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September 25, 2022

Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Subject: GRAS Notification –
β-agarase DagA As a Processing Aid in the Production of a Food Ingredient

To whom it may concern,

On behalf of Dyne Bio Inc., we are submitting a GRAS notification for β-agarase DagA as a processing aid (in the production of neoagarooligosaccharides, a food ingredient). The enclosed document provides the notice of a claim that the use of β-agarase DagA as a processing aid in the production of a food ingredient 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 a food processing aid. We believe that this determination and notification are in compliance with Pursuant to 21 C.F.R. Part 170, subpart E.

We submit a pdf file for your review. Please feel free to contact me if additional information or clarification is needed as you proceed with the review. We would appreciate your kind attention to this matter.

Sincerely,

[redacted]

Susan Cho, Ph.D.
Susanscho1@yahoo.com or scho@aceoners.com
Agent for Dyne Bio Inc.

DETERMINATION OF
THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF β-agarase DagA
AS A PROCESSING AID

Prepared for:
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Appendices C to F are presented in Part B

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List of Abbreviations

ADME = absorption, distribution, metabolism, and elimination
ALD = approximate lethal dose
ANI = average nucleotide identity
bw = body weight
CAS = chemical abstract service
CFR = Code of Federal Regulations
cGMP = current good manufacturing practice
DDH = DNA-DNA hybridization
DNS = dinitrosalicylic acid
EC = Enzyme Commission
EDI = estimated dietary intake
FAO = Food and Agriculture Organization
FARRP = Food Allergy Research and Resource Program
FCC = Food Chemicals Codex
FD&C = Food, Drug, and Cosmetic
FDA = Food and Drug Administration
FOIA = Freedom of Information Act
FSIS = Food Safety and Inspection Service
GRAS = Generally Recognized as Safe
h = hour
HACCP = Hazard Analysis and Critical Control Point
HDPE = high-density polyethylene
ICR = Imprinting Control Region
IUBMB = International Union of Biochemistry and Molecular Biology
MNPCE = polychromatic erythrocyte with micronuclei
NAO = neoagaro-oligosaccharide
NOAEL = no-observed-adverse-effect-level
PCE = polychromatic erythrocyte
QC = quality control
RBC = red blood cell
rDNA = ribosomal deoxyribonucleic acid
SD = Sprague-Dawley
TOS = total organic solid
U.S.C. = United States Code
USDA = United States Department of Agriculture
UV = ultraviolet
WGS = whole genome sequencing
WHO = World Health Organization

WT = wild type

PART 1. SIGNED STATEMENTS AND A CERTIFICATION

1.A.

Pursuant to 21 Code of Federal Regulations (CFR) Part 170, subpart E, Company (hereinafter referred to as ‘Comp’) submits a Generally Recognized as Safe (GRAS) notice and claims that the use of beta-agarase in foods, as described in Parts 2 through 7 of this GRAS notice, is not subject to premarket approval requirements of the Food, Drug, and Cosmetic (FD&C) Act based on its conclusion that the substance is GRAS under the conditions of its intended use.

1.B. Name and Address of the Notifier

Contact: Je Hyeon Lee, Ph.D.
Company: Dyne Bio Inc. Inc.
Address: B-B205 14 Sagimakgol-ro 45beon-gil Jungwon-gu
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Phone number: +82-10-5356-4439

1.C. Common or Trade Name

β-agarase DagA

1.D. Applicable Conditions of Use of the Notified Substance

1.D.1. Foods in Which the Substance is to be Used

The β-agarase DagA will be used as a processing aid for hydrolysis of agar-agar during production of neoagaro-oligosaccharides (NAO), a food ingredient. However, the enzyme used during the production of NAO is not expected to exert any unintentional enzymatic activity in the final food ingredient due to the following factors: 1) removal (approximately 99.8%) of enzyme during the purification process of NAO; 2) denaturation of the enzymes during processing, and 3) lack of water activity, etc.

1.D.2. Levels of Use in Such Foods

β-agarase DagA is not added to final foodstuffs, but is used as a processing aid during conversion of agar-agar to a food ingredient NAO. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and per requirements for normal production following current good manufacturing practices (cGMP). The following is the maximum suggested use level for the production of NAO: up to 20 mg total organic solid (TOS) or 10,000 U/g raw material, agar-agar on a dry weight basis. After purification steps during the production of a food ingredient NAO, it is expected that residual β-agarase DagA level will be 0.04 mg TOS/g NAO.

1.D.3. Purpose for Which the Substance is Used

The β-agarase DagA will be used as a processing aid in the production of NAO, i.e., the hydrolysis of agar-agar into NAO, a food ingredient.

1.D.4. Description of the Population Expected to Consume the Substance

It is not expected that members of the general population would consume foods with no functional β-agarase DagA activity. The NAO produced by the β-agarase DagA will be purified by a series of purification processes including ultra-filtration and micro-filtration to remove >99.8% of residual enzyme.

Even if a small amount of residual enzyme residue is present in the finished food ingredient, the enzyme is not expected to exert a function in the final food for the reasons described in 1.C.1. Consequently, the presence of residues of food enzymes in the final food does not lead to any effect in or on the final food. The enzyme action is expected to be over in the food product when available to consumers.

1.E. Basis for the GRAS Determination

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.F. Availability of Information

The data and information that are the basis for this GRAS conclusion will be made available to Food and Drug Administration (FDA) upon request by contacting Susan Cho at AceOne RS, Inc. at the address below. The data and information will be made available to FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

1.G. Availability of Freedom of Information Act (FOIA) Exemption

None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the FOIA Act, 5 United States Code (U.S.C.) §552.

1.H. Certification

Dyne Bio Inc. certifies that, to the best of its knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by Dyne Bio Inc., including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of its β-agarase DagA.

1.I. Name, Position/Title of Responsible Person Who Signs Dossier, and Signature

[redacted]

Name: Je Hyeon Lee, Ph.D. Date: 9/23/22
Title: CEO

Address correspondence to
Susan S. Cho, Ph.D., AceOne RS, Inc.
Agent for Dyne Bio Inc.
301-875-6454;
scho@aceoners.com or sscho397@yahoo.com

1.J. Food Safety and Inspection Service (FSIS)/United States Department of Agriculture (USDA) Statement

Dyne Bio does not intend to add β-agarase DagA to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

[redacted]

PART 2. IDENTITY, MANUFACTURING, SPECIFICATIONS, AND TECHNICAL EFFECTS

2.A.1. Identity of the Notified Substance

2.A.1.1. Common Name

IUBMB Enzyme nomenclature Classification: β-agarase DagA
IUB No.: EC 3.2.1.81

2.A.1.2. Chemical Names of Main Component

2.A.1.3. Chemical Abstract Service (CAS) Registry Number

2.A.1.4. Empirical Formula:

β-agarase DagA derived from *Streptomyces coelicolor* A3(2) M22-2C43 is composed of 309 amino acids and its amino acid sequence is as follows:

MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQLLPSHSDDFNYTGKPQ
TFRGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGYVTSRTPVEYPLYTEVLMV
SGLKLSSNFWLLSRDDVNEIDVIECYGNESLHGKHMNTAYHIFQRNPFTELARSQKGYFADGSYGYNGET
GQVFGDGAGQPLLRNGFHRYGVHWISATEFDFYFNGRLVRRLNRSNDLRDPRSRFFDQPMHLILNTESH
QWRVDRGIEPTDAELADPSINNIYYRWVRTYQAV

Amino acid sequence of β-agarase DagA derived from *S. coelicolor* A3(2) M22-2C43 does not have homology with known allergenic proteins. Details are presented in Appendix A.

2.A.1.5. Structural Formula:

2.A.1.6. Molecular Weight: Approximately 32kD

2.A.1.7. Background

β-agarase is designated as EC 3.2.1.81 according to the Enzyme Commission (EC) enzyme tree system shown below (available at: https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.81).

EC enzyme tree
└ 3. Hydrolases
  └ 3.2 Glycosylases
    └ 3.2.1 Glycosidases, i.e. enzymes that hydrolyse *O*- and *S*-glycosyl compounds
      └ 3.2.1.81 beta-agarase

β-agarases (DagA) derived from *S. coelicolor* A3(2) M22-2C43 (which can be often abbreviated as M22-2C43), an ultraviolet (uv)-treated mutant strain of *S. coelicolor* A3(2) M22-2C43-wild type [WT] (or strain M22-2C43-WT), is used to produce a food ingredient NAO.

Diverse carbohydrases are used as processing aids or for production of carbohydrate-ingredients. Agarose can be hydrolyzed into into oligomers (agarooligosaccharides and NAO) by enzymes, and further saccharification of oligomers by enzymatic reactions into monomers (3,6-anhydro-L-galactose and D-galactose). Chemical liquefaction of agarose with acid under mild conditions produced predominantly agarooligosaccharides due to the preferential cleavage of α-1,3-glycosidic bonds. While enzymatic liquefaction using endo-type β-agarase, even-numbered NAO are produced due to the cleavage of β-1,4-glycosidic bonds (Yun et al., 2017). There are two types of β-agarases, DagA and DagB (Temuujin et al., 2011, 2012). β-agarase DagA degraded agar into neoagarotetraose (NA4) and neoagarohexaose (NA6), and β-agarase DagB degraded agar into neoagarobiose (NA2). Dyne Bio Inc.'s β-agarase DagA is derived from *S. coelicolor* A3(2) M22-2C43, an uv-treated mutant of *S. coelicolor* A3(2) M22-2C43-WT and is an endo-type.

2.A.2. Potential Toxicants in the Source of the Notified Substance

Not applicable. The enzyme preparation does not contain any potential toxicant.

2.A.3. Particle Size

Not applicable

2.B. Method of Manufacture

Raw Materials

The raw materials used during the manufacturing process are all standard ingredients allowed in the food industry. The quality control (QC) department of Dyne Bio Inc. Inc., samples the materials on arrival and monitors all the analyses to ensure their qualities.

Table 1 lists the raw materials used in fermentation and their CAS numbers and regulatory status. The raw materials conform to Food Chemicals Codex (FCC) specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with the FCC requirements.

Table 1. List of Raw Materials Used in the Production of β-Agarase DagA and Their Regulatory Status

Material	CAS No.	Regulatory Status
Raw materials for fermentation		
Glucose	50-99-7	21CFR § 184.1865
Yeast extract	8013-01-2	21CFR § 184.1983
Magnesium chloride hexahydrate	7791-18-6	21CFR § 184.1426
Agar powder	9002-18-0	21CFR§ 184.1115
Sodium hydroxide	1310-73-2	21CFR § 184.1763
Processing aid		
Polysulfone (UF)	25135-51-7	21CFR § 177.1655
Polyvinylidene fluoride (MF)	24937-79-9	21CFR § 177.2510

Manufacturing Process consists of two parts: fermentation and recovery/purification process.

Fermentation Process

The β-agarase DagA under this notification is prepared by fermentation of *S. coelicolor* A3(2) M22-2C43 cells. All fermenters and other equipment used during the manufacturing process were designed and constructed to ensure the absence of contamination of foreign microorganisms during fermentation.

Table 1. lists the raw materials used in fermentation and their CAS numbers and regulatory status. The fermentor is equipped with control valves and an agitation system to continuously mix the reaction solution.

Recovery and Purification Process

The recovery process starts immediately after the completion of the fermentation process that consists of centrifuge, filtration, concentration, and standardization. The final product is standardized according to the product specification.

Steps Involved in Manufacturing Processes

1) Seed culture
    a) For the 1st seed culture, the *S. coelicolor* A3(2) M22-2C43 stock solution is streaked onto the RSM3 agar medium (containing 1.5% agar, 0.5% $MgCl_2 \cdot 6H_2O$ and 1.1% yeast extract) plate and incubated at 30 ℃ for 120 hours (h).
    b) For the 2nd seed culture, four colonies of the *S. coelicolor* A3(2) M22-2C43 are isolated, transferred into 100 mL of RSM3 liquid medium (containing 0.3% agar, 0.5% $MgCl_2 \cdot 6H_2O$, 1.1% yeast extract and 1% glucose), and incubated at 30 ℃ for 72 h with 200 rpm in a shaking incubator.
    c) For the 3rd seed culture, the 2nd seed culture solution is transferred to 300 mL of of RSM3 liquid medium and incubated at 30 ℃ for 72 h with 200 rpm in a shaking incubator.

2) Main culture (Working Volume: 30 L)
    a) 30 L of of RSM3 liquid medium is placed into the 50 L fermenter (Model NO.: KS B 6231, KOBIOTECH), followed by sterilization, and cooling.
    b) The 3rd culture medium is aseptically inoculated into the sterilized, cooled fermentation medium and incubated at 30 ℃ for 24 h under 1 volume of air (liter)/volume of medium (liter)/minute (vvm), 2.9 pounds-per-square-inch (psi) and 200 rotation per minute (rpm).
3) Main culture (Working Volume: 300 L)
    a) 300 L of RSM3 liquid medium is placed into the 500 L fermenter (Model NO.: KS B 6231, KOBIOTECH), followed by sterilization, and cooling.
    b) The culture medium from the 50 L fermenter is transferred to the sterilized, cooled 300 L fermentation medium in the 500 L fermenter and incubated at 30 ℃ for 24 h under 1 vvm, 2.9 psi, and 200 rpm.
4) Main culture (Working Volume: 3,000 L)
    a) 3000 L of RSM3 liquid medium is placed into the 5000 L fermenter (Model NO.: KS B 6231, KOBIOTECH), followed by sterilization, and cooling.
    b) The culture medium from the 500 L fermenter is transferred to the sterilized, cooled 3,000 L fermentation medium in the 5,000 L fermenter and incubated at 30 ℃ for 24 h under 1 vvm, 2.9 psi and 80 rpm.

5) Centrifugation

After the main culture in the 5,000 L fermenter, cells are removed by continuous centrifugation (Model No.: MBPX 810 SGV-34CL, Alfa Laval).

6) Concentration

The enzyme activity of the β-agarase DagA enzyme of supernatant is measured, and then is concentrated by ultrafiltration (filter module: SIP-3023 (MWCO: 6,000), Pall Co.) at below 43.5 psi, 15 ℃. This process is repeated 10 to 20 times.

7) Filtration

Concentrated enzyme solution is subjected to membrane filters (filter module: CVHL72TP3 (0.45 um), Merck Millipore.) at below 29 psi at 15 ℃.

8) Formulation

β-agarase DagA activity is adjusted to make the final activity unit of 10,000 U/mL. No additive such as glucose or potassium sorbate, is added.

Figure 1 presents flow diagram of β-Agarase DagA manufacturing process.

Quality Assurance Procedure

The quality of the product is monitored according to the designated product specification. Periodically, samples from the fermenter are taken out to analyze under the microscope to confirm the stain identity by staining, and the absence of foreign microorganisms in the fermenter is regularly tested under the microscope by plating on a nutrient agar plate for overnight incubation at 37°C. The fermentation is declared "contaminated" if one of the following conditions are fulfilled: 1) contamination is observed in 2 or more samples by microscopy and 2) contamination is observed in two successive agar plates at a minimum interval of 8 hours. Any contamination observed will be rejected.

β-agarase DagA is manufactured under cGMP using common food industry materials and processes. Dyne Bio observes the principles of Hazard Analysis and Critical Control Point (HACCP)-controlled manufacturing process and rigorously tests its final production batches to verify adherence to quality control specifications. All processing aids used in the manufacturing process are food grade. Process tanks and lines are cleaned with sodium hydroxide and hydrogen peroxide following standard procedures common to the food industry.

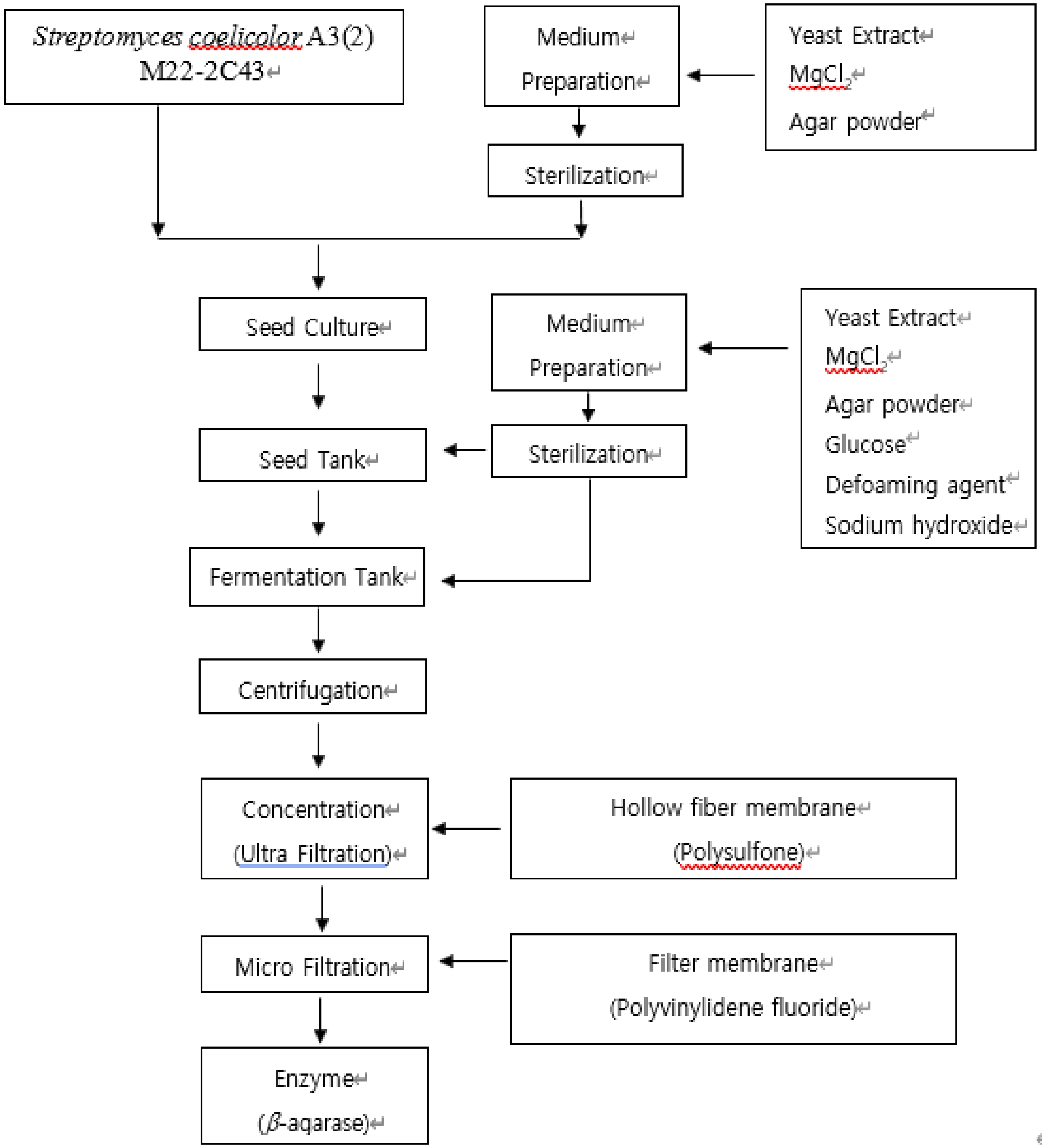

Figure 1. Flow Chart of β-Agarase DagA Production Process

2.C. Specifications and Composition of β-Agarase DagA

Tables 2 to 4 show the specifications and composition of Dyne Bio Inc.'s β-agarase DagA. Tables 3 and 4 presents the analytical values for three non-consecutive lots of β-agarase DagA. The β-agarase DagA enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in *FCC*. In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives in Compendium of Food Additive Specifications (JECFA, 2006). The β-agarase DagA preparation, that is the subject of this notification, does not contain a potential food allergen (such as soy) in the fermentation media. Certificates of analysis are presented in Appendix B.

The β-agarase DagA activity is measured by a modified 3,5-dinitrosalicylic acid method, as reported in Sengupta et al. (2000). Briefly, 0.125 mL of the partially purified enzyme solution was mixed with 0.125 mL of 10 mM phosphate buffer (pH 7.0) containing 1% dissolved agar. After the reaction for 10 min at 45°C, 0.75 mL of dinitrosalicylic acid (DNS) reagent (6.5 g DNS, 2 M NaOH 325 mL, glycerol 45 mL per 1 L distilled water) was added and boiled for 5 min. After cooling, 2 ml of water is added and the optical density at 540 nm (OD540) of the mixture was measured using UV-is spectrophotometer (Mega-U600, Sinco, Seoul, Korea). One unit of enzyme activity is the amount of enzyme that produces reducing sugars equivalent to 1 µmol of glucose per minute from a substrate.

Table 2. Specifications for Dyne Bio Inc.'s β-Agarase DagA

Parameter	Specification	Method of analysis
Activity unit, unit/mL	4.9 - 9.0	Food additive process, 9th, 2018, MHLW(Japan)
TOS, g/100 mL	>1.05	By calculation
Total plate count	≤1,000 cfu/g	AOAC 990.12
Total yeast & mold	≤ 100 cfu/g	AOAC 977.02
Staphylococcus aureus	<10/g	AOAC 975.55
Coliform	<10 cfu/g	AOAC 991.14
Escherichia coli	Absent in 25g	ISO 7251.2005
Listeria monocytogenes	Absent in 25g	AOAC 992.19
Salmonella	Absent in 25g	AOAC 991.13
Mercury (Hg), ppm	≤0.1	ASTM D-6722-01/ CV-AAS
Arsenic (As), ppm	≤0.1	AOAC 2013.06 / ICP-MS
Lead (Pb), ppm	≤0.1	AOAC 2013.06 / ICP-MS
Cadmium (Cd), ppm	≤0.1	AOAC 2013.06 /ICP-MS

cfu = colony forming unit.

Table 3. Summary of Analytical Values for Dyne Bio Inc.'s β-Agarase DagA

Parameter	Batch Number			Mean
	21-NBE0723	21-NBE0917	22-NBE0218	
Activity unit, U/g	7.02	6.97	7.17	7.05
TOS, g/100 g	1.42	1.28	1.39	1.36
Total plate count, cfu/g	<10	<10	<10	<10
Yeast & mold, cfu/g	<10	<10	<10	<10
Staphylococcus aureus, /g	<10	<10	<10	<10
Coliform, cfu/g	<10	<10	<10	<10
Escherichia coli, /25 g	ND	ND	ND	ND
Listeria monocytogenes in 25 g	ND	ND	ND	ND
Salmonella in 25 g	ND	ND	ND	ND
Arsenic (As), ppm	<0.02	<0.02	<0.02	<0.02
Cadmium (Cd), ppm	<0.007	<0.02	<0.007	<0.007
Lead (Pb), ppm	<0.02	<0.02	<0.02	<0.02
Mercury (Hg), ppm	<0.02	<0.02	<0.02	<0.02

cfu = colony forming unit.
ND = Not Detected

Table 4. Other Components Present in Dyne Bio Inc.'s β-Agarase DagA

Component	Batch Number			Method of Analysis
	21-NBE0723	21-NBE0917	22-NBE0218	
Moisture, %	98.47	98.60	98.46	AOAC 935.29
Ash, %	0.11	0.12	0.15	AOAC 923.03
Magnesium (Mg), mg/100 g	69.0	62.0	69.6	AOAC 2013.06
Calcium (Ca), mg/100 g	<20	<20	<20	AOAC 2013.06
Iron (Fe), mg/100 g	<2	<2	<2	AOAC 2013.06
Zinc (Zn), mg/100 g	<2	<2	<2	AOAC 2013.06
Sodium (Na), mg/100 g	16.7	15.0	31.5	AOAC 2013.06
Potassium (K), mg/100 g	68.5	55.1	62.9	AOAC 2013.06
Phosphorus (P), mg/100 g	18.0	50.5	7.35	AOAC 2013.06

*Total Organic Solids is defined as: 100% - water - ash.

Dyne Bio Inc.'s β-agarase DagA is derived from *S. coelicolor* A3(2) M22-2C43 and does not contain any residual production microorganism. Details of safety of the production microorganism and proof for the absence of residual production microorganism are presented in Appendices C and D, respectively.

2.D. Intended Technical Effects

The enzyme β-agarase DagA is used as a processing aid in the manufacture of a food ingredient (NAO) and is not added directly to the final foodstuffs. In particular, β-agarase hydrolyzes the 1,6 β-glucosidic bonds and β-agarase DagA degrades agarose into NA4 and NA6. The typical food processes, where β-agarase DagA is used, include the production of NAO from agar-agar derived from red algae, the subject to 21CFR 184.1115. It is expected that the finished NAO ingredient does not have significant amount of residual β-agarase DagA (less than 0.04 mg TOS/ g NAO) because sequential purification processes are employed for the production of NAO.

2.E. Stability of β-Agarase DagA

The β-agarase DagA activity was tested for stability at two temperatures, and the following microbiological tests were done: aerobic plate counts, yeasts and molds, coliform, *Escherichia coli, Staphylococcus aureus*, and Salmonella. The study was performed with three 100 mL batches of β-agarase DagA in high-density polyethylene (HDPE) bottles. In the accelerated condition, the analysis was performed every 7 days for 42 days at 4°C. In addition, the analysis was performed every 2 months for 12 months at -20°C to check the long term stability at a frozen condition. The tests were done using the AOAC test methods listed in Table 5-1. Each test was done in triplicate. Results are presented in Tables 5-2 to 5-8.

The results showed that the enzyme was stable for 6 weeks at 4°C and for 12 months at -20°C.

Table 5-1. Analytical Methods for β-Agarase DagA Employed in the Stability Tests

Parameter		Specification	Method of analysis
Physical and chemical Test	Appearance	Light brown or dark brown colored liquid	Visual
	Enzyme Activity	4.9 ~ 9.0 Unit/mL	Food additive process, 9th, 2018, MHLW(Japan)
Microbiological Tests	Aerobic plate Count	≤1,000 cfu/g	AOAC OMA 990.12 (Petri film AC)
	Total Yeast & Mold	≤100 cfu/g	AOAC 997.02 (Petri film YM)
	Coliform	<10 cfu/g	AOAC 991.14 (Petri film EC)
	Staphylococcus aureus	<10 cfu/g	AOAC 2003.07 (Petri film STX+Disk)

	Salmonella	Absent in 25 g	BAM chapter 5

Appearance

During the storage period, no detectable changes in appearance were noted.

Enzyme activity of β-agarase DagA

The analytical results of the β-agarase DagA activity confirmed that there were no significant changes in the enzyme activity regardless of the storage period (Tables 5-2 and 5-3).

Table 5-2. Analysis of the β-Agarase DagA Activity at 4°C

	Activity (U/mL)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 days	7.11 ± 0.48	6.87 ± 0.36	7.66 ± 0.43
7 days	7.05 ± 0.35	6.45 ± 0.49	7.54 ± 0.40
14 days	6.95 ± 0.49	6.82 ± 0.45	7.74 ± 0.48
21 days	7.12 ± 0.24	6.67 ± 0.36	7.42 ± 0.51
28 days	7.22 ± 0.30	6.78 ± 0.39	7.68 ± 0.31
42 days	7.03 ± 0.63	6.82 ± 0.28	7.44 ± 0.47

Table 5-3. Analysis of the β-Agarase DagA Activity at -20°C

	Activity (U/mL)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 months	7.11 ± 0.48	6.77 ± 0.46	7.60 ± 0.34
2 months	7.17 ± 0.22	6.54 ± 0.57	7.58 ± 0.57
4 months	6.99 ± 0.58	6.72 ± 0.47	7.54 ± 0.43
6 months	7.06 ± 0.51	6.84 ± 0.35	7.39 ± 0.25
8 months	7.02 ± 0.34	6.64 ± 0.58	7.54 ± 0.58
12 months	7.18 ± 0.44	6.73 ± 0.47	7.50 ± 0.31

Microbiological Tests of the β-agarase DagA

As shown in Tables 5-4 to 5-8, no changes in microbiological parameters during the storage conditions.

Table 5-4. Aerobic Plate Counts of the β-Agarase DagA During Storage Conditions

	cfu/g at 4°C			cfu/g at -20°C			
	20-NBE1120	21-NBE0122	21-NBE0226		20-NBE 1120	21-NBE 0122	21-NBE 0226
0 days	<10	<10	<10	0 months	<10	<10	<10
7 days	<10	<10	<10	2 months	<10	<10	<10
14 days	<10	<10	<10	4 months	<10	<10	<10
21 days	<10	<10	<10	6 months	<10	<10	<10
28 days	<10	<10	<10	8 months	<10	<10	<10
42 days	<10	<10	<10	12 months	<10	<10	<10

Table 5-5. Total Yeast and Mold Counts of the β-Agarase DagA During Storage Conditions

	cfu/g at 4°C			cfu/g at -20°C			
	20-NBE1120	21-NBE0122	21-NBE0226		20-NBE 1120	21-NBE 0122	21-NBE 0226
0 days	<10	<10	<10	0 months	<10	<10	<10
7 days	<10	<10	<10	2 months	<10	<10	<10
14 days	<10	<10	<10	4 months	<10	<10	<10
21 days	<10	<10	<10	6 months	<10	<10	<10
28 days	<10	<10	<10	8 months	<10	<10	<10
42 days	<10	<10	<10	12 months	<10	<10	<10

Table 5-6. Coliforms Counts of the β-Agarase DagA During Storage Conditions

	cfu/g at 4°C			cfu/g at -20°C			
	20-NBE1120	21-NBE0122	21-NBE0226		20-NBE 1120	21-NBE 0122	21-NBE 0226
0 days	<10	<10	<10	0 months	<10	<10	<10
7 days	<10	<10	<10	2 months	<10	<10	<10
14 days	<10	<10	<10	4 months	<10	<10	<10
21 days	<10	<10	<10	6 months	<10	<10	<10
28 days	<10	<10	<10	8 months	<10	<10	<10
42 days	<10	<10	<10	12 months	<10	<10	<10

Table 5-7. *Staphylococcus aureus* Counts of the β-Agarase DagA During Storage Conditions

	cfu/g at 4°C			cfu/g at -20°C			
	20-NBE1120	21-NBE0122	21-NBE0226		20-NBE 1120	21-NBE 0122	21-NBE 0226
0 days	<10	<10	<10	0 months	<10	<10	<10
7 days	<10	<10	<10	2 months	<10	<10	<10
14 days	<10	<10	<10	4 months	<10	<10	<10
21 days	<10	<10	<10	6 months	<10	<10	<10
28 days	<10	<10	<10	8 months	<10	<10	<10
42 days	<10	<10	<10	12 months	<10	<10	<10

Table 5-8. *Salmonella* Counts of the β-Agarase DagA During Storage Conditions

	cfu/25 g at 4°C			cfu/25 g at -20°C			
	20-NBE1120	21-NBE0122	21-NBE0226		20-NBE 1120	21-NBE 0122	21-NBE 0226
0 days	Absent	Absent	Absent	0 months	Absent	Absent	Absent
7 days	Absent	Absent	Absent	2 months	Absent	Absent	Absent
14 days	Absent	Absent	Absent	4 months	Absent	Absent	Absent
21 days	Absent	Absent	Absent	6 months	Absent	Absent	Absent
28 days	Absent	Absent	Absent	8 months	Absent	Absent	Absent
42 days	Absent	Absent	Absent	12 months	Absent	Absent	Absent

Conclusion

The results showed that β-agarase DagA was stable for 6 weeks at 4°C and for 12 months at -20°C with no changes in the appearance and counts of undesirable microorganisms.

PART 3. DIETARY EXPOSURE

3.A. Estimated Dietary Intakes (EDIs) of β-Agarase DagA Under the Intended Use

It is expected that no significant amount of residual enzyme will be retained in the final NAO ingredient, because approximately 99.8% of enzyme is removed during the manufacturing (purification) process of NAO after enzymatic hydrolysis of the raw material, agar-agar.

NAO contains approximately 70% of dietary fiber. Even if NAO would be the only source of dietary fiber in American diet which provides approximately 16 g of dietary fiber per person per day (King et al., 2012), per capita EDI of NAO would be no more than 23 g/day. Because the residual enzyme content would be 0.04 mg TOS/g NAO, the maximum per capita EDI of β-agarase DagA is expected to be 0.92 mg TOS/day or approximately 0.0131 mg/kg body weight (bw)/day. From a subchronic toxicity study of β-agarase DagA, the no-observed-adverse-effect-level (NOAEL) was set at 159 mg TOS/kg bw/day. After a safety margin of 100, the safe intake level would be 1.59 mg TOS/kg bw/day or approximately 111 mg TOS/person/day, assuming an average American adult weighs 70 kg. The EDI is within the safe intake level. However, this EDI is overly inflated because it is not expected that NAO could be the only source of dietary fiber in the American diet.

3.B. Food Sources of β-Agarase DagA

Not applicable.

3.C. EDIs of β-Agarase DagA from Diet

Not applicable.

3.D. Total EDIs of β-Agarase DagA from Diet and Under the Intended Use

Not applicable.

3.E. EDIs of Other Substances Under the Intended Use

Not applicable.

Summary of Consumption Data

Under the intended use of β-agarase DagA as a processing aid in the manufacture of a food ingredient NAO, the maximum EDI would be approximately 0.013 mg TOS/kg bw/day. The EDI is less than 100th of the safe intake level of 1.59 mg TOS/kg bw/day, which was determined to be safe in a subchronic toxicity study of β-agarase DagA in rats after the consideration of a safety margin of 100.

PART 4. SELF LIMITING LEVELS OF USE

The use of the β-agarase DagA enzyme preparation to produce NAO is limited by the level that can economically be added to maximize the enzyme efficiency in the production of the ingredient.

PART 5. HISTORY OF CONSUMPTION

Carbohydrase enzyme preparations derived from *Streptomyces coelicolor* A3(2) M22-2C43 were not in common use in food prior to January 1, 1958.

PART 6. BASIS FOR GRAS DETERMINATION

6.A. Current Regulatory Status

There is no regulatory status for β-agarase DagA. However, bacterially-derived carbohydrase enzyme preparation is subjected to the 21 CFR §184.1148.

6.B. Review of Safety Data

6.B.1. Absorption, Distribution, Metabolism, and Elimination (ADME)

As discussed in Part 3, it is expected that no biologically significant amount of enzyme will be ingested under the intended use of β-agarase DagA as a processing aid. Even if a small amount of residual enzyme residue is present in the finished food ingredient, the enzyme action is expected to be over in the food product when available to consumers. Thus, ADME is not discussed in this GRAS determination.

6.B.2. Safety of β-Agarase DagA

6.B.2.1. Homology of Amino Acid Sequence of β-Agarase DagA with Those of Known Allergenic Proteins

As stated in Part 2.A.1.4, Dyne Bio Inc. compared the amino acid sequence of β-agarase DagA with those of known allergenic proteins using the Food Allergy Research and Resource Program (FARRP) allergen protein database (http://allergenonline.org). No significant homology was found between the β-agarase DagA and any of the allergens in the databases mentioned above. Details are described in Appendix A.

6.B.2.2. Mutagenicity and Genotoxicity Studies

Hong et al. (2019) evaluated the safety of Dyne Bio Inc.'s β-agarase DagA from *S. coelicolor* A3(2) M22-2C43 in genotoxicity tests, including bacterial reverse mutation assay, chromosomal aberration assay, and *in vivo* micronucleus assay. For safety evaluation, an extracellular protein containing DagA (crDagA) was prepared from the culture broth of *S. coelicolor* A3(2) M22-2C43, an ultraviolet -treated mutant of *S.coelicolor* A3(2). The β-agarase DagA evaluated in this study contained 1.59±0.02% of TOS and 7.39 units/mL of β-agarase DagA activity.

As summarized in Table 6, Dyne Bio Inc.'s β-agarase DagA was not mutagenic or genotoxic under the test conditions.

Table 6. Summary of Mutagenicity and Genotoxicity Studies of Dyne Bio Inc.'s β-Agarase DagA Preparation

Test System	β-agarase DagA Dose	Result	Reference
S. typhimurium TA98, TA100, TA1535, TA1537, and *E. coli* WP2 *uvr*A ± S9	0, 62, 185, 556, 1,670, or 5,000 µg/plate (or up to 79.5 µg TOS/plate)	Not mutagenic	Hong et al., 2019
Chinese hamster ovary fibroblast-derived cells ± S9	0, 1,250, 2,500, or 5,000 µg/mL (up to 79.5 µg TOS/mL)	No chromosomal aberrations	
Micronucleus from male ICR mice	0, 500, 1,000, or 2,000 mg/kg bw (up to 31.8 mg TOS/kg bw)	Not mutagenic	

bw = body weight; TOS = total organic solids.

Bacterial Reverse Mutation Test

In the bacterial reverse mutation assay, *S. typhimurium* TA98, TA100, TA1535, and TA1537, and *E. coli* WP2 *uvr*A were exposed to β-agarase DagA concentrations at 0, 62, 185, 556, 1,670, or 5,000 µg/plate (or up to 79.5 µg TOS/plate) in the absence or presence of S9 metabolic activation (Hong et al., 2019). The positive controls were sodium azide, 9-aminoacridine, 4-nitroquinoline-N-oxide, 2-nitrofluorene, 2-aminoanthracene, and benzo(α)pyrene. The number of revertant colonies were determined. Mutagenicity was deemed positive if the test substance increased the number of revertant colonies to at least twice of the negative control in a dose-dependent manner. Regardless to S9, β-agarase DagA doses up to 5,000 µg/plate (up to 79.5 µg TOS/plate) did not induce reverse mutation in the *S. typhimurium* and *E. coli* strains and, therefore, it was considered as not mutagenic under the test conditions.

In Vitro Chromosome Aberration Test

In an *in vitro* chromosome aberration test, Chinese hamster ovary fibroblast-derived cells were exposed to 0, 1,250, 2,500, or 5,000 µg/mL β-agarase DagA (up to 79.5 µg TOS/mL) in the presence and absence of S9. Minimum essential medium served as the negative control, and cyclophosphamide and mitomycin as the positive controls. Chromosome aberrations were scored. Chromosomal anomalies, such as structural and numerical anomalies, were determined in a sample of 300 cells. There were no significant changes in structural or numerical chromosomal aberrations under the test conditions.

***In Vivo* Mouse Micronucleus Test**

In the *in vivo* micronucleus assay, male Imprinting Control Region (ICR) mice were divided into 4 groups: 0, 500, 1,000, or 2,000 mg/kg bw β-agarase DagA (up to 31.8 mg TOS/kg bw) (Hong et al., 2019). Mitomycin C served as the positive control. The bone marrow cells from the femur were collected after 24 hours of the last administration. The frequency of polychromatic erythrocytes with micronuclei (MNPCEs) per total polychromatic erythrocytes (PCEs) in 4,000 PCEs and the ratio of PCE/red blood cell (RBC) in 500 RBC were determined. The result was deemed positive if the increase was statistically dose-dependent and reproducible. No deaths and no clinical symptoms were observed. There were no significant changes in ratios of MNPCE/5,000 PCE and PCE/RBC. The results demonstrated that β-agarase DagA doses up to 2,000 mg/kg (up to 31.8 TOS/kg bw) was not mutagenic under the test conditions.

6.B.2.3. Toxicity Studies of β-Agarase DagA Preparation in Animals

Table 7 summarizes the animal toxicity studies of β-agarase DagA Preparation in Animals.

Table 7. Summary of Animal Toxicity Studies of Dyne Bio Inc.'s β-Agarase DagA Preparation

Animal	β-agarase DagA Dose	Duration	Results	Reference
Healthy SD rats (n=5/sex/group)	0 or 20,000 mg/kg bw (or 318 mg TOS/kg bw)	Single dose, monitored for 14 d	ALD >20,000 mg/kg bw (or 318 mg TOS/kg bw)	Hong et al., 2019
Healthy SD rats (n=5/sex/group)	0, 2,500, 5,000, or 10,000 mg/kg bw/d (or up to 159 mg TOS/kg bw/d)	28 d	No effects up to 10,000 mg/kg bw/d (or up to 159 mg TOS/kg bw/d)	
Healthy SD rats (n=10/sex/group)	0, 2,500, 5,000, or 10,000 mg/kg bw/d (or up to 159 mg TOS/kg bw/d)	90 d	NOAEL = 10,000 mg/kg bw/d (159 mg TOS/kg bw/d)	

ALD = approximate lethal dose; bw = body weight; d = days; NOAEL = no-observed-adverse-effect-level; TOS=total organic solids.

Acute Toxicity Study of Dyne Bio Inc.’s β-Agarase DagA Preparation

In an acute toxicity study, fasted healthy Sprague-Dawley (SD) rats (age not specified; 191-256 g) were orally administered 0 or 20,000 mg/kg bw/day β-agarase DagA (or 318 mg TOS/kg bw) as a single dose. General behavior, changes in body weight, signs of toxicity, and death were examined for 14 days. No deaths, no significant changes in body weight, and no specific symptoms were observed. The approximate lethal dose (ALD) of the β-agarase DagA was more than 20 g/kg bw (or 318 mg TOS/kg bw) for rats.

Subacute Toxicity Study of Dyne Bio Inc.’s β-agarase DagA Preparation

A dose range finding study was conducted to determine the tolerable dose range for a long-term toxicity study (Hong et al., 2019). SD rats orally received 0, 2,500, 5,000, or 10,000 mg/kg bw/day β-agarase DagA (or up to 159 mg TOS/kg bw/day) for 28 days. General health status, morbidity, mortality, clinical abnormalities, and total physical examination were examined. No mortality and abnormal behavior were observed as well as no significant differences in food intake, bw change, hematology, blood biochemistry, clinical signs, and organ weights. The no-observed-adverse-effect-level (NOAEL) was determined to be 10,000 mg/kg bw/day β-agarase DagA (or 159 mg TOS/kg bw/day) for both male and female rats. Based on the results from this subacute toxicity study in rats, the highest dose level for the long-term toxicity study in rats was set as 10,000 mg/kg bw/day (or 150 mg TOS/kg bw/day).

Subchronic Toxicity Study of Dyne Bio Inc.’s β-agarase DagA Preparation in SD rats

Healthy SD rats (n=10/sex/group; age, not specified; 143.1-204.0 g) were orally administered 0, 2,500, 5,000, or 10,000 mg/kg bw/day β-agarase DagA (up to 159 mg TOS/kg bw/day) by gavage for 90 days (Hong et al., 2019). Blood samples were collected at 91th day after fasting for 17 hours. Food intake, body weight, clinical signs, ophthalmology abnormalities, urinalysis (total urine volume, urine color, urine sediment, and presence of leukocytes, RBCs, epithelial cells, and casts), hematology, serum biochemistry, and histopathology were examined. The organ weights of spleen, kidney, heart, lung, brain, liver, adrenal gland, pituitary grand, thymus, prostate (males), testis (males), epididymis (males, and ovaries (females) were determined. There were no deaths and abnormal behaviors as well as no treatment-related abnormalities in body weight, food intake, clinical signs, ophthalmological parameters, and urinalysis. No treatment-related abnormalities were found in hematology, blood chemistry, and organ weight parameters in both female and male rats (Tables 8-1 to 10-4).

The differences in organ weights included decreases in the absolute weights of left adrenal gland and prostate gland and the relative weights of left adrenal gland (0.0051 vs 0.0060% to body weight, $P<0.05$) and prostate (0.113 vs 0.147% to body weight, $P<0.05$) in the male low-dose group, increase in the relative weights of brain in female mid-dose group (0.749 vs 0.697% to body weight, $P<0.05$), and increase in the relative weight of spleen in the female high-dose group (0.244 vs 0.217% to body weight, $P<0.05$). These differences were not dose-dependent and within the normal range, and thus, deemed not to be of toxicological significance. In the male control and low-dose groups, redness of the thymus was found. Redness of the lung was observed in a male of the high-dose group. In the female control, mid- and high-dose groups, retention of clear liquid in the uterus were observed. A male in the high-dose group displayed “severe” atrophy of the testis and loss of sperm in the epididymis. In the male high-dose group, “minimal” infiltration of inflammatory cells into the prostate and heart, and basophilic tubule of kidney were found. “Minimal” inflammatory cells infiltration into the liver was observed in a female in the high-dose group. These findings were not test substance-related because they were found in the control group and/or had low incidence.

No significant changes were observed in necropsy factors. The observed macroscopic autopsy changes included redness of the thymus in a male control and a male low-dose groups, redness of the lung in a male high-dose group, and retention of clear liquid in the uterus in a female control and a female mid- and high-dose groups. These changes were considered as non-test substance-derived changes since they were also observed in the control groups and/or the incidence was low.

The results of the histopathological examination were labeled according to their severity: minimal, slight, moderate, and severe. In males, “severe” atrophy of the testis, “severe” loss of sperm in the epididymis, “minimal” infiltration of inflammatory cells into the prostate and heart, and “minimal” basophilic tubule of kidney were found in the high-dose group. In females, “minimal” infiltration of inflammatory cells into the liver was found in the high-dose group. These histopathological findings were considered as non-test substance-derived changes because they were also found in the control groups and the incidence was low.

Overall, the NOAEL of crude β-agarase DagA preparation was set at 10,000 mg/kg bw/day (159 mg TOS/kg bw/day), the highest level tested, in male and female SD rats.

Table 8-1. Hematological Analysis of Male Rats Consuming β-Agarase DagA for 90 Days

Hematological values	Group (mg β-agarase DagA /kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	0	39.75	79.5	159
RBC* (10^6/μL)	8.99 ± 0.370	8.70 ± 0.458	8.71 ± 0.295	8.93 ± 0.282
HGB (g/dL)	15.3 ± 0.63	15.0 ± 0.83	15.4 ± 0.39	15.1 ± 0.92
HCT (%)	45.2 ± 1.26	44.2 ± 2.17	44.7 ± 1.21	44.4 ± 1.60
MCV (fL)	50.4 ± 2.02	50.9 ± 2.63	51.3 ± 0.99	49.8 ± 2.20
MCH (pg)	17.0 ± 0.90	17.2 ± 1.08	17.7 ± 0.58	16.9 ± 1.28
MCHC (g/dL)	33.8 ± 0.78	33.9 ± 0.67	34.4 ± 0.63	33.9 ± 1.16
RDW (%)	13.3 ± 0.63	13.4 ± 0.55	13.2 ± 0.38	13.1 ± 0.62
HDW (g/dL)	2.81 ± 0.314	2.85 ± 0.267	2.74 ± 0.115	2.81 ± 0.286
RET (%)	1.57 ± 0.310	1.64 ± 0.205	1.55 ± 0.191	1.60 ± 0.346
PLT (10^3/μL)	1,006 ± 148.2	1,049 ± 150.8	958 ± 94.4	948 ± 83.8
MPV (fL)	7.1 ± 0.21	7.2 ± 0.28	7.1 ± 0.36	7.0 ± 0.48
WBC (10^3/μL)	4.12 ± 1.385	4.75 ± 1.315	3.86 ± 1.293	3.33 ± 0.995
NEU (10^3/μL)	1.01 ± 0.616	1.34 ± 0.447	0.88 ± 0.524	0.86 ± 0.357
LYM (10^3/μL)	2.89 ± 0.942	3.16 ± 1.082	2.77 ± 0.862	2.28 ± 0.680
MONO (%)	3.1 ± 0.78	2.9 ± 0.97	2.8 ± 0.90	2.8 ± 1.27
EOS (%)	1.5 ± 0.35	1.5 ± 0.53	1.6 ± 0.42	2.0 ± 0.79
BASO (%)	0.1 ± 0.07	0.1 ± 0.05	0.2 ± 0.07	0.1 ± 0.08
LUC (%)	0.7 ± 0.27	0.9 ± 0.38	0.9 ± 0.50	0.8 ± 0.53

BASO = basophil; bw = body weight; d = days; EOS = eosinophil; HCT = hematocrit; HDW = hemoglobin distribution width; HGB = hemoglobin; LUC = large unstained cell; LYM = lymphocyte; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; MONO = monocyte; MPV = mean platelet volume; NEU = neutrophil; PLT = platelet; RBC = red blood cells; RDW = red cell distribution width; RET = reticulocyte; WBC = white blood cells.

Table 8-2. Hematological Analysis of Female Rats Consuming β-Agarase DagA for 90 Days

Hematological values	Group (mg β-agarase DagA/kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	0	39.75	79.5	159
RBC* (10^6/μL)	7.95 ± 0.326	7.89 ± 0.274	7.84 ± 0.379	8.05 ± 0.397
HGB (g/dL)	14.7 ± 0.51	14.7 ± 0.45	14.4 ± 0.70	14.7 ± 0.47
HCT (%)	42.3 ± 1.29	41.9 ± 1.24	41.7 ± 1.66	42.7 ± 0.94
MCV (fL)	53.2 ± 0.89	53.2 ± 1.19	53.2 ± 1.69	53.1 ± 1.78
MCH (pg)	18.5 ± 0.53	18.6 ± 0.77	18.4 ± 0.86	18.3 ± 0.97
MCHC (g/dL)	34.8 ± 0.64	35.0 ± 0.81	34.6 ± 0.68	34.5 ± 0.93
RDW (%)	11.7 ± 0.54	11.6 ± 0.43	12.0 ± 0.69	11.8 ± 0.75
HDW (g/dL)	2.42 ± 0.116	2.42 ± 0.081	2.42 ± 0.137	2.51 ± 0.206

RET (%)	1.59 ± 0.371	1.69 ± 0.339	1.81 ± 0.405	1.73 ± 0.442
PLT ($10^3/\mu L$)	1051 ± 102.6	998 ± 108.6	1051 ± 133.1	999 ± 121.7
MPV (fL)	7.3 ± 0.32	7.3 ± 0.15	7.3 ± 0.20	7.4 ± 0.25
WBC ($10^3/\mu L$)	1.66 ± 0.529	1.32 ± 0.266	1.77 ± 0.507	1.87 ± 0.646
NEU ($10^3/\mu L$)	0.32 ± 0.280	0.41 ± 0.280	0.34 ± 0.166	0.38 ± 0.204
LYM ($10^3/\mu L$)	1.25 ± 0.342	0.83 ± 0.340	1.33 ± 0.462	1.39 ± 0.538
MONO (%)	2.6 ± 1.21	3.3 ± 4.17	2.4 ± 0.68	2.2 ± 1.41
EOS (%)	2.2 ± 0.85	1.9 ± 1.30	2.6 ± 1.29	1.9 ± 0.65
BASO (%)	0.2 ± 0.11	0.1 ± 0.09	0.3 ± 0.13	0.2 ± 0.12
LUC (%)	0.6 ± 0.41	0.4 ± 0.28	0.7 ± 0.34	0.8 ± 0.29

BASO = basophil; bw = body weight; d = days; EOS = eosinophil; HCT = hematocrit; HDW = hemoglobin distribution width; HGB = hemoglobin; LUC = large unstained cell; LYM = lymphocyte; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; MONO = monocyte; MPV = mean platelet volume; NEU = neutrophil; PLT = platelet; RBC = red blood cells; RDW = red cell distribution width; RET = reticulocyte; WBC = white blood cells.

Table 9-1. Blood Biochemistry of Male Rats Consuming β-Agarase DagA for 90 Days

Biochemical test	Group (mg β-agarase DagA/kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	39.75	79.5	159	39.75
AST (U/L)*	90 ± 19.4	85 ± 18.7	87 ± 18.5	94 ± 27.1
ALT (U/L)	27 ± 3.8	26 ± 3.2	27 ± 3.4	26 ± 3.5
ALP (U/L)	217 ± 31.1	190 ± 36.0	211 ± 25.4	195 ± 26.8
CPK (U/L)	290 ± 196.3	265 ± 130.8	263 ± 133.6	325 ± 280.9
BIL (mg/dL)	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02
GLU (mg/dL)	166 ± 19.3	168 ± 13.6	162 ± 16.2	165 ± 34.5
CHO (mg/dL)	80 ± 13.6	80 ± 19.9	89 ± 9.7	86 ± 12.1
TG (mg/dL)	66 ± 39.1	57 ± 19.2	62 ± 20.3	59 ± 19.6
ALB (g/dL)	3.3 ± 0.11	3.3 ± 0.05	3.3 ± 0.07	3.3 ± 0.10
A/G ratio	1.2 ± 0.05	1.1 ± 0.06	1.1 ± 0.05	1.1 ± 0.07
BUN (mg/dL)	15.3 ± 1.70	14.0 ± 1.89	14.5 ± 2.17	14.3 ± 1.37
CRE (mg/dL)	0.7 ± 0.03	0.7 ± 0.05	0.7 ± 0.06	0.7 ± 0.03
GGT (U/dL)	3.9 ± 0.82	3.8 ± 0.66	3.8 ± 0.61	4.3 ± 0.37
IP (mg/dL)	6.1± 0.64	5.61 ± 0.54	5.6 ± 0.85	5.5 ± 0.69
PRO (g/dL)	6.1 ± 0.24	6.2 ± 0.18	6.2 ± 0.22	6.2 ± 0.23
Ca^{2+} (mg/dL)	10.3 ± 0.41	10.3 ± 0.17	10.4 ± 0.31	10.2 ± 0.23
Na^+ (mmol/L)	139.2 ± 1.26	138.1 ± 1.07	138.4 ± 0.80	139.4 ± 1.39
K^+ (mmol/L)	4.3 ± 0.35	4.4 ± 0.19	4.3 ± 0.18	4.4 ± 0.38
Cl^- (mmol/L)	104.2 ± 1.79	104.7 ± 1.23	104.8 ± 1.01	104.8 ± 1.58

A/G = albumin/globulin; ALB = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BIL = bilirubin; BUN = blood urea nitrogen; bw = body weight; CHO =

cholesterol; CPK = creatine phosphokinase; CRE = creatinine; d = day; GGT = gamma glutamyl-transferase; GLU = glucose; IP = inorganic phosphorus; PRO = protein; TG = triglyceride; TP = total protein.

