Safety and Toxicological Documentation

Product

SEACODE™ *marine ingredient*

Product code

BI040

Date

September 2013

Revision

1

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Product information

Trade name	SEACODE™ *marine ingredient*
Product code	BI040

INGREDIENTS

INCI name	CAS No	EINECS No
WATER (AQUA)	7732-18-5	231-791-2
PSEUDOALTEROMONAS FERMENT EXTRACT	820959-16-8	-
SALICYLIC ACID	69-72-7	200-712-3
CITRIC ACID	5949-29-1	201-069-1
SODIUM SALICYLATE	54-21-7	200-198-0

Toxicology Tests

In order to assess the safety of the product SEACODE™ *marine ingredient*, the following tests were performed.

ORAL IRRITATION TEST

Date	2013
Test method	Cell viability and histological analysis were assessed in a 3-dimensional reconstruction of Human Gingival Epidermal tissue. This tissue culture forms epithelial tissue without stratum corneum, histologically similar to the outer cell layers of the human gum, thus providing a suitable model for evaluating oral irritation *in vitro*.
Results	SEACODE™ *marine ingredient* was tested at 5%. No significant effects on cell viability were observed, with an observed ET_{50} value of more than 3 hours.

Toxicological data - Ingredients

Ingredient:	**SALICYLIC ACID**
Acute oral toxicity	LD_{50} ~ 891 mg/kg (rat)
Skin irritation	Repeated or prolonged contact may cause slight irritation (pure substance)
Sensitisation	Non-sensitising
Mutagenicity	No genotoxic potential

Ingredient:	**CITRIC ACID**
Acute oral toxicity	LD_{50}/mouse: 5400 mg/kg LD_{50}/rabbit > 7000 mg/kg LD_{50}/rat > 6730 mg/kg
Acute intraperitoneal toxicity	LD_{50}/rat: > 975 mg/kg
Repeated dose toxicity	NOAEL: 4000 mg/kg/d (oral, rat, 5 d)
Skin irritation	Moderately irritating (rabbit)
Eye irritation	Strongly irritant (rabbit)
Mutagenicity	Not mutagenic
Carcinogenicity	Not carcinogenic (rat) Not carcinogenic (mouse)
Reproduction toxicity	Not teratogenic
Note	*Citric acid is an itermediate product of human metabolism (citric acid cycle)*

Ingredient:	**SODIUM SALICYLATE**
Acute oral toxicity	LD_{50}/rat: 930 mg/kg
Skin irritation	Irritant for skin and mucous membranes
Eye irritation	Irritant effect
Sensitisation	No sensitizing effect known

Conclusions

All the raw materials involved in the formulation of SEACODE™ are regarded as safe for their use in a cosmetic product at the recommended dosage. The safety of the product is ensured by the safety of its ingredients.

Annex

The toxicity summary of active ingredient Pseudoalteromonas Ferment Extract (as Glycoprotein and Exopolysaccharide) is attached.

Summary of Toxicology Tests

INCI name

PSEUDOALTEROMONAS FERMENT EXTRACT (Glycoprotein)

Date

January 2013

Revision

0

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Toxicology Tests

The following charts are a summary of the tests performed in order to assess the safety of Pseudoalteromonas Ferment Extract (Glycoprotein).

TESTS PERFORMED

IN VITRO
Skin Primary Irritation Test
Bacterial Reverse Mutation Test (Ames Test)
Acute Toxicity Test
Red Blood Cell Toxicity (Haemolysis Test)
Acute Toxicity Test
Cytotoxicity Test on Fibroblasts
Cytotoxicity Test on Keratinocytes
Hen's Egg Test- Chorioallantoic Membrane (HET-CAM) Test
Skin Sensitisation (Hypoallergenicity)

