S. cerevisiae* research strain S288c. The published *ASP3* gene sequence of S288c was taken from the Saccharomyces Genome Database (SGD) (Appendix 1) and sent to GenScript USA Inc. (www.genscript.com) for synthesis.

The *ASP3* gene is 1089 nucleotides long and encodes for the periplasmic enzyme L-asparaginase II, which is involved in the cleavage of asparagine into aspartate and ammonia (Figure 2). The protein sequence of the *ASP3* gene is given in Appendix 2. The haploid genome of yeast contains four identical copies of the *ASP3* gene (hence a normally diploid strain would contain 8 copies of *ASP3*). The genes are located near a ribosomal DNA cluster region on chromosome XII and are activated during nitrogen limited conditions (Dunlop *et al*., 1980; Kim *et al*., 1988). Thus, *ASP3* gene is nitrogen regulated (nitrogen catabolite repression [NCR]).

2.3 The functionally-enhanced microorganism

2.3.1 Final gene constructs used in the integration strategy

The final three constructs comprised of *S. cerevisiae* genetic sequences were inserted into the *S. cerevisiae* commercial baker yeast strain. The KanMX marker present in the original cassette was removed from the yeast genome via homologous Acrylamide-Preventing Yeast strain only contains the three *S. cerevisiae ASP3* genes, under control of the *S. cerevisiae* constitutive *PGK1* promoter and *PGK1* terminator signals. As the fragments of the DNA insert present in the Acrylamide-Preventing Yeast

strain were all obtained from *S. cerevisiae* (*i.e.*, no foreign genetic material has been incorporated into the yeast strain), the FT Acrylamide-Preventing Yeast is *non-transgenic.*

2.3.1.1 LEU2-KanMX-ASP3 integration cassette construction strategy

The LEU2-KanMX-ASP3 gene construct used for the first transformation of the host organism was isolated from the LEU2-KanMX-ASP3-pMK plasmid (Figure 3). The construction of plasmid LEU2-KanMX-ASP3-pMK is the result of successive cloning events. Sequences of primers used in PCR amplification reactions are listed in Table 1. Plasmids used in the cloning events are listed in Table 2.

The *ASP3* gene was synthesized by GenScript USA Inc. (www.genscript.com) and inserted into the pUC57 plasmid and given the name ASP3-pUC57 (Figure 4). Using the ASP3-pUC57 plasmid as the template, the *ASP3* gene was amplified by PCR using a high fidelity, proof-reading DNA polymerase (KOD Hot Start DNA polymerase, Novagen) and the following primers 5′-ACAACGCGTATGAGATCTTTAAATACCCTTTTAC-3′ and 5′-TCAATGCTAGCTTAACCACCGTAGACGCCAGAA-3′, with built in restriction enzyme sites for MluI and BmtI, respectively (underlined). The resulting ASP3 amplicon was subsequently digested with the restriction enzymes MluI and BmtI and purified. Concurrently, the plasmid LEU2-KanMX-pMK, a previously synthesized DNA cassette (Mr. Gene GmbH, Regensburg, Germany), was also digested with MluI and BmtI. The LEU2-KanMX-pMK contains the LEU2 flanking sequences, KanMX marker, and PGK1 promoter/terminator sequences. Plasmid LEU2-KanMX-ASP3-pMK was obtained by subcloning the MluI/BmtI digested ASP3 PCR amplicon into the linear LEU2-KanMX-pMK vector. The complete LEU2-KanMX-ASP3 integration cassette was excised from the pMK vector by digestion with the restriction endonuclease blunt cutter SwaI. Figure 3 is a schematic of the linear LEU2-KanMX-ASP3 cassette and the cassette as it is located within the plasmid LEU2-KanMX-ASP3-pMK. The sequence for the linear LEU2-KanMX-ASP3 cassette is given in FASTA format in Appendix 3.

2.3.1.1.1 Detailed description of the final LEU2-KanMX-ASP3 construct

The LEU2-KanMX-ASP3 integration cassette is 4784 bp long (Figure 3). The sequence contains the KanMX ORF (1810 bp) on the 5′-end and the *ASP3* ORF (2047 bp) on the 3′-end of the cassette. The *LEU2* flanking sequences of 390 bp and 518 bp, are upstream and downstream of the ORFs, respectively. The KanMX marker is an antibiotic resistance marker that conveys resistance to the antibiotic G418. The use of the antibiotic marker provides a direct screening method for yeast cells containing the *ASP3* gene cassette. Both ORFs contain the constitutive *PGK1* promoter, which means that the KanMX gene is between the two repeat *PGK1* promoter sequences. The *PGK1*p direct repeat sequences help facilitate the loss of the KanMX marker via homologous recombination. The final recombinant gene cassette in the *LEU2* locus of the FT Acrylamide-Preventing Yeast strain contains only *S. cerevisiae* DNA and is composed of three parts, *ASP3* gene (1089 bp), *PGK1* promoter (759 bp), and *PGK1* (188 bp) terminator sequences. These three elements allow for the proper expression of the *ASP3* gene.

2.3.1.2 URA3-ASP3 integration cassette construction strategy

The recombinant gene insert, URA3-KanMX-ASP3, used for the second transformation of the host organism was isolated from plasmid URA3-ASP3-pMA (Figure 6). The LEU2-KanMX-ASP3 gene cassette was use in the construction of plasmid URA3-KanMX-ASP3-pMA.

The fragment, KanMX-ASP3, containing the KanMX ORF and the *ASP3* ORF was excised from the LEU2-KanMX-ASP3-pMK vector (Figure 3) using the restriction enzymes EagI and SacII. Concurrently, the plasmid URA3-pMK (Figure 5), containing the *URA3* flanking sequences (synthesized by Mr. Gene GmbH, Regensburg, Germany), was also digested with EagI and SacII. The EagI-SacII digested KanMX-ASP3 fragment was subcloned into the linear URA3-pMK vector. The complete URA3-KanMX-ASP3 integration cassette was excised from the pMA vector by digestion with the restriction enzyme AfeI. Figure 6 is a schematic of the linear URA3-KanMX-ASP3 cassette and the cassette as it is located within the plasmid URA3-KanMX-ASP3-pMK. The sequence for the linear URA3-KanMX-ASP3 cassette is given in FASTA format in Appendix 5.

2.3.1.2.1 Detailed description of the final URA3-KanMX-ASP3 construct

The URA3-KanMX-ASP3 integration cassette is 4657 bp long (Figure 6). The sequence contains the KanMX ORF (1810 bp) on the 5-end and the *ASP3* ORF (2047 bp) on the 3-end of the cassette. The *URA3* flanking sequences of 416 bp and 403 bp, are upstream and downstream of the ORFs, respectively. The KanMX marker is an antibiotic resistance marker that conveys resistance to the antibiotic G418. The use of the antibiotic marker provides a direct screening method for yeast cells containing the *ASP3* gene cassette. Both ORFs contain the constitutive *PGK1* promoter, which means that the KanMX gene is between the two repeat *PGK1* promoter sequences. The *PGK1* promoter direct repeat sequences help facilitate the loss of the KanMX marker via homologous recombination. The final recombinant gene cassette in the *URA3* loci of the FT Acrylamide-Preventing Yeast strain contains only *S. cerevisiae* DNA and is composed of three parts, *ASP3* gene (1089 bp), *PGK1* promoter (759 bp), and *PGK1* terminator (188 bp) sequences. These three elements allow for the proper expression of the *ASP3* gene.

2.3.1.3 TRP1-ASP3 integration cassette construction strategy

The recombinant gene insert, TRP1-KanMX-ASP3, used for the third transformation of the host organism was isolated from plasmid TRP1-KanMX-ASP3-pUC18 (Figure 7). Sequences of primers used in PCR amplification reactions are listed in Table 1. Plasmids used in the cloning events are listed in Table 2. All PCR amplifications were carried out using a high fidelity, proof-reading DNA polymerase (KOD Hot Start DNA polymerase, Novagen) and genomic DNA from the host strain was used as the template for TRP1 gene amplification.

The construction of plasmid TRP1-KanMX-ASP3-pMK is the result of successive cloning events. Firstly, the 5-TRP1 flanking region was amplified using primers 5-GAGCATGCAGCGCTTGTCACCTTACGTACAATCTTG-3-with built in restriction enzyme site for SphI (underlined) and 5-GTGGATCCCGGCCGCAATGCCGTAATCATTGACCAG-3-with

built in restriction enzyme sites BamHI and EagI (underlined). The resulting 5□TRP1 PCR amplicon and pUC18 plasmid were both digested with the restriction enzymes SphI and BamHI and purified. Subsequently, the SphI□BamHI digested 5□TRP1fragment was subcloned into the linear pUC18 plasmid resulting in the plasmid 5□TRP1-pUC18. Secondly, the 3□TRP1 flanking region was PCR amplified using primers 5□ GAGGTACCCCGCGGTCGTGGCAAGAATACCAAGAG-3□with built in restriction enzyme sites KpnI and SacII (underlined) and 5□GTGAATTCAGCGCTAGCAAGAATCGGGTCATTG-3□with built in restriction enzyme sites EcoRI (underlined). The resulting 3□TRP1 PCR amplicon and 5□TRP1-pUC18 vector were digested with KpnI and EcoRI and purified. Subsequently, the KpnI□EcoRI digested 3□TRP1 fragment was subcloned into the linearized 5□TRP1-pUC18 plasmid resulting in the new plasmid TRP1-pUC18. With the flanking sequences in the pUC18 plasmid, the next step was just inserting the KanMX and ASP3 gene cassettes between the flanking sequences. The fragment, KanMX-ASP3, containing the KanMX ORF and the ASP3 ORF was excised from the LEU2-KanMX-ASP3-pMK vector (Figure 3) using the restriction enzymes EagI and SacII. Concurrently, the plasmid TRP1-pUC18 was also digested with EagI and SacII. The EagI□SacII digested KanMX-ASP3 fragment was subcloned into the linearized TRP1-pUC18 plasmid. The complete TRP1-KanMX-ASP3 integration cassette was excised from the pUC18 vector by digestion with the restriction enzyme AfeI. Figure 7 is a schematic of the linear TRP1-KanMX-ASP3 cassette and the cassette as it is located within the plasmid TRP1-KanMX-ASP3-pUC18. The sequence for the linear TRP1-KanMX-ASP3 cassette is given in FASTA format in Appendix 7.

2.3.1.3.1 Detailed description of the final TRP1-KanMX-ASP3 construct

The TRP1-KanMX-ASP3 integration cassette is 4948 bp long (Figure 7). The sequence contains the KanMX ORF (1810 bp) on the 5□end and the *ASP3* ORF (2047 bp) on the 3□end of the cassette. The *TRP1* flanking sequences of 441 bp and 631 bp, are upstream and downstream of the ORFs, respectively. The KanMX marker is an antibiotic resistance marker that conveys resistance to the antibiotic G418. The use of the antibiotic marker provides a direct screening method for yeast cells containing the ASP3 gene cassette. Both ORFs contain the constitutive *PGK1* promoter, which means that the KanMX gene is between the two repeat *PGK1* promoter sequences. The *PGK1* promoter direct repeat sequences help facilitate the loss of the KanMX marker via homologous recombination. The final recombinant gene cassette in the *TRP1* locus of the FT Acrylamide-Preventing Yeast strain contains only *S. cerevisiae* DNA and is composed of three parts, *ASP3* gene (1089 bp), *PGK1* promoter (759 bp), and *PGK1* terminator (188 bp) sequences. These three elements allow for the proper expression of the *ASP3* gene.

2.3.2 The transformation event

2.3.2.1 Genetic material used for the transformation method

The linear LEU2-KanMX-ASP3, URA3-KanMX-ASP3, and TRP1-KanMX-ASP3 cassettes excised from the vector LEU2-KanMX-ASP3-pMK, URA3-KanMX-ASP3-pMK, TRP1-KanMX-ASP3-pUC18 (respectively) were the only DNA sequences used in the transformation method.

2.3.2.2 Screening method for transformants

After the transformation of each of the ASP3 integration cassettes the cells were plated onto G418 containing media in order to select for transformed cells. A single colony was chosen and streaked for purity and subsequently sub-cultured until the KanMX marker was lost through homologous recombination. The colonies sensitive to G418 was retransformed with the next ASP3 recombinant gene cassette and the selection steps repeated.

2.3.3 Genetic characterization of the modified microorganism

2.3.3.1 The loss of the antibiotic resistance genes

Loss of the KanMX antibiotic resistance gene was confirmed by streaking the FT Acrylamide-Preventing Yeast onto G418 containing media and confirming sensitivity. Figure 9 clearly shows that the FT Acrylamide-Preventing Yeast is sensitive to the G418 antibiotic like its parental strain. The loss of the KanMX marker gene was also confirmed genetically by Southern analysis and by sequencing the *ASP3* gene cassettes in the *LEU2*, *URA3*, *and TRP1* loci.

2.3.3.2 KanMX Southern blot analysis

The absence of the KanMX marker was confirmed by probing the FT Acrylamide-Preventing Yeast genomic DNA with the KanMX gene. Isolated genomic DNA from FT Acrylamide-Preventing Yeast and its parental strain was digested with KpnI restriction enzyme and separated by electrophoresis in a 0.7□ agarose gel. Following electrophoresis, the DNA was blotted onto positively charged nylon membranes, fixed, and probed with a KanMX probe. The KanMX probe (810 bp) was generated by PCR using primers 5□ATGGGTAAGGAAAAGACTCACG-3□and 5□ TTAGAAAAACTCATCGAGCATCAAATGA-3□and the pUC18-KanMX plasmid as template. No signal was detected in either the parent strain or the FT Acrylamide-Preventing Yeast strain for the KanMX gene that encodes for G418 resistance (Figure 10). A positive band was detected in the positive control lane of the parent strain containing the KanMX marker. This indicated that the KanMX markers used in the transformation process were removed from the genome of the FT Acrylamide-Preventing Yeast strain.

2.3.3.3 DNA sequencing of the integrated ASP3 gene cassettes

The *ASP3* ORFs and surrounding flanking sequences from the FT Acrylamide-Preventing Yeast strain were sequenced. The sequence results confirmed that all three cassettes integrated into the targeted LEU2, URA3, and TRP1 loci. The sequence results also confirmed the Southern blot analysis that the KanMX markers, initially present in all three ASP3 integration cassettes, were successfully removed via homologous recombination and only the *ASP3* ORF (PGK1 promoter, ASP3 gene, and PGK1 terminator sequences) remained in the LEU2, URA3, and TRP1 loci.

The *ASP3* ORFs and flanking sequences were shown to contain only DNA from *Saccharomyces cerevisiae* and that KanMX markers were absent. The sequence data also confirmed that the ASP3 gene cassette successfully integrated into the LEU2, URA3, and TRP1 loci of the commercial strain FT Acrylamide-Preventing Yeast.

2.3.4 Absence of difference between genetic profiles of the transformed and the host strain

The genome of *Saccharomyces cerevisiae* contains long terminal repeat sequences known as δ elements. These δ elements are the remnants of Ty1 transposon integration events. The number and location of these δ elements are specific to a strain and has been used to fingerprint and differentiate between strains of *Saccharomyces cerevisiae*. Using PCR and primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' to amplify these δ sequences we have verified the genetic relationship between the parent strain and FT Acrylamide-Preventing Yeast strain (Figure 11). This technique is commonly employed to differentiate between industrial *Saccharomyces cerevisiae* strains.

We therefore conclude that the Functional Technologies Acrylamide-Preventing Yeast strain is no different than the parent strain. No major DNA reorganization event has occurred upon integration of the *ASP3* cassettes into the FT Acrylamide-Preventing Yeast strain.