Table 9-2. Blood Biochemistry of Female Rats Consuming β-Agarase DagA for 90 Days

Biochemical test	Group (mg β-agarase DagA/kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	0	39.75	79.5	159
AST (U/L)*	96 ± 25.3	95 ± 17.0	96 ± 19.0	98 ± 23.5
ALT (U/L)	22 ± 2.9	20 ± 2.9	22 ± 3.3	22 ± 3.4
ALP (U/L)	146 ± 29.4	146 ± 26.8	148 ± 25.8	155 ± 25.6
CPK (U/L)	290 ± 227.7	294 ± 116.8	310 ± 160.2	291 ± 234.4
BIL (mg/dL)	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.03
GLU (mg/dL)	125 ± 14.5	119 ± 13.6	117 ± 15.6	119 ± 7.5
CHO (mg/dL)	82 ± 17.4	78 ± 19.5	87 ± 23.3	86 ± 31.9
TG (mg/dL)	29 ± 5.3	30 ± 6.1	28 ± 4.5	28 ± 5.9
ALB (g/dL)	3.3 ± 0.17	3.3 ± 0.14	3.3 ± 0.13	3.3 ± 0.09
A/G ratio	1.3 ± 0.08	1.3 ± 0.09	1.3 ± 0.07	1.3 ± 0.14
BUN (mg/dL)	18.2 ± 1.15	18.1 ± 2.12	19.0 ± 2.25	17.9 ± 2.38
CRE (mg/dL)	0.7 ± 0.04	0.7 ± 0.05	0.7 ± 0.04	0.7 ± 0.05
GGT (U/dL)	3.5 ± 0.63	3.9 ± 1.60	3.7 ± 0.64	3.8 ± 0.69
IP (mg/dL)	5.4 ± 0.58	4.9 ± 0.47	5.0 ± 0.60	5.4 ± 0.37
PRO (g/dL)	5.9 ± 0.27	5.8 ± 0.30	5.9 ± 0.21	5.8 ± 0.21
Ca^{2+} (mg/dL)	10.1 ± 0.18	10.0 ± 0.27	10.0 ± 0.20	10.1 ± 0.24
Na^+ (mmol/L)	138.0 ± 0.88	138.8 ± 0.95	138.4 ± 1.05	138.7 ± 1.14
K^+ (mmol/L)	4.1 ± 0.42	4.2 ± 0.13	4.1 ± 0.32	4.0 ± 0.20
Cl^- (mmol/L)	106.9 ± 1.68	107.7 ± 1.48	107.7 ± 1.31	107.1 ± 1.96

A/G = albumin/globulin; ALB = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BIL = bilirubin; BUN = blood urea nitrogen; bw = body weight; CHO = cholesterol; CPK = creatine phosphokinase; CRE = creatinine; d = day; GGT = gamma glutamyl-transferase; GLU = glucose; IP = inorganic phosphorus; PRO = protein; TG = triglyceride; TP = total protein.

Table 10-1. Absolute Organ Weights of Male Rats Consuming β-Agarase DagA for 90 Days

Parameters (g)	Group (mg β-agarase DagA/kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	0	39.75	79.5	159
BW (g)	528.5 ± 43.54	512.7 ± 22.14	535.7 ± 43.68	512.3 ± 38.74
Adrenal gland-left	0.0297 ± 0.0037	0.0250 ± 0.0040	0.0267 ± 0.0037	0.0277 ± 0.0026

Adrenal gland-right	0.0288 ± 0.0034	0.0264 ± 0.0038	0.0258 ± 0.0039	0.0281 ± 0.0017
Pituitary gland	0.0125 ± 0.0010	0.0121 ± 0.0020	0.0129 ± 0.0014	0.0125 ± 0.0015
Thymus	0.493 ± 0.064	0.452 ± 0.058	0.516 ± 0.094	0.484 ± 0.085
Spleen	0.869 ± 0.099	0.909 ± 0.107	0.876 ± 0.110	0.895 ± 0.146
Kidney-left	1.437 ± 0.137	1.431 ± 0.108	1.452 ± 0.112	1.426 ± 0.154
Kidney-right	1.458 ± 0.155	1.446 ± 0.103	1.472 ± 0.135	1.454 ± 0.163
Heart	1.36 ± 0.08	1.40 ± 0.21	1.40 ± 0.10	1.37 ± 0.08
Lung	1.76 ± 0.15	1.70 ± 0.17	1.74 ± 0.18	1.66 ± 0.11
Brain	2.13 ± 0.12	2.09 ± 0.11	2.06 ± 0.17	2.12 ± 0.09
Liver	13.28 ± 2.07	13.73 ± 0.90	13.78 ± 1.50	13.04 ± 1.79
Prostate gland	0.73 ± 0.08	0.55 ± 0.13	0.70 ± 0.14	0.59 ± 0.14
Testis-left	1.98 ± 0.20	1.96 ± 0.33	1.98 ± 0.15	1.82 ± 0.29
Testis-right	1.94 ± 0.23	1.98 ± 0.38	1.94 ± 0.13	1.87 ± 0.24
Epididymis-left	0.692 ± 0.070	0.663 ± 0.098	0.678± 0.061	0.692 ± 0.098
Epididymis-right	0.691 ± 0.070	0.677 ± 0.088	0.680 ± 0.057	0.656 ± 0.099

Table 10-2. Relative Organ Weights of Male Rats Consuming β-Agarase DagA for 90 Days

Parameters (% to BW unless noted otherwise)	Group (mg β-agarase DagA/kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	0	39.75	79.5	159
BW (g)	528.5 ± 43.5	512.7 ± 22.1	535.7 ± 43.7	512.3 ± 38.7
Adrenal gland-left	0.0060 ± 0.0009	0.0051 ± 0.0009*	0.0053 ± 0.0010	0.0057 ± 0.0007
Adrenal gland-right	0.0058 ± 0.0009	0.0054 ± 0.0009	0.0051 ± 0.0009	0.0058 ± 0.0006
Pituitary gland	0.0025 ± 0.0002	0.0025 ± 0.0004	0.0026 ± 0.0003	0.0026 ± 0.0002
Thymus	0.100 ± 0.018	0.092± 0.013	0.101 ± 0.018	0.099 ± 0.014
Spleen	0.175 ± 0.025	0.186 ± 0.028	0.173 ± 0.026	0.184 ± 0.025
Kidney-left	0.288 ± 0.021	0.292 ± 0.022	0.286 ± 0.012	0.293 ± 0.020
Kidney-right	0.292 ± 0.020	0.295 ± 0.022	0.290 ± 0.026	0.298 ± 0.021
Heart	0.272 ± 0.016	0.285 ± 0.040	0.276 ± 0.022	0.283 ± 0.017
Lung	0.352 ± 0.023	0.347 ± 0.036	0.344 ± 0.046	0.342 ± 0.009
Brain	0.427 ± 0.026	0.427 ± 0.027	0.406 ± 0.039	0.438 ± 0.033
Liver	2.64 ± 0.22	2.80 ± 0.20	2.71 ± 0.21	2.67 ± 0.21
Prostate gland	0.147 ± 0.017	0.113 ± 0.028*	0.139 ± 0.030	0.123 ± 0.033
Testis-left	0.399 ± 0.046	0.401 ± 0.078	0.391 ± 0.030	0.374 ± 0.055
Testis-right	0.390 ± 0.048	0.405 ± 0.088	0.384 ± 0.031	0.384 ± 0.045
Epididymis-left	0.139 ± 0.016	0.136 ± 0.023	0.134 ± 0.018	0.143 ± 0.020
Epididymis-right	0.139 ± 0.014	0.139 ± 0.022	0.134 ± 0.012	0.135 ± 0.019

*Represents a significant difference at $p < 0.05$ level compared with the vehicle control.

Table 10-3. Absolute Organ Weights of Female Rats Consuming β-Agarase DagA for 90 Days

Parameter (g)	Group (mg β-agarase DagA/kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	0	39.75	79.5	159
BW (g)	297.0 ± 13.0	289.7 ± 18.0	286.1 ± 16.5	295.6 ± 24.8
Adrenal gland-left	0.0380 ± 0.0044	0.0375 ± 0.0067	0.0361 ± 0.0034	0.0357 ± 0.0034
Adrenal gland-right	0.0391 ± 0.0041	0.0392 ± 0.0061	0.0355 ± 0.0022	0.0361 ± 0.0038
Pituitary gland	0.0162 ± 0.0012	0.0160 ± 0.0024	0.0165 ± 0.0017	0.0152 ± 0.0009
Thymus	0.295 ± 0.089	0.301 ± 0.057	0.288 ± 0.064	0.341 ± 0.072
Spleen	0.605 ± 0.053	0.593 ± 0.078	0.617 ± 0.069	0.666 ± 0.075
Kidney-left	0.856 ± 0.051	0.826 ± 0.067	0.839 ± 0.063	0.855 ± 0.045
Kidney-right	0.850 ± 0.054	0.855 ± 0.048	0.839 ± 0.077	0.882 ± 0.034
Heart	0.892 ± 0.048	0.846 ± 0.072	0.808 ± 0.040	0.838 ± 0.082
Lung	1.33 ± 0.06	1.29 ± 0.10	1.22 ± 0.08	1.32 ± 0.06
Brain	1.94 ± 0.06	1.97 ± 0.13	1.99± 0.07	1.98 ± 0.07
Liver	6.69 ± 0.42	6.73 ± 0.63	6.79 ± 0.59	6.90 ± 0.64
Ovary-left	0.0636 ± 0.0119	0.0589 ± 0.0157	0.0570 ± 0.0166	0.068 ± 0.015
Ovary-right	0.0598 ± 0.0110	0.0621 ± 0.0121	0.0549 ± 0.0186	0.068 ± 0.011

*Represents a significant difference at p <0.05 level compared with the vehicle control.

Table 10-4. Relative Organ Weights of Female Rats Consuming β-Agarase DagA for 90 Days

Parameter (% to BW unless noted otherwise)	Group (mg β-agarase DagA/kg bw/d)			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (10,000)
	Group (mg TOS /kg bw/d)			
	0	39.75	79.5	159
BW (g)	297.0 ± 13.01	289.7 ± 18.01	286.1 ± 16.54	295.6 ± 24.81
Adrenal gland-left	0.0136 ± 0.0015	0.0138 ± 0.0022	0.0136 ± 0.0016	0.0131 ± 0.0013
Adrenal gland-right	0.0140 ± 0.0013	0.0145 ± 0.0021	0.0134 ± 0.0010	0.0133 ± 0.0015
Pituitary gland	0.0058 ± 0.0004	0.0059 ± 0.0007	0.0062 ± 0.0008	0.0056 ± 0.0004
Thymus	0.105 ± 0.029	0.112 ± 0.022	0.109 ± 0.024	0.124 ± 0.0205
Spleen	0.217 ± 0.023	0.219 ± 0.025	0.233 ± 0.025	0.244 ± 0.0273*
Kidney-left	0.307 ± 0.020	0.306 ± 0.027	0.316 ± 0.020	0.313 ± 0.0190
Kidney-right	0.305 ± 0.020	0.318 ± 0.031	0.316 ± 0.026	0.324 ± 0.0232
Heart	0.320 ± 0.021	0.314 ± 0.023	0.304 ± 0.013	0.306 ± 0.010
Lung	0.478 ± 0.035	0.479 ± 0.033	0.461 ± 0.028	0.486 ± 0.038
Brain	0.697 ± 0.046	0.729 ± 0.059	0.749 ± 0.037*	0.725 ± 0.042

Liver	2.40 ± 0.134	2.49 ± 0.182	2.56 ± 0.155	2.52 ± 0.184
Ovary-left	0.0229 ± 0.0050	0.0216 ± 0.0050	0.0215 ± 0.0063	0.0248 ± 0.0054
Ovary-right	0.0215 ± 0.0042	0.0229 ± 0.0040	0.0207 ± 0.0070	0.0250 ± 0.0043

*Represents a significant difference at p <0.05 level compared with the vehicle control.

Conclusion

Based on the studies summarized above, for purposes of safety evaluation, a NOAEL of 10,000 mg/kg bw/day (or 159 mg TOS/kg bw/day), the highest level tested, was chosen for rats.

6.B.3. Safety of Production Microorganism

6.B.3.1. Characteristics of *Streptomyces coelicolor* A3(2)

Streptomyces belongs to the group of Gram-positive eubacteria. *Streptomyces* are mostly isolated from soil or water, and very few strains are pathogenic. *Streptomyces coelicolor* is the representative of the genus *Streptomyces*. According to the genomic information (Bentley et al., 2002), *S. coelicolor* has 819 potential secreted proteins (10.5% of total proteins), including hydrolases such as proteases and peptidases, chitosanases and chitinases, cellulases, amylases, two pectate lyases, xylanase, lipase, and agarases.

The original name of *S. coelicolor* was *Actinomyces coelicolor* and was identified from a discarded tap water sample as a decomposer of agar (Hong et al., 2019). *Streptomyces coelicolor* A3(2) is a model representative of a group of soil-dwelling organism. Dyne Bio Inc. obtained *S. coelicolor* A3(2) M22-2C43-WT strain which was acknowledged as *S. coelicolor* A3(2) from the John Innes Foundation, United Kingdom. In other words, *S. coelicolor* which was acknowledged as A3(2) at the John Innes Foundation, United Kingdom, was named as *S. coelicolor* A3(2) M22-2C43-WT by Dyne Bio. To increase the production yield of β-agarase DagA, *S. coelicolor* A3(2) M22-2C43 WT was subjected to an uv treatment and an uv-treated mutant was named as *S. coelicolor* M22-2C43. This mutant is often abbreviated as M22-2C43.

There are 2 types of β-agarases, DagA and DagB, from *S. coelicolor* (Temuujin et al., 2011, 2012). β-agarase DagA degrades agar into neoagarotetraose and neoagarohexaose, and β-agarase DagB degrades agar into neoagarobiose. Dyne Bio Inc.'s β-agarase DagA is derived from *S. coelicolor* A3(2) M22-2C43. In the safety evaluation by Hong et al. (2019), this strain was used in the production of β-agarase DagA.

6.B.3.2. Identification of Production Microorganism

16S ribosomal deoxyribonucleic acid (rDNA) Sequencing

Pure cultures of *S. coelicolor* A3(2) M22-2C43 were grown on minimal agar at 30°C for 72-96 hours. The pellet was sent to Chunlab (Chunlab, Seoul, Korea) for bi-directional 16S rDNA sequencing. Bi-directional sequencing results were assembled using Codon Code Aligner (Codon Code Corporation, USA) and compared with reference sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/Blastn/) (Camacho et al., 2009). The 16S rDNA sequence of *S. coelicolor* A3(2) M22-2C43 strain is given in Table 11. The sequence was analyzed and compared using blastN and its identity was confirmed as *S. coelicolor* A3(2) M22-2C43 (Table 12). Taxonomic Classification of *Streptomyces coelicolor* A3(2) M22-2C43 is shown in Table 13.

Table 11. Partial Sequence of *S. coelicolor* A3(2) M22-2C43 16S rDNA

Strain	Sequence
S. coelicolor M22-2C43	CTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGG

Table 12. 16S rDNA Sequence NCBI blast Matching Results for *S. coelicolor* A3(2) M22-2C43

Sample Name	Description	Similarity
S. coelicolor A3(2)M22-2C43	*Streptomyces coelicolor* strain M1154/pAMX4/pGP1416 chromosome, complete genome	100%
	Streptomyces sp. strain MJM14745 16S ribosomal RNA gene, partial sequence	100%

Table 13. Taxonomic Classification of *Streptomyces coelicolor* A3(2) M22-2C43

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales
Family	Streptomycetaceae
Genus	*Streptomyces*
Species	*Streptomyces coelicolor*
Strain	*Streptomyces coelicolor* A3(2) M22-2C43

Comparative genome analysis of *Streptomyces coelicolor* Strains

Comparative genome analysis of *S. coelicolor* A3(2) M22-2C43 was performed to understand the taxonomic similarity with the parent strain *S. coelicolor* A3(2) M22-2C43-WT (or M22-2C43-WT) strain. The A3 (2) strain has 2 plasmids, but both *S. coelicolor* A3(2) M22-2C43-WT and M22-2C43 strains do not have any. Genomic size was reduced from 8,667,507 to 7,438,186 bp during an uv-treatment and the M22-2C43 strain consists of 84 tRNAs with a GC content of 72.1 mol% (Table 14). Details are described in Appendix C.

DNA-DNA hybridization (DDH) values have been used by bacterial taxonomists since the 1960s to determine relatedness between strains and are still the most reliable criterion in the delineation of bacterial species. Most recently, the average nucleotide identity (ANI), calculated from pairwise comparisons of all sequences shared between any two strains, has been proposed as the new metrics for bacterial species classification. Goris et al. (2007) reported 95% similarity of calculated ANI based on WGS corresponding to 70% of DNA -DNA hybridization (DDH) which is considered as a gold standard value of species delineation. The comparative ANI value of *S. coelicolor A3(2) M22-2C43* and M22-2C43- WT strains was calculated using WGS ANI calculating algorithm (Yoon et al., 2017). The M22-2C43-WT genome had an 8,668,266 bp linear genome, and the M22-2C43 genome had a 7,438,186 bp circular genome. Comparative genome analysis between the WT and mutated (via adaptive laboratory evolution) strains revealed that both ends of the WT genome were deleted. And 2 large structural variations (1 deletion and 1 insertion) and 29 genome modifications (2 small insertions, 22 substitutions, and 5 small deletions) were observed in the mutant strain. The ortho ANI analysis showed a 100% match which proves a strong similarity between these strains (Tables 14).

Table 14. WGS of *S. coelicolor* A3(2) M22-2C43 in Comparison with *S. coelicolor* A3(2) M22-2C43-WT

Contents	M22-2C43	M22-2C43 WT
Status	Complete	Complete
Number of contigs	1 circular	1 linear
Plasmids	0	0
Genome size, bp	7,438,186	8,668,266
DNA G+C content, %	72.1	72.1
Number of CDSs	6,604	7,711
Number of rRNAs (tRNA)	18 (84)	18 (88)
Similarity with *S. coelicolor* M22-2C43-WT by OrthoANI analysis, %	100%	-

6.B.3.3 The Analysis of WGS of *S. coelicolor* A3(2) M22-2C43

The safety of the *S. coelicolor* A3(2) M22-2C43 strain was evaluated through four bioinformatic analyses to identify antibiotics resistance genes, virulence factor genes, allergen genes, and biosynthetic gene clusters. The analyses showed that the *S. coelicolor* A3(2) M22-2C43 strain did not have any virulence factors and potential allergens. And it also did not contain biosynthetic gene clusters for producing widely used antibiotics. In the case of antimicrobial resistance (AMR) genes, the M22-2C43 strain was shown to have three antibiotic resistance genes (dldHA2X, erm(O), tet) from the ResFinder analysis. However, comparative AMR gene analysis of *S. coelicolor* species indicated that the genomes of all known *S. coelicolor* strains also contained these three antibiotic resistance genes. Therefore, these genes might be considered intrinsic genes of *S. coelicolor* strains. Additional safety-related analyses (Virulence Factor, Allergen, and BGC) of *S. coelicolor* A3(2) M22-2C43 strain did not find any homology with known virulence and allergenic genes. Based on the results from the WGS analysis, it was concluded that *S. coelicolor* A3(2) M22-2C43 was non-pathogenic and non-toxigenic. Details are described in Appendix C.

6.C. Potential Adverse Effects

A wide variety of enzymes are used in food processing, and enzyme proteins do not generally raise safety concerns (Pariza and Foster, 1983; Pariza and Johnson, 2001). Pariza and Johnson (2001) noted that very few toxic agents have enzymatic properties. From the investigation on possible allergenicity of 19 different commercial enzymes used in the food industry, Bindslev-Jensen et al. (2006) concluded that ingestion of food enzymes, in general, is not likely to be a concern with regard to food allergy.

A review by Ladics and Sewalt (2018) also summarizes the safety of food industrial enzymes as follows:

1) Enzymes, in general, do not produce acute toxicity, dermal sensitization, genotoxicity, or repeated dose oral toxicity.
2) Several hundred mutagenicity studies have been conducted on bacterial and mammalian cells using a variety of enzymes. No positive findings were observed.
3) Over 225 90-day studies have reported no adverse findings, including in the bone marrow. The data showing no adverse effects for enzyme preparations also confirms that the microbial metabolites and fermentation materials lack toxicity as well.
4) Exposure to enzyme products is also minimal as recommended use levels are low, generally <0.1% (wt/wt). The weight-of-evidence indicates that there are no concerns for oral toxicity of enzymes, in general, nor genotoxicity.
5) Therefore, continued routine practice of performing genotoxicity and 90-day studies on enzyme preparations as a part of the approval requirements is questionable, and establishing general health-based guidance values for enzymes may be considered.

Based on these toxicological data of β-agarase DagA and enzymes, in general, it is concluded that the β-agarase DagA is safe for its intended use as a processing aid for the hydrolysis of agar-agar to a food ingredient NAO.

6.D. Safety Determination

The subject of the present GRAS notice is β-agarase DagA from *S. coelicolor* A3(2) M22-2C43. This GRAS determination is based on the data and information generally available about the safety of β-agarase DagA. There is broad-based and widely disseminated knowledge concerning the safety of β-agarase DagA and its production microorganism.

The following safety evaluation fully considers the composition, intake, and microbiological and toxicological properties of Dyne Bio Inc.'s β-agarase DagA and its microorganism as well as appropriate corroborative data.

(A) The β-agarase DagA is used for hydrolysis of agar-agar during production of a food ingredient NAO. β-agarase DagA is not added to final foodstuffs, but is used as a processing aid during manufacturing of NAO. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and per requirements for normal production following cGMP. During the purification/production of NAO, more than 99.8% of enzyme is removed. Thus, residual enzyme present in the NAO is approximately 0.04 mg TOS/g NAO.

(B) Dyne Bio Inc. follows the principles of cGMP and HACCP-controlled manufacturing process and rigorously tests its final production batches to verify adherence to quality control parameters and specifications.

(C) FDA has determined that mixed carbohydrase and protease enzyme preparations derived from *B. subtilis* are GRAS (21 CFR 184.1148 and 21 CFR 184.1150). In addition, a review by Ladics and Sewalt (2018) summarizes that there are no concerns for oral toxicity of enzymes, in general, nor genotoxicity.

(D) Mutagenicity and genotoxicity studies of Dyne Bio Inc.'s β-agarase DagA found that it was not mutagenic or genotoxic under the test conditions. Additional subchronic oral toxicity study of β-agarase DagA DagA preparations suggested that the enzyme was well tolerated in rats with no side effects. In a 90-day subchronic toxicity study, the NOAEL was determined to be 159 mg TOS/kg bw/day (or 10,000 mg/kg bw/day), the highest level tested. After a safety margin of 100, the safe intake level would be 1.59 mg TOS/kg bw/day or approximately 111 mg TOS/person/day, assuming an average American adult weighs 70 kg.

(E) Under the assumption that the maximum daily use of NAO will be 23 g and that residual enzyme level in the finished ingredient is expected to be 0.04 mg TOS/g NAO, the theoretical maximum daily intake of consumers of the food enzyme would be 0.92 mg TOS/person/day or 0.013 mg TOS/kg bw/day. The EDI is within the safe intake level. However, this EDI is overly inflated because it is not expected that NAO could be the only source of dietary fiber in the American diet. In addition, the presence of the enzyme residues in the final food is not expected to have any effect in or on the final food, and the enzyme action is expected to be over before the food product is available for consumers.

(F) The enzyme β-agarase DagA is free of the production microorganism. In addition, production microorganism, *S. coelicolor* A3(2) M22-2C43, for β-agarase DagA is absent of allergenic genes and virulent genes.

Thus, it is concluded that the β-agarase DagA enzyme preparation derived from *S. coelicolor* A3(2) M22-2C43 is safe and Generally Recognized as Safe (GRAS) for its intended use as a processing aid (in the manufacture of a food ingredient NAO).

6.E. Conclusions and General Recognition of the Safety of β-Agarase DagA

6.E.1. Common Knowledge Element of the GRAS Determination

β-agarase DagA is classified as a degrading enzyme that specifically hydrolyzes β-1,4-glucosidic bonds in agar-agar. β-agarase DagA is a naturally occurring enzyme in many microbial species. The safety of carbohydrases is well acknowledged by authorities. A review by Ladics and Sewalt (2018) also summarizes that food industrial enzymes, in general, do not produce acute toxicity, dermal sensitization, genotoxicity, or repeated dose oral toxicity. Because this safety evaluation was based on generally available and widely accepted data and information, it satisfies the so-called “common knowledge” element of a GRAS determination.

6.E.2. Technical Element of the GRAS Determination (Safety Determination)

The intended use of β-agarase DagA has been determined to be safe though scientific procedures as set forth in 21 CFR 170.3(b); thus, satisfying the so-called “technical” element of the GRAS determination. The β-agarase DagA is used for hydrolysis of agar-agar to NAO. However, the enzyme used during processing is not expected to exert any unintentional enzymatic activity in the final food when using the β-agarase DagA in the production of NAO, a food ingredient. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and per requirements for normal production following cGMP. No toxicants have been detected from β-agarase DagA enzyme preparations.

Literature or publicly available data did not identify safety or toxicity concerns related to β-agarase DagA or its production microorganisms. A battery of toxicity studies of Dyne Bio Inc.'s β-agarase DagA found no adverse effects of β-agarase. In addition, residual β-agarase DagA level will be approximately 0.04 mg TOS/g NAO, resulting in the theoretical maximum EDI of 0.92 mg/person/day or 0.0131 mg/ TOS/kg bw/day. After consideration of a safety margin of 100, the EDI would be less than 100th of the safe intake level determined from a 90 day oral toxicity study in rats. This evidence is sufficient to support the safety and GRAS status of the proposed use of β-agarase DagA as a processing aid for the manufacture of foods.

Dyne Bio Inc. has concluded that Dyne Bio Inc.'s β-agarase DagA is GRAS under the intended conditions of use on the basis of scientific procedures, and other experts qualified to assess the safety of food ingredients would concur with these conclusions. Therefore, it is excluded from the definition of a food additive and may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR. Therefore, Dyne Bio Inc. has concluded that β-agarase DagA, when used as described in this dossier, is GRAS based on scientific procedures.

6.F. Discussion of Information Inconsistent with GRAS Determination

Dyne Bio Inc. has reviewed the available data and information and is not aware of any data and information that are, or may appear to be, inconsistent with its conclusion of the GRAS status.

PART 7. REFERENCES

7.A. References that are Generally Available

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7.B. References that are Not Generally Available

Not applicable

Appendix A. Amino Acid Sequence of β-Agarase DagA and its Homology with Those of Known Allergenic Proteins

Authors: Hye-Jeong KO, Eun Joo Kim, Je Hyeon Lee

Institution: Dyne Bio Inc., Gyeonggi-Do, 13209, Republic of Korea

Date: November 03, 2021

OBJECTIVE

The aim of this study was to determine if β-agarase DagA has any allergenic potential. The amino acid sequence of β-agarase DagA was determined and compared with those of known allergenic proteins.

1. Brief overview of methods

Following the guidelines developed by FAO/WHO (2001) and modified by Codex Alimentarius Commission (2003), the whole amino acid sequence of β-agarase DagA (in FASTA format) was compared to allergens from the Food Allergy Research and Resource Program (FARRP) allergen protein database (http://allergenonline.org). There was no significant homology found between β-agarase DagA and any of the allergens in the database mentioned above.

2. Amino acid sequence of β-agarase DagA (in FASTA format)

> β-agarase DagA

```
MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQL
LPSHSDDFNYTGKPQTFRGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRA
PDGRVYCGYVTSRTPVEYPLYTEVLMRVSGLKLSSNFWLLSRDDVNEIDVIECYGNES
LHGKHMNTAYHIFQRNPFTELARSQKGYFADGSYGYNGETGQVFGDGAGQPLLRNG
FHRYGVHWISATEFDFYFNGRLVRRLNRSNDLRDPRSRFFDQPMHLILNTESHQWRV
DRGIEPTDAELADPSINNIYYRWVRTYQAV
```

3. Homologies search

The allergenicity between β-agarase DagA and allergenic proteins included in the publicly available database was analyzed through the online tool, AllergenOnline, with three methods (http://www.allergenonline.org/).

3.1 Known allergens

The FARRP AllergenOnline.org database (http://www.allergenonline.org/) has been updated to version 21 on February 14, 2021. Version 21 contains a comprehensive list (2,233 protein [amino acid]) sequence entries that are categorized into 912 taxonomic-protein groups of unique proven or putative allergens (food, airway, venom/salivary, and contact). Its annual update process includes collecting new sequences designated as “allerg*” in reference files from the NCBI protein database (compiled from GenBank, RefSeq, and TPA databases as well as protein sequences from SwissProt, PIR, PRF, and PDB databases).

3.2 Methodology (http://www.allergenonline.org/) including the criteria to identify it as allergenic potential (the methodology overview has been adopted from Ladics [2008])

Typically, sequence homology searches comparing the structure of novel proteins to known allergens in a database are conducted using various algorithms, such as FASTA, to predict overall structural similarities. As recommended by FAO/WHO (2001), IgE cross-reactivity between a novel protein and a known allergen is considered a possibility when there is more than 35% identity over a segment of 80 or greater amino acids. It should also be pointed out, however, that for cross-reactivity to occur, Aalberse (2000) has reported that a high degree of homology is needed, likely in excess of 50–60%, over significant spans of the target protein and allergen. In addition, step-wise contiguous identical amino acid segment searches are also performed to identify amino acid sequences that may represent linear IgE binding epitopes. IgE binding epitopes, however, have only been identified for a few allergens. Therefore, in the absence of an IgE binding epitope database, potential epitopes can be evaluated by producing all overlapping peptides of the allergens contained in a particular database and comparing them in a pair-wise manner to all same-size potential peptides of a novel protein using bioinformatic tools. Eight contiguous amino acid matches between a novel protein and a known allergen(s) are routinely used to identify sequences that may represent linear epitopes. The 2001 FAO/WHO consultation has suggested using a six amino acid match for this type of analysis. The use of a contiguous amino acid match of <8 occurs too commonly between unrelated proteins and, therefore, is not a reliable criterion for predicting allergenic potential. Many random matches that are very unlikely to indicate potential IgE epitopes are observed using such a short sequence.

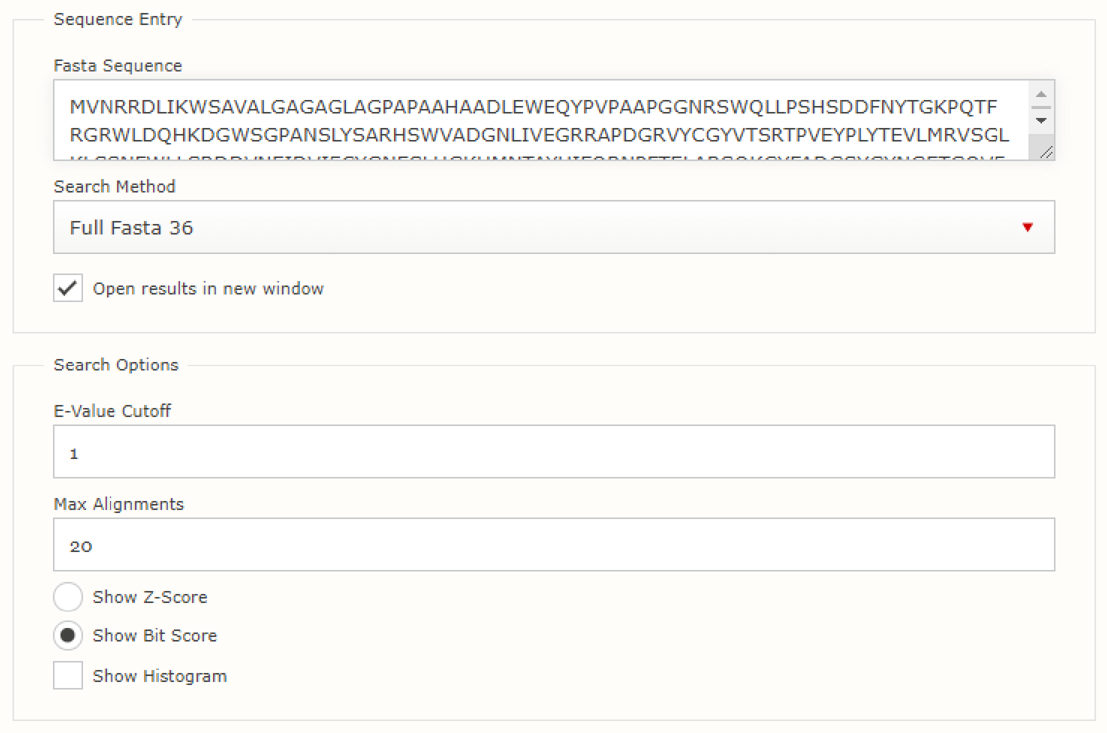
In our analysis, three methodologies with default parameters were used and described below:

Sequence search routines

1) Search for full-length alignments by FASTA: The most predictive search is the overall FASTA alignment (see FASTA Help Page), with identity matches greater than 50% indicating possible cross-reactivity (Aalberse, 2000).
2) Search for 80 amino acid alignments by FASTA: A precautionary search using a sliding window of 80 amino acid segments of each protein to find identities greater than 35% (according to CODEX Alimentarius Guidelines [2003]).
3) Search for 8 amino acid exact match: An 8-amino acid short-sequence identity search is provided since some regulatory authorities demand results of this extremely precautionary search. Our scientific opinion is that there is no evidence that an 8 amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%). In our experience, isolated identity matches of 8 contiguous amino acids occur by chance alone at some modest rate, matches of 7 and 6 occur more commonly. Experience (published and unpublished) demonstrates that two proteins sharing only a single short identity match of 6 to 8 contiguous amino acids do not share IgE binding in the absence of more extensive identity alignments (at least >35% identity over 80 or more amino acids). Sequences sharing less than 50% identity over their full-lengths are rarely cross-reactive. Thus, we recommend not using these short identity matches as there is no scientific evidence that they predict IgE cross-reactivity and they do not predict shared clinical activities.

4. Results

4.1 Input and output of search for full-length alignments by FASTA



Sequence Entry

Fasta Sequence

```
MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQLLPSHSDDFNYTGKPQTF
RGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGYVTSRTPVEYPLYTEVLMRVSGL
```

Search Method: Full Fasta 36

☑ Open results in new window

Search Options

E-Value Cutoff: 1

Max Alignments: 20

○ Show Z-Score

● Show Bit Score

☐ Show Histogram

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

%_id 1 = 100% identity, alen=alignment length

AllergenOnline Database v21 (February 14, 2021)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

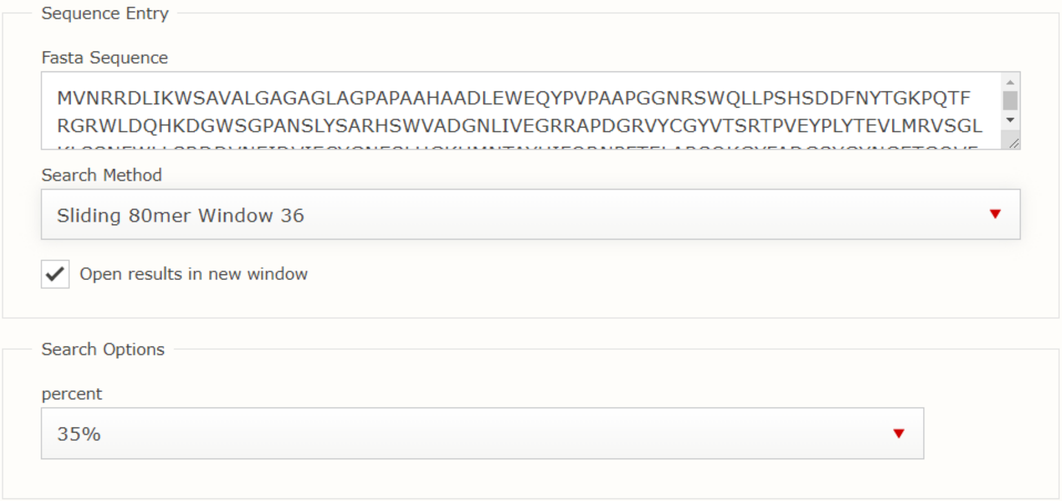
fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:₩Windows₩Temp₩allB85A.tmp version2136.fasta
User Query #1 >query

User Query #1

```
>query
MVNRRDLIKW SAVALGAGAG LAGPAPAAHA ADLEWEQYPV PAAPGGNRSW QLLPSHSDDF NYTGKPQTFR GRWLDQHKDG WSGPANSLYS ARHSWVADGN LIVEGRRAPD GRVYCGYVTS RTPVEYPLYT EVLMRVSGLK LSSNFWLLSR DDVNEIDVIE
CYGNESLHGK HMNTAYHIFQ RNPFTELARS QKGYFADGSY GYNGETGQVF GDGAGQPLLR NGFHRYGVHW ISATEFDFYF NGRLVRRLNR SNDLRDPRSR FFDQPMHLIL NTESHQWRVD RGIEPTDAEL ADPSINNIYY RWVRTYQAV
```

```
# fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:₩Windows₩Temp₩allB85A.tmp version2136.fasta
FASTA searches a protein or DNA sequence data bank
 version 36.3.8g Oct, 2018
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:₩Windows₩Temp₩allB85A.tmp
  1>>>query - 309 aa
Library: version2136.fasta
   540227 residues in  2233 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 5.6083+/-0.00722; mu= 15.8432+/- 0.381
 mean_var=39.6118+/-10.066, 0's: 0 Z-trim(80.4): 28  B-trim: 0 in 0/36
 Lambda= 0.203780
 statistics sampled from 443 (443) to 443 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
 ktup: 2, E-join: 1 (0.499), E-opt: 0.2 (0.198), width:  16
 Scan time:  0.000

The best scores are:                                         opt bits E(2233) %_id  %_sim  alen
gi|1079717864|gid|3017|PREDICTED: collagen alpha-1(I (1449)   87 30.9     0.51 0.379 0.500   66

>>>query, 309 aa vs version2136.fasta library

>>gi|1079717864|gid|3017|PREDICTED: collagen alpha-1(I) chain-like isofor  (1449 aa)
 initn:  94 init1:  57 opt:  87  Z-score: 110.8  bits: 30.9 E(2233): 0.51
Smith-Waterman score: 88; 37.9% identity (50.0% similar) in 66 aa overlap (20-85:602-658)

                                    10        20        30        40        50
query                       MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQLLPSHSDD
                                               :  ::: ::  :  . :: :: . ::    .: ::. .
notag| PGVMGFPGPKGAAGDAGKPGERGPGGASGPVGAPGKDGDVGAPGPAGAAGPAGEKGEQGPV-GPPG----FQGLPGPQGA
             570       580       590       600       610       620            630

      60        70        80        90       100       110       120       130
query  FNYTGKPQTFRGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGYVTSRTPVEYPLYTEVLMRVSGL
        . ::::    :.     . :  :::
notag| TGETGKP----GEQGAPGEAGPPGPAGPRGDRGFPGERGAPGITGPVGARGAPGPAGSDGPKGEPGAAGAPGGQGAPGMQ
        640           650       660       670       680       690       700       710

>>>///

309 residues in 1 query   sequences
540227 residues in 2233 library sequences
 Scomplib [36.3.8g Oct, 2018]
 start: Mon Sep 13 01:46:36 2021 done: Mon Sep 13 01:46:36 2021
 Scan time:  0.000 Display time:  0.000

Function used was FASTA [36.3.8g Oct, 2018]
```

4.2 Input and output of search for 80 amino acid alignments by FASTA



Sequence Entry

Fasta Sequence

MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQLLPSHSDDFNYTGKPQTF
RGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGYVTSRTPVEYPLYTEVLMRVSGL

Search Method

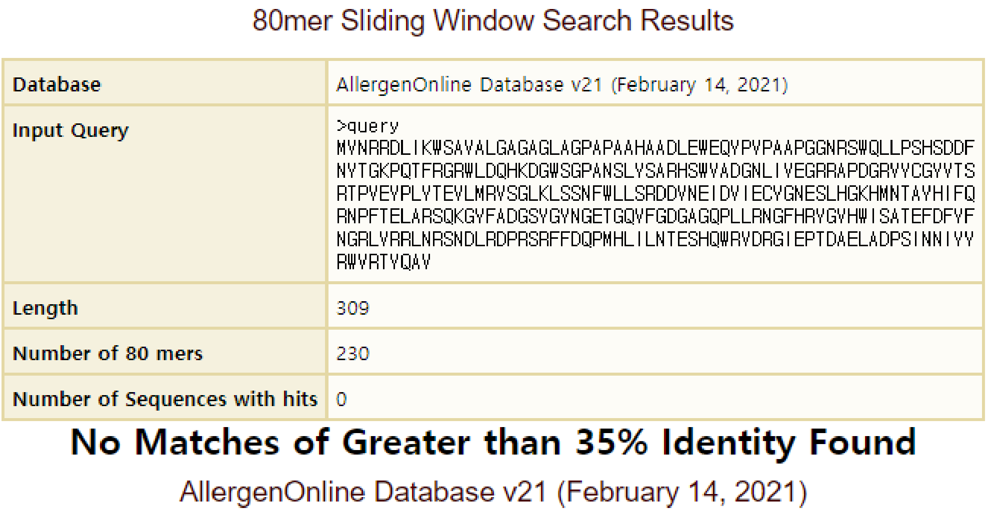
Sliding 80mer Window 36

☑ Open results in new window

Search Options

percent

35%



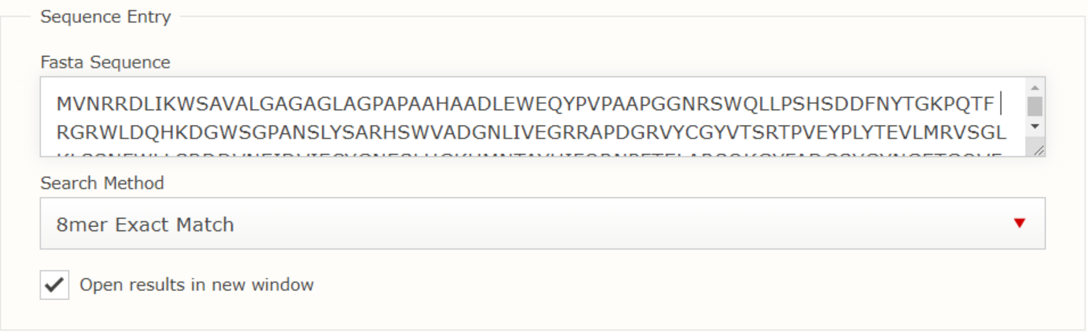
80mer Sliding Window Search Results

Database	AllergenOnline Database v21 (February 14, 2021)
Input Query	>query MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQLLPSHSDDF NYTGKPQTFRGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGYVTS RTPVEYPLYTEVLMRVSGLKLSSNFWLLSRDDVNEIDVIECYGNESLHGKHMNTAYHIFQ RNPFTELARSQKGYFADGSYGYNGETGQVFGDGAGQPLLRNGFHRYGVHWISATEFDFYF NGRLVRRLNRSNDLRDPRSRFFDQPMHLILNTESHQWRVDRGIEPTDAELADPSINNIYY RWVRTYQAV
Length	309
Number of 80 mers	230
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

AllergenOnline Database v21 (February 14, 2021)

4.3 Input and output of search for 8 amino acid exact match



Sequence Entry

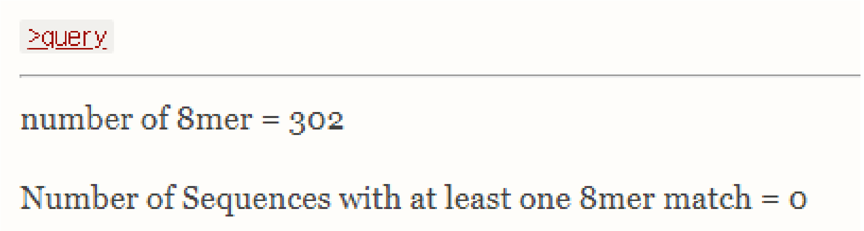
Fasta Sequence

MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQLLPSHSDDFNYTGKPQTF
RGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGYVTSRTPVEYPLYTEVLMRVSGL

Search Method

8mer Exact Match

☑ Open results in new window



```
>query

number of 8mer = 302

Number of Sequences with at least one 8mer match = 0
```

5. Conclusion

Therefore, there is no allergenicity between β-agarase DagA and allergenic proteins included in the publicly available database through the usage of all of three validation methods provided by AllergenOnline.org.

6. References

1. Aalberse RC. Structural biology of allergens. J Allergy Clin Immunol. 2000;106(2):228-38. Review.

2. Codex Alimentarious Commission, 2003. Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarious Commission, Twenty-Fifth Session, Rome, Italy, 30 June–5 July, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, and Appendix IV, Annex on the assessment of possible allergenicity, pp. 47–60.

3. FAO/WHO, 2001. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, January 22–25, 2001, Rome, Italy

4. Ladics GS. Current codex guidelines for assessment of potential protein allergenicity. Food Chem Toxicol. 2008 Oct;46 Suppl 10:S20-3.

Appendix B. Certificate of Analysis

CERTIFICATE OF ANALYSIS

PRODUCT NAME: β-agarase DagA

LOT NUMBER: 21-NBE0723

DATE MANUFACTURED 2021-07-23

Parameter	Specification	Method of analysis	Result
Activity unit, unit/ml	4.9 ~ 9.0	Food additive process, 9th, 2018, MHLW(Japan)	7.02
Total plate count	≤1,000 cfu/g	AOAC 990.12	<10
Yeast & Mold	≤ 100 cfu/g	AOAC 977.02	<10
Staphylococcus aureus	<10 cfu/g	AOAC 975.55	<10
Coliform	<10 cfu/g	AOAC 991.14	<10
Escherichia coli	Absent in 25g	ISO 7251.2005	Not detected
Listeria monocytogenes	Absent in 25g	AOAC 992.19	Not detected
Salmonella	Absent in 25g	AOAC 991.13	Not detected
Arsenic (As)	≤0.1	AOAC 2013.06 / ICP-MS	<0.02
Cadmium (Cd)	≤0.1	AOAC 2013.06 /ICP-MS	<0.007
Lead (Pb)	≤0.1	AOAC 2013.06 / ICP-MS	<0.02
Mercury (Hg)	≤0.1	ASTM D-6722-01/ CV-AAS	<0.02

CONCLUSION : Qualified

21-NBE0723

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AR-22-HX-007634

Analytical Report

Analytical Report No. **AR-22-HX-007634** Date 25-Apr-2022

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002360		
Sample Description:	β-agarase		
Lot NO.	21-NBE0723		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	uidelines
HX027	Appearance Method: Food code, Visual examination			
	Appearance AUG	Have own appearance without other odor and color.		

SIGNATURE

[redacted]

Kyuhee Oh
Technical manager

EXPLANATORY NOTE

Not Detected means not detected at or above the Limit of Quantification (LOQ)
☆ means the test is subcontracted within Eurofins group
● means the test is subcontracted outside Eurofins group
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The tests are identified by a five-digit code, their description is available on request.

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www.eurofins.co.kr

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21-NBE0723

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AR-22-HX-002847-01

Analytical Report

Analytical Report No. **AR-22-HX-002847-01** Date 22-Feb-2022

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00012729 / 984-2021-12003824
Sample Description:	β-agarase
Lot NO.	21-NBE0723
Reception Date:	27-Dec-2021
Test Purpose	Voluntary testing
Product Type	Liquid

Test Result(s):

			Results	Unit	Guideline
HX164	β-Agarase	Method: Food additives code, MHLW(Japan), Enzymatic-spectrophotometry			
	β-Agarase		7.02	U/ml	

SIGNATURE

[redacted]

Kyuhee Oh
Technical manager

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21-NBE0723

Page 1/1
AR-22-HX-010525-R2

Analytical Report

Analytical Report No. **AR-22-HX-010525-R2** **Date 23-Sep-2022**

(*this report cancels and replaces the previous one, numbered AR-22-HX-010525-R1/984-2022-06000024 dated 25/08/2022 which must be destroyed)

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00017885 / 984-2022-06000024		
Sample Description:	β-agarase		
Test Purpose	Voluntary testing		
Reception Date:	02-Jun-2022		
Manufacture Date	21-NBE0723		
Product Type	liquid	**Sample Weight**	100ml

Test Result(s):

			Results	Unit	uideline
HX1EX	Coliforms count	Method: AOAC 991.14, D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms		<10	cfu/g	

SIGNATURE

[redacted]

Kyuhee Oh
Technical manager

EXPLANATORY NOTE

Not Detected means not detected at or above the Limit of Quantification (LOQ)
☆ means the test is subcontracted within Eurofins group
⊛ means the test is subcontracted outside Eurofins group
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Results have been obtained and reported in accordance with our general sales conditions available on request.
When declaring compliance or non-compliance, the uncertainty associated with the result has been added or subtracted in order to obtain a result that can be compared to regulatory limits or specifications.The uncertainty has not been taken into account for standards that already include measurement uncertainty.
The tests are identified by a five-digit code, their description is available on request.

