

GRAS Notice (GRN) No. 526

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

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ORIGINAL SUBMISSION

000001



# UNIQUE BIOTECH LIMITED

June 20, 2014

# 526  
GRN 000526

Office of Food Additive Safety (HFS-255)  
Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5100 Paint Branch Parkway  
College Park, MD 20740-3835

**Subject: GRAS Notification for *Bacillus coagulans* Unique IS2**

Dear Sir/Madam:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), Unique Biotech Limited, India, hereby provides notice of a claim that the food ingredient *Bacillus coagulans* Unique IS2 spore preparation described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures.

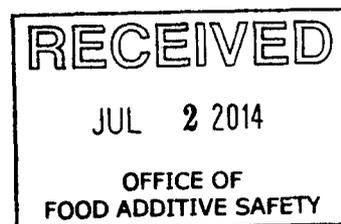
As required, please find enclosed three copies of the notification. If you have any questions or require additional information, please feel free to contact me by phone Phone: +91 -40-23751346/47 or Fax: +91 -40-23751345 or by E-mail: [sudha@uniquebiotech.com](mailto:sudha@uniquebiotech.com). Alternatively, in the US you can also contact Dr. Soni who assisted us with this notice. You can reach him by phone at +1-772-299-0746 or by email at [sonim@bellsouth.net](mailto:sonim@bellsouth.net).

Sincerely,

(b) (6)

Ratna Sudha, Ph.D.  
Managing Director  
Unique Biotech Ltd

Enclosure: Three copies of the GRAS notice



000002



## GRAS NOTIFICATION

### I. Claim of GRAS Status

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

Unique Biotech Limited (the notifier) has determined that *Bacillus coagulans* Unique IS2 spore preparation is Generally Recognized As Safe, 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, under the conditions of its intended use as a food ingredient. Therefore, the use of *Bacillus coagulans* Unique IS2 spore preparation is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Date 20.06.2014

Ratna Sudha, Ph.D.  
Managing Director  
Unique Biotech Ltd

**B. Name and Address of Notifier:**

Ratna Sudha, Ph.D.  
Unique Biotech Ltd  
G - 43, Madhura Nagar, Yousufguda,  
Hyderabad - 500034, A.P.  
INDIA.  
Phone: +91 -40-23751346/47  
Fax: +91 -40-23751345  
E-mail: sudha@uniquebiotech.com

**C. Common or Usual Name of the Notified Substance:**

The common name of the substance of this notification is *Bacillus coagulans* Unique IS2. The preparation contains spores.

**D. Conditions of Intended Use in Food**

*Bacillus coagulans* Unique IS2 spore preparation, is intended for use as a probiotic in the following food categories: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups at a maximum level of approximately  $2 \times 10^9$  colony forming units (CFU)/serving (reference amounts customarily consumed, 21CFR 101.12). *B. coagulans* Unique IS2 spore preparation is not proposed for uses in foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as it is not intended for use in meat and poultry products that come under USDA jurisdictions. The intended use of *B. coagulans* Unique IS2 spore in the above mentioned food categories, is estimated to result in a maximum daily intake of  $36.4 \times 10^9$  cfu/day.

**E. Basis for GRAS Determination:**

In accordance with 21 CFR 170.30, the intended use of *B. coagulans* Unique IS2 spore preparation has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. The determination is supported by the opinion of the Expert Panel. A comprehensive search of the scientific literature was also utilized for this determination. There exists sufficient qualitative and quantitative scientific evidence, including human and animal data to determine safety-in-use for *B. coagulans* Unique IS2 spore preparation. The safety determination of *B. coagulans* Unique IS2 spore is based on the totality of available evidence.

There are different strains of *Bacillus coagulans* that are used as a probiotic to improve and maintain ecological balance of the intestinal microflora. In the published literature several experimental studies, including subchronic toxicity, chronic toxicity, reproduction toxicity, *in*

*vitro* and *in vivo* genotoxicity and human clinical trials, have appeared. All these studies support the safety in use of *B. coagulans* at the intended use levels.

**F. Availability of Information:**

The data and information that forms the basis for this GRAS determination will be provided to Food and Drug Administration upon request or will be available for FDA review and copying at reasonable times at the above mentioned offices of the notifier (Section I, B) or in the US by contacting one of the Expert Panel members: Madhu G. Soni, PhD, FATS, Soni & Associates Inc., 749 46<sup>th</sup> Square, Vero Beach, FL 32068; Telephone: +1-772-299-0746; Email: sonim@bellsouth.net

**II. Detailed Information About the Identity of the Notified Substance:**

*Bacillus coagulans* Unique IS2 spore preparation is a standardized brown colored powder (200 billion cfu/g product) or white to beige colored powder (15 billion cfu/g product). It is a member of a subgroup of *Bacillus* spp. and is isolated from human fecal soil (*soil* contaminated with *human feces*).

**A. Common or Usual Name:**

*Bacillus coagulans* Unique IS2.

**B. Identity of Microorganism:**

*B. coagulans* Unique IS2 is a gram-positive, catalase-positive, spore forming, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. The strain has been deposited with the American Type Culture Collection (ATCC) - assigned number ATCC PTA-11748 and with the Microbial Type Culture Collection (MTCC) and Gene Bank with an accession number of *B. coagulans* Unique IS-2 (MTCC 5260). The NCBI accession number of *B. coagulans* Unique IS-2 is FN675759 (NCBI database).

The subject strain of present GRAS assessment, *B. coagulans* Unique IS2 spore preparation is selected from a total number of 29 isolates of *Bacillus* spp that were microscopically observed and biochemically tested. *B. coagulans* Unique IS2 has been extensively studied for phenotypic and genotypic characteristics. The biochemical tests employed for identification includes: catalase (positive), indole test (negative), gelatin hydrolysis (negative), gram reaction (positive), and lactic acid production (positive). The genotypic identification of *B. coagulans* Unique IS2 was carried out using the following tests: (A) by mole G+C content of DNA, (B) 16S rRNA gene sequence and Phylogenetic analysis, and (C) DNA-DNA hybridization.

The mole % G+C content of 46 for *B. coagulans* Unique IS2 was similar to 45.5% noted for *B. coagulans* ATCC 7050 (well characterized strain). Additionally, the *B. coagulans* Unique IS2 was confirmed at the genus and species level by specific PCR assays as well as by 16S rRNA sequencing and was subjected to a battery of tests as per FAO/WHO guidelines. The DNA-DNA hybridization shows a high level of DNA-DNA homology (88%) between *B. coagulans* Unique IS2 and *B. coagulans* ATCC 7050. Thus the available information

supports the characterization and identity of *B. coagulans* Unique IS2, the subject of this GRAS assessment.

### C. Typical Specifications

Typical food grade specifications of *Bacillus coagulans* Unique IS2 preparation are presented in Tables II-C.1. Analytical data from five non-consecutive lots with strength  $200 \times 10^9$  cfu/g and five lots with strength  $15 \times 10^9$  cfu/g are presented in Appendix I. These data suggest that *B. coagulans* Unique IS2 is consistently manufactured to meet the standard product specifications.

**Table II-C.1. Food Grade Specifications of *Bacillus coagulans* Unique IS2 Preparations**

<b>Parameter</b>	<b>Characteristics (Unique Biotech, 2014)*</b>
Appearance	Brown colored powder (200 billion cfu/g product) White to beige colored powder (15 billion cfu/g product)
Description	Characteristic odor and slightly sweet in taste
Microscopy	The spores are seen as small terminal oval shape refractile bodies at the end of each vegetative cell
Lactic acid producing capacity (ml)	Not less than 10 ml of 0.05 N NaOH should be consumed
Assay (cfu/g)	Not less than 200 Billion cfu/g Not less than 15 Billion cfu/g
Loss on drying (%)	Not more than 5%
Sieve test	100% through 40 mesh 98% through 80 mesh
<b>Heavy metals</b>	
Arsenic	NMT 1 ppm
Lead	NMT 2 ppm
Mercury	NMT 0.5 ppm
Cadmium	NMT 1 ppm
<b>Microbiological assays</b>	
Yeast & Mould count	Not more than 50 cfu/g
<b>Pathogens</b>	
<i>Escherichia coli</i>	Absent/10 g
<i>Salmonella</i>	Absent/10 g
<i>Pseudomonas aeruginosa</i>	Absent/1 g
<i>Staphylococcus aureus</i>	Absent/1 g
<i>Listeria monocytogenes</i>	Absent/25 g

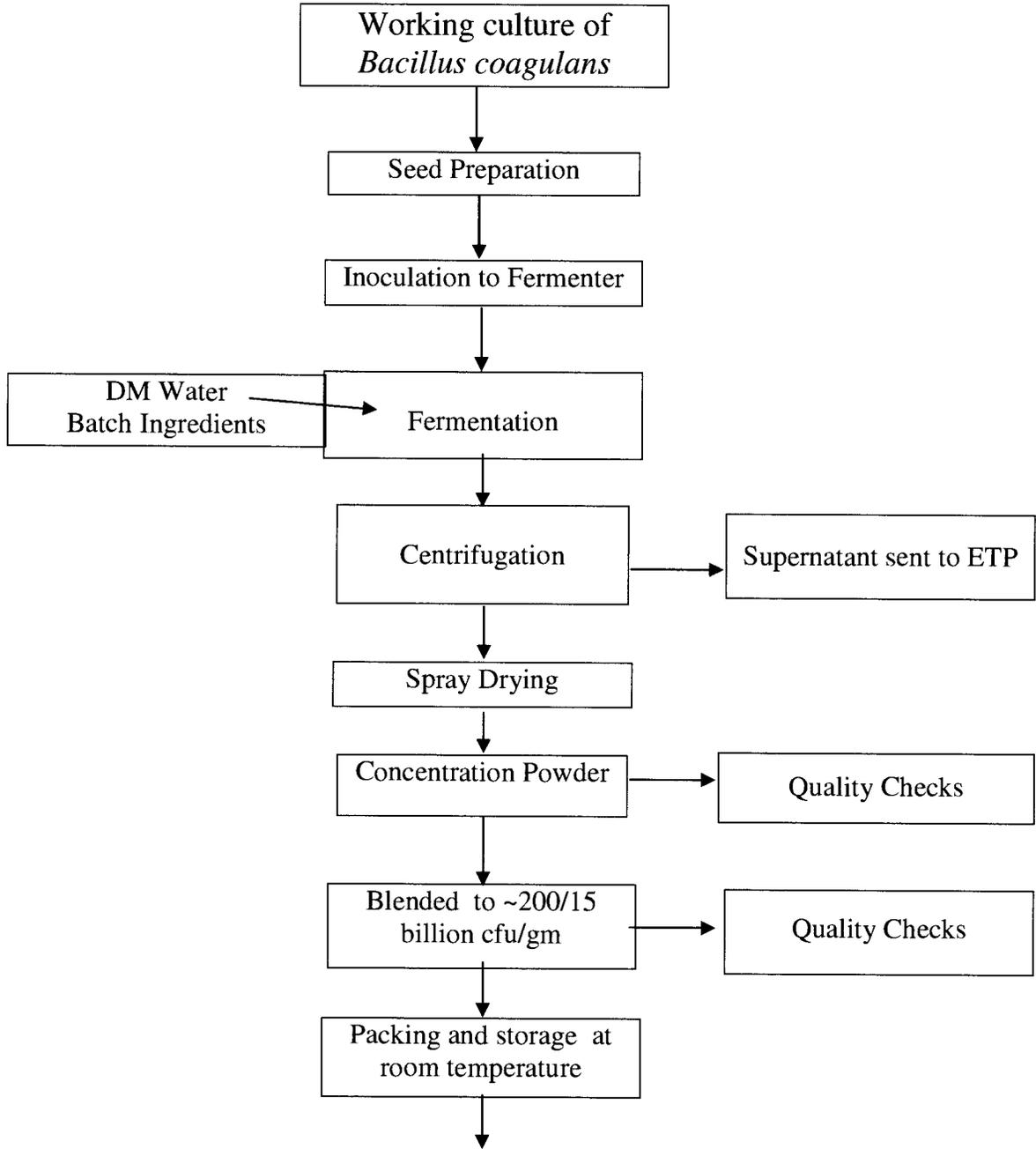
\*Based on information provided by Unique Biotech; NMT = Not more than; cfu = colony forming units; ppm = parts per million

#### **D. Manufacturing process**

*B. coagulans* Unique IS2 is manufactured according to current good manufacturing practices (GMP), as summarized in Figure 3. The production of *B. coagulans* Unique IS2 is achieved through a specific time and temperature controlled fermentation of suitable ingredients. The stock culture of *B. coagulans* Unique IS2 is checked annually for genetic stability. The raw material mixture for fermentation is sterilized, cooled and inoculated with *B. coagulans* Unique IS-2. The inoculum is allowed to incubate to the fermentation endpoint under constant temperature and aeration. After the required incubation period, the biomass is collected by centrifugation. Subsequently, the concentrated suspension is dried by spray drying. The dried culture is then placed and stored in a dry condition environment at room temperature. The processing aids, fermentation medium and diluents used in the manufacturing of *B. coagulans* Unique IS2 are either approved as food additives or are GRAS substances. The manufacturing facility is ISO 9001:2008 and WHO GMP certified.

The finished product is prepared from the approved concentrated product, by diluting with food grade diluents such as maltodextrin and/or microcrystalline cellulose powder (MCCP) and/or fructooligosaccharides (FOS). These diluents are safe for the intended uses. Use of maltodextrin is GRAS as per 21 CFR 184.1444. The other two diluents, MCCP and FOS have also been reviewed for their safety. The Select Committee on GRAS Substances (SCOGS) as part of the report on ethyl cellulose reviewed MCCP and it is considered as GRAS. Similarly, FOS was the subject of a GRAS Notice to FDA (GRN 44). The Expert Panel reviewed the intended use of all these diluents by Unique Biotech and considered them to be safe.

### E. Manufacturing process diagram



**Figure II-E.1. Manufacturing process of *Bacillus coagulans* Unique IS2 (DM water = Distilled mineral water; ETP = Effluent treatment plant)**

### **III. Summary of the Basis for the Notifier's Determination that *Bacillus coagulans* Unique IS2 is GRAS**

The determination that *B. coagulans* Unique IS2 spore preparation is GRAS is based on scientific procedures. A comprehensive search of the scientific literature for safety and toxicity information on *Bacillus coagulans* was conducted through May 2014<sup>1</sup> and was utilized for this assessment. Based on a critical evaluation of the pertinent data and information summarized here and employing scientific procedures, it is determined that the addition of *B. coagulans* Unique IS2 to the selected foods described in this notice and at a maximum use level of approximately  $2 \times 10^9$  colony forming units (cfu)/serving (in accordance with established reference amounts customarily consumed, 21 CFR 101.12) meeting the specification cited above and manufactured according to current Good Manufacturing Practice, is GRAS under the conditions of intended use as specified herein.