2.3.5 Method of manufacture of the Functional Technologies Acrylamide-Preventing Yeast.

No selective media needs to be used for the propagation of the FT Acrylamide-Preventing Yeast strain. The FT Acrylamide-Preventing Yeast active dry yeast can therefore be manufactured in the exact manner as all active dry yeast used in bakery, brewing or winemaking, as described in the literature (*e.g.*, see Reed and Nagodawithana, 1991). A brief description of the manufacturing of FT Acrylamide-Preventing Yeast active dry yeast is given in Figure 13.

3. Information on any self-limiting levels of use

Functional Technologies Corp. sees no reason why the inoculation rate should be different than the one recommended as indicated in Section 1.3.3.

4. Determination that the use of the active dry yeast obtained from the Functional Technologies Acrylamide-Preventing Yeast strain in in food and beverage products is exempt from the pre-market approval requirements of the act because such use is GRAS

4.1 Safety assessment of the host strain

The host yeast strain is an industrial strain of *Saccharomyces cerevisiae* known as baker's yeast that is commonly used in commercial bread production in the US. *S. cerevisiae* is an organism that 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 responsible for spontaneous fermentation of grape juice (Lodder and Kreger-van-Rij, 1967). The Bureau of Alcohol, Tobacco and Firearms rates yeast or yeast cultures grown in juice of the same kind of fruit (here grapes) as permitted material added in the production of natural wines (27 CRF 24.176).

S. cerevisiae is considered GRAS through its use in the brewing, baking and winemaking industry. Its genome has been sequenced, and it has been determined that the yeast is free of known pathogenicity traits. Genetically modified yeast strains such as the malolactic yeast (Wine Research Centre, UBC), and Phyterra's self-cloned ECMo01 strain have been previously granted GRAS status by the USFDA (GRAS Notice No. 000175). Phyterra's most recent low hydrogen sulfide producing self-cloned P1Y0 strain has also been granted GRAS status by the USFDA (GRAS Notice No. GRN 000350).

In the 27th report of The Scientific Committee for Human Food of the European Community the authors stated that *Saccharomyces cerevisiae* has a safe history of use in food and belongs to a species that is known not to produce toxins. In addition, the Environment Protection Agency (EPA) has included *S. cerevisiae* as a recipient microorganism for exemptions from EPA review and expedited EPA review (40 CFR 725.420). This exemption was made because this species is found to have little adverse effects. They also determined that the introduction of genetic material would 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.

The Functional Technologies Acrylamide-Preventing Yeast strain belongs to the *Saccharomyces cerevisiae* species which have been used for more than 5,000 years by humans in bread and fermented beverages. It can therefore be concluded that species will remain GRAS, even after genetic transformation, as long as no harmful DNA material is added to it.

4.2 Safety assessment of the genetic material used to construct the modified organism

4.2.1 Foreign genetic material source and product

No foreign (non-*Saccharomyces*) genetic material is in the Functional Technologies Acrylamide-Preventing Yeast strain.

4.2.2 Native genetic material source and product: *ASP3* sequences

4.2.2.1 The ASP3 gene

Source: *Saccharomyces cerevisiae*

The published *ASP3* gene sequence of S288c was synthesized by GenScript USA Inc. as described in Section 2.2.2.

Product: L-asparaginase II

Sequence analyses showed that the inserted ASP3 ORFs in the FT Acrylamide-Preventing Yeast strain only contained the *PGK1* promoter, *ASP3* gene, and *PGK1* terminator. The KanMX ORFs were removed from the genome during selection via homologous recombination. Thus, **only DNA from *S. cerevisiae* is present in the ASP3 integration cassette of the FT Acrylamide-Preventing Yeast strain and its *ASP3* gene product is also *S. cerevisiae.***

4.2.3 DNA integration cassettes

The three DNA integration cassettes (Figures 3, 6, and 7) were inserted into targeted loci on the yeast's genome. Construction of the three DNA integration cassettes is explained in Section 2.3. The LEU2-KanMX-ASP3 DNA integration cassette contained flanking sequences that are homologous to the *LEU2* gene for targeted integration into the *LEU2* locus. The URA3-KanMX-ASP3 DNA integration cassette contained flanking sequences that are homologous to the *URA3* gene for targeted integration into the *URA3* locus. The TRP1-KanMX-ASP3 DNA integration cassette contained flanking sequences that are homologous to the *TRP1* gene for targeted integration into the *TRP1* locus.

4.2.4 Transformation of the host strain with the integration cassette

The three linear gene cassettes: LEU3-KanMX-ASP3, URA3-KanMX-ASP3, and TRP1-KanMX-ASP3 were the only DNA sequences used in the transformation method. Figure 8 is a summary of the transformation event of the host strain with the LEU2-KanMX-ASP3 integration cassette. The other two cassettes were transformed in the same manner. All three DNA cassettes contained the antibiotic resistance marker

KanMX that convey resistance to the antibiotics G418. The KanMX marker provides a direct screening method for transformed yeast cells containing the integration cassette.

After transformation with the LEU2-KanMX-ASP3 DNA cassette, the cells were plated onto G418 containing media in order to select for transformed cells. A single colony was chosen and streaked onto G418 containing media and a single colony was picked and inoculated into liquid yeast growth media. The culture was continuously sub-cultured into fresh media everyday and incubated at 30°C. After two weeks, standard microbiological methods were employed to verify that the KanMX antibiotic marker was eliminated from the genome of the FT Acrylamide-Preventing Yeast strain. These methods relied on the fact that the FT Acrylamide-Preventing Yeast strain had lost its ability to grow on a G418 containing media, indicating the absence of the KanMX resistance gene (Figure 9). A sensitive colony was selected and re-transformed with the URA3-KanMX-ASP3 DNA cassette and plated onto G418 containing media. A pure colony resistant to G418 was sub-cultured in the same manner as above to lose the KanMX marker. Once a sensitive colony was found, it was transformed with the third DNA cassette, TRP1-KanMX-ASP3, and the whole process of losing was KanMX marker was repeated again.

G418 sensitivity screening, DNA sequencing, and Southern blot analysis all confirmed that the KanMX marker is not present in the FT Acrylamide-Preventing Yeast strain.

4.3 Safety assessment of the modified organism

4.3.1 Characterization of the transformation event

The genome of *S. cerevisiae* contains long terminal repeat sequences known as δ elements. These δ elements are the remnants of Ty1 transposon integration events. The number and location of these δ elements are specific to a strain and has been used to fingerprint and differentiate between strains of *S. cerevisiae*. Using PCR and primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' to amplify these δ sequences we have verified the genetic relationship between the host strain and P1Y0 (Figure 11).

Loss of the antibiotic resistance genes was confirmed by streaking the FT Acrylamide-Preventing Yeast strain onto G418 containing media and confirming sensitivity. Figure 9 clearly shows that FT Acrylamide-Preventing Yeast Strain is sensitive to the G418 antibiotic like the host strain. The loss of the KanMX marker was also genetically verified by DNA sequencing and Southern blot Analysis (Figure 10). In addition, DNA sequencing verified that the LEU2-ASP3, URA3-ASP3, and TRP1-ASP3 cassettes integrated into the targeted *LEU2*, *URA3*, and *TRP1* loci.

Genetic characterization of the FT Acrylamide-Preventing Yeast strain confirmed that it contains no DNA sequences other than those present in the isolated *ASP3* integration DNA. No KanMX marker sequence was present in the genetic material of the FT Acrylamide-Preventing Yeast strain. Therefore, to the best of our knowledge, the FT Acrylamide-Preventing Yeast strain contains only *S. cerevisiae* DNA.

4.3.2 L-Asparaginase II

The published *ASP3* gene sequence from the *S. cerevisiae* strain S288c was used in the construction for all three transformation cassettes.

Thus, there should be no differences in the amino acid sequences of the asparaginase II enzyme synthesized by the FT Acrylamide-Preventing Yeast and the published amino acid sequence of L-asparaginase II. The length of the amino acid sequence is 362 (Appendix 2) and the molecular weight of this protein has been determined to be 38,686 Da.

The presence of the ASP3 cassette in the FT Acrylamide-Preventing Yeast does not compromise the safety of the microorganism and will in fact enhance the final product (i.e. bread, biscuits, etc) by reducing acrylamide formation.

4.3.3 Consequence of the genetic modification on the physiology of the FT Acrylamide-Preventing Yeast strain

4.3.3.1 Overview of the function of the ASP3 gene

The *ASP3* gene encodes for the exoenzyme aspraginase II. The *ASP3* gene is induced in response to nitrogen limited conditions. Asparaginase II hydrolyzes the free asparagine into aspartate and acrylamide.

4.3.3.2 Manufacture of active dry yeast during fed-batch aerobic cultures

Section 2.3.5 gave an overview of the method of manufacture of the FT Acrylamide-Preventing Yeast strain in the form of fresh or active dry yeast. This method of manufacture is identical to that used for the current commercial manufacture of fresh or active dry yeast obtained with the host strain.

4.3.3.3 Cell growth of FT Acrylamide-Preventing Yeast and Parent strain

Growth rates of the FT Acrylamide-Preventing Yeast and its parent strain were monitored in the laboratory by absorbance readings. Results show that the FT Acrylamide-Preventing Yeast and its parent strain have similar growth rates as illustrated in Figure 12. Thus, the presence of the three ASP3 gene cassettes does not give the FT Acrylamide-Preventing Yeast any growth advantage, even though the strain has enhanced asparagine catabolic abilities.

Genetic studies have shown that the FT Acrylamide-Preventing Yeast strain is genetically similar to the host strain, except the enhanced asparagine catalytic activity. This leads to the conclusion that the FT Acrylamide-Preventing Yeast strain can be regarded as substantially equivalent to its host strain. Physiological studies carried out in different culture conditions indicate that no difference between the FT Acrylamide-Preventing Yeast strain and host strains can be seen excepted for the enhanced asparagine catabolic activity. The three *ASP3* gene cassettes do not confer any significant growth advantage to the host strain. Therefore, it can be concluded that the FT Acrylamide-Preventing Yeast strain is substantially equivalent to the host strain except for the FT Acrylamide-Preventing Yeast strain's increased ability to degrade asparagine.

4.4 Safety assessment of the product derived from the functionally-enhanced organism

4.4.1 Changes in the processing procedures as a consequence of the Functional Technologies Acrylamide-Preventing Yeast addition

The use of the FT Acrylamide-Preventing Yeast leads to no changes in the processes where baker's yeast strains are generally employed (example, baking). In processes that have never incorporated yeast strains, the addition of acrylamide-preventing yeast may alter the process and the degree of modification would depend on the food/beverage. Any such modification would not affect the safety of the product but it is recognized that sensory characteristics may be affected if the dose of yeast were at a high concentration.

Reduction of asparagine in food

In addition to completing experiments using laboratory media containing asparagine, we have also completed simulated food preparation trials using typical ingredients used in the production of grain-based-foods and vegetable-based food products. Under normal food processing conditions we achieved significant reductions in asparagine immediately after the addition of the FT Acrylamde-Preventing Yeast. If the food processing method allowed additional contact time prior to heat inactivation, the asparagine levels would dramatically decrease to non-detectable levels, generally within a one to two hour period (depending on dose of yeast, temperature, water activity, type of product etc.)

Hence, the FT Acrylamide-Preventing Yeast strain reduces more asparagine than the parental strain, which would result in reducing acrylamide formation in the final food product.

Other undesirable substances

The *ASP3* integration cassette present in the FT Acrylamide-Preventing Yeast strain as the result of the genetic modification does not code for either toxic or allergenic proteins, nor proteins implicated in the formation of undesirable compounds.

These considerations lead us to conclude that allergenic or toxic risks related to the presence of the *ASP3* cassette within the new FT Acrylamide-Preventing Yeast strain are negligible.

Therefore, to the best of our knowledge, the use of the FT Acrylamide-Preventing Yeast strain in food and beverages products will not lead to the release or the enhancement of the presence of undesirable compounds in these products. Moreover, the potentially carcinogenic compound, acrylamide is significantly reduced in many food products using the FT Acrylamide-Preventing Yeast strain.

Figures

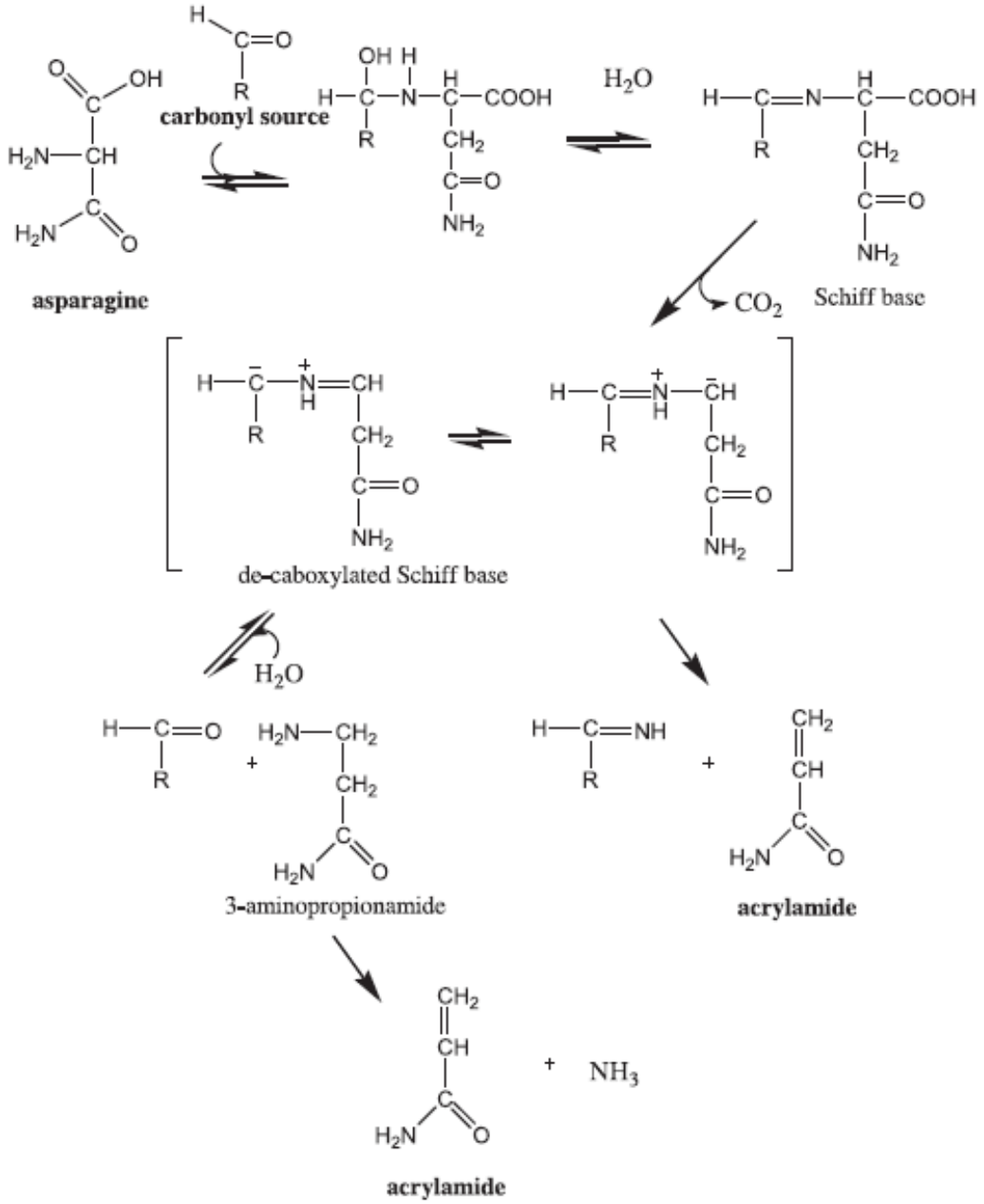


Figure 1: Acrylamide formation in the Millard Reaction (Claus *et al.*, 2008).