END OF REPORT

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EK-FM-QP-1609(3)r01 2020.06.15(REV.01)

21-NBE0723

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Analytical Report

Analytical Report No. **AR-22-HX-007247-02**

Date 23-Sep-2022

(*this report cancels and replaces the previous one, numbered AR-22-HX-007247-01/984-2022-03002364 dated 20/04/2022 which must be destroyed)

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002364		
Sample Description:	β-agarase		
Lot NO.	21-NBE0723		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	uideline
HX0SB	Total plate count_AOAC 990.12 Method: AOAC 990.12, E-Cultural technique (media film)			
	Total plate count	<10	cfu/g	
HX0SC	S.aureus_AOAC 975.55 Method: AOAC 975.55, E-Cultural technique (chromogenic media)			
	Staphylococcus aureus	<10	cfu/g	
HX0SD	Yeast and mold_AOAC 977.02 Method: AOAC 997.02, E-Cultural technique (non-chromogenic media)			
	Yeast & mould (count)	<10	cfu/g	
HX0SF	L.monocytogenes_AOAC 992.19 Method: AOAC 992.19, D-Cultural technique (chromogenic media)			
	Listeria monocytogenes nn/25g	Not Detected	/25 g	
HX0SG	Salmonella.spp_AOAC 991.13 Method: AOAC 991.13, Biochemical tests			
	Salmonella	Not Detected	/25 g	
HX1BX	E.coli Method: ISO 7251:2005 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Escherichia coli	Not Detected	/25 g	
HX1DX	Coliforms Method: ISO 4831:2006 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	Not Detected	/25 g	

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Kyuhee Oh
Technical manager

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EXPLANATORY NOTE

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21-NBE0723

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Page 1/1
AR-22-HX-007252-02

Analytical Report

Analytical Report No. **AR-22-HX-007252-02** **Date 14-Jun-2022**

(*this report cancels and replaces the previous one, numbered AR-22-HX-007252-01/984-2022-03002362 dated 22/04/2022 which must be destroyed)

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002362		
Sample Description:	β-agarase		
Lot NO.	21-NBE0723		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	Guideline
HX0SH	As (Arsenic)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS **Arsenic (As)**	<0.02	mg/kg	
HX0SI	Cd (Cadmium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS **Cadmium (Cd)**	<0.007	mg/kg	
HX0SJ	Pb (Lead)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS **Lead (Pb)**	<0.02	mg/kg	
HX0SS	Hg (Mercury)_ASTM D-6722-01 Mod. Method: ASTM D-6722-01 mod., CV-AAS **Mercury (Hg)**	<0.02	mg/kg	

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Kyuhee Oh
Technical manager

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Eurofins Korea Analytic Service Co., Ltd.
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EK-FM-QP-1609(3)r01 2020.06.15(REV.01)

21-NBE0723

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Page 1/1
AR-22-HX-010686

Analytical Report

Analytical Report No. **AR-22-HX-010686** **Date 14-Jun-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00016957 / 984-2022-05004176		
Sample Description:	β-agarase		
Lot NO.	21-NBE0723		
Reception Date:	12-May-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	100 ml

Test Result(s):

		Results	Unit	Guideline
HX18X	Ash Method: AOAC 923.03, Gravimetry			
	Ash	**0.11**	%	
HX1J1	Moisture Method: AOAC 935.29, Gravimetry			
	Moisture	**98.47**	%	

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Technical manager

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EK-FM-QP-1609(3)r01 2020.06.15(REV.01)

21-NBE0723

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AR-22-HX-007191-01

Analytical Report

Analytical Report No. **AR-22-HX-007191-01** **Date 20-Apr-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002361		
Sample Description:	β-agarase		
Lot NO.	21-NBE0723		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

	Results	Unit	Guidelines
HX0SK Mg (Magnesium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Magnesium (Mg)**	69.0	mg/100 g	
HX0SL Ca (Calcium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Calcium (Ca)**	<20	mg/100 g	
HX0SM Fe (Iron)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Iron (Fe)**	<2	mg/100 g	
HX0SN Zn (Zinc)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Zinc (Zn)**	<2	mg/100 g	
HX0SP Na (Sodium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Sodium (Na)**	16.7	mg/100 g	
HX0SQ K (Potassium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Potassium (K)**	68.5	mg/100 g	
HX0SR P (Phosphorus)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Phosphorus (P)**	18.0	mg/100 g	

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Kyuhee Oh
Technical manager

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EK-FM-QP-1609(3)r01 2020.06.15(REV.01)

21-NBE0723

Analytical Report

Analytical Report No. **AR-22-HX-007043-01** **Date 19-Apr-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014586 / 984-2022-04000114		
Sample Description:	β-agarase		
Lot NO.	21-NBE0723		
Reception Date:	04-Apr-2022		
Sample Weight	600 ml		
Product Type	liquid	**Test Purpose**	Voluntary testing

Test Result(s):

			Results	Unit	LOQ Guideline
HX01G	Pesticides Screening	Method: Food code, GC-MS/MS			
	Screened pesticides		**Not Detected**		
HX01H	Pesticides Screening	Method: Food code, LC-MS/MS			
	Screened pesticides		**Not Detected**		

List of screened and not detected molecules (* = limit of quantification)

HX01G **Pesticides Screening** (LOQ* mg/kg)

(a) Acrinathrin (0.01)	(a) Alachlor (0.01)	(a) Aldrin (0.01)	(a) Ametoctradin (0.01)	(a) Anilofos (0.01)	(a) Azaconazole (0.01)
(a) Benfuresate (0.01)	(a) Bifenox (0.01)	(a) Bifenthrin (0.01)	(a) BROMOBUTIDE (0.01)	(a) Bromopropylate (0.01)	(a) Butachlor (0.01)
(a) Butafenacil (0.01)	(a) Carbophenothion (0.01)	(a) Chlorantraniliprole (0.01)	(a) Chlordane (0.01)	(a) Chlorfenapyr (0.01)	(a) Chlorfenvinphos (0.01)
(a) Chlorfluazuron (0.01)	(a) Chlorobenzilate (0.01)	(a) Chlorpropham (0.01)	(a) Chlorpyrifos-methyl (0.01)	(a) Cyfluthrin (0.01)	(a) Cyhalothrin (0.01)
(a) Cypermethrin (sum of isomers) (0.01)	(a) Cyprodinil (0.01)	(a) DDT (total) (0.01)	(a) Deltamethrin (0.01)	(a) Diclofop-methyl (0.01)	(a) Dicloran (0.01)
(a) Dicofol, p,p- (0.01)	(a) Dieldrin (0.01)	(a) Difenoconazole (0.01)	(a) Dimethoate (0.01)	(a) Dimethylvinphos (0.01)	(a) Diphenylamine (0.01)
(a) Disulfoton (0.01)	(a) Endosulfan (total) (0.01)	(a) Endrin (0.01)	(a) EPN (0.01)	(a) Epoxiconazole (0.01)	(a) Ethalfluralin (0.01)
(a) Ethion (0.01)	(a) Etridiazole (0.01)	(a) Fenclorim (0.01)	(a) Fenitrothion (0.01)	(a) Fenothiocarb (0.01)	(a) Fenoxanil (0.01)
(a) Fenpropathrin (0.01)	(a) Fenthion (0.01)	(a) Fenvalerate (0.01)	(a) Fipronil (0.01)	(a) Flucythrinate (0.01)	(a) Flumioxazin (0.01)
(a) Fluopyram (0.01)	(a) Fonofos (0.01)	(a) Halfenprox (0.01)	(a) HCH (sum) (0.01)	(a) Heptachlor/Heptachloroepoxide (0.01)	(a) Imibenconazole (0.01)
(a) Indanofan (0.01)	(a) Indoxacarb (sum, R+S isomers) (0.01)	(a) Iprodione (0.01)	(a) Isazophos (0.01)	(a) Isofenphos (0.01)	(a) Lindane (gamma-HCH) (0.01)
(a) Mecarbam (0.01)	(a) Methidathion (0.01)	(a) Metolachlor (0.01)	(a) Metribuzin (0.01)	(a) Oxyfluorfen (0.01)	(a) Parathion-ethyl (0.01)
(a) Parathion-methyl (0.01)	(a) Pendimethalin (0.01)	(a) Penthiopyrad (0.01)	(a) Permethrin (sum of isomers) (0.01)	(a) Phenothrin (0.01)	(a) Phorate (0.01)
(a) Phosalone (0.01)	(a) Phthalide (0.01)	(a) Picoxystrobin (0.01)	(a) Piperonyl butoxide (0.01)	(a) Pirimiphos-ethyl (0.01)	(a) Pretilachlor (0.01)
(a) Prochloraz (0.01)	(a) Procymidone (0.01)	(a) Promecarb (0.01)	(a) Prometryn (0.01)	(a) Propachlor (0.01)	(a) Propazine (0.01)
(a) Propiconazole (sum of isomers) (0.01)	(a) propisochlor (0.01)	(a) Propyzamide (0.01)	(a) Prothiofos (0.01)	(a) Pyridalyl (0.01)	(a) Quintozene (0.01)
(a) Silafluofen (0.01)	(a) Simazine (0.01)	(a) Simeconazole (0.01)	(a) Simetryn (0.01)	(a) Spiromesifen (0.01)	(a) Tebupirimfos (0.01)
(a) Tefluthrin (0.01)	(a) Terbufos (0.01)	(a) Terbutryn (0.01)	(a) Tetradifon (0.01)	(a) Thifluzamide (0.01)	(a) Tolclofos-methyl (0.01)
(a) Triadimenol (0.01)	(a) Tri-allate (0.01)	(a) Trifluralin (0.01)	(a) Vinclozolin (0.01)	(a) Zoxamide (0.01)	

In case of Agricultural product, performed according to the MFDS' guideline 'Korean food code, attached table 4, Pesticides MRL of each agricultural product'

HX01H **Pesticides Screening** (LOQ* mg/kg)

(a) Abamectin (0.01)	(a) Acephate (0.01)	(a) Acetamiprid (0.01)	(a) Aldicarb (0.01)	(a) Amisulbrom (0.01)	(a) Azimsulfuron (0.01)
(a) Azinphos-methyl (0.01)	(a) Azoxystrobin (0.01)	(a) Bendiocarb (0.01)	(a) Bensulfuron methyl (0.01)	(a) Benthiavalicarb, isopropyl- (0.01)	(a) Benzobicyclon (0.01)
(a) Benzoximate (0.01)	(a) Bitertanol (0.01)	(a) Boscalid (0.01)	(a) Bromacil (0.01)	(a) Buprofezin (0.01)	(a) Cadusafos (0.01)
(a) Cafenstrole (0.01)	(a) Carbaryl (0.01)	(a) Carbendazim (0.01)	(a) Carbofuran (0.01)	(a) Carboxin (0.01)	(a) Carfentrazone-ethyl (0.01)
(a) Carpropamid (0.01)	(a) Chlorpyrifos (0.01)	(a) Chlorsulfuron (0.01)	(a) Chromafenozide (0.01)	(a) Clethodim (0.01)	(a) Clofentezine (0.01)
(a) Clomazone (0.01)	(a) Clothianidin (0.01)	(a) Cyazofamid (0.01)	(a) Cyclosulfamuron (0.01)	(a) Cyflufenamid (0.01)	(a) Cyhalofop-butyl (0.01)
(a) Cymoxanil (0.01)	(a) Cyproconazole (0.01)	(a) DAIMURON (0.01)	(a) Demeton-S-methyl (0.01)	(a) Diazinon (0.01)	(a) Dichlorvos (0.01)
(a) Diethofencarb (0.01)	(a) Diflubenzuron (0.01)	(a) Dimepiperate (0.01)	(a) Dimethametryn (0.01)	(a) Dimethenamid including other mixtures of constitue (0.01)	(a) Dimethomorph (0.01)
(a) Diniconazole (0.01)	(a) Dinotefuran (0.01)	(a) Diphenamid (0.01)	(a) DITHIOPYR (0.01)	(a) Diuron (0.01)	(a) Edifenphos (0.01)
(a) ESPROCARB (0.01)	(a) Ethaboxam (0.01)	(a) Ethiofencarb (0.01)	(a) Ethoprophos (0.01)	(a) Ethoxysulfuron (0.01)	(a) Etofenprox (0.01)
(a) Etoxazole (0.01)	(a) Etrimfos (0.01)	(a) Famoxadone (0.01)	(a) Fenamiphos (0.01)	(a) Fenarimol (0.01)	(a) Fenazaquin (0.01)
(a) Fenbuconazole (sum of constituent enantiomers) (0.01)	(a) Fenhexamid (0.01)	(a) Fenobucarb (0.01)	(a) Fenoxaprop-ethyl (0.01)	(a) Fenoxycarb (0.01)	(a) Fenpyroximate (0.01)

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(a) FENTRAZAMIDE (0.01)	(a) Ferimzone (Z) (0.01)	(a) Flonicamid (0.01)	(a) Fluacrypyrim (0.01)	(a) Flubendiamide (0.01)	(a) Flucetosulfuron (0.01)
(a) Fludioxonil (0.01)	(a) Flufenacet (0.01)	(a) Flufenoxuron (0.01)	(a) Fluopicolid (0.01)	(a) Fluquinconazole (0.01)	(a) Flusilazole (0.01)
(a) Flutolanil (0.01)	(a) Fluxapyroxad (0.01)	(a) Forchlorfenuron (0.01)	(a) Fosthiazate (0.01)	(a) Furathiocarb (0.01)	(a) Gibberellic Acid (0.01)
(a) Halosulfuron-methyl (0.01)	(a) Haloxyfop (0.01)	(a) Hexaconazole (0.01)	(a) Hexaflumuron (0.01)	(a) Hexazinone (0.01)	(a) Hexythiazox (0.01)
(a) Imazalil (any ratio of constituent isomers) (0.01)	(a) Imazosulfuron (0.01)	(a) Imicyafos (0.01)	(a) Imidacloprid (0.01)	(a) INABENFIDE (0.01)	(a) Iprobenfos (0.01)
(a) Iprovalicarb (0.01)	(a) Isoprocarb (0.01)	(a) Isoprothiolane (0.01)	(a) Isopyrazam (0.01)	(a) Kresoxim-methyl (0.01)	(a) Linuron (0.01)
(a) Lufenuron (0.01)	(a) Malathion (0.01)	(a) Mandipropamid (any ratio of constituent isomers) (0.01)	(a) Mefenacet (0.01)	(a) Mepanipyrim (0.01)	(a) Mepronil (0.01)
(a) Metalaxyl (0.01)	(a) Metamifop (0.01)	(a) Metazosulfuron (0.01)	(a) Metconazole (0.01)	(a) Methabenzthiazuron (0.001)	(a) Methiocarb (0.01)
(a) Methomyl (0.01)	(a) Methoxyfenozide (0.01)	(a) Metobromuron (0.01)	(a) Metolcarb (0.01)	(a) Metrafenone (0.01)	(a) Mevinphos (0.01)
(a) Milbemectin (sum) (0.01)	(a) Molinate (0.01)	(a) Monocrotophos (0.01)	(a) Myclobutanil (sum of constituent isomers) (0.01)	(a) Napropamide (0.01)	(a) Nicosulfuron (0.01)
(a) Novaluron (0.01)	(a) Nuarimol (0.01)	(a) Ofurace (0.01)	(a) Omethoate (0.01)	(a) Oxadiazon (0.01)	(a) Oxadixyl (0.01)
(a) Oxamyl (0.01)	(a) Oxaziclomefone (0.01)	(a) Paclobutrazol (0.01)	(a) Penconazole (sum of constituent isomers) (0.01)	(a) Pencycuron (0.01)	(a) Penoxsulam (0.01)
(a) PENTOXAZONE (0.01)	(a) Phenthoate (0.01)	(a) Phosphamidon (0.01)	(a) Phoxim (0.01)	(a) Piperophos (0.01)	(a) Pirimicarb (0.01)
(a) Pirimiphos-methyl (0.01)	(a) Probenazole (0.01)	(a) Profenofos (0.01)	(a) Propamocarb (Sum of propamocarb and its salts, exp (0.01)	(a) Propanil (0.01)	(a) Propaquizafop (0.01)
(a) Propoxur (0.01)	(a) Pyraclofos (0.01)	(a) Pyraclostrobin (0.01)	(a) PYRAZOLYNATE (0.01)	(a) Pyrazophos (0.01)	(a) Pyribenzoxim (0.01)
(a) PYRIBUTICARB (0.01)	(a) Pyridaben (0.01)	(a) Pyridaphenthion (0.01)	(a) Pyrifluquinazon (0.01)	(a) PYRIFTALID (0.01)	(a) Pyrimethanil (0.01)
(a) Pyrimidifen (0.01)	(a) Pyriminobac-methyl (0.01)	(a) Pyrimisulfan (0.01)	(a) Pyriproxyfen (0.01)	(a) Pyroquilon (0.01)	(a) Quinalphos (0.01)
(a) Quinmerac (0.01)	(a) Quinoclamine (0.01)	(a) Quizalofop ethyl (0.01)	(a) Saflufenacil (0.01)	(a) Sethoxydim (0.01)	(a) Spinetoram (sum) (0.01)
(a) Spirodiclofen (0.01)	(a) Spirotetramat (0.01)	(a) Sulfoxaflor (0.01)	(a) Tebuconazole (0.01)	(a) Tebufenozide (0.01)	(a) Tebufenpyrad (0.01)
(a) Teflubenzuron (0.01)	(a) Terbuthylazine (0.01)	(a) Tetraconazole (0.01)	(a) Thenylchlor (0.01)	(a) Thiabendazole (0.01)	(a) Thiacloprid (0.01)
(a) Thiamethoxam (0.01)	(a) THIAZOPYR (0.01)	(a) Thidiazuron (0.01)	(a) Thifensulfuron-methyl (0.01)	(a) Thiobencarb (0.01)	(a) Thiodicarb (0.01)
(a) Tiadinil (0.01)	(a) Triadimefon (0.01)	(a) Triazophos (0.01)	(a) Tricyclazole (0.01)	(a) Trifloxystrobin (0.01)	(a) Triflumizole (0.01)
(a) Triflumuron (0.01)	(a) Uniconazole (0.01)	(a) Vamidothion (0.01)			

In case of Agricultural product, performed according to the MFDS' guideline 'Korean food code, attached table 4, Pesticides MRL of each agricultural product'

SIGNATURE

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Kyuhee Oh
Technical manager

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END OF REPORT

CERTIFICATE OF ANALYSIS

PRODUCT NAME:	β-agarase DagA
LOT NUMBER:	21-NBE0917
DATE MANUFACTURED	2021-09-17

Parameter	Specification	Method of analysis	Result
Activity unit, unit/ml	4.9 ~ 9.0	Food additive process, 9th, 2018, MHLW(Japan)	6.97
Total plate count	≤1,000 cfu/g	AOAC 990.12	<10
Yeast & Mold	≤ 100 cfu/g	AOAC 977.02	<10
Staphylococcus aureus	<10/g	AOAC 975.55	<10
Coliform	<10g	AOAC 991.14	<10
Escherichia coli	Absent in 25g	ISO 7251.2005	Not detected
Listeria monocytogenes	Absent in 25g	AOAC 992.19	Not detected
Salmonella	Absent in 25g	AOAC 991.13	Not detected
Arsenic (As)	≤0.1	AOAC 2013.06 / ICP-MS	<0.02
Cadmium (Cd)	≤0.1	AOAC 2013.06 /ICP-MS	<0.007
Lead (Pb)	≤0.1	AOAC 2013.06 / ICP-MS	<0.02
Mercury (Hg)	≤0.1	ASTM D-6722-01/ CV-AAS	<0.02

CONCLUSION : Qualified

21-NBE0917

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AR-22-HX-007635

Analytical Report

Analytical Report No. **AR-22-HX-007635** Date 25-Apr-2022

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002365		
Sample Description:	β-agarase		
Lot NO.	21-NBE0917		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	Guidelines
HX027 Appearance	Method: Food code, Visual examination			
Appearance AUG		**Have own appearance without other odor and color.**		

SIGNATURE

[redacted]

Kyuhee Oh
Technical manager

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EK-FM-QP-1609(3)r01 2020.06.15(REV.01)

21-NBE0917

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AR-22-HX-002848-01

Analytical Report

Analytical Report No. **AR-22-HX-002848-01** **Date 22-Feb-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00012729 / 984-2021-12003825
Sample Description:	β-agarase
Lot NO.	21-NBE0917
Reception Date:	27-Dec-2021
Test Purpose	Voluntary testing
Product Type	Liquid

Test Result(s):

			Results	Unit	Guideline
HX164	β-Agarase	Method: Food additives code, MHLW(Japan), Enzymatic-spectrophotometry			
	β-Agarase		**6.97**	U/ml	

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Kyuhee Oh
Technical manager

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21-NBE0917

Analytical Report

Analytical Report No. **AR-22-HX-010526-R2**

Date 23-Sep-2022

(*this report cancels and replaces the previous one, numbered AR-22-HX-010526-R1/984-2022-06000025 dated 25/08/2022 which must be destroyed)

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00017885 / 984-2022-06000025		
Sample Description:	β-agarase		
Test Purpose	Voluntary testing		
Reception Date:	02-Jun-2022		
Manufacture Date	21-NBE0917		
Product Type	liquid	**Sample Weight**	100ml

Test Result(s):

		Results	Unit	Guidelines
HX1EX	Coliforms count Method: AOAC 991.14, D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	<10	cfu/g	

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Kyuhee Oh
Technical manager

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21-NBE0917

Analytical Report

Analytical Report No. **AR-22-HX-007248-02**

Date 23-Sep-2022

(*this report cancels and replaces the previous one, numbered AR-22-HX-007248-01/984-2022-03002369 dated 20/04/2022 which must be destroyed)

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002369		
Sample Description:	β-agarase		
Lot NO.	21-NBE0917		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	Guidelines
HX0SB	Total plate count_AOAC 990.12 Method: AOAC 990.12, E-Cultural technique (media film)			
	Total plate count	<10	cfu/g	
HX0SC	S.aureus_AOAC 975.55 Method: AOAC 975.55, E-Cultural technique (chromogenic media)			
	Staphylococcus aureus	<10	cfu/g	
HX0SD	Yeast and mold_AOAC 977.02 Method: AOAC 997.02, E-Cultural technique (non-chromogenic media)			
	Yeast & mould (count)	<10	cfu/g	
HX0SF	L.monocytogenes_AOAC 992.19 Method: AOAC 992.19, D-Cultural technique (chromogenic media)			
	Listeria monocytogenes nn/25g	Not Detected	/25 g	
HX0SG	Salmonella.spp_AOAC 991.13 Method: AOAC 991.13, Biochemical tests			
	Salmonella	Not Detected	/25 g	
HX1BX	E.coli Method: ISO 7251:2005 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Escherichia coli	Not Detected	/25 g	
HX1DX	Coliforms Method: ISO 4831:2006 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	Not Detected	/25 g	

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Kyuhee Oh
Technical manager

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AR-22-HX-007253-02

Analytical Report

Analytical Report No. **AR-22-HX-007253-02** Date 14-Jun-2022

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Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002367		
Sample Description:	β-agarase		
Lot NO.	21-NBE0917		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	uidelines
HX0SH	As (Arsenic)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS			
	Arsenic (As)	<0.02	mg/kg	
HX0SI	Cd (Cadmium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS			
	Cadmium (Cd)	<0.02	mg/kg	
HX0SJ	Pb (Lead)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS			
	Lead (Pb)	<0.02	mg/kg	
HX0SS	Hg (Mercury)_ASTM D-6722-01 Mod. Method: ASTM D-6722-01 mod., CV-AAS			
	Mercury (Hg)	<0.02	mg/kg	

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Kyuhee Oh
Technical manager

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21-NBE0917

Page 1/1
AR-22-HX-014194

Analytical Report

Analytical Report No. **AR-22-HX-014194** **Date 20-Jul-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00018964 / 984-2022-07001498		
Sample Description:	β-agarase		
Lot NO.	21-NBE0917		
Reception Date:	11-Jul-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	100ml

Test Result(s):

			Results	Unit	Guideline
HX18X	Ash	Method: AOAC 923.03, Gravimetry			
	Ash		**0.12**	%	
HX1J1	Moisture	Method: AOAC 935.29, Gravimetry			
	Moisture		**98.60**	%	

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Kyuhee Oh
Technical manager

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21-NBE0917

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AR-22-HX-007192-01

Analytical Report

Analytical Report No. **AR-22-HX-007192-01** **Date 20-Apr-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002366		
Sample Description:	β-agarase		
Lot NO.	21-NBE0917		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	Guidelines
HX0SK	Mg (Magnesium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES			
	Magnesium (Mg)	62.0	mg/100 g	
HX0SL	Ca (Calcium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES			
	Calcium (Ca)	<20	mg/100 g	
HX0SM	Fe (Iron)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES			
	Iron (Fe)	<2	mg/100 g	
HX0SN	Zn (Zinc)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES			
	Zinc (Zn)	<2	mg/100 g	
HX0SP	Na (Sodium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES			
	Sodium (Na)	15.0	mg/100 g	
HX0SQ	K (Potassium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES			
	Potassium (K)	55.1	mg/100 g	
HX0SR	P (Phosphorus)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES			
	Phosphorus (P)	50.5	mg/100 g	

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[redacted]
Technical manager

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EK-FM-QP-1609(3)r01 2020.06.15(REV.01)

21-NBE0917

Analytical Report

Analytical Report No. **AR-22-HX-007044-01** **Date 19-Apr-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014586 / 984-2022-04000115		
Sample Description:	β-agarase		
Lot NO.	21-NBE0917		
Reception Date:	04-Apr-2022		
Sample Weight	600 ml		
Product Type	liquid	**Test Purpose**	Voluntary testing

Test Result(s):

			Results	Unit	LOQ	Guideline
HX01G	Pesticides Screening	Method: Food code, GC-MS/MS				
	Screened pesticides		Not Detected			
HX01H	Pesticides Screening	Method: Food code, LC-MS/MS				
	Screened pesticides		Not Detected			

List of screened and not detected molecules (* = limit of quantification)

HX01G **Pesticides Screening** (LOQ* mg/kg)

(a) Acrinathrin (0.01)
(a) Alachlor (0.01)
(a) Aldrin (0.01)
(a) Ametoctradin (0.01)
(a) Anilofos (0.01)
(a) Azaconazole (0.01)
(a) Benfuresate (0.01)
(a) Bifenox (0.01)
(a) Bifenthrin (0.01)
(a) BROMOBUTIDE (0.01)
(a) Bromopropylate (0.01)
(a) Butachlor (0.01)
(a) Butafenacil (0.01)
(a) Carbophenothion (0.01)
(a) Chlorantraniliprole (0.01)
(a) Chlordane (0.01)
(a) Chlorfenapyr (0.01)
(a) Chlorfenvinphos (0.01)
(a) Chlorfluazuron (0.01)
(a) Chlorobenzilate (0.01)
(a) Chlorpropham (0.01)
(a) Chlorpyrifos-methyl (0.01)
(a) Cyfluthrin (0.01)
(a) Cyhalothrin (0.01)
(a) Cypermethrin (sum of isomers) (0.01)
(a) Cyprodinil (0.01)
(a) DDT (total) (0.01)
(a) Deltamethrin (0.01)
(a) Diclofop-methyl (0.01)
(a) Dicloran (0.01)
(a) Dicofol, p.p- (0.01)
(a) Dieldrin (0.01)
(a) Difenoconazole (0.01)
(a) Dimethoate (0.01)
(a) Dimethylvinphos (0.01)
(a) Diphenylamine (0.01)
(a) Disulfoton (0.01)
(a) Endosulfan (total) (0.01)
(a) Endrin (0.01)
(a) EPN (0.01)
(a) Epoxiconazole (0.01)
(a) Ethalfluralin (0.01)
(a) Ethion (0.01)
(a) Etridiazole (0.01)
(a) Fenclorim (0.01)
(a) Fenitrothion (0.01)
(a) Fenothiocarb (0.01)
(a) Fenoxanil (0.01)
(a) Fenpropathrin (0.01)
(a) Fenthion (0.01)
(a) Fenvalerate (0.01)
(a) Fipronil (0.01)
(a) Flucythrinate (0.01)
(a) Flumioxazin (0.01)
(a) Fluopyram (0.01)
(a) Fonofos (0.01)
(a) Halfenprox (0.01)
(a) HCH (sum) (0.01)
(a) Heptachlor/Heptachloroepoxide (0.01)
(a) Imibenconazole (0.01)
(a) Indanofan (0.01)
(a) Indoxacarb (sum, R+S isomers) (0.01)
(a) Iprodione (0.01)
(a) Isazophos (0.01)
(a) Isofenphos (0.01)
(a) Lindane (gamma-HCH) (0.01)
(a) Mecarbam (0.01)
(a) Methidathion (0.01)
(a) Metolachlor (0.01)
(a) Metribuzin (0.01)
(a) Oxyfluorfen (0.01)
(a) Parathion-ethyl (0.01)
(a) Parathion-methyl (0.01)
(a) Pendimethalin (0.01)
(a) Penthiopyrad (0.01)
(a) Permethrin (sum of isomers) (0.01)
(a) Phenothrin (0.01)
(a) Phorate (0.01)
(a) Phosalone (0.01)
(a) Phthalide (0.01)
(a) Picoxystrobin (0.01)
(a) Piperonyl butoxide (0.01)
(a) Pirimiphos-ethyl (0.01)
(a) Pretilachlor (0.01)
(a) Prochloraz (0.01)
(a) Procymidone (0.01)
(a) Promecarb (0.01)
(a) Prometryn (0.01)
(a) Propachlor (0.01)
(a) Propazine (0.01)
(a) Propiconazole (sum of isomers) (0.01)
(a) propisochlor (0.01)
(a) Propyzamide (0.01)
(a) Prothiofos (0.01)
(a) Pyridalyl (0.01)
(a) Quintozene (0.01)
(a) Silafluofen (0.01)
(a) Simazine (0.01)
(a) Simeconazole (0.01)
(a) Simetryn (0.01)
(a) Spiromesifen (0.01)
(a) Tebupirimfos (0.01)
(a) Tefluthrin (0.01)
(a) Terbufos (0.01)
(a) Terbutryn (0.01)
(a) Tetradifon (0.01)
(a) Thifluzamide (0.01)
(a) Tolclofos-methyl (0.01)
(a) Triadimenol (0.01)
(a) Tri-allate (0.01)
(a) Trifluralin (0.01)
(a) Vinclozolin (0.01)
(a) Zoxamide (0.01)

In case of Agricultural product, performed according to the MFDS' guideline 'Korean food code, attached table 4, Pesticides MRL of each agricultural product'

HX01H **Pesticides Screening** (LOQ* mg/kg)

(a) Abamectin (0.01)
(a) Acephate (0.01)
(a) Acetamiprid (0.01)
(a) Aldicarb (0.01)
(a) Amisulbrom (0.01)
(a) Azimsulfuron (0.01)
(a) Azinphos-methyl (0.01)
(a) Azoxystrobin (0.01)
(a) Bendiocarb (0.01)
(a) Bensulfuron methyl (0.01)
(a) Benthiavalicarb, isopropyl- (0.01)
(a) Benzobicyclon (0.01)
(a) Benzoximate (0.01)
(a) Bitertanol (0.01)
(a) Boscalid (0.01)
(a) Bromacil (0.01)
(a) Buprofezin (0.01)
(a) Cadusafos (0.01)
(a) Cafenstrole (0.01)
(a) Carbaryl (0.01)
(a) Carbendazim (0.01)
(a) Carbofuran (0.01)
(a) Carboxin (0.01)
(a) Carfentrazone-ethyl (0.01)
(a) Carpropamid (0.01)
(a) Chlorpyrifos (0.01)
(a) Chlorsulfuron (0.01)
(a) Chromafenozide (0.01)
(a) Clethodim (0.01)
(a) Clofentezine (0.01)
(a) Clomazone (0.01)
(a) Clothianidin (0.01)
(a) Cyazofamid (0.01)
(a) Cyclosulfamuron (0.01)
(a) Cyflufenamid (0.01)
(a) Cyhalofop-butyl (0.01)
(a) Cymoxanil (0.01)
(a) Cyproconazole (0.01)
(a) DAIMURON (0.01)
(a) Demeton-S-methyl (0.01)
(a) Diazinon (0.01)
(a) Dichlorvos (0.01)
(a) Diethofencarb (0.01)
(a) Diflubenzuron (0.01)
(a) Dimepiperate (0.01)
(a) Dimethametryn (0.01)
(a) Dimethenamid including other mixtures of constitue (0.01)
(a) Dimethomorph (0.01)
(a) Diniconazole (0.01)
(a) Dinotefuran (0.01)
(a) Diphenamid (0.01)
(a) DITHIOPYR (0.01)
(a) Diuron (0.01)
(a) Edifenphos (0.01)
(a) ESPROCARB (0.01)
(a) Ethaboxam (0.01)
(a) Ethiofencarb (0.01)
(a) Ethoprophos (0.01)
(a) Ethoxysulfuron (0.01)
(a) Etofenprox (0.01)
(a) Etoxazole (0.01)
(a) Etrimfos (0.01)
(a) Famoxadone (0.01)
(a) Fenamiphos (0.01)
(a) Fenarimol (0.01)
(a) Fenazaquin (0.01)
(a) Fenbuconazole (sum of constituent enantiomers) (0.01)
(a) Fenhexamid (0.01)
(a) Fenobucarb (0.01)
(a) Fenoxaprop-ethyl (0.01)
(a) Fenoxycarb (0.01)
(a) Fenpyroximate (0.01)

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(a) FENTRAZAMIDE (0.01)	(a) Ferimzone (Z) (0.01)	(a) Flonicamid (0.01)	(a) Fluacrypyrim (0.01)	(a) Flubendiamide (0.01)	(a) Flucetosulfuron (0.01)
(a) Fludioxonil (0.01)	(a) Flufenacet (0.01)	(a) Flufenoxuron (0.01)	(a) Fluopicolid (0.01)	(a) Fluquinconazole (0.01)	(a) Flusilazole (0.01)
(a) Flutolanil (0.01)	(a) Fluxapyroxad (0.01)	(a) Forchlorfenuron (0.01)	(a) Fosthiazate (0.01)	(a) Furathiocarb (0.01)	(a) Gibberellic Acid (0.01)
(a) Halosulfuron-methyl (0.01)	(a) Haloxyfop (0.01)	(a) Hexaconazole (0.01)	(a) Hexaflumuron (0.01)	(a) Hexazinone (0.01)	(a) Hexythiazox (0.01)
(a) Imazalil (any ratio of constituent isomers) (0.01)	(a) Imazosulfuron (0.01)	(a) Imicyafos (0.01)	(a) Imidacloprid (0.01)	(a) INABENFIDE (0.01)	(a) Iprobenfos (0.01)
(a) Iprovalicarb (0.01)	(a) Isoprocarb (0.01)	(a) Isoprothiolane (0.01)	(a) Isopyrazam (0.01)	(a) Kresoxim-methyl (0.01)	(a) Linuron (0.01)
(a) Lufenuron (0.01)	(a) Malathion (0.01)	(a) Mandipropamid (any ratio of constituent isomers) (0.01)	(a) Mefenacet (0.01)	(a) Mepanipyrim (0.01)	(a) Mepronil (0.01)
(a) Metalaxyl (0.01)	(a) Metamifop (0.01)	(a) Metazosulfuron (0.01)	(a) Metconazole (0.01)	(a) Methabenzthiazuron (0.001)	(a) Methiocarb (0.01)
(a) Methomyl (0.01)	(a) Methoxyfenozide (0.01)	(a) Metobromuron (0.01)	(a) Metolcarb (0.01)	(a) Metrafenone (0.01)	(a) Mevinphos (0.01)
(a) Milbemectin (sum) (0.01)	(a) Molinate (0.01)	(a) Monocrotophos (0.01)	(a) Myclobutanil (sum of constituent isomers) (0.01)	(a) Napropamide (0.01)	(a) Nicosulfuron (0.01)
(a) Novaluron (0.01)	(a) Nuarimol (0.01)	(a) Ofurace (0.01)	(a) Omethoate (0.01)	(a) Oxadiazon (0.01)	(a) Oxadixyl (0.01)
(a) Oxamyl (0.01)	(a) Oxaziclomefone (0.01)	(a) Paclobutrazol (0.01)	(a) Penconazole (sum of constituent isomers) (0.01)	(a) Pencycuron (0.01)	(a) Penoxsulam (0.01)
(a) PENTOXAZONE (0.01)	(a) Phenthoate (0.01)	(a) Phosphamidon (0.01)	(a) Phoxim (0.01)	(a) Piperophos (0.01)	(a) Pirimicarb (0.01)
(a) Pirimiphos-methyl (0.01)	(a) Probenazole (0.01)	(a) Profenofos (0.01)	(a) Propamocarb (Sum of propamocarb and its salts, exp (0.01)	(a) Propanil (0.01)	(a) Propaquizafop (0.01)
(a) Propoxur (0.01)	(a) Pyraclofos (0.01)	(a) Pyraclostrobin (0.01)	(a) PYRAZOLYNATE (0.01)	(a) Pyrazophos (0.01)	(a) Pyribenzoxim (0.01)
(a) PYRIBUTICARB (0.01)	(a) Pyridaben (0.01)	(a) Pyridaphenthion (0.01)	(a) Pyrifluquinazon (0.01)	(a) PYRIFTALID (0.01)	(a) Pyrimethanil (0.01)
(a) Pyrimidifen (0.01)	(a) Pyriminobac-methyl (0.01)	(a) Pyrimisulfan (0.01)	(a) Pyriproxyfen (0.01)	(a) Pyroquilon (0.01)	(a) Quinalphos (0.01)
(a) Quinmerac (0.01)	(a) Quinoclamine (0.01)	(a) Quizalofop ethyl (0.01)	(a) Saflufenacil (0.01)	(a) Sethoxydim (0.01)	(a) Spinetoram (sum) (0.01)
(a) Spirodiclofen (0.01)	(a) Spirotetramat (0.01)	(a) Sulfoxaflor (0.01)	(a) Tebuconazole (0.01)	(a) Tebufenozide (0.01)	(a) Tebufenpyrad (0.01)
(a) Teflubenzuron (0.01)	(a) Terbuthylazine (0.01)	(a) Tetraconazole (0.01)	(a) Thenylchlor (0.01)	(a) Thiabendazole (0.01)	(a) Thiacloprid (0.01)
(a) Thiamethoxam (0.01)	(a) THIAZOPYR (0.01)	(a) Thidiazuron (0.01)	(a) Thifensulfuron-methyl (0.01)	(a) Thiobencarb (0.01)	(a) Thiodicarb (0.01)
(a) Tiadinil (0.01)	(a) Triadimefon (0.01)	(a) Triazophos (0.01)	(a) Tricyclazole (0.01)	(a) Trifloxystrobin (0.01)	(a) Triflumizole (0.01)
(a) Triflumuron (0.01)	(a) Uniconazole (0.01)	(a) Vamidothion (0.01)			

In case of Agricultural product, performed according to the MFDS' guideline 'Korean food code, attached table 4, Pesticides MRL of each agricultural product'

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Kyuhee Oh
Technical manager

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END OF REPORT

CERTIFICATE OF ANALYSIS

PRODUCT NAME: β-agarase DagA

LOT NUMBER: 22-NBE0218

DATE MANUFACTURED 2022-02-18

Parameter	Specification	Method of analysis	Result
Activity unit, unit/ml	4.9 ~ 9.0	Food additive process, 9th, 2018, MHLW(Japan)	7.17
Total plate count	≤1,000 cfu/g	AOAC 990.12	<10
Yeast & Mold	≤ 100 cfu/g	AOAC 977.02	<10
Staphylococcus aureus	<10/g	AOAC 975.55	<10
Coliform	<10g	AOAC 991.14	<10
Escherichia coli	Absent in 25g	ISO 7251.2005	Not detected
Listeria monocytogenes	Absent in 25g	AOAC 992.19	Not detected
Salmonella	Absent in 25g	AOAC 991.13	Not detected
Arsenic (As)	≤0.1	AOAC 2013.06 / ICP-MS	<0.02
Cadmium (Cd)	≤0.1	AOAC 2013.06 /ICP-MS	<0.007
Lead (Pb)	≤0.1	AOAC 2013.06 / ICP-MS	<0.02
Mercury (Hg)	≤0.1	ASTM D-6722-01/ CV-AAS	<0.02

CONCLUSION : Qualified

22-NBE0218

Analytical Report

Analytical Report No. **AR-22-HX-007637** **Date 25-Apr-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002375		
Sample Description:	β-agarase		
Lot NO.	22NBE0218		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

			Results	Unit	Guideline
HX027	Appearance	Method: Food code, Visual examination			
	Appearance AUG		**Have own appearance without other odor and color.**		

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Kyuhee Oh Technical manager

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22-NBE0218

Analytical Report

Analytical Report No. **AR-22-HX-007492-01** **Date 22-Apr-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002354		
Sample Description:	β-agarase		
Lot NO.	22NBE0218		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	1 ml

Test Result(s):

			Results	Unit	Guidelines
HX164	β-Agarase	Method: Food additives code, MHLW(Japan), Enzymatic-spectrophotometry			
	β-Agarase		7.17	U/ml	

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Kyuhee Oh
Technical manager

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Page 1/1
AR-22-HX-010528-R2

Analytical Report

Analytical Report No. **AR-22-HX-010528-R2** Date 23-Sep-2022

(*this report cancels and replaces the previous one, numbered AR-22-HX-010528-R1/984-2022-06000027 dated 25/08/2022 which must be destroyed)

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00017885 / 984-2022-06000027		
Sample Description:	β-agarase		
Test Purpose	Voluntary testing		
Reception Date:	02-Jun-2022		
Manufacture Date	22-NBE0218		
Product Type	liquid	**Sample Weight**	100ml

Test Result(s):

		Results	Unit	Guideline
HX1EX	Coliforms count Method: AOAC 991.14, D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	**<10**	cfu/g	

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Kyuhee Oh
Technical manager

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22-NBE0218

Analytical Report

Analytical Report No. **AR-22-HX-007250-02** **Date 23-Sep-2022**

(*this report cancels and replaces the previous one, numbered AR-22-HX-007250-01/984-2022-03002379 dated 20/04/2022 which must be destroyed)

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002379		
Sample Description:	β-agarase		
Lot NO.	22NBE0218		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	Guideline:
HX0SB	Total plate count_AOAC 990.12 Method: AOAC 990.12, E-Cultural technique (media film)			
	Total plate count	**<10**	cfu/g	
HX0SC	S.aureus_AOAC 975.55 Method: AOAC 975.55, E-Cultural technique (chromogenic media)			
	Staphylococcus aureus	**<10**	cfu/g	
HX0SD	Yeast and mold_AOAC 977.02 Method: AOAC 997.02, E-Cultural technique (non-chromogenic media)			
	Yeast & mould (count)	**<10**	cfu/g	
HX0SF	L.monocytogenes_AOAC 992.19 Method: AOAC 992.19, D-Cultural technique (chromogenic media)			
	Listeria monocytogenes nn/25g	**Not Detected**	/25 g	
HX0SG	Salmonella.spp_AOAC 991.13 Method: AOAC 991.13, Biochemical tests			
	Salmonella	**Not Detected**	/25 g	
HX1BX	E.coli Method: ISO 7251:2005 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Escherichia coli	**Not Detected**	/25 g	
HX1DX	Coliforms Method: ISO 4831:2006 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	**Not Detected**	/25 g	

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Kyuhee Oh
Technical manager

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AR-22-HX-007255-02

Analytical Report

Analytical Report No. **AR-22-HX-007255-02** **Date 14-Jun-2022**

(*this report cancels and replaces the previous one, numbered AR-22-HX-007255-01/984-2022-03002377 dated 22/04/2022 which must be destroyed)

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002377		
Sample Description:	β-agarase		
Lot NO.	22NBE0218		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	Guideline
HX0SH	As (Arsenic)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS			
	Arsenic (As)	<0.02	mg/kg	
HX0SI	Cd (Cadmium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS			
	Cadmium (Cd)	<0.007	mg/kg	
HX0SJ	Pb (Lead)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-MS			
	Lead (Pb)	<0.02	mg/kg	
HX0SS	Hg (Mercury)_ASTM D-6722-01 Mod. Method: ASTM D-6722-01 mod., CV-AAS			
	Mercury (Hg)	<0.02	mg/kg	

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Kyuhee Oh
Technical manager

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AR-22-HX-013291

Analytical Report

Analytical Report No.	**AR-22-HX-013291**	**Date 06-Jul-2022**

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00018361 / 984-2022-06002006		
Sample Description:	β-agarase		
Test Purpose	Voluntary testing		
Reception Date:	16-Jun-2022		
Manufacture Date	22-NBE0218		
Product Type	liquid	**Sample Weight**	100ml

Test Result(s):

			Results	Unit	uidelines
HX18X	Ash	Method: AOAC 923.03, Gravimetry			
	Ash		**0.15**	%	
HX1J1	Moisture	Method: AOAC 935.29, Gravimetry			
	Moisture		**98.46**	%	

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Kyuhee Oh
Technical manager

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EK-FM-QP-1609(3)r01 2020.06.15(REV.01)

22-NBE0218

Analytical Report

Analytical Report No. **AR-22-HX-007194-01** Date 20-Apr-2022

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002376		
Sample Description:	β-agarase		
Lot NO.	22NBE0218		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

	Results	Unit	Guidelines
HX0SK Mg (Magnesium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Magnesium (Mg)**	69.6	mg/100 g	
HX0SL Ca (Calcium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Calcium (Ca)**	<20	mg/100 g	
HX0SM Fe (Iron)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Iron (Fe)**	<2	mg/100 g	
HX0SN Zn (Zinc)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Zinc (Zn)**	<2	mg/100 g	
HX0SP Na (Sodium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Sodium (Na)**	31.5	mg/100 g	
HX0SQ K (Potassium)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Potassium (K)**	62.9	mg/100 g	
HX0SR P (Phosphorus)_AOAC 2013.06 Mod. Method: AOAC 2013.06 mod., ICP-OES **Phosphorus (P)**	7.35	mg/100 g	

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22-NBE0218

Analytical Report

Analytical Report No. **AR-22-HX-007046-01** **Date 19-Apr-2022**

Dynebio INC
B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014586 / 984-2022-04000117		
Sample Description:	β-agarase		
Lot NO.	22NBE0218		
Reception Date:	04-Apr-2022		
Sample Weight	600 ml		
Product Type	liquid	**Test Purpose**	Voluntary testing

Test Result(s):

			Results	Unit	LOQ Guidelines
HX01G	Pesticides Screening	Method: Food code, GC-MS/MS			
	Screened pesticides		Not Detected		
HX01H	Pesticides Screening	Method: Food code, LC-MS/MS			
	Screened pesticides		Not Detected		

List of screened and not detected molecules (* = limit of quantification)

HX01G **Pesticides Screening** (LOQ* mg/kg)

(a) Acrinathrin (0.01)	(a) Alachlor (0.01)	(a) Aldrin (0.01)	(a) Ametoctradin (0.01)	(a) Anilofos (0.01)	(a) Azaconazole (0.01)
(a) Benfuresate (0.01)	(a) Bifenox (0.01)	(a) Bifenthrin (0.01)	(a) BROMOBUTIDE (0.01)	(a) Bromopropylate (0.01)	(a) Butachlor (0.01)
(a) Butafenacil (0.01)	(a) Carbophenothion (0.01)	(a) Chlorantraniliprole (0.01)	(a) Chlordane (0.01)	(a) Chlorfenapyr (0.01)	(a) Chlorfenvinphos (0.01)
(a) Chlorfluazuron (0.01)	(a) Chlorobenzilate (0.01)	(a) Chlorpropham (0.01)	(a) Chlorpyrifos-methyl (0.01)	(a) Cyfluthrin (0.01)	(a) Cyhalothrin (0.01)
(a) Cypermethrin (sum of isomers) (0.01)	(a) Cyprodinil (0.01)	(a) DDT (total) (0.01)	(a) Deltamethrin (0.01)	(a) Diclofop-methyl (0.01)	(a) Dicloran (0.01)
(a) Dicofol, p.p- (0.01)	(a) Dieldrin (0.01)	(a) Difenoconazole (0.01)	(a) Dimethoate (0.01)	(a) Dimethylvinphos (0.01)	(a) Diphenylamine (0.01)
(a) Disulfoton (0.01)	(a) Endosulfan (total) (0.01)	(a) Endrin (0.01)	(a) EPN (0.01)	(a) Epoxiconazole (0.01)	(a) Ethalfluralin (0.01)
(a) Ethion (0.01)	(a) Etridiazole (0.01)	(a) Fenclorim (0.01)	(a) Fenitrothion (0.01)	(a) Fenothiocarb (0.01)	(a) Fenoxanil (0.01)
(a) Fenpropathrin (0.01)	(a) Fenthion (0.01)	(a) Fenvalerate (0.01)	(a) Fipronil (0.01)	(a) Flucythrinate (0.01)	(a) Flumioxazin (0.01)
(a) Fluopyram (0.01)	(a) Fonofos (0.01)	(a) Halfenprox (0.01)	(a) HCH (sum) (0.01)	(a) Heptachlor/Heptachloroepoxide (0.01)	(a) Imibenconazole (0.01)
(a) Indanofan (0.01)	(a) Indoxacarb (sum, R+S isomers) (0.01)	(a) Iprodione (0.01)	(a) Isazophos (0.01)	(a) Isofenphos (0.01)	(a) Lindane (gamma-HCH) (0.01)
(a) Mecarbam (0.01)	(a) Methidathion (0.01)	(a) Metolachlor (0.01)	(a) Metribuzin (0.01)	(a) Oxyfluorfen (0.01)	(a) Parathion-ethyl (0.01)
(a) Parathion-methyl (0.01)	(a) Pendimethalin (0.01)	(a) Penthiopyrad (0.01)	(a) Permethrin (sum of isomers) (0.01)	(a) Phenothrin (0.01)	(a) Phorate (0.01)
(a) Phosalone (0.01)	(a) Phthalide (0.01)	(a) Picoxystrobin (0.01)	(a) Piperonyl butoxide (0.01)	(a) Pirimiphos-ethyl (0.01)	(a) Pretilachlor (0.01)
(a) Prochloraz (0.01)	(a) Procymidone (0.01)	(a) Promecarb (0.01)	(a) Prometryn (0.01)	(a) Propachlor (0.01)	(a) Propazine (0.01)
(a) Propiconazole (sum of isomers) (0.01)	(a) propisochlor (0.01)	(a) Propyzamide (0.01)	(a) Prothiofos (0.01)	(a) Pyridalyl (0.01)	(a) Quintozene (0.01)
(a) Silafluofen (0.01)	(a) Simazine (0.01)	(a) Simeconazole (0.01)	(a) Simetryn (0.01)	(a) Spiromesifen (0.01)	(a) Tebupirimfos (0.01)
(a) Tefluthrin (0.01)	(a) Terbufos (0.01)	(a) Terbutryn (0.01)	(a) Tetradifon (0.01)	(a) Thifluzamide (0.01)	(a) Tolclofos-methyl (0.01)
(a) Triadimenol (0.01)	(a) Tri-allate (0.01)	(a) Trifluralin (0.01)	(a) Vinclozolin (0.01)	(a) Zoxamide (0.01)	

In case of Agricultural product, performed according to the MFDS' guideline 'Korean food code, attached table 4, Pesticides MRL of each agricultural product'

HX01H **Pesticides Screening** (LOQ* mg/kg)

(a) Abamectin (0.01)	(a) Acephate (0.01)	(a) Acetamiprid (0.01)	(a) Aldicarb (0.01)	(a) Amisulbrom (0.01)	(a) Azimsulfuron (0.01)
(a) Azinphos-methyl (0.01)	(a) Azoxystrobin (0.01)	(a) Bendiocarb (0.01)	(a) Bensulfuron methyl (0.01)	(a) Benthiavalicarb, isopropyl- (0.01)	(a) Benzobicyclon (0.01)
(a) Benzoximate (0.01)	(a) Bitertanol (0.01)	(a) Boscalid (0.01)	(a) Bromacil (0.01)	(a) Buprofezin (0.01)	(a) Cadusafos (0.01)
(a) Cafenstrole (0.01)	(a) Carbaryl (0.01)	(a) Carbendazim (0.01)	(a) Carbofuran (0.01)	(a) Carboxin (0.01)	(a) Carfentrazone-ethyl (0.01)
(a) Carpropamid (0.01)	(a) Chlorpyrifos (0.01)	(a) Chlorsulfuron (0.01)	(a) Chromafenozide (0.01)	(a) Clethodim (0.01)	(a) Clofentezine (0.01)
(a) Clomazone (0.01)	(a) Clothianidin (0.01)	(a) Cyazofamid (0.01)	(a) Cyclosulfamuron (0.01)	(a) Cyflufenamid (0.01)	(a) Cyhalofop-butyl (0.01)
(a) Cymoxanil (0.01)	(a) Cyproconazole (0.01)	(a) DAIMURON (0.01)	(a) Demeton-S-methyl (0.01)	(a) Diazinon (0.01)	(a) Dichlorvos (0.01)
(a) Diethofencarb (0.01)	(a) Diflubenzuron (0.01)	(a) Dimepiperate (0.01)	(a) Dimethametryn (0.01)	(a) Dimethenamid including other mixtures of constitue (0.01)	(a) Dimethomorph (0.01)
(a) Diniconazole (0.01)	(a) Dinotefuran (0.01)	(a) Diphenamid (0.01)	(a) DITHIOPYR (0.01)	(a) Diuron (0.01)	(a) Edifenphos (0.01)
(a) ESPROCARB (0.01)	(a) Ethaboxam (0.01)	(a) Ethiofencarb (0.01)	(a) Ethoprophos (0.01)	(a) Ethoxysulfuron (0.01)	(a) Etofenprox (0.01)
(a) Etoxazole (0.01)	(a) Etrimfos (0.01)	(a) Famoxadone (0.01)	(a) Fenamiphos (0.01)	(a) Fenarimol (0.01)	(a) Fenazaquin (0.01)
(a) Fenbuconazole (sum of constituent enantiomers) (0.01)	(a) Fenhexamid (0.01)	(a) Fenobucarb (0.01)	(a) Fenoxaprop-ethyl (0.01)	(a) Fenoxycarb (0.01)	(a) Fenpyroximate (0.01)

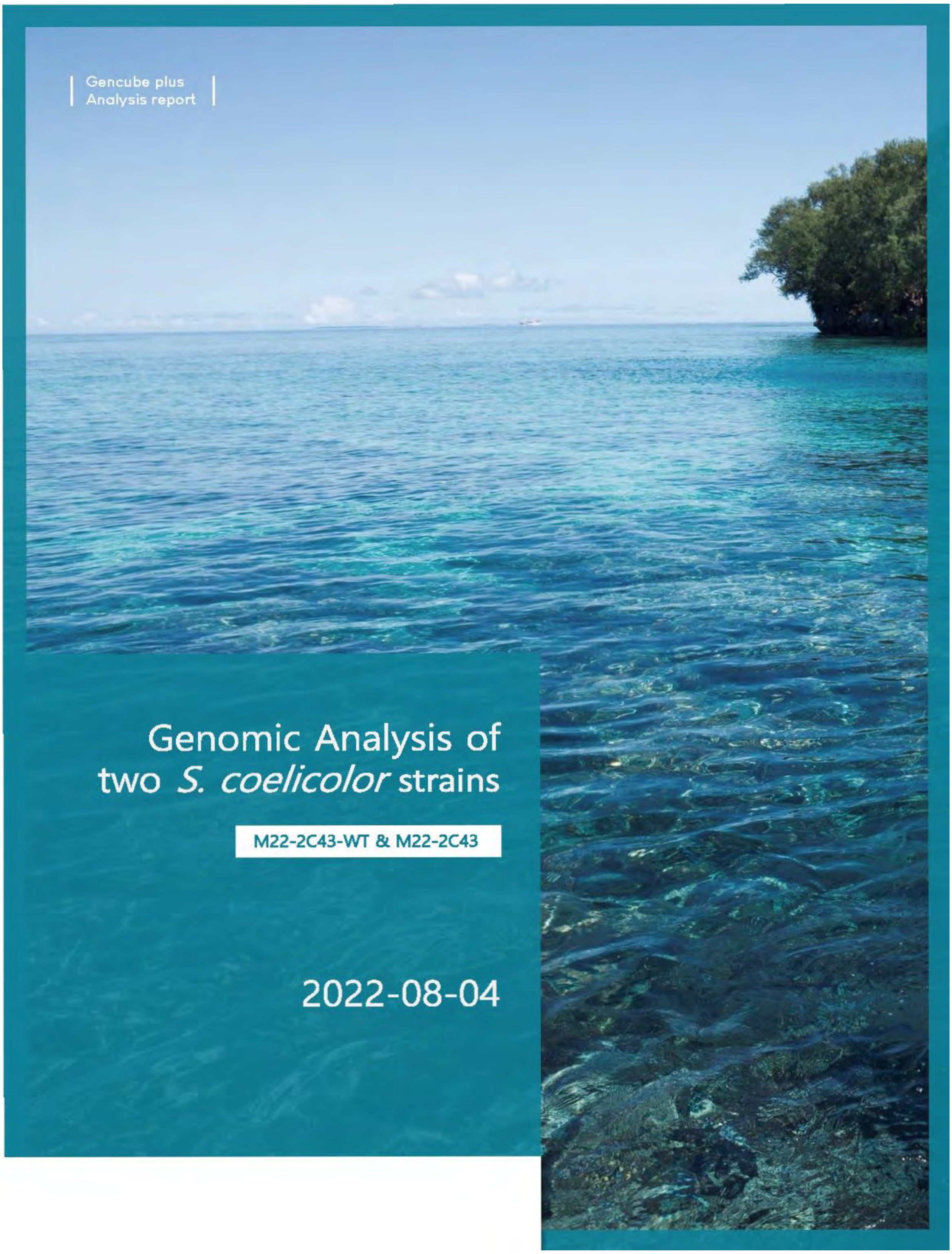
This test report is not related to accreditation by Korea Laboratory Accreditation Scheme and ISO/IEC 17025.