In coming to this decision that *B. coagulans* Unique IS2 spore preparation is GRAS, Unique Biotech Limited relied upon the conclusions that *B. coagulans* Unique IS2 does not pose any toxicological hazards or safety concerns at the intended use levels, as well as on published toxicology studies and other articles relating to the safety of the product. Other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

### **IV. Basis for a Conclusion that *Bacillus coagulans* Unique IS2 is GRAS for its Intended Use.**

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened to determine the safety of *B. coagulans* Unique IS2 spore preparation used as a food ingredient. Based on a critical evaluation of the pertinent data and information summarized herein, the Expert Panel members have individually and collectively determined by scientific procedures that the addition of *B. coagulans* Unique IS2 spore preparation

baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups at a maximum use level of approximately  $2 \times 10^9$  colony forming units (cfu)/serving (reference amounts customarily consumed, 21CFR 101.12) when not otherwise precluded by a Standard of Identity as described here and resulting in the estimated daily intake of  $36.4 \times 10^9$  cfu *B. coagulans* spores/day is GRAS. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion (see attached Expert Panel Statement).

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<sup>1</sup> The updated database searches performed subsequent to the Expert Panel review of the *B. coagulans* Unique IS2 GRAS assessment in February 2014 did not reveal any significant findings that will affect the panel conclusion.

# GRAS NOTIFICATION

## I. Claim of GRAS Status

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

Unique Biotech Limited (the notifier) has determined that *Bacillus coagulans* Unique IS2 spore preparation is Generally Recognized As Safe, 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, under the conditions of its intended use as a food ingredient. Therefore, the use of *Bacillus coagulans* Unique IS2 spore preparation is exempt from the requirement of premarket approval.

Signed,

\_\_\_\_\_ Date \_\_\_\_\_

Ratna Sudha, Ph.D.  
Managing Director  
Unique Biotech Limited

**B. Name and Address of Notifier:**

Ratna Sudha, Ph.D.  
Unique Biotech Ltd  
G - 43, Madhura Nagar, Yousufguda,  
Hyderabad - 500034, A.P.  
INDIA.

Phone: +91 -40-23751346/47  
Fax: +91 -40-23751345  
E-mail: sudha@uniquebiotech.com

**C. Common or Usual Name of the Notified Substance:**

The common name of the substance of this notification is *Bacillus coagulans* Unique IS2. The preparation contains spores.

**D. Conditions of Intended Use in Food**

*Bacillus coagulans* Unique IS2 spore preparation, is intended for use as a probiotic in the following food categories: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups at a maximum level of approximately  $2 \times 10^9$  colony forming units (CFU)/serving (reference amounts customarily consumed, 21CFR 101.12). *B. coagulans* Unique IS2 spore preparation is not proposed for uses in foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as it is not intended for use in meat and poultry products that come under USDA jurisdictions. The intended use of *B. coagulans* Unique IS2 spore in the above mentioned food categories, is estimated to result in a maximum daily intake of  $36.4 \times 10^9$  cfu/day.

**E. Basis for GRAS Determination:**

In accordance with 21 CFR 170.30, the intended use of *B. coagulans* Unique IS2 spore preparation has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. The determination is supported by the opinion of the Expert Panel. A comprehensive search of the scientific literature was also utilized for this determination. There exists sufficient qualitative and quantitative scientific evidence, including human and animal data to determine safety-in-use for *B. coagulans* Unique IS2 spore preparation. The safety determination of *B. coagulans* Unique IS2 spore is based on the totality of available evidence.

There are different strains of *Bacillus coagulans* that are used as a probiotic to improve and maintain ecological balance of the intestinal microflora. In the published literature several experimental studies, including subchronic toxicity, chronic toxicity, reproduction toxicity, *in*

*vitro* and *in vivo* genotoxicity and human clinical trials, have appeared. All these studies support the safety in use of *B. coagulans* at the intended use levels.

#### **F. Availability of Information:**

The data and information that forms the basis for this GRAS determination will be provided to Food and Drug Administration upon request or will be available for FDA review and copying at reasonable times at the above mentioned offices of the notifier (Section I, B) or in the US by contacting one of the Expert Panel members: Madhu G. Soni, PhD, FATS, Soni & Associates Inc., 749 46<sup>th</sup> Square, Vero Beach, FL 32068; Telephone: +1-772-299-0746; Email: sonim@bellsouth.net

### **II. Detailed Information About the Identity of the Notified Substance:**

*Bacillus coagulans* Unique IS2 spore preparation is a standardized brown colored powder (200 billion cfu/g product) or white to beige colored powder (15 billion cfu/g product). It is a member of a subgroup of *Bacillus* spp. and is isolated from human fecal soil (*soil* contaminated with *human feces*).

#### **A. Common or Usual Name:**

*Bacillus coagulans* Unique IS2.

#### **B. Identity of Microorganism:**

*B. coagulans* Unique IS2 is a gram-positive, catalase-positive, spore forming, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. The strain has been deposited with the American Type Culture Collection (ATCC) - assigned number ATCC PTA-11748 and with the Microbial Type Culture Collection (MTCC) and Gene Bank with an accession number of *B. coagulans* Unique IS-2 (MTCC 5260). The NCBI accession number of *B. coagulans* Unique IS-2 is FN675759 (NCBI database).

The subject strain of present GRAS assessment, *B. coagulans* Unique IS2 spore preparation is selected from a total number of 29 isolates of *Bacillus* spp that were microscopically observed and biochemically tested. *B. coagulans* Unique IS2 has been extensively studied for phenotypic and genotypic characteristics. The biochemical tests employed for identification includes: catalase (positive), indole test (negative), gelatin hydrolysis (negative), gram reaction (positive), and lactic acid production (positive). The genotypic identification of *B. coagulans* Unique IS2 was carried out using the following tests: (A) by mole G+C content of DNA, (B) 16S rRNA gene sequence and Phylogenetic analysis, and (C) DNA-DNA hybridization.

The mole % G+C content of 46 for *B. coagulans* Unique IS2 was similar to 45.5% noted for *B. coagulans* ATCC 7050 (well characterized strain). Additionally, the *B. coagulans* Unique IS2 was confirmed at the genus and species level by specific PCR assays as well as by 16S rRNA sequencing and was subjected to a battery of tests as per FAO/WHO guidelines. The DNA-DNA hybridization shows a high level of DNA-DNA homology (88%) between *B. coagulans* Unique IS2 and *B. coagulans* ATCC 7050. Thus the available information

supports the characterization and identity of *B. coagulans* Unique IS2, the subject of this GRAS assessment.

### C. Typical Specifications

Typical food grade specifications of *Bacillus coagulans* Unique IS2 preparation are presented in Tables II-C.1. Analytical data from five non-consecutive lots with strength  $200 \times 10^9$  cfu/g and five lots with strength  $15 \times 10^9$  cfu/g are presented in Appendix I. These data suggest that *B. coagulans* Unique IS2 is consistently manufactured to meet the standard product specifications.

**Table II-C.1. Food Grade Specifications of *Bacillus coagulans* Unique IS2 Preparations**

Parameter	Characteristics (Unique Biotech, 2014)*
Appearance	Brown colored powder (200 billion cfu/g product) White to beige colored powder (15 billion cfu/g product)
Description	Characteristic odor and slightly sweet in taste
Microscopy	The spores are seen as small terminal oval shape refractile bodies at the end of each vegetative cell
Lactic acid producing capacity (ml)	Not less than 10 ml of 0.05 N NaOH should be consumed
Assay (cfu/g)	Not less than 200 Billion cfu/g Not less than 15 Billion cfu/g
Loss on drying (%)	Not more than 5%
Sieve test	100% through 40 mesh 98% through 80 mesh
<b>Heavy metals</b>	
Arsenic	NMT 1 ppm
Lead	NMT 2 ppm
Mercury	NMT 0.5 ppm
Cadmium	NMT 1 ppm
<b>Microbiological assays</b>	
Yeast & Mould count	Not more than 50 cfu/g
<b>Pathogens</b>	
<i>Escherichia coli</i>	Absent/10 g
<i>Salmonella</i>	Absent/10 g
<i>Pseudomonas aeruginosa</i>	Absent/1 g
<i>Staphylococcus aureus</i>	Absent/1 g
<i>Listeria monocytogenes</i>	Absent/25 g

\*Based on information provided by Unique Biotech; NMT = Not more than; cfu = colony forming units; ppm = parts per million

## D. Manufacturing process

*B. coagulans* Unique IS2 is manufactured according to current good manufacturing practices (GMP), as summarized in Figure 3. The production of *B. coagulans* Unique IS2 is achieved through a specific time and temperature controlled fermentation of suitable ingredients. The stock culture of *B. coagulans* Unique IS2 is checked annually for genetic stability. The raw material mixture for fermentation is sterilized, cooled and inoculated with *B. coagulans* Unique IS-2. The inoculum is allowed to incubate to the fermentation endpoint under constant temperature and aeration. After the required incubation period, the biomass is collected by centrifugation. Subsequently, the concentrated suspension is dried by spray drying. The dried culture is then placed and stored in a dry condition environment at room temperature. The processing aids, fermentation medium and diluents used in the manufacturing of *B. coagulans* Unique IS2 are either approved as food additives or are GRAS substances. The manufacturing facility is ISO 9001:2008 and WHO GMP certified.

The finished product is prepared from the approved concentrated product, by diluting with food grade diluents such as maltodextrin and/or microcrystalline cellulose powder (MCCP) and/or fructooligosaccharides (FOS). These diluents are safe for the intended uses. Use of maltodextrin is GRAS as per 21 CFR 184.1444. The other two diluents, MCCP and FOS have also been reviewed for their safety. The Select Committee on GRAS Substances (SCOGS) as part of the report on ethyl cellulose reviewed MCCP and it is considered as GRAS. Similarly, FOS was the subject of a GRAS Notice to FDA (GRN 44). The Expert Panel reviewed the intended use of all these diluents by Unique Biotech and considered them to be safe.

## E. Manufacturing process diagram

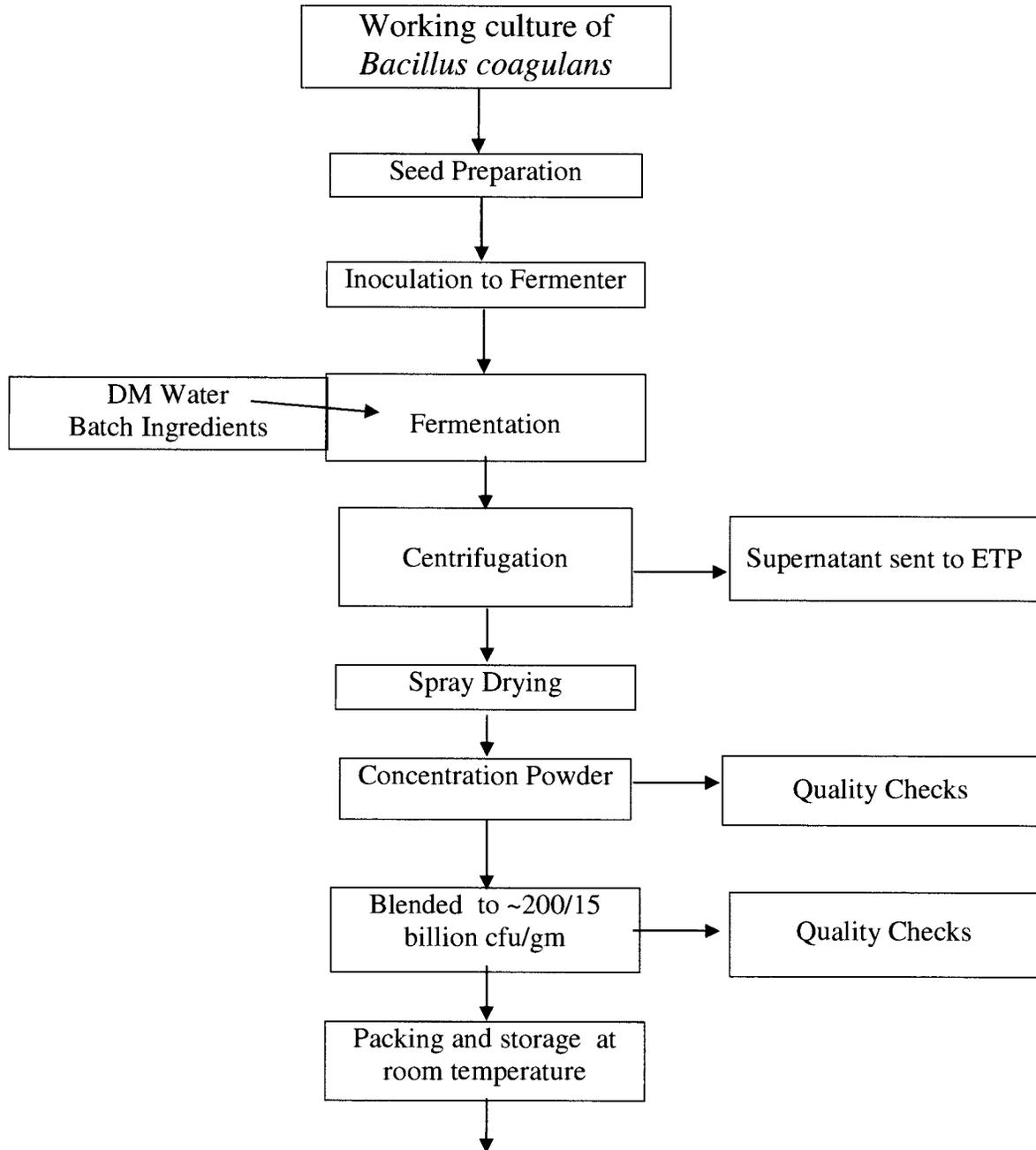


Figure II-E.1. Manufacturing process of *Bacillus coagulans* Unique IS2 (DM water = Distilled mineral water; ETP = Effluent treatment plant)

### III. Summary of the Basis for the Notifier's Determination that *Bacillus coagulans* Unique IS2 is GRAS

The determination that *B. coagulans* Unique IS2 spore preparation is GRAS is based on scientific procedures. A comprehensive search of the scientific literature for safety and toxicity information on *Bacillus coagulans* was conducted through May 2014<sup>1</sup> and was utilized for this assessment. Based on a critical evaluation of the pertinent data and information summarized here and employing scientific procedures, it is determined that the addition of *B. coagulans* Unique IS2 to the selected foods described in this notice and at a maximum use level of approximately  $2 \times 10^9$  colony forming units (cfu)/serving (in accordance with established reference amounts customarily consumed, 21 CFR 101.12) meeting the specification cited above and manufactured according to current Good Manufacturing Practice, is GRAS under the conditions of intended use as specified herein.