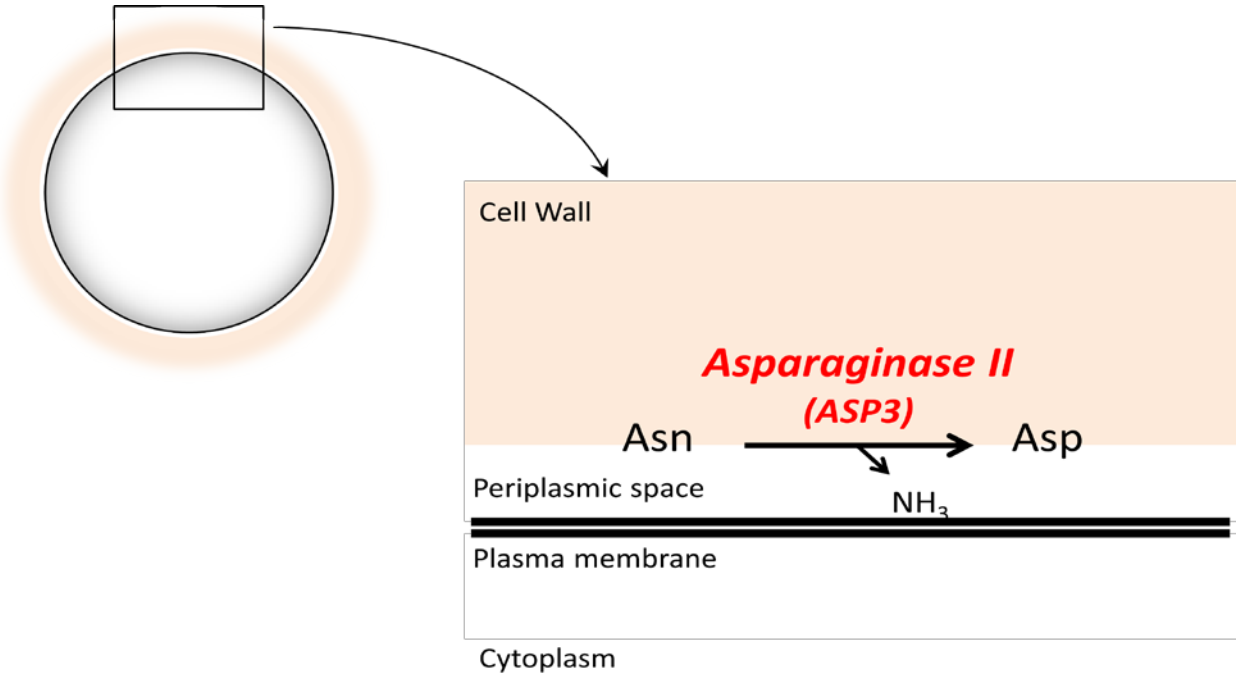
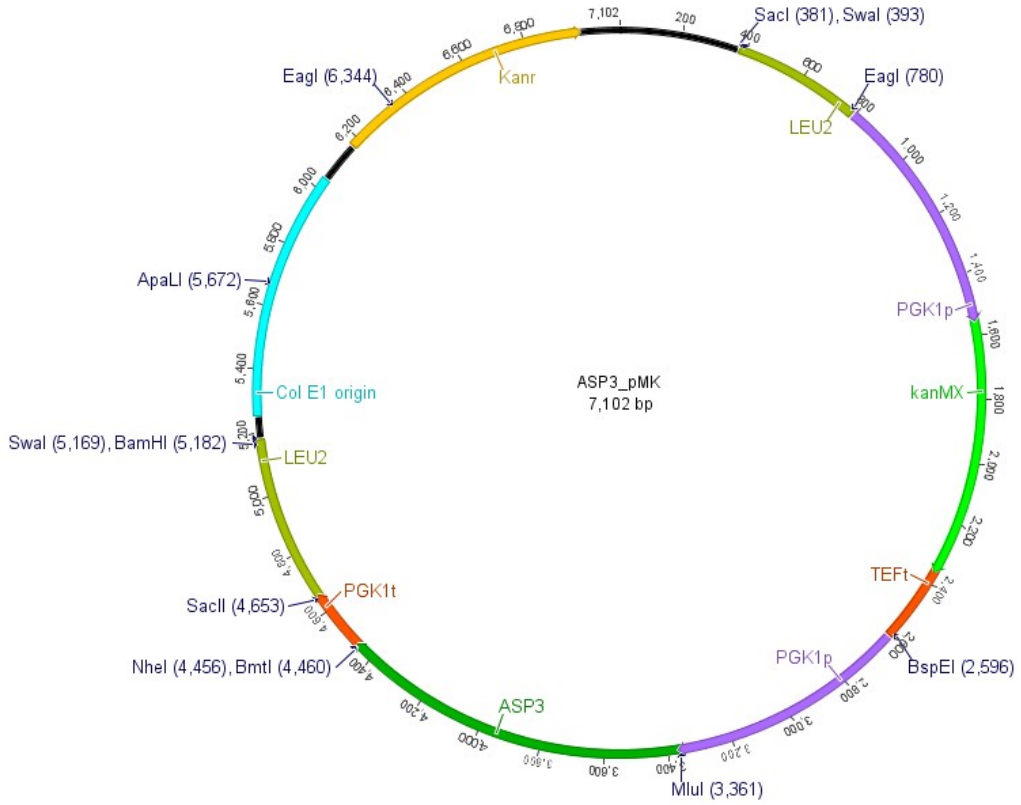


Figure 2: The catalytic activity of the periplasmic enzyme asparaginase II in yeast.



(a)

(b)

(c)

Figure 3: LEU2-ASP3 cassette

(a) A schematic of the plasmid vector LEU2-ASP3-pMK (7102 bp), (b) the linear cassette without the vector plasmid backbone (4784 bp), and (c) the cassette (2960 bp) after the removal of the KanMX ORF via homologous recombination.

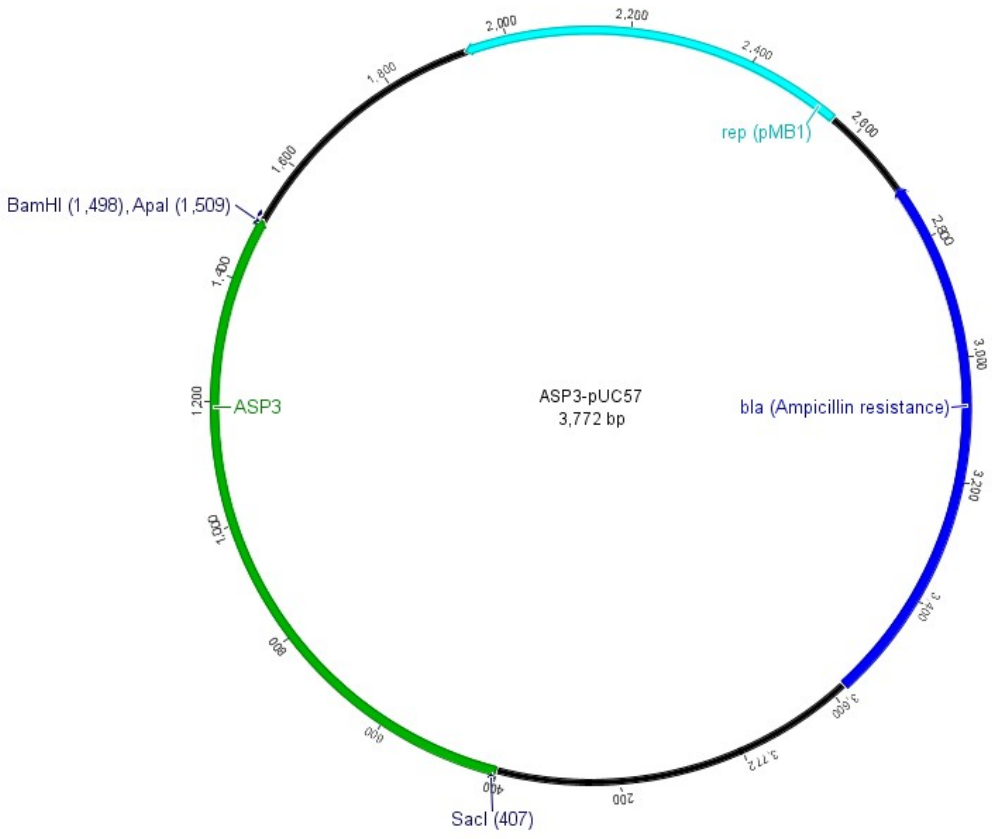


Figure 4: A schematic of the plasmid ASP3-pUC57.

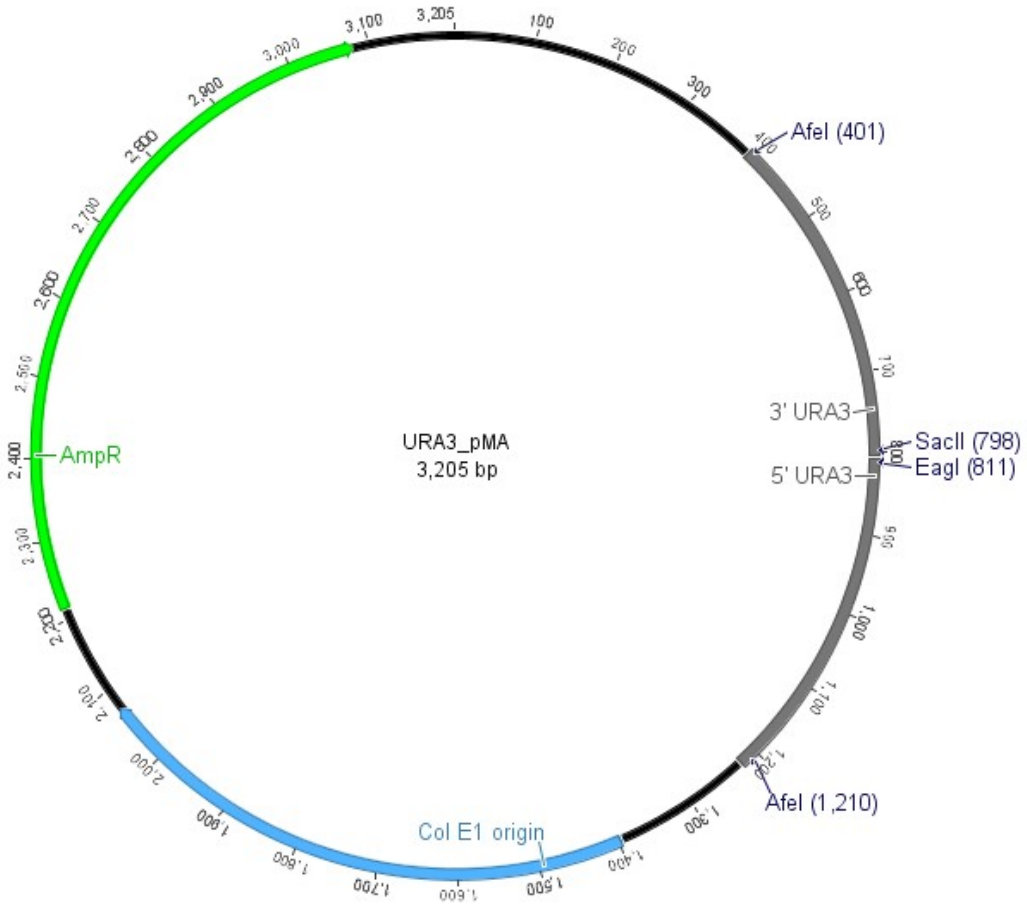
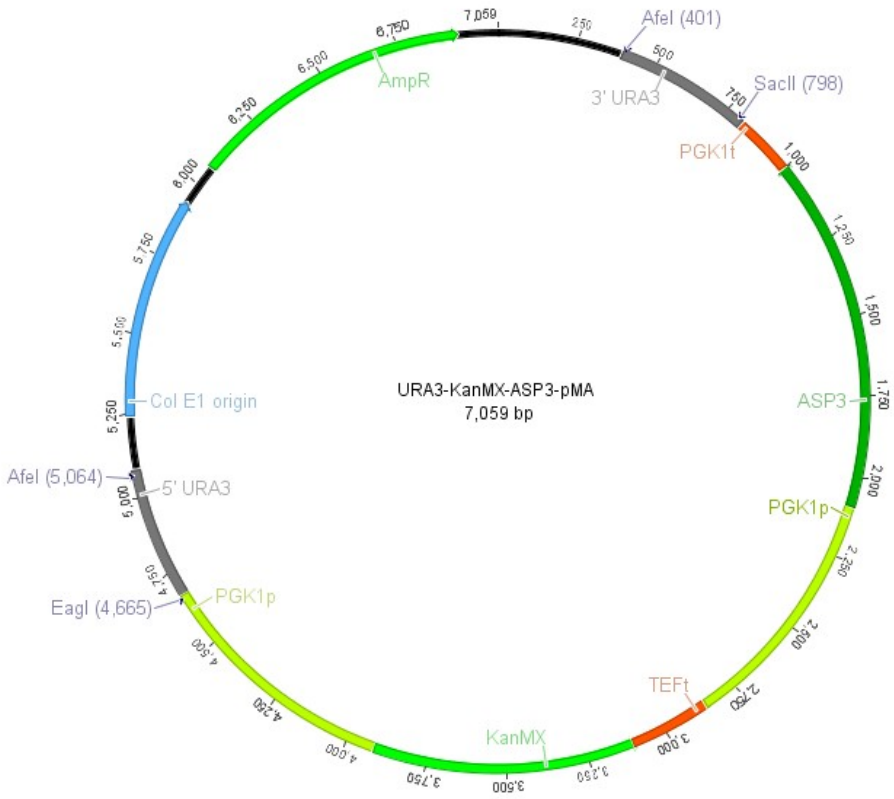
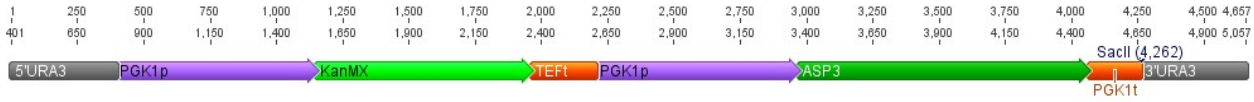


Figure 5: A schematic of the plasmid vector URA3-pMA.