DETERMINATION OF
THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF β-agarase DagA
AS A PROCESSING AID

Part B: Appendices C to F

Appendix C. Genomic Analysis of Two *Streptomyces coelicolor* Strains

(*S. coelicolor* A3(2) and its UV-treated mutant A3(2) M22-2C43 strains, which are abbreviated as M22-2C43-WT and M22-2C43, respectively)



Gencube plus
Analysis report

Genomic Analysis of
two *S. coelicolor* strains

M22-2C43-WT & M22-2C43

2022-08-04

Author: Woori Kwak

Institution: Gencube Plus, Seoul, 08592, Korea

Date: August 04, 2022

1. Abstract

In this analysis, two complete genomes of the *Streptomyces coelicolor* strains (*S. coelicolor* A3(2) M22-2C43 WT and its UV-treated mutant A3(2) M22-2C43 strains, which are abbreviated as M22-2C43-WT and M22-2C43, respectively) were determined, and the potential risk of the M22-2C43 strain was evaluated based on its genome information. Dyne Bio Inc. obtained M22-2C43-WT strain which was acknowledged as *S. coelicolor* A3(2) from the John Innes Foundation, United Kingdom. The M22-2C43-WT genome had an 8,668,266 bp linear genome, and the M22-2C43 genome had a 7,438,186 bp circular genome. Comparative genome analysis between the WT and mutated (via adaptive laboratory evolution) strains revealed that both ends of the WT genome were deleted. Two large structural variations (1 deletion and 1 insertion) and 29 genome modifications (2 small insertions, 22 substitutions, and 5 small deletions) were observed in the mutant strain. The safety of the M22-2C43 strain was evaluated through four bioinformatic analyses to identify antibiotics resistance genes, virulence factor genes, allergen genes, and biosynthetic gene clusters.

The analyses concluded that the M22-2C43 strain did not have any virulence factors and potential allergens. It also does not contain biosynthetic gene clusters for producing widely used antibiotics. In the case of anti-microbial resistance (AMR) genes, the M22-2C43 strain was confirmed to have three antibiotic resistance genes (dldHA2X, erm(O), tet) from the ResFinder analysis. However, comparative AMR gene analysis of *S. coelicolor* strains indicated that the genomes of all known *S. coelicolor* strains also contain these three genes. Therefore, these genes might be considered as intrinsic genes of the *S. coelicolor* strains. Additional safety-related analyses (virulence factor, allergen, and BGC) did not find any known genomic contents related to the safety in the genome of the *S. coelicolor* M22-2C43 strain.

Based on all the analysis results of *S. coelicolor* A3(2) M22-2C43, no specific issue related to the safety of this strain was observed, and it can be judged that there is no critical reason to limit the use of this strain.

1.1 Project Information

Sample Information

Name	M22-2C43-WT and M22-2C43
Species	*Streptomyces coelicolor*
Sample Type	Pallet
Total Count	2

Customer Information

Name	Je Hyeon Lee
Species	Dyne Bio Inc.

Data Information

Platform	NanoporeFlongle, Minion
Read type	Single-end, Paired-end
Read Length	Long and Short Hybrid

1.2 Research Workflow

Data Generation Workflow

1) DNA Extraction
OMEGA bio: Mag-Bind Universal Pathogen Kit

↓

2) Library Construction
Nanopore Library Construction: Oxford Nanopores SQK-LSK09
Illumina Library Construction: TruSeq Nano DNA Sample Preparation Kit

↓

3) Sequencing Flow Cell
Oxford nanopore Flow Cell: FLO-FLG001 Illumina
Miseq Flow Cell

↓

4) Sequencing
Oxford Nanopore MinKNOW 4.5.5 with basecalling
Illumina 300 bp paired-end mode

Genome Construction Workflow

1) Basecalling
Guppy_basecaller v 6.0.1: Basecalling and Adapter Trimming

2) Genome Assembly
Canu v 2.1.1: Microbial Genome Assembly

3) Genome Polishing
Medaka v 1.3.3: polishing genome using read
Homopolish v 0.3.3: polishing genome using homologous

4) Genome Evaluation
BUSCO v 5.1.1: Genome assembly evaluation using
Single Copy Orthologous gene

5) Annotation
Prokka v .14.6: Automated genome assembly pipeline for prokaryote

Genome Analysis Workflow

1) Genome Modification
GSalign v 1.0.22: Genome to genome alignment

2) AMR gene Analysis
Staramr: offline version of ResFinder from Center for Genomic Epidemiology (CGE)
(heep://cge. cbs.dtu.dk/services/ResFinder/)

3) Virulence Factor Analysis
CFDB full set: Database for known virulence factor protein
Diamond: Sequence similarity search
VirulenceFinder 2.0: Virulence analysis program of CGE
(http://cge.cbs.dtu.dk/services/VirulenceFinder/)

4) Allergen Analysis
COMPARE database: Database for known allergen
Diamond: Sequences similarity search

5) BGCs Analysis
antiSMASH v6: Identify biosynthetic gene clusters in the genome

2. Analysis Methods

2.1. Whole Genome Sequencing and Genome Construction

The genomic DNAs of the *S. coelicolor* strains (M22-2C43-WT and M22-2C43) were extracted using Omega bio Mag-Bind Universal Pathogen Kit following the manufacturer's protocol. Sequencing libraries for Nanopore Flongle and Illumina Miseq were constructed using the Nanopore SQK-LSK109 kit and the Illumina TruSeq Nano DNA Sample Preparation Kit, respectively. Three hundred bp paired-end sequencing was conducted using Illumina Miseq. Basecalling for the generated signal from Nanopore was conducted using guppy v6.0.6 with a high-accuracy model and CUDA acceleration. Quality pass reads from guppy were assembled using canu v2.1.1 [1] with the default parameters and appropriate genome size of the strain. For the analysis of the genome sequence of the M22-2C43 strain, which has a circular chromosome, the constructed genome sequence was manually trimmed using circular suggestion information from the canu assembler. To obtain a more accurate genome sequence, hybrid polishing was conducted using Nanopore long reads and Illumina short reads. Using the Nanopore reads, medaka v1.6.1 was used for initial polishing with a high accuracy calling model. After medaka polishing, Homopolish v.0.3.3 [2] was used for a second-level polishing with -m R9.4.pkl parameter. The final polishing process was conducted using pilon v.1.24 [3]. For the pilon polishing process, the sequencing artifacts and low-quality bases from Illumina Miseq were removed using Trimmomatic v0.39 [4] with the ILLUMINACLIP:TruSeq3-PE.fa:2:30:10:2:True LEADING:5 TRAILING:20 MINLEN:250 parameter. QC passed reads were mapped to the assembled genome using Bowtie 2 [5] with the no-mixed option (only proper pair read mapping) for more reliable read mapping. Generated bam files were used as inputs of the pilon polishing. After the final polishing, genome orientation and direction were identified using the ACT program v17.0.1 [6] in the Artemis program, and the complete genome of the *S. coelicolor* A3(2) M22-2C43 strain was manually finalized. Gene prediction and annotation of the determined genome sequences were conducted using Prokka v1.14.6 [7] with the default option, and the closest neighbor of the assembled genome was identified using the JSpeciesWS system. Genome assembly completeness was evaluated using BUSCO v5 [8] with streptomycetales_odb10.

2.2 Comparative Genome Analysis

To identify the sequence changes in the genome of the M22-2C43 strain as compared to the M22-2C43-WT strain during the ALE process, GSalign v1.0.22 [9], which allows sequence alignment and comparison of genome sequences, was used with the default option. The assembled genome sequence of the M22-2C43 strain was mapped to the genome sequence of the parental strain (M22-2C43-WT). Large genomic variants, which were not identified by GSalign, were manually identified based on the ACT analysis information. OrthoVenn2 [10] was used for gene content comparison.

2.3 AMR Gene Analysis

AMR gene analysis was conducted using staramr v0.7.2 [11] with the 2021 EFSA database (2022-05-24) [12]. Staramr is a convenient local version of ResFinder from CGE (https://cge.cbs.dtu.dk). The detection parameters were set to the same criteria as the default ResFinder EFSA criteria (Identify > 90% and minimum subject coverage > 60%) using ResFinder 60 parameters. The web-version of ResFinder EFSA was also employed with the default option except for the select species option (Other) to predict the phenotypic antibiotics resistance.

2.4 Virulence Factor Gene Analysis

Analysis of known virulence factors of *S. coelicolor* A3(2) M22-2C43 was identified using the virulence factor database from VFDB [13]. The full set protein sequences of VFDB (2022-06-29) were downloaded, and the protein sequences of *S. coelicolor* A3(2) M22-2C43 from the Prokka annotation were matched to VFDB using DIAMOND [14] with -e^0.00001, --max-target-seqs 1 parameter. The matched results were filtered using universal criteria of AMR gene detection used in CGE to remove false positives. Identity > 90% and subject coverage > 60% were used for filtering DIAMOND Blastp result. In addition, VirulenceFinder 2.0 [15] was also used to confirm the analysis with default parameters.

2.5 Allergen Gene Analysis

To identify the genes which might cause allergenic responses, COMprehensive Protein Allergen REsource database (COMPARE, https://comparedatabase.org/) [16] was used. Allergen protein sequences (2022-01-26) were downloaded, and DIAMOND Blastp search was conducted while virulence factor genes were searched. The resulting alignments were also filtered using the same criteria (identity > 90% and min subject coverage 60%), which was used for identifying virulence factor genes.

2.6 Biosynthetic Gene Clusters (BCG) Analysis

To identify the genes capable of producing antibiotics and secondary metabolites of the *S. coelicolor* A3(2) M22-2C43 strain, antiSMASH v6.0 (https://antismash.secondarymetabolites.org) [17] was used for the analysis. Detection strictness was set to strict and relaxed.

3. Results

3.1. Assembled Genome Information (M22-2C43-WT)

Sequence Filename

WT_pilon.fasta

Sequence lengths

8,668,266 (bp) – 1 Chromosome

Residue Counts

Number of A’s	1,213,364	14.00%
Number of C’s	3,130,174	36.11%
Number of G’s	3,121,255	36.01%
Number of T’s	1,203,473	13.88%
Number of N’s	-	-
Total	8,668,266	

3.2. Assembled Genome Information (M22-2C43)

Sequence Filename

ALE_pilon.fasta

Sequence lengths

7,438,186 (bp) – 1 Chromosome

Residue Counts

Number of A’s	1,041,207	14.00%
Number of C’s	2,691,359	36.18%
Number of G’s	2,675,749	35,97%
Number of T’s	1,029,871	13.85%
Number of N’s	-	-
Total	7,438,186	

3.3. BUSCO Evaluation Result

Used Database

Streptomycetales_odb10

Total Summary

	M22-2C43-WT	M22-2C43
Complete Single	1,565	1,557
Complete duplicated	9	8
Fragmented	2	2
Missing	3	12
Total Completeness	1,574(99.7%)	1,565(99.1%)

3.4. Complete Genome Sequence of *S. coelicolor* A3(2) M22-2C43

The complete genomes of the two strains (M22-2C43-WT and M22-2C43) were successfully constructed using Nanopore sequencing. Table 1 shows the overview assembly statistics of two assemblies based on the genome sequence of *S. coelicolor* A3(2), the closest reference genome of the M22-2C43 strain. Both the assembled genomes contained one chromosome, and they did not contain any additional plasmid genomes. However, the topology of the two genomes was different. The M22-2C43-WT strain had a linear genome, whereas the M22-2C43 strain had a circular genome. Approximately 1.2 Mb genomic region was deleted in the M22-2C43 strain, and the linear genome is expected to be circularized during the ALE process. To confirm the species name of the assembled genome, tetra correlation search was conducted using JSpeciesWS. The tetra correlation search identified that the closest genome with the M22-2C43-WT and M22-2C43 strains was *S. coelicolor* A3(2) (Z-Score of 0.99993 and 099975, respectively). ANIb result showed that the two genomes showed 100% ANIb value to *S. coelicolor* A3(2) strain (one direction).

Table 1. Whole genome sequence overview of two strains with reference genome

Strain	M22-2C43-WT	M22-2C43	A3 (2)
Specie Name	*Streptomyces coelicolor*		
NCBI Taxonomy ID	1902		
Domain	Bacteria		
Taxonomy	Bacteria; Terrabacteria group; Actinobacteria; Actinobacteria; Streptomycetales; Streptomycetaceae; Streptomyces; Streptomyces albidoflavus group; *Streptomyces coelicolor*		
Genome Size (bp)	8,668,266	7,438,186	8,667,507
GC content in the DNA	72.12 mol% G+C	72.15 mol% G+C	72.12 mol% G+C
Number of Genome Sequences	1 Linear (Single chromosomal DNA without plasmid)	1 Circular (Single chromosomal DNA without plasmid)	1 Linear (Single chromosomal DNA with 2 plasmids)
Number of Plasmids	0	0	2

Number of Coding Sequences	7,711	6,604	7,711
Number of RNAs rRNA(tRNA)	18 (88)	18 (84)	18 (88)
Homology with *S. coelicolor* A3 (2) by JSpeciesWS (ANIb)	100%	100%	-

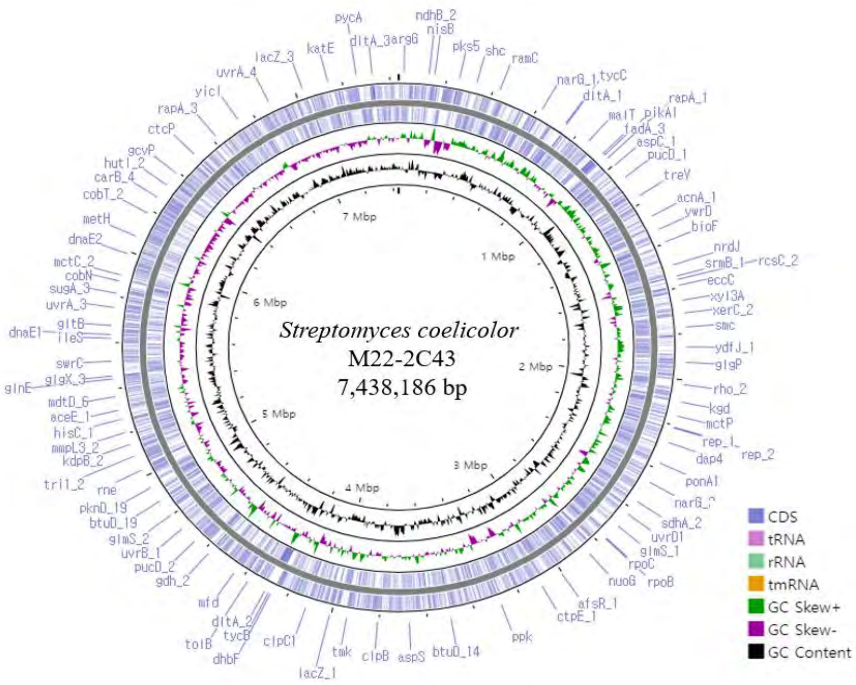


Figure 1. Genome map of *S. coelicolor* M22-2C43

Figure 1 shows the circular genome map of the M22-2C43 strain, and Table 1 shows the summary statistics of gene annotation using Prokka. Because of the large deletion (approximately 1.2 Mb) in the M22-2C43 strain, the number of annotated genes in the M22-2C43 genome was smaller than the M22-2C43-WT strain. As a result, 1,107 coding sequences and 4 tRNAs were not found in the genome of the M22-2C43 strain as compared to that of the M22-2C43-WT strain. However, the number of rRNAs remained the same.

3.5. Comparative Genome Structure between WT and ALE Strains

S. coelicolor A3(2) M22-2C43 is an ALE strain of M22-2C43-WT, and its genome was expected to be circularized during the ALE process. To identify the genome modification during the ALE process, genome to genome comparison was conducted using ACT included in the Artemis program. Figure 2 shows the genome alignment between the wild-type and ALE strains.

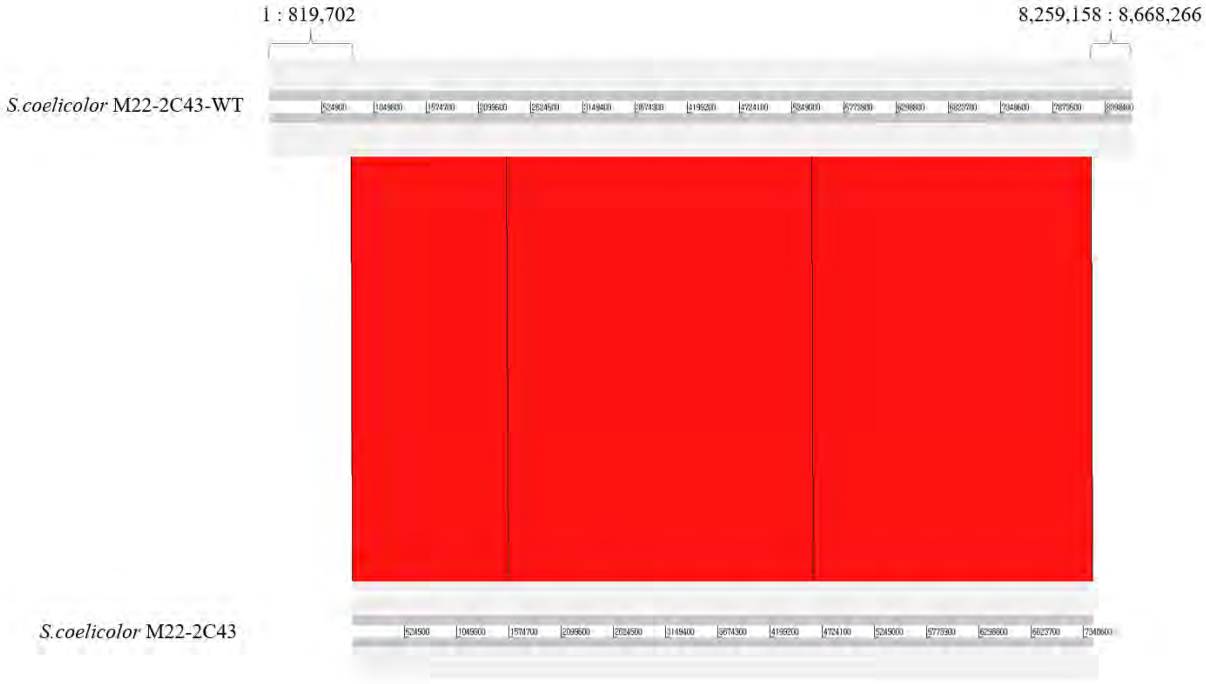


Figure 2. Visualized genome alignment of two strains using ACT

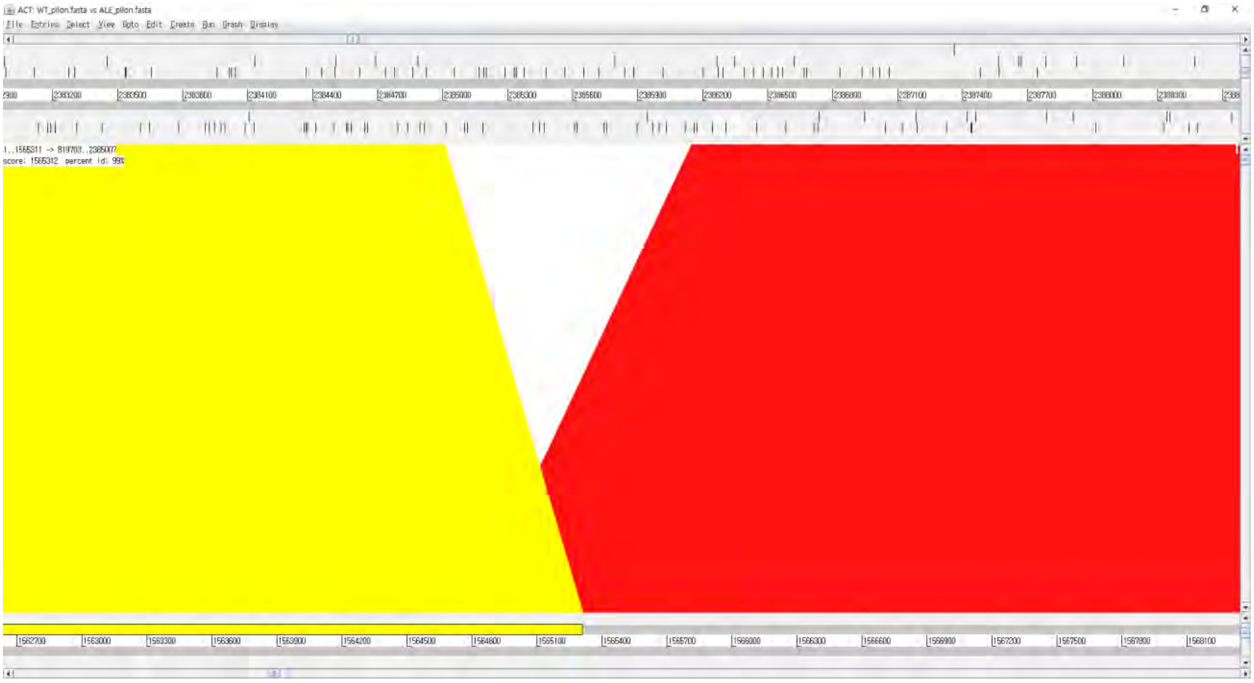
3.6. Comparative Genome Analysis between Two Strains

Genome to genome alignment using ACT identified that both ends of the linear genome of the M22-2C43-WT strain were deleted in the circularized genome of the M22-2C43 strain. Divided alignment blocks also showed that several genomic variants also exist in the genome of the ALE strain (M22-2C43). To identify the detailed genome modification during the ALE process, the genome sequence of the ALE strain was aligned to the WT genome using GSalign. GSalign identified a total of 29 genome modifications including 2 insertions, 22 substitutions, and 5 deletions. Details of the modified genome information are shown in Table 2. As Gsalign cannot identify the large structural variation, candidates of large structural variants were identified in the ACT alignment manually by mapping short reads from the M22-2C43 to M22-2C43-WT genomes (Figure 3-6). The genome of the ALE strain contained a large insert of “CTCGGTG” motif repeatedly like the known *S. coelicolor* genome A3(2).

Table 2. Identified genome modification in M22-2C43 strain during ALE process

Position	M22-2C43-WT	M22-2C43	Type
905,148	T	TCCAGGT	Insertion
1,965,208	G	C	Substitution
2,017,567	T	C	Substitution
2,195,934	T	A	Substitution
3,017,572	A	C	Substitution
3,160,480	G	A	Substitution
3,501,695	T	A	Substitution
3,578,704	G	A	Substitution
3,747,815	G	T	Substitution
4,135,838	GCG	G	Deletion
4,135,850	AC	A	Deletion
4,139,966	A	C	Substitution
4,164,504	G	C	Substitution
4,171,818	T	C	Substitution
4,184,603	A	C	Substitution
4,184,678	T	C	Substitution
4,217,212	G	A	Substitution
4,816,001	C	G	Substitution
4,825,130	G	T	Substitution
4,879,611	C	T	Substitution
5,370,317	GC	G	Deletion
5,453,740	G	C	Substitution
5,667,409	C	A	Substitution
5,838,792	G	GG	Insertion
5,838,793	C	T	Substitution
6,205,337	CCC	C	Deletion

6,365,139	A	G	Substitution
7,640,134	T	A	Substitution
8,203,175	CC	C	Deletion



```
1..1565311 -> 819703..2385007
score: 1565312  percent id: 99%
```

Figure 3. Large deletion in M22-2C43 during ALE process. (2,385,008 : 2,386,149)

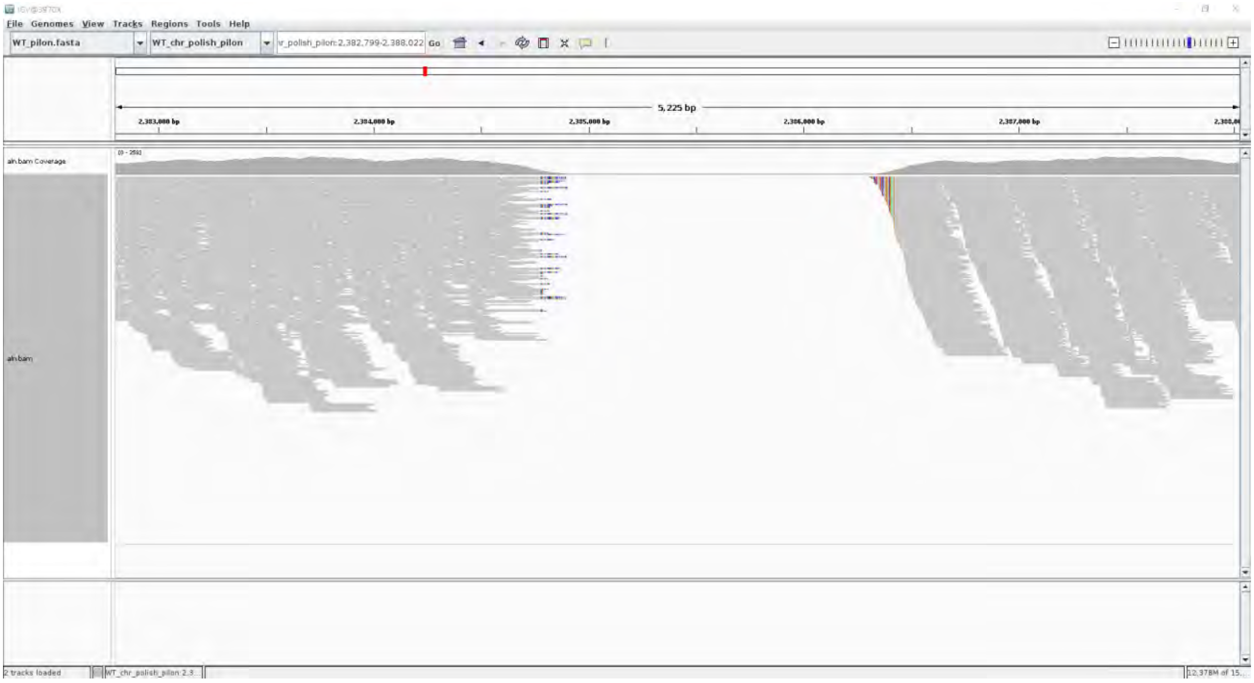
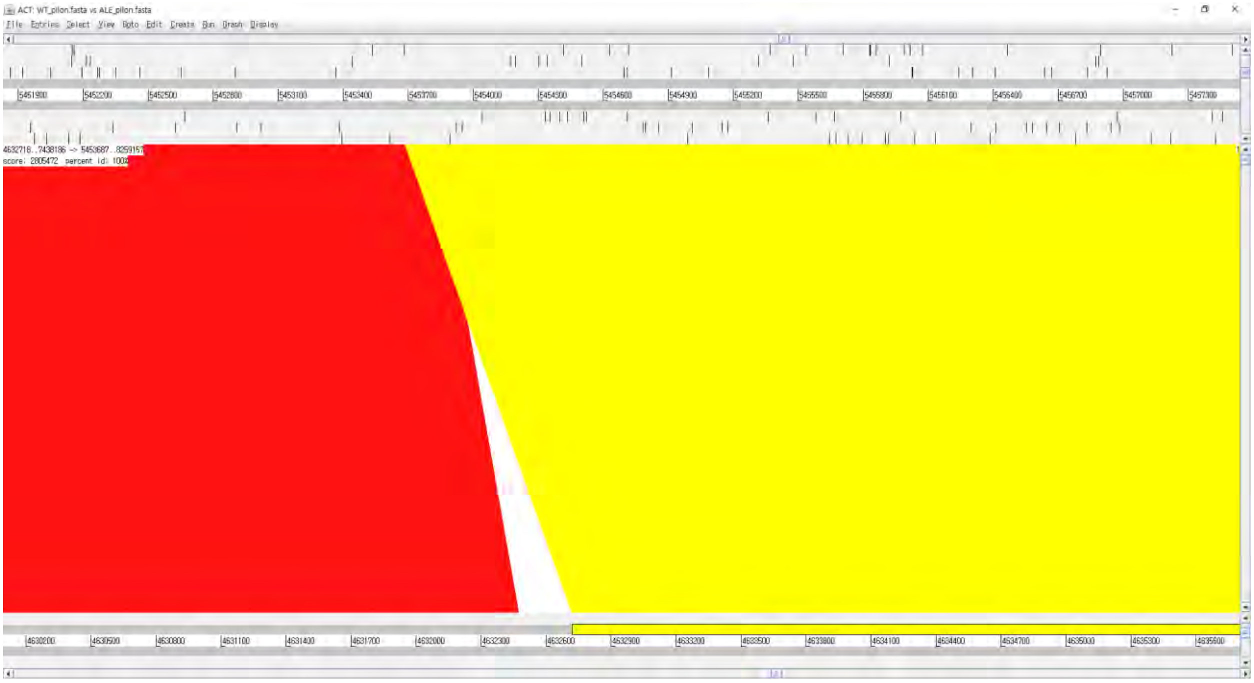


Figure 4. Short read mapping confirmation on the large deletion region



4632718..7438186 -> 5453687..8259157
score: 2805472 percent id: 100%

Figure 5. Large insertion in M22-2C43 during ALE process (4,632,473 : 4,632,717)

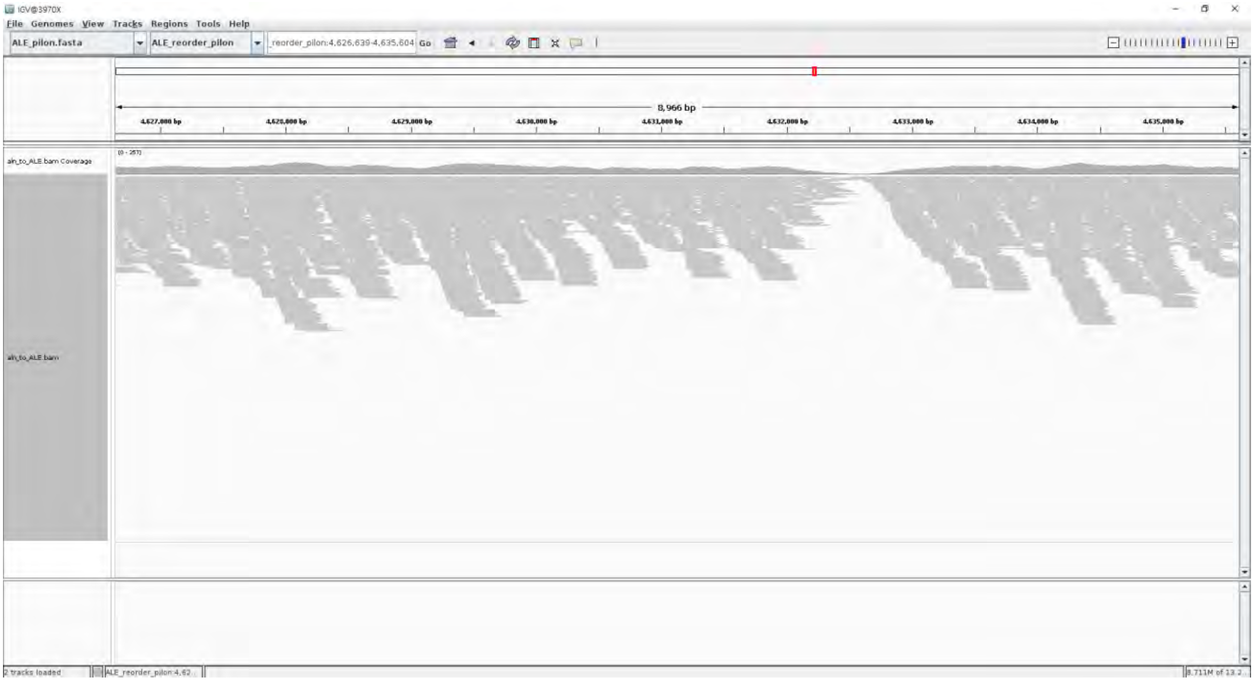
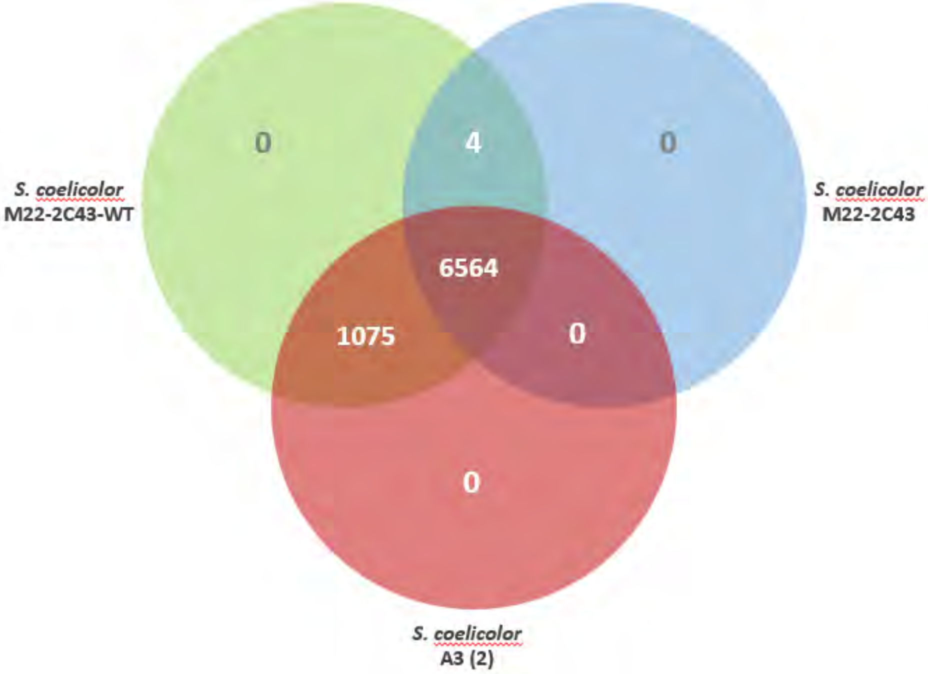


Figure 6. Short read mapping confirmation on the large deletion region

To compare the specific gene contents and their functions of the M22-2C43-WT and M22-2C43 strains with a reference strain of *S. coelicolor* A3 (2), OrthoVenn2 was employed. Protein sequences from the Prokka annotation of the three genomes were compared. Figure 7 shows the result of the gene cluster comparison among the three strains.



Comparative gene cluster analysis using OrthoVenn2 showed that only 4 gene clusters were unique in the constructed genomes (M22-2C43-WT and M22-2C43) as compared to the genome of *S. coelicolor* A3(2). OrthoVenn2 analysis also identified singleton genes in each strain (M22-2C43-WT: 3, M22-2C43: 5, A3(2): 9).

Table 3 shows the details genes of the unique clusters of the M22-2C43-WT and M22-2C43 strains, and the singletons genes in M22-2C43-WT and M22-2C43 were shown in Table 4 and Table 5, respectively.

Table 3. Genes in 4 unique gene clusters of M22-2C43-WT and M22-2C43 strains

Cluster	M22-2C43-WT	M22-2C43	Gene Symbol	Annotation
Cluster6602	JGJLPFEM_04406	LDEOFFGE_03641	-	Hypothetical Protein
Cluster6710	JGJLPFEM_07227	LDEOFFGE_06460	-	Hypothetical Protein
Cluster6711	JGJLPFEM_05236	LDEOFFGE_04469	-	Hypothetical Protein
Cluster6712	JGJLPFEM_02182	LDEOFFGE_01419	-	Hypothetical Protein

Table 4. Identified unique genes in the M22-2C43-WT genome using OrthoVenn2

Gene ID	Gene Symbol	Annotation
JGJLPFEM_03527	-	Hypothetical Protein
JGJLPEM_04717	-	Hypothetical Protein
JGJLPFEM_07627	-	Hypothetical Protein

Table 5. Identified unique genes in the M22-2C43 genome using OrthoVenn2

Gene ID	Gene Symbol	Annotation
LDEOFFGE_00155	-	Hypothetical Protein
LDEOFFGE_00664	-	Hypothetical Protein
LDEOFFGE_03008	IS5-	IS5 family transposase ISSgr2
LDEOFFGE_03879		Hypothetical Protein
LDEOFFGE_06651		Hypothetical Protein

No specific gene ontology function of the unique clusters and genes of the M22- 2C43-WT and M22-2C43 strains were identified in the comparative genome analysis using OrthoVenn2. It is because all the unique genes were hypothetical proteins except one in the M22-2C43-WT and M22-2C43 strains. The only gene identified for its function was the IS5 family transposase ISSgr2. The IS5 gene has not been reported to be related to the pathogenicity of bacteria.

Overall, the genome strains constructed in this analysis did not have any additional functional gene clusters compared to the reference strain, A3(2). These strains have no additional genes that might cause safety issues compared to the reference genome, *S. coelicolor* A3(2).

To identify the deleted genes during the ALE process, the gene ontology biological process of the deleted genes in the ALE strain was identified. Table 6 shows the GO terms related to the deleted genes in the genome of the ALE strain.

Table 6. Biological process related to the deleted gene in the ALE strain using OrthoVenn2

Slimmed_GO	Biological Process	Gene Count
GO:0008152	metabolic process	165
GO:0008150	biological process	158
GO:0044237	cellular metabolic process	136
GO:0006807	nitrogen compound metabolic process	73
GO:0009987	cellular process	69
GO:0044238	primary metabolic process	60
GO:0006725	cellular aromatic compound metabolic process	58
GO:0043170	macromolecule metabolic process	56
GO:0046483	heterocycle metabolic process	48
GO:0006082	organic acid metabolic process	44
GO:0006139	nucleobase-containing compound metabolic process	43
GO:0065007	biological regulation	38
GO:0006810	transport	33
GO:0016070	RNA metabolic process	33
GO:0051234	establishment of localization	33
GO:0005975	carbohydrate metabolic process	31
GO:0050896	response to stimulus	22
GO:0006629	lipid metabolic process	21
GO:0044255	cellular lipid metabolic process	18

GO:0005976	polysaccharide metabolic process	15
GO:0051186	cofactor metabolic process	15
GO:0006793	phosphorus metabolic process	12
GO:0006805	xenobiotic metabolic process	12
GO:0006464	cellular protein modification process	8
GO:0009117	nucleotide metabolic process	7
GO:0017144	drug metabolic process	7
GO:0006811	ion transport	6
GO:0019748	secondary metabolic process	6
GO:0032502	developmental process	6
GO:0051704	multi-organism process	6
GO:0006066	alcohol metabolic process	5
GO:0006508	proteolysis	5
GO:0006518	peptide metabolic process	5
GO:0032196	transposition	5
GO:0042180	cellular ketone metabolic process	5
GO:0042440	pigment metabolic process	5
GO:0006091	generation of precursor metabolites and energy	4
GO:0019538	protein metabolic process	4
GO:0043603	cellular amide metabolic process	4
GO:0051179	localization	4
GO:0006259	DNA metabolic process	3
GO:0006865	amino acid transport	3
GO:0007154	cell communication	3
GO:0009116	nucleoside metabolic process	3
GO:0006081	cellular aldehyde metabolic process	2
GO:0006766	vitamin metabolic process	2
GO:0008643	carbohydrate transport	2
GO:0015031	protein transport	2
GO:0016043	cellular component organization	2
GO:0044419	interspecies interaction between organisms	2
GO:0000003	reproduction	1
GO:0006112	energy reserve metabolic process	1
GO:0006119	oxidative phosphorylation	1
GO:0006260	DNA replication	1
GO:0006412	translation	1
GO:0006818	hydrogen transport	1
GO:0006914	autophagy	1
GO:0007049	cell cycle	1
GO:0007155	cell adhesion	1

GO:0009225	nucleotide-sugar metabolic process	1
GO:0009308	amine metabolic process	1
GO:0015833	peptide transport	1
GO:0015849	organic acid transport	1
GO:0016032	viral process	1
GO:0032501	multicellular organismal process	1
GO:0032989	cellular component morphogenesis	1
GO:0043094	cellular metabolic compound salvage	1
GO:0043412	macromolecule modification	1
GO:0043449	cellular alkene metabolic process	1
GO:0045333	cellular respiration	1
GO:0050877	neurological system process	1
GO:0065003	macromolecular complex assembly	1
GO:0071555	cell wall organization	1

3.7. AMR Genes Analysis of the M22-2C43 Strain

The AMR genes in the *S. coelicolor* A3(2) M22-2C43 genome were analyzed using staramr, and it is the local standalone version of ResFinder. Staramr scans bacterial genomes against the ResFinder, PointFinder, and PlasmidFinder databases (used by the ResFinder web-service and other webservices offered by the CGE). Tables 7 and 8 show the identified AMR genes in the *S. coelicolor* A3(2) M22-2C43 genome and the prediction of its AMR phenotype. In the AMR gene analysis, 3 genes (dldHA2X, erm(O), and tet) were identified.

Table 7. Identified AMR genes in the *S. coelicolor* A3(2) M22-2C43 genome

AMR gene	dldHA2X	erm(O)	tet
Predicted Phenotype	Unknown	erythromycin azithromycin	tetracycline
% Identity	100	99.62	98.54
% Overlap	100	100	100
HSP Length / Total Length	2,670/2,670	783/783	1,920/1,920
Start Position	3,877,021	1,159,440	7,017,352
End Position	3,874,352	1,158,658	7,015,445
Accession	AL939117	M74717	M74049

Table 8. Predicted antibiotics resistance of *S. coelicolor* A3(2) M22-2C43 genome using Resfinder EFSA

Antimicrobial	Class	WGS-predicted phenotype	Genetic Background

clindamycin	lincosamide	Resistant	erm(O) (erm(O)_M74717)
doxycycline	tetracycline	Resistant	tet (tet_M74049)
erythromycin	macrolide	Resistant	erm(O) (erm(O)_M74717)
lincomycin	lincosamide	Resistant	erm(O) (erm(O)_M74717)
minocycline	tetracycline	Resistant	tet (tet_M74049)
pristinamycin IA	streptogramin b	Resistant	erm(O) (erm(O)_M74717)
quinupristin	streptogramin b	Resistant	erm(O) (erm(O)_M74717)
tetracycline	tetracycline	Resistant	tet (tet_M74049)
vancomycin	glycopeptide	Resistant	dldHA2X (dldHA2X_AL939117)
virginiamycin s	streptogramin b	Resistant	erm(O) (erm(O)_M74717)

Table 9. Predicted antibiotics resistance of *S. coelicolor* A3(2) M22-2C43 genome using Resfinder EFSA

Isolate ID	Data	%Identity	%Overlap	HSP Length/Total Length	Contig	Start	End	Accession
GCF_008124905.1	aac(3)-IV	100	100	777/777	ref|NZ_VNIC01000017.1|	151	927	DQ241380
GCF_008124905.1	dldHA2X	100	100	2670/2670	ref|NZ_VNIC01000003.1|	416882	414213	AL939117
GCF_008124905.1	erm(O)	99.62	100	783/783	ref|NZ_VNIC01000017.1|	120418	119636	M74717
GCF_008124905.1	tet	98.54	100	1920/1920	ref|NZ_VNIC01000004.1|	230103	228196	M74049
GCF_008124915.1	dldHA2X	100	100	2670/2670	ref|NZ_VNID01000003.1|	416882	414213	AL939117
GCF_008124915.1	erm(O)	99.62	100	783/783	ref|NZ_VNID01000009.1|	208284	207502	M74717
GCF_008124915.1	tet	98.54	100	1920/1920	ref|NZ_VNID01000004.1|	219613	221520	M74049
GCF_008124975.1	aac(3)-IV	100	100	777/777	ref|NZ_VNIG01000017.1|	208034	207258	DQ241380
GCF_008124975.1	dldHA2X	100	100	2670/2670	ref|NZ_VNIG01000003.1|	63243	65912	AL939117
GCF_008124975.1	erm(O)	99.62	100	783/783	ref|NZ_VNIG01000017.1|	87767	88549	M74717
GCF_008124975.1	tet	98.54	100	1920/1920	ref|NZ_VNIG01000004.1|	219613	221520	M74049
GCF_008124985.1	aac(3)-IV	100	100	777/777	ref|NZ_VNIF01000016.1|	148	924	DQ241380
GCF_008124985.1	dldHA2X	100	100	2670/2670	ref|NZ_VNIF01000003.1|	63244	65913	AL939117
GCF_008124985.1	erm(O)	99.62	100	783/783	ref|NZ_VNIF01000016.1|	120415	119633	M74717
GCF_008124985.1	tet	98.54	100	1920/1920	ref|NZ_VNIF01000004.1|	219613	221520	M74049
GCF_008125035.1	aac(3)-IV	100	100	777/777	ref|NZ_VNIH01000016.1|	208034	207258	DQ241380
GCF_008125035.1	dldHA2X	100	100	2670/2670	ref|NZ_VNIH01000003.1|	63243	65912	AL939117

GCF_008125035.1	erm(O)	99.62	100	783/783	ref|NZ_VNIH01000016.1|	87767	88549	M74717
GCF_008125035.1	tet	98.54	100	1920/1920	ref|NZ_VNIH01000004.1|	230103	228196	M74049
GCF_008931305.1	dldHA2X	100	100	2670/2670	ref|NZ_CP042324.1|	3971328	3973997	AL939117
GCF_008931305.1	erm(O)	99.62	100	783/783	ref|NZ_CP042324.1|	6688040	6688822	M74717
GCF_008931305.1	tet	98.54	100	1920/1920	ref|NZ_CP042324.1|	829910	831817	M74049
GCF_013307045.1	IncY	100	100	765/765	ref|NZ_JAATOK010000003.1|	254327	253563	K02380
GCF_013307045.1	aph(3')-Ia	100	100	816/816	ref|NZ_JAATOK010000003.1|	247960	248775	V00359
GCF_013307045.1	dldHA2X	100	100	2670/2670	ref|NZ_JAATOK010000003.1|	469021	466352	AL939117
GCF_013307045.1	erm(O)	99.62	100	783/783	ref|NZ_JAATOK010000009.1|	319538	318756	M74717
GCF_013307045.1	tet	98.54	100	1920/1920	ref|NZ_JAATOK010000005.1|	219645	221552	M74049
GCF_013317105.1	IncFIA	100	100	388/388	ref|NZ_CP050522.1|	4148963	4148576	AP001918
GCF_013317105.1	IncY	100	100	765/765	ref|NZ_CP050522.1|	4161605	4162369	K02380
GCF_013317105.1	aph(3')-IIa	99.62	100	795/795	ref|NZ_CP050522.1|	4152422	4151628	V00618
GCF_013317105.1	aph(3')-Ia	100	100	816/816	ref|NZ_CP050522.1|	4167973	4167158	V00359
GCF_013317105.1	catA1	99.7	100	660/660	ref|NZ_CP050522.1|	4150783	4151442	V00622
GCF_013317105.1	dldHA2X	100	100	2670/2670	ref|NZ_CP050522.1|	3864322	3866991	AL939117
GCF_013317105.1	erm(O)	99.62	100	783/783	ref|NZ_CP050522.1|	6675094	6675876	M74717
GCF_013317105.1	tet	98.54	100	1920/1920	ref|NZ_CP050522.1|	808256	810163	M74049

In the comparative AMR gene analysis using all available RefSeq genomes of the *S. coelicolor* strains, three identified AMR genes in the *S. coelicolor* A3(2) M22-2C43 commonly existed in all available RefSeq genomes of the same species. Table 9 shows the identified AMR gene in all available RefSeq genomes. The identified 3 AMR genes in the M22-2C43-WT and M22-2C43 strains also existed in all available RefSeq genomes of the same species. Even though the number of currently available RefSeq genomes is limited (8), these 3 AMR genes can be considered as intrinsic AMR genes in the *S. coelicolor* species. This indicates that the AMR genes in the M22-2C43 strain might not be additionally acquired by event, such as horizontal gene transfer.

3.8. Virulence Factor Genes Analysis of M22-2C43 Strain

To identify the virulence factor genes in the *S. coelicolor* A3(2) M22-2C43 genome, DIAMOND search of the M22-2C43 strain proteins to the VFDB full set protein database was conducted, and a raw DIAMOND Blastp search identified 905 protein matches. However, every match showed low identity with known virulence factor genes, and no virulence factors remained when the filtering criteria of VirulenceFinder 2.0 (identify >90%, subject coverage > 60%) was applied. The raw match result is shown in Appendix 1. In addition, the search result from the VirulenceFinder 2.0 server showed that no virulence factors were found in the genome of the M22-2C43 strain.

VirulenceFinder-2.0 Server - Results

Organism(s): *S. aureus,Escherichia coli,Listeria,Enterococcus*

Hostimm genes for S. aureus

Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
No hit found						

Exoenzyme genes for S. aureus

Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
No hit found						

Toxin genes for S. aureus

Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
No hit found						

Virulence genes for Escherichia coli

Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
No hit found						

Shiga-toxin genes

Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
No hit found						

Virulence genes for Listeria

Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
No hit found						

Virulence genes for Enterococcus

Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
No hit found						

Input Files: *ALE_pilon.fasta*

CITATIONS

For publication of results, please cite:

- Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli.
  Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM.
  J. Clin. Micobiol. 2014. 52(5): 1501-1510.
  View the abstract

3.9. Allergen Genes Analysis of *S. coelicolor* A3(2) M22-2C43 Strain

To identify the genes coding for a potential allergen in the genome of the *S. coelicolor* M22-2C43 strain, COMPARE database was used for the DIAMOND search. Raw DIAMOND search reported 84 matches, but same as the virulence factor search case, all matches showed low identity. The minimum identity was 23.7%, and the maximum identity was 55.4%. The raw match result to the COMPARE database is shown in Appendix 2. Based on the filtering criteria used in the virulence factor analysis, no known allergenic genes were identified in the genome of the *S. coelicolor* A3(2) M22-2C43 strain.

3.10. BCG Analysis

To identify the BCGs in the genome of *S. coelicolor* A3(2) M22-2C43 strain, antiSMASH was used. Figure 7 shows the identified BCGs in the ALE strain.

