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(54) **Title**: METHOD OF PRODUCING BIOSURFACTANTS

(57) **Abstract**: The present invention relates to a method of producing biosurfactants, such as surfactin, comprising culturing at least one biosurfactant-producing microbe in a liquid culture medium comprising vinasse as a carbon source. Methods of using the crude biosurfactant containing culture broth in tertiary oil recovery and as antibacterial compositions in tertiary oil recovery are also described. Methods of using the culture broth residue after isolation of the biosurfactants as fertilizer and compositions for this use are also described.

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Method of producing biosurfactants

Field of the Invention

The present invention relates to a method of producing biosurfactants, such as surfactin, comprising culturing at least one biosurfactant-producing microbe in a liquid culture medium comprising vinasse as a carbon source. Methods of using the crude biosurfactant containing culture broth in tertiary oil recovery and as antibacterial compositions in tertiary oil recovery are also described. Methods of using the culture broth residue after isolation of the biosurfactants as fertilizer and compositions for this use are also described.

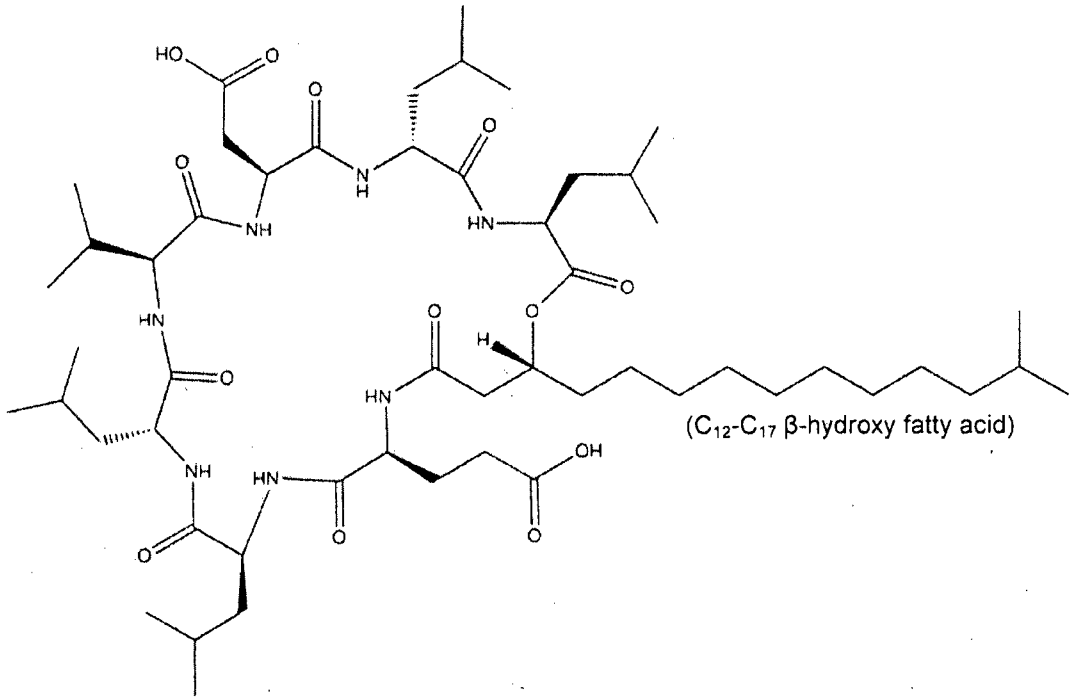
Background of the Invention

There has been significant interest in the production of biosurfactants, such as surfactin, as they are very powerful surfactants being able to alter the interfacial tension of liquids at very low concentrations. Furthermore, many biosurfactants have antimicrobial activity.

Cyclic lipopeptides, such as surfactin, have a cyclic peptide moiety and a moiety derived from a fatty acid. Surfactin has a cyclic peptide of seven amino acids including both D- and L-amino acids, Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu, linked from the N-terminus to the C-terminus to form a cyclic moiety by a C_{12}-C_{17} β-hydroxy fatty acid as shown below.