In coming to this decision that *B. coagulans* Unique IS2 spore preparation is GRAS, Unique Biotech Limited relied upon the conclusions that *B. coagulans* Unique IS2 does not pose any toxicological hazards or safety concerns at the intended use levels, as well as on published toxicology studies and other articles relating to the safety of the product. Other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

### IV. Basis for a Conclusion that *Bacillus coagulans* Unique IS2 is GRAS for its Intended Use.

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened to determine the safety of *B. coagulans* Unique IS2 spore preparation used as a food ingredient. Based on a critical evaluation of the pertinent data and information summarized herein, the Expert Panel members have individually and collectively determined by scientific procedures that the addition of *B. coagulans* Unique IS2 spore preparation

baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups at a maximum use level of approximately  $2 \times 10^9$  colony forming units (cfu)/serving (reference amounts customarily consumed, 21CFR 101.12) when not otherwise precluded by a Standard of Identity as described here and resulting in the estimated daily intake of  $36.4 \times 10^9$  cfu *B. coagulans* spores/day is GRAS. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion (see attached Expert Panel Statement).

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<sup>1</sup> The updated database searches performed subsequent to the Expert Panel review of the *B. coagulans* Unique IS2 GRAS assessment in February 2014 did not reveal any significant findings that will affect the panel conclusion.

## **EXPERT PANEL STATEMENT**

### **DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *BACILLUS COAGULANS* UNIQUE IS-2 FOR USE IN FOOD**

Unique Biotech Limited  
Plot No. 2, Phase-II  
Alexandria Knowledge Park  
Kolthur Village, Shameerpet Mandal  
Ranga Reddy Dist  
Hyderabad-500 078, AP  
INDIA

#### **Panel Members**

Douglas Archer, Ph.D.  
Robert L. Martin, Ph.D.  
Madhusudan G. Soni, Ph.D., F.A.T.S.

April, 2014

**000017**

## EXPERT PANEL STATEMENT

### DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *Bacillus coagulans* Unique IS-2 FOR USE IN FOOD

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**000019**

# DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *Bacillus coagulans* Unique IS-2 FOR USE IN FOOD

## 1. INTRODUCTION

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)<sup>1</sup>, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Soni & Associates Inc., at the request of Unique Biotech Limited, India (Unique Biotech), to determine the Generally Recognized As Safe (GRAS) status of *Bacillus coagulans* Unique IS-2 spores in baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups at a maximum use level of approximately  $2 \times 10^9$  colony forming units (cfu)/serving. A comprehensive search of the scientific literature for safety and toxicity information on *Bacillus coagulans* was conducted through February 2014 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Unique Biotech and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on April 28, 2014 and unanimously agreed to the decision described herein.

### 1.1. Background

As defined by an expert panel convened in 2002 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are “live microorganisms administered in adequate amounts that confer a beneficial health effect on the host” (FAO/WHO, 2002). For over 50 years, *Bacillus* species have been used as probiotics and in recent years the scientific interest in *Bacillus* species as probiotics has significantly increased (Mazza, 1994; Sanders et al., 2003; Hong et al., 2005; Cutting, 2011). These species are spore forming bacteria that are commonly found in nature. As the *Bacillus* spores are stable at high temperatures and in acidic conditions, spores are gaining significant attention. Compared to non-spore formers, such as *Lactobacillus* species, spores provide advantages as the product can be stored at room temperature in a desiccated form without any deleterious effect on viability; spores are capable of surviving the low pH of the gastric barrier (Barbosa et al., 2005; Spinosa et al., 2000; Tuohy et al., 2007); and, the entire dose of ingested bacteria can reach the small intestine intact and germinate. Additionally, it can survive industrial food manufacturing conditions and ensures long term viability (Sanders et al., 2001).

*Bacillus coagulans* is a lactic-acid producing species that has typical characteristics of both *Lactobacillus* and *Bacillus* genera. It was first isolated and described in 1932 by Horowitz and Wlassowa. At the time it was named as *Lactobacillus sporogenes* (Bergey et al., 1939). As *B. coagulans* exhibits characteristics typical of both genera *Lactobacillus* and *Bacillus*, its

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<sup>1</sup>Modeled after that described in section 201(s) of the Federal Food, Drug, and Cosmetic Act, As Amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.

taxonomic position was debated for some time and, in 1957, this microorganism was reclassified in Bergey's Manual of Determinative Bacteriology based on its biochemical properties. The current correct nomenclature is *Bacillus coagulans*. This microorganism possesses a protective, spore-like protein coating (endospores) that protects it from the acidic conditions in the stomach. Thus, it can reach the small intestine, germinate, and multiply. It is also an economically important species, frequently used in the production of optically pure lactic acid, coagulin, and other thermostable enzymes (Su et al., 2012). *B. coagulans* has been marketed as a probiotic to maintain the ecological balance of the intestinal microflora and normal gut function. Given the potential benefits of *B. coagulans*, Unique Biotech intends to market *B. coagulans* Unique IS2 strain for use as a food ingredient in selected foods as described in this dossier.

## 1.2. Identity and Description

### 1.2.1. Name and Source of GRAS Organism

The specific bacterial strain which is the subject of this Generally Recognized As Safe (GRAS) assessment is *Bacillus coagulans* Unique IS2. It is a member of a subgroup of *Bacillus* spp. and is isolated from human fecal soil<sup>2</sup>.

### 1.2.2. Description of GRAS Organism

General descriptive parameters and properties of the *B. coagulans* Unique IS2 preparations manufactured by Unique Biotech are summarized in Table 1. *B. coagulans* Unique IS2 is a gram-positive, catalase-positive, spore forming, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. *B. coagulans* Unique IS2, the subject of this present GRAS determination, has been deposited with the American Type Culture Collection (ATCC) - assigned number ATCC PTA-11748 and with the Microbial Type Culture Collection (MTCC) and Gene Bank with an accession number of *B. coagulans* Unique IS-2 (MTCC 5260). The NCBI accession number of *B. coagulans* Unique IS-2 is FN675759 (NCBI database). As mentioned earlier, *B. coagulans* is a highly resilient bacterium commonly found in the soil, air and dust. This bacterial species can grow in a highly alkaline environment and the spores can also withstand the stomach acidic environment. The spores of *B. coagulans* can withstand temperatures in excess of 100°C, while the vegetative cells can grow at temperatures as high as 65°C. The hierarchical classification of *B. coagulans* Unique IS2 is presented in Table 2.

**Table 1. General Descriptive Characteristics of *Bacillus coagulans* Unique IS2**

Parameter	Description *
Organism	<i>Bacillus coagulans</i> Unique IS-2
Origin	Isolated from human fecal soil.
Physical characteristics	White to beige (15 billion product); Brown powder (200 billion product)
Taste	Slightly sweet in taste
Odor	Characteristic
Shelf life	36 months

\*Based on information provided by Unique Biotech

<sup>2</sup>Soil contaminated with human feces.

**Table 2. Classification of *Bacillus coagulans* Unique IS2**

Bacteria
Endospore-Forming Bacteria
Gram-Positive Endospore-Forming Bacteria
Firmicutes
Gram-Positive Endospore-Forming Rods
Bacillaceae
Bacillus
<i>Bacillus coagulans</i>
<i>Bacillus coagulans</i> Unique IS-2

**1.2.3. Identification and Characterization**

The subject of this present GRAS determination, *B. coagulans* Unique IS2, was selected following a search for new probiotics from a total number of 29 isolates of *Bacillus* spp. from the local region (Hyderabad, India) and was selected based on colony morphology. Among the preliminary isolates, 11 isolates were tentatively identified as *Bacillus* based on their microscopic observation and motility testing. All twenty-nine isolates were biochemically tested for their identification. The biochemical characterization included sugar fermentation as well as testing for catalase, indole, gelatin hydrolysis and lactic acid production. Based on this testing, only one isolate (*B. coagulans* Unique IS2) was selected. The phenotypic characteristics of *B. coagulans* Unique IS2 are summarized in Tables 3 and 4. As described below, the selected isolate was further characterized genetically. The isolate was designated as *B. coagulans* Unique IS2.

**Table 3. Sugar Fermentation Test for the Identification of *Bacillus coagulans* Unique IS2\***

Carbohydrates	Acid	Gas	Carbohydrates	Acid	Gas
Glucose	+	-	Arabinose	+	-
Lactose	+	-	Rhamnose	-	-
Inulin	-	-	Maltose	+	-
Maltose	+	-	Dextrose	+	-
Mannitol	+	-	Raffinose	-	+
Sorbital	-	-	Fructose	+	-
Sucrose	+	-	Galactose	+	-
Xylose	-	+			

Adapted from Sudha et al. (2010)

**Table 4: Biochemical Tests Employed for the Identification of *Bacillus coagulans* Unique IS2\***

Parameter	Finding
Catalase	+
Indole test	-
Gelatin hydrolysis	-
Gram reaction	+
Lactic acid production	+

\*Adapted from Sudha et al. (2010)

### 1.2.4. Genotypic Identification

In an attempt to further characterize *B. coagulans* Unique IS2, genotypic identification was carried out using the following tests: (A) by mole G+C content of DNA, (B) 16S rRNA gene sequence and Phylogenetic analysis, and (C) DNA-DNA hybridization. In order to identify and characterize *B. coagulans* Unique IS2 strain, the molecular characteristics were compared with known standard cultures such as: *B. coagulans* strain NCIM 2030, *B. coagulans* strain MTCC 492 and ATCC 7050, and *B. subtilis*. Standard, and well established, procedures were followed to grow the cultures and isolate DNA for molecular characterization. The purified DNA was used for G+C content, PCR (16S rRNA sequence) and for DNA-DNA hybridization studies. All these procedures are described in a recent article published by Sudha et al. (2010). DNA-DNA hybridization was performed by the membrane filter method of Tourova and Antonov (1987) and essentially involves three steps: (A) Radioactive labeling of DNA to be used as a probe, (B) Immobilization of the DNA, and (C) Hybridization between the immobilized DNA and the radioactive DNA. For phylogenic analysis, the reference sequences required for comparison were downloaded from the EMBL database<sup>3</sup>. All the sequences aligned using the multiple sequence alignment program CLUSTAL W (Sudha et al., 2010). For RAPD analysis, genomic DNA was amplified using a set of 18 random primers.

The results of G+C content are summarized in Table 5. These results indicated that *B. coagulans* Unique IS2 had a mole % G + C content of DNA with value of 46 which is in accordance with what has been reported for *B. coagulans* (44 to 50%) (Sneath, 1986). The mole % G+C content of *B. coagulans* ATCC 7050 was found to be 45.5 which further support the identity of *B. coagulans* Unique IS2 strain (Table 3). The *B. coagulans* Unique IS2 was confirmed at the genus and species level by specific PCR assays as well as by 16S rRNA sequencing and was subjected to a battery of tests as per FAO/WHO guidelines.

**Table 5. The Mole % G+C Content of DNA of *Bacillus* Strains\***

<b>Bacillus Strains</b>	<b>Mole % G + C of DNA</b>
<i>B. coagulans</i> (Unique Biotech Isolate, IS2)	46.08
<i>B. coagulans</i> (ATCC 7050, MTCC 492)	45.50
<i>B. coagulans</i> (NCIM 2030)	35.10
<i>B. subtilis</i> (IICT)	42.90

\*Adapted from Sudha et al. (2010)

For the identity and phylogenic position of *B. coagulans* Unique IS2, the 1.5 kb 16S rRNA gene was amplified by PCR along with the genes of *B. coagulans* (NCIM 2030) and *B. coagulans* (MTCC 492) and sequenced (Sudha et al., 2010). The 16S rDNA sequences of 1470 bp compared between the three strains of *B. coagulans* and closely related species of the genus *Bacillus*. The 16S rDNA level *B. coagulans* Unique IS2 was closely related to two strains of *B. coagulans* namely *B. coagulans* (MTCC 492) and *B. coagulans* (JCM 2257T) and the similarity was 99.4 and 98.8% respectively (Table 6). Further, *B. coagulans* Unique IS2 differed from the other species of *Bacillus* by a minimum of 2.1 with *Bacillus badius* to a maximum of 10% with *B. azotoformans* (Table 6). It appeared more closely related to *Bacillus subtilis*. The phylogenic

<sup>3</sup> Available at: <http://www.ncbi.nlm.nih.gov/Genbank>

tree construction by UPGMA based on the 16S rRNA gene sequence (Figure 1) using Clustal W further confirms that the *B. coagulans* Unique IS2 is indeed *Bacillus coagulans* and it forms a coherent cluster with two other strains of *B. coagulans* namely *B. coagulans* ATCC 7050 and *B. coagulans* JCM 2257T which is a type strain of *Bacillus coagulans*. The greater than 70% bootstrap values between the three strains further confirms that the *B. coagulans* Unique IS2 is phylogenetically very close to the other *B. coagulans* strains implying that it is yet another strain of *Bacillus coagulans* (Sudha et al., 2010).

**Table 6: Dissimilarity Matrix Showing the Distance Between *B. coagulans* Unique IS2 and Related Species of the Genus *Bacillus* at the 16S rRNA Gene Level\***

Isolate	1	2	3	4	5	6	7	8	9	10	11	12
<i>B. subtilis</i> NCDO 1769												
<i>B. licheniformis</i> DSM 13	1.74											
<i>B. coagulans</i> Unique IS2 (Unique Biotech)	7.57	7.19										
<i>B. coagulans</i> (ATCC 7050, MTCC 492)	7.33	7.12	0.62									
<i>B.adius</i> ATCC 14574	7.68	7.46	2.10	1.44								
<i>B. coagulans</i> JCM 2257	7.50	7.52	1.16	0.43	1.31							
<i>B. smithii</i> DSM 4216	7.25	6.82	6.15	6.08	6.71	6.60						
<i>B. lentus</i> IAM 12466	6.47	6.75	6.86	6.78	6.48	6.61	5.89					
<i>B. firmus</i> IAM 12464	5.30	5.03	6.81	6.73	6.81	6.79	6.19	3.75				
<i>B. coagulans</i> (NCIM 2030)	6.36	6.71	7.84	7.54	8.22	7.81	8.07	6.34	6.12			
<i>B. circulans</i> IAM 12462	6.36	6.0	6.96	6.89	7.0	6.97	5.66	3.66	3.90	6.21		
<i>B. azotoformans</i> DSM 1046	7.79	7.74	10.01	10.05	10.88	10.18	9.72	7.74	7.02	8.45	6.73	
<i>P. citreus</i> FO-074a	8.27	8.28	9.10	9.43	9.67	9.45	8.07	7.58	6.82	7.84	6.62	10.28

\*Adapted from Sudha et al. (2010).