(a)

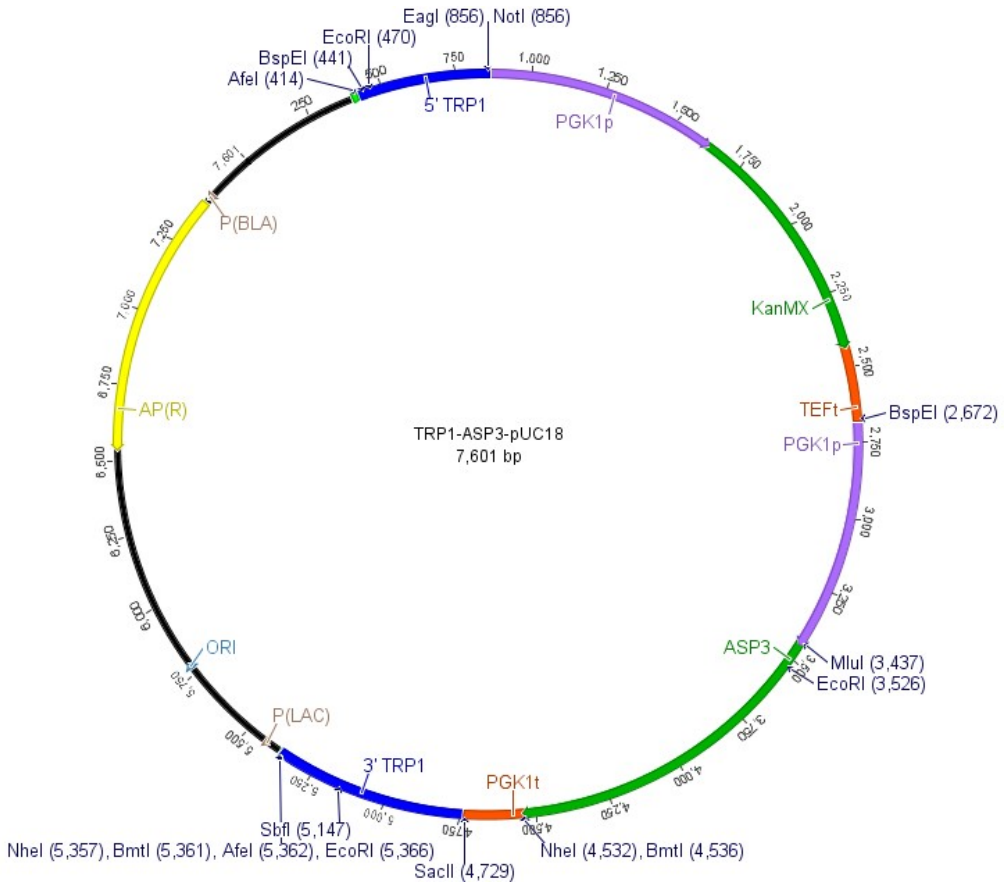


(b)

(c)

Figure 6: URA3-ASP3 cassette

(a) A schematic of the plasmid vector URA3-ASP3-pMK (7059 bp), (b) the linear cassette without the vector plasmid backbone (4657 bp), and (c) the cassette (2847 bp) after the removal of the KanMX ORF via homologous recombination.



(a)

(b)

(c)

Figure 7: TRP1-ASP3 cassette

(a) A schematic of the plasmid vector TRP1-ASP3-pMK (7601 bp), (b) the linear cassette without the vector plasmid backbone (4948 bp), and (c) the cassette (3138 bp) after the removal of the KanMX ORF via homologous recombination.

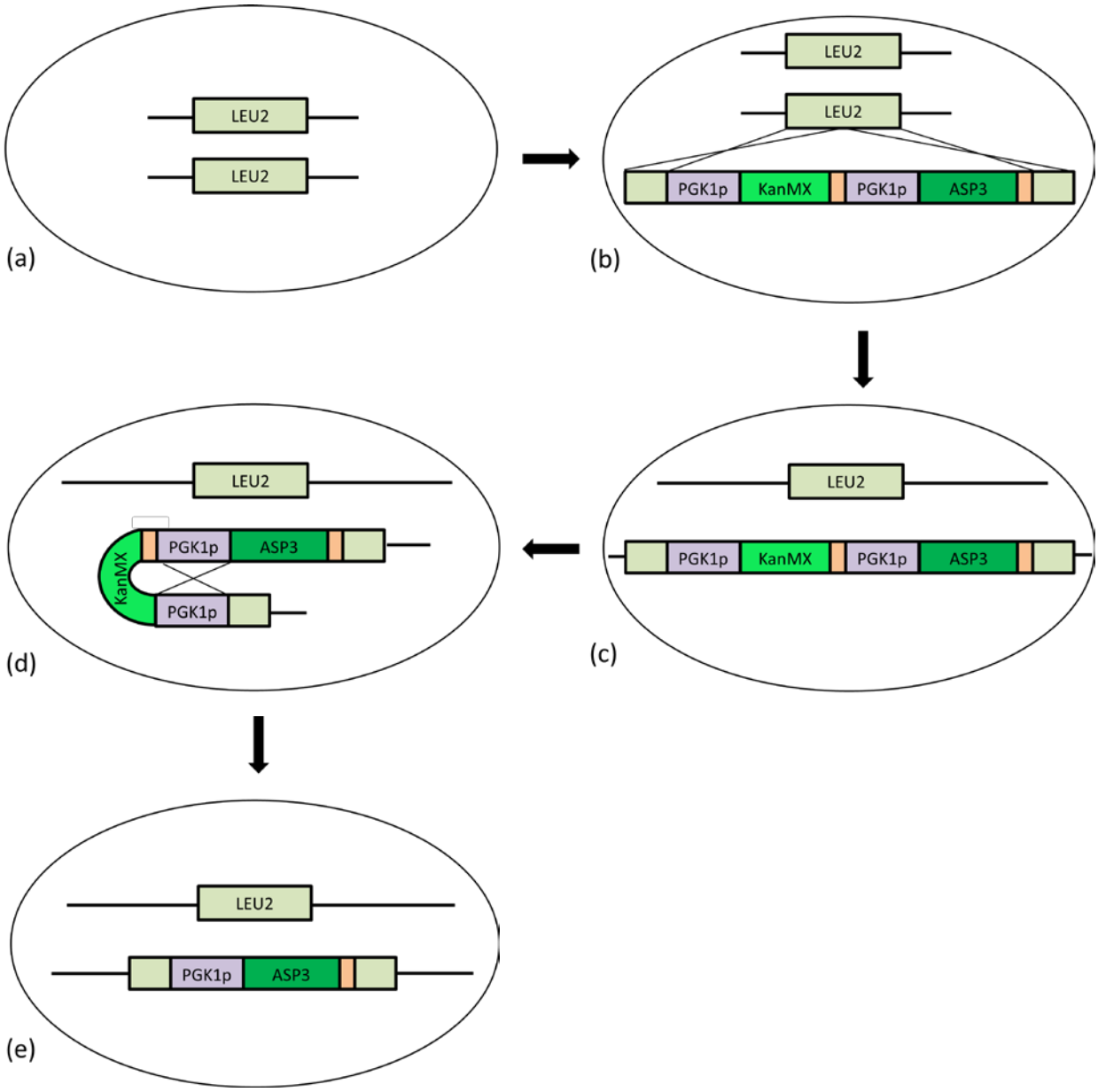


Figure 8: Transformation events of ASP3 cassette into host strain.

Schematic diagram of the LEU2-ASP3 cassette transformation into the host strain. The URA3-ASP3 and TRP1-ASP3 cassette was inserted into the URA3 and TRP1 loci (respectively) in the same manner. (a) Host strain containing two copies of the *LEU2* gene. (b) ASP3 cassette transformed into the host strain. (e) Transformed cell with the ASP3 cassette integrated into the *LEU2* locus via homologous recombination. The flanking sequences of the ASP3 cassette have 100□ homology to the *LEU2* gene. (d) Through sub-culturing of the transformed cell the KanMX marker is removed via homologous recombination. (e) The Functional Technologies Acrylamide Reducing Yeast strain containing the ASP3 cassette and no KanMX marker.

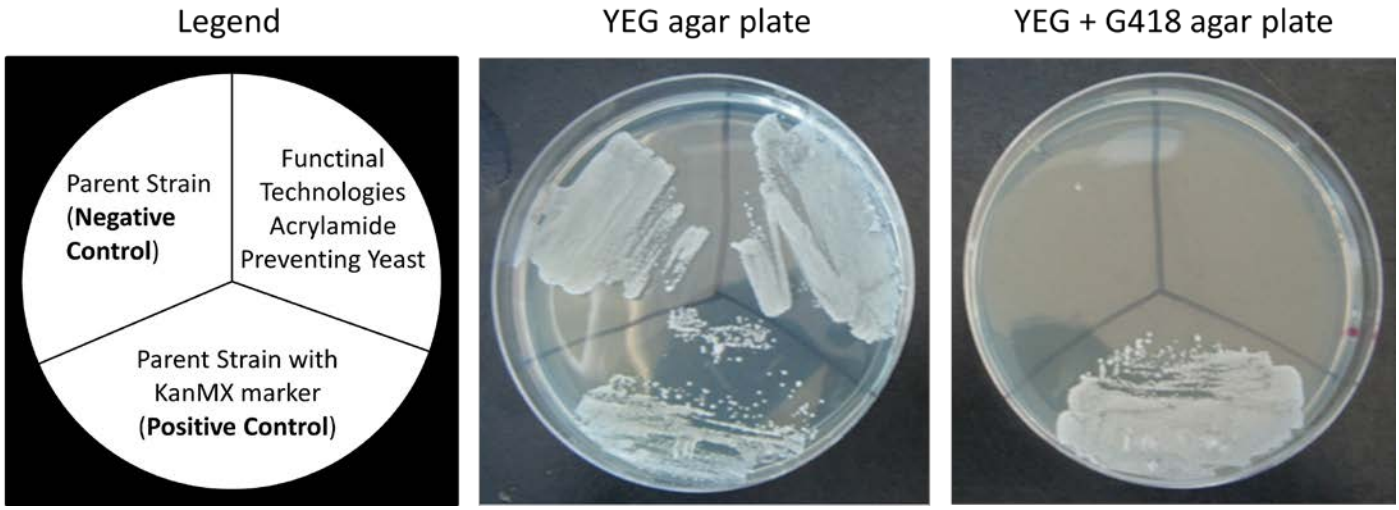


Figure 9: Confirmation of the loss of the KanMX marker that conveys resistance to the antibiotic G418.

The base media is 10 g□l Yeast extract, 20 g□l of Glucose and 15 g□l of agar (YEG). The concentration of the G418 antibiotic was 500 ug□l.

Figure 10: Southern blot analysis of the KanMX gene that encodes for Geneticin G418 resistance.

Southern blot analysis of genomic DNA digested with *KpnI* restriction enzyme and probed with the KanMX gene. (A) genomic DNA of parent strain (B) genomic DNA of the FT Acryalmide preventing yeast strain (C) genomic DNA of parent strain with *KanMX* gene cassette integrated into chromosomal DNA (positive control).

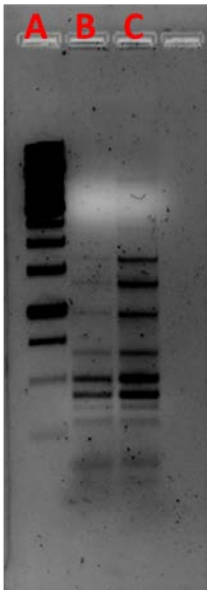


Figure 11: PCR of genetic patterns of the host baker's yeast and Functional Technologies Acrylamide-Preventing Yeast strains.

(A) DNA ladder, (B) parent strain, (C) Functional Technologies Acrylamide-Preventing Yeast strain. PCR was performed using primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' on genomic DNA isolated from the host and FT Acrylamide Yeast strains. This technique is described by Schuller *et al*. (2004).

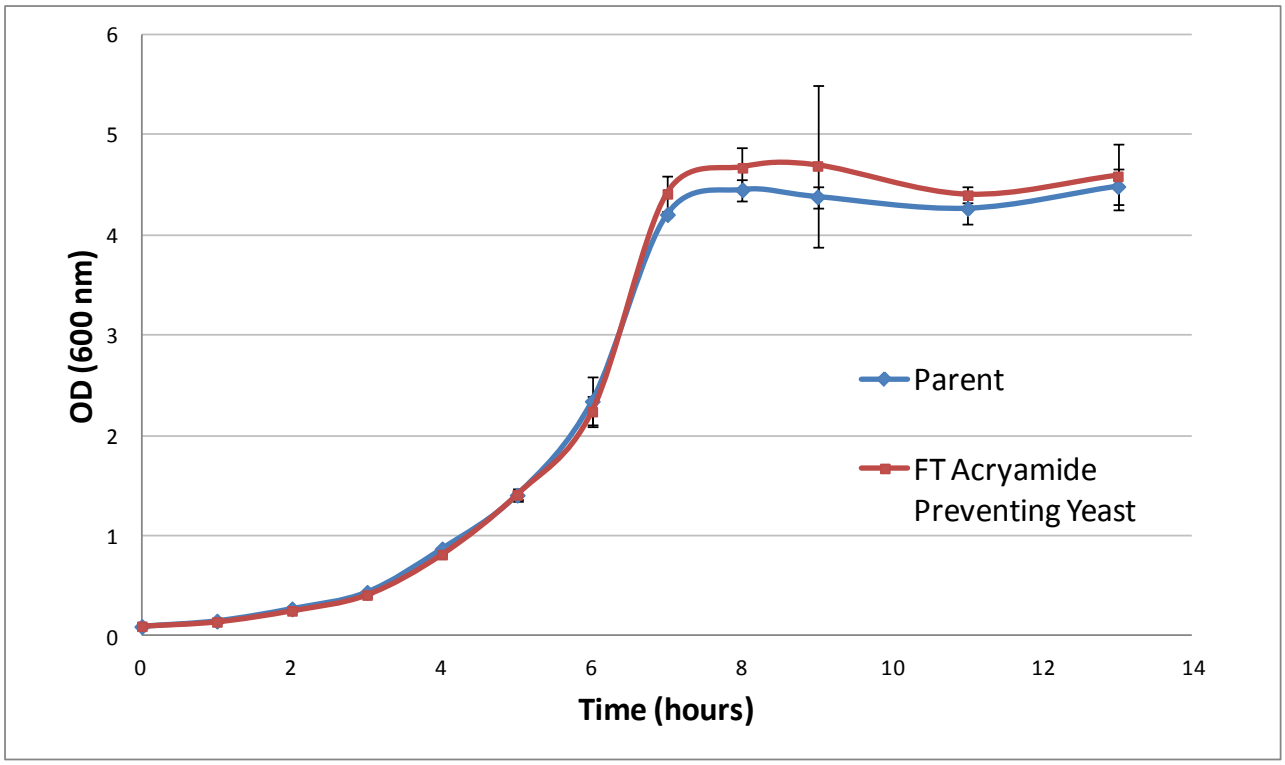


Figure 12: Growth curve of Functional Technologies Acrylamide-Preventing Yeast strain versus the parent strain.

Each strain was inoculated in triplicates at an OD_{600nm} of 0.1 and incubated on a 200 rpm shaker at 30°C for 13 hours. Readings were taken at each hour and the average OD reading of the triplicate cultures and its standard deviation were plotted.