Lichenysin has a similar structure with the amino acid sequence differing from surfactin, Gln-Leu-D-Leu-Val-Asp-D-Leu-Ile, linked from the N-terminus to the C-terminus to form a cyclic moiety by a C_{12}-C_{17} β-hydroxy fatty acid.

Fengycin is a cyclic lipopeptide having the sequence Glu-D-Orn-Tyr-D-Allo-Thr-Glu-D-Ala-Pro-Glu-D-Tyr-Ile where the peptide is cyclized between the tyrosine phenoxy group of position 3 and the C-terminus of the Ile at position 10, the fatty acid is attached to the peptide forming an amide with the N-terminus.



Surfactin

Iturin refers to a group of cyclic peptides with the sequence Asn-D-Tyr-D-Asn-Gln-Pro-D-Asn-Ser in which the N-terminus and C-terminus are connected by a β-amino fatty acid of varying length.

Methods of producing these biosurfactants have focussed on the identification of high yielding strains of *Bacillus subtilis* [US 3030789, Mulligan *et al.*, 1989, *Applied Microbiology and Biotechnology* **31**:486-489], or by adjusting culture conditions such as culturing in a magnetic field [JP-A-6-121668], high iron concentrations [Wei *et al.*, *Enz. Microbial. Technol.* 1989, **22**:724-728], in the presence of peat [Sheppard *et al.* 1989, *Appl. Microbial. Biotechnol.* **27**:486-489] or reduced oxygen [Kim *et al.*, *J. Ferment. Bioeng.* 1997, **84**:41-46].

In most cases, the culturing medium contains food products or commodities as a catabolizable carbon source, for example, glucose, maltose, sucrose, hydroylzed starch, molasses, potato extract, malt, peat, vegetable oil, corn steep liquor, fructose, syrup, sugar,

liquid sugar, invert sugar, alcohol, organic acids and their salts or alkanes [US 7011959]. However, many of these carbon sources are valuable commodities in other commercial areas or are food products.

Vinasse is a by-product of the sugar industry. Sugar cane or sugar beet is processed to product crystalline sugar, pulp and molasses. The molasses is then processed by fermentation to produce ethanol, ascorbic acid and other products. After the fermentation and isolation of the desired product, the remaining residue is vinasse. Vinasse is a waste product which is often disposed of by burning [Cortez & Perez, *Brazilian Journal of Chemical Engineering*, 1997, 14] or dumping into rivers.

Vinasse is a viscous, black-reddish liquid with total solids content of 2-4% when obtained directly from sugar cane juice or 5-10% solids when obtained from molasses. It has a high biological oxygen demand (BOD) (30 000 – 40 000) and high acidity (pH 4-5).

The dumping of Vinasse in rivers causes damage to aquatic life because of its high BOD. Combustion is an expensive means of disposal.

Vinasse has sometimes been used as a fertilizer. However, its high acidity limits its usefulness to particular types of soils.

Microbes and the biosurfactants they produce have been used in tertiary oil recovery (microbial enhanced oil recovery, MEOR). To enhance oil recovery from wells near the end of their production, either

i) compositions of nutrients that stimulate endogenous bacteria to produce biosurfactants, break down heavy oil to lighter oil, reduce oil viscosity, increase reservoir (well) pressure and control mobility of oil during sweeping;

ii) or compositions of exogenous microbes that can perform these functions in oil well conditions of high pressure, high temperature and high salinity;

have been used. However, a disadvantage of MEOR is the biological production of hydrogen sulphide (also referred to as souring), which may result in corrosion of piping and machinery used in the recovery process.

Biosurfactants can be used to reduce interfacial tension between the oil and rock surfaces in wells. The forces affecting the flow of petroleum in porous rock reservoirs include gravity and capillary pressure. Capillary pressure is a function of interfacial tension between the oil/water and rock surface therefore, a reduction in interfacial tension facilitates the flow of trapped oil in the porous rock by reducing the coherent energy barrier at the elastic interface layer of the two phases. The rock becomes water-wet.