The results of DNA-DNA hybridization studies between *B. coagulans* Unique IS2 and other strains of *B. coagulans* and *B. subtilis* indicate a high level of DNA-DNA homology (88%) between *B. coagulans* IS2 and *B. coagulans* ATCC 7050 (Sudha et al., 2010). However, it showed very little homology with *B. coagulans* NCIM 2030 and *B. subtilis* IICT isolate (Table 7 and Figure 2).

**Table 7: Percent Homology of *B. coagulans* Unique IS2 with Other Strains of *B. coagulans* and *B. subtilis* by DNA-DNA Hybridization.**

Bacterial Strain	Hybridization (%)
<i>B. coagulans</i> Unique IS2 (Unique Biotech Isolate)	100.0
<i>B. coagulans</i> (ATCC 7050, MTCC 492)	88.0
<i>B. coagulans</i> (NCIM 2030)	5.6
<i>B. subtilis</i> (IICT Isolate)	5.2

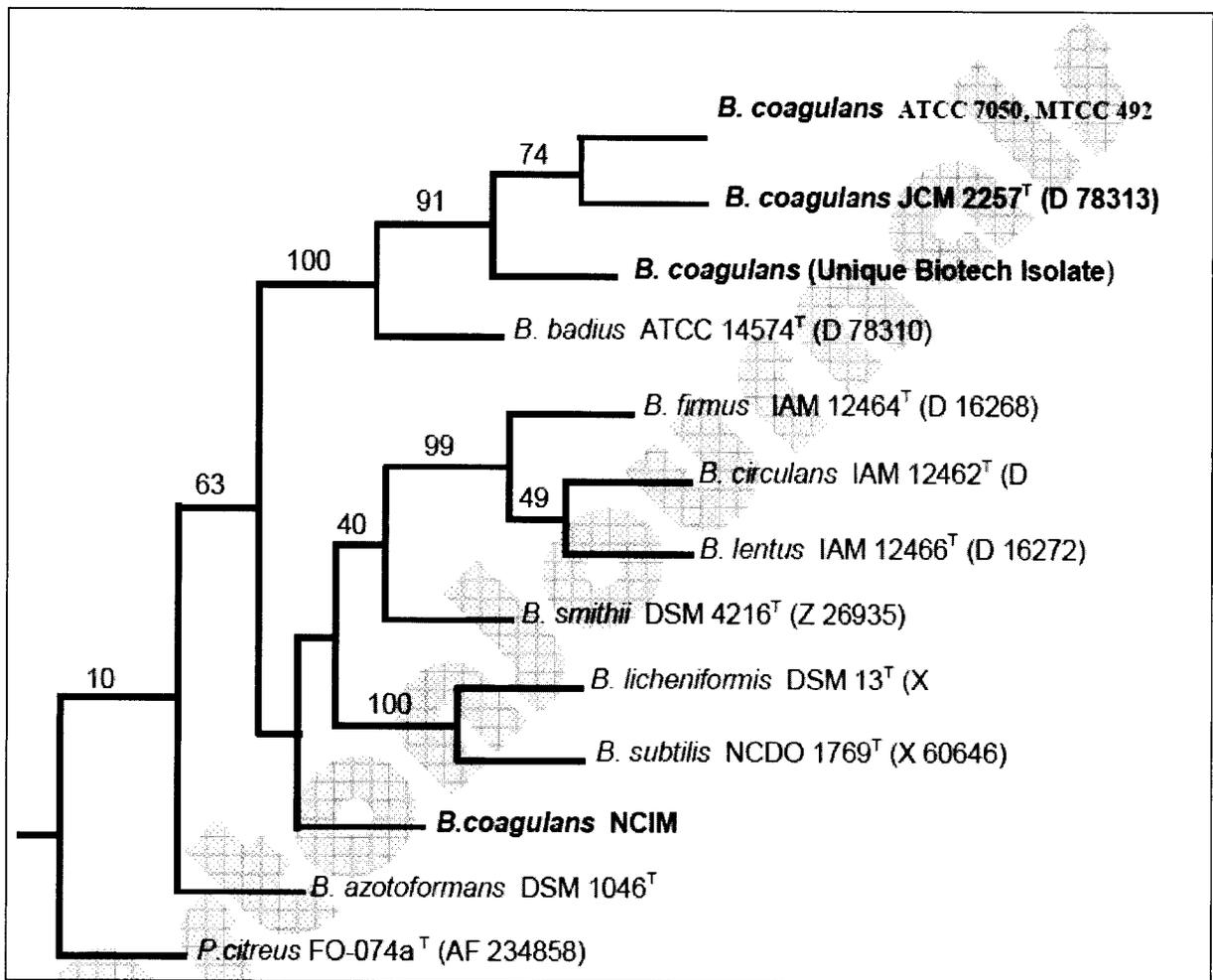


Figure 1: UPGMA Phenogram Showing the Phylogenetic Relationship Between *B. coagulans* Unique IS2 and Related Species of *Bacillus* and *Planococcus citreus* (adapted from Sudha et al., 2010)

The RAPD profile evaluated with PCR amplification of genomic DNA of *B. coagulans* NCIM2030, *B. coagulans* Unique IS2, *B. coagulans* ATCC 7050, and *B. subtilis* (Sudha et al., 2010). On electrophoresis, amplified DNA bands were visible only when OPA-7, OPA-9, OPA-10, OPA-11, OPA-13, OPA-14, OPA-16, OPA-20, OPB-7 and OPB-12 were used as the primers. The number of DNA bands varied between 1 to 6 depending on the primer used and the bacterial strain are representative RAPD profiles of the *Bacillus* strains. The primers that did not yield any amplified product were not utilized. For phylogenetic analysis 75 bands generated by 8 random primers and DNA from *B. coagulans* NCIM 2030, *B. coagulans* ATCC 7050, *B. coagulans* Unique IS2 and *B. subtilis* were considered. The data converted to binary digits that formed the source of data for phylogenetic analysis. The phylogenetic tree based on UPGMA analysis of the RAPD data comprising 75 bands indicated a similarity between all the 4 strains at the 22% level (Figure 3). Nevertheless, it was interesting to note that *B. coagulans* Unique IS2 and *B. coagulans* ATCC 7050 had a higher degree of similarity (35%). These results clearly indicate that the *B. coagulans* Unique IS2 is a distinctly different strain of *B. coagulans*. The results are in accordance with De Clerck et al (2004).

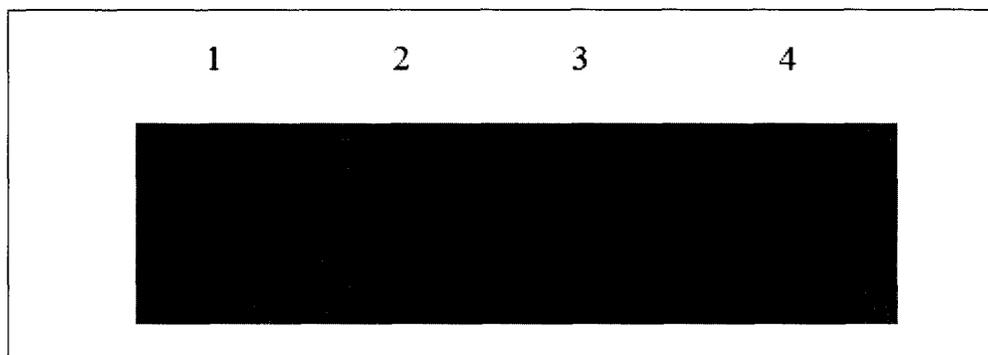


Figure 2: DNA-DNA Hybridization of *B. coagulans* Unique IS2 with (1) *B. coagulans* (NCL), (2) *B. subtilis*, (3) *B. coagulans* Unique IS2 and (4) *B. coagulans* (ATCC 7050, MTCC 492) (Sudha et al., 2010)

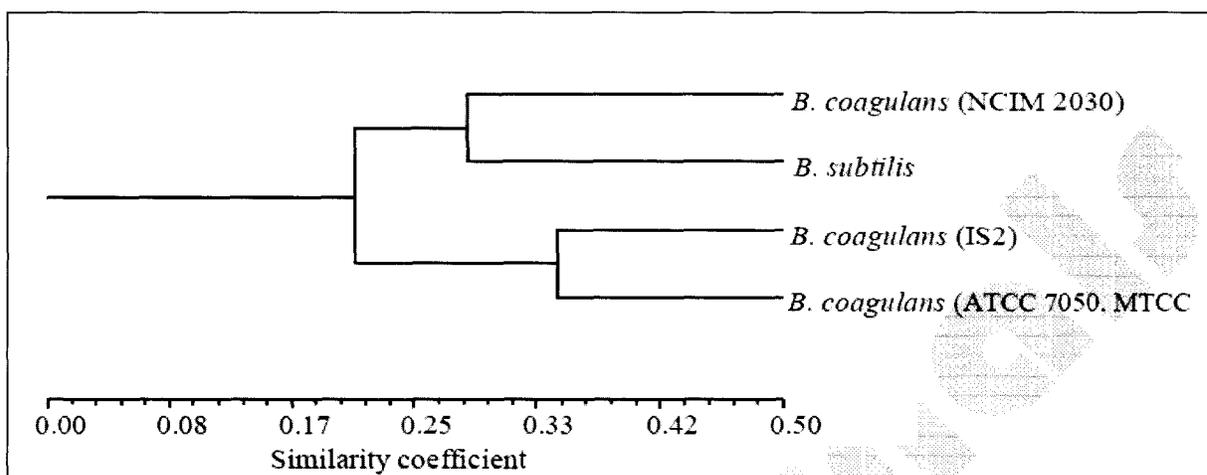


Figure 2: Phylogenetic Relationship of *B. coagulans* Unique IS2 with Reference to the Standard Strains Based on RAPD Analysis (adapted from Sudha et al., 2010)

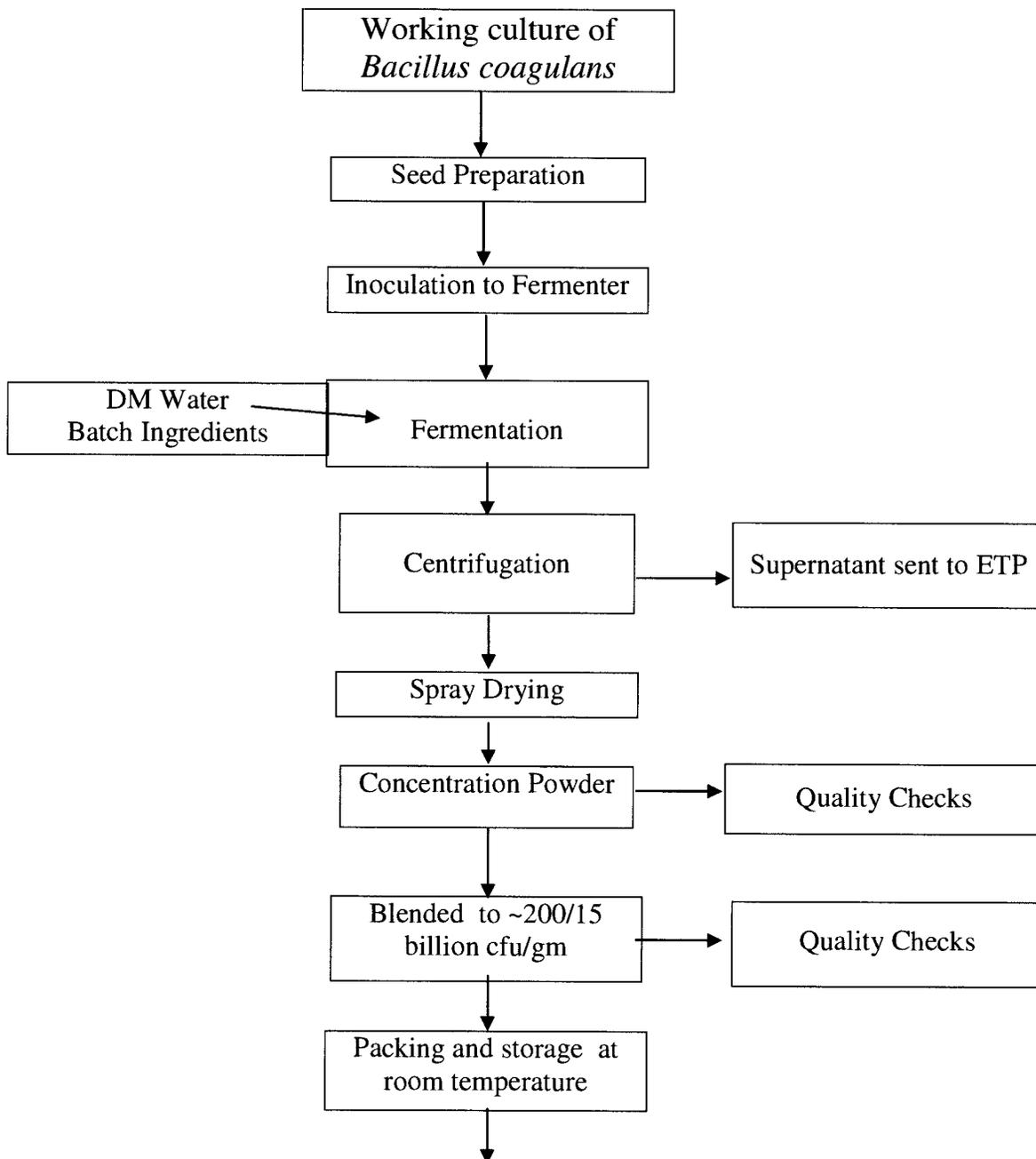
### 1.3. Manufacturing Process

*B. coagulans* Unique IS2 is manufactured according to current good manufacturing practices (GMP), as summarized in Figure 3. The manufacturing procedure assures a consistent and high-quality product that meets the specifications (Table 8). The processing aids, fermentation medium and diluents used in the manufacturing of *B. coagulans* Unique IS2 are either approved as food additives or are GRAS substances. The manufacturing facility is ISO 9001:2008 and WHO GMP certified.