Figure 6. Identified BCGs in the M22-2C43 genome using antiSMASH 6.0

A total of 21 BCG regions were identified, and the details of the identified BCGs regions are shown in Table 10.

Table 10. Detailed information of identified BGCs in the *S.coelicolor* A3(2) M22-2C43 genome

Region	Type	From	To	Most similar known cluster		Similarity
Region 1	lanthipeptide-class-i	137,871	164,324			
Region 2	T1PKS	228,983	275,023	arsono-polyketide	Polyketide	95%
Region 3	terpene	315,248	341,057	hopene	Terpene	100%
Region 4	lanthipeptide-class-iii	414,909	437,623	SapB	RiPP:Lanthipeptide	100%
Region 5	thioamide-NRP,NRPS	704,919	759,102	nogalamycin	Polyketide	40%
Region 6	T1PKS,butyrolactone	895,829	966,031	coelimycin P1	Polyketide:Modular type I	100%
Region 7	siderophore	992,937	1,004,666	enduracidin	NRP	6%
Region 8	terpene	1,171,142	1,190,462	geosmin	Terpene	100%
Region 9	RiPP-like	1,205,135	1,214,872			
Region 10	T1PKS,prodigiosin	1,370,924	1,417,817	undecylprodigiosin	NRP + Polyketide	100%
Region 11	siderophore	1,500,925	1,511,277			
Region 12	T2PKS	2,024,028	2,093,584	spore pigment	Polyketide	66%
Region 13	terpene	2,155,431	2,176,444	albaflavenone	Terpene	100%
Region 14	T2PKS	2,281,174	2,353,668	actinorhodin	Polyketide:Type II	100%
Region 15	NRPS	4,243,305	4,322,384	CDA1b / CDA2a / CDA2b / CDA3a / CDA3b / CDA4a / CDA4b	NRP:Ca+-dependent lipopeptide	87%
Region 16	siderophore	4,801,572	4,813,359	desferrioxamin B / desferrioxamine E	Other	83%
Region 17	melanin	4,897,379	4,907,948	istamycin	Saccharide	4%
Region 18	ectoine	5,841,361	5,851,759	ectoine	Other	100%
Region 19	T3PKS	6,548,512	6,589,636	herboxidiene	Polyketide	8%
Region 20	RiPP-like	7,045,463	7,055,678	informatipeptin	RiPP:Lanthipeptide	42%
Region 21	NRPS	7,302,342	7,353,273	coelichelin	NRP	100%

Among 21 BGCs, 12 BGCs show high similarities with known BGCs. However, there is no BGC related to widely used antibiotics.

4. Conclusion

In this analysis, two complete genomes of the *S. coelicolor* strains (A3(2) M22-2C43-WT and A3(2) M22-2C43, which are often abbreviated as M22-2C43-WT and M22-2C43) were successfully constructed and compared. During the ALE process, a large portion of the genomic regions was deleted, and the linear genome of the parental strain was circularized in the ALE strain.

Three antibiotic resistance genes that can affect the safety of strain were identified in the AMR gene analysis using ResFinder. Although three AMR genes were found in the M22-2C43 genome, the comparative AMR gene analysis confirmed that these three resistance genes identified in the *S. coelicolor* A3(2) M22-2C43 were common to all publicly available RefSeq genomes of this species. Therefore, it is expected that they can be regarded as intrinsic AMR genes, and they are not expected to be additionally acquired through horizontal gene transfer, etc.

Additional safety-related analyses (virulence factor, allergen, and BGC) did not find any known genomic contents related to the safety in the genome of the *S. coelicolor* A3(2) M22-2C43 strain.

Based on all the analysis results of *S. coelicolor* A3(2) M22-2C43, no specific issue related to the safety of this strain was observed, and it can be judged that there is no critical reason to limit the use of this strain.

5. Declare of Transparency

It is confirmed that the contents contained in the results of this study have not been intentionally altered or manipulated to achieve the desired results, and all analyses were performed exactly as described in the materials and methods section.

CEO of Gencube plus

Ki Hwan Kim, Ph.D.

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7. Appendices-Available upon request

Appendix 1. Raw DIAMOND Search Result to VFDB Database

DIAMOND Blastp result: VFDB_diamond.xlsx

Appendix 2. Raw DIAMOND Search Result to COMPARE Database

DIAMOND Blastp result: Compare_diamond.xlsx

Appendix 3. A list of Screened Virulence Genes

Virulence Genes in VFDB: VFs. xlsx

Appendix 4. A List of Screened Allergen Genes

Known Allergens in COMPARE Database: Compare_2022.xlsx

Appendix D. Proof of β-Agarase DagA Enzyme Removal from NAO

(Quantitative analysis of DagA protein using LC-MS/MS)

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Date: September 28, 2021

Abstract

Agar, a polysaccharide complex extracted from the cell walls of red algae, is a food ingredient in Asia (e.g., Korea, Japan, and China), a GRAS food additive in the USA, and an approved food additive in Europe. Agar can be hydrolyzed by β-agarase DagA at the β-1,4 glycosidic linkage to produce neoagaro-oligosaccharides (NAO). An industrial process for manufacturing NAO from agar was developed using microbial β-agarase DagA from *Streptomyces coelicolor* A3(2)M22-2C43. The microbial enzyme is removed by a series of purification processes after the production of NAO. The purpose of this study was to measure the residual amount of DagA, the raw material used in NAO production. The quantitative method for analysis of the residual β-agarase DagA (DagA, Sco3471) was developed using SDS gel electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis), followed by hydrolysis of the enzyme to peptides, and a parallel reaction monitoring (PRM) method using an LC/MS/MS (Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer).

To select an appropriate peptide as the standard material for PRM quantitative analysis of the DagA enzyme, the selected peptides were 7-25 amino acids long, with no possibility of post-translation modifications (PTM) and with a large number of peptide spectrum matches (PSMs) specific peptides. As a result, it was found that more than 99.8% of the DagA enzyme was removed during purification of the NAO product.

Keywords: NAO, DagA, LC-MS/MS, PRM

1. Introduction

The process for manufacturing neoagaro-oligosaccharides (NAO) involves hydrolysis of agar from agar-agar (seaweed) by a microbial enzyme (DagA). The latter is removed in a series of purification steps after production of the NAO. The purpose of this study was to measure the residual amount of DagA, the raw material used in NAO production. Samples collected at each stage of the production process were separated using SDS-PAGE in-gel digestion, and then peptides with trypsin were used in analysis by LC/MS/MS (a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer) (Figure 1).

The Q-Exactive plus mass spectrometer is an analyzer that has both quadrupole and Orbitrap, enabling both sincerity and quantitative analysis. It has MS resolution up to 140,000 and scan speed up to 12 Hz, making it suitable for metabolic analysis. Molecular weight up to 6,000 m/z can be detected, allowing detection of metabolites having greater variety of molecular weights, and detecting materials having different polarities in a single operation with a fast polarity switching function. With this instrument, qualitative and quantitative analysis and targeted metabolic quantitative analysis techniques can be applied to complexes of various types.

To select an appropriate peptide as the standard material for PRM quantitative analysis of the DagA enzyme, the selected peptides were 7-25 amino acids long, with no possibility of PTM, and with a large number of PSM specific peptides. The amount of residual DagA protein was analyzed using mass spectrometry (LC-MS/MS) based on a quantitative analysis method using parallel reaction monitoring (PRM). This was done at the KBIO Osong Medical Innovation Foundation to measure the remaining amount of DagA enzymes [1]. PRM is an ion monitoring technique derived from multiple reaction monitoring (MRM), which can also simultaneously perform relative or absolute quantitative detection of multiple target proteins in complex biological samples.

PRM is based on high-resolution, high-precision mass spectrometers (such as the Q-Exactive HF-X). PRM uses the selective detection capability of a quadrupole mass analyzer to detect the precursor ion information of the target peptide. The precursor ions are then fragmented in a collision cell. Finally, a high-resolution, high-quality precision Orbitrap analyzer is used to detect all fragments in the selected precursor ion window in the secondary mass spectrum. PRM is based on Q-Orbitrap as the representative quadrupole-high resolution mass spectrum platform. First, PRM uses the quadrupole (Q1) to select the precursor ion, and the selection window is usually m/z ≤ 2. Then, the precursor ion is fragmented in the collision cell (Q2), and finally, Orbitrap replaces Q3, then scans all product ions with high resolution and high accuracy. The principle of this technique is comparable to SRM/MRM, but it is more convenient for absolute quantification of proteins and peptides. It is most suitable for quantification of multiple proteins in complex samples with attomole-level detection [2, 3].

2. Methods

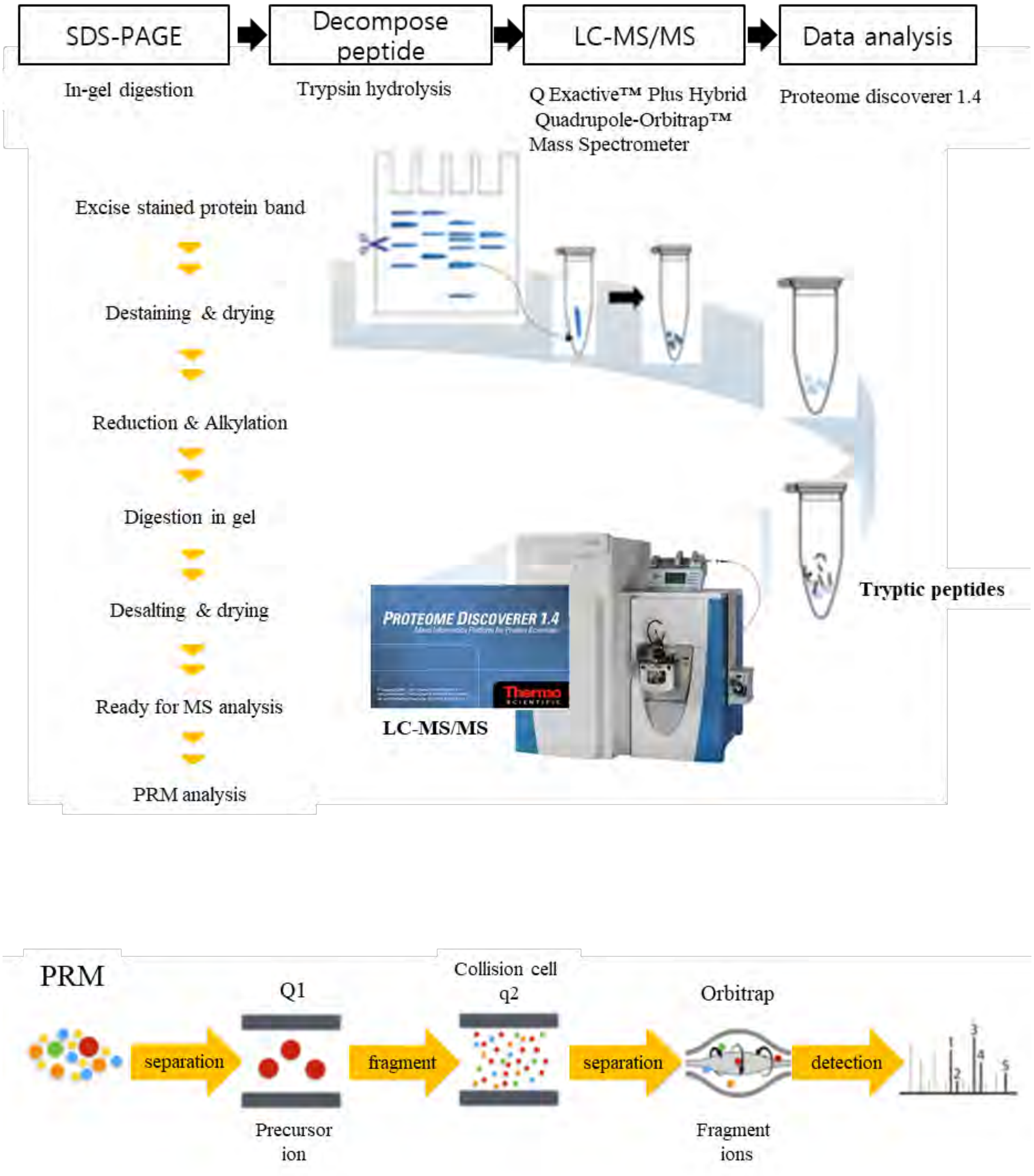


Figure 1. Flow diagram of LC-MS/MS analysis process

2.1 Materials

2.1.1 Sample information (Figure 2)

No.	Samples		Production Strain
1	For calibration curve	β-agarase DagA (DagA, Positive control)	*Streptomyces lividans* TK24
2	Without substrate(agar)	β-agarase DagA (DagA, standard)	*Streptomyces coelicolor* A3(2) M22-2C43
3		β-agarase1 _enzyme reaction	
4		β-agarase 2_enzyme inactivation	
5		β-agarase3 _filtration	
6		β-agarase4_concentration	
7	With substrate(agar)	NAO1_enzyme reaction	
8		NAO2_enzyme inactivation	
9		NAO3_filtration	
10		NAO4_concentration	

1) Sample 1: The *S. lividans* TK24 strain is a recombinant strain (GMO) with inserted DagA gene (Sco3471) from the *S. coelicolor* A3(2) M22-2C43 strain. Due to better expression of DagA and the vector in which the DagA (Sco3471) gene was inserted, it was used as a sample to quantify DagA as a positive control.

2) Sample 2: DagA enzyme used in the NAO manufacturing process. Performed at concentration 100× higher than in the real process due to the LC-MS/MS detection limit.

3) Samples 3 to 6: To confirm the degradation and removal of DagA when performing the NAO manufacturing process, conditions such as temperature and process time were applied to the enzyme itself without a substrate (agar). This enzyme process was conducted at a concentration 100× higher than in the real process due to the LC-MS/MS detection limit.

4) Samples 7 to 10: Sampling during each step of the NAO manufacturing process with substrate (agar). After the reaction in the standard process was completed, samples were concentrated 100 times.
Samples 3 and 7 were hydrolyzed by DagA for 16 h at 43-46±5°C. Samples 4 and 8 were inactivated for 1 h at 95±5°C. Samples 5 and 9 were filtered with a 0.5 mm polypropylene (PP) filter. Samples 6 and 10 were concentrated to 1/10 the original volume using a vacuum pump (65°C, 5 h) (Figure 3).

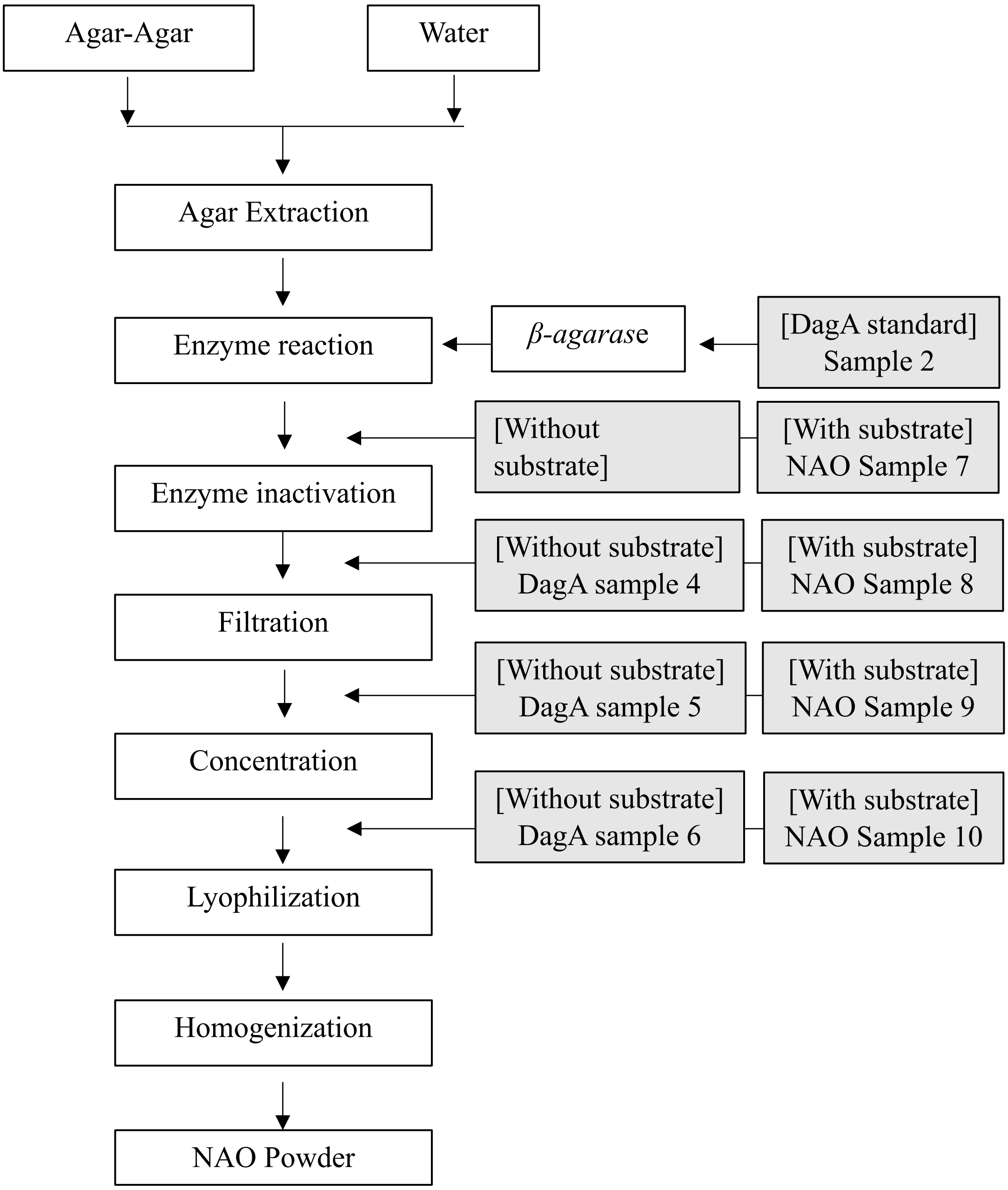


Figure 2. Flow chart of NAO production and product sampling processes

2.1.2 Materials

Reagent	Cat No.	Manufacturer
Acetonitrile (ACN)	AH015-4	Honeywell
0.1% FA in Acetonitrile	LS120-212	Fisher chemical
0.1% FA in Water	LS118-4	Fisher chemical
Formic acid	F0507-100ML	Sigma
Ammonium bicarbonate (NH_4HCO_3)	A6141	Sigma
Dithiothreitol (DTT)	A62251	Sigma
Iodoacetamide (IAM)	A62250	Sigma
Urea	U0631	Sigma
Trypsin/Lys-C Protease Mix	V5073	Promega
OASIS SPE cartridge	WAT094225	Waters
Ziptip µ-C18	ZTC18M096	Millipore
NuPAGE 4-12% Bis-Tris Gel	NP0321BOX	Invitrogen
NuPAGE sample buffer (4X)	PN 1771559	Invitrogen
Precision Plus Protein Kaleidoscope Standards	161-0375	BIO-RAD
eStain protein staining pads R-250	PN L02011	GenScript
Analytical column: Acclaim® Pepmap RSLC C18 50 µm × 15 cm, nanoViper	PN 164943	Thermo Fisher Scientific
Trapping column: Acclaim® Pepmap 100 C18 75 µm × 2 cm, nanoViper	PN 164535	Thermo Fisher Scientific

2.2 Equipment

Equipment	Cat no.	Manufacturer
Thermo Scientific Sorvall Legend Micro 21 Centrifuge	TSS-21	Thermo Fisher Scientific
SAVANT SpeedVac	SPD2010	Thermo Fisher Scientific
EASY-nLC 1200	LC140	Thermo Fisher Scientific
Nano Trap Column, packed with PepMap™ RSLC, nanoViper™ Fittings	164535	Thermo Fisher Scientific
EASY-Spray 50 cm × 75 µm PepMap RSLC C18 2 µm	ES803A	Thermo Fisher Scientific

Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer	IQLAAEGAAPFALGMBDK	Thermo Fisher Scientific

3. Methods

3.1 Sample preparation

To determine the amount of DagA in a sample, 25 µL of each sample was loaded into an SDS-PAGE well and after running the electrophoresis, a gel slice of each sample was cut out from the area of the DagA (32.24 kDa) band. After the gel slice was peptized, a PRM relative quantitative analysis was performed using LC-MS/MS. When performing this analysis, a calibration curve was created using sample 1, and experimental samples 2 to 10 were subjected to a quantitative experiment with results compared to the calibration curve.

3.2 In-gel digestion

A portion (25 µL) of the 4X SDS loading buffer was added to 25 µL of each sample. To this, 100 µL of water was added, so that it became 1X concentration. This was boiled at 95°C for 3 min. Then, the samples were loaded into the wells of the SDS-PAGE and electrophoresis was performed at 200 V for 35 min. CBB staining was performed using eStain protein staining pads (GenScript). The 32 kDa location of the DagA gel band to be analyzed was cut out and sized to 1 × 1 mm. Then, the sample was de-stained. The gel was sufficiently washed using 50 mM NH_4HCO_3 and the gel dried using 100% acetonitrile (ACN). The gel was immersed in 10 mM DTT and reacted at 56°C for 1 h. All of the supernatant was discarded, and the gel immersed in 55 mM IAM for 1 h to react in the dark and at room temperature (this reaction is sensitive to light, so it must be conducted under dark conditions). All of the supernatant was discarded, and the gel was sufficiently washed using 50 mM NH_4HCO_3 and then dried using 100% ACN. The gel was immersed in 10 ng/µL trypsin to react in a Thermomixer at 37°C for 16 h. Only the supernatant (peptide sample) was transferred to a new tube, purified with a zip tip (µ-C18), and dried.

3.3 LC-MS/MS analysis

The dried sample was dissolved in a solution of 0.1% FA at the concentration of 0.5 µg/µL. A peptide sample of 2 µL was injected into a reverse phase (Aclam®Peppmap RSLC C18) column and eluted for 60 min at a gradient concentration of 5–40% ACN (Buffer B) (see Table 1 and 2).

Table 1. Nano LC condition

Nano LC Gradient			
Time (min)	A (%)	B (%)	Flow Rate (nL/min)
0	95	5	300
10	95	5	
40	60	40	
43	5	95	
47	5	95	
50	95	5	
60	95	5	
Column Temp.			50°C
Sample Temp.			4°C

Table 2. Mass spectrometry condition

Full MS condition	
Resolution	70,000
Scan Range	350-2000 m/z
Maximum IT	120 ms
Polarity	Positive
MS/MS condition (DDA: data dependent accusation method)	
Resolution	17,500
AGC	5.00E+0.5
Isolation width	1.2 m/z
Top N	20
NCE (%)	25
Maximum IT	80 ms
Dynamic Exc.	30s

4. Results

4.1 Quantitative analysis for the DagA (Sco3471) protein

Peptizing β-agarase DagA (sample 1) as an analytical standard was performed by mass spectrometry. As shown in Table 3, a total of 16 peptides were analyzed to identify DagA. Among them, the peptide DGWSGPANSLYSAR was suitable for quantitative analysis regarding the length of amino acids affecting ionization, the probability of PTMs, and the number of PSMs (Figure 3).

Table 3. Identified tryptic peptides

Description	Coverage	#Unique Peptides	#Peptides	#PSMs[a]	Score		# AAs[b]	MW [kDa]	calc. pI[c]
Sequence of β-agarase DagADagA(Sco3471) “Sco3471”	64.08	16	16	113	516.16		309	35.1	7.36
	#PSMs	# Protein Groups	Protein Group Accessions	Modifications	MH+ [Da]	A4	XCorr A4	Probability A4	Missed Cleavages
GYFADGSYGYNGETGQVFGDGAGQP LLR	15	1	"Sco3471"		2896.3074	High	7.89	0	0
FFDQPMHLILNTESHQWR	3	1	"Sco3471"		2299.0992	High	7.65	0	0
SRFFDQPMHLILNTESHQWR	3	1	"Sco3471"		2542.2328	High	7.58	0	1
WLDQHKDGWSGPANSLYSAR	1	1	"Sco3471"		2288.0758	High	7.43	0	1
SRFFDQPMHLILNTESHQWR	2	1	"Sco3471"	M8(Oxidation)	2558.225	High	7.29	0	1
LSSNFWLLSRDDVNEIDVIECYGNESL HGK	1	1	"Sco3471"	C21(Carbamidomethyl)	3509.6525	High	7.17	0	1
VDRGIEPTDAELADPSINNIYYR	3	1	"Sco3471"		2621.2752	High	7.13	0	1
SWQLLPSHSDDFNYTGKPQTFR	5	1	"Sco3471"		2624.2377	High	6.98	0	0
DDVNEIDVIECYGNESLHGK	4	1	"Sco3471"	C11(Carbamidomethyl)	2306.0164	High	6.75	0	0
GIEPTDAELADPSINNIYYR	2	1	"Sco3471"		2251.0801	High	6.31	0	0
FFDQPMHLILNTESHQWR	5	1	"Sco3471"	M6(Oxidation)	2315.0963	High	6	0	0
HSWVADGNLIVEGR	49	1	"Sco3471"		1552.7804	High	5.09	0	0
HMNTAYHIFQR	1	1	"Sco3471"		1417.6767	High	4.98	0	0
TPVEYPLYTEVLMR	5	1	"Sco3471"		1710.8725	High	4.65	0	0
TPVEYPLYTEVLMR	7	1	"Sco3471"	M13(Oxidation)	1726.8644	High	4.15	0	0
DGWSGPANSLYSAR	3	1	"Sco3471"		1480.6763	High	3.71	0	0
LSSNFWLLSR	1	1	"Sco3471"		1222.6539	High	3.64	0	0
NPFTELAR	2	1	"Sco3471"		947.49237	High	3.03	0	0
SNDLRDPR	1	1	"Sco3471"		972.48242	High	1.1	0	1

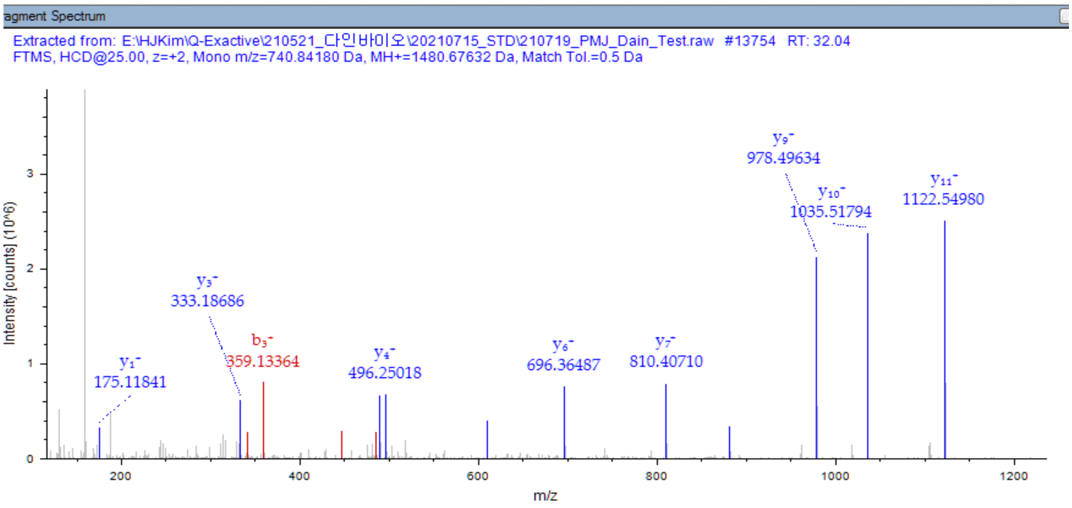
[a] PSM, peptide spectrum match, means the number of spectra that resulted in 1% FDR identification.

[b] #AA is the number of the amino acids in a protein.

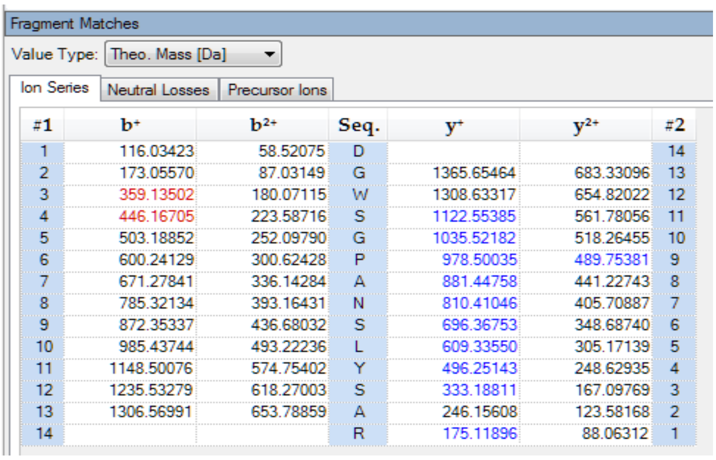
[c]Calc. pI shows the calculated pI.

Only peptides that could be quantified without overlapping with other proteins were selected. Target peptides were required to be between 7 and 25 amino acids long and were selected based on uniqueness and anticipated chemical stability. Peptides containing cysteine or methionine residues were not excluded. There was no possibility of PTM, and the selected peptide was one with a large number of PSMs.

Two peptides ("DGWSGPANSLYSAR" and "HSWVADGNLIVEGR") suitable for the above conditions were selected. After making the calibration curves for these two peptides, it was determined that the R^2 value of "HSWVADGNLIVEGR" was 0.97828, and the R^2 value of "DGWSGPANSLYSAR" was 0.9984. "DGWSGPANSLYSAR" was the final selection because it is suitable only when the R^2 value is 0.99 or higher.



Extracted from: E:\HJKim\Q-Exactive\210521_다인바이오\20210715_STD\210719_PMJ_Dain_Test.raw #13754 RT: 32.04
FTMS, HCD@25.00, z=+2, Mono m/z=740.84180 Da, MH+=1480.67632 Da, Match Tol.=0.5 Da



Value Type: Theo. Mass [Da]

#1	b⁺	b²⁺	Seq.	y⁺	y²⁺	#2
1	116.03423	58.52075	D			14
2	173.05570	87.03149	G	1365.65464	683.33096	13
3	359.13502	180.07115	W	1308.63317	654.82022	12
4	446.16705	223.58716	S	1122.55385	561.78056	11
5	503.18852	252.09790	G	1035.52182	518.26455	10
6	600.24129	300.62428	P	978.50035	489.75381	9
7	671.27841	336.14284	A	881.44758	441.22743	8
8	785.32134	393.16431	N	810.41046	405.70887	7
9	872.35337	436.68032	S	696.36753	348.68740	6
10	985.43744	493.22236	L	609.33550	305.17139	5
11	1148.50076	574.75402	Y	496.25143	248.62935	4
12	1235.53279	618.27003	S	333.18811	167.09769	3
13	1306.56991	653.78859	A	246.15608	123.58168	2
14			R	175.11896	88.06312	1

Figure 3. Manually verified identified peptides (DGWSGPANSLYSAR, Charge: +2, Monoisotopic m/z: 740.84180 Da)

4.2 Development of a quantitative method for analysis of DagA (Sco3471)

Analysis was developed using the PRM mode for the peptide DGWSGPANSLYSAR by Q-Exactive plus LC-MS/MS (Figure 4). The top 5 transition was selected and is shown in Table 4.

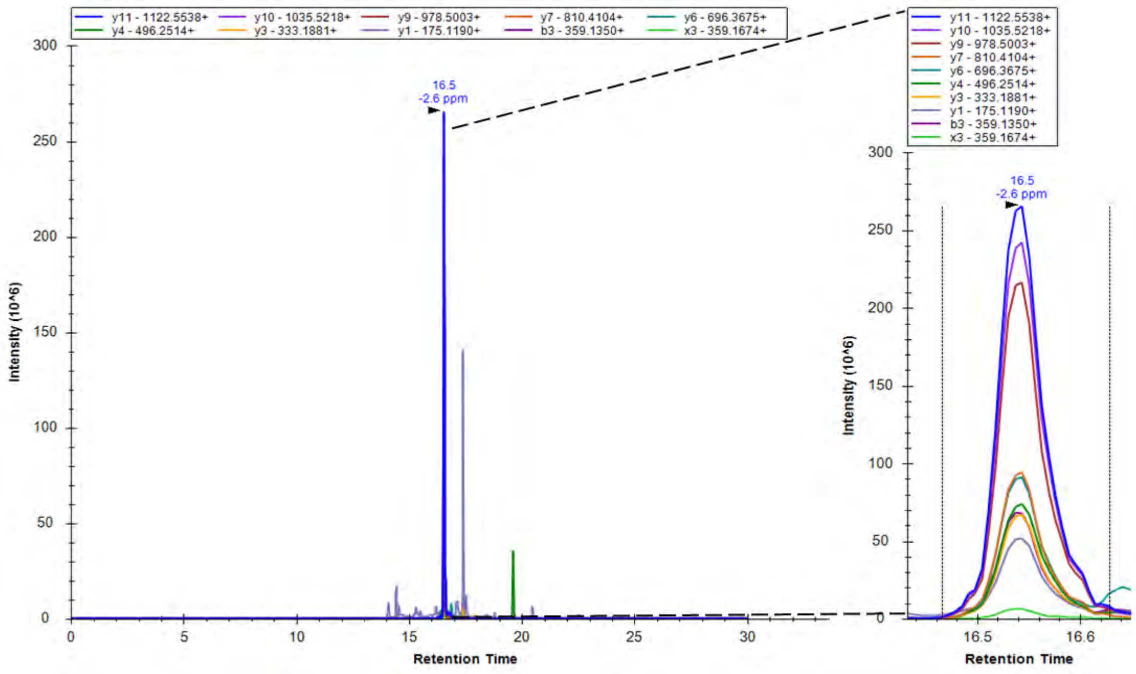


Figure 4. Extracted ion chromatograms

Table 4. PRM transition list

Q1	Q3	Retention Time (min)	Peptides	Protein	Ion
740.8444	978.5003	25.1	DGWSGPANSLYSAR	Sco3471	y9
740.8444	881.4476	25.1	DGWSGPANSLYSAR	Sco3471	y8
740.8444	810.4104	25.1	DGWSGPANSLYSAR	Sco3471	y7
740.8444	696.3675	25.1	DGWSGPANSLYSAR	Sco3471	y6
740.8444	609.3355	25.1	DGWSGPANSLYSAR	Sco3471	y5

4.3 Verification of the PRM analysis method

The standard β-agarase DagA (sample 1) was subjected to serial dilution twice to provide a calibration curve. Starting with 2,000 ng, for the analysis method, evaluation was done at absolute quantity of 1,000, 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, and 0.977 ng. For each concentration, three repetitive analyses were performed. For the calibration curve, the data selected were < 20% CV. The peptide DGWSGPANSLYSAR satisfied the "acceptance criteria" in the range of 3.9-2,000 ng. The coefficient of determination of linearity (R^2) was 0.998, and the LLOQ was 3.9 ng (Table 5, Figure 5). This relative quantitative analysis method is different from the absolute quantitative method performed by synthesizing standard peptides. It is necessary to interpret it in consideration of the fact that this relative quantitative value is not an absolute quantitative value.

Table 5. Results of the precision analysis of peptide (DGWSGPANSLYSAR)

Protein	Peptide	Precursor Charge	Precursor Mz	Replicate	ng	Average	STDEV	CV
Sco3471	DGWSGPANSLYSAR	2	740.844404	Cal01	0.977	1603476	11989.34	1%
				Cal02	1.953	1701879	450613.1	26%
				Cal03	3.906	3142442	330276.5	11%
				Cal04	7.813	30933920	3986708	13%
				Cal05	15.625	1.43E+08	6703728	5%
				Cal06	31.25	3.93E+08	15636031	4%
				Cal07	62.5	8.24E+08	11192395	1%
				Cal08	125	1.61E+09	22932395	1%
				Cal09	250	2.58E+09	1.69E+08	7%
				Cal10	500	3.63E+09	3.25E+08	9%
				Cal11	1,000	4.3E+09	68510351	2%
				Cal12	2,000	5.29E+09	31455041	1%

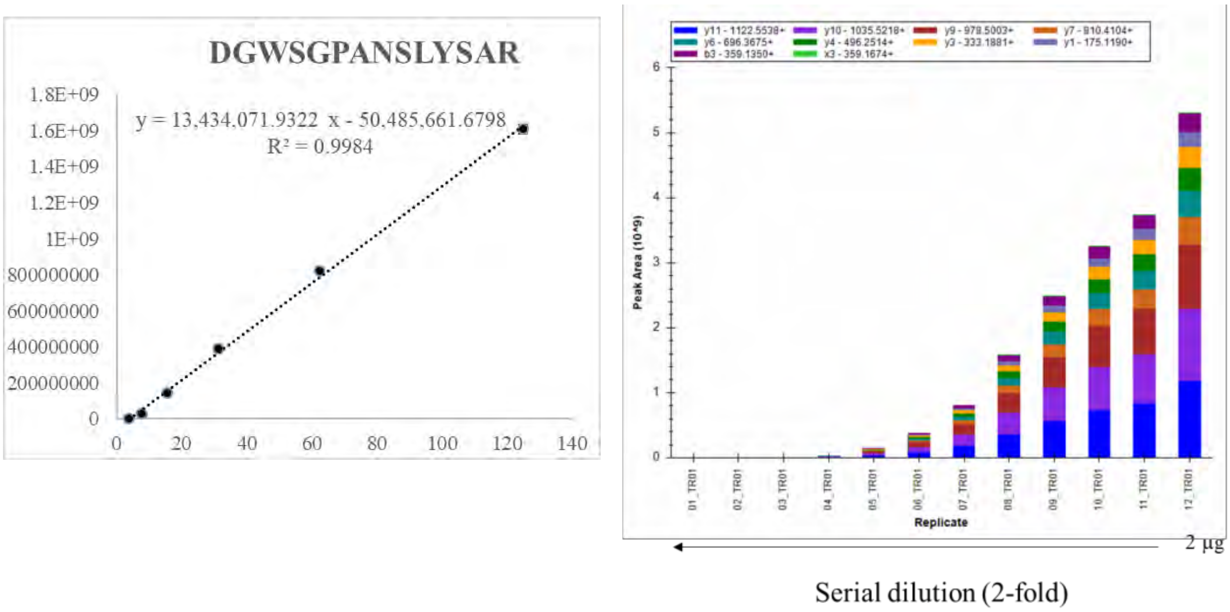


Figure 5. Calibration curve of the selected peptide (DGWSGPANSLYSAR)

4.4 Measurement of the residual amount of DagA (Sco3471) in samples

The amount of DagA present at each step of the manufacturing process was measured using the established analysis method as described above. After loading the SDS-PAGE with 25 µL in each well, PRM was used to analyze the in-gel digested peptide (Figure 6). It was determined that DagA has a molecular weight of 32.24 kDa (mature form) and a pI value of 6.39. For the full sequence (i.e., including the signal peptide), the molecular weight is 35.17

kDa. DagA is composed of 309 amino acids, and the full sequence is shown in Figure 7. The data in Table 6 (DagA remaining and removed) were obtained using the PRM analysis method.

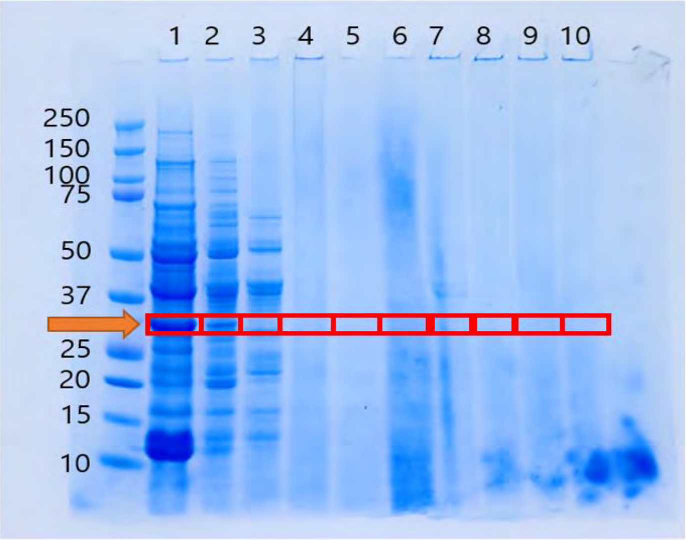


Figure 6. CBB staining for the SDS-PAGE analysis

MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQYPVPAAPGGNRSWQLLPSHSDDFNYTGKP
QTFRGRWLDQHKDGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGYVTSRTPVEYPLYTEVL
MRVSGLKLSSNFWLLSRDDVNEIDVIECYGNESLHGKHMNTAYHIFQRNPFTELARSQKGYFADGSYGY
NGETGQVFGDGAGQPLLRNGFHRYGVHWISATEFDFYFNGRLVRRLNRSNDLRDPRSRFFDQPMHLIL
NTESHQWRVDRGIEPTDAELADPSINNIYYRWVRTYQAV

Figure 7. Amino acid sequences of β-agarase DagADagA (Sco3471) of *S. coelicolor* A3(2) M22-2C43: The DagA signal peptide is first: MVNRRDLIKWSAVALGAGAGLAGPAPAAHA.

4.5 Quantitative analysis of one peptide (DGWSGPANSLYSAR) in each sample

The concentration of one peptide (DGWSGPANSLYSAR) is shown in Table 3, and the chromatograms shown in Figure 8 were drawn from the results of two repeat analyses (n = 2). The amount of DagA in each sample is shown in Figure 9.

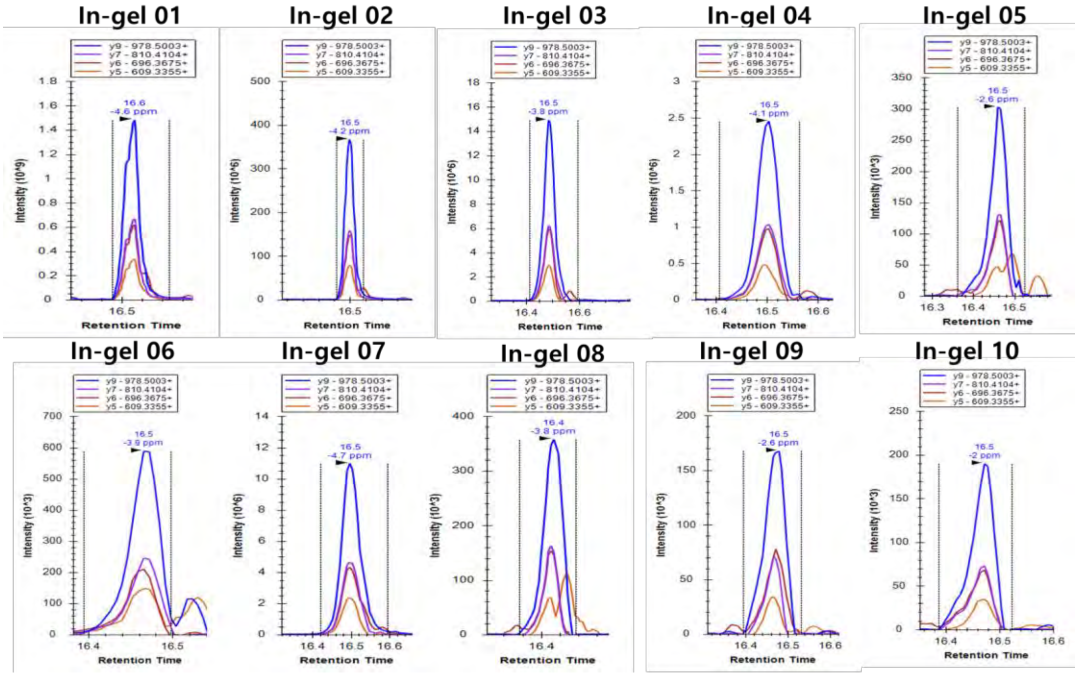


Figure 8. Extracted ion chromatograms

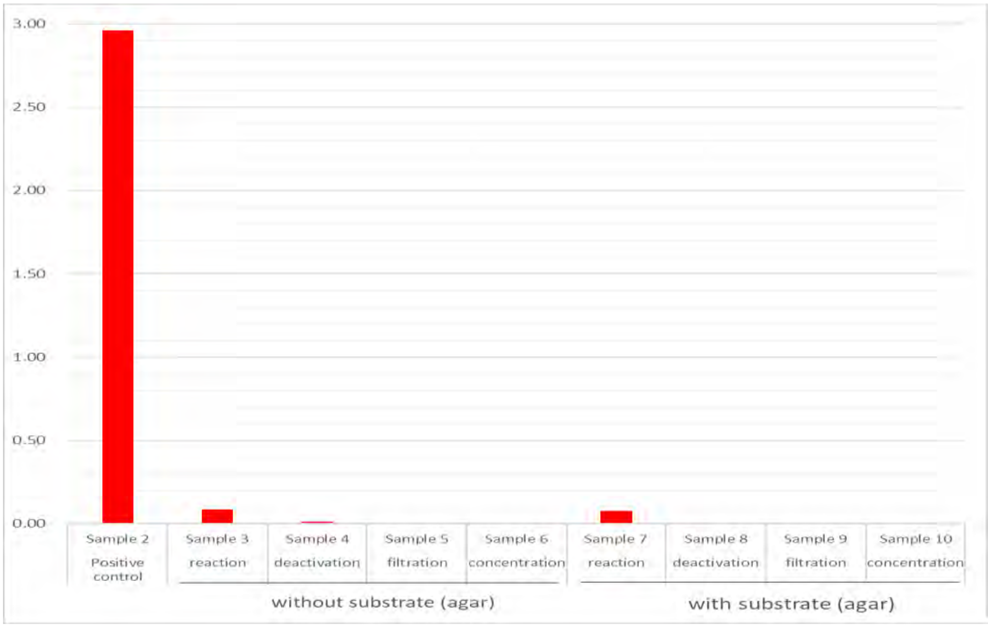


Figure 9. Presence of DagA in a sample from each process (n = 2)

Table 6. Amount of DagA protein remaining and removal rate for each sample

No.	Sample		Analyzed amount of DagA* [ng/25 μL]	Calculated Amount of DagA in NAO [pg/μL]	Removal rate [%]
2	β-agarase DagA (Positive Control)		301.1	120.4	100.00
3	Without substrate (agar)	β-agarase1 _enzyme reaction	13.7	5.5	95.45
4		β-agarase2 _enzyme inactivation	6.5	2.6	97.84
5		β-agarase3_filtration	5.2	2.1	98.27
6		β-agarase4 _10× concentration**	0.53	0.2	99.82
7	With substrate (agar)	NAO1 _enzyme reaction	12.7	5.1	95.78
8		NAO2 _enzyme inactivation	5.2	2.1	98.27
9		NAO3_filtration	5.12	2.0	98.31
10		NAO4 _10× concentration**	0.51	0.2	99.83

*Result from analyzing 25 μL loaded into SDS-PAGE for in-gel digestion

**Samples 6 and 10 were concentrated to 1/10 the original volume.

5. Discussion

We established a method for quantifying the DagA enzyme used in the NAO manufacturing process. To track quantitatively the dynamics of the DagA enzyme protein used in the NAO manufacturing process, the analysis was performed based on the PRM method using LC-MS/MS. We confirmed that, in each step in the NAO manufacturing process using the target peptide (DGWSGPANSLYSAR), the enzyme DagA has a linearity coefficient R^2 of 0.998 and LLOQ of 3.9 ng. Moreover, we confirmed that nearly all of the enzymes used in the NAO manufacturing process were removed. As a result, DagA protein remaining in the final product NAO was 0.17% of the initial amount (i.e., 99.83% was removed).

6. References

1) Hye-Jung Kim, De Lin, Hyoung-Joo Lee, Ming Li, and Daniel C. Liebler (2015) Quantitative Profiling of Protein Tyrosine Kinases in Human Cancer Cell Lines by Multiplexed Parallel Reaction Monitoring Assays, Technological Innovation and Resources, 682-691

2) Prakash A, Rezai T, et al. (2012) Interlaboratory reproducibility of selective reaction monitoring assays using multiple upfront analyte enrichment strategies. Journal of proteome research, 11(8): 3986-3995.

3) Bourmaud A, Gallien S. (2016) Parallel reaction monitoring using quadrupole-Orbitrap mass spectrometer: principle and applications. Proteomics. 16(15-16):2146-2159.

Appendix E. Residual β-Agarase DagA Level in NAO

Authors: Hye-Jeong KO, Eun Joo Kim, Je Hyeon Lee

Institution: Dyne Bio Inc., Gyeonggi-Do, 13209, Republic of Korea

Date: October 21,2021

1. Abstract

The β-agarase DagA production strain is a *Streptomyces coelicolor* A3(2) wild type UV mutant. The largest β-agarase DagA activity in the microbial community that survived UV treatment was named *S. coelicolor* A3(2) M22-2C43. After incubating β-agarase DagA, centrifugation was performed to remove most of the production microorganisms. After concentrating the enzyme in the ultrafilter (UF, MWCO: 6,000) process, proceed to 2 steps of microfiltration (MF, 0.45 µm, 0.2 µm). The final products, β-agarase DagA and neoagaro-oligosaccharide (NAO), were analyzed by selecting 3 lots to test for any remnants of the production strains. Colonies with different phenotypes were selected from the plate of each sample, and PCR analysis was performed by Macrogen Co., Ltd. *Streptomyces* genus was analyzed in the positive control group, and nothing was detected in the negative control group. In the final products of β-agarase DagA and NAO, *Bacillus* genus was generally analyzed, and *Streptomyces* genus was not detected. The results confirmed that there were no residues of the productive microorganisms in the final products, β-agarase DagA and NAO.

2. Introduction

Agar, a polysaccharide complex extracted from the cell walls of red algae, is considered a food ingredient in Asia (Korea, Japan, and China, among others), a GRAS food additive in the USA, and an approved food additive in Europe. An industrial process for manufacturing NAO from agar was developed using microbial β-agarase DagA from *Streptomyces coelicolor* A3(2)M22-2C43. The production strain (the bacteria) is removed from products by a series of purification processes.

After incubating β-agarase DagA, centrifugation was performed to remove most of the production microorganisms. The absence of the production microorganisms in the enzyme was confirmed using the polymerase chain reaction (PCR) method. PCR tests were performed on 3 lots of β-agarase DagA enzymes to demonstrate that Dyne Bio Inc.'s β-agarase DagA was free from contamination by host microorganisms throughout the manufacturing process.

3. Materials

Reagent	Cat No.	Manufacture
Difco^{TM} Nutrient broth	AH015-4	BD
Micro agar	LS120-212	Duchefa Biochemie

A nutrient broth medium consisting of 3 g/L beef extract, 5 g/L peptone, and 1.5% agarose was used. To make a nutrient agar (NA) plate, 8 g of the powder was suspended in 1 L of purified water and mixed thoroughly. This mixture was heated with frequent agitation and then boiled for 1 minute (min) to completely dissolve the powder, after which 1.5% micro agar was added. This was autoclaved at 121°C for 15 min and then poured (20 to 25 mL) into a petri-dish.

4. Methods

4.1 Sample selection

In the enzyme production process, it was sampled at each stage and used as a positive control group. The positive control was based on three kinds of samples: incubation, centrifugation (CF), and ultra-filtration (UF). The enzyme manufacturing process cultivates strains in a culture medium. This kind of sampling involves incubation. After incubation, the cells are removed through CF and only the supernatant is recovered for CF sampling. Then, UF is carried out to concentrate, and sampling is performed when concentration is completed. The remnants of the production strains are removed from the concentrated sample in a two-stage microfiltration (MF) process. MF is the final step in the enzyme manufacturing process. What results from this process is called β-agarase and is an end product (Figure 1).

There is also a negative control with two parts: sterilized deionized water (SDW) and culture media (RSM3 Media). The sterilized water and culture media, which are expected to be free of bacterial strains, were used as the negative controls. The RSM3 media consist of yeast extract, aga-agar powder, and magnesium chloride hexahydrate.

The final products, β-agarase and NAO, were analyzed by selecting 3 lots of each (20-NBE0128, 20-NBE0217, and 20-NBE0323) to test for any remnants of the production strains. The NAO manufactured using the enzyme as a raw material was also sampled by selection of 3 lots (20FN0301, 20FN0401, and 20FN0801) to determine if residuals of the production strains were present. The β-agarase was used in a crude solution. The NAO, a powder, was dissolved to provide a concentration of 10 mg/mL. The samples were spread on a plate and two to three colonies with different phenotypes were selected to identify strains using the PCR method (Figure 2).