There is a need for methods of producing high yields of biosurfactant while avoiding consumption of valuable commercial and/or food commodities. There is also a need for effective means of use of vinasse byproducts.

Summary of the Invention

The present invention is predicated in part on the discovery that vinasse can be used as a carbon source in the microbial production of biosurfactants to achieve good yield at low cost.

In one aspect of the present invention there is provided a method of producing biosurfactants comprising culturing at least one biosurfactant-producing microbe in a liquid culture medium comprising vinasse as a carbon source, wherein the culturing occurs at pH 6 to 8 and a temperature of 25°C to 40°C.

In some embodiments, the at least one biosurfactant-producing microbe is selected from *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus popilliae* and mixtures thereof, especially *Bacillus subtilis* subspecies *subtilis* NRRL B-3383 (US Department of Agriculture, Agricultural Research Service, ARS Culture Collection NRRL). In some embodiments, the at least one biosurfactant-producing microbe is a mixture of *Bacillus subtilis* and *Bacillus licheniformis*. In some embodiments,

the vinasse is sugar cane vinasse. In some embodiments, the vinasse is present in the liquid culture medium in an amount of from 3% to 10% w/v. In some embodiments, the biosurfactant produced is selected from surfactin, lichenysin, fengycin, iturin and mixtures thereof, especially surfactin, lichenysin and mixtures thereof, more especially surfactin.

In some embodiments, the temperature of the culturing process is 30°C to 35°C. In some embodiments, the pH is between 6.4 and 7.2. In some embodiments, the liquid culture medium further comprises a catabolizable nitrogen source especially salts of an ammonium ions and/or nitrate ions, such as ammonium nitrate. In some embodiments, the liquid culture medium further comprises at least one inorganic salt, such as salts of phosphates, sulfates, iron, manganese, magnesium and calcium. In some embodiments, the sulphate salts are minimised or omitted.

In some embodiments, the method further comprises aerating the liquid culture medium during culturing and collecting foamate produced during aeration. In some embodiments, aeration is begun before inoculation of the culture medium. In some embodiments, aeration is begun at the time of inoculation of the culture medium.

In another aspect of the invention there is provided a biosurfactant produced by the method described above, especially where the biosurfactant is selected from surfactin, lichenysin and mixtures thereof.

In some embodiments, the biosurfactant is produced in a purity of at least 50%, especially at least 60%, 70%, 80% or above 90%, more especially at least 95%, for example, about 98% purity.

In some embodiments, the biosurfactant is retained in the culture broth. In other embodiments, the biosurfactant is isolated from the culture broth.

In another aspect of the invention there is provided a composition comprising at least one biosurfactant-producing microbe and vinasse residue, wherein the vinasse residue is

formed by decomposition of vinasse by the at least one biosurfactant-producing microbe during a fermentation process.

In some embodiments, the composition further comprises at least one biosurfactant, such as surfactin, lichenysin, iturin, fengycin or mixtures thereof, especially surfactin, lichenysin and mixtures thereof, more especially surfactin.

In some embodiments, the composition contains trace amounts of biosurfactant, for example, less than 30 μmol of biosurfactant. In other embodiments, the composition comprises biosurfactant in the range of about 750 mg/L to 2000 mg/L.

In some embodiments, the composition further comprises an added food source such as molasses, glycerine or the residue of high fructose corn syrup.

In another aspect of the invention there is provided a use of the compositions described above in tertiary oil recovery.

In another aspect of the invention there is provided a use of the composition described above as an antibacterial composition to protect equipment from corrosion during tertiary oil recovery or natural gas high pressure well processing.

In yet another aspect of the invention there is provided a use of the composition described above as a fertilizer.

Detailed Description of the Invention

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will

be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

In a first aspect of the invention there is provided a method of producing a biosurfactant comprising culturing at least one biosurfactant-producing microbe in a liquid culture medium comprising vinasse as a carbon source, wherein the culturing occurs at pH 6to 8 and a temperature of 25°C to 40°C.