The product is manufactured through a specific time and temperature controlled fermentation of suitable ingredients with *B. coagulans* Unique IS2. The “stock culture” of *B. coagulans* Unique IS2 is checked annually for genetic stability. Prior to addition of *B. coagulans* Unique IS2, the raw material mixture is sterilized and cooled. The solution is then inoculated with *B. coagulans* Unique IS-2 and allowed to incubate to the fermentation endpoint under constant temperature and aeration. After the required incubation period, the biomass is collected by centrifugation. Subsequently, the concentrated suspension is dried by spray drying. The dried culture is then placed and stored in a dry condition environment at room temperature.

Release of the product is based on established specifications and is approved by Quality Control Department of Unique Biotech Limited. The finished product is prepared from the

approved concentrated product, by diluting with food grade diluents such as maltodextrin and/or microcrystalline cellulose powder (MCCP) and/or fructooligosaccharides (FOS). These diluents are safe for the intended uses. Use of maltodextrin is GRAS as per 21 CFR 184.1444. The other two diluents, MCCP and FOS have also been reviewed for their safety. The Select Committee on GRAS Substances (SCOGS) as part of the report on ethyl cellulose reviewed MCCP and it is considered as GRAS. Similarly, FOS was the subject of a GRAS Notice to FDA (GRN 44). The Expert Panel reviewed the intended use of all these diluents by Unique Biotech and considered them to be safe.



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**Figure 3. Manufacturing process of *Bacillus coagulans* Unique IS2**

#### 1.4. Specifications

Food grade specifications of *Bacillus coagulans* Unique IS2 preparation have been established by Unique Biotech and are summarized in Table 8. Analytical results from ten non-consecutive lots (Appendix I) with strength of  $200 \times 10^9$  (five lots) and  $15 \times 10^9$  (five lots) cfu/g demonstrate that *B. coagulans* Unique IS2 is consistently manufactured to meet the standard product specifications. The *B. coagulans* Unique IS2 strain used in this determination was found, through DNA Hybridization Analysis, to be over 88% similar to *B. coagulans* type strain ATCC 7050. The standardized product will be marketed under the trade name, *Bacillus coagulans* Unique IS2.

<b>Table 8. Specifications of <i>Bacillus coagulans</i> Unique IS2 Preparation</b>	
<b>Parameter</b>	<b>Characteristics (Unique Biotech, 2014)*</b>
Appearance	Brown colored powder (200 billion cfu/g product) White to beige colored powder (15 billion cfu/g product)
Description	Characteristic odor and slightly sweet in taste
Microscopy	The spores are seen as small terminal oval shape refractile bodies at the end of each vegetative cell
Lactic acid producing capacity (ml)	Not less than 10 ml of 0.05 N NaOH should be consumed
Assay (cfu/g)	Not less than 200 Billion cfu/g Not less than 15 Billion cfu/g
Loss on drying (%)	Not more than 5%
Sieve test	100% through 40 mesh 98% through 80 mesh
<b>Heavy metals</b>	
Arsenic	NMT 1 ppm
Lead	NMT 2 ppm
Mercury	NMT 0.5 ppm
Cadmium	NMT 1 ppm
<b>Microbiological assays</b>	
Yeast & Mould count	Not more than 50 cfu/g
<b>Pathogens</b>	
<i>Escherichia coli</i>	Absent/10 g
<i>Salmonella</i>	Absent/10 g
<i>Pseudomonas aeruginosa</i>	Absent/1 g
<i>Staphylococcus aureus</i>	Absent/1 g
<i>Listeria monocytogenes</i>	Absent/25 g

\*Based on information provided by Unique Biotech; NMT = Not more than; ppm = parts per million

#### 1.5. Uses and Regulatory Status

In recent years, among the spore-forming bacteria, *B. coagulans* and *B. subtilis* are marketed as dietary supplement probiotics for human consumption (Sanders et al., 2003; Hong et al., 2008). A simple internet search revealed that *B. coagulans* is sold as a dietary supplement under different names such as Nature's Plus, GanedenBC3°, Pit-Stop, Fresh Start Bolus, GutFlora (VSL-3), Tarm-X Balans™, Sanvita, Ampilac, Sprolac®, Bactlyte, etc. In addition to this, *B. coagulans* is also marketed as a constituent of other dietary supplements. In products like these, *B. coagulans* is formulated with other ingredients, including bifidobacteria, lactobacilli,

minerals, vitamins (particularly B complex), or prebiotics. The available information product labels indicate that the recommended dose for *B. coagulans* ranges from  $3.6 \times 10^8$  to  $1.5 \times 10^9$  cfu/capsule, two or three times *per day* for a healthy adult.

In addition to its use as a dietary supplement, the US FDA has received and filed a GRAS notice (GRN 399) for the use of *B. coagulans* strain in conventional foods (Ganedan, 2011). In this GRAS notice, use of *B. coagulans* was proposed for addition to a wide variety of foods at levels up to approximately  $2 \times 10^9$  cfu/serving. The notifier determined the acceptable daily intake (ADI) as  $93.8 \times 10^9$  cfu/person/day and FDA did not question the proposed uses (FDA, 2012). The US FDA (2001) has also approved the use of *B. coagulans* in the production of enzymes that can be used in the manufacturing of foods. *B. coagulans* (a nonpathogenic and nontoxicogenic microorganism) is recognized as GRAS (21 CFR 184.1372) in the production of insoluble glucose isomerase enzyme. Similar to the US FDA, the Health Canada has permitted the use of *B. coagulans* in the production of glucose isomerase enzyme. Furthermore, the FDA's Center for Veterinary Medicine has also recognized the use of *B. coagulans* as GRAS for veterinary purposes. The ATCC has classified *B. coagulans* as Biosafety Level 1, indicating that this species is not known to cause disease in healthy humans.

In other countries, the European Food Safety Authority (EFSA) has granted Qualified Presumption of Safety (QPS) status of *B. coagulans*, since 2008 (EFSA, 2012). This suggests that *B. coagulans* can be safely used in European countries. The Japanese Ministry of Health and Welfare has approved the use of *B. coagulans* products for improvement in symptoms caused by abnormalities in the intestinal flora or in dysbiosis (Majeed and Prakash, 1998). The available information suggest that *B. coagulans* has been used as part of the fermenting process for the production of a protein-rich food known as “ugba” that is commonly consumed in Nigeria (Isu and Njoku, 1997).

## 1.6. Intended Uses and Food Categories

Unique Biotech intends to use a *B. coagulans* Unique IS2 as a food ingredient, in multiple food categories. The proposed use of *B. coagulans* Unique IS2 is in the same food categories and at the same levels ( $2 \times 10^9$  cfu/serving) to those mentioned in the GRAS notice (GRN 399) (FDA, 2012). Unique Biotech has not proposed any new food uses for *B. coagulans* Unique IS2. The intended uses are as a food ingredient in food product categories such as baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups (excluding meat and poultry products). *B. coagulans* Unique IS2 will not be used in any foods for which food standards would preclude its use.

The intended use of *B. coagulans* Unique IS2 in the same food categories and at the same use levels ( $2 \times 10^9$  cfu/serving) as those described in GRN 399 is unlikely to affect the intake of *B. coagulans* Unique IS2 in the diet of the public from introduction into the market by another supplier who will have to compete in essentially the same market and foods. In determining the estimated daily intake, it was assumed that males aged 51 and older consume the

largest number (18.2) of servings of food in a day. Using this number of servings/day and the use levels of  $2 \times 10^9$  cfu/serving, in GRN 399, it was estimated that the daily intake of *B. coagulans* spores will be  $36.4 \times 10^9$  cfu/day. The US FDA (2012) did not question the proposed use levels and the resulting intake.

### 1.7. Common Knowledge of Safe Use

The available evidence suggests that various lactic acid producing bacteria have been consumed in food for centuries. These bacteria have been used in foods and are generally considered as harmless (Lee and Salminen, 1995). These are industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits. They are commonly used as starter cultures for fermentation in the dairy, meat and other food industries. Several strains of bacteria selected for such uses have been previously associated or are endogenously found in humans. These uses also ensure the inherent safety of these bacteria for their continued uses in food. The available evidence also indicates that these bacteria can also improve the safety, shelf life, nutritional value, flavor and quality of the product. These bacteria can be used as cell factories for the production of food additives or enzyme preparations and can also function as probiotics.

The role of lactic acid bacteria has been extensively studied in the intestinal microecology. These bacteria play an important role in maintaining the healthy digestive tract (Adams, 1999; Soomro et al., 2002; Ouwehand et al., 2004). Among the different lactic acid producing bacteria, *B. coagulans* has a long history of use for its potential health benefits. Sanders et al. (2003) reviewed several *Bacillus* species for their potential probiotic characteristics. These investigators noted that *B. coagulans* has been evaluated for probiotic functionality and sold worldwide for both human and animal uses. In another article, Catanzaro and Green (1997) considered *B. coagulans* as beneficial bacteria. This microorganism was first isolated in 1932 (Horowitz-Wlassowa and Nowotelnow, 1932). The potential gastrointestinal benefits of *B. coagulans* and other spore-forming bacteria have been investigated during 1958 and 1959 (Guida et al., 1958; Guida and Guida, 1959). The available information indicate that *B. coagulans* has been in use for over five decades.

#### 1.7.1. Human Food Uses

In Africa, consumption of fermented foods has a long history (Okonko et al., 2006). In Nigeria, among several fermented food, *ugba* is a popular protein-rich solid, flavorful alkaline food consumed by the Ibo ethnic group. In the preparation of this food, *B. coagulans* has been identified as one of the species. In the production of *ugba*, African oil bean are fermented with *B. coagulans*. Consumption of this protein rich food is known to result in the intake of *B. coagulans* and its spores (Isu and Njoku, 1997). In another article, Onofiok et al. (1996) reported that a large proportion of the population (76%) consume *ugba* as a snack. Based on the available information, the likely consumption of *B. coagulans* from its presence in *ugba* may be over  $1 \times 10^9$  cfu/day. All this information provides support for the consumption and traditional uses of *B. coagulans* in food.

## 2. SAFETY RELATED DATA

In a series of preclinical and clinical studies conducted by Unique Biotech, the acute and short-term effects of *B. coagulans* Unique IS2 have been investigated. In addition to safety assessment, in these studies, efficacy of *B. coagulans* Unique IS2 for its probiotic effects were

investigated. In a series of *in vitro* investigations, *B. coagulans* Unique IS2, along with other strains, was tested for survival in simulated acid, bile tolerance, adherence to cell surface (hydrophobicity), antibiotic sensitivity and antibacterial activity. The efficacy studies are reviewed as part of the safety evaluation, as in addition to efficacy, relevant safety endpoints were also included. This monograph also reviews other animal and human studies performed to evaluate the safety and probiotic effects of *B. coagulans*. The assessment of efficacy studies is limited to a review of the results related to safety and tolerability.

## **2.1. Specific Studies with *B. coagulans* Unique IS2**

### **2.1.1. Acid and Bile Tolerance - *B. coagulans* Unique IS2**

Sudha et al. (2010) investigated the survival of *B. coagulans* Unique IS2 strain at high acidic environment as per the method described by Clark et al. (1993). Low pH solutions were prepared in sterile sodium chloride solution (0.5%) adjusting the pH to 1.5, 2.0 and 3.0. The tolerance capacity of *B. coagulans* Unique IS2 strain for high bile concentration was checked as per Chung et al. (1999). High bile percentage solution was prepared by suspending bile salts (1, 2 and 3%) in a sterile sodium chloride solution (0.5%). In order to investigate the tolerance level of *B. coagulans* Unique IS2 strain, pour plating was done at 0, 1, 2 and 3 hour interval from both acid and bile solutions. The results of these investigations revealed that *B. coagulans* Unique IS2 strain survived well even at pH 1.5 (Sudha et al., 2011a). Only two log cycle reductions were noted at this pH, which is thought to be very drastic condition for all microorganisms. At pH 2 and pH 3 only one log cycle reduction was noted. As regards bile sensitivity, two log cycle reductions were noted for 3% bile, while only one log cycle reduction was observed in bile 1% and 2% (Sudha et al., 2010). The high degree of survivability of *B. coagulans* under gastrointestinal condition could be attributed to the spore forming ability of the bacteria that prevents the bacteria from harsh environment.

### **2.1.2. Adherence Capacity - *B. coagulans* Unique IS2**

In another study, Sudha et al. (2010) studied the capability of *B. coagulans* Unique IS2 to adhere to the inner lining of the gastrointestinal tract, following the method of Wiencek et al. (1998) and Doyle and Rosenberg (1997). The adherence ability of the strains was evaluated based on cell surface hydrophobicity tests with different hydrocarbons. For this assessment, N-hexadecane was used for evaluating the adherence ability of *B. coagulans* Unique IS2. It showed cell surface hydrophobicity 32%, which conferred the survival and growth of *B. coagulans* Unique IS2 in gastrointestinal tract (Sudha et al., 2010). Additional evidence for the adherence of Unique IS2 is found in other clinical trials. Specifically, Majeed and Prakash (1998) reported that upon discontinuation of ingestion, *B. coagulans* spores are excreted in feces for approximately 7 days.

### **2.1.3. Antibiotic and Antimicrobial Activity - *B. coagulans* Unique IS2**

In order to assess the susceptibility of *B. coagulans* IS2, Sudha et al. (2010) employed the disk diffusion method published by Charteris et al. (1998). *B. coagulans* Unique IS2 was assayed against twenty-one antibiotics and the diameter of zone of inhibition around antibiotic discs measured (Sudha et al., 2011a) and sensitivity level was determined using high performance standards for antimicrobial disc susceptibility of clinical and laboratory standard institute (NCCLS, 2005). Results of the antibiotic sensitivity profile are summarized in Table 9. The results of these investigations suggest that *B. coagulans* Unique IS2 was found to be

sensitive to majority of antibiotics tested with the exceptions of Bacitracin, Colistin, Methicilin and Metranidazole and Streptomycin, while intermediate for Clindamycin, Doxycycline, Erythromycin, Penicillin and Tetracycline.