Summary of toxicology tests

SKIN PRIMARY IRRITATION TEST

Date	2001
Test method	Male albino rabbits weighing between 2.0 and 2.5 Kg were used to examine potential dermal irritation induced by Pseudoalteromonas Ferment Extract (Glycoprotein). The dorsal hair of the rabbits was clipped 24 hours before the application of the sample on a surface of 10 by 10 cm. The test preparations were applied to both undamaged hairless skin and to hairless skin eroded with thin cuts (without bleeding). The dorsal zone was covered with a sterile pad which was fixed to the animal´s back. Erithema and edema were evaluated for each site according to the modification of Draize's method and graded from 0 to 8. An index was calculated from adding up all the scores and dividing by the number of animals.
Results	Pseudoalteromonas Ferment Extract (Glycoprotein) obtained an index of 0 out of 8, meaning no irritation, redness or edema was observed after seven days and therefore is considered as non irritant to the skin.

BACTERIAL REVERSE MUTATION TEST (AMES TEST)

Date	1998
Test method	The aim of this study was to assess the mutagenic potential of Pseudoalteromonas Ferment Extract (Glycoprotein) using four strains of *Salmonella typhimurium* in the presence and absence of a metabolic activation system[1]. The four strains of *S. typhimurium* (TA-97, TA-98, TA-100 and TA-102) are histidine-dependent and mutagenicity is detected through a significant rise in the number of revertants in comparison with the spontaneous reversions. Using five bacterial strains, different types of mutagens can be detected. The metabolic activation system (S9) was used to determine whether Pseudoalteromonas Ferment Extract (Glycoprotein) needs to be metabolised in order to produce mutations. [1] OECD guideline for testing of chemicals: Bacterial Reverse Mutation, nº 471, Updated Guideline, 21 July 1997.
Results	Pseudoalteromonas Ferment Extract (Glycoprotein) was tested at 5 µg/plate, 10 µg/plate, 0.05 mg/plate, and 0.1 mg/plate and proved to be non mutagenic for all test strains, either in the presence or absence of metabolic activation under the experimental conditions used.

RED BLOOD CELL TOXICITY (HAEMOLYSIS TEST)

Date	2002
Test method	The haemolysis test was used to assess red blood cell toxicity by measuring the percentage of haemolysis provoked by the test item. Rabbits were bled from the ear vein. Blood was collected in plastic tubes containing 0.1 mL of sodium citrate solution (3.8% concentration) per mL of blood. These samples were diluted 4 times with NaCl (0.9%), centrifuged in a bench top centrifuge at 500x g for 5 min at room temperature to obtain the erythrocytes, which were then rinsed by successive centrifugations with sodium chloride (0.9%) solution. Finally erythrocytes were diluted 4 times in NaCl 0.9%. Controls: 400 µL of the cell suspension was pipetted into a test tube, into which 4.6 mL of NaCl 0.9% was added. It was incubated for 30 min. at room temperature and split into two fractions, a negative control of 2.5 mL (C-) and a positive control of 2.3 mL plus 200 µL of Triton X-100 (10%) – (which will lyse all the cells – C+). Samples: 400 µL aliquots of the cell suspension were pipetted into test tubes, into which different volumes (X) of the sample were added as well as NaCl 0.9% so that the final volume was kept constant (5 mL). Incubations were carried out at room temperature for 30 min and, as stated above, each sample was split into two aliquots: sample of 2.5 mL (Sx) and a sample of 2.3 mL plus 200 µL of 10% Triton X-100 (S+x) which is used a positive control per each sample, in order to discard errors due to the haematocrit content. In addition, a blank is prepared in 0.9% NaCl with the same volume of sample used in each case (X), topped up to 5 mL, and an aliquot of 2.5 mL is kept (Bx). All tubes (C-, C+, Sx, S+x, Bx) were centrifuged at 10000 rpm for 10 minutes, the supernatants separated and their absobance read at 540 nm in order to quantify haemoglobin. The percentage of haemolysis was quantified estimating 100% haemolysis for the samples treated with Triton X-1 00 (10%). Values represented the means of three independent experiments.
Results	Pseudoalteromonas Ferment Extract (Glycoprotein) tested negative for haemolysis.