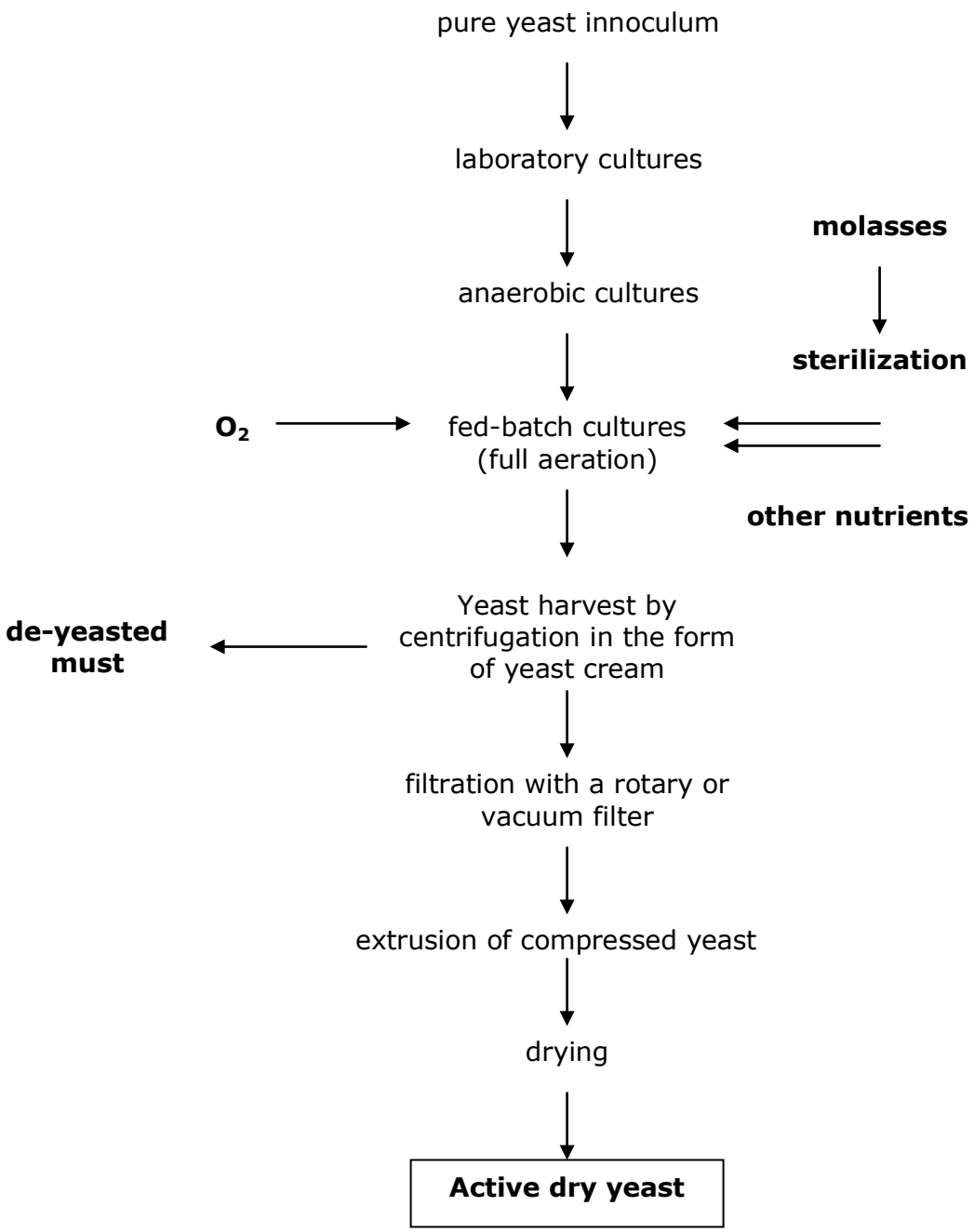


Figure 13: Flow diagram describing the manufacture of active dry yeast.

Tables

Table 1: PCR primers used for the ASP3 integration cassette construction

Designation	DNA sequences (5'→ 3')	Description
ASP3-F	ACAACGCGTATGAGATCTTTAAATACCCTTTTAC	Forward primer for the *ASP3* gene with the MluI site (underlined).
ASP3-R	TCAATGCTAGCTTAACCACCGTAGACGCCAGAA	Reverse primer for the *ASP3* gene with the BmtI site (underlined).
TRP1-F1	GAGCATGCAGCGCTTGTCACCTTACGTACAATCTTG	Forward primer for the 5'half of the *TRP1* gene with the SphI site (underlined).
TRP1-R1	GTGGATCCCGGCCGCAATGCCGTAATCATTGACCAG	Reverse primer for the 5'half of the *TRP1* gene with the BamHI and EagI sites (underlined).
TRP1-F2	GAGGTACCCCGCGGTCGTGGCAAGAATACCAAGAG	Forward primer for the 3'half of the *TRP1* gene with the KpnI and SacII sites (underlined).
TRP1-R2	GTGAATTCAGCGCTAGCAAGAATCGGGTCATTG	Reverse primer for the 3'half of the *TRP1* gene with the EcoRI site (underlined).
KanMX-F	ATGGGTAAGGAAAAGACTCACG	Forward primer used to amplify the KanMX gene.
KanMX-R	TTAGAAAAACTCATCGAGCATCAAATGA	Reverse primer used to amplify the KanMX gene.
δ1	GTGGATTTTTATTCCAAC	Primer used to amplify δ elements of Ty1 retro-transposons for δ sequence typing
δ1,2	TCAACAATGGAATCCCAAC	Primer used to amplify δ elements of Ty1 retro-transposons for δ sequence typing

Table 2: Plasmids used for the construction and integration of the integration cassette.

Designation	Description	Source
ASP3-pUC57	pUC57 is a commonly used plasmid that is a derivative of pUC19. The DNA synthesized *ASP3* gene was subcloned between the SacI and BamHI restriction enzyme sites.	GenScript
LEU2-KanMX-pMK	pMK vector contains the LEU2 flanking sequences, KanMX marker, and PGK1 promoter and terminator sequences.	Mr. Gene GmbH, Regensburg, Germany
LEU2-ASP3-pMK	The synthesized *ASP3* gene was subcloned into the MluI and BmtI restriction enzyme sites of the LEU2-KanMX-pMK vector.	This study
URA3-pMA	pMA vector contains the URA3 flanking sequences.	Mr. Gene GmbH, Regensburg, Germany
URA3-ASP3-pMA	A fragment containing the KanMX and ASP3 ORFs was excised from the LEU2-ASP3-pMK plasmid and subcloned into the EagI and SacII restriction enzyme sites of the URA3-KanMX-pMA vector.	This study
5□TRP1-pUC18	pUC18 vector contains the 5□TRP1 flanking sequence.	This study
TRP1-pUC18	pUC18 vector contains both the 5□and 3□TRP1 flanking sequences	This study
TRP1-ASP3-pUC18	pUC18 vector contains the TRP1 flanking sequences, KanMX marker, *ASP3* gene, and PGK1 promoter and terminator sequences.	This study

Appendix

Appendix 1. Genomic sequence of the *ASP3* gene (1089 nucleotides) in FASTA format.

```
>YLR155C  Chr 12

ATGAGATCTTTAAATACCCTTTTACTTTCTCTCTTTGTCGCAATGTCCAGTGGTGCTCCA
CTACTAAAAATTCGTGAAGAGAAGAATTCTTCTTTGCCATCAATCAAAATTTTTGGTACC
GGCGGTACTATCGCTTCCAAGGGTTCGACAAGTGCAACAACGGCGGGTTATAGCGTGGGA
TTAACCGTAAATGATTTAATAGAAGCCGTCCCATCTTTAGCTGAGAAGGCAAATCTGGAC
TATCTTCAAGTGTCTAACGTTGGTTCAAATTCTTTAAACTATACGCATCTGATCCCATTG
TATCACGGTATCTCCGAGGCACTAGCCTCTGATGACTACGCTGGTGCGGTTGTCACTCAT
GGGACCGACACTATGGAGGAGACAGCTTTCTTCTTAGATTTGACCATAAATTCAGAGAAG
CCAGTATGTATCGCAGGCGCTATGCGTCCAGCCACTGCCACGTCTGCTGATGGCCCAATG
AATTTATATCAAGCAGTGTCTATTGCTGCTTCTGAGAAATCACTGGGTCGTGGCACGATG
ATCACTCTAAACGATCGTATTGCCTCTGGGTTTTGGACAACGAAAATGAATGCCAACTCT
TTAGATACATTCAGAGCGGATGAACAGGGATATTTAGGTTACTTTTCAAATGATGACGTG
GAGTTTTACTACCCACCAGTCAAGCCAAATGGATGGCAATTTTTTGACATTTCCAACCTC
ACAGACCCTTCGGAAATTCCAGAAGTCATTATTCTGTACTCCTATCAAGGCTTGAATCCT
GAGCTAATAGTAAAGGCCGTCAAGGACCTGGGCGCAAAAGGTATCGTGTTGGCGGGTTCT
GGAGCTGGTTCCTGGACTGCTACGGGTAGTATTGTAAACGAACAACTTTATGAAGAGTAT
GGTATACCAATTGTTCACAGCAGAAGAACAGCAGATGGTACAGTTCCTCCAGATGATGCC
CCAGAGTACGCCATTGGATCTGGCTACCTAAACCCTCAAAAATCGCGTATTTTGCTACAA
TTATGTTTGTACTCCGGCTACGGCATGGATCAGATTAGGTCTGTTTTTTCTGGCGTCTAC
GGTGGTTAA
```

Appendix 2. Protein sequence of the *ASP3* gene in FASTA format.

```
>YLR155C
MRSLNTLLLSLFVAMSSGAPLLKIREEKNSSLPSIKIFGTGGTIASKGSTSATTAGYSVG
LTVNDLIEAVPSLAEKANLDYLQVSNVGSNSLNYTHLIPLYHGISEALASDDYAGAVVTH
GTDTMEETAFFLDLTINSEKPVCIAGAMRPATATSADGPMNLYQAVSIAASEKSLGRGTM
ITLNDRIASGFWTTKMNANSLDTFRADEQGYLGYFSNDDVEFYYPPVKPNGWQFFDISNL
TDPSEIPEVIILYSYQGLNPELIVKAVKDLGAKGIVLAGSGAGSWTATGSIVNEQLYEEY
GIPIVHSRRTADGTVPPDDAPEYAIGSGYLNPQKSRILLQLCLYSGYGMDQIRSVFSGVY
GG*
```

Appendix 3: Nucleotide sequence of the linear LEU2-KanMX-ASP3 cassette in FASTA format.

```
1         10         20         30         40         50
  |         |          |          |          |          |
  ATTTAAATCATTTAATTGGTGGTGCTGCTATCGATGCTACAGGTGTTCCA
  CTTCCAGATGAGGCGCTGGAAGCCTCCAAGAAGGCTGATGCCGTTTTGTT
  AGGTGCTGTGGGTGGTCCTAAATGGGGTACCGGTAGTGTTAGACCTGAAC
  AAGGTTTACTAAAAATCCGTAAAGAACTTCAATTGTACGCCAACTTAAGA
  CCATGTAACTTTGCATCCGACTCTCTTTTAGACTTATCTCCAATCAAGCC
  ACAATTTGCTAAAGGTACTGACTTCGTTGTTGTCAGAGAATTAGTGGGAG
  GTATTTACTTTGGTAAGAGAAAGGAAGACGATGGTGATGGTGTCGCTTGG
  GATAGTGAACAATACACCGTTCCAGAAGTGCAAAGAATCACGGCCGCAAC
  TCAAGACGCACAGATATTATAACATCTGCATAATAGGCATTTGCAAGAAT
  TACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATACCTGCATTTAAA
  GATGCCGATTTGGGCGCGAATCCTTTATTTTGGCTTCACCCTCATACTAT
  TATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCCTTCTTGAATTGATGTTA
  CCCTCATAAAGCACGTGGCCTCTTATCGAGAAAGAAATTACCGTCGCTCG
  TGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACACGCTCGAC
  TTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACAAGGT
  CCTAGCGACGGCTCACAGGTTTTGTAACAAGCAATCGAAGGTTCTGGAAT
  GGCGGGAAAGGGTTTAGTACCACATGCTATGATGCCCACTGTGATCTCCA
  GAGCAAAGTTCGTTCGATCGTACTGTTACTCTCTCTCTTTCAAACAGAAT
  TGTCCGAATCGTGTGACAACAACAGCCTGTTCTCACACACTCTTTTCTTC
  TAACCAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTGAAACTTACATTTA
  CATATATATAAACTTGCATAAATTGGTCAATGCAAGAAATACATATTTGG
  TCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGATGCTTTCTTTTTCTCT
  TTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAATATA
  AAACAATGGGTAAGGAAAAGACTCACGTTTCGAGGCCGCGATTAAATTCC
  AACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGG
  GCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAG
  AGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGAT
  GAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCAT
  CAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGA
  TCCCCGGCAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGT
  GAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGAT
  TCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTC
  AGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGAT
  GACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAA
  GCTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCAC
  TTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTT
  GGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAA
  CTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAAT
  ATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTC
  GATGAGTTTTTCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAAG
  AACTTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAATC
  AAATGTTAGCGTGATTTATATTTTTTTTCGCCTCGACATCATCTGCCCAG
  ATGCGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGA
  ATGCTGGTCGCTATACTGCTGTCGATTCGATACTAACGCCGCCATCCAGT
  GTCGACTCCGGACAACTCAAGACGCACAGATATTATAACATCTGCATAAT
  AGGCATTTGCAAGAATTACTCGTGAGTAAGGAAAGAGTGAGGAACTATCG
  CATACCTGCATTTAAAGATGCCGATTTGGGCGCGAATCCTTTATTTTGGC
  TTCACCCTCATACTATTATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCCT
  TCTTGAATTGATGTTACCCTCATAAAGCACGTGGCCTCTTATCGAGAAAG
  AAATTACCGTCGCTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAA
  CCCAGACACGCTCGACTTCCTGTCTTCCTATTGATTGCAGCTTCCAATTT
```

CGTCACACAACAAGGTCCTAGCGACGGCTCACAGGTTTTGTAACAAGCAA
TCGAAGGTTCTGGAATGGCGGGAAAGGGTTTAGTACCACATGCTATGATG
CCCACTGTGATCTCCAGAGCAAAGTTCGTTCGATCGTACTGTTACTCTCT
CTCTTTCAAACAGAATTGTCCGAATCGTGTGACAACAACAGCCTGTTCTC
ACACACTCTTTTCTTCTAACCAAGGGGGTGGTTTAGTTTAGTAGAACCTC
GTGAAACTTACATTTACATATATATAAACTTGCATAAATTGGTCAATGCA
AGAAATACATATTTGGTCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGA
TGCTTTCTTTTTCTCTTTTTTACAGATCATCAAGGAAGTAATTATCTACT
TTTTACAACAAATATAAAACAACGCGTATGAGATCTTTAAATACCCTTTT
ACTTTCTCTCTTTGTCGCAATGTCCAGTGGTGCTCCACTACTAAAAATTC
GTGAAGAGAAGAATTCTTCTTTGCCATCAATCAAAATTTTTGGTACCGGC
GGTACTATCGCTTCCAAGGGTTCGACAAGTGCAACAACGGCGGGTTATAG
CGTGGGATTAACCGTAAATGATTTAATAGAAGCCGTCCCATCTTTAGCTG
AGAAGGCAAATCTGGACTATCTTCAAGTGTCTAACGTTGGTTCAAATTCT
TTAAACTATACGCATCTGATCCCATTGTATCACGGTATCTCCGAGGCACT
AGCCTCTGATGACTACGCTGGTGCGGTTGTCACTCATGGGACCGACACTA
TGGAGGAGACAGCTTTCTTCTTAGATTTGACCATAAATTCAGAGAAGCCA
GTATGTATCGCAGGCGCTATGCGTCCAGCCACTGCCACGTCTGCTGATGG
CCCAATGAATTTATATCAAGCAGTGTCTATTGCTGCTTCTGAGAAATCAC
TGGGTCGTGGCACGATGATCACTCTAAACGATCGTATTGCCTCTGGGTTT
TGGACAACGAAAATGAATGCCAACTCTTTAGATACATTCAGAGCGGATGA
ACAGGGATATTTAGGTTACTTTTCAAATGATGACGTGGAGTTTTACTACC
CACCAGTCAAGCCAAATGGATGGCAATTTTTTGACATTTCCAACCTCACA
GACCCTTCGGAAATTCCAGAAGTCATTATTCTGTACTCCTATCAAGGCTT
GAATCCTGAGCTAATAGTAAAGGCCGTCAAGGACCTGGGCGCAAAAGGTA
TCGTGTTGGCGGGTTCTGGAGCTGGTTCCTGGACTGCTACGGGTAGTATT
GTAAACGAACAACTTTATGAAGAGTATGGTATACCAATTGTTCACAGCAG
AAGAACAGCAGATGGTACAGTTCCTCCAGATGATGCCCCAGAGTACGCCA
TTGGATCTGGCTACCTAAACCCTCAAAAATCGCGTATTTTGCTACAATTA
TGTTTGTACTCCGGCTACGGCATGGATCAGATTAGGTCTGTTTTTTCTGG
CGTCTACGGTGGTTAAGCTAGCATTGAATTGAATTGAAATCGATAGATCA
ATTTTTTTCTTTTCTCTTTCCCCATCCTTTACGCTAAAATAATAGTTTAT
TTTATTTTTTGAATATTTTTTATTTATATACGTATATATAGACTATTATT
TATCTTTTAATGATTATTAAGATTTTTATTAAAAAAAAATTCGCTCCTCT
TTTAATGCCTCCGCGGCAAGAATGGCCGCTTTCATGGCCCTACAACATGA
GCCACCATTGCCTATTTGGTCCTTGGATAAAGCTAATGTTTTGGCCTCTT
CAAGATTATGGAGAAAAACTGTGGAGGAAACCATCAAGAACGAATTCCCT
ACATTGAAGGTTCAACATCAATTGATTGATTCTGCCGCCATGATCCTAGT
TAAGAACCCAACCCACCTAAATGGTATTATAATCACCAGCAACATGTTTG
GTGATATCATCTCCGATGAAGCCTCCGTTATCCCAGGTTCCTTGGGTTTG
TTGCCATCTGCGTCCTTGGCCTCTTTGCCAGACAAGAACACCGCATTTGG
TTTGTACGAACCATGCCACGGTTCTGCTCCAGATTTGCCAAAGAATAAGG
TCAACCCTATCGCCACTATCTTGTCTGCTGCAATGATGTTGAAATTGTCA
TTGAACTTGCCTGAAGAAGGTAAGGCCATTGAAGATGCAGTTAAAAAGGT
TTTGGATGCAGGTATCAGAACTGGTGATTTAAAT