In a recent publication by EFSA (2008), concerns with disc diffusion methods are highlighted. These concerns include effect of media components on antibiotics, diffusion of antibiotic, unknown amount of cell concentration, etc. Latest description from the EFSA (2008) and other researchers suggest that the microdilution method is effective. In this method a uniform concentration of antibiotics throughout the growth medium and a known amount of cells must be taken for the study. Considering these factors, microdilution method is specified to study antibiotic sensitivity pattern of probiotics. In an attempt to further understand antibiotic resistance noted following disk diffusion method, microdilution method was employed to investigate susceptibility of the *B. coagulans* Unique IS2 strain (Table 10). The minimum inhibitory concentration (MIC) of the test antibiotics was found to fall within the breakpoint limit concentration proposed by EFSA (2008). The results of these investigations suggest that *B. coagulans* Unique IS2 strain is safe and is unlikely to possess transferrable antibiotic resistance genes.

**Table 9. Antibiotics Resistance Pattern for *B. coagulans* Unique IS2\* by Disk Diffusion**

Name of the Antibiotic	Finding	Name of the Antibiotic	Finding
Bacitracin	Resistant	Methicilin	Resistant
Cefaclor	Sensitive	Metronidazole	Resistant
Cephoxitin	Sensitive	Nalidixic Acid	Sensitive
Chloramphenicol	Sensitive	Penicillin	Intermediate
Ciprofloxacin	Sensitive	Polymixin B	Sensitive
Clindamycin	Intermediate	Rifampcin	Sensitive
Colistin	Resistant	Streptomycin	Resistant
Doxycycline	Intermediate	Trimethoprim	Sensitive
Erythromycin	Intermediate	Novobiocin	Sensitive
Gentamycin	Sensitive	Tetracycline	Intermediate
Kanamycin	Sensitive		

\*Adapted from Sudha et al. (2010)

**Table 10. Antibiotic Resistance as Evaluated by Microdilution Method**

Name of the Antibiotic	Results (sensitive/Resistance)*
Ampicilin	Sensitive
Streptomycin	Sensitive
Erythromycin	Sensitive
Ciprofloxacin	Sensitive
Chloramphenicol	Sensitive
Kanamycin	Sensitive
Tetracycline	Sensitive
Trimethoprim	Sensitive
Gentamycin	Sensitive
Clindamycin	Sensitive

\*Sensitivity or resistance was determined as per EFSA (2008) recommendation

Probiotics have the capacity to suppress the pathogens by natural ways to maintain a normal balance in the intestine. The antimicrobial activity of probiotics may be due to different organic acids such as lactic acid, acetic acid along with H<sub>2</sub>O<sub>2</sub> and bacteriocin. In an *in vitro* study, antimicrobial potentials of *B. coagulans* Unique IS2 against three pathogens such as *Escherichia coli* NCDC 135, *Shigella flexneri* MTCC 1457 and *B. cereus* NCDC 240 was investigated (Sudha et al., 2010). The qualitative analysis of antibiotic sensitivity done with around 25 different broad spectrums and/or commonly used antibiotics for profiling the resistance behavior of the test strains towards different antibiotic agents. On evaluating the antibacterial activity of *B. coagulans* Unique IS2, the maximum zone of inhibition was found against *Bacillus cereus* NCDC 240 (14 mm) followed by *E. coli* NCDC 135 (12 mm). However, the strain was not found to be effective in inhibiting *Shigella flexneri* MTCC 1457 (Sudha et al., 2010). In a published study, Yilma et al. (2006) also found the same type of results.

#### **2.1.4. Animal Studies of *B. coagulans* Unique IS2**

##### **2.1.4.1. Acute Toxicity of *B. coagulans* Unique IS2**

In an acute toxicity study, Sprague Dawley rats (6/sex/group) were divided into three groups (Sudha et al., 2011a). *B. coagulans* Unique IS2 ( $5 \times 10^9$  cfu/g) dissolved in water was administered through oral route at dose levels of 0 (control, vehicle), 3250 and 6500 mg/kg body weight (bw). The animals were observed for 14 days. Observations were focused on behavioral pattern during 1, 7, 14 days and ophthalmoscopy during first and 14<sup>th</sup> day of the study. Clinical signs and mortality were observed daily. The body weights and feed intake were recorded on day 1, 7, 10 and 14. All animals were euthanized before necropsy. The selected dose level in rats for acute toxicity testing was 25X and 50X more of the intended exposure to human studies. Administration of single dose of *B. coagulans* Unique IS2 did not produce any treatment-related changes in any of the animal. As compared to control group, no significant changes in body weight were noted. Similarly, no treatment-related changes were observed in clinical signs, feed intake and gross pathology. The results of this study show that the LD<sub>50</sub> of *B. coagulans* Unique IS2 is greater than 6500 mg/kg bw ( $32.5 \times 10^9$  cfu/kg bw).

##### **2.1.4.2. Repeat-dose Toxicity of *B. coagulans* Unique IS2**

In a short-term, repeat-dose study, toxicity of *B. coagulans* Unique IS2 was investigated in rats (Sudha et al., 2011a). In this dose-response study, a total of 48 rats (24/sex) were divided randomly into four groups (6/sex/group) i.e., control (Group I; vehicle), low dose (Group II), medium dose (Group III) and high dose (Group IV). Rats were administered orally (gavage) with 0, 130, 650, 1300 mg *B. coagulans* Unique IS2 preparation/kg bw/day for 14 consecutive days. The *B. coagulans* Unique IS2 preparation contained  $5 \times 10^9$  cfu/g. The resulting in daily dose of *B. coagulans* Unique IS2 for each group was 0.0,  $0.65 \times 10^9$ ,  $3.25 \times 10^9$  and  $6.50 \times 10^9$  cfu/kg bw/day, respectively. All animals were observed daily for clinical signs and mortality. The body weights, detailed clinical examination were recorded weekly. Urine analysis was performed during the last week of the study and fasting blood samples were collected for the hematological analysis and biochemical estimations on the day of necropsy. Half of the animals from each group were euthanized on day 15, while remaining animals (recovery group) were euthanized on day 28 and observed for gross lesions. All organs such as adrenals, bone marrow, brain, colon, esophagus, heart, kidneys, liver, lungs, ovaries, spleen, stomach, testes, thymus, trachea, uterus and site of injection were collected and preserved for histopathological examination. Organs

such as spleen, testis with epididymis (male), heart, kidneys with adrenals, liver, brain and lungs were weighed before preservation. No treatment-related changes were observed in clinical signs, bodyweights, feed intake, urine parameters, hematological examinations, clinical chemistry, gross pathology and histopathology. Based on the results of this study, the investigators concluded that *B. coagulans* Unique IS2 was clinically well tolerated at doses up to 1300 mg or  $6.5 \times 10^9$  cfu/kg bw/day, when administered orally to Sprague Dawley rats for 14 days consecutively. The No Observed Adverse Effect Level (NOAEL) for *B. coagulans* Unique IS2 was determined as 1300 mg ( $6.5 \times 10^9$  cfu)/kg bw/day, the highest dose tested (Sudha et al., 2011a).

### 2.1.5. Human Studies of *B. coagulans* Unique IS2

Sudha et al. (2011b) investigated the effects of *B. coagulans* Unique IS2, on serum cholesterol levels in hypercholesterolemic subjects. In this study, 30 hyperlipidemic (serum cholesterol levels between 200 to 240 mg/dl) subjects (15 male and 15 female; age 42-53 years) were divided into 3 groups (n=10). Subjects in two groups were allotted to receive a daily dose of two capsules containing *B. coagulans* Unique IS2 ( $10 \times 10^9$  cfu/capsule (Group A) and  $20 \times 10^9$  cfu/capsule (Group B) and the subjects in third group received standard medication. Serum lipid profile was analyzed at 0, 30 and 60<sup>th</sup> day of the study period. At the end of study there were slight reductions in total cholesterol (11%) and LDL (0.8%), whereas an increase in HDL cholesterol levels (3.6%) was noted. No adverse effects were reported.

In another study, Sudha et al. (2012a) investigated the effects of oral supplementation of probiotic capsules containing *B. coagulans* Unique IS2 in subjects with bacterial vaginosis. The study participants included 40 Indian women diagnosed with bacterial vaginosis by the presence of symptoms including white discharge, pH greater than 4.7, burning micturation, itching, soreness and redness at vulva. The subjects were divided into two groups probiotic (n = 20) and control (n = 20) based on age (control group:  $33 \pm 3$  years; probiotic group:  $32.5 \pm 3$  years), history of previous vaginosis (control group: 75% or 15/20; probiotic group: 75% or 15/20) and severity of current vaginosis infection (burning micturation and itching, 35% in each group). The control group patients were given standard vaginosis treatment alone (Ofloxacin–Ornidazole with a strength of 200–500 mg per capsule/day for 5 days along with co-kimaxazol vaginal peccaries for 3 days). The probiotic group patients took standard vaginosis treatment plus two capsules of *B. coagulans* Unique IS2 ( $1 \times 10^9$  cfu/capsule) twice a day before meals for 90 days. All the subjects returned for follow-up visit at 90<sup>th</sup> day. Follow up evaluation included, diagnosis for bacterial vaginosis symptoms (described above) and a report of adverse events. At the end of the treatment, 80% of the probiotic group subjects showed significant positive response as revealed by reduction of vaginosis symptoms as compared to the control group which exhibited reduction in 45% subjects only. No adverse effects of the treatment were reported. The results of present study indicate that strain *B. coagulans* Unique IS2 can provide benefits to women being treated with antibiotics to cure an infectious condition.

In yet another study, Sudha et al. (2012b) evaluated the efficacy and safety of *B. coagulans* Unique IS2 in the treatment of patients with acute diarrhea. In this prospective, phase I clinical trial, a total of 28 patients with acute diarrhea were included. The study participants were between ages 18 to 55 years with  $\geq 3$  loose motions in last 24 hours for less than 7 days. All participants received capsule (containing 2 billion or  $2 \times 10^9$  cfu of *B. coagulans* Unique IS2) two times a day for 10 days. Efficacy was evaluated by assessment of duration of diarrhea and frequency of defecation, abdominal pain and consistency of stool. Safety aspects of *B. coagulans*

Unique IS2 were evaluated by assessment of incidence, type of adverse events, physical examination, and clinical laboratory test values (CBC, SGPT, serum creatinine, stool routine and microscopy). Treatment with *B. coagulans* Unique IS2 decreased mean values for duration of diarrhea, frequency of defecation, and abdominal pain, while consistency of stool improved. Significant reduction in counts of RBC, WBC, and content of serum creatinine was observed, however the values were found to be in the normal range. No other significant changes in safety parameters were observed during the treatment. The investigators concluded that use of *B. coagulans* Unique IS2 is effective and safe in the treatment of patients with acute diarrhea.

## **2.2. Studies with Other Strains of *B. coagulans***

### **2.2.1. Antimicrobial Resistance – Other Strains**

Moldenhauer et al. (1996) investigated the antimicrobial resistance potentials of *B. coagulans* and two other *Bacillus* species. In this study, resistance to thirty antimicrobial agents was investigated. Trypticase Soy Agar (TSA) plates were swabbed with *B. coagulans* ATCC 51232 spores, and then individual discs were impregnated with an antimicrobial agent dispensed onto the surface to determine a zone of inhibition of the growth of *B. coagulans* by the antibiotic. *B. coagulans* was found to be susceptible to 28 antibiotics tested suggesting that *B. coagulans* is susceptible to commonly used antibiotics. Hong et al. (2008) summarized unpublished work from another laboratory in which 33 isolates of *Bacillus* strains were tested and over half showed resistance to clindamycin. Hong et al. (2008) speculated that clindamycin resistance may be an intrinsic characteristic of *Bacillus* species. However, as reported earlier (Section 2.1.3.), *Bacillus coagulans* Unique IS2 was susceptible to clindamycin.

The available information indicates that *B. coagulans* does not produce antibiotics. FDA has listed the use of *B. coagulans* in the production of glucose isomerase enzyme. In its list of enzyme preparations used in food, FDA has stated that, “Insoluble glucose isomerase enzyme preparations are derived from recognized species of precisely classified, nonpathogenic, and nontoxicogenic microorganisms, including *Streptomyces rubiginosus*, *Actinoplanes missouriensis*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes* and *Bacillus coagulans* grown in a pure culture fermentation that produces no antibiotic.”<sup>4</sup>

### **2.2.2. Virulence – Other Strains**

Lactic acid bacteria that occur naturally have an excellent safety profile and it is well recognized that these bacteria are non-pathogenic to human (Fooks and Gibson, 2002; Doron and Gorbach, 2006). The available literature did not reveal any major safety issues or health risks to humans related to the use of lactic acid producing bacteria (Holzapfel et al., 1995; Salminen et al., 1996). Compared to commonly marketed and used *Lactobacillus* and *Bifidobacterium* species, *B. coagulans* has a longer safe history of use. There is no evidence of significant pathogenic or opportunistic illness related to *B. coagulans* consumption.

In a published study, Banerjee et al. (1998) reported 24 episodes of *Bacillus* bacteremias in 18 febrile patients during January 1978 to June 1986. Of the 24 episodes, only one was identified as related to *B. coagulans*. Twelve of the 24 episodes of *Bacillus* bacteremia were

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<sup>4</sup> Available at:

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/MicroorganismsMicrobialDerivedIngredients/default.htm>

considered possible infections. Of the twelve patients, 4 had clinically documented sites of infection at the time of the bacteremic episodes, but specific microbiologic documentation of the offending pathogen(s) was not obtained. The remaining eight patients did not have a clear cause for the *Bacillus* bacteremia, nor had a clinical site of infection. This information indicate that *B. coagulans* is likely only an opportunistic bacteria, and this bacteria may only be opportunistic in a highly immuno-compromised population, and would not be defined as virulent. There is no direct evidence that *B. coagulans* causes infection following oral ingestion, however it is recognized that like most other bacteria, *B. coagulans* could translocate from the gut. However, no harmful effects would be expected in all but severely immunocompromised individuals (Cotton et al., 1987). In studies published over a decade ago, the identification of *B. coagulans* may be suspect, as newer genetic methods have demonstrated that possibility.