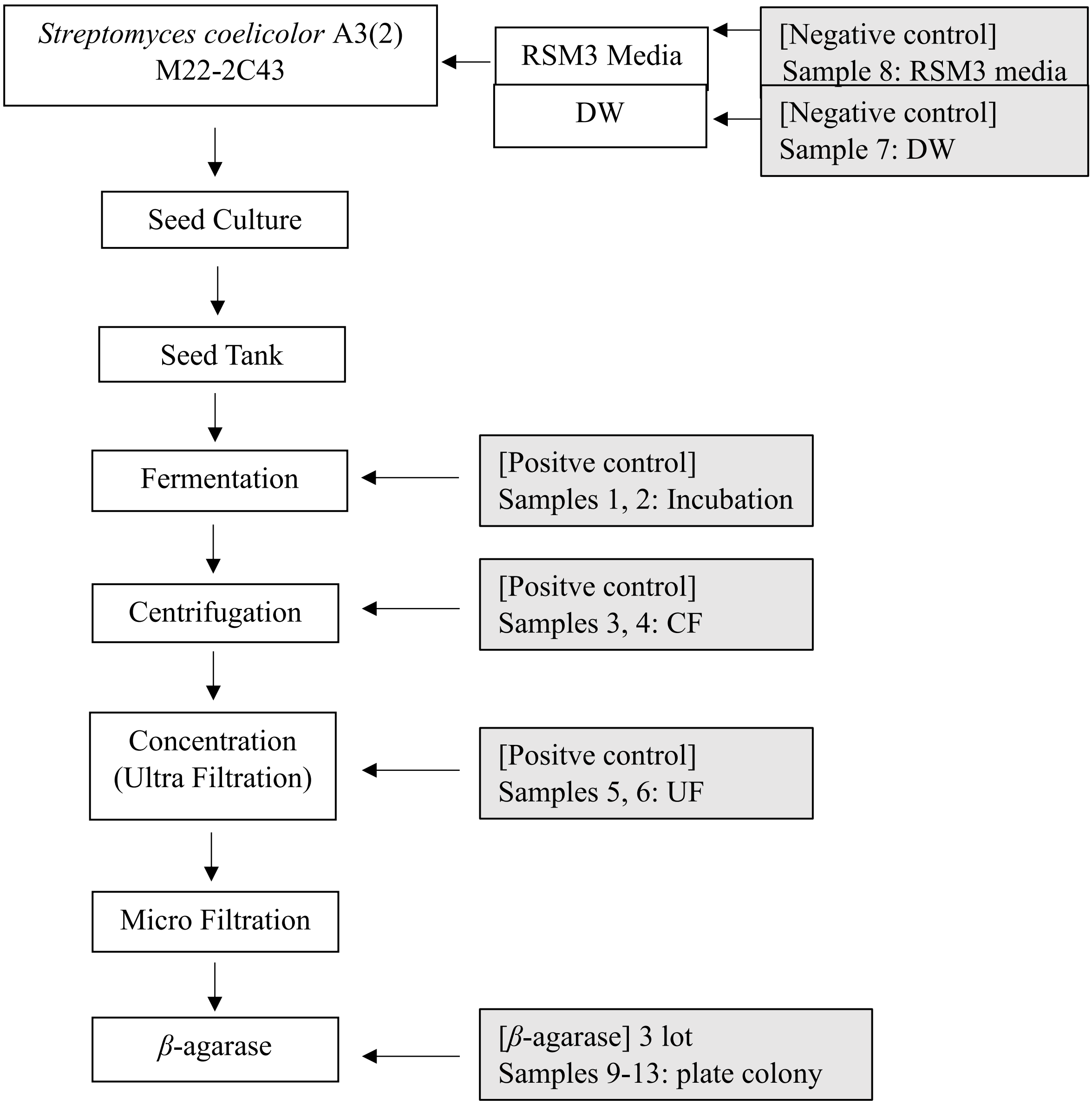


Figure 1*. Flow Chart of the β-Agarase DgA Production Process

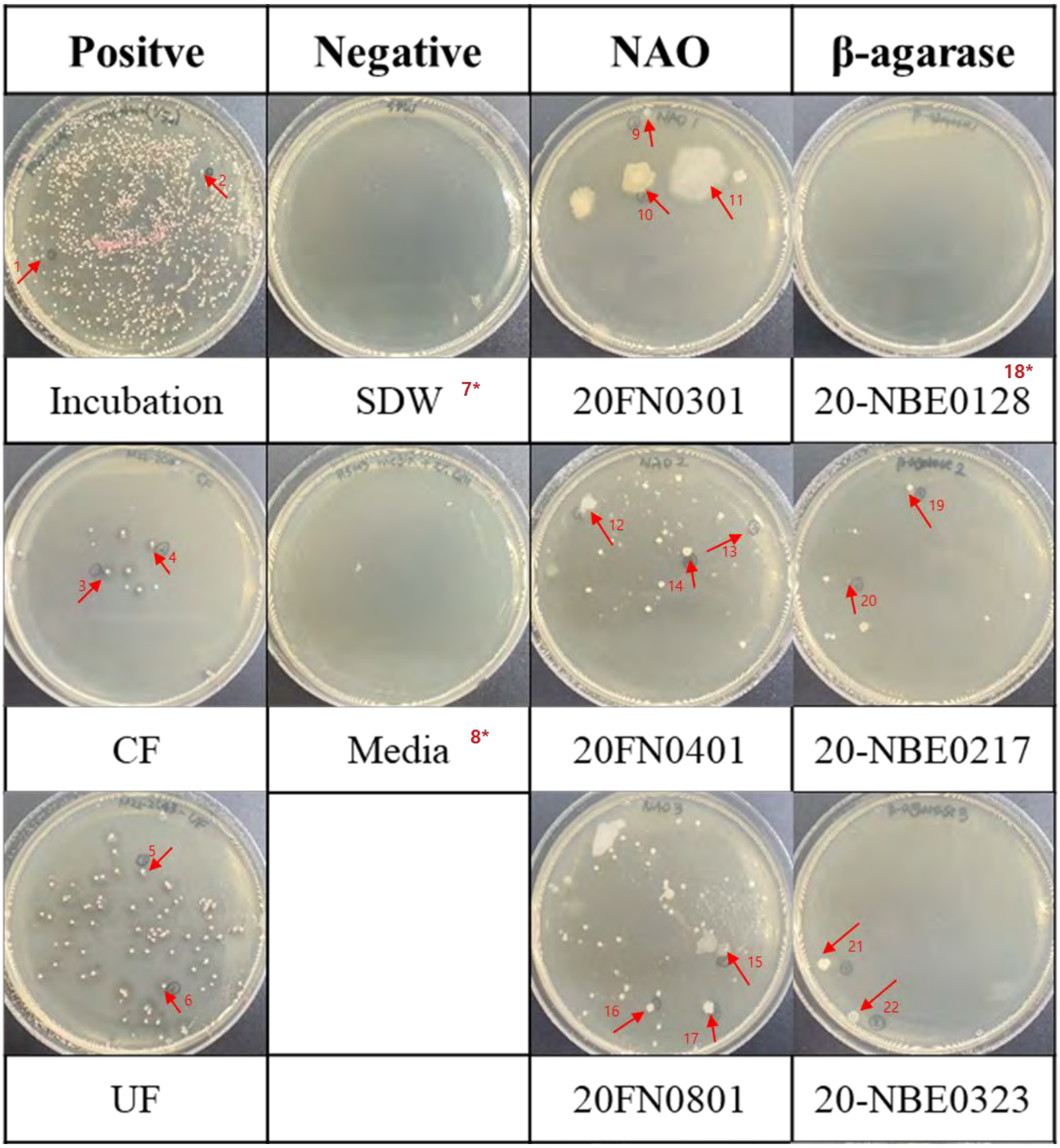


Figure 2. Sample information: Identification of microorganisms in nutrient agar (NA) plates of NAO and β-agarase DagA. *Colonies were not detected on the plate, so liquid was provided for analysis, but nothing was detected using the PCR.

4.2 Microbial identification using PCR analysis

Colonies with different phenotypes were selected from the plates for the PCR analysis requested by Macrogen Co., Ltd. The PCR analysis was performed using the universal PCR primer. The PCR primer and primer sequences were 27F 5' AGA GTT TGA TCM TGG CTC AG 3' and 1492R 5' TAC GGY TAC CTT GTT ACG ACT T 3'. The sequencing primer and primer sequences were 785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CMT TTR AGT TT 3' (Table 2). The analysis was done using the Basic Local Alignment Search Tool (BLAST) in the NCBI database to search for sequences that were obtained in sequencing and to provide BLAST reports (Table 1).

Table 1. Primer Information

PCR primer and primer sequences	
27F	5' AGA GTT TGA TCM TGG CTC AG 3'
1492R	5' TAC GGY TAC CTT GTT ACG ACT T 3'
Sequencing primer and primer sequences	
785F	5' GGA TTA GAT ACC CTG GTA 3'
907R	5' CCG TCA ATT CMT TTR AGT TT 3'

5. Results

In the positive control sample, *S. coelicolor* A3(2) M22-2C43 was analyzed with similar species, *Streptomyces coelescens* and *Streptomyces tricolor* (Figures 3–8).

The negative control samples (DW and media) and β-agarase (20-NBE0128) showed no colonies on the plates. It was confirmed that there were no remnants of the production strain (*Streptomyces* species) in the final product. No colonies were detected on the plate, so it was provided in a liquid form for analysis, but nothing was detected using the PCR.

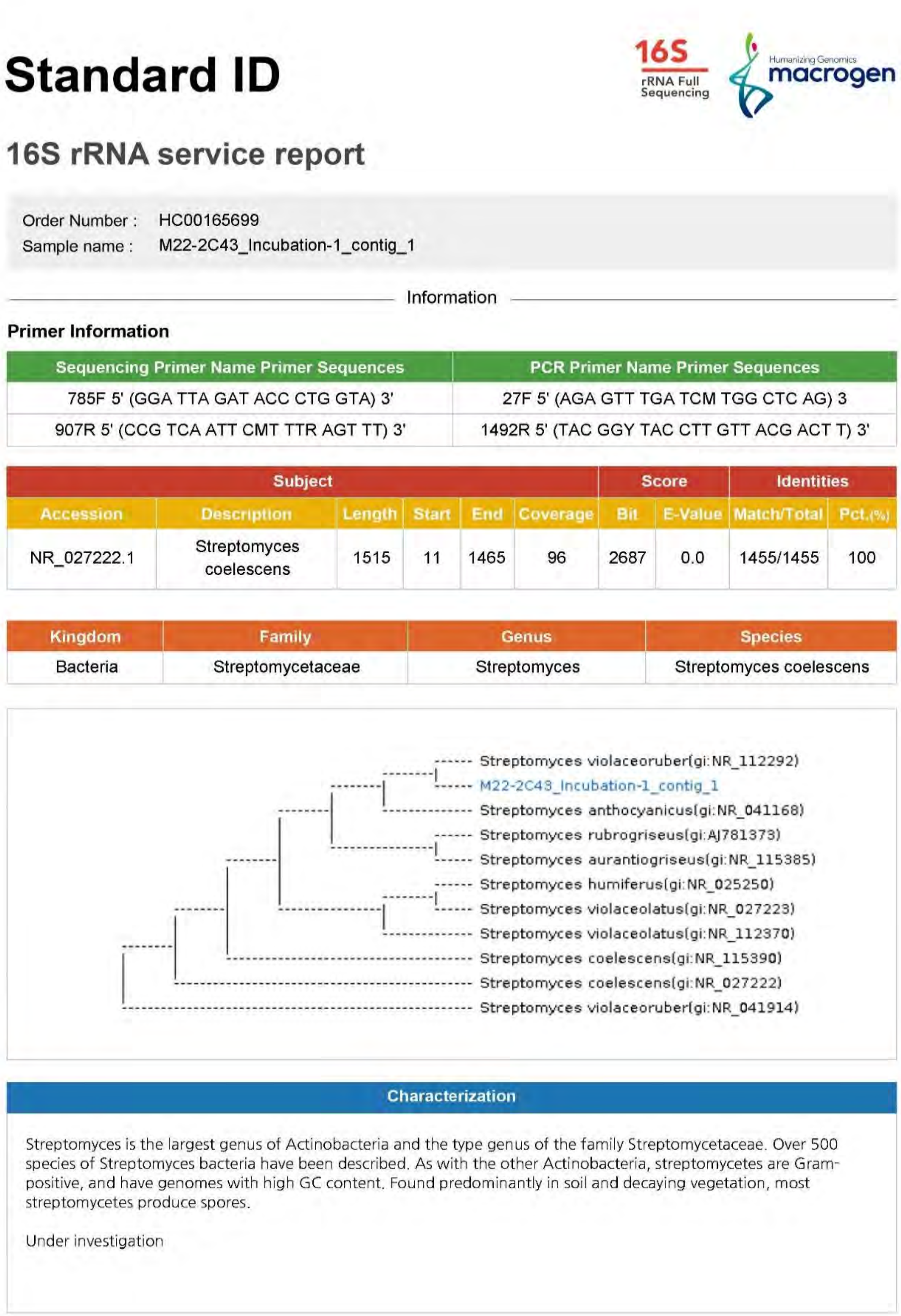
In the NAO sample, *Streptomyce*s species, the production strains, were not found; only *Bacillus* species were found (Figures 9-17). It was confirmed that the production microorganisms had been removed.

In the β-agarase sample, *Streptomyce*s species (production microorganisms) were not found, but *Bacillus* and *Pseudomonas* species were found (Figure 18-21). It was confirmed that the production microorganisms had been removed by the MF process, the last step of the β-agarase process (Table 2).

Table 2. Identification of microorganisms using the PCR method

No.	Sample lot		Sample name	Species
1	Positive control		Incubation-1	*Streptomyces coelescens*
2			Incubation-2	*Streptomyces coelescens*
3			CF-1	*Streptomyces tricolor*
4			CF-2	*Streptomyces coelescens*
5			UF-1	*Streptomyces coelescens*
6			UF-2	*Streptomyces coelescens*
7	Negative control		DW*	*Not detected*
8			Media*	*Not detected*
9	NAO	20FN0301	NAO1-1	*Bacillus wiedmannii*
10			NAO1-2	*Bacillus flexus*
11			NAO1-3	*Bacillus flexus*
12		20FN0401	NAO2-1	*Bacillus subtilis*
13			NAO2-2	*Bacillus subtilis*
14			NAO2-3	*Bacillus megaterium*
15		20FN0801	NAO3-1	*Bacillus siamensis*
16			NAO3-2	*Bacillus megaterium*
17			NAO3-3	*Bacillus flexus*
18	β-agarase	20-NBE0128	β-agarase1*	*Not detected*
19		20-NBE0217	β-agarase2-1	*Bacillus flexus*
20			β-agarase2-2	*Bacillus nealsonii*
21		20-NBE0323	β-agarase3-1	*Pseudomonas rhodesiae*
22			β-agarase3-2	*Pseudomonas rhodesiae*

*Colonies were not detected on the plate, so liquid was provided for analysis, but nothing was detected using the PCR.



Standard ID

16S rRNA service report

Order Number : HC00165699
Sample name : M22-2C43_Incubation-1_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_027222.1	Streptomyces coelescens	1515	11	1465	96	2687	0.0	1455/1455	100

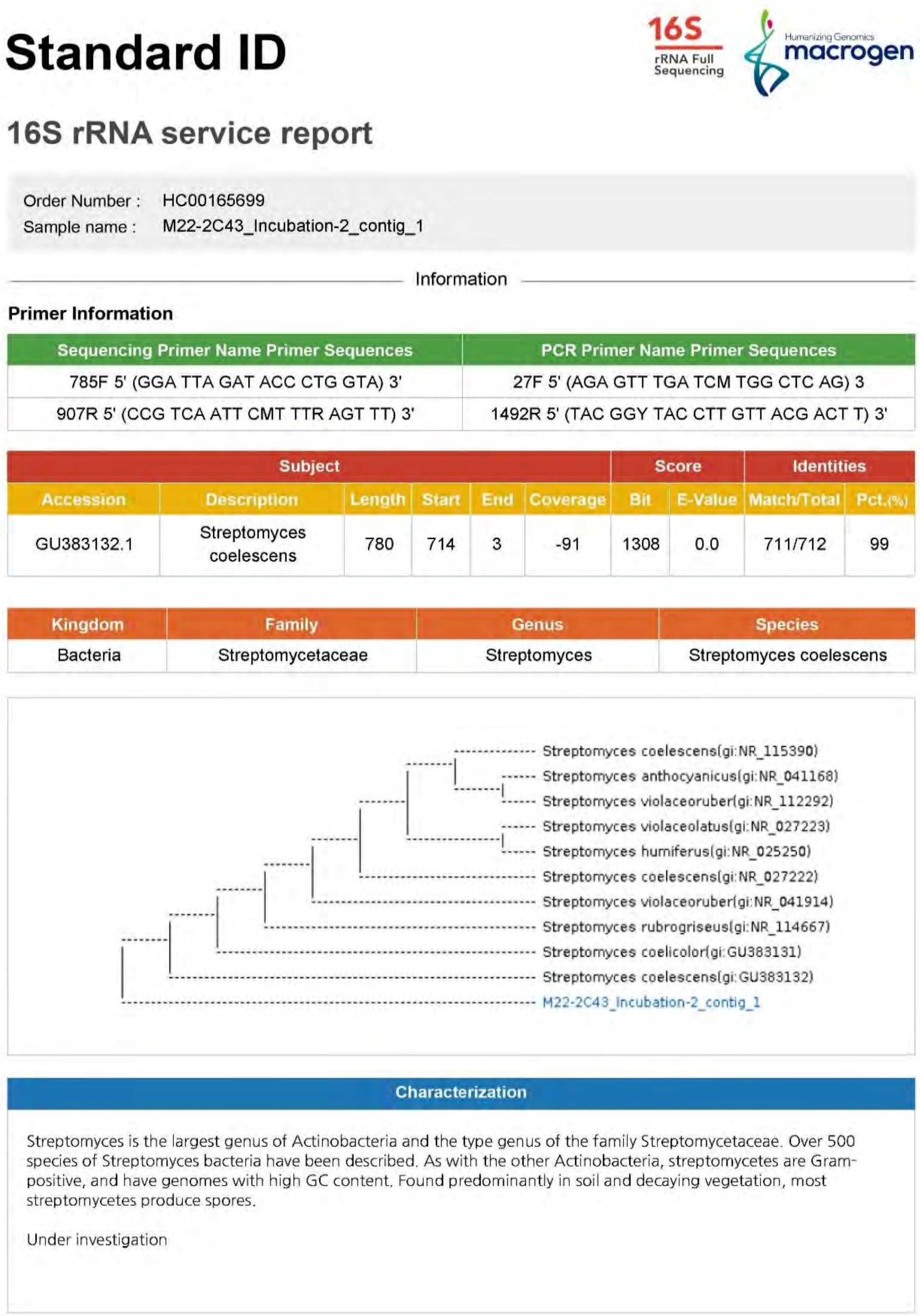
Kingdom	Family	Genus	Species
Bacteria	Streptomycetaceae	Streptomyces	Streptomyces coelescens

Characterization

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae. Over 500 species of Streptomyces bacteria have been described. As with the other Actinobacteria, streptomycetes are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores.

Under investigation

Figure 3. PCR analysis result of positive control Sample 1



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : M22-2C43_Incubation-2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
GU383132.1	Streptomyces coelescens	780	714	3	-91	1308	0.0	711/712	99

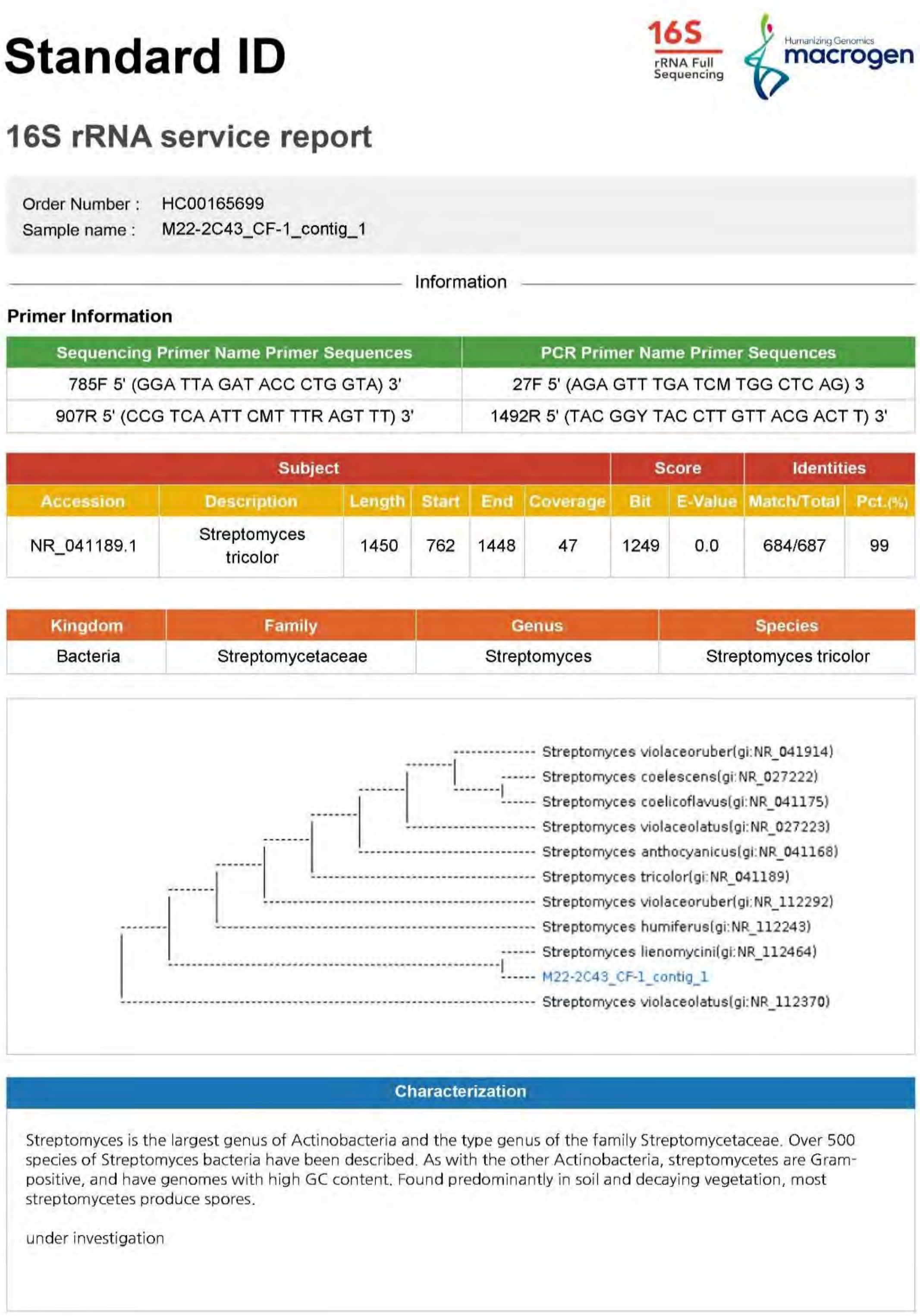
Kingdom	Family	Genus	Species
Bacteria	Streptomycetaceae	Streptomyces	Streptomyces coelescens

Characterization

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae. Over 500 species of Streptomyces bacteria have been described. As with the other Actinobacteria, streptomycetes are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores.

Under investigation

Figure 4. PCR analysis result for positive control Sample 2



Standard ID

16S rRNA service report

Order Number : HC00165699
Sample name : M22-2C43_CF-1_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_041189.1	Streptomyces tricolor	1450	762	1448	47	1249	0.0	684/687	99

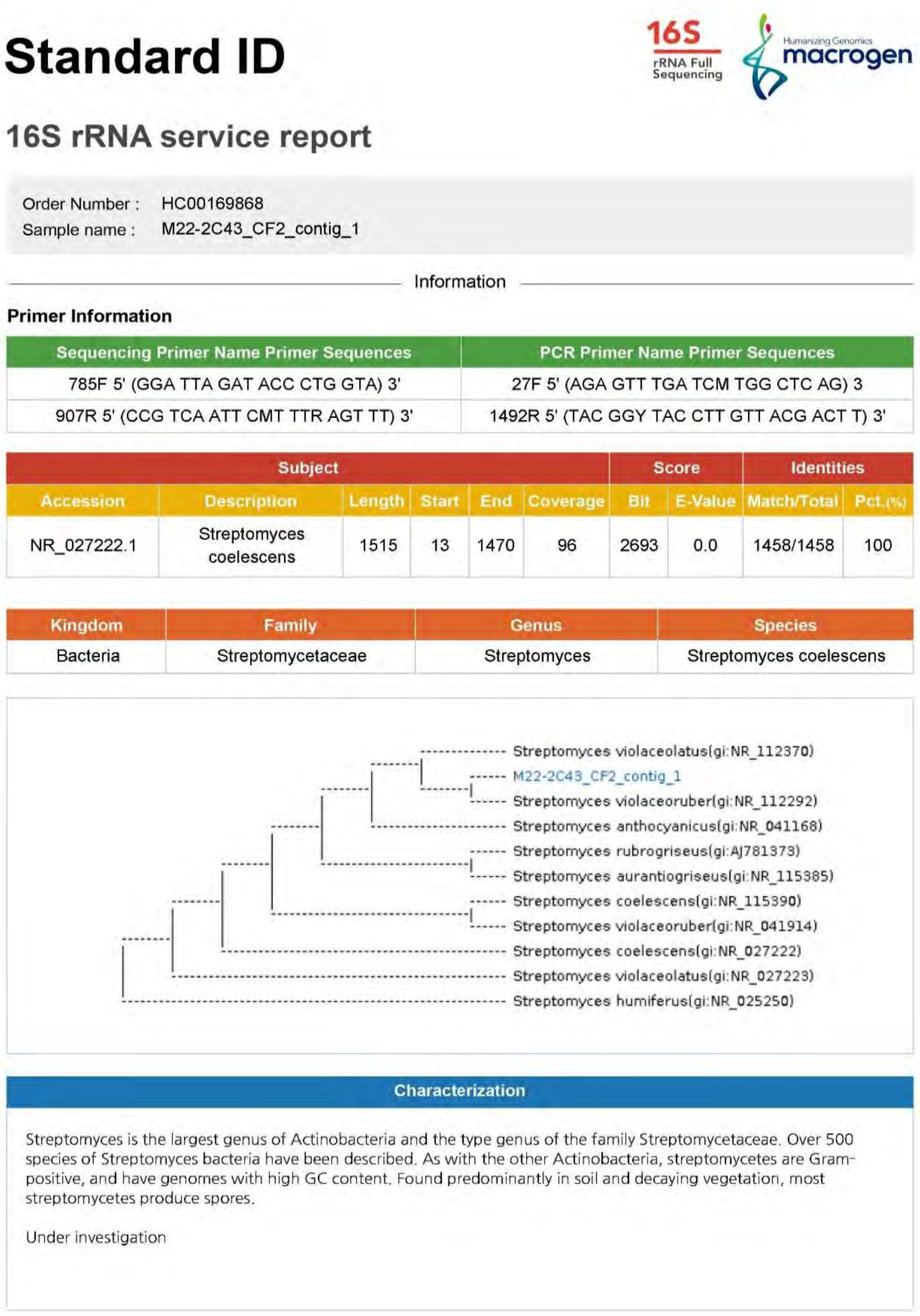
Kingdom	Family	Genus	Species
Bacteria	Streptomycetaceae	Streptomyces	Streptomyces tricolor

Characterization

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae. Over 500 species of Streptomyces bacteria have been described. As with the other Actinobacteria, streptomycetes are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores.

under investigation

Figure 5. PCR analysis result of positive control Sample 3



Standard ID

16S rRNA service report

Order Number : HC00169868
Sample name : M22-2C43_CF2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_027222.1	Streptomyces coelescens	1515	13	1470	96	2693	0.0	1458/1458	100

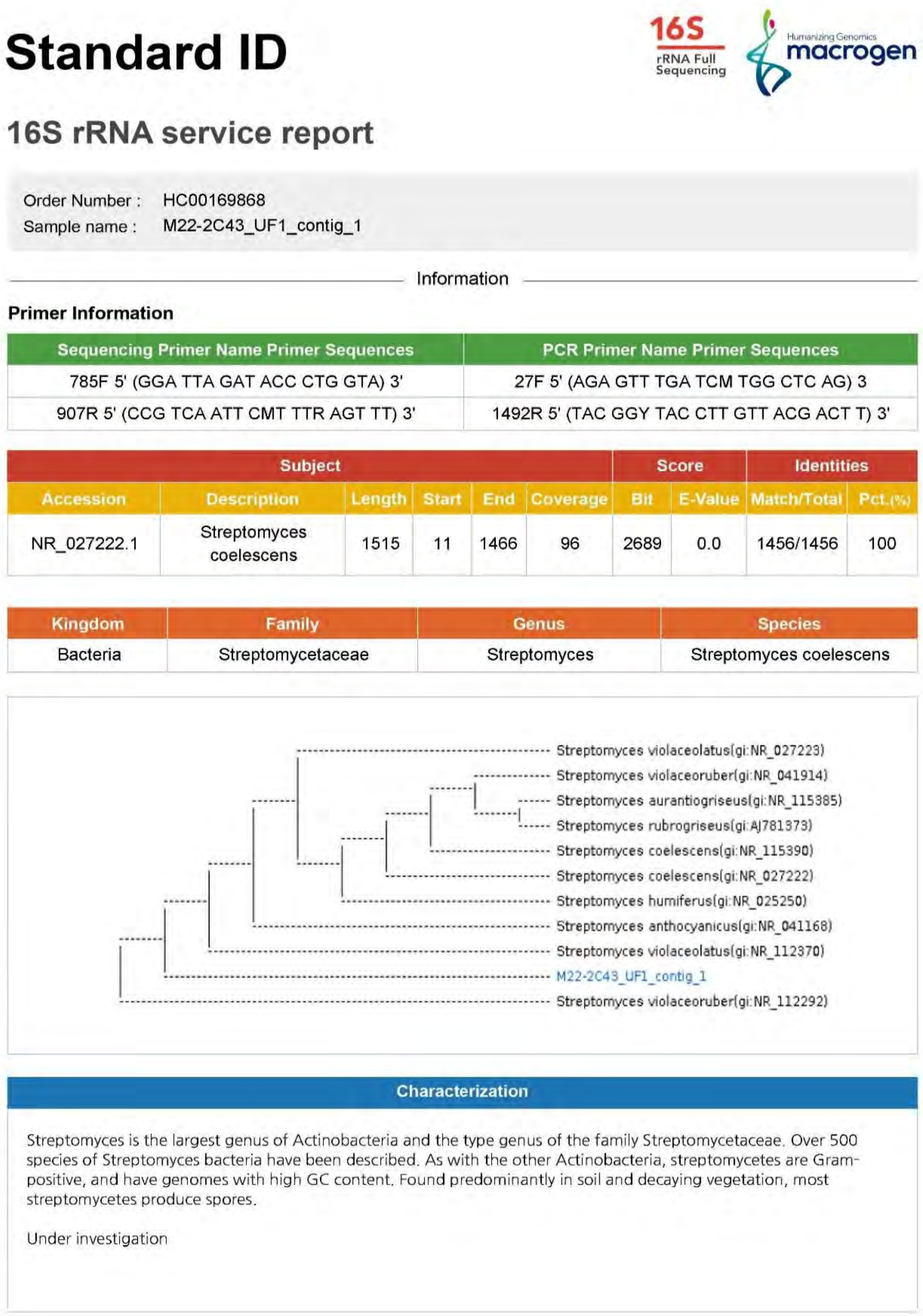
Kingdom	Family	Genus	Species
Bacteria	Streptomycetaceae	Streptomyces	Streptomyces coelescens

Characterization

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae. Over 500 species of Streptomyces bacteria have been described. As with the other Actinobacteria, streptomycetes are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores.

Under investigation

Figure 6. PCR analysis result of positive control Sample 4



Standard ID

16S rRNA service report

Order Number : HC00169868
Sample name : M22-2C43_UF1_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_027222.1	Streptomyces coelescens	1515	11	1466	96	2689	0.0	1456/1456	100

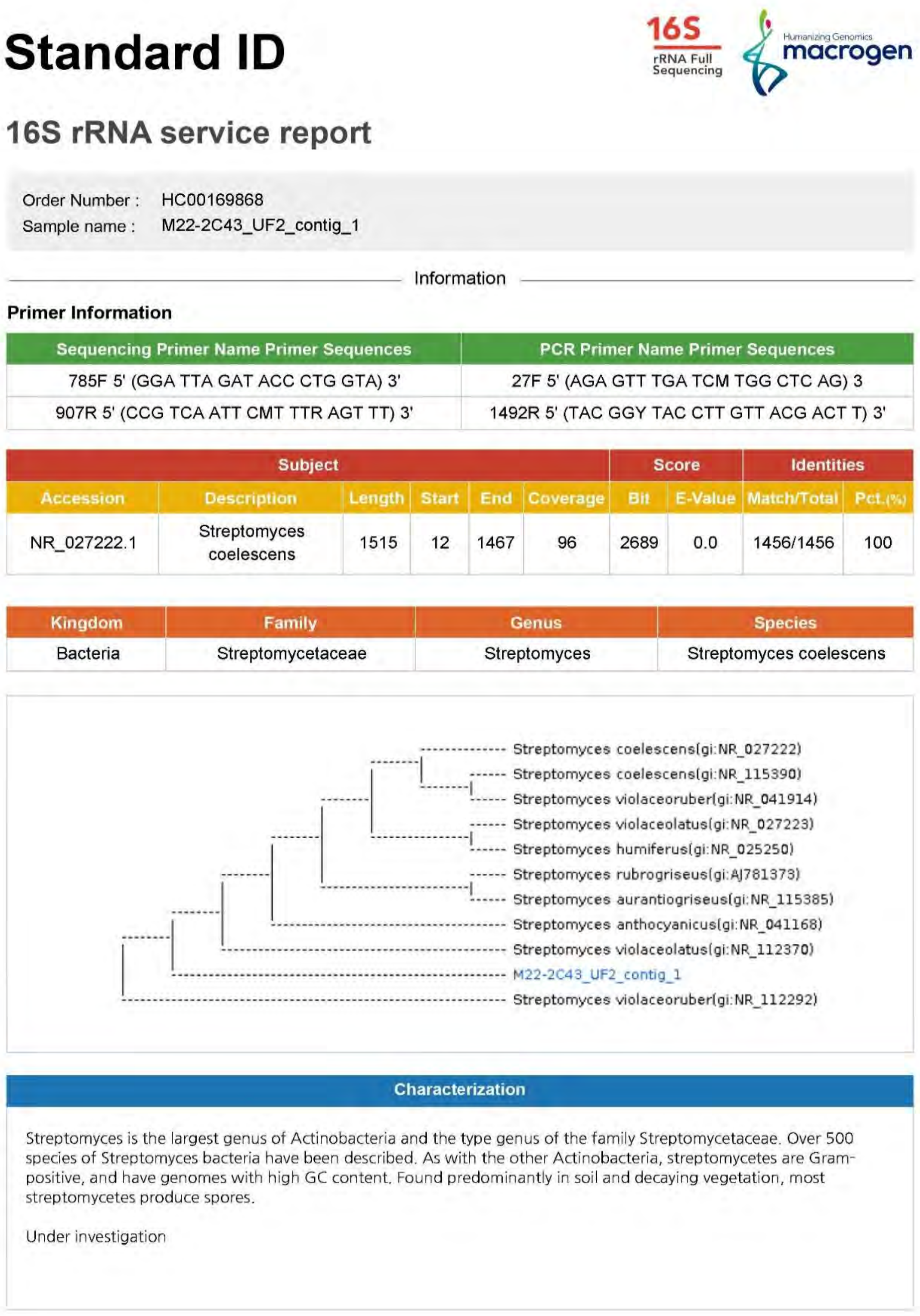
Kingdom	Family	Genus	Species
Bacteria	Streptomycetaceae	Streptomyces	Streptomyces coelescens

Characterization

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae. Over 500 species of Streptomyces bacteria have been described. As with the other Actinobacteria, streptomycetes are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores.

Under investigation

Figure 7. PCR analysis result of positive control Sample 5



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00169868
Sample name : M22-2C43_UF2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_027222.1	Streptomyces coelescens	1515	12	1467	96	2689	0.0	1456/1456	100

Kingdom	Family	Genus	Species
Bacteria	Streptomycetaceae	Streptomyces	Streptomyces coelescens

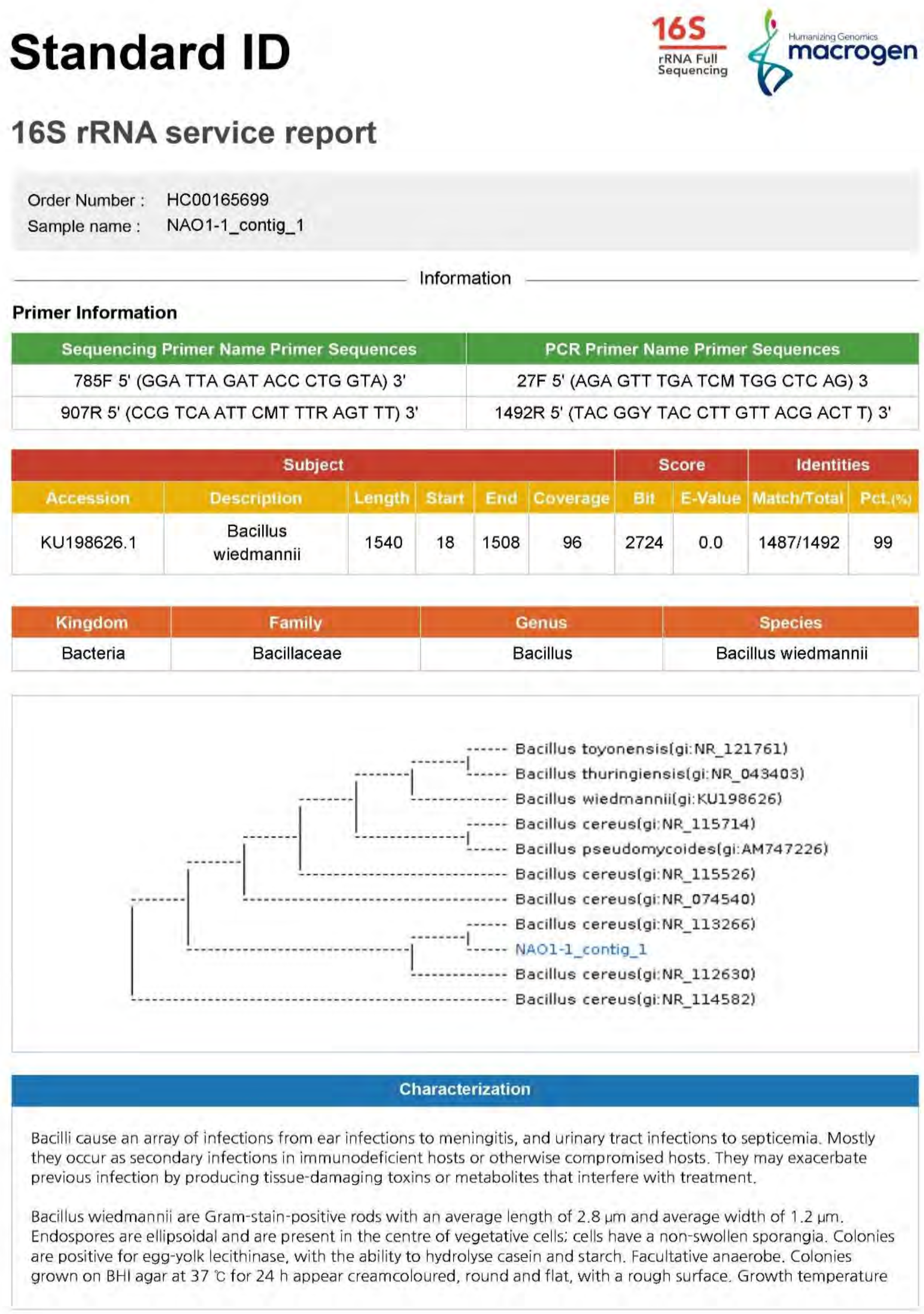
Streptomyces coelescens(gi:NR_027222)
Streptomyces coelescens(gi:NR_115390)
Streptomyces violaceoruber(gi:NR_041914)
Streptomyces violaceolatus(gi:NR_027223)
Streptomyces humiferus(gi:NR_025250)
Streptomyces rubrogriseus(gi:AJ781373)
Streptomyces aurantiogriseus(gi:NR_115385)
Streptomyces anthocyanicus(gi:NR_041168)
Streptomyces violaceolatus(gi:NR_112370)
M22-2C43_UF2_contig_1
Streptomyces violaceoruber(gi:NR_112292)

Characterization

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae. Over 500 species of Streptomyces bacteria have been described. As with the other Actinobacteria, streptomycetes are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores.

Under investigation

Figure 8. PCR analysis result of positive control Sample 6



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : NAO1-1_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
KU198626.1	Bacillus wiedmannii	1540	18	1508	96	2724	0.0	1487/1492	99

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus wiedmannii

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Bacillus wiedmannii are Gram-stain-positive rods with an average length of 2.8 μm and average width of 1.2 μm. Endospores are ellipsoidal and are present in the centre of vegetative cells; cells have a non-swollen sporangia. Colonies are positive for egg-yolk lecithinase, with the ability to hydrolyse casein and starch. Facultative anaerobe. Colonies grown on BHI agar at 37 ℃ for 24 h appear creamcoloured, round and flat, with a rough surface. Growth temperature

Figure 9. PCR analysis result of NAO Sample 9

Standard ID

16S rRNA Full Sequencing

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16S rRNA service report

Order Number : HC00165699
Sample name : NAO1-2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_024691.1	Bacillus flexus	1529	8	1507	98	2760	0.0	1499/1501	99

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus flexus

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Gram-variable, rods, 0.9um mean cell width, presenting centrally/paracentrally, ellipsoidal endospores in unswollen sporangia.

Figure 10. PCR analysis result of NAO Sample 10

Standard ID

16S rRNA Full Sequencing

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16S rRNA service report

Order Number : HC00165699
Sample name : NAO1-3_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_024691.1	Bacillus flexus	1529	8	1502	97	2743	0.0	1493/1496	99

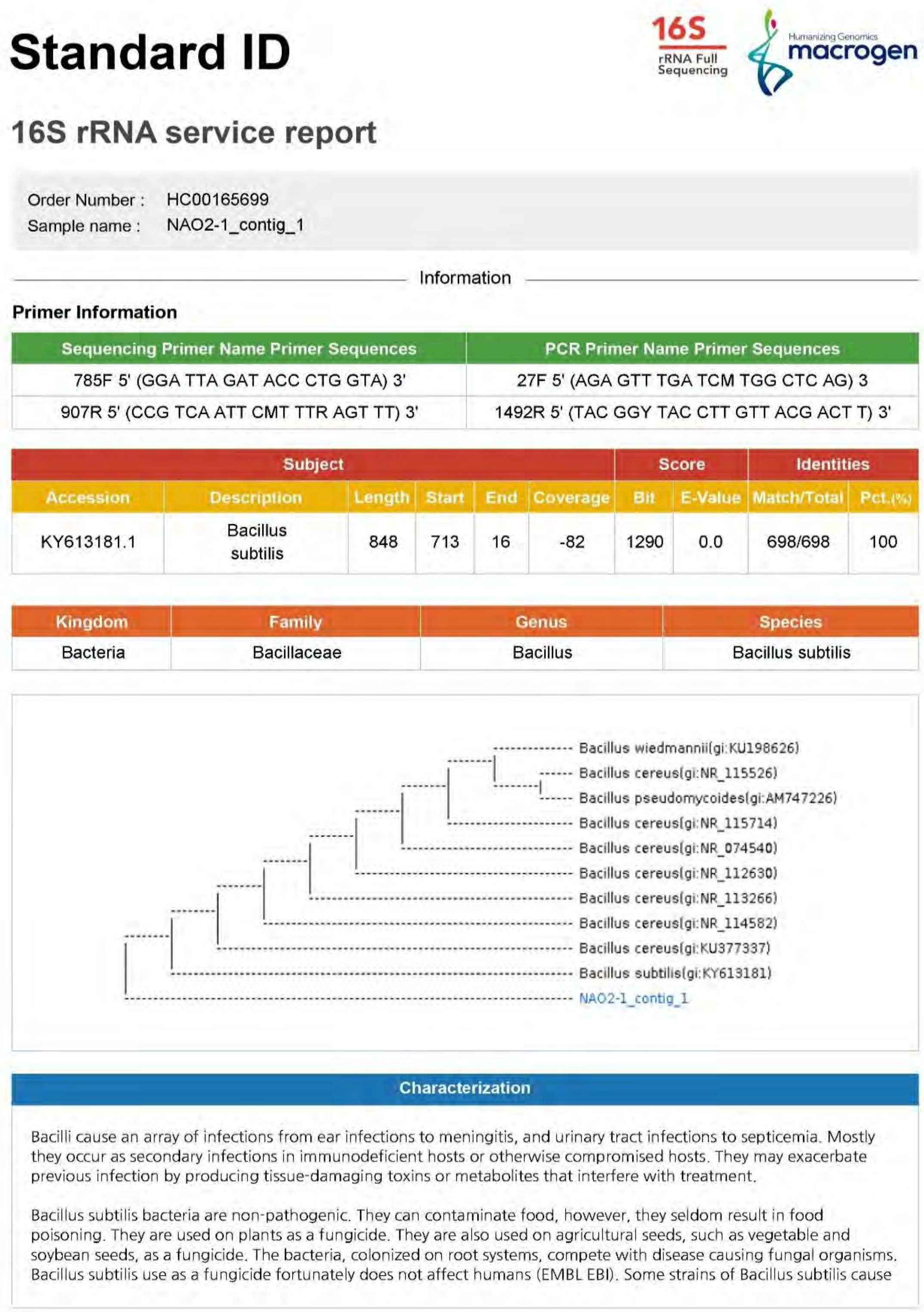
Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus flexus

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Gram-variable, rods, 0.9um mean cell width, presenting centrally/paracentrally, ellipsoidal endospores in unswollen sporangia.

Figure 11. PCR analysis result of NAO Sample 11



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : NAO2-1_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
KY613181.1	Bacillus subtilis	848	713	16	-82	1290	0.0	698/698	100

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus subtilis

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Bacillus subtilis bacteria are non-pathogenic. They can contaminate food, however, they seldom result in food poisoning. They are used on plants as a fungicide. They are also used on agricultural seeds, such as vegetable and soybean seeds, as a fungicide. The bacteria, colonized on root systems, compete with disease causing fungal organisms. Bacillus subtilis use as a fungicide fortunately does not affect humans (EMBL EBI). Some strains of Bacillus subtilis cause

Figure 12. PCR analysis result of NAO Sample 12

Standard ID

16S rRNA service report

Order Number : HC00165699
Sample name : NAO2-2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP020102.1	Bacillus subtilis	4215607	97214	96453	0	1402	0.0	762/763	99

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus subtilis

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Bacillus subtilis bacteria are non-pathogenic. They can contaminate food, however, they seldom result in food poisoning. They are used on plants as a fungicide. They are also used on agricultural seeds, such as vegetable and soybean seeds, as a fungicide. The bacteria, colonized on root systems, compete with disease causing fungal organisms. Bacillus subtilis use as a fungicide fortunately does not affect humans (EMBL EBI). Some strains of Bacillus subtilis cause

Figure 13. PCR analysis result of NAO Sample 13

Standard ID

16S rRNA service report

Order Number : HC00165699
Sample name : NAO2-3_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP009920.1	Bacillus megaterium	5343114	2157087	2156324	0	1349	0.0	757/769	98

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus megaterium

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Bacillus megaterium is a rod-like, Gram-positive, mainly aerobic spore forming bacterium found in widely diverse habitats. With a cell length of up to 4 ?m and a diameter of 1.5 ?m, B. megaterium is amongst the biggest known bacteria. The cells often occur in pairs and chains, where the cells are joined together by polysaccharides on the cell walls.

Figure 14. PCR analysis result of NAO Sample 14

Standard ID

16S
rRNA Full Sequencing

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macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : NAO3-1_contig_1

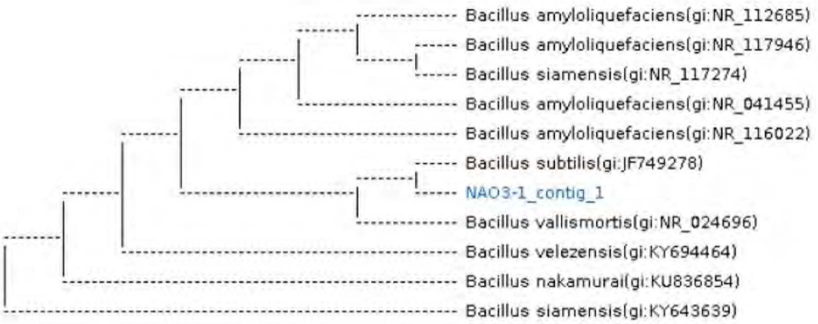
Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
KY643639.1	Bacillus siamensis	1492	764	2	-51	1288	0.0	747/770	97

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus siamensis

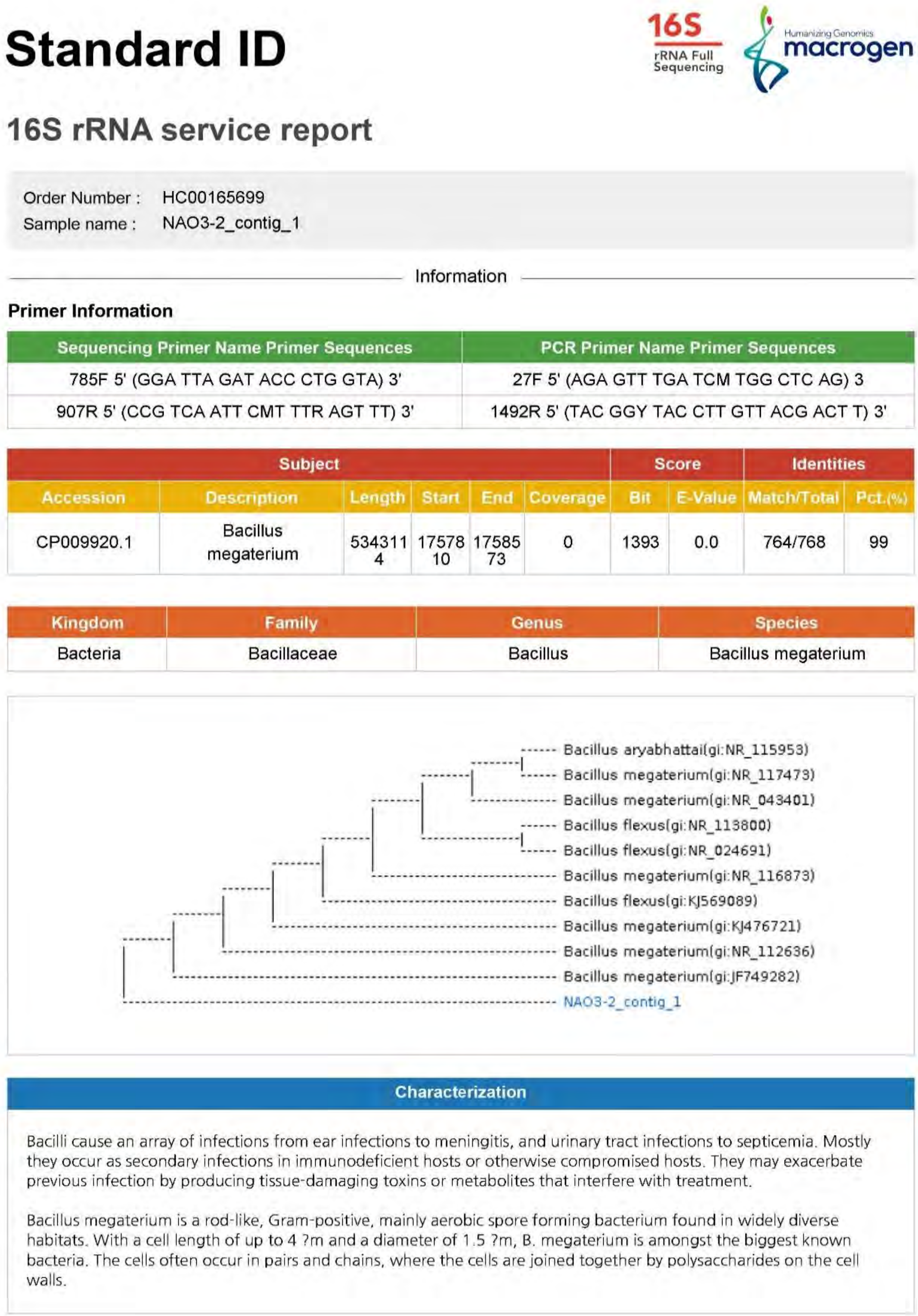


Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Bacillus siamensis, Cells are Gram-positive, facultatively anaerobic and rod-shaped, measuring 0.3?0.6×1.5?3.5 um. Cells occur singly, in pairs and occasionally in short chains. They are motile with peritrichous flagella. Ellipsoidal endospores are produced at central or subterminal positions in swollen sporangia. Colonies are creamy white, mucoid, translucent, raised, have an entire margin and are 3?4 mm in diameter after 2 days incubation at 37 C on TSA. In liquid

Figure 15. PCR analysis result of NAO Sample 15



Standard ID

16S rRNA service report

Order Number : HC00165699
Sample name : NAO3-2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP009920.1	Bacillus megaterium	5343114	1757810	1758573	0	1393	0.0	764/768	99

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus megaterium

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Bacillus megaterium is a rod-like, Gram-positive, mainly aerobic spore forming bacterium found in widely diverse habitats. With a cell length of up to 4 ?m and a diameter of 1.5 ?m, B. megaterium is amongst the biggest known bacteria. The cells often occur in pairs and chains, where the cells are joined together by polysaccharides on the cell walls.