While the at least one biosurfactant-producing microbe may be any microbe known to produce biosurfactants, in particular embodiments, the at least one biosurfactant-producing microbe is from the genus *Bacillus*, for example, they may be selected from *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus popilliae* and mixtures thereof. In some embodiments, one biosurfactant-producing microbe is present in the liquid culture medium. In other embodiments, two biosurfactant-producing microbes are present in the liquid culture medium. In yet another embodiment, three biosurfactant-producing microbes are present in the liquid culture medium. In still further embodiments, four biosurfactant-producing microbes are present in the liquid culture medium. In some embodiments, the at least one biosurfactant-producing microbe is a mixture of five biosurfactant-producing microbes. The at least one biosurfactant-producing microbe may be a strain of microbe known to produce biosurfactants in improved yields. For example, many species of *Bacillus* produce biosurfactants, however, *Bacillus subtilis* and *Bacillus licheniformis* are known to produce significant quantities of biosurfactants. Furthermore, specific strains of *Bacillus subtilis* are known to produce improved yields of biosurfactants such as *B. subtilis* ATCC 21331, *B. subtilis* ATCC 21332, *B. subtilis* SD901 (FERM BP.7666), *B. Subitilis* NRRL B-3383 and *B. subtilis* RSA-203 or mixtures thereof. Many strains of biosurfactant-producing microbes are commercially or publicly available. In some embodiments, the at least one biosurfactant-producing microbe is *B. subtilis* strain RSA-203.

RSA-203 is a microorganism that is a strain of *Bacillus subtilis*. It is a rod-shaped, aerobic, Gram-positive, β-hemolytic microbe capable of forming endospores. Nucleic acid

sequence analysis confirms it is a strain of *B. subtilis*. A sample of this microorganism was deposited at ATCC depository, 10801 University Boulevard, Manassas, Virginia 20110-2209, United States of America on 9 January 2013, and has been allocated Accession No. _____.

RSA-203 produces significant amounts of the biosurfactant surfactin. If culture conditions include foamate removal during culture, surfactin may be produced in amounts of 250 mg/L to 1000 mg/L in the culture medium and 850 mg/L to 2 g/L in the foamate.

In a particular embodiment, the at least one biosurfactant-producing microbe is *B. subtilis* NRRL B-3383 which is publicly available. In other particular embodiments, the at least one biosurfactant-producing microbe is *B. subtilis* strain RSA-203.

In some embodiments, the at least one biosurfactant-producing microbe is a mixture of *B. subtilis* and *B. licheniformis*. In other embodiments, the at least one biosurfactant-producing microbe is a mixture of *B. subtilis*, B. licheniformis, *B. amyloliquefaciens*, *B. pumilus* and *Bacillus popilliae*. In these embodiments, the ratio of each microbe may be adjusted to determine the amount of different biosurfactants produced. In some embodiments, the *B. subtilis* is present in a mixture of biosurfactant-producing microbes in about 50-98% of the mixture, especially 60-95%, 70-95%, 80-95%, more especially about 90%.

The carbon source used in the liquid culture medium is vinasse. Vinasse is a by-product of the sugar industry obtained from the processing of sugar cane or sugar beet. The molasses produced during sugar processing is fermented to produce ethanol and ascorbic acid. The residue left after this fermentation is referred to as vinasse. Vinasse is a viscous liquid with a total solids content of 2-10%, high acidity pH 4-5 and high BOD (30 000 – 40 000).

In some embodiments, the amount of vinasse in the liquid culture medium is from 3 to 20% w/v, especially 3 to 15% w/v, more especially 3 to 12% w/v or 3 to 10% w/v, most

especially about 10% w/v. In some embodiments, the amount of vinasse is varied to obtain a desired concentration of biosurfactant in the culture broth.

In some embodiments, a further carbon source is added in addition to the vinasse. Suitable carbon sources include carbohydrate sources such as molasses, dextrose, glucose, glycerine and the like. The further carbon source may be present in the liquid culture medium in an amount of 0 to 15% w/v especially 0 to 10% w/v.

In some embodiments, the culturing method takes place at a sugar processing plant, for example, a sugar cane processing plant. Advantageously, this reduces the costs involved in biosurfactant production as if the vinasse is required to be transported to another facility, the vinasse may need to be dehydrated to remove excess water before transport and dehydration and transport costs add to the cost of the biosurfactant.