Appendix 4: Nucleotide sequence of the linear LEU2-ASP3 cassette in FASTA format.

```
1         10         20         30         40         50
  |         |          |          |          |          |
  ATTTAAATCATTTAATTGGTGGTGCTGCTATCGATGCTACAGGTGTTCCA
  CTTCCAGATGAGGCGCTGGAAGCCTCCAAGAAGGCTGATGCCGTTTTGTT
  AGGTGCTGTGGGTGGTCCTAAATGGGGTACCGGTAGTGTTAGACCTGAAC
  AAGGTTTACTAAAAATCCGTAAAGAACTTCAATTGTACGCCAACTTAAGA
  CCATGTAACTTTGCATCCGACTCTCTTTTAGACTTATCTCCAATCAAGCC
  ACAATTTGCTAAAGGTACTGACTTCGTTGTTGTCAGAGAATTAGTGGGAG
  GTATTTACTTTGGTAAGAGAAAGGAAGACGATGGTGATGGTGTCGCTTGG
  GATAGTGAACAATACACCGTTCCAGAAGTGCAAAGAATCACGGCCGCAAC
  TCAAGACGCACAGATATTATAACATCTGCATAATAGGCATTTGCAAGAAT
  TACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATACCTGCATTTAAA
  GATGCCGATTTGGGCGCGAATCCTTTATTTTGGCTTCACCCTCATACTAT
  TATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCCTTCTTGAATTGATGTTA
  CCCTCATAAAGCACGTGGCCTCTTATCGAGAAAGAAATTACCGTCGCTCG
  TGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACACGCTCGAC
  TTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACAAGGT
  CCTAGCGACGGCTCACAGGTTTTGTAACAAGCAATCGAAGGTTCTGGAAT
  GGCGGGAAAGGGTTTAGTACCACATGCTATGATGCCCACTGTGATCTCCA
  GAGCAAAGTTCGTTCGATCGTACTGTTACTCTCTCTCTTTCAAACAGAAT
  TGTCCGAATCGTGTGACAACAACAGCCTGTTCTCACACACTCTTTTCTTC
  TAACCAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTGAAACTTACATTTA
  CATATATATAAACTTGCATAAATTGGTCAATGCAAGAAATACATATTTGG
  TCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGATGCTTTCTTTTTCTCT
  TTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAATATA
  AAACAACGCGTATGAGATCTTTAAATACCCTTTTACTTTCTCTCTTTGTC
  GCAATGTCCAGTGGTGCTCCACTACTAAAAATTCGTGAAGAGAAGAATTC
  TTCTTTGCCATCAATCAAAATTTTTGGTACCGGCGGTACTATCGCTTCCA
  AGGGTTCGACAAGTGCAACAACGGCGGGTTATAGCGTGGGATTAACCGTA
  AATGATTTAATAGAAGCCGTCCCATCTTTAGCTGAGAAGGCAAATCTGGA
  CTATCTTCAAGTGTCTAACGTTGGTTCAAATTCTTTAAACTATACGCATC
  TGATCCCATTGTATCACGGTATCTCCGAGGCACTAGCCTCTGATGACTAC
  GCTGGTGCGGTTGTCACTCATGGGACCGACACTATGGAGGAGACAGCTTT
  CTTCTTAGATTTGACCATAAATTCAGAGAAGCCAGTATGTATCGCAGGCG
  CTATGCGTCCAGCCACTGCCACGTCTGCTGATGGCCCAATGAATTTATAT
  CAAGCAGTGTCTATTGCTGCTTCTGAGAAATCACTGGGTCGTGGCACGAT
  GATCACTCTAAACGATCGTATTGCCTCTGGGTTTTGGACAACGAAAATGA
  ATGCCAACTCTTTAGATACATTCAGAGCGGATGAACAGGGATATTTAGGT
  TACTTTTCAAATGATGACGTGGAGTTTTACTACCCACCAGTCAAGCCAAA
  TGGATGGCAATTTTTTGACATTTCCAACCTCACAGACCCTTCGGAAATTC
  CAGAAGTCATTATTCTGTACTCCTATCAAGGCTTGAATCCTGAGCTAATA
  GTAAAGGCCGTCAAGGACCTGGGCGCAAAAGGTATCGTGTTGGCGGGTTC
  TGGAGCTGGTTCCTGGACTGCTACGGGTAGTATTGTAAACGAACAACTTT
  ATGAAGAGTATGGTATACCAATTGTTCACAGCAGAAGAACAGCAGATGGT
  ACAGTTCCTCCAGATGATGCCCCAGAGTACGCCATTGGATCTGGCTACCT
  AAACCCTCAAAAATCGCGTATTTTGCTACAATTATGTTTGTACTCCGGCT
  ACGGCATGGATCAGATTAGGTCTGTTTTTTCTGGCGTCTACGGTGGTTAA
  GCTAGCATTGAATTGAATTGAAATCGATAGATCAATTTTTTTCTTTTCTC
  TTTCCCCATCCTTTACGCTAAAATAATAGTTTATTTTATTTTTTGAATAT
  TTTTTATTTATATACGTATATATAGACTATTATTTATCTTTTAATGATTA
  TTAAGATTTTTATTAAAAAAAAATTCGCTCCTCTTTTAATGCCTCCGCGG
  CAAGAATGGCCGCTTTCATGGCCCTACAACATGAGCCACCATTGCCTATT
  TGGTCCTTGGATAAAGCTAATGTTTTGGCCTCTTCAAGATTATGGAGAAA
```

AACTGTGGAGGAAACCATCAAGAACGAATTCCCTACATTGAAGGTTCAAC
ATCAATTGATTGATTCTGCCGCCATGATCCTAGTTAAGAACCCAACCCAC
CTAAATGGTATTATAATCACCAGCAACATGTTTGGTGATATCATCTCCGA
TGAAGCCTCCGTTATCCCAGGTTCCTTGGGTTTGTTGCCATCTGCGTCCT
TGGCCTCTTTGCCAGACAAGAACACCGCATTTGGTTTGTACGAACCATGC
CACGGTTCTGCTCCAGATTTGCCAAAGAATAAGGTCAACCCTATCGCCAC
TATCTTGTCTGCTGCAATGATGTTGAAATTGTCATTGAACTTGCCTGAAG
AAGGTAAGGCCATTGAAGATGCAGTTAAAAAGGTTTTGGATGCAGGTATC
AGAACTGGTGATTTAAAT

Appendix 5: Nucleotide sequence of the linear URA3-KanMX-ASP3 cassette in FASTA format.

```
1         10         20         30         40         50
  |         |          |          |          |          |
  GCTGGCCGCATCTTCTCAAATATGCTTCCCAGCCTGCTTTTCTGTAACGT
  TCACCCTCTACCTTAGCATCCCTTCCCTTTGCAAATAGTCCTCTTCCAAC
  AATAATAATGTCAGATCCTGTAGAGACCACATCATCCACGGTTCTATACT
  GTTGACCCAATGCGTCTCCCTTGTCATCTAAACCCACACCGGGTGTCATA
  ATCAACCAATCGTAACCTTCATCTCTTCCACCCATGTCTCTTTGAGCAAT
  AAAGCCGATAACAAAATCTTTGTCGCTCTTCGCAATGTCAACAGTACCCT
  TAGTATATTCTCCAGTAGCTAGGGAGCCCTTGCATGACAATTCTGCTAAC
  ATCAAAAGGCCTCTAGGTTCCTTTGTTACTTCTTCCGCCGCCTCAACTCA
  AGACGCACAGATATTATAACATCTGCATAATAGGCATTTGCAAGAATTAC
  TCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATACCTGCATTTAAAGAT
  GCCGATTTGGGCGCGAATCCTTTATTTTGGCTTCACCCTCATACTATTAT
  CAGGGCCAGAAAAAGGAAGTGTTTCCCTCCTTCTTGAATTGATGTTACCC
  TCATAAAGCACGTGGCCTCTTATCGAGAAAGAAATTACCGTCGCTCGTGA
  TTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACACGCTCGACTTC
  CTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACAAGGTCCT
  AGCGACGGCTCACAGGTTTTGTAACAAGCAATCGAAGGTTCTGGAATGGC
  GGGAAAGGGTTTAGTACCACATGCTATGATGCCCACTGTGATCTCCAGAG
  CAAAGTTCGTTCGATCGTACTGTTACTCTCTCTCTTTCAAACAGAATTGT
  CCGAATCGTGTGACAACAACAGCCTGTTCTCACACACTCTTTTCTTCTAA
  CCAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTGAAACTTACATTTACAT
  ATATATAAACTTGCATAAATTGGTCAATGCAAGAAATACATATTTGGTCT
  TTTCTAATTCGTAGTTTTTCAAGTTCTTAGATGCTTTCTTTTTCTCTTTT
  TTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAATATAAAA
  CAATGGGTAAGGAAAAGACTCACGTTTCGAGGCCGCGATTAAATTCCAAC
  ATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCA
  ATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGT
  TGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAG
  ATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAA
  GCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCC
  CCGGCAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAA
  AATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCC
  TGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGG
  CGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGAC
  GAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCT
  TTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTG
  ATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGA
  CGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTG
  CCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATG
  GTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGAT
  GAGTTTTTCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAAC
  TTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAA
  TGTTAGCGTGATTTATATTTTTTTTCGCCTCGACATCATCTGCCCAGATG
  CGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATG
  CTGGTCGCTATACTGCTGTCGATTCGATACTAACGCCGCCATCCAGTGTC
  GACTCCGGACAACTCAAGACGCACAGATATTATAACATCTGCATAATAGG
  CATTTGCAAGAATTACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCAT
  ACCTGCATTTAAAGATGCCGATTTGGGCGCGAATCCTTTATTTTGGCTTC
  ACCCTCATACTATTATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCCTTCT
  TGAATTGATGTTACCCTCATAAAGCACGTGGCCTCTTATCGAGAAAGAAA
  TTACCGTCGCTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCC
```

AGACACGCTCGACTTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGT
CACACAACAAGGTCCTAGCGACGGCTCACAGGTTTTGTAACAAGCAATCG
AAGGTTCTGGAATGGCGGGAAAGGGTTTAGTACCACATGCTATGATGCCC
ACTGTGATCTCCAGAGCAAAGTTCGTTCGATCGTACTGTTACTCTCTCTC
TTTCAAACAGAATTGTCCGAATCGTGTGACAACAACAGCCTGTTCTCACA
CACTCTTTTCTTCTAACCAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTG
AAACTTACATTTACATATATATAAACTTGCATAAATTGGTCAATGCAAGA
AATACATATTTGGTCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGATGC
TTTCTTTTTCTCTTTTTTACAGATCATCAAGGAAGTAATTATCTACTTTT
TACAACAAATATAAAACAACGCGTATGAGATCTTTAAATACCCTTTTACT
TTCTCTCTTTGTCGCAATGTCCAGTGGTGCTCCACTACTAAAAATTCGTG
AAGAGAAGAATTCTTCTTTGCCATCAATCAAAATTTTTGGTACCGGCGGT
ACTATCGCTTCCAAGGGTTCGACAAGTGCAACAACGGCGGGTTATAGCGT
GGGATTAACCGTAAATGATTTAATAGAAGCCGTCCCATCTTTAGCTGAGA
AGGCAAATCTGGACTATCTTCAAGTGTCTAACGTTGGTTCAAATTCTTTA
AACTATACGCATCTGATCCCATTGTATCACGGTATCTCCGAGGCACTAGC
CTCTGATGACTACGCTGGTGCGGTTGTCACTCATGGGACCGACACTATGG
AGGAGACAGCTTTCTTCTTAGATTTGACCATAAATTCAGAGAAGCCAGTA
TGTATCGCAGGCGCTATGCGTCCAGCCACTGCCACGTCTGCTGATGGCCC
AATGAATTTATATCAAGCAGTGTCTATTGCTGCTTCTGAGAAATCACTGG
GTCGTGGCACGATGATCACTCTAAACGATCGTATTGCCTCTGGGTTTTGG
ACAACGAAAATGAATGCCAACTCTTTAGATACATTCAGAGCGGATGAACA
GGGATATTTAGGTTACTTTTCAAATGATGACGTGGAGTTTTACTACCCAC
CAGTCAAGCCAAATGGATGGCAATTTTTTGACATTTCCAACCTCACAGAC
CCTTCGGAAATTCCAGAAGTCATTATTCTGTACTCCTATCAAGGCTTGAA
TCCTGAGCTAATAGTAAAGGCCGTCAAGGACCTGGGCGCAAAAGGTATCG
TGTTGGCGGGTTCTGGAGCTGGTTCCTGGACTGCTACGGGTAGTATTGTA
AACGAACAACTTTATGAAGAGTATGGTATACCAATTGTTCACAGCAGAAG
AACAGCAGATGGTACAGTTCCTCCAGATGATGCCCCAGAGTACGCCATTG
GATCTGGCTACCTAAACCCTCAAAAATCGCGTATTTTGCTACAATTATGT
TTGTACTCCGGCTACGGCATGGATCAGATTAGGTCTGTTTTTTCTGGCGT
CTACGGTGGTTAAGCTAGCATTGAATTGAATTGAAATCGATAGATCAATT
TTTTTCTTTTCTCTTTCCCCATCCTTTACGCTAAAATAATAGTTTATTTT
ATTTTTTGAATATTTTTTATTTATATACGTATATATAGACTATTATTTAT
CTTTTAATGATTATTAAGATTTTTATTAAAAAAAAATTCGCTCCTCTTTT
AATGCCTCCGCGGGCTTCAAACCGCTAACAATACCTGGGCCCACCACACC
GTGTGCATTCGTAATGTCTGCCCATTCTGCTATTCTGTATACACCCGCAG
AGTACTGCAATTTGACTGTATTACCAATGTCAGCAAATTTTCTGTCTTCG
AAGAGTAAAAAATTGTACTTGGCGGATAATGCCTTTAGCGGCTTAACTGT
GCCCTCCATGGAAAAATCAGTCAAGATATCCACATGTGTTTTTAGTAAAC
AAATTTTGGGACCTAATGCTTCAACTAACTCCAGTAATTCCTTGGTGGTA
CGAACATCCAATGAAGCACACAAGTTTGTTTGCTTTTCGTGCATGATATT
AAATAGCTTGGCAGCAACAGGACTAGGATGAGTAGCAGCACGTTCCTTAT
ATGTAGC