Figure 16. PCR analysis result of NAO Sample 16

Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : NAO3-3_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_024691.1	Bacillus flexus	1529	10	1502	97	2739	0.0	1490/1493	99

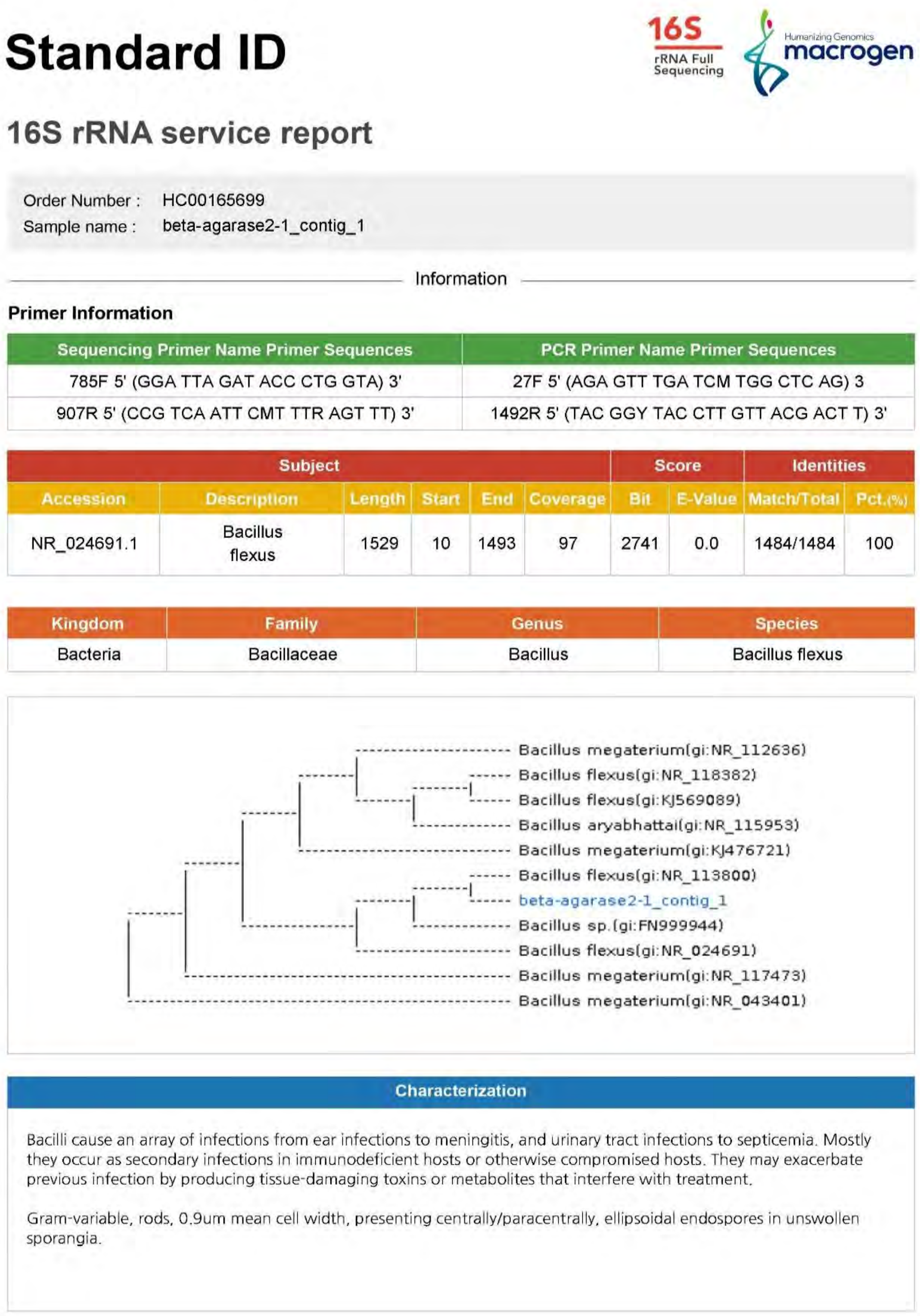
Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus flexus

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Gram-variable, rods, 0.9um mean cell width, presenting centrally/paracentrally, ellipsoidal endospores in unswollen sporangia.

Figure 17. PCR analysis result of NAO Sample 17



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : beta-agarase2-1_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_024691.1	Bacillus flexus	1529	10	1493	97	2741	0.0	1484/1484	100

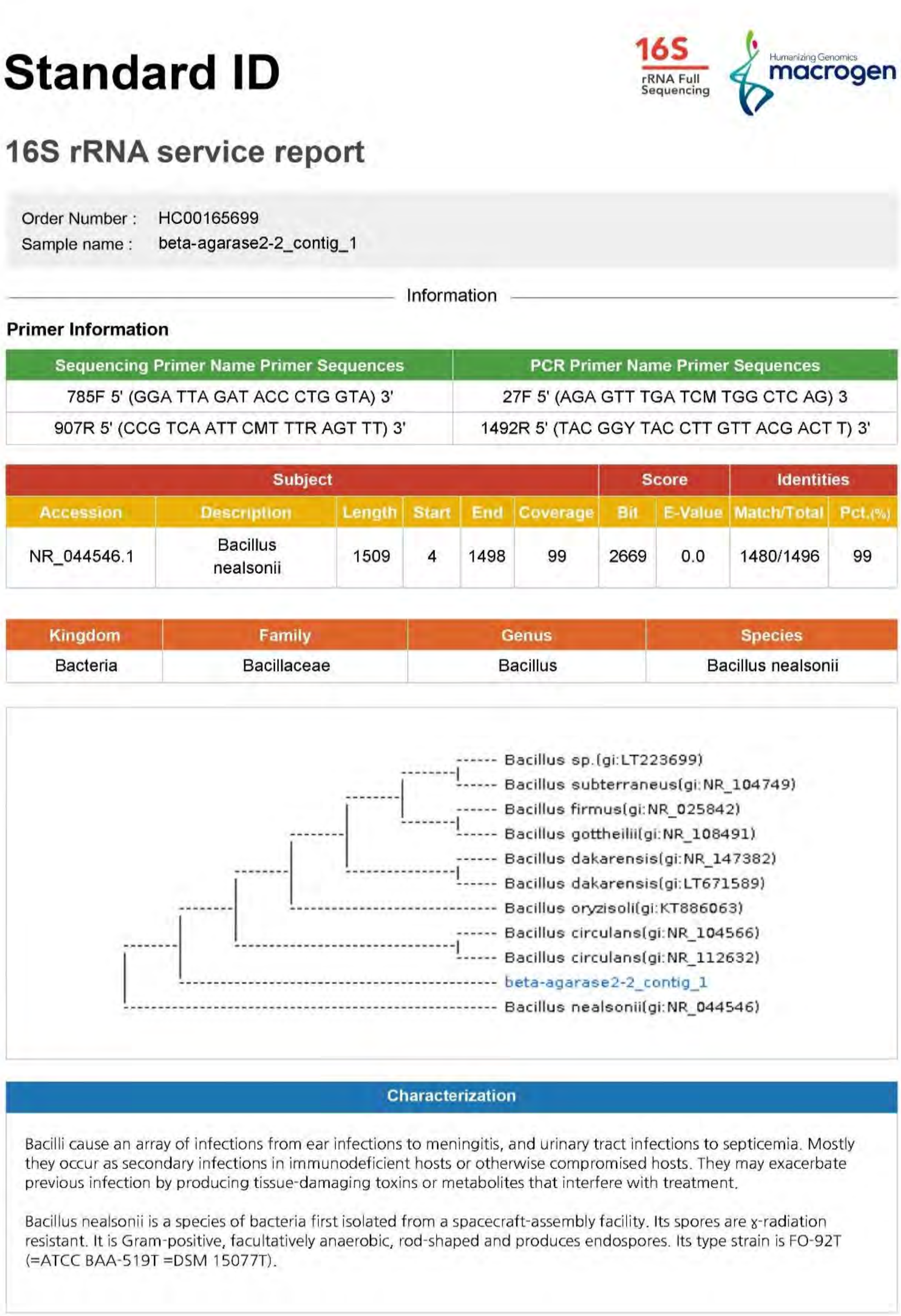
Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus flexus

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Gram-variable, rods, 0.9um mean cell width, presenting centrally/paracentrally, ellipsoidal endospores in unswollen sporangia.

Figure 18. PCR analysis result of β-agarase Sample 19



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : beta-agarase2-2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_044546.1	Bacillus nealsonii	1509	4	1498	99	2669	0.0	1480/1496	99

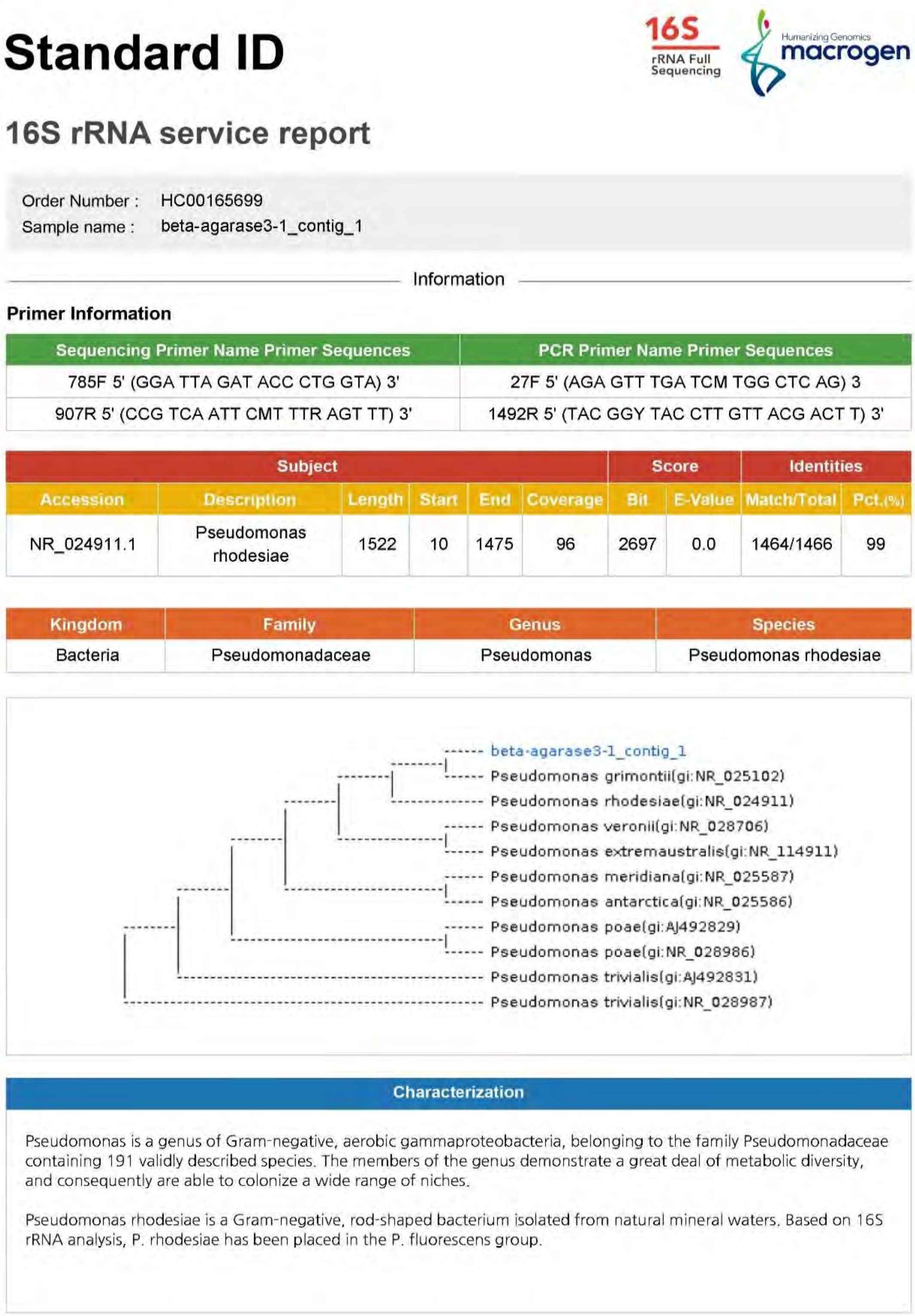
Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus nealsonii

Characterization

Bacilli cause an array of infections from ear infections to meningitis, and urinary tract infections to septicemia. Mostly they occur as secondary infections in immunodeficient hosts or otherwise compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment.

Bacillus nealsonii is a species of bacteria first isolated from a spacecraft-assembly facility. Its spores are γ-radiation resistant. It is Gram-positive, facultatively anaerobic, rod-shaped and produces endospores. Its type strain is FO-92T (=ATCC BAA-519T =DSM 15077T).

Figure 19. PCR analysis result of β-agarase Sample 20



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : beta-agarase3-1_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_024911.1	Pseudomonas rhodesiae	1522	10	1475	96	2697	0.0	1464/1466	99

Kingdom	Family	Genus	Species
Bacteria	Pseudomonadaceae	Pseudomonas	Pseudomonas rhodesiae

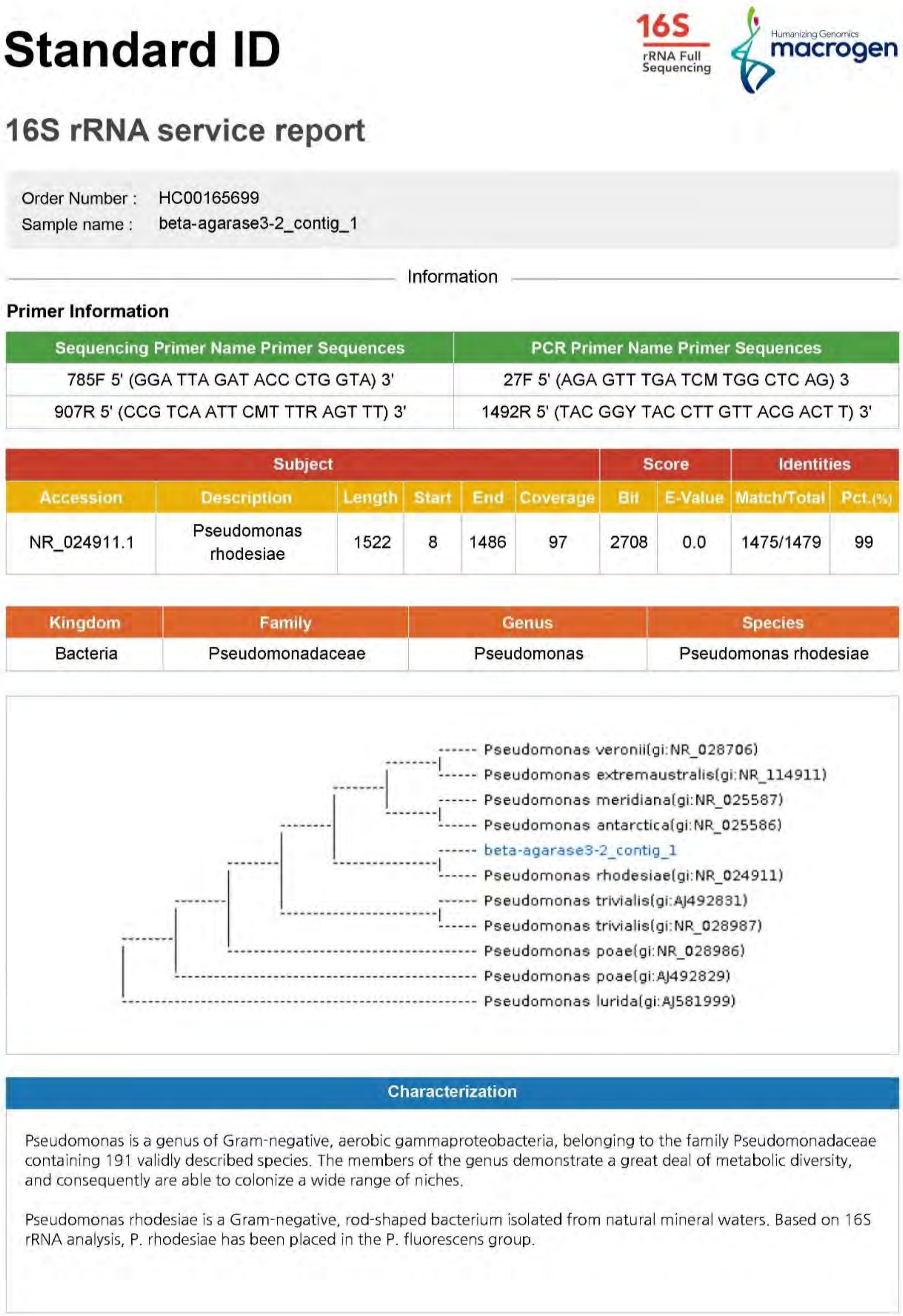
beta-agarase3-1_contig_1
Pseudomonas grimontii(gi:NR_025102)
Pseudomonas rhodesiae(gi:NR_024911)
Pseudomonas veronii(gi:NR_028706)
Pseudomonas extremaustralis(gi:NR_114911)
Pseudomonas meridiana(gi:NR_025587)
Pseudomonas antarctica(gi:NR_025586)
Pseudomonas poae(gi:AJ492829)
Pseudomonas poae(gi:NR_028986)
Pseudomonas trivialis(gi:AJ492831)
Pseudomonas trivialis(gi:NR_028987)

Characterization

Pseudomonas is a genus of Gram-negative, aerobic gammaproteobacteria, belonging to the family Pseudomonadaceae containing 191 validly described species. The members of the genus demonstrate a great deal of metabolic diversity, and consequently are able to colonize a wide range of niches.

Pseudomonas rhodesiae is a Gram-negative, rod-shaped bacterium isolated from natural mineral waters. Based on 16S rRNA analysis, P. rhodesiae has been placed in the P. fluorescens group.

Figure 20. PCR analysis result of β-agarase Sample 21



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : HC00165699
Sample name : beta-agarase3-2_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_024911.1	Pseudomonas rhodesiae	1522	8	1486	97	2708	0.0	1475/1479	99

Kingdom	Family	Genus	Species
Bacteria	Pseudomonadaceae	Pseudomonas	Pseudomonas rhodesiae

Characterization

Pseudomonas is a genus of Gram-negative, aerobic gammaproteobacteria, belonging to the family Pseudomonadaceae containing 191 validly described species. The members of the genus demonstrate a great deal of metabolic diversity, and consequently are able to colonize a wide range of niches.

Pseudomonas rhodesiae is a Gram-negative, rod-shaped bacterium isolated from natural mineral waters. Based on 16S rRNA analysis, P. rhodesiae has been placed in the P. fluorescens group.

Figure 21. PCR analysis result of β-agarase Sample 22

6. Conclusion

Streptomyces genus was analyzed in the positive control group, and nothing was detected in the negative control group. In the final products of β-agarase and NAO, *Bacillus* genus were generally analyzed, and *Streptomyce*s genus was not detected. The results confirmed that there were no residues of the productive microorganisms in the final products, β-agarase and NAO.

Appendix F. Stability of β-Agarase DagA

Authors: Hye-Jeong KO, Eun Joo Kim, Je Hyeon Lee

Institution: Dyne Bio Inc., Gyeonggi-Do, 13209, Republic of Korea

Date: April 18, 2022

1. Abstract

The purpose of this study was to verify the stability of β-agarase DagA secreted by *Streptomyces coelicolor* A3(2) M22-2C43. The β-agarase DagA activity was tested for stability at two temperatures. The following microbiological tests were done to test for pathogenic microorganisms: total plate count and counts (cfu/g) of yeast and mold, coliform and *Escherichia coli, Staphylococcus aureus*, and *Salmonella*. The analysis was performed for 42 days at 4°C in an accelerated-term test and for 12 months at -20°C in a long-term test. The study was performed with three 100 mL batches of β-agarase DagA in high-density polyethylene (HDPE) bottles. The results show that the enzyme activity in the acceleration test was maintained for 42 days. Microorganisms were not analyzed from the beginning to the last sample because the samples themselves were expected to be completely eradicated. In the long-term test, enzyme activity was maintained the same as the result in the acceleration test, and microorganisms were not analyzed during the long-term test. The results confirm that microorganisms remained eradicated, and the enzyme activity was maintained during the acceleration and long-term tests of β-agarase DagA. Therefore, the stability during the test period can be guaranteed.

2. Introduction

The enzyme, β-agarase DagA, is derived from a non-genetically modified *Streptomyces coelicolor* A3(2) M22-2C43 strain, an ultraviolet-treated mutant strain from the wild type of *S. coelicolor* A3(2). β-Agarase DagA is used in various food industries, especially in different applications by Dyne Bio Inc. This enzyme is used to produce neoagaro-oligosaccharide (NAO); β-agarase DagA decomposes the polysaccharides of agar into complex monosaccharides. To be used as a food additive, it is necessary to verify the stability of β-agarase. It is usually frozen and stored. This study observed whether the β-agarase enzyme activity was maintained for a certain period in frozen (-20°C) and refrigerated conditions (4°C) and tested for the presence of pathogenic microorganisms. Microbiological tests were done to determine the total plate count and counts (cfu/g) of yeast and fungi, coliform and *E. coli*, *Staphylococcus aureus*, and *Salmonella*.

3. Materials

3.1. Sample

The sample used three discontinuous batches produced by the GMP facility of Dyne Bio Inc. The study was performed using three 100 mL batches of β-agarase DagA in high density polyethylene (HDPE) bottles. Other than β-agarase DagA, no other additives were included.

Table 1. Batch information

Batch No.	20-NBE1120	21-NBE0122	21-NBE0226
Manufacturer	Jeonbuk Institute for Food-Bioindustry		
Date of manufacture	Nov 20th, 2020	Jan 22th, 2021	Feb 26th, 2021
Site of manufacture	Pilot Plant	Pilot Plant	Pilot Plant
Scale of manufacture	Pilot Scale	Pilot Scale	Pilot Scale
Batch size	180 L	180 L	180 L
Date of packaging	Nov 20th, 2020	Jan 22th, 2021	Feb 26th, 2021

3.2. Sample test date

In the acceleration test, the analysis was performed every 7 days for 42 days at 4°C, and in the long-term test, the analysis was performed every 2 months for 12 months at -20°C (Table 2). It was confirmed that the temperature was maintained well during the test period.

Table 2. Analysis schedule and temperature

Batch No.	Temperature	Storage period Testing frequency
accelerated-term test	4°C± 3°C	0, 7, 14, 21, 28, 35 and 42 days
long-term test	-20°C± 5°C	0, 2, 4, 6, 8 and 12 months

4. Methods

The test for reviewing the stability of β-agarase DagA was done using the AOAC test method, and the content analysis was done according to its own test method (Table 3). Each test measurement was done three times. Table 4 shows the standard specifications for each test of β-agarase DagA. It was stipulated that it is no longer stable if it deviates from the relevant standard.

Table 3. Analysis method

		Analysis	Method
1	Physical and chemical Data	Appearance	Visual examination
2		Enzyme Activity	Food additive process, 9th, 2018, MHLW(Japan)
3	Microbiological Data	Total plate Count	AOAC OMA 990.12 (Petri film AC)
4		Total Yeast & Mold	AOAC 997.02 (Petri film YM)
5		Coliform & *Escherichia coli*	AOAC 991.14 (Petri film EC)
6		*Staphylococcus aureus*	AOAC 2003.07 (Petri film STX+Disk)
7		*Salmonella*	FDA BAM chapter 5

Table 4. Specification

	Analysis	Specification
1	Appearance	Light brown or dark brown colored liquid
2	Enzyme Activity	4.9 - 9.0 Unit/mL
3	Total plate Count	≤1,000 cfu/g
4	Total Yeast & Mold	≤100 cfu/g
5	Coliform & *Escherichia coli*	<10 cfu/g
6	*Staphylococcus aureus*	<10 cfu/g
7	*Salmonella*	Absent in 25 g

4.1. Enzyme activity method

4.1.1. Enzyme activity test solution

1) Substrate solution: After adding phosphate buffer (pH 7.0, 0.01 M) to agarose, melt completely until it becomes clear. Add phosphate buffer that was previously warmed in a 45±0.5°C water bath, then shake it slowly, and keep warm in a 45±0.5°C water bath.

2) Phosphate buffer (pH 7.0, 0.01 M).

3) 3,5-Dinitrosalicylic acid (DNS) reagent solution: Dissolve DNS with sodium hydroxide. Add glycerol to this, and add water to fill up. This liquid is stored at room temperature while blocking the light and used within one month.

4.1.2. Experiment method

The substrate solution and test solution kept warm in a 45±0.5°C water bath are well mixed and incubated in a 45±0.5°C water bath; then, the DNS reagent solution is added to stop the reaction (enzyme test solution). Put this enzyme test solution in boiling water for 5 minutes, immediately cool it down with running water for 10 minutes, and dilute with distilled water. After that, measure the absorbance of the supernatant at a wavelength of 540 nm (AT value).

4.1.3. Enzyme control sample

Mix the substrate solution, test solution, and DNS solution as a control experiment. The mixed solution without reaction is used for the enzyme blank test, and the absorbance is measured (AT0). The enzyme blank test solution is treated the same way as the enzyme test solution. Convert the amount of glucose (mg) corresponding to AT and AT0 into GT and GT0.

4.1.4. Preparation of the calibration curve

Prepare a standard solution of different concentrations by dissolving glucose into distilled water by concentration. Put each standard solution into a test tube, add the DNS solution, and mix. Heat this solution in boiling water for 5 minutes, immediately cool it down with running water for 10 minutes, and then dilute with distilled water. In addition, measure each absorbance at a wavelength of 540 nm with a control solution (0 mg/mL) of distilled water instead of the standard solution and draw a calibration curve with the absorbance for the amount of glucose (mg).

4.1.5. Calculate the enzyme titer according to the following formula

Titer (units/g or mL) = (GT – GT0) $\times \frac{1}{10} \times \frac{1}{0.18} \times \frac{a}{b} \times \frac{1}{\mathrm{W}}$

- GT: The amount of glucose in the enzyme solution obtained from the calibration curve (mg)

- GT0: The amount of glucose in the enzyme blank solution obtained from the calibration curve (mg)

- 10: Reaction time (minutes)

- 0.18: 1 μmol (mg) glucose
- a: Amount of enzyme reaction mixture (substrate and sample) (mL)
- b: Amount of sample (mL)
- W: The amount of sample in 1 mL of the test solution (g)

4.2. Microbiology method

4.2.1 AOAC official method 990.12 aerobic plate count for foods (total plate)

Place the dry-film aerobic count plate on a flat surface. Lift top film and inoculate 1 mL of the test suspension onto the center of the film base. Carefully place the top film down onto the inoculum. Distribute the suspension over the prescribed growth area with downward pressure in the center of the plastic spreader device (recessed side down). Leave the plate undisturbed for 1 min to permit the gel to solidify. Incubate the plates for 48 ± 3 h at 35 ± 1°C. In an incubator, place the plates in a horizontal position, clear side up, in stacks not exceeding 20 units. Count the red colonies promptly after the incubation period. Use a standard colony counter for counting purposes. Colonies are stained in various shades of red. Count all colonies in a countable range (30–300 colonies). To compute the bacterial count, multiply the total number of colonies per plate (or average number of colonies per plate if counting duplicate plates of the same dilution) by reciprocal of the dilution used.

4.2.2. AOAC official method 997.02, counts of yeast and mold in foods

Place the plates in an incubator in a horizontal position, clear side up, in stacks not exceeding 20 units. Incubate the plates for 5 days at 20–25°C. Count the yeast and mold colonies promptly after the incubation period. Yeast appears as blue-green or off-white in color and forms small defined colonies. Mold colonies are usually blue but may also assume their natural pigmentation (e.g., black, yellow, or green). They tend to be larger and more diffuse than yeast colonies.

4.2.3. AOAC official method 991.14, counts of coliform and *Escherichia coli* in foods

(a) Coliform count—Place the dry-film *E. coli* count plate or coliform count plate on a flat surface. Lift the top film and inoculate 1 mL of the test suspension onto the center of the film base. Carefully place the top film down onto the inoculum. Distribute the test suspension over the prescribed growth area with downward pressure on the center of the plastic spreader device (flat side down). Leave the plate undisturbed for 1 min to permit the gel to solidify. Incubate the plates for 24 ± 2 h at 35 ± 1°C. Count all colonies in a countable range (15–150 colonies). Red colonies with outgas bubbles are not counted as coliform organisms.

(b) *E. coli* count—Use the *E. coli* count plate and proceed as in (a). Incubate for an additional 24 ± 2 h (48 ± 4 h total). *E. coli* colonies appear as blue colonies associated with gas bubbles; other coliforms appear as red colonies with gas.

4.2.4. AOAC official method 2003.07, enumeration of *Staphylococcus aureus* in selected types of processed and prepared foods

Place Petrifilm Staph Express Count plate on a flat surface. Lift the top film and inoculate 1 mL of the test suspension onto the center of the bottom film. Incubate the plates at 37 ± 1°C for 24 ± 2 h. Observe the colony colors. If no colonies or only red-violet colonies are present after 24 ± 2 h, count the red-violet colonies on the plate as *S. aureus*; the test is complete. If any colony colors other than red-violet are present, use a Petrifilm Staph Express disk. Insert the Petrifilm Staph Express disk into the plate. Apply pressure by sliding a gloved finger firmly across the entire disk area (including edges) to ensure uniform contact of the disk with the gel and to eliminate any air bubbles. Incubate the plates for at least 60 min and no longer than 3 h at 37 ± 1°C. Enumerate the pink zones as *S. aureus* regardless of whether the colonies are present. Pink zones are usually associated with *S. aureus* but may indicate *S. hyicus* or *S. intermedius*. Colonies not associated with a pink zone are not *S. aureus* and should not be counted.

4.2.5. BAM chapter 5, *Salmonella* in Foods

Aseptically weigh 25 g of the sample into a sterile flask and add 225 mL of buffered peptone water (M192) and completely soak the contents without any homogenization. Incubate for 24 ± 2 h at 35°C. Transfer 0.1 mL of the mixture to 10 mL of Rappaport-Vassiliadis (RV) medium (M132) and repeat the above method three times. Incubate the RV medium for 24 ± 2 h at 42 ± 0.2°C. Repeat 3 times with a loopful (10 µL) of the RV medium and incubate the plates for 24 ± 2 h at 35°C. Examine the plates for the presence of colonies suspected to be *Salmonella*.

5. Result

5.1. Appearance

During the storage period, no change in appearance took place. There was no change in color or scent.

5.2. Enzyme activity of β-agarase

The analysis results of the β-agarase DagA activity confirmed that there was no significant change regardless of the storage period.

Table 5. Analysis of the β-agarase DagA activity at 4°C

4°C	Activity (U/mL)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 days	7.11 ± 0.48	6.87 ± 0.36	7.66 ± 0.43
7 days	7.05 ± 0.35	6.45 ± 0.49	7.54 ± 0.40
14 days	6.95 ± 0.49	6.82 ± 0.45	7.74 ± 0.48
21 days	7.12 ± 0.24	6.67 ± 0.36	7.42 ± 0.51
28 days	7.22 ± 0.30	6.78 ± 0.39	7.68 ± 0.31
42 days	7.03 ± 0.63	6.82 ± 0.28	7.44 ± 0.47

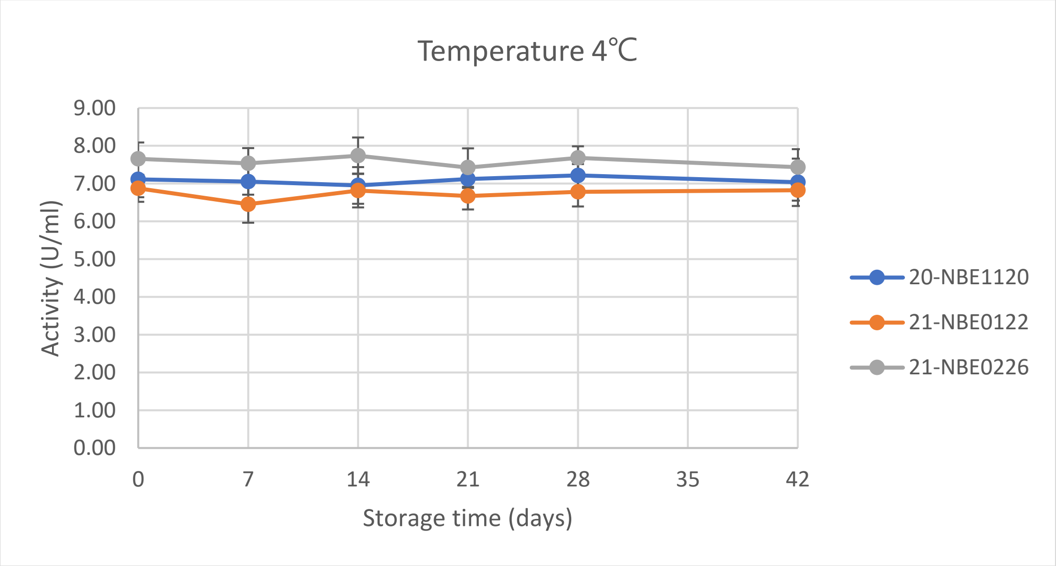


Figure 1. Analysis of the β-agarase DagA activity at 4°C

Table 6. Analysis of the β-agarase DagA activity at -20°C

-20°C	Activity (U/mL)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 months	7.11 ± 0.48	6.77 ± 0.46	7.60 ± 0.34
2 months	7.17 ± 0.22	6.54 ± 0.57	7.58 ± 0.57
4 months	6.99 ± 0.58	6.72 ± 0.47	7.54 ± 0.43
6 months	7.06 ± 0.51	6.84 ± 0.35	7.39 ± 0.25
8 months	7.02 ± 0.34	6.64 ± 0.58	7.54 ± 0.58
12 months	7.18 ± 0.44	6.73 ± 0.47	7.50 ± 0.31

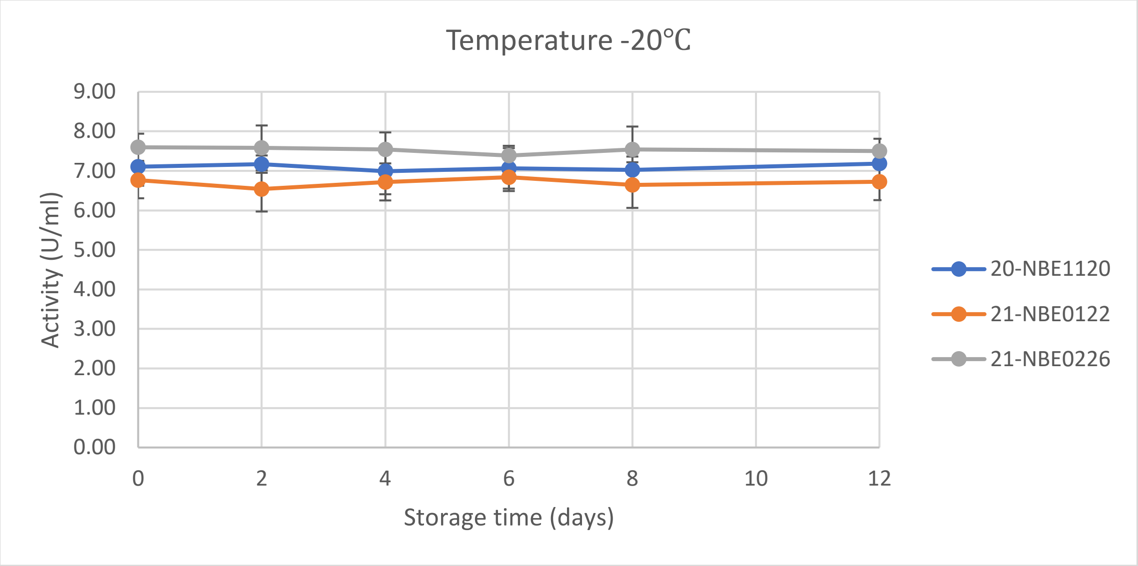


Figure 2. Analysis of the β-agarase DagA activity at -20°C

5.3. Microbiological tests of the β-agarase DagA samples

5.3.1. Aerobic plate count (AOAC OMA 990.12 [Petri film AC])

The total number of colonies on the aerobic plate count did not change during the storage period.

Table 7. Aerobic plate count of the β-agarase DagA samples at 4°C

4°C	Aerobic plate Count (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 days	<10	<10	<10
7 days	<10	<10	<10
14 days	<10	<10	<10
21 days	<10	<10	<10
28 days	<10	<10	<10
42 days	<10	<10	<10

Table 8. Aerobic plate count of the β-agarase DagA samples at -20°C

-20°C	Aerobic plate Count (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 months	<10	<10	<10
2 months	<10	<10	<10
4 months	<10	<10	<10
6 months	<10	<10	<10
8 months	<10	<10	<10
12 months	<10	<10	<10

5.3.2. Total yeast count and mold (AOAC 997.02 [petri film YM])

The total number of yeast and mold did not change during the storage period.

Table 8. Total yeast and mold count of the β-agarase DagA samples at 4°C

4°C	Total Yeast Count & Mold (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 days	<10	<10	<10
7 days	<10	<10	<10
14 days	<10	<10	<10
21 days	<10	<10	<10
28 days	<10	<10	<10
42 days	<10	<10	<10

Table 9. Total yeast and mold count of the β-agarase DagA samples at -20°C

-20°C	Total Yeast Count & Mold (cfu/g)		
(months)	20-NBE1120	21-NBE0122	21-NBE0226
0 months	<10	<10	<10
2 months	<10	<10	<10
4 months	<10	<10	<10
6 months	<10	<10	<10
8 months	<10	<10	<10
12 months	<10	<10	<10

5.3.3. Coliforms and *E. coli* (AOAC 991.14 [petri film CC])

No coliforms and *E. coli* were detected during the storage period.

Table 10. Coliforms and *E. coli* count of the β-agarase DagA samples at 4°C

4°C	Coliforms & *E. coli* (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 days	ND	ND	ND
7 days	ND	ND	ND
14 days	ND	ND	ND
21 days	ND	ND	ND
28 days	ND	ND	ND
42 days	ND	ND	ND

Table 11. Coliforms and *E. coli* count of the β-agarase DagA samples at -20°C

-20°C	Coliforms & *E. coli* (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 months	ND	ND	ND
2 months	ND	ND	ND
4 months	ND	ND	ND
6 months	ND	ND	ND
8 months	ND	ND	ND
12 months	ND	ND	ND

5.3.4. *Staphylococcus aureus* (AOAC 2003.08 [petri film STX+Disk])

No *Staphylococcus aureus* was detected during the storage period.

Table 12. *Staphylococcus aureus* count of the β-agarase DagA samples at 4°C

4°C	*Staphylococcus aureus* (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 days	ND	ND	ND
7 days	ND	ND	ND
14	ND	ND	ND
21	ND	ND	ND
28	ND	ND	ND
42	ND	ND	ND

Table 13. *Staphylococcus aureus* count of the β-agarase DagA samples at -20°C

-20°C	*Staphylococcus aureus* (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 months	ND	ND	ND
2 months	ND	ND	ND
4 months	ND	ND	ND
6 months	ND	ND	ND
8 months	ND	ND	ND
12 months	ND	ND	ND

5.3.5. *Salmonella* (AOAC 991.13)

No *Salmonella* were detected during the storage period.

Table 14. *Salmonella* count of the β-agarase DagA samples at 4°C

4°C	*Salmonella* (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 days	ND	ND	ND
7 days	ND	ND	ND
14 days	ND	ND	ND
21 days	ND	ND	ND
28 days	ND	ND	ND
42 days	ND	ND	ND

Table 15. *Salmonella* count of the β-agarase DagA samples at -20°C

-20°C	*Salmonella* (cfu/g)		
	20-NBE1120	21-NBE0122	21-NBE0226
0 months	ND	ND	ND
2 months	ND	ND	ND
4 months	ND	ND	ND
6 months	ND	ND	ND
8 months	ND	ND	ND
12 months	ND	ND	ND

6. Conclusion

This stability report shows the primary stability data of three batches of β-agarase DagA (100 mL) packed in high-density polyethylene (HDPE) bottles. The stability tests performed were done for accelerated-term (42 days at 4°C) and long term (12 months at -20°C) tests. The analysis performed according to the AOAC method showed a stable β-agarase DagA activity. There was no change in the appearance, and pathogenic microorganisms (coliforms and *E. coli*, *Staphylococcus aureus*, and *Salmonella*) were not detected. The β-agarase DagA tested in this study is a stable product; there were only slight changes during storage. Therefore, its stability during the test period can be guaranteed.

7. Reference

1. ASEAN guidelines on stability study and shelf-life of health supplements.

2. Official Methods of Analysis of AOAC 990.12 in March 2002 (Aerobic plate).

3. Official Methods of Analysis of AOAC 997.02 in March 2002 (Yeast & Mold).

4. Official Methods of Analysis of AOAC 991.14 in March 2002 (Coliform & *E. coli*).

5. Official Methods of Analysis of AOAC 2003.07 in April 2006 (*Staphylococcus aureus*)

6. BAM chapter 5, *Salmonella*, FDA

Viebrock, Lauren

From:	Susan S Cho <susanscho1@yahoo.com>
Sent:	Monday, March 18, 2024 10:18 AM
To:	Viebrock, Lauren
Subject:	[EXTERNAL] GRN 001109 Beta-agarase
Attachments:	GRN 1109 Amendment 3-15-24 submitted to FDA r.pdf

Dear Dr. Viebrock,

Good morning!
Please find the attached Amendment to GRN 001109. If you have any questions, please let me know. We would appreciate your kind attention to this matter. Have a nice day!

Sincerely,
Susan
Susan Cho, Ph.D.
AceOne RS, Inc. (formerly NutraSource, Inc.)
Agent for Dyne Bio Inc. +1-301-875-6454 (C)

March 15, 2024

To: Dr. Lauren Viebrock, FDA

Subject: GRN 001109, Beta-agarase: Amendment

From: Susan Cho, AceOne RS, Inc.

Dear Dr. Viebrock,

In response to FDA's GRAS request, Dyne Bio Inc. has prepared its amendment as follows:

1. Please clarify if the beta-agarase DagA is secreted in the fermentation media.

Dyne Bio' s Response :
β-agarase DagA is an enzyme that is secreted from the cell. When *Streptomyces coelicolor* A3(2) M22-2C43 strain is cultured in fermentation media, the β-agarase DagA enzyme is secreted, so the enzyme can be recovered without breaking the cells of the production microorganism.

2. Please confirm that the methods used for establishing the specifications for beta-agarase DagA have been validated for their intended purpose.

Dyne Bio' s Response :
The analytical methods used for establishing the specifications for β -agarase DagA have been validated for their intended purpose. An analytical method for β-agarase DagA has been validated at Dyne Bio Inc.'s lab. All other analytical values supporting the enzyme specifications were based on the methods validated at Eurofins Korea Analytical Service Co., Ltd., an institution capable of analysis under the International Standards, for intended purpose.

3. In Table 2, you stated that total yeast and mold was measured using AOAC method 977.02. However, AOAC 977.02 is validated for Nitrogen (Total) (Crude protein) in plants. Please provide results from an appropriate method such as AOAC 997.02.

Dyne Bio' s Response :
We apologize for the typo. Total yeast and molds were measured using AOAC method 997.02. A typo in certificates of analysis has been corrected by Eurofins and the new COA issued by Eurofins is shown in Appendix A. The revised specifications for β-agarase DagA are presented below.

Revised Table 1. Specifications for Dyne Bio Inc.'s β-agarase DagA

Parameter	Specification	Method of analysis
Activity unit, unit/mL	4.9 - 9.0	Food additive process, 9th, 2018, MHLW(Japan)
TOS, g/100 mL	>1.05	By calculation
Total plate count	≤1,000 cfu/g	AOAC 990.12
Total yeast & mold	≤ 100 cfu/g	AOAC 997.02
Staphylococcus aureus	<10/g	AOAC 975.55
Coliform	<10 cfu/g	AOAC 991.14
Escherichia coli	Absent in 25g	ISO 7251.2005
Listeria monocytogenes	Absent in 25g	AOAC 992.19
Salmonella	Absent in 25g	AOAC 991.13
Mercury (Hg), ppm	≤0.1	ASTM D-6722-01/ CV-AAS
Arsenic (As), ppm	≤0.1	AOAC 2013.06 / ICP-MS
Lead (Pb), ppm	≤0.1	AOAC 2013.06 / ICP-MS
Cadmium (Cd), ppm	≤0.1	AOAC 2013.06 /ICP-MS

cfu = colony forming unit.

4. Please provide the CAS number for the beta-agarase enzyme.

Dyne Bio' s Response :

β-agarase CAS No. is 37288-57-6

5. Please indicate how the sequence integrity of the beta-agarase enzyme was confirmed.

Dyne Bio' s Response :

Monitoring amino acid sequence over time is the most ideal way of confirming the sequence integrity of the β-agarase. However, it is not practical as a QC method. Thus, the sequence of the gene encoding β-agarase enzyme was monitored using PCR as a proxy for the enzyme sequence to ensure sequence integrity of the β-agarase enzyme. The sequence integrity of the β-agarase enzyme of the production strain was confirmed by monitoring no changes in the sequence of the gene encoding β-agarase DagA.

>DNA sequence of gene encoding the enzyme β-agarase DagA (930 bp) is listed below:

GTGGTCAACCGACGTGATCTCATCAAGTGGAGTGCCGTCGCACTCGGAGCGGGTG
CGGGGCTCGCGGGTCCCGCACCCGCCGCTCATGCCGCAGACCTCGAATGGGAACA
GTACCCCGTGCCGGCCGCCCCTGGCGGAAACAGGTCCTGGCAGCTTCTCCCCAGC
CATTCGGACGACTTCAACTACACCGGCAAGCCTCAAACCTTCAGGGGCAGATGGC
TGGACCAGCACAAGGATGGCTGGTCGGGCCCGGCCAACAGCCTCTACAGTGCGC
GCCATTCCTGGGTGGCTGACGGAAATCTCATCGTCGAGGGCCGCAGGGCGCCGGA
CGGGAGGGTCTACTGCGGCTACGTGACCTCCCGCACCCCAGTCGAGTACCCTCTC
TATACCGAAGTACTCATGCGTGTGAGCGGGCTGAAG CTCTCATCGA ATTTCTGGCT

CCTGAGCAGAGACGACGTCAACGAGATTGACGTGATCGAATGCTACGGCAACGA
GTCATTGCACGGAAAGCACATGAACACCGCCTACCACATATTCCAGCGGAACCCC
TTCACTGAACTGGCGAGAAGCCAGAAGGGGTATTTCGCAGATGGGAGCTACGGGT
ACAATGGTGAGACTGGGCAGGTGTTTGGGGACGGCGCCGGGCAACCTCTTCTTCG
GAATGGATTCCACCGCTACGGCGTGCACTGGATAAGCGCCACCGAATTCGATTTCT
ACTTCAACGGCAGGTTGGTGCGCCGGCTGAACCGGTCGAACGACCTCAGGGACC
CCCGGAGCCGGTTCTTCGACCAGCCAATGCATCTGATCCTCAACACCGAGAGTCA
TCAGTGGCGCGTCGACCGAGGTATCGAACCCACGGACGCGGAACTCGCAGACCC
CAGCATCAACAACATCTACTACCGCTGGGTCAGGACGTATCAGGCCGTGTAG

6. On page 14, the notifier states that the final activity of the beta-agarase is adjusted to 10,000 U/mL and in Table 2 the notifier states a specification for activity of 4.9-9.0 U/mL. Please clarify the discrepancy.

Dyne Bio' s Response :
We apologize for causing confusion. There has been a change in the enzyme analysis method. The enzyme unit of 10,000 U/mL is based on the old method (Temujin U et al., 2011), and 4.9-9.0 U/mL is based on the current analytical method, an officially certified Japanese method (Food additive process, 9th, 2018, MHLW, Japan). The key differences between these methods lie in the substrate composition, calculation methodologies, and definition of enzyme units. Because the Temujin et al. (2011) is not used any longer, we will delete the enzyme unit number, 10,000 U/mL, which may correspond to approximately 5 U/mL by the Japanese MHLW method. It now reads as follows:
8) Formulation

> β-agarase DagA activity is adjusted to make the final activity unit of 5-9 U/mL.

In addition, we would like to correct additional typos: in the second paragraph of page 16, 'Sengupta et al. (2000)' should be replaced with 'the officially certified Japanese agarase enzyme activity method (Food Additive Process, 9th Edition, 2018, Ministry of Health, Labor and Welfare, Japan.' It now reads as follows:

> The β-agarase DagA activity is measured by a modified 3,5-dinitrosalicylic acid method, as reported in the officially certified Japanese agarase enzyme activity method (Food Additive Process, 9th Edition, 2018, Ministry of Health, Labour and Welfare, Japan.

References

Temuujin U, Chi WJ, Lee SY, Chang YK, Hong SK. Overexpression and biochemical characterization of DagA from *Streptomyces coelicolor* A3(2): an endo-type β-agarase DagAproducing neoagarotetraose and neoagarohexaose. Appl Microbiol Biotechnol. 2011;92:749-59.

7. On page 14, Dyne Bio states that the beta-agarase enzyme preparation is formulated to a final activity of 10,000 U/mL and does not contain glucose or potassium sorbate. Please clarify whether the final enzyme preparation only contains total organic solids from the manufacturing process and water.

Dyne Bio' s Response :
We apologize for the typo with the reason explained in 6. A final activity of '10,000 U/mL' should be replaced with a final activity of '4.9-9 U/mL'. It now reads as follows:

8) Formulation
β-agarase DagA activity is adjusted to make the final activity unit of 4.9-9 U/mL.

The final enzyme preparation only contains total organic solids from the manufacturing process and water. No stabilizers or additives, such as potassium sorbate, have been added to the final enzyme.

8. Please confirm that the final beta-agarase enzyme preparation does not contain any major food allergens.

Dyne Bio' s Response :
No raw materials are allergens or derived from allergenic ingredients. Thus, it is expected that β-agarase DagA preparation does not contain a food allergen.

9. In Appendix 2, the notifier presents analysis for pesticides. Please clarify why pesticides are expected to be present in the beta-agarase enzyme preparation.

Dyne Bio's Response :
For Korean Ministry of Food and Drug Safety (MFDA) food additive approval, a pesticide report is often required when the production microorganism was isolated from soil. This is an analysis based on Korean MFDA standards.

10. On p. 105 of the notice (in Appendix C), you describe your bioinformatics approach for identifying potential allergenic sequences in the *S. coelicolor* M22- 2C43 strain. Please confirm your sequence search routine was for full-length alignments, and please clarify whether an 80 amino acid alignment search was also performed. Additionally, you noted that 84 matches were reported. Please provide additional details on any matches with a minimum identity greater than 50%, provided these results are from a full-length alignment search.

Dyne Bio's Response ;

Our sequence search routine was for full-length alignments and an 80 amino acid alignment search.

We performed the full-length alignment for the amino acid sequences of *Streptomyces coelicolor* A3(2) M22-2C43 from prokka gene annotation to 2,463 known allergen amino acid sequences in the COMPARE database using diamond alignment program with default parameters. 84 matches were reported in the diamond search and the attached document includes the raw diamond match result to the COMPARE database. Among the 84 matches, one protein was matched to the COMPARE database with a minimum identity greater than 50%. It was enolase and it was matched to CBL79146.1 (enolase from Salmo sala [Atlantic salmon]) with 55.4% identity.

To check the potential allergenicity of this enolase protein, we additionally employed the sequence search tool in the AllergenOnline.org database (http://allergenonline.org). Following the guidelines developed by FAO/WHO(2001) and modified by Codex Alimentarius Commission (2003), the whole amino acid sequence of enolase from M22-2C43 was compared to allergens from the FARRP allergen protein database (http://allergenonline.org).