The biosurfactant produced is preferably a cyclic lipopeptide biosurfactant such as surfactin, lichenysin, iturin, fengycin and mixtures thereof. Each of these biosurfactants may contain mixtures of compounds varying in the chain length of the fatty acid moiety of the lipopeptide. Addition of specific amino acids and/or hydrocarbon fatty acids to the culture broth may enable the production of biosurfactants with varying ratios of lipid fatty acid chain lengths.

In some embodiments, the biosurfactant produced is selected from surfactin and lichenysin and mixtures thereof. In other embodiments, the biosurfactant produced is surfactin.

The temperature of the culturing process is 25°C to 40°C, especially 30°C to 40°C, more especially about 30°C to 35°C. The temperature used may depend on the identity of the biosurfactant-producing microbe. For example, the temperature is one that produces growth of the microbes to a stress point which limits motility because of the production of chemically produced microbial markers within the broth. This allows for the maximum biosurfactant production for a given microbial population. A person skilled in the art could

determine appropriate temperature for a given bacterial population by routine trial methods.

The pH of the culture medium is maintained between 6 and 8, especially 6 and 7.5, more especially 6.5 to 7.2. In a particular embodiment, the culture medium is buffered at about pH 7 by monobasic and dibasic phosphate buffer adjusted to pH 7 with hydroxide such as sodium or potassium hydroxide.

The inoculum of at least one biosurfactant-producing microbe is a culture of at least one biosurfactant-producing microbe in a mid-log phase of growth. The inoculum is added to the culture medium to provide an initial optical density at 600_{nm} (OD_{600nm}) of 0.1 to 0.15.

In some embodiments the liquid culture medium further comprises a catabolizable nitrogen source. In some embodiments, the catabolizable nitrogen source is selected from a nitrogen containing inorganic salt or nitrogen-containing organic compound for example, ammonium salts, nitrate salts, urea, peptone, meat extract, yeast extract, soybean cake, corn steep liquor, peptone, or flour derived from legumes such as soybean, adzuki bean, pea, broad bean, chick pea, lentil and string bean or extracts of such a flour. In particular embodiments, the catabolizable nitrogen source is an inorganic salt such as an ammonium salt or nitrate salt, especially ammonium nitrate, ammonium chloride, ammonium acetate, ammonium carbonate, ammonium bicarbonate, potassium nitrate, sodium nitrate, magnesium nitrate, and calcium nitrate. In particular embodiments, the catabolizable nitrogen source is ammonium nitrate.

The amount of catabolizable nitrogen source present in the liquid culture medium will depend on the nature of the source and the availability of the nitrogen within the source. For example, the nitrogen source may be present in an amount of 1 to 20 g/L. When the nitrogen source is an inorganic nitrogen source, it may be present in an amount of 1 to 10g/L, especially 2 to 7 g/L, more especially 3.5 to 4.5 g/L.

In some embodiments, the liquid culture medium further comprises at least one inorganic salt, such as sulfates, phosphates, chlorides, especially of metals such as manganese, iron, sodium, potassium, magnesium and calcium. In some embodiments the inorganic salts are selected from sulfates, chlorides and phosphates of ions such as manganese, sodium, potassium and iron or mixtures of such salts. In a particular embodiment, the at least one inorganic salt is selected from manganese sulfate, sodium phosphate, calcium chloride, magnesium sulfate, ferrous sulfate and mixtures thereof, especially sodium phosphate, manganese sulfate and ferrous sulfate or mixtures thereof. In other embodiments, the inorganic salts present are not sulfates. For example, in some embodiments, the inorganic salts present are phosphates or chlorides. This embodiment reduces the amount of sulfate present in compositions that may be used in tertiary oil recovery where the presence of sulfates may result in production of hydrogen sulfide.