ACUTE TOXICITY TEST

Date	1998
Test method	Swiss CD-1 mice were separated into three groups. Each group received a different dose: 10, 100 or 500 mg/kg body weight and each animal received 0.3 ml Pseudoalteromonas Ferment Extract (Glycoprotein) intravenously. The animals were monitored for a fortnight. The animals remained healthy and no signs of toxicity were observed.
Results	After 11 days survival was 100% at all doses which means Pseudoalteromonas Ferment Extract (Glycoprotein) presents no toxicity at the administered doses.

CYTOTOXICITY TEST ON FIBROBLASTS

Date	2001
Test method	The assay is performed on HDF (Human Dermal Fibroblasts) and is based on the determination of the mitochondrial reductase activity. It is well documented that the first cellular components which are affected by a cytotoxic process are mitochondria, responsible for the cellular levels of NADH. The measurement of this enzymatic activity is carried out using tetrazolium salts. Living cells reduce tetrazolium salts to coloured formazan compounds via the "succinate-tetrazolium reductase" system which belongs to the respiratory chain of mitochondria. This system is active only in metabolically active cells, that is, living cells. The tetrazolium salt (for instance, WST-1, Life Technologies) is applied to the cell culture, samples are taken after 4 h and their absorbance measured in order to detect the formazan. The amount of formazan is proportional to the concentration of salt modified by the mitochondrial reductases and therefore indicates living cells. Three controls are used: a population of non treated cells, a negative control of cells treated with a non cytotoxic product (0.01 M NaCl), and a positive control of cells treated with a cytotoxic product (0.02% SDS – Sodiumdodecylsulphate)
Results	Comparison with the controls shows that Pseudoalteromonas Ferment Extract (Glycoprotein) used in concentrations from 0.01 ng/mL to 1 mg/mL shows no cytotoxic effect on Human Dermal Fibroblasts.

CYTOTOXICITY TEST ON KERATINOCYTES

Date	2001
Test method	The assay is performed on cocultures of HEK (Human Epidermal Keratinocytes) over HDF and is based on the determination of the mitochondrial reductase activity. It is well documented that the first cellular components which are affected by a cytotoxic process are mitochondria, responsible for the cellular levels of NADH. The measurement of this enzymatic activity is carried out using tetrazolium salts. Living cells reduce tetrazolium salts to coloured formazan compounds via the "succinate-tetrazolium reductase" system which belongs to the respiratory chain of mitochondria. This system is active only in metabolically active cells, that is, living cells. The tetrazolium salt (for instance, WST-1, Life Technologies) is applied to the cell culture, samples are taken after 4 h and their absorbance measured in order to detect the formazan. The amount of formazan is proportional to the concentration of salt modified by the mitochondrial reductases and therefore indicates living cells. Three controls are used: a population of non treated cells, a negative control of cells treated with a non cytotoxic product (0.01 M NaCl), and a postive control of cells treated with a cytotoxic product (0.02% SDS – Sodiumdodecylsulphate)
Results	Comparison with the controls shows that Pseudoalteromonas Ferment Extract (Glycoprotein) used in concentrations from 0.01 ng/mL to 1 mg/mL shows no cytotoxic effect on Human Dermal Keratinocytes.

HEN'S EGG TEST-CHORIOALLANTOIC MEMBRANE (HET-CAM) TEST

Date	2002
Test method	Chemicals are placed directly onto the chorionallantoic membrane of the hen's egg. The occurrence of vascular injury or coagulation in response to a compound is the basis for employing this technique as an indication of the potential of a chemical to damage mucous membranes (in particular the eye) in vivo. Hen's eggs are rotated in an incubator for 9 days after which time any defective eggs are discarded. The shell around the air cell is removed and the inner membranes are extracted to reveal the chorioallantoic membrane. Test chemicals are added to the membrane and left in contact for 5 min. The membrane is examined for vascular damage and the time taken for injury to occur is recorded. Irritancy is scored according to the severity and speed at which damage occurs.
Results	Pseudoalteromonas Ferment Extract (Glycoprotein) is non irritant under the conditions of the study.