Appendix 6: Nucleotide sequence of the linear URA3-ASP3 cassette in FASTA format.

```
1         10         20         30         40         50
  |         |          |          |          |          |
  GCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAA
  GCTATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCTTCATTGG
  ATGTTCGTACCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCC
  AAAATTTGTTTACTAAAAACACATGTGGATATCTTGACTGATTTTTCCAT
  GGAGGGCACAGTTAAGCCGCTAAAGGCATTATCCGCCAAGTACAATTTTT
  TACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATACAGTCAAATTG
  CAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGAA
  TGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCCGGCCG
  CAACTCAAGACGCACAGATATTATAACATCTGCATAATAGGCATTTGCAA
  GAATTACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATACCTGCATT
  TAAAGATGCCGATTTGGGCGCGAATCCTTTATTTTGGCTTCACCCTCATA
  CTATTATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCCTTCTTGAATTGAT
  GTTACCCTCATAAAGCACGTGGCCTCTTATCGAGAAAGAAATTACCGTCG
  CTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACACGCT
  CGACTTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACA
  AGGTCCTAGCGACGGCTCACAGGTTTTGTAACAAGCAATCGAAGGTTCTG
  GAATGGCGGGAAAGGGTTTAGTACCACATGCTATGATGCCCACTGTGATC
  TCCAGAGCAAAGTTCGTTCGATCGTACTGTTACTCTCTCTCTTTCAAACA
  GAATTGTCCGAATCGTGTGACAACAACAGCCTGTTCTCACACACTCTTTT
  CTTCTAACCAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTGAAACTTACA
  TTTACATATATATAAACTTGCATAAATTGGTCAATGCAAGAAATACATAT
  TTGGTCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGATGCTTTCTTTTT
  CTCTTTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAA
  TATAAAACAACGCGTATGAGATCTTTAAATACCCTTTTACTTTCTCTCTT
  TGTCGCAATGTCCAGTGGTGCTCCACTACTAAAAATTCGTGAAGAGAAGA
  ATTCTTCTTTGCCATCAATCAAAATTTTTGGTACCGGCGGTACTATCGCT
  TCCAAGGGTTCGACAAGTGCAACAACGGCGGGTTATAGCGTGGGATTAAC
  CGTAAATGATTTAATAGAAGCCGTCCCATCTTTAGCTGAGAAGGCAAATC
  TGGACTATCTTCAAGTGTCTAACGTTGGTTCAAATTCTTTAAACTATACG
  CATCTGATCCCATTGTATCACGGTATCTCCGAGGCACTAGCCTCTGATGA
  CTACGCTGGTGCGGTTGTCACTCATGGGACCGACACTATGGAGGAGACAG
  CTTTCTTCTTAGATTTGACCATAAATTCAGAGAAGCCAGTATGTATCGCA
  GGCGCTATGCGTCCAGCCACTGCCACGTCTGCTGATGGCCCAATGAATTT
  ATATCAAGCAGTGTCTATTGCTGCTTCTGAGAAATCACTGGGTCGTGGCA
  CGATGATCACTCTAAACGATCGTATTGCCTCTGGGTTTTGGACAACGAAA
  ATGAATGCCAACTCTTTAGATACATTCAGAGCGGATGAACAGGGATATTT
  AGGTTACTTTTCAAATGATGACGTGGAGTTTTACTACCCACCAGTCAAGC
  CAAATGGATGGCAATTTTTTGACATTTCCAACCTCACAGACCCTTCGGAA
  ATTCCAGAAGTCATTATTCTGTACTCCTATCAAGGCTTGAATCCTGAGCT
  AATAGTAAAGGCCGTCAAGGACCTGGGCGCAAAAGGTATCGTGTTGGCGG
  GTTCTGGAGCTGGTTCCTGGACTGCTACGGGTAGTATTGTAAACGAACAA
  CTTTATGAAGAGTATGGTATACCAATTGTTCACAGCAGAAGAACAGCAGA
  TGGTACAGTTCCTCCAGATGATGCCCCAGAGTACGCCATTGGATCTGGCT
  ACCTAAACCCTCAAAAATCGCGTATTTTGCTACAATTATGTTTGTACTCC
  GGCTACGGCATGGATCAGATTAGGTCTGTTTTTTCTGGCGTCTACGGTGG
  TTAAGCTAGCATTGAATTGAATTGAAATCGATAGATCAATTTTTTTCTTT
  TCTCTTTCCCCATCCTTTACGCTAAAATAATAGTTTATTTTATTTTTTGA
  ATATTTTTTATTTATATACGTATATATAGACTATTATTTATCTTTTAATG
  ATTATTAAGATTTTTATTAAAAAAAAATTCGCTCCTCTTTTAATGCCTCC
  GCGGAGGCGGCGGAAGAAGTAACAAAGGAACCTAGAGGCCTTTTGATGTT
```

AGCAGAATTGTCATGCAAGGGCTCCCTAGCTACTGGAGAATATACTAAGG
GTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTATCGGCTTTATT
GCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTAT
GACACCCGGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGT
ATAGAACCGTGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTT
GGAAGAGGACTATTTGCAAAGGGAAGGGATGCTAAGGTAGAGGGTGAACG
TTACAGAAAAGCAGGCTGGGAAGCATATTTGAGAAGATGCGGCCAGC

Appendix 7: Nucleotide sequence of the linear TRP1-KanMX-ASP3 cassette in FASTA format.

```
1         10         20         30         40         50
  |         |          |          |          |          |
  GCTTGTCACCTTACGTACAATCTTGATCCGGAGCTTTTCTTTTTTTGCCG
  ATTAAGAATTCGGTCGAAAAAAGAAAAGGAGAGGGCCAAGAGGGAGGGCA
  TTGGTGACTATTGAGCACGTGAGTATACGTGATTAAGCACACAAAGGCAG
  CTTGGAGTATGTCTGTTATTAATTTCACAGGTAGTTCTGGTCCATTGGTG
  AAAGTTTGCGGCTTGCAGAGCACAGAGGCCGCAGAATGTGCTCTAGATTC
  CGATGCTGACTTGCTGGGTATTATATGTGTGCCCAATAGAAAGAGAACAA
  TTGACCCGGTTATTGCAAGGAAAATTTCAAGTCTTGTAAAAGCATATAAA
  AATAGTTCAGGCACTCCGAAATACTTGGTTGGCGTGTTTCGTAATCAACC
  TAAGGAGGATGTTTTGGCTCTGGTCAATGATTACGGCATTGCGGCCGCAA
  CTCAAGACGCACAGATATTATAACATCTGCATAATAGGCATTTGCAAGAA
  TTACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATACCTGCATTTAA
  AGATGCCGATTTGGGCGCGAATCCTTTATTTTGGCTTCACCCTCATACTA
  TTATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCCTTCTTGAATTGATGTT
  ACCCTCATAAAGCACGTGGCCTCTTATCGAGAAAGAAATTACCGTCGCTC
  GTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACACGCTCGA
  CTTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACAAGG
  TCCTAGCGACGGCTCACAGGTTTTGTAACAAGCAATCGAAGGTTCTGGAA
  TGGCGGGAAAGGGTTTAGTACCACATGCTATGATGCCCACTGTGATCTCC
  AGAGCAAAGTTCGTTCGATCGTACTGTTACTCTCTCTCTTTCAAACAGAA
  TTGTCCGAATCGTGTGACAACAACAGCCTGTTCTCACACACTCTTTTCTT
  CTAACCAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTGAAACTTACATTT
  ACATATATATAAACTTGCATAAATTGGTCAATGCAAGAAATACATATTTG
  GTCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGATGCTTTCTTTTTCTC
  TTTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAATAT
  AAAACAATGGGTAAGGAAAAGACTCACGTTTCGAGGCCGCGATTAAATTC
  CAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCG
  GGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCA
  GAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGA
  TGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCA
  TCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCG
  ATCCCCGGCAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGG
  TGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGA
  TTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCT
  CAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGA
  TGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATA
  AGCTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCA
  CTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGT
  TGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGA
  ACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAA
  TATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCT
  CGATGAGTTTTTCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAA
  GAACTTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAAT
  CAAATGTTAGCGTGATTTATATTTTTTTTCGCCTCGACATCATCTGCCCA
  GATGCGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTG
  AATGCTGGTCGCTATACTGCTGTCGATTCGATACTAACGCCGCCATCCAG
  TGTCGACTCCGGACAACTCAAGACGCACAGATATTATAACATCTGCATAA
  TAGGCATTTGCAAGAATTACTCGTGAGTAAGGAAAGAGTGAGGAACTATC
  GCATACCTGCATTTAAAGATGCCGATTTGGGCGCGAATCCTTTATTTTGG
  CTTCACCCTCATACTATTATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCC
  TTCTTGAATTGATGTTACCCTCATAAAGCACGTGGCCTCTTATCGAGAAA
```

GAAATTACCGTCGCTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAA
ACCCAGACACGCTCGACTTCCTGTCTTCCTATTGATTGCAGCTTCCAATT
TCGTCACACAACAAGGTCCTAGCGACGGCTCACAGGTTTTGTAACAAGCA
ATCGAAGGTTCTGGAATGGCGGGAAAGGGTTTAGTACCACATGCTATGAT
GCCCACTGTGATCTCCAGAGCAAAGTTCGTTCGATCGTACTGTTACTCTC
TCTCTTTCAAACAGAATTGTCCGAATCGTGTGACAACAACAGCCTGTTCT
CACACACTCTTTTCTTCTAACCAAGGGGGTGGTTTAGTTTAGTAGAACCT
CGTGAAACTTACATTTACATATATATAAACTTGCATAAATTGGTCAATGC
AAGAAATACATATTTGGTCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAG
ATGCTTTCTTTTTCTCTTTTTTACAGATCATCAAGGAAGTAATTATCTAC
TTTTTACAACAAATATAAAACAACGCGTATGAGATCTTTAAATACCCTTT
TACTTTCTCTCTTTGTCGCAATGTCCAGTGGTGCTCCACTACTAAAAATT
CGTGAAGAGAAGAATTCTTCTTTGCCATCAATCAAAATTTTTGGTACCGG
CGGTACTATCGCTTCCAAGGGTTCGACAAGTGCAACAACGGCGGGTTATA
GCGTGGGATTAACCGTAAATGATTTAATAGAAGCCGTCCCATCTTTAGCT
GAGAAGGCAAATCTGGACTATCTTCAAGTGTCTAACGTTGGTTCAAATTC
TTTAAACTATACGCATCTGATCCCATTGTATCACGGTATCTCCGAGGCAC
TAGCCTCTGATGACTACGCTGGTGCGGTTGTCACTCATGGGACCGACACT
ATGGAGGAGACAGCTTTCTTCTTAGATTTGACCATAAATTCAGAGAAGCC
AGTATGTATCGCAGGCGCTATGCGTCCAGCCACTGCCACGTCTGCTGATG
GCCCAATGAATTTATATCAAGCAGTGTCTATTGCTGCTTCTGAGAAATCA
CTGGGTCGTGGCACGATGATCACTCTAAACGATCGTATTGCCTCTGGGTT
TTGGACAACGAAAATGAATGCCAACTCTTTAGATACATTCAGAGCGGATG
AACAGGGATATTTAGGTTACTTTTCAAATGATGACGTGGAGTTTTACTAC
CCACCAGTCAAGCCAAATGGATGGCAATTTTTTGACATTTCCAACCTCAC
AGACCCTTCGGAAATTCCAGAAGTCATTATTCTGTACTCCTATCAAGGCT
TGAATCCTGAGCTAATAGTAAAGGCCGTCAAGGACCTGGGCGCAAAAGGT
ATCGTGTTGGCGGGTTCTGGAGCTGGTTCCTGGACTGCTACGGGTAGTAT
TGTAAACGAACAACTTTATGAAGAGTATGGTATACCAATTGTTCACAGCA
GAAGAACAGCAGATGGTACAGTTCCTCCAGATGATGCCCCAGAGTACGCC
ATTGGATCTGGCTACCTAAACCCTCAAAAATCGCGTATTTTGCTACAATT
ATGTTTGTACTCCGGCTACGGCATGGATCAGATTAGGTCTGTTTTTTCTG
GCGTCTACGGTGGTTAAGCTAGCATTGAATTGAATTGAAATCGATAGATC
AATTTTTTTCTTTTCTCTTTCCCCATCCTTTACGCTAAAATAATAGTTTA
TTTTATTTTTTGAATATTTTTTATTTATATACGTATATATAGACTATTAT
TTATCTTTTAATGATTATTAAGATTTTTATTAAAAAAAAATTCGCTCCTC
TTTTAATGCCTCCGCGGTCGTGGCAAGAATACCAAGAGTTCCTCGGTTTG
CCAGTTATTAAAAGACTCGTATTTCCAAAAGACTGCAACATACTACTCAG
TGCAGCTTCACAGAAACCTCATTCGTTTATTCCCTTGTTTGATTCAGAAG
CAGGTGGGACAGGTGAACTTTTGGATTGGAACTCGATTTCTGACTGGGTT
GGAAGGCAAGAGAGCCCCGAAAGCTTACATTTTATGTTAGCTGGTGGACT
GACGCCAGAAAATGTTGGTGATGCGCTTAGATTAAATGGCGTTATTGGTG
TTGATGTAAGCGGAGGTGTGGAGACAAATGGTGTAAAAGACTCTAACAAA
ATAGCAAATTTCGTCAAAAATGCTAAGAAATAGGTTATTACTGAGTAGTA
TTTATTTAAGTATTGTTTGTGCACTTGCCTGCAGGCCTTTTGAAAAGCAA
GCATAAAAGATCTAAACATAAAATCTGTAAAATAACAAGATGTAAAGATA
ATGCTAAATCATTTGGCTTTTTGATTGATTGTACAGGAAAATATACATCG
CAGGGGGTTGACTTTTACCATTTCACCGCAATGGAATCAAACTTGTTGAA
GAGAATGTTCACAGGCGCATACGCTACAATGACCCGATTCTTGCTAGC