Donskey et al. (2001) investigated the effect of oral administration of *B. coagulans* on the density of vancomycin-resistant enterococci (VRE) in fecal matter in mice. In this study, male CF-1 mice were subcutaneously injected clindamycin at a dose of 1.4 mg/day two days. The mice received gastric inoculation of one of three different strains of VRE (VanB-1, Van A and VanB-2;  $1 \times 10^8$  cfu/mouse) before and three days after clindamycin treatment. The results showed that four days of oral *B. coagulans* therapy resulted in a statistically significant reduction in the density of stool VRE in mice colonized with one VanB strain, but not in a second VanB strain or in a VanA strain. *B. coagulans* remained detectable in the stool of mice four days after completion of therapy. These results indicate that *B. coagulans* treatment may reduce the density of colonization with some strains of VRE.

### **2.3. Human Studies – Other Strains**

The available information suggests that following oral administration, spores of *B. coagulans* pass through the stomach and reach the intestine. In about four hours after administration, these spores germinate and multiply rapidly in the intestine. Following discontinuation of treatment *B. coagulans* spores can be found in feces for up to seven days of its ingestion. In multiple clinical trials, no adverse effects of *B. coagulans* administration were noted. In a long term study in children, daily administration of a *B. coagulans* preparation at a daily dose level of  $1 \times 10^8$  spores for 12 months did not result in adverse effects. The findings from some of the clinical studies of *B. coagulans* are summarized in Table 11.

**Table 11. Summary of Published Clinical Studies of *B. coagulans***

Reference	No. of subjects	Treatment	Condition	Findings
Cui et al. (2002)	204 (103 and 101/group)	$3 \times 10^8$ cfu of <i>B. coagulans</i> or <i>Bifidobacterium longum</i> (for 3-7 days and 14-21 days)	Acute or chronic diarrhea	No treatment-related adverse effects; effective in the treatment
Mandel et al. (2010)	45 (23 and 22/group)	$2 \times 10^9$ cfu daily for sixty days	Rheumatoid arthritis	Appeared to be a safe. There were no serious adverse reactions reported throughout this study.
Dutta et al. (2010)	148 (78 and 70/group)	$0.24 \times 10^9$ cfu daily for 5 days	Diarrhea in children	No therapeutic impact on management of acute dehydrating diarrhea
Kalman et al. (2009)	61 (30 and 31/group)	$2.0 \times 10^9$ cfu/capsule daily for 4 weeks	Gastrointestinal symptoms	Significant improvement in GSRS abdominal pain subscore was noted. No adverse effects were reported.
Hun (2009)	44 (22/group)	$0.8 \times 10^9$ cfu daily for eight weeks	IBS- Abdominal pain and bloating patients	Improvements from baseline in abdominal pain and bloating scores in the treatment group. May be a safe and effective

### 2.3.1. Studies in Infants and Children – Other Strains

In a clinical trial, published as an abstract, Dhongade and Anjaneyulu (1977) investigated the effects *B. coagulans* in the treatment of neonatal diarrhea. In this study, 60 infants with neonatal diarrhea were treated with  $1.5 \times 10^7$  *B. coagulans* spores/day. Within 2 days of the administration of *B. coagulans*, 49 infants responded to the treatment. No adverse effects of the treatment were noted. Additional details of the study were not available.

In another randomized, double-blind, placebo-controlled, cross-over trial in infants with symptomatic gastroesophageal reflux (GER), Labalestra et al. (2008) investigated the effect of a combination of symethicone and *B. coagulans* on the gastric emptying time (GET) and relief of symptoms. In this study, 19 consecutive children, younger than one year of age, (11 female, 8 male; mean age: 5.5 months) with symptomatic GER were given the combination as an oral solution as well as a placebo for seven days administered four times daily. The wash-out period was seven days. In the group receiving the combination of symethicone and *B. coagulans* final GET was significantly shorter as compared to the placebo group. Additionally, the group receiving the combination treatment showed a stronger improvement of the GER symptoms as compared to the placebo group. No adverse effects were reported.

In yet another randomized, double-blind, placebo-controlled trial, La Rosa et al. (2003) studied the effects of *B. coagulans* and fructo-oligosaccharides (prebiotic/probiotic) preparation in the prevention of antibiotic-related diarrhea in children. In this study, 120 children with active infections requiring antibiotics were divided in two groups (60/group) and were treated orally with probiotic/prebiotic preparation or a placebo. The treatment group received daily mixture containing *B. coagulans* ( $5.5 \times 10^8$  cfu) and fructo-oligosaccharide (250 mg), while control group received placebo. Of the 98 evaluable subjects, no diarrhea was noted in the 71% of the subjects in the treatment group receiving the prebiotic/probiotic as compared to 38% in the placebo group.

In the treatment group, the duration of diarrhea was significantly lower (0.7 days) as compared to the placebo group (1.6 days). The investigators concluded that prophylaxis with the prebiotic/probiotic treatment significantly reduced the number of days and duration of events in children with antibiotic-induced diarrhea. No adverse effects of treatment were reported.

### 2.3.2. Infection-related Studies – Other Strains

In a review article, Losada and Olleros (2002) discussed utility and advantages of *B. coagulans* from various studies. These investigators also reported that *B. coagulans* exhibits a high degree of safety. In another review article, Doron et al. (2008) undertook meta-analyses of clinical trials in which probiotics were used in the prevention of antibiotic associated diarrhea. Based on this analysis, these investigators noted that *B. coagulans* strains are effective and generally safe. In yet another review article, Johnston et al. (2007) assessed the efficacy and safety of probiotics for the prevention of antibiotic-associated diarrhea in children. For this assessment, 10 clinical trials with *Lactobacilli* spp., *Bifidobacterium* spp., *Streptococcus* spp., or *Saccharomyces boulardii* alone or in combination, *Lactobacillus* GG, *B. coagulans*, *Saccharomyces boulardii* at  $0.5 \times 10^{10}$  to  $4 \times 10^{10}$  cfu/day were evaluated. The investigators noted that the most promising probiotics were considered as *Lactobacillus* GG, *B. coagulans*, *Saccharomyces boulardii* at 5 to 40 billion cfu/day).

In a randomized, double-blind trial, Cui et al. (2004) evaluated the effects of *B. coagulans* in subjects suffering from acute and chronic diarrhea. In this study, 204 subject were divided in to control group (n = 101; 51 - acute diarrhea; 50 - chronic diarrhea) and treatment group (n = 103; 51 - acute diarrhea; 52 - chronic diarrhea). The control group received Golden Bifid (*Bifidobacterium longum*) at a dose level of  $1 \times 10^8$  cfu three times daily for 3-7 days (acute diarrhea) and 14-21 days (chronic diarrhea), while treatment group was treated with *B. coagulans* at a dose of  $1 \times 10^8$  cfu, three times daily for 3-7 days (acute diarrhea) and 14-21 days (chronic diarrhea). No treatment-related adverse effects were noted in both the groups. In the gut of both groups, significant increase in *Bifidobacterium* and *Lactobacillus* species was noted. The investigators concluded that the *B. coagulans* is an effective agent in the treatment of acute and chronic diarrhea and that its efficacy and safety are similar to that of Golden Bifid.

### 2.3.3. Additional Clinical Evidence – Other Strains

The available information indicates that following oral administration, *B. coagulans* passes through the stomach in spore form to the duodenum, where it germinates and multiplies rapidly (Losada and Olleros, 2002). Following oral ingestion, it takes about 4 hours for the spores to travel to the duodenum and start germination. Following germination, *B. coagulans* becomes metabolically active as part of the facultative anaerobes and produces lactic acid. The available evidence indicate that *B. coagulans* resides temporarily the human intestinal tract. Upon discontinuation of the ingestion, spores of *B. coagulans* are excreted for approximately 7 days in the feces (Majeed and Prakash, 1998). *B. coagulans* has been claimed to improve gastrointestinal ecology by replenishing the quantity of desirable obligatory bacteria and antagonizing pathogenic microorganism (Anonymous, 2002).

Kajimoto et al. (2005) studied the effects *B. coagulans* SANK70258 in subjects with seasonal allergic rhinitis. In this randomized, placebo controlled, double-blind clinical trial, 55 healthy male and female volunteers (age 20-65 years) with a history of Japanese cedar pollinosis were randomized to receive either test food containing  $4 \times 10^8$  viable *B. coagulans* SANK70258

cells (n=29) or placebo (n=26) for eight weeks. The subjects were monitored for improvements from allergy and for safety related commonly measured hematology and clinical chemistry parameters. Additional, monitoring included adverse effects such as gastrointestinal symptoms and skin symptoms. No significant changes in the physical examination, hematology and clinical chemistry parameters were noted between the groups. Similarly, no adverse reactions were noted in the group receiving test food. *B. coagulans* SANK70258 at a daily dose level of  $4 \times 10^8$  cfu for eight weeks was found to be safe for human consumption.

In an open label study, Mohan et al. (1990a; 1990b) investigated the effects of *B. coagulans* on serum lipid levels in hypercholesterolemic patients. In this study, administration of *B. coagulans* spores at a dose level of  $3.6 \times 10^8$  cfu/day for 12 weeks to 17 patients with type II hyperlipidemia was found to result in a significant decrease in the total cholesterol vs. HDL cholesterol ratios by 24%, while LDL vs. HDL ratios decreased by 33%. Additionally improvement in total cholesterol to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol ratios was noted without any change in the serum triglyceride levels. No adverse effects of *B. coagulans* treatment were noted.

Ara et al. (2002) evaluated the effects of *B. coagulans* on intestinal flora and decomposition products in the intestine, as well as on various dermal characteristics in 20 volunteers. The subjects were monitored for six weeks: two weeks before administration, two weeks during administration of *B. coagulans* ( $1 \times 10^8$  cfu/day) and two weeks after treatment. Stool samples were collected and analyzed for decomposition products, with the volunteers recording their defecation frequency and assessing their fecal characteristics throughout the study period. Ingestion of *B. coagulans* revealed improvement in the fecal shape, change of fecal color from dark brown to yellowish brown, decrease of fecal odor, the fecal pH, and an increase in defecation frequency of persons whose frequency was relatively low. Following administration and as compared to the values before the intake, the number of intestinal bifidobacteria was found to be significantly increased, whereas the number of intestinal *C. perfringens* significantly decreased. The levels of intestinal ammonia, indole and p-cresol were decreased. *B. coagulans* administration was found to improve the intestinal environment, defecation frequency, fecal characteristics and dermal characteristics.

In another study, Ara et al. (2002) investigated the effects of *B. coagulans* in 23 female volunteers (20 to 40 years old), with a tendency for constipation, on dermal characteristics as a result of the changes in the intestinal environment. The total study period was 12 weeks, with four weeks before administration, four weeks of administration of *B. coagulans* SANK 70258 ( $1 \times 10^8$  cfu/day), and four weeks of placebo administration. In this study, the volunteers recorded the defecation frequency and fecal characteristics and skin characteristics (number of comedones). The skin was analyzed every two weeks by counting the number of skin eruptions (flares and papules). A significantly greater stool defecation frequency was noted following *B. coagulans* administration and as compared to before the intake. Seventy two percent of the subjects that complained of constipation or diarrhea before intake reported significant improvements after the treatment with *B. coagulans*. No adverse effects of *B. coagulans* treatment were noted.

Iino et al. (1997a, 1997b) studied the effects of *B. coagulans* on intestinal microflora in two separate studies. In the first study, Iino et al. (1997a) investigated the effects of *B. coagulans* on stool color, stool shape, stool frequency, defecation feeling and stool odor in 28 adult healthy Japanese women. The subjects ingested one sachet (containing lactose and  $1 \times 10^8$  *B. coagulans*

cells/g) per day for two weeks. Improvements in stool properties (color, shape), along with increase in defecation frequency, was noted. No adverse effects of *B. coagulans* were reported. In the second study, Iino et al. (1997b) investigated the effects of *B. coagulans* on intestinal flora, decayed products and stool property. In this study, 18 healthy adult women were divided in three groups to receive  $0.2 \times 10^8$ ,  $1.0 \times 10^8$  and  $2.0 \times 10^8$  cells of *B. coagulans* per day for two weeks. No subject complained of gas generation, diarrhea or continuous abdominal pain problems due to ingestion of *B. coagulans*. The investigators suggested that consumption of *B. coagulans* improves the bacterial flora and improves the health.

In a randomized, double-blind, placebo-controlled clinical trial, 55 volunteers (including patients with diarrhea-predominant irritable bowel syndrome- IBS-D) received either *B. coagulans* (GBI-30, 6086;  $2 \times 10^9$  cfu) (n=26; 7 male, 19 female) or placebo (n=29; 6 male, 23 female) once a day for 8 weeks (Dolin, 2009). During the course of study, adverse events reported for the most part were mild to moderate and generally self limiting. Five patients who received *B. coagulans* reported six adverse effects and six patients receiving placebo reported six adverse effects. In the placebo group one severe adverse effect (headache) was reported. In general, *B. coagulans* was well tolerated. The results of this study suggest that *B. coagulans* (GBI-30, 6086) is safe and effective for reducing daily bowel movements in patients with IBS-D.

Kalman et al. (2009) evaluated the effects of *B. coagulans* on gastrointestinal symptoms in adults with post-prandial intestinal gas-related symptoms (abdominal pain, distention, flatulence). In this randomized double-blind, placebo-controlled clinical trial, 61 adults volunteers (age  $36.5 \pm 12.6$  years) were randomized to receive  $2.0 \times 10^9$  cfu *B. coagulans*/day *B. coagulans* GBI-30, 6086 preparation (n=30) or placebo (n=31) for four weeks. In addition to hemodynamics (standard biochemical safety) and adverse event monitoring, the subjects were evaluated every two weeks and during each visit, the participants were asked to fill a series of questionnaires. Additional details of the hemodynamic parameters measured were not mentioned in the publication. The investigators concluded that the *B. coagulans* was effective in improving the quality of life and reducing gastrointestinal symptoms in adults with post prandial intestinal gas-related symptoms and no GI diagnoses (Kalman et al., 2009).

#### **2.4. Animal Studies – Other Strains**

In an acute toxicity study, administration of *B. coagulans* GBI-30, 6086 cell mass at a dose level of 5 g/kg bw ( $5.2 \times 10^{11}$  cfu/kg bw) to Wistar rats did not result in mortality or adverse effects (Endres et al., 2009). The results of this study suggest that the LD<sub>50</sub> of the cell mass containing *B. coagulans* is greater than 5 g/kg bw. Endres et al. (2009) also investigated subchronic toxicity potentials of *B. coagulans*. In this study performed as per OECD guidelines, *B. coagulans* (GanedenBC<sup>30</sup>™) cell mass ( $1.36 \times 10^{11}$  cfu/g) was orally administered (gavage) to Wistar rats (10/sex/group) at dose levels of 0, 100, 300 and 1000 mg/kg bw/day for 90 consecutive days. The highest dose treated animals received a dose of  $1.36 \times 10^{11}$  cfu *B. coagulans*/kg bw/day. No deaths or treatment-related signs were observed throughout the study period in any of the groups. Appearance and behavior of the animals were similar for all groups. No toxicologically significant differences between the treatment and control groups with respect to feed consumption, water consumption, sensory reactivity, general and behavioral conditions, hematological and clinical chemistry evaluations was noted. At termination, no treatment-related macroscopic or microscopic changes in the organs were noted. The NOAEL for both males and females was determined to be >1000 mg ( $1.36 \times 10^{11}$  cfu)/kg bw/day, the highest dose tested (Endres et al., 2009).