SKIN SENSITISATION (HYPOALLERGENICITY)

Date	2003
Test method	Fifty human volunteers, both male and female, aged between 18 and 70 were used in the test. Pseudoalteromonas Ferment Extract (Glycoprotein) is applied on the back of the volunteers via a 7 mm Finn Chamber (an aluminium patch containing paper disks soaked in the sample). The patch is left in contact with the skin for 24 hours. Then, it is removed and cutaneous reactions are evaluated after 15 minutes, 1 hour and 24 hours. In the following 3 weeks the volunteers undergo 3 other patch tests, the last one of which is taken as an indicator for skin sensitization. The skin is assessed separately for erythema and oedema in a scale from 0 (non existent) to 4 (serious). For each patch test, all values for Erythema at 24 h are averaged and the same is done for Oedema. Both values are in turn averaged giving an overall score for each patch test.
Results	A product is classified as irritating if the overall score for a patch test is over 0.5. Pseudoalteromonas Ferment Extract (Glycoprotein) scored 0.18, 0.10, 0.08 so it can be classified as non-irritant. Sensitisation (Hypoallergenicity) is evaluated on the last patch test and classified as follows: low sensitization (0 volunteers out of 50 show sensitisation), medium sensitization (0 to 5 volunteers out of 50 show sensitisation), high sensitisation (more than 5 volunteers out of 50 show sensitisation). Pseudoalteromonas Ferment Extract (Glycoprotein) did not cause sensitisation in any volunteer so it can be classified as Low Sensitisation.

Summary of Toxicology Tests

INCI name

PSEUDOALTEROMONAS FERMENT EXTRACT (Exopolysaccharide)

Date

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Revision

0

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Toxicology Tests

The following charts are a summary of the tests performed in order to assess the safety of Pseudoalteromonas Ferment Extract (Exopolysaccharide).

TESTS PERFORMED

IN VITRO
Cytotoxicity test on human epidermal keratinocytes
Cytotoxicity test on 3T3 fibroblasts
Ocular Irritation (HET-CAM test)
NRU Phototoxicity test
Bacterial reverse mutation test (Ames test)

IN VIVO
Skin sensitisation and cutaneous compatibility test

Summary of toxicology tests

CYTOTOXICITY TEST ON HUMAN EPIDERMAL KERATINOCYTES

Date	2009
Test method	The cytotoxicity is evaluated by a fluorescence-based cell viability method. Live cells are distinguished by the presence of intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent cell-permeate calcein-AM to the intensely fluorescent calcein, which is retained within live cells and imparts an intense green fluorescence.
Results	Pseudoalteromonas Ferment Extract (Exopolysaccharide) proved not to have significant cytotoxic effects on human epidermal keratinocyte cell cultures at the tested concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 10 mg/mL), showing an IC_{50} = n.d. (>10 mg/mL) (>7.26 mM).

CYTOTOXICITY TEST ON 3T3 FIBROBLASTS

Date	2009
Test method	The cytotoxicity is evaluated by a fluorescence-based cell viability method. Live cells are distinguished by the presence of intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent cell-permeate calcein-AM to the intensely fluorescent calcein, which is retained within live cells and imparts an intense green fluorescence.
Results	Pseudoalteromonas Ferment Extract (Exopolysaccharide) proved not to have significant cytotoxic effects on 3T3 fibroblast cell cultures at the tested concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 10 mg/mL), showing an IC_{50} = n.d. (>10 mg/mL) (>7.26 mM).