Appendix 8: Nucleotide sequence of the linear TRP1-ASP3 cassette in FASTA format.

```
1         10         20         30         40         50
  |         |          |          |          |          |
  GCTTGTCACCTTACGTACAATCTTGATCCGGAGCTTTTCTTTTTTTGCCG
  ATTAAGAATTCGGTCGAAAAAAGAAAAGGAGAGGGCCAAGAGGGAGGGCA
  TTGGTGACTATTGAGCACGTGAGTATACGTGATTAAGCACACAAAGGCAG
  CTTGGAGTATGTCTGTTATTAATTTCACAGGTAGTTCTGGTCCATTGGTG
  AAAGTTTGCGGCTTGCAGAGCACAGAGGCCGCAGAATGTGCTCTAGATTC
  CGATGCTGACTTGCTGGGTATTATATGTGTGCCCAATAGAAAGAGAACAA
  TTGACCCGGTTATTGCAAGGAAAATTTCAAGTCTTGTAAAAGCATATAAA
  AATAGTTCAGGCACTCCGAAATACTTGGTTGGCGTGTTTCGTAATCAACC
  TAAGGAGGATGTTTTGGCTCTGGTCAATGATTACGGCATTGCGGCCGTCC
  GGACAACTCAAGACGCACAGATATTATAACATCTGCATAATAGGCATTTG
  CAAGAATTACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATACCTGC
  ATTTAAAGATGCCGATTTGGGCGCGAATCCTTTATTTTGGCTTCACCCTC
  ATACTATTATCAGGGCCAGAAAAAGGAAGTGTTTCCCTCCTTCTTGAATT
  GATGTTACCCTCATAAAGCACGTGGCCTCTTATCGAGAAAGAAATTACCG
  TCGCTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACAC
  GCTCGACTTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACA
  ACAAGGTCCTAGCGACGGCTCACAGGTTTTGTAACAAGCAATCGAAGGTT
  CTGGAATGGCGGGAAAGGGTTTAGTACCACATGCTATGATGCCCACTGTG
  ATCTCCAGAGCAAAGTTCGTTCGATCGTACTGTTACTCTCTCTCTTTCAA
  ACAGAATTGTCCGAATCGTGTGACAACAACAGCCTGTTCTCACACACTCT
  TTTCTTCTAACCAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTGAAACTT
  ACATTTACATATATATAAACTTGCATAAATTGGTCAATGCAAGAAATACA
  TATTTGGTCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGATGCTTTCTT
  TTTCTCTTTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAAC
  AAATATAAAACAACGCGTATGAGATCTTTAAATACCCTTTTACTTTCTCT
  CTTTGTCGCAATGTCCAGTGGTGCTCCACTACTAAAAATTCGTGAAGAGA
  AGAATTCTTCTTTGCCATCAATCAAAATTTTTGGTACCGGCGGTACTATC
  GCTTCCAAGGGTTCGACAAGTGCAACAACGGCGGGTTATAGCGTGGGATT
  AACCGTAAATGATTTAATAGAAGCCGTCCCATCTTTAGCTGAGAAGGCAA
  ATCTGGACTATCTTCAAGTGTCTAACGTTGGTTCAAATTCTTTAAACTAT
  ACGCATCTGATCCCATTGTATCACGGTATCTCCGAGGCACTAGCCTCTGA
  TGACTACGCTGGTGCGGTTGTCACTCATGGGACCGACACTATGGAGGAGA
  CAGCTTTCTTCTTAGATTTGACCATAAATTCAGAGAAGCCAGTATGTATC
  GCAGGCGCTATGCGTCCAGCCACTGCCACGTCTGCTGATGGCCCAATGAA
  TTTATATCAAGCAGTGTCTATTGCTGCTTCTGAGAAATCACTGGGTCGTG
  GCACGATGATCACTCTAAACGATCGTATTGCCTCTGGGTTTTGGACAACG
  AAAATGAATGCCAACTCTTTAGATACATTCAGAGCGGATGAACAGGGATA
  TTTAGGTTACTTTTCAAATGATGACGTGGAGTTTTACTACCCACCAGTCA
  AGCCAAATGGATGGCAATTTTTTGACATTTCCAACCTCACAGACCCTTCG
  GAAATTCCAGAAGTCATTATTCTGTACTCCTATCAAGGCTTGAATCCTGA
  GCTAATAGTAAAGGCCGTCAAGGACCTGGGCGCAAAAGGTATCGTGTTGG
  CGGGTTCTGGAGCTGGTTCCTGGACTGCTACGGGTAGTATTGTAAACGAA
  CAACTTTATGAAGAGTATGGTATACCAATTGTTCACAGCAGAAGAACAGC
  AGATGGTACAGTTCCTCCAGATGATGCCCCAGAGTACGCCATTGGATCTG
  GCTACCTAAACCCTCAAAAATCGCGTATTTTGCTACAATTATGTTTGTAC
  TCCGGCTACGGCATGGATCAGATTAGGTCTGTTTTTTCTGGCGTCTACGG
  TGGTTAAGCTAGCATTGAATTGAATTGAAATCGATAGATCAATTTTTTTC
  TTTTCTCTTTCCCCATCCTTTACGCTAAAATAATAGTTTATTTTATTTTT
  TGAATATTTTTTATTTATATACGTATATATAGACTATTATTTATCTTTTA
  ATGATTATTAAGATTTTTATTAAAAAAAAATTCGCTCCTCTTTTAATGCC
```

TCCGCGGTCGTGGCAAGAATACCAAGAGTTCCTCGGTTTGCCAGTTATTA
AAAGACTCGTATTTCCAAAAGACTGCAACATACTACTCAGTGCAGCTTCA
CAGAAACCTCATTCGTTTATTCCCTTGTTTGATTCAGAAGCAGGTGGGAC
AGGTGAACTTTTGGATTGGAACTCGATTTCTGACTGGGTTGGAAGGCAAG
AGAGCCCCGAAAGCTTACATTTTATGTTAGCTGGTGGACTGACGCCAGAA
AATGTTGGTGATGCGCTTAGATTAAATGGCGTTATTGGTGTTGATGTAAG
CGGAGGTGTGGAGACAAATGGTGTAAAAGACTCTAACAAAATAGCAAATT
TCGTCAAAAATGCTAAGAAATAGGTTATTACTGAGTAGTATTTATTTAAG
TATTGTTTGTGCACTTGCCTGCAGGCCTTTTGAAAAGCAAGCATAAAAGA
TCTAAACATAAAATCTGTAAAATAACAAGATGTAAAGATAATGCTAAATC
ATTTGGCTTTTTGATTGATTGTACAGGAAAATATACATCGCAGGGGGTTG
ACTTTTACCATTTCACCGCAATGGAATCAAACTTGTTGAAGAGAATGTTC
ACAGGCGCATACGCTACAATGACCCGATTCTTGCTAGC

5. References

1. Barlow, S., and Schlatter, J. 2010. Risk assessment of carcinogens in food. *Toxicology and Applied Pharmacology*. **243**: 180□190.

2. Codon, A.C., Benitez, T., Korhola, M. 1997. Chromosomal reorganization during meiosis of *Saccharomyces cerevisiae* baker□s yeast. *Curent Genetics*. **32**: 247-259.

3. Dunlop, P.C., Meyer, G.M., Roon, R.J. 1980. Nitrogen catabolite repression of asparaginase II in Saccharomyces cerevisiae. *J. Bacteriol.* **143**: 422□426.

4. Evans, I.H. Yeast strains for baking: recent developments. In: *Yeast Technology*, J.F.T. Spencer, D.M. Spencer, eds., Heidelber: Springer-Verlag, 1990, pp 13-54.

5. Friedman, M. 2003. Chemistry, biochemistry, and safety of acrylamide. A review. *J. Agric. Food Chem.* **51**: 4504-4526.

6. Food and Drug Administration, Department of Health and Human Services. 2009. Acrylamide in food; request for comments and food scientific data and information. Federal Register, **74**(164): 43134-43138.

7. Granby, K., Nielsen, N.J., Hedegaard, R.V., Christenen, T., Kann, M., and Skibsted, L.H. 2008. Acrylamide-asparagine relationship in baked□toasted wheat and rye breads. *Food Addit. Contam.* **8**: 921-929.

8. Health Canada Website http:□□www.hc-sc.gc.ca□fn-an□securit□chem-chim□food-aliment□acrylamide□index-eng.php

9. Hogervorst, J. G., Schouten, L. J., Konings, E. J., Goldbohm, R. A. 2008. Van Den Brandt, P. A. Dietary acrylamide intake and the risk of renal cell, bladder, and prostate cancer. *Am. J. Clin. Nutr.* **87**: 1428□1438.

10. Hogervorst, J. G., Schouten, L. J., Konings, E. J., Goldbohm, R. A., Van Den Brandt, P. A., A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer. *Cancer Epidemiol. Biomarkers Prev.* 2008, **16**: 2304□2313.

11. IARC. 1994. Monographs on the evaluation of carcinogenic risks to humans. Volume **60**. Lyon, France: *International Agency for Research on Cancer*.

12. Kim, K.W., Kamerud, J.Q., Livingston, D.M., Roon, R.J. 1988. Asparaginase II of Saccharomyces cerevisiae. Characterization of the ASP3 gene. *J. Biol. Chem.* **263**: 11948□11953.

13. Lingnert, H., Grivas, S., J□gerstad, M., Skog, K., T□rnqvist, M., and □man, P. 2002. Acrylamide in food: Mechanisms of formation and influencing factors during heating of foods. *Scand. J. Nutr*. **46**:159-172.

14. Mottram, D. S., Wedzicha, B. I., Dodson, A. T. 2002. Acrylamide is formed in the Maillard reaction. *Nature.* **419**: 448–449.

15. Olesen, P.T., Olsen, A., Frandsen, H., Frederiksen, K., Overvad, K., Tjonneland, A. 2008. Acrylamide exposure and incidence of breast cancer among postmenopausal women in the Danish Diet, Cancer and Health Study. *Int. J. Cancer.* **122**(9): 2094-100.

16. Reed R., Nagodawithana T.W. *Yeast Technology*. 2nd edition. New York : Van Nostrand Reinhold. 1991. 454 pp.

17. Rice, J.M., 2005. The carcinogenicity of acrylamide. *Mutation Research*. **530**: 3-20.

18. Rydberg, P., Eriksson, S., Tareke, E., Karlsson, P., Ehrenberg, L., and Törnquist, M. 2003. Investigations of factors that will influence the acrylamide content of heated foodstuffs. *J. Agric. Food Chem.* **51**:7012-7018.

19. Stadler, R. H., Blank, I., Varga, N., Robert, F. *et al*. 2002. Acrylamide from Maillard reaction products. *Nature.* **419**: 449–450.

20. Surdyk, N., Rosén, J., Anderson, R., Aman, P. 2004. Effects of asparagine, fructose and baking conditions on acrylamide content in yeast-leavened wheat bread. *Journal of Agriculture and Food Chemistry*. **52**: 2047-2057.

21. Svensson, K., Abramsson, L., Becker, W., Glynn, A., Hellenäs, K.-E., Lind, Y., and Rosén, J. 2003. Dietary intake of acrylamide in Sweden. *Food Chem. Toxicol*. **41**:1581-1586.

22. Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., Törnqvist, M. 2002. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *Journal of Agricultural and Food Chemistry*. **50**: 4998–5006.

23. Wilson, K.M., Mucci, L.A., Rosner, B.A., Willett, W.C. 2010. A prospective study of dietary acrylamide intake and the risk of breast, endometrial, and ovarian cancers. *Cancer Epidemiol Biomarkers Prev.* **19**(10): 2503-2515.

24. Yaylayan, V. A., Wnorowski, A., Locas, C. P. 2003. Why asparagine needs carbohydrates to generate acrylamide. *J. Agric. Food Chem.* **51**: 1753–1757.

25. Zyzak, D. V., Sanders, S. A., Stojanovic, M., Tallmadge, D. H. *et al*. 2003. Acrylamide formation mechanism in heated foods. *J. Agric. Food Chem.* **51**: 4782–4787.

Farias, Bianca *

From: John Husnik [jihusnik.functionaltechcorp@gmail.com]
Sent: Wednesday, February 29, 2012 8:45 PM
To: Ramos-Valle, Moraima
Cc: Farias, Bianca *
Subject: RE: GRAS submission
Attachments: GRAS Exemption Claim - Page 7.docx

Hello Moraima,

Please find attached a revised page 7 of our submission that contains the GRAS Exemption Claim as we discussed.

Please let me know if this is okay.

Thank you,

John

John Husnik, Ph.D.
Senior Research Scientist
Phyterra Yeast Inc./Functional Technologies Corp.

This e-mail, any attachments and their respective contents are CONFIDENTIAL and are intended only for the use of the person to whom it is addressed. Any distribution, copying or other use by anyone else is strictly prohibited. If you have received this message in error, please telephone or e-mail us immediately and delete this message. Thank you.

From: Ramos-Valle, Moraima [mailto:Moraima.Ramos-Valle@fda.hhs.gov]
Sent: February-29-12 8:25 AM
To: Ramos-Valle, Moraima; 'John Husnik'; 'jihusnik.functionaltechcorp@gmail.com'
Cc: Farias, Bianca *
Subject: RE: GRAS submission

Dear Dr. Husnik,

This message is to follow up with our phone conversation regarding the GRAS submission on "functionally -enhanced baker's yeast" and the revisions that we discussed in order to make your submission filable.

Please call me or email me at your earliest convenience.

Moraima

Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration
Phone: 240-402-1248
Email: Moraima.Ramos-Valle@fda.hhs.gov

From: John Husnik [mailto:john.husnik@phyterra.com]
Sent: Thursday, January 21, 2010 8:36 AM
To: Ramos-Valle, Moraima
Cc: Farias, Bianca
Subject: RE: GRAS submission

Dear Moraima,

Thank you for the assistance and we will shortly prepare an updated submission for your review.

Thank you,

John

From: Ramos-Valle, Moraima [mailto:Moraima.Ramos-Valle@fda.hhs.gov]
Sent: January 20, 2010 5:36 PM
To: john.husnik@phyterra.com
Cc: Farias, Bianca; Ramos-Valle, Moraima
Subject: GRAS submission

Date: 1/20/2010
Phone: 902-892-1074

Dear Dr. Husnik,

This email is to follow up our earlier conversation between:
Ms. Bianca Farias (FDA)
Mrs. Moraima Ramos (FDA)
And
Dr. John Husnik (Phyterra)

As I mentioned we have received your GRAS submission dated January 7, 2009 for the use of "Modified Yeast P1Y0" in foods, but we can not file it as it is since we could not locate all the items of the GRAS exemption claim.

The issues discussed during our conversation were the following:

1. The GRAS exemption claim statement is missing from the submission
2. We could not locate the section with the signature of the notifier
3. we need two additional copies (three copies of the submission are required)
4. We clarified that Biofuels is not under the Food and Drug Administration purview.
5. We requested clarification about the intended use: food categories and use levels.
6. Who was the best contact person and phone number?

Below are some helpful links that provide information on what to include in a GRAS exemption claim.

The link to "How to submit a GRAS notice":
http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083062.htm

Below is some text extracted from the GRAS proposed Rule, related to the GRAS exemption claim section:

```
1. GRAS Exemption Claim
    A GRAS determination must comply with the provisions of Sec. 170.30
and the person making such determination is responsible for ensuring
such compliance, regardless of whether that person notifies the agency
about the determination. Accordingly, proposed Sec. 170.36(c)(1)
requires that the notice include a claim (hereinafter referred to as
the ``GRAS exemption claim''), dated and signed by the notifier, that a
particular use of a substance is exempt from the premarket approval
requirements of the act because the notifier has determined that such
```

use is GRAS. Proposed Sec. 170.36(c)(1) would distinguish the notification procedure, in which the notifier explicitly accepts responsibility for the GRAS determination, from the GRAS petition process, in which the notifier is requesting that the agency affirm the GRAS status of use of a substance.

If you wish to see the entire proposed rule it is accessible online at: http://www.gpo.gov/fdsys/pkg/FR-1997-04-17/html/97-9706.htm

We also have recently added to our inventory list the releasable sections of some GRAS notices, so this is helpful to notifiers because they can use them as a guide to prepare their packages: http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing

Please let me know if you have any questions before sending your submission.

Sincerely,
Moraima

Moraima J. Ramos Valle
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration
Phone: 301-436-1248
Email: Moraima.Ramos-Valle@fda.hhs.gov

1. GRAS Exemption Claim

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)

As defined herein, Functional Technologies' Acrylamide-Preventing Yeast, has been determined by Functional Technologies Corporation to be Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This determination is based on scientific procedures as described in the following sections, and on the consensus opinion of an appropriately convened panel of Experts qualified by scientific training and expertise to evaluate the safety of Acrylamide-Preventing yeast under the conditions of intended use in foods. Therefore, the use of Acrylamide-Preventing Yeast in food as described herein is exempt from the requirement of premarket approval (Section 409 of the Federal Food, Drug and Cosmetic Act).

1.1 Name and address of the notifier

Functional Technologies Corporation
Suite 1500 - 885 West Georgia Street
Vancouver, British Columbia, V6C 3E8
Canada

Telephone: (604) 648-2200
Facsimile: (604) 648-2201

Scientific Manager: Dr. John Husnik
jihusnik.functionaltechcorp@gmail.com

Regulatory: Dr. John Husnik
jihusnik.functionaltechcorp@gmail.com

The research and development of an Acrylamide-Preventing yeast strain was completed in the research laboratory of Functional Technologies Corp., Charlottetown, PEI, Canada. The technology is based on the *ASP3* gene that encodes for the enzyme L-asparaginase II which is involved in asparagine catabolism.

SUBMISSION END