In a subsequent one-year feeding study, Endres et al. (2011) studied the long-term effects of *B. coagulans* in rats. The study was conducted as a combined study to investigate chronic oral toxicity along with one-generation reproductive toxicity. *B. coagulans* preparation was fed to Wistar rats (20/sex/group) in their diet at levels of 0, 10,000, 20,000 and 33,300 mg/kg feed, corresponding to a dose level of 0, 600, 1200 and 2000 mg/kg bw/day, respectively, for 52 to 53 weeks. The intake at 2000 mg/kg bw/day will be equivalent to approximately  $2.6 \times 10^{13}$  cfu/person/day spores. No mortality was noted in the treatment groups. Clinical observations did not reveal any toxic signs related to the test article. No *B. coagulans* treatment-related changes in body weight, body weight gain, or feed consumption were noted during the study. Blood samples from 3 week and 3, 6 or 12 months did not reveal any toxicological relevant changes in hematological, clinical chemistry or urine parameters. Statistically significant changes noted were either not dose-related, or were well within the historical background range or not correlated with other hematological or histopathological changes. Termination, macroscopic and microscopic examinations did not reveal treatment-related lesions. The NOAEL in male and female rats was determined as 1948 and 2525 mg/kg bw/day, respectively, the highest dose tested (Endres et al., 2011).

In the one-generation reproduction toxicity study by Endres et al. (2011), Wistar rats were divided in to four groups (10/sex/group) and were fed a diet containing *B. coagulans* preparation at a dose levels of 0, 600, 1200 and 2000 mg/kg bw/day. For this study, male rats were fed the diet for 70 days before mating and during the three-week mating period, while female rats were fed for ten weeks prior to mating, during the three-week mating period, throughout pregnancy and lactation and up to weaning of the F1 offspring. There were no signs of treatment-related toxicity on the F0 (parental) generation (male or female). The NOEL for the parental group (reproductive performance) male and female rats was established as 2372 and 3558 mg/kg bw/day, respectively. The NOEL for the F1 offspring was determined as 3558 mg/kg bw/day (Endres et al., 2011).

Cavazzoni et al. (1998) investigated the effects of *B. coagulans* during the first seven weeks of life of chicken. In this study, 75 male Ross strain chickens were randomly assigned to three treatment groups: Group C- received the standard diet without any additive; Group A- received the antibiotic virginiamycin (10 ppm) contained in the daily diet; and Group P- received *B. coagulans* daily at a dose level of  $1.6 \times 10^{10}$  cfu/kg/day (1000 ppm) for the first seven days of life, then fed  $4.0 \times 10^9$  cfu/kg/day (250 ppm) during days 8-49. The investigators noted that *B. coagulans* became integrated in the enteric microflora and did not interfere with other bacterial groups in this animal model. *B. coagulans* was found to be transient, without any adhesion to the intestinal epithelium. Presence of *B. coagulans* was detected in the feces after one week treatment.

### 3. SUMMARY AND DISCUSSION

In recent years, because of certain advantages, such as resistance to heat and intestinal fluids, spore forming bacteria, including *B. coagulans*, are gaining importance for their potential health benefits. Unique Biotech intends to use a well-characterized *B. coagulans* Unique IS2 strain as a food ingredient in foods for human consumption. *B. coagulans* is a gram-positive, rod-shaped, catalase positive, spore-forming, motile, a facultative anaerobe. *B. coagulans* Unique IS2 is a white to beige to brown colored powder depending on its concentration with characteristic odor and slightly sweet taste. The identity and specifications of the product containing *B.*

*coagulans* Unique IS2 has been fully developed. The bacterial strain is an isolate of *B. coagulans* obtained human fecal soil sample. The identity of *B. coagulans* Unique IS2 has been fully explored and confirmed by phenotypic, genotypic and genomic analysis.

Unique Biotech intends to use *B. coagulans* Unique IS2 at a maximum use level of  $2 \times 10^9$  cfu/serving in a variety of foods such as baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups (excluding meat and poultry products).

In 1932, *B. coagulans* was first isolated and described. As this bacteria forms spores, it possesses high heat and acid resistance. This microorganism is used as a probiotic to improve and maintain ecological balance of the intestinal microflora. In African countries, *B. coagulans* is used in the production of a protein-rich food known as *ugba*. The use of *B. coagulans* in the preparation of a traditional Nigerian food (*ugba*) suggests the dietary consumption of this microorganism. The US FDA has approved the use of *B. coagulans* in the production of glucose isomerase enzyme. This approval supports the conclusion that this microorganism is nonpathogenic and nontoxicogenic. In 2011, FDA issued a “no questions” letter in response to a GRAS notice (GRN 399) for the use of *B. coagulans* strain in conventional foods resulting in a daily intake of  $9.38 \times 10^{10}$  cfu/person/day. The available published animal and human scientific studies of *B. coagulans* further supports its safe-in-use by humans. The available information suggest that *B. coagulans* is well-tolerated, non-pathogenic and non-toxicogenic.

The safety of *B. coagulans* Unique IS2 is supported by *in vitro*, animal toxicity and human tolerance studies. All these studies are published in peer reviewed scientific journals. The *in vitro* studies includes survival in simulated acid, bile tolerance, adherence to cell surface (hydrophobicity), antibiotic sensitivity and antibacterial activity. In addition to chemical characteristics, *B. coagulans* Unique IS2 has been characterized by genotypic analysis. In an acute toxicity study in rats, oral LD<sub>50</sub> of *B. coagulans* Unique IS2 was reported as greater  $32.5 \times 10^9$  cfu/kg bw. In a 14-day repeat dose study, oral administration of *B. coagulans* Unique IS2 spores to rats suggest a NOAEL of  $6.5 \times 10^9$  cfu/kg bw/day, the highest dose tested. In three separate human studies with *B. coagulans* Unique IS2, no adverse effects were reported.

In addition to the above mentioned specific studies of *B. coagulans* Unique IS2, the available information from published studies with other strains of *B. coagulans* further supports the safety. Administration of *B. coagulans* to chickens at a dose level of  $4.0 \times 10^9$  cfu/kg bw/day for up to 7 weeks did not reveal any adverse effects. Additional studies in rats show that *B. coagulans* spore preparation does not induce acute, subchronic, chronic, or reproductive toxicity following consumption of up to 2000 mg/kg bw/day (equivalent to  $2.6 \times 10^{13}$  cfu/person/day) spores. All this information supports the conclusion that oral ingestion of *B. coagulans* is unlikely to cause adverse effects. Furthermore, human studies suggest that following oral administration, *B. coagulans* passes through the stomach and germinates in the intestine within four hours. Upon discontinuation of oral *B. coagulans* administration, spores of this microorganism were noted in feces for up to seven days. Oral administration of *B. coagulans* to infants at a dose of  $1 \times 10^8$  cfu/day for 12 months did not reveal any adverse effects. Similarly,

oral administration of *B. coagulans* at a dose of  $1 \times 10^8$  cfu, three times daily to acute and chronic diarrhea subjects did not reveal any adverse effects. In hyperlipidemic subjects daily oral administration of  $3.6 \times 10^8$  cfu of *B. coagulans* spores for 12 weeks was found to be safe.

The safety determination of *B. coagulans* Unique IS2 is based on the totality of available evidence, including phenotypic and genotypic characterization, and animal and human studies, including those for other similar strains. The evidence of *B. coagulans* safety is supported by:

- History of use of products containing *B. coagulans* species. Use in the production of a traditional protein-rich food known as *ugba*.
- Full identity characterization by phenotypic and genotypic means. No pathogenic and toxicogenic effects noted.
- Lack of antimicrobial activity. Susceptibility to antibiotics.
- Transient nature of *B. coagulans* in the gastrointestinal tract without any cumulative.
- No toxicity reported in animal studies at very high doses.
- No adverse effects noted in several human studies, including studies of up to one year duration and in susceptible groups (children).
- Corroboration of safety from studies with substantially equivalent/similar strains

In summary, on the basis of scientific procedures<sup>5</sup> including knowledge from a history of exposure to *B. coagulans* Unique IS2, the consumption of *B. coagulans* Unique IS2 as an added food ingredient from its intended uses at levels up to  $2 \times 10^9$  cfu/serving in a variety of specified foods and resulting in estimated daily intake of  $36.4 \times 10^9$  cfu *B. coagulans* spores/day is considered safe. The intended uses are compatible with current regulations, *i.e.*, *B. coagulans* Unique IS2 is used in specified foods (described in this document) and is produced according to current good manufacturing practices (cGMP).

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<sup>5</sup> 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

#### 4. CONCLUSION

Based on a critical evaluation of the publicly available data summarized herein, the Expert Panel members whose signatures appear below, have individually and collectively concluded that a *Bacillus coagulans* Unique IS2 preparation, meeting the specifications cited above, and when used at maximum use levels of up to  $2 \times 10^9$  cfu/serving (reference amounts customarily consumed, 21 CFR 101.12) in specific foods (baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups) when not otherwise precluded by a Standard of Identity as described in this dossier and resulting in estimated daily intake of  $36.4 \times 10^9$  cfu *B. coagulans* spores/day is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that the use of this *B. coagulans* preparation in the foods at the levels specified above is GRAS.

#### Signatures

(b) (6)

[Redacted Signature]

Douglas Archer, Ph.D.

May 12, 2014  
Date

(b) (6)

[Redacted Signature]

Robert L. Martin, Ph.D.

May 10, 2014  
Date

(b) (6)

[Redacted Signature]

Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S.

May 16, 2014  
Date

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## 6. APPENDIX I

### Analytical data from five manufacturing lots of 200 billion and 15 billion cfu/g batches

**Appendix I-A:** Specifications of *B. coagulans* Unique IS2 (200 billion) from five non-consecutive manufacturing lots

Parameter	Standard specifications*	Batch Numbers of 200 billion cfu/g				
		UB/12/12/122	UB/13/01/151	UB/12/08/057	UB/13/08/100	UB/13/04/001
Description	Brown colored powder with characteristic odor and slightly sweet in taste.	Complies	Complies	Complies	Complies	Complies
Microscopy	The spores are seen as small terminal oval shape refractile bodies at the end of each vegetative cell	Complies	Complies	Complies	Complies	Complies
Lactic acid producing capacity (ml)	Not less than 10 ml of 0.05 N NaOH should be consumed	14.8	12.4	14.7	12.1	14.5
Assay (billion cfu/g)	Not less than 200 Billion cfu/g	207	210	209	220	220
Loss on drying (%)	Not more than 5%	3.5	2.3	3.6	3.8	4.5
<b>Heavy Metals</b>						
Arsenic	NMT 1 ppm	0.5	0.54	0.31	0.28	0.46
Lead	NMT 2 ppm	0.8	1.4	1.14	0.18	0.12
Mercury	NMT 0.5 ppm	0.26	0.24	0.22	0.18	0.14
Cadmium	NMT 1 ppm	0.45	0.21	0.18	0.51	0.44
Yeast & Mould count	Not more than 50 cfu/g	< 10 cfu/g	10 cfu/g	10 cfu/g	10 cfu/g	<10 cfu/g
<b>Specified Pathogens</b>						
<i>Escherichia coli</i>	Absent/10 g	Absent	Absent	Absent	Absent	Absent
<i>Salmonella</i>	Absent/10 g	Absent	Absent	Absent	Absent	Absent
<i>Pseudomonas aeruginosa</i>	Absent/1 g	Absent	Absent	Absent	Absent	Absent
<i>Staphylococcus aureus</i>	Absent/1 g	Absent	Absent	Absent	Absent	Absent
<i>Listeria monocytogenes</i>	Absent/25 g	Absent	Absent	Absent	Absent	Absent

\*Standard specifications for marketed product

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**Appendix I-B: Specifications of *B. coagulans* Unique IS2 (15 billion) from five non-consecutive manufacturing lots**

Parameter	Standard specifications*	Batch Numbers of 15 billion cfu/g				
		UB/12/06/030	UB/12/06/031	UB/12/06/035	UB/12/07/046	UB/12/08/070
Description	White to beige colored powder with characteristic odor and slightly sweet in taste	Complies	Complies	Complies	Complies	Complies
Microscopy	The spores are seen as small terminal oval shape refractile bodies at the end of each vegetative cell	Complies	Complies	Complies	Complies	Complies
Lactic acid producing capacity (ml)	Not less than 10 ml of 0.05 N NaOH should be consumed	14.3	14.5	12.7	14.3	14.7
Assay (Billion cfu/g)	Not less than 15 Billion cfu/g	17.5	17.5	18.0	18.1	18.2
Loss on drying (%)	Not more than 5 %	4.0	4.6	4.8	3.2	3.7
<b>Heavy Metals</b>						
Arsenic	NMT 1 ppm	0.05	0.05	0.12	0.08	0.16
Lead	NMT 2 ppm	0.8	0.38	0.29	0.92	0.31
Mercury	NMT 0.5 ppm	0.06	0.04	0.22	0.18	0.14
Cadmium	NMT 1 ppm	0.25	0.21	0.18	0.36	0.18
Yeast & Mould count	Not more than 50 cfu/g	<10 cfu/g	<10 cfu/g	20 cfu/g	20 cfu/g	20 cfu/g
<b>Specified Pathogens</b>						
<i>Escherichia coli</i>	Absent/10 g	Absent	Absent	Absent	Absent	Absent
<i>Salmonella</i>	Absent/10 g	Absent	Absent	Absent	Absent	Absent
<i>Pseudomonas aeruginosa</i>	Absent/1 g	Absent	Absent	Absent	Absent	Absent
<i>Staphylococcus aureus</i>	Absent/1 g	Absent	Absent	Absent	Absent	Absent
<i>Listeria monocytogenes</i>	Absent/25 g	Absent	Absent	Absent	Absent	Absent

\*Standard specifications for marketed product

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SUBMISSION END

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