OCULAR IRRITATION (HET-CAM TEST)

Date	2009
Test method	The HET-CAM test (Hen's Egg Chorio – Allantoic Membrane) uses fragile membrane lining a living hen's egg. The chorioallantoic membrane (CAM) is an extra-embryonic membrane which serves as a gas exchange surface. Since it mediates gas exchanges with the extra-embryonic environment until hatching, it has a very thick capillary network that forms a continuous surface in direct contact with the shell. The CAM blood vessels network is useful for evaluation of the potential of a chemical to damage mucous membranes (in particular the eye) *in vivo*. The occurrence of vascular injury or coagulation in response to a compound is the basis for employing this technique as an indication of ocular irritation.
Results	Pseudoalteromonas Ferment Extract (Exopolysaccharide) showed no ocular irritation at 10 mg/mL (1%).

NRU PHOTOTOXICITY TEST

Date	2009
Test method	The *in vitro* 3T3 NRU phototoxicity test[1] is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red (NR) when measured 24 hours after treatment with the test chemical and irradiation. NR is a weak cationic dye that readily penetrates cell membranes by non-diffusion, accumulating intracellularly in lysosomes. Alterations of the cell surface of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged or dead cells. Substances identified by this test are likely to be phototoxic *in vivo* following systemic application and distribution to the skin, or after topical application. [1] OECD guideline for testing of chemicals: in vitro 3T3 NRU phototoxicity test, nº 432, adopted 13 April 2004.
Results	Pseudoalteromonas Ferment Extract (Exopolysaccharide) proved not to present phototoxicity at the tested concentrations (0.001, 0.01, 0.1, 1, 10, 100, 1000 and 10000 µg/mL) on 3T3 fibroblast cell cultures, showing a PIF = n.d. and a MPE = -0.02.

BACTERIAL REVERSE MUTATION TEST (AMES TEST)

Date	2009
Test method	The aim of this study was to assess the mutagenic potential of Pseudoalteromonas Ferment Extract (Exopolysaccharide) using five strains of *Salmonella typhimurium* in the presence and absence of a metabolic activation system[1]. The five strains of *S. typhimurium* (TA-1535, TA-1537, TA-98, TA-100 and TA-102) are histidine-dependent and mutagenicity is detected through a significant rise in the number of revertants in comparison with the spontaneous reversions. Using five bacterial strains, different types of mutagens can be detected. The metabolic activation system (S9) was used to determine whether Pseudoalteromonas Ferment Extract (Exopolysaccharide) needs to be metabolised in order to produce mutations. [1] OECD guideline for testing of chemicals: Bacterial Reverse Mutation, nº 471, Updated Guideline, 21 July 1997.
Results	Pseudoalteromonas Ferment Extract (Exopolysaccharide) was tested at 0.5, 1.5, 5, 15 and 50 mg/mL and proved to be non mutagenic for all test strains, either in the presence or absence of metabolic activation under the experimental conditions used.

SKIN SENSITISATION AND CUTANEOUS COMPATIBILITY TEST

Date	2010
Test method	The aim of this test was to check the absence of irritant and sensitising cutaneous potentials of Pseudoalteromonas Ferment Extract (Exopolysaccharide), by repeated applications to the skin under occlusive patch in the healthy adult subject. It is a sensitisation and cutaneous compatibility study[1] under dermatological control. The volunteers undergo an induction period with 9 consecutive applications of the product to the same area (48 – 72 h) for 3 consecutive weeks. After a rest period of 15 days, there is a final challenge phase that consists of a single application (48 h). Clinical examinations after both the induction and challenge phases are carried out and the readings are performed by comparison with a negative control. The evaluation of cutaneous irritation and sensitisation is performed according to a numerical scale. [1] Marzulli and Maibach's Method: Human Repeated Insult Patch Test.
Results	Under the experimental conditions adopted the repeated applications of the raw material 0.25% Pseudoalteromonas Ferment Extract (Exopolysaccharide), under occlusive patch, induced no reaction of irritation and the raw material has a very god skin compatibility. Moreover no allergic reaction was detected. Thus, the raw material may be considered as hypoallergenic, under this specific context.