The amino acid sequence of enolase from M22-2C43 is as follows:

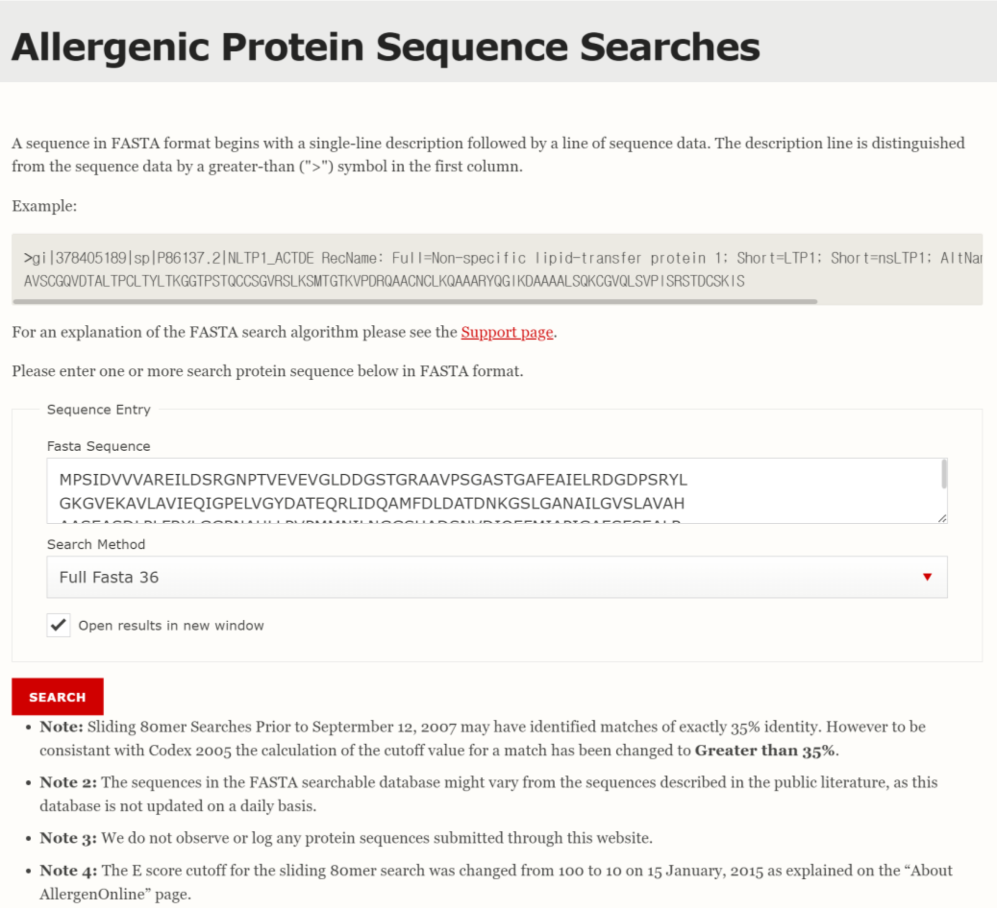
>LDEOFFGE_03967 Enolase

MPSIDVVVAREILDSRGNPTVEVEVGLDDGSTGRAAVPSGASTGAFEAIELRDGDPSR
YLGKGVEKAVLAVIEQIGPELVGYDATEQRLIDQAMFDLDATDNKGSLGANAILGVS
LAVAHAASEASDLPLFRYLGGPNAHLLPVPMMNILNGGSHADSNVDIQEFMIAPIGAE
SFSEALRWGAEVYHTLKKVLKNKGLATGLGDEGGFAPNLGSNREALDLILEAIKEAG
YTPGEQIALALDVAASEFYKDGSYAFEGKNRSAAEMTEYYAELVEAYPLVSIEDPLFE
DDWDGWNTITAKLGDKVQLVGDDLFVTNPERLARGIEENSANALLVKVNQIGSLTET
LDAVELAQRNGFKCMMSHRSGETEDVTIADLAVATNCGQIKTGAPARSERVAKYNQL
LRIEEILDDAAVYAGRSAFPRFKG

The potential allergenicity between Enolase and allergenic proteins included in the publicly available databases was analyzed through the online tool AllergenOnline with two methods (http://www.allergenonline.org/). The two methods used for the search are described below:

- Search for full-length alignments by FASTA: The most predictive search is the overall FASTA alignment (see FASTA Help Page), with identity matches greater than 50% indicating possible cross-reactivity (Aalberse, 2000).
- Search for 80 amino acid alignments by FASTA: A precautionary search using a sliding window of 80 amino acid segments of each protein to find identities greater than 35% (according to CODEX Alimentarius guidelines, 2003).

Input and output of search for full-length alignments by FASTA.



Allergenic Protein Sequence Searches

A sequence in FASTA format begins with a single-line description followed by a line of sequence data. The description line is distinguished from the sequence data by a greater-than (">") symbol in the first column.

Example:

```
>gi|378405189|sp|P86137.2|NLTP1_ACTDE RecName: Full=Non-specific lipid-transfer protein 1; Short=LTP1; Short=nsLTP1; AltNa
AVSCGQVDTALTPCLTYLTKGGTPSTQCCSGVRSLKSMTGTKVPDRQAACNCLKQAAARYQGIKDAAAALSQKCGVQLSVPISRSTDCSKIS
```

For an explanation of the FASTA search algorithm please see the Support page.

Please enter one or more search protein sequence below in FASTA format.

Sequence Entry

Fasta Sequence

```
MPSIDVVVAREILDSRGNPTVEVEVGLDDGSTGRAAVPSGASTGAFEAIELRDGDPSRYL
GKGVEKAVLAVIEQIGPELVGYDATEQRLIDQAMFDLDATDNKGSLGANAILGVSLAVAH
```

Search Method: Full Fasta 36

☑ Open results in new window

- **Note:** Sliding 80mer Searches Prior to Septermber 12, 2007 may have identified matches of exactly 35% identity. However to be consistant with Codex 2005 the calculation of the cutoff value for a match has been changed to **Greater than 35%**.
- **Note 2:** The sequences in the FASTA searchable database might vary from the sequences described in the public literature, as this database is not updated on a daily basis.
- **Note 3:** We do not observe or log any protein sequences submitted through this website.
- **Note 4:** The E score cutoff for the sliding 80mer search was changed from 100 to 10 on 15 January, 2015 as explained on the "About AllergenOnline" page.

A total of 19 matches were identified from the AllergenOnline database using full-length alignment and the result is below:

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

%_id 1 = 100% identity, alen=alignment length

AllergenOnline Database v22 (May 25, 2023)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:₩Windows₩Temp₩all3790.tmp version2236.fasta
User Query #1 >query

User Query #1

```
>query
MPSIDVVVAR EILDSRGNPT VEVEVGLDDG STGRAAVPSG ASTGAFEAIE LRDGDPSRYL GKGVEKAVLA VIEQIGPELV GYDATEQRLI DQAMFDLDAT DNKGSLGANA ILGVSLAVAH AASEASDLPL FRYLGGPNAH LLPVPMMNIL
NGGSHADSNV DIQEFMIAPI GAESFSEALR WGAEVYHTLK KVLKNKGLAT GLGDEGGFAP NLGSNREALD LILEAIKEAG YTPGEQIALA LDVAASEFYK DGSYAFEGKN RSAAEMTEYY AELVEAYPLV SIEDPLFEDD WDGWNTITAK
LGDKVQLVGD DLFVTNPERL ARGIEENSAN ALLVKVNQIG SLTETLDAVE LAQRNGFKCM MSHRSGETED VTIADLAVAT NCGQIKTGAP ARSERVAKYN QLLRIEEILD DAAVYAGRSA FPRFKG
```

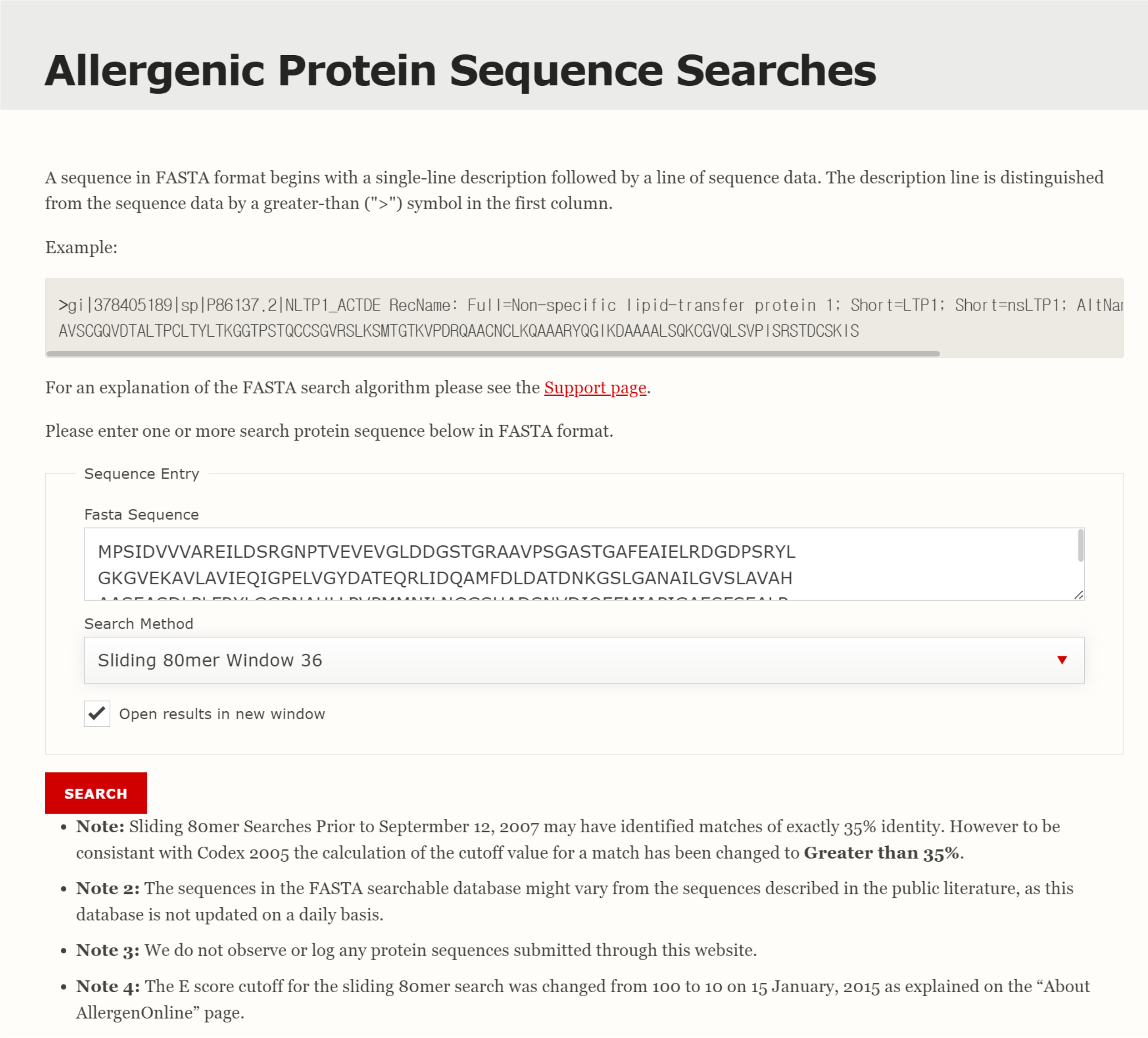
```
# fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:₩Windows₩Temp₩all3790.tmp version2236.fasta
FASTA searches a protein or DNA sequence data bank
 version 36.3.8g Oct, 2018
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:₩Windows₩Temp₩all3790.tmp
  1>>>query - 426 aa
Library: version2236.fasta
   558897 residues in  2291 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 6.1783+/-0.00659; mu= 17.3514+/- 0.334
 mean_var=59.5936+/-19.137, 0's: 1 Z-trim(80.4): 20  B-trim: 4 in 1/32
 Lambda= 0.166140
 statistics sampled from 430 (449) to 430 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
 ktup: 2, E-join: 1 (0.528), E-opt: 0.2 (0.196), width:  16
 Scan time:  0.000

The best scores are:                                      opt bits E(2291) %_id  %_sim  alen
gi|385145180|gid|1959|enolase [Salmo salar]          ( 432) 1194 293.7 1.6e-080 0.551 0.796  432
gi|576011129|gid|1955|RecName: Full=Alpha-enolase; A ( 432) 1148 282.7 3.4e-077 0.542 0.796  432
gi|46048765|gid|2710|beta-enolase [Gallus gallus]    ( 434) 1130 278.4 6.7e-076 0.533 0.781  430
gi|1503219495|gid|3047|beta-enolase [Pangasianodon h ( 341) 1125 277.2 1.2e-075 0.528 0.810  337
gi|197632415|gid|1959|enolase 3-2 [Salmo salar]      ( 434) 1110 273.6 1.9e-074 0.537 0.779  430
gi|232054|gid|396|Enolase 1 (2-phosphoglycerate dehy ( 440) 1020 252.0 5.9e-068 0.548 0.789  427
gi|30314940|gid|103|enolase [Rhodotorula mucilaginos ( 439)  993 245.5 5.2e-066 0.533 0.781  433
gi|2325204249|gid|3371|Per a 14 allergen [Periplanet ( 433)  991 245.1 7.2e-066 0.539 0.774  434
gi|13991101|gid|249|enolase [Penicillium citrinum]   ( 438)  944 233.8 1.8e-062 0.535 0.748  437
gi|14423684|gid|66|Enolase (2-phosphoglycerate dehyd ( 438)  934 231.4 9.4e-062 0.529 0.741  433
gi|83288046|gid|329|Enolase (2-phosphoglycerate dehy ( 438)  930 230.4 1.8e-061 0.538 0.745  435
gi|13925873|gid|329|enolase [Aspergillus fumigatus]  ( 438)  925 229.2 4.2e-061 0.536 0.747  435
gi|14585753|gid|1031|enolase [Curvularia lunata]     ( 440)  846 210.3 2.1e-055 0.508 0.729  435
gi|9581744|gid|586|enolase, isoform 1 [Hevea brasili ( 445)  739 184.7 1.1e-047 0.552 0.782  435
gi|2321485072|gid|3380|enolase [Platanus x hispanica ( 445)  731 182.7 4.2e-047 0.553 0.788  438
gi|14423687|gid|586|Enolase 2 (2-phosphoglycerate de ( 445)  730 182.5  5e-047 0.549 0.777  435
gi|6015094|gid|491|Enolase (2-phosphoglycerate dehyd ( 440)  688 172.4 5.3e-044 0.526 0.749  435
gi|467660|gid|491|enolase; phosphopyruvate hydratase ( 440)  681 170.8 1.7e-043 0.522 0.745  435
gi|344049993|gid|2295|enolase, partial [Fenneropenae ( 117)  299 79.2 1.6e-016 0.487 0.765  119
```

Input and output of search for 80 amino acid alignments by FASTA.



Allergenic Protein Sequence Searches

A sequence in FASTA format begins with a single-line description followed by a line of sequence data. The description line is distinguished from the sequence data by a greater-than (">") symbol in the first column.

Example:

```
>gi|378405189|sp|P86137.2|NLTP1_ACTDE RecName: Full=Non-specific lipid-transfer protein 1; Short=LTP1; Short=nsLTP1; AltNa
AVSCGQVDTALTPCLTYLTKGGTPSTQCCSGVRSLKSMTGTKVPDRQAACNCLKQAAARYQGIKDAAAALSQKCGVQLSVPISRSTDCSKIS
```

For an explanation of the FASTA search algorithm please see the Support page.

Please enter one or more search protein sequence below in FASTA format.

Sequence Entry

Fasta Sequence

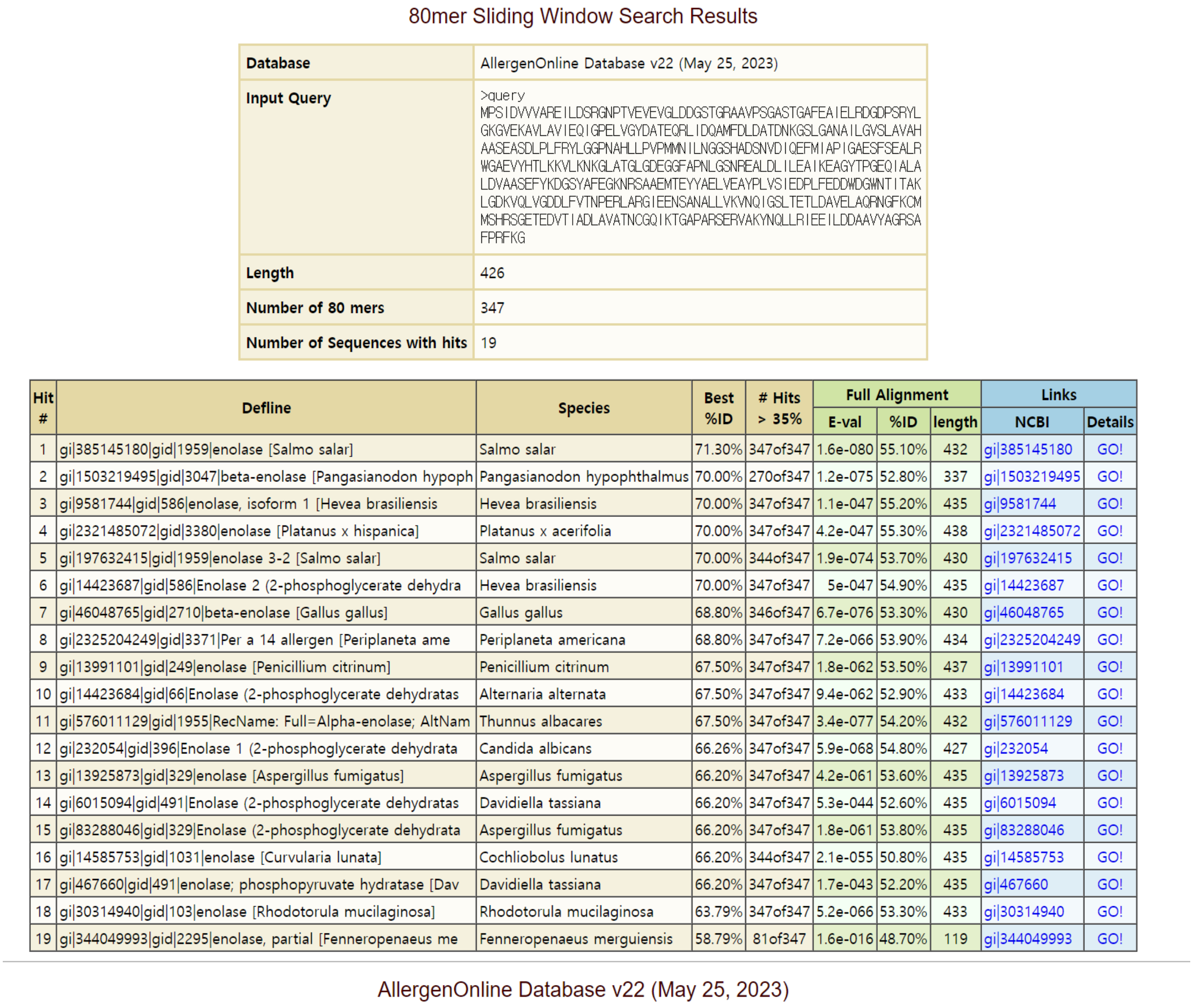
```
MPSIDVVVAREILDSRGNPTVEVEVGLDDGSTGRAAVPSGASTGAFEAIELRDGDPSRYL
GKGVEKAVLAVIEQIGPELVGYDATEQRLIDQAMFDLDATDNKGSLGANAILGVSLAVAH
```

Search Method: Sliding 80mer Window 36

☑ Open results in new window

- **Note:** Sliding 80mer Searches Prior to Septermber 12, 2007 may have identified matches of exactly 35% identity. However to be consistant with Codex 2005 the calculation of the cutoff value for a match has been changed to **Greater than 35%**.
- **Note 2:** The sequences in the FASTA searchable database might vary from the sequences described in the public literature, as this database is not updated on a daily basis.
- **Note 3:** We do not observe or log any protein sequences submitted through this website.
- **Note 4:** The E score cutoff for the sliding 80mer search was changed from 100 to 10 on 15 January, 2015 as explained on the "About AllergenOnline" page.

Same as full-length alignment, a total of 19 matches were identified from AllergenOnline database using 80-mer sliding window search and the result is below :



80mer Sliding Window Search Results

<table>
<tr><td>Database</td><td>AllergenOnline Database v22 (May 25, 2023)</td></tr>
<tr><td>Input Query</td><td>>query
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GKGVEKAVLAVIEQIGPELVGYDATEQRLIDQAMFDLDATDNKGSLGANAILGVSLAVAH
AASEASDLPLFRYLGGPNAHLLPVPMMNILNGGSHADSNVDIQEFMIAPIGAESFSEALR
WGAEVYHTLKKVLKNKGLATGLGDEGGFAPNLGSNREALDLILEAIKEAGYTPGEQIALA
LDVAASEFYKDGSYAFEGKNRSAAEMTEYYAELVEAYPLVSIEDPLFEDDWDGWNTITAK
LGDKVQLVGDDLFVTNPERLARGIEENSANALLVKVNQIGSLTETLDAVELAQRNGFKCM
MSHRSGETEDVTIADLAVATNCGQIKTGAPARSERVAKYNQLLRIEEILDDAAVYAGRSA
FPRFKG</td></tr>
<tr><td>Length</td><td>426</td></tr>
<tr><td>Number of 80 mers</td><td>347</td></tr>
<tr><td>Number of Sequences with hits</td><td>19</td></tr>
</table>

<table>
<tr><th rowspan="2">Hit #</th><th rowspan="2">Define</th><th rowspan="2">Species</th><th rowspan="2">Best %ID</th><th rowspan="2"># Hits > 35%</th><th colspan="3">Full Alignment</th><th colspan="2">Links</th></tr>
<tr><th>E-val</th><th>%ID</th><th>length</th><th>NCBI</th><th>Details</th></tr>
<tr><td>1</td><td>gi|385145180|gid|1959|enolase [Salmo salar]</td><td>Salmo salar</td><td>71.30%</td><td>347of347</td><td>1.6e-080</td><td>55.10%</td><td>432</td><td>gi|385145180</td><td>GO!</td></tr>
<tr><td>2</td><td>gi|1503219495|gid|3047|beta-enolase [Pangasianodon hypoph</td><td>Pangasianodon hypophthalmus</td><td>70.00%</td><td>270of347</td><td>1.2e-075</td><td>52.80%</td><td>337</td><td>gi|1503219495</td><td>GO!</td></tr>
<tr><td>3</td><td>gi|9581744|gid|586|enolase, isoform 1 [Hevea brasiliensis</td><td>Hevea brasiliensis</td><td>70.00%</td><td>347of347</td><td>1.1e-047</td><td>55.20%</td><td>435</td><td>gi|9581744</td><td>GO!</td></tr>
<tr><td>4</td><td>gi|2321485072|gid|3380|enolase [Platanus x hispanica]</td><td>Platanus x acerifolia</td><td>70.00%</td><td>347of347</td><td>4.2e-047</td><td>55.30%</td><td>438</td><td>gi|2321485072</td><td>GO!</td></tr>
<tr><td>5</td><td>gi|197632415|gid|1959|enolase 3-2 [Salmo salar]</td><td>Salmo salar</td><td>70.00%</td><td>344of347</td><td>1.9e-074</td><td>53.70%</td><td>430</td><td>gi|197632415</td><td>GO!</td></tr>
<tr><td>6</td><td>gi|14423687|gid|586|Enolase 2 (2-phosphoglycerate dehydra</td><td>Hevea brasiliensis</td><td>70.00%</td><td>347of347</td><td>5e-047</td><td>54.90%</td><td>435</td><td>gi|14423687</td><td>GO!</td></tr>
<tr><td>7</td><td>gi|46048765|gid|2710|beta-enolase [Gallus gallus]</td><td>Gallus gallus</td><td>68.80%</td><td>346of347</td><td>6.7e-076</td><td>53.30%</td><td>430</td><td>gi|46048765</td><td>GO!</td></tr>
<tr><td>8</td><td>gi|2325204249|gid|3371|Per a 14 allergen [Periplaneta ame</td><td>Periplaneta americana</td><td>68.80%</td><td>347of347</td><td>7.2e-066</td><td>53.90%</td><td>434</td><td>gi|2325204249</td><td>GO!</td></tr>
<tr><td>9</td><td>gi|13991101|gid|249|enolase [Penicillium citrinum]</td><td>Penicillium citrinum</td><td>67.50%</td><td>347of347</td><td>1.8e-062</td><td>53.50%</td><td>437</td><td>gi|13991101</td><td>GO!</td></tr>
<tr><td>10</td><td>gi|14423684|gid|66|Enolase (2-phosphoglycerate dehydratas</td><td>Alternaria alternata</td><td>67.50%</td><td>347of347</td><td>9.4e-062</td><td>52.90%</td><td>433</td><td>gi|14423684</td><td>GO!</td></tr>
<tr><td>11</td><td>gi|576011129|gid|1955|RecName: Full=Alpha-enolase; AltNam</td><td>Thunnus albacares</td><td>67.50%</td><td>347of347</td><td>3.4e-077</td><td>54.20%</td><td>432</td><td>gi|576011129</td><td>GO!</td></tr>
<tr><td>12</td><td>gi|232054|gid|396|Enolase 1 (2-phosphoglycerate dehydrata</td><td>Candida albicans</td><td>66.26%</td><td>347of347</td><td>5.9e-068</td><td>54.80%</td><td>427</td><td>gi|232054</td><td>GO!</td></tr>
<tr><td>13</td><td>gi|13925873|gid|329|enolase [Aspergillus fumigatus]</td><td>Aspergillus fumigatus</td><td>66.20%</td><td>347of347</td><td>4.2e-061</td><td>53.60%</td><td>435</td><td>gi|13925873</td><td>GO!</td></tr>
<tr><td>14</td><td>gi|6015094|gid|491|Enolase (2-phosphoglycerate dehydratas</td><td>Davidiella tassiana</td><td>66.20%</td><td>347of347</td><td>5.3e-044</td><td>52.60%</td><td>435</td><td>gi|6015094</td><td>GO!</td></tr>
<tr><td>15</td><td>gi|83288046|gid|329|Enolase (2-phosphoglycerate dehydrata</td><td>Aspergillus fumigatus</td><td>66.20%</td><td>347of347</td><td>1.8e-061</td><td>53.80%</td><td>435</td><td>gi|83288046</td><td>GO!</td></tr>
<tr><td>16</td><td>gi|14585753|gid|1031|enolase [Curvularia lunata]</td><td>Cochliobolus lunatus</td><td>66.20%</td><td>344of347</td><td>2.1e-055</td><td>50.80%</td><td>435</td><td>gi|14585753</td><td>GO!</td></tr>
<tr><td>17</td><td>gi|467660|gid|491|enolase; phosphopyruvate hydratase [Dav</td><td>Davidiella tassiana</td><td>66.20%</td><td>347of347</td><td>1.7e-043</td><td>52.20%</td><td>435</td><td>gi|467660</td><td>GO!</td></tr>
<tr><td>18</td><td>gi|30314940|gid|103|enolase [Rhodotorula mucilaginosa]</td><td>Rhodotorula mucilaginosa</td><td>63.79%</td><td>347of347</td><td>5.2e-066</td><td>53.30%</td><td>433</td><td>gi|30314940</td><td>GO!</td></tr>
<tr><td>19</td><td>gi|344049993|gid|2295|enolase, partial [Fenneropenaeus me</td><td>Fenneropenaeus merguiensis</td><td>58.79%</td><td>81of347</td><td>1.6e-016</td><td>48.70%</td><td>119</td><td>gi|344049993</td><td>GO!</td></tr>
</table>

AllergenOnline Database v22 (May 25, 2023)

We hope we have properly responded to the FDA’s questions. If you have any further questions or clarifications, please contact me.

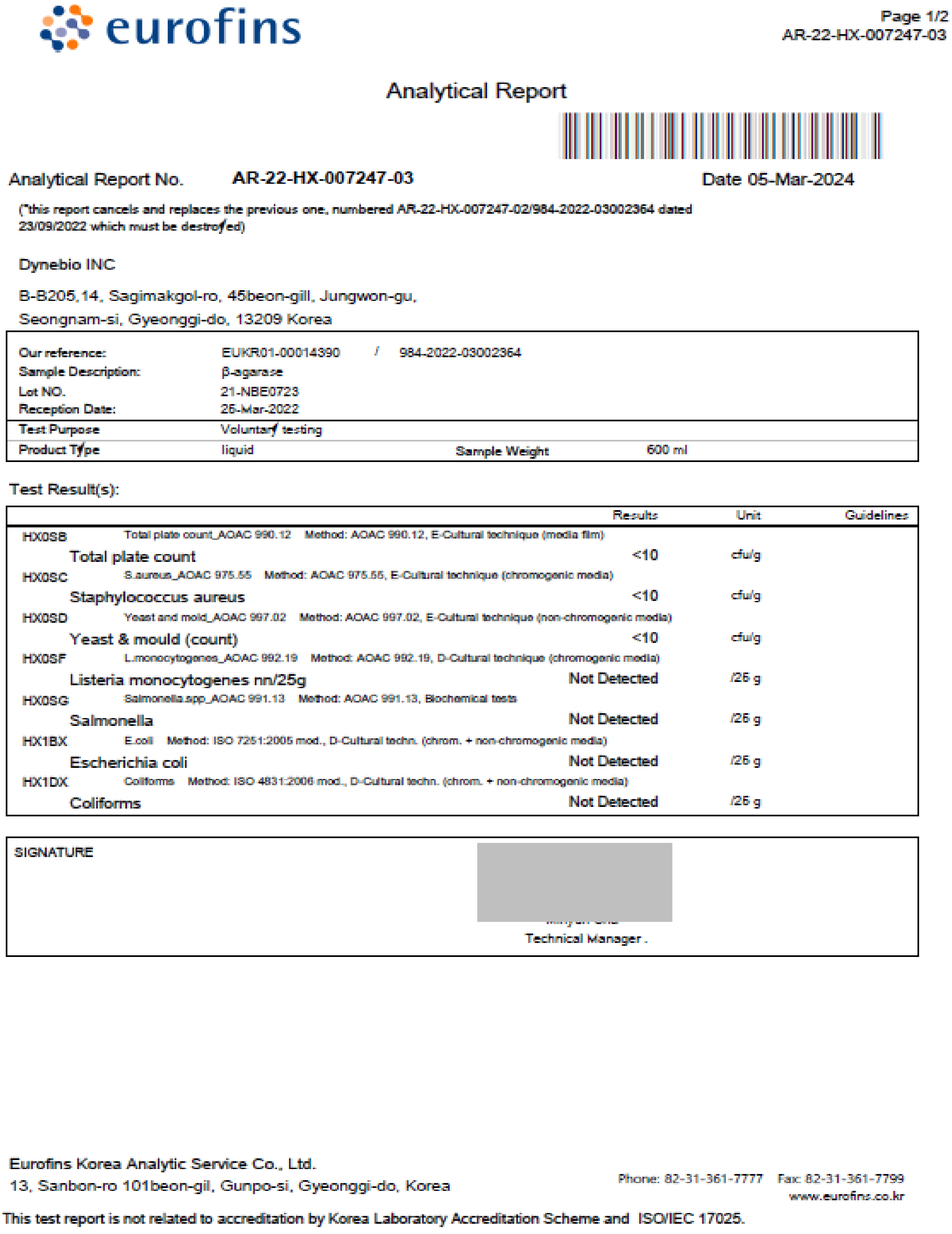
Sincerely,

[redacted]

Susan
Susan Cho, Ph.D.
AceOne RS, Inc., Agent for Dyne Bio Inc.
(301) 875-6454; Susanscho1@yahoo.com or scho@aceoners

Appendix A. Revised Certification of Analysis for Total Yeasts and Mold in β-agarase Enzyme beta-agarase DagA

21-NBE0723



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AR-22-HX-007247-03

Analytical Report

Analytical Report No. **AR-22-HX-007247-03** Date 05-Mar-2024

(*this report cancels and replaces the previous one, numbered AR-22-HX-007247-02/984-2022-03002364 dated 23/09/2022 which must be destroyed)

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002364		
Sample Description:	β-agarase		
Lot NO.	21-NBE0723		
Reception Date:	26-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	Sample Weight	600 ml

Test Result(s):

		Results	Unit	Guidelines
HX0SB	Total plate count_AOAC 990.12 Method: AOAC 990.12, E-Cultural technique (media film)			
	Total plate count	<10	cfu/g	
HX0SC	S.aureus_AOAC 975.55 Method: AOAC 975.55, E-Cultural technique (chromogenic media)			
	Staphylococcus aureus	<10	cfu/g	
HX0SD	Yeast and mold_AOAC 997.02 Method: AOAC 997.02, E-Cultural technique (non-chromogenic media)			
	Yeast & mould (count)	<10	cfu/g	
HX0SF	L.monocytogenes_AOAC 992.19 Method: AOAC 992.19, D-Cultural technique (chromogenic media)			
	Listeria monocytogenes nn/25g	Not Detected	/25 g	
HX0SG	Salmonella spp_AOAC 991.13 Method: AOAC 991.13, Biochemical tests			
	Salmonella	Not Detected	/25 g	
HX1BX	E.coli Method: ISO 7251:2005 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Escherichia coli	Not Detected	/25 g	
HX1DX	Coliforms Method: ISO 4831:2006 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	Not Detected	/25 g	

SIGNATURE

[redacted]

Technical Manager .

Eurofins Korea Analytic Service Co., Ltd.
13, Sanbon-ro 101beon-gil, Gunpo-si, Gyeonggi-do, Korea

Phone: 82-31-361-7777 Fax: 82-31-361-7799
www.eurofins.co.kr

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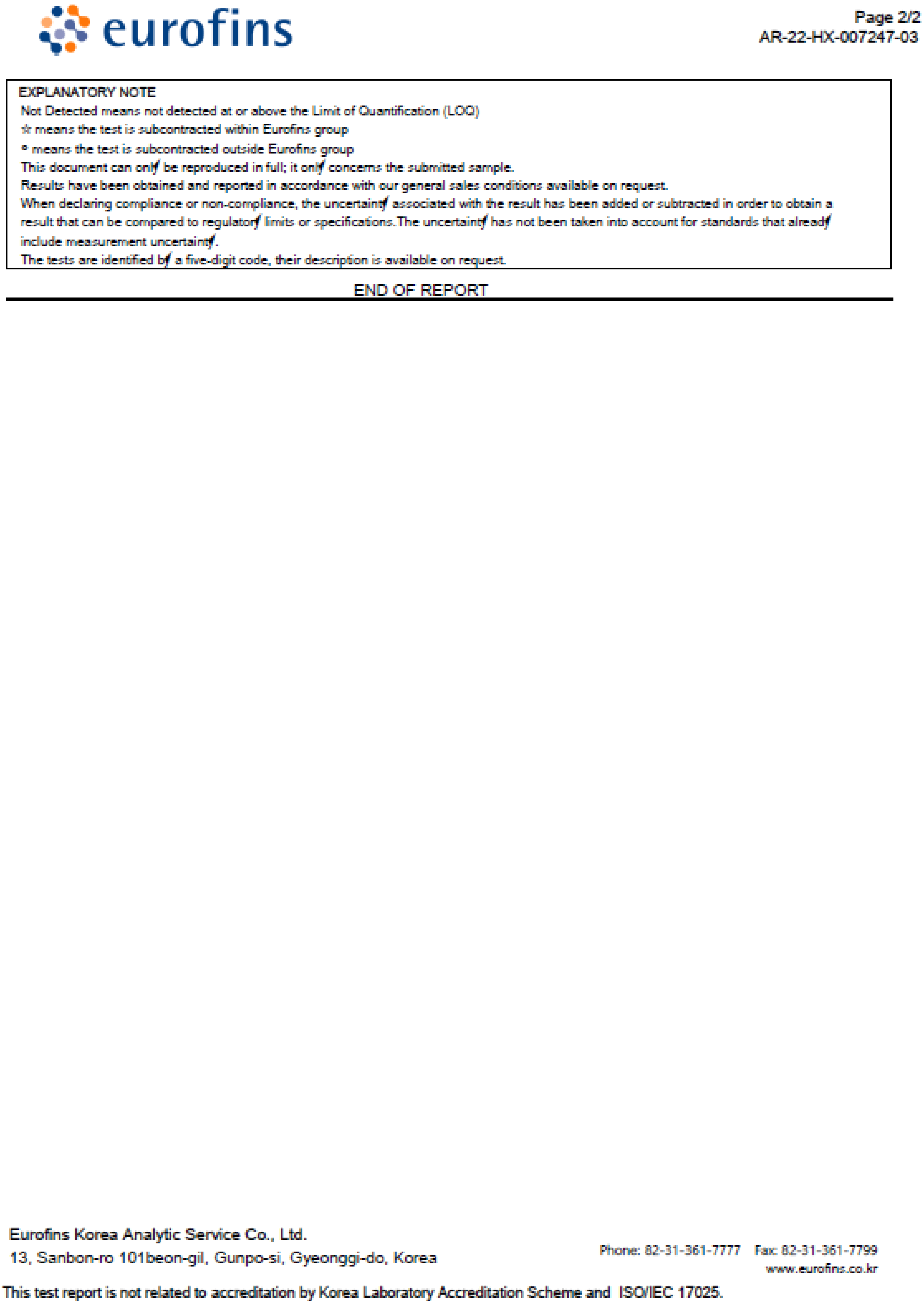

EXPLANATORY NOTE

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☆ means the test is subcontracted within Eurofins group
* means the test is subcontracted outside Eurofins group
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21-NBE0917

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AR-22-HX-007248-03

Analytical Report

Analytical Report No. **AR-22-HX-007248-03** Date 05-Mar-2024

(*this report cancels and replaces the previous one, numbered AR-22-HX-007248-02/984-2022-03002369 dated 23/09/2022 which must be destroyed)

Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002369		
Sample Description:	β-agarase		
Lot NO.	21-NBE0917		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	**Sample Weight**	600 ml

Test Result(s):

		Results	Unit	Guidelines
HX0SB	Total plate count_AOAC 990.12 Method: AOAC 990.12, E-Cultural technique (media film)			
	Total plate count	<10	cfu/g	
HX0SC	S.aureus_AOAC 975.55 Method: AOAC 975.55, E-Cultural technique (chromogenic media)			
	Staphylococcus aureus	<10	cfu/g	
HX0SD	Yeast and mold_AOAC 997.02 Method: AOAC 997.02, E-Cultural technique (non-chromogenic media)			
	Yeast & mould (count)	<10	cfu/g	
HX0SF	L.monocytogenes_AOAC 992.19 Method: AOAC 992.19, D-Cultural technique (chromogenic media)			
	Listeria monocytogenes nn/25g	Not Detected	/25 g	
HX0SG	Salmonella.spp_AOAC 991.13 Method: AOAC 991.13, Biochemical tests			
	Salmonella	Not Detected	/25 g	
HX1BX	E.coli Method: ISO 7251:2005 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Escherichia coli	Not Detected	/25 g	
HX1DX	Coliforms Method: ISO 4831:2006 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	Not Detected	/25 g	

SIGNATURE

[redacted]

Mihyun Cha
Technical Manager .

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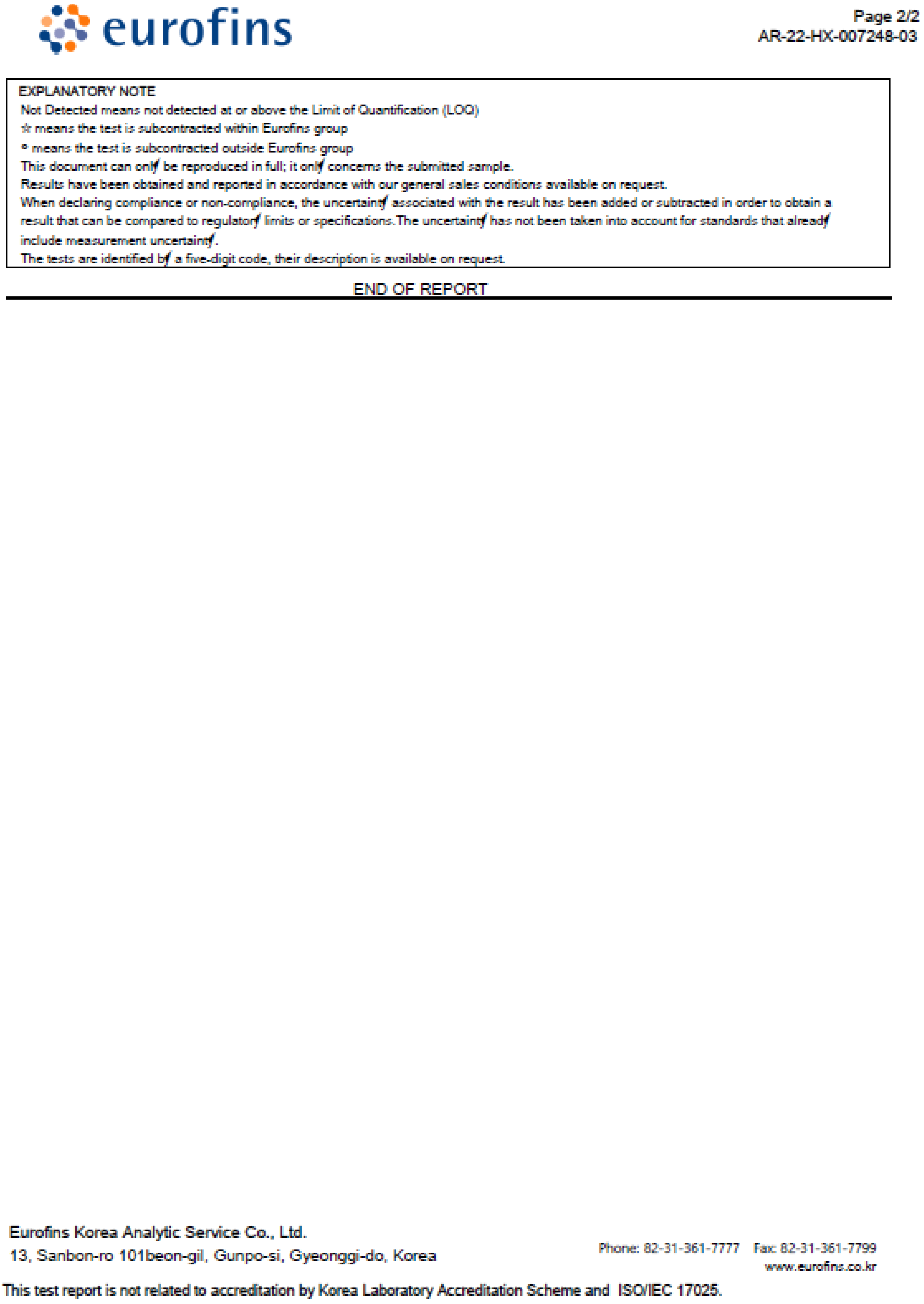

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AR-22-HX-007248-03

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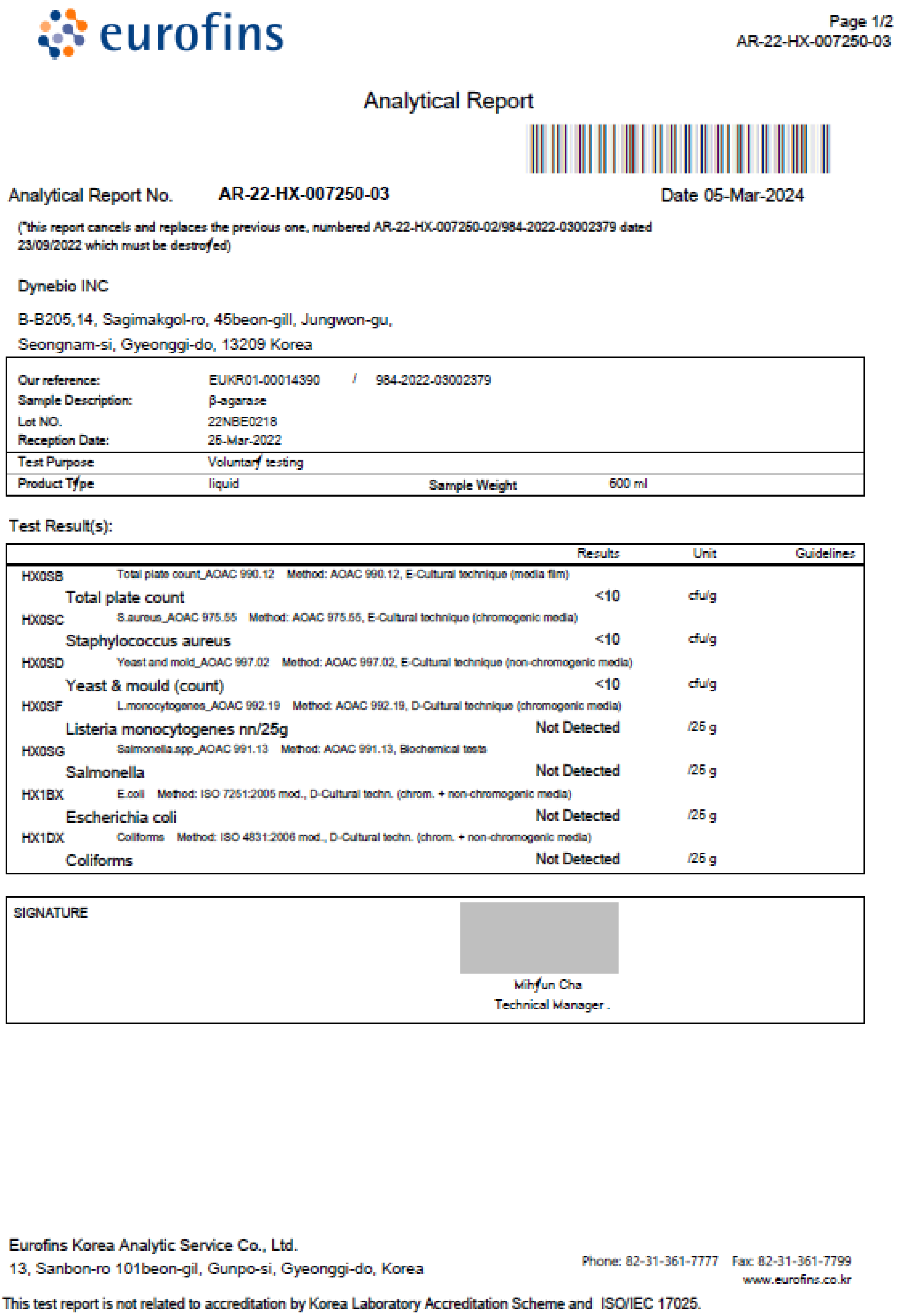
21-NBE0218



Page 1/2
AR-22-HX-007250-03

Analytical Report

Analytical Report No. **AR-22-HX-007250-03** Date 05-Mar-2024

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Dynebio INC

B-B205,14, Sagimakgol-ro, 45beon-gill, Jungwon-gu,
Seongnam-si, Gyeonggi-do, 13209 Korea

Our reference:	EUKR01-00014390 / 984-2022-03002379		
Sample Description:	β-agarase		
Lot NO.	22NBE0218		
Reception Date:	25-Mar-2022		
Test Purpose	Voluntary testing		
Product Type	liquid	Sample Weight	600 ml

Test Result(s):

		Results	Unit	Guidelines
HX0SB	Total plate count_AOAC 990.12 Method: AOAC 990.12, E-Cultural technique (media film)			
	Total plate count	<10	cfu/g	
HX0SC	S.aureus_AOAC 975.55 Method: AOAC 975.55, E-Cultural technique (chromogenic media)			
	Staphylococcus aureus	<10	cfu/g	
HX0SD	Yeast and mold_AOAC 997.02 Method: AOAC 997.02, E-Cultural technique (non-chromogenic media)			
	Yeast & mould (count)	<10	cfu/g	
HX0SF	L.monocytogenes_AOAC 992.19 Method: AOAC 992.19, D-Cultural technique (chromogenic media)			
	Listeria monocytogenes nn/25g	Not Detected	/25 g	
HX0SG	Salmonella spp_AOAC 991.13 Method: AOAC 991.13, Biochemical tests			
	Salmonella	Not Detected	/25 g	
HX1BX	E.coli Method: ISO 7251:2005 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Escherichia coli	Not Detected	/25 g	
HX1DX	Coliforms Method: ISO 4831:2006 mod., D-Cultural techn. (chrom. + non-chromogenic media)			
	Coliforms	Not Detected	/25 g	

SIGNATURE

[redacted]

Mihyun Cha
Technical Manager .

Eurofins Korea Analytic Service Co., Ltd.
13, Sanbon-ro 101beon-gil, Gunpo-si, Gyeonggi-do, Korea
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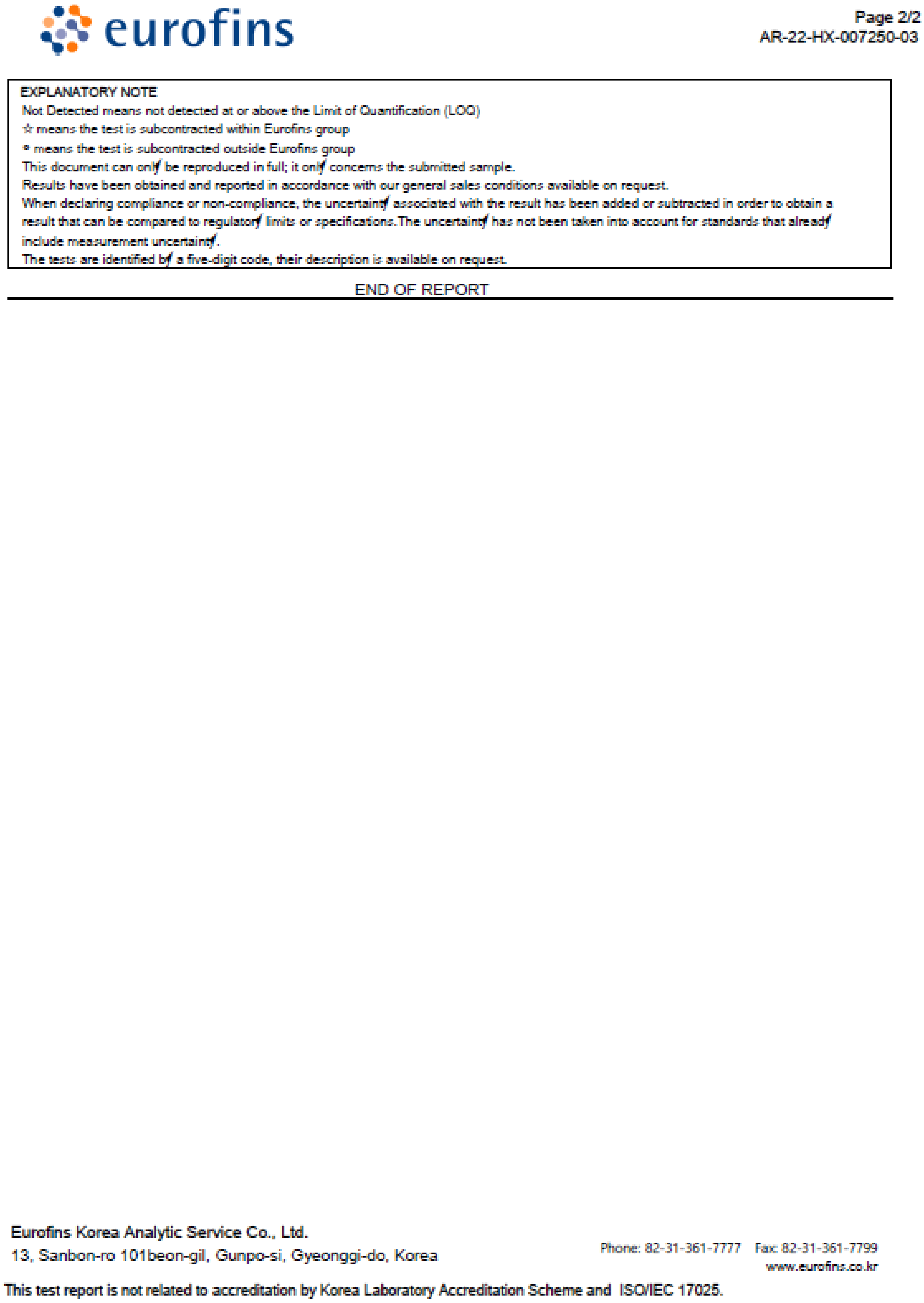

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