The inorganic salts vary in amount depending on the salts used. If a source of phosphate is present, it may be present in an amount of about 1 to 10 g/L, especially 2 to 7 g/L, more especially 4 to 7 g/L, most especially 5 to 6 g/L. Where inorganic salts are added to provide trace elements such as iron, manganese, and calcium, the amounts will vary between 1 mg/L and 5 g/L, for example, calcium salts may be added in an amount of 0.5 g/L to 1 g/L, iron and manganese salts may be added in an amount of 1 to 10 mg/L, manganese salts may be added in an amount of 0.5 to 1 g/L, magnesium salts may be added in an amount of 0.5 g/L to 5 g/L.

In some embodiments, the culture medium further comprises a chelating agent. Particular chelating agents include amino carboxylic acids and salts thereof, such as ethylene diamine tetraacetic acid (EDTA), hydroxyethylethylenediamine triacetic acid, 1,2-diamino-cyclohexane tetraacetic acid, ethylene glycol-bis([beta]-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), diethylenetriamine-pentaacetic acid (DPTA), triethylenetetraamine hexaacetic acid (TTG), aminodiacetic acid and hydroxyethyl aminodiacetic acid. Particular chelating agents are salts and mixed salts of EDTA such as dipotassium, ammonium, calcium, disodium, trisodium and tetrasodium salts, most preferably disodium or tetrasodium salts of EDTA, especially disodium EDTA. The

chelating agent is present in amount of between 0.1 and 5 mg/L, especially 0.5 to 3 mg/L, more especially 1 to 2.5 mg/L of culture medium.

The culturing method may occur on a small scale in laboratory flasks in an incubator or may occur on larger scale, such as industrial scale in a bioreactor. The method is conducted under aerobic conditions.

The duration of the culturing process will depend on the use of the culture broth. In some embodiments the culturing process has a duration of 8 to 120 hours, especially 8 to 72 hours, 8 to 48 hours or 8 to 24 hours, for example 10 to 14 hours. The duration of the culturing process is dependent on achieving a cell density greater than OD_{600nm} of ~1.2 to 1.4, especially about ~1.3, where biosurfactant production begins and the length of time taken to reach the stationary phase of growth, ~OD_{600nm} of 1.8 to 2.5, where biosurfactant production ceases.

In some embodiments, the process further comprises aeration of the culture medium to provide dissolved oxygen. Typically, this involves bubbling air through the culture medium at a rate of between 1 L/minute to 3 L/minute, especially about 1.5 L/minute. The rate of aeration may be readily determined by a person skilled in the art. Aeration may occur from the beginning of the culturing process or may begin after the culturing process has begun or may begin prior to inoculation at the beginning of the culturing process, especially from the beginning of the culturing process or before inoculation. In particular embodiments, aeration maintains a dissolved oxygen concentration of about 20 to 40%, especially 25 to 35%. In some embodiments, the dissolved oxygen concentration is maintained at about 30% during the culturing process.

Once biosurfactant production has begun, the culture medium may foam because of the presence of biosurfactant. In some embodiments, the foam production may be controlled by spraying the foamate with a mixture of alcohol such as ethanol, and solvent such as dichloromethane or acetone. In some embodiments, the bioreactor in which the fermentation is done is explosion proof. In some embodiments, the foam collecting

equipment is explosion proof. The extent of pressure for which equipment must withstand is determined by the pump pressure and flow rate into the foam column.

In some embodiments, the production of foamate is encouraged and the foamate is collected from the culturing vessel. The foamate comprises the biosurfactant produced together with small amounts of culture medium. The foam may be collected via a rotary valve into a tank with a slight vacuum or a tank with a spray column to break the foam. The biosurfactant may be isolated from the foamate collected. In some embodiments, the biosurfactant is isolated by acidification followed by liquid/liquid extraction and then evaporation of the liquids. In other embodiments, the biosurfactant is isolated by centrifugation followed by liquid/liquid extraction and evaporation or distillation of the liquids.

In some embodiments, the biosurfactant is isolated from the culture broth after the culturing process is complete. For example, the crude culture broth may be centrifuged to remove biomass. The supernatant is then acidified to acidic pH, for example, pH 2 with acid, such as HCl. The acidic pH results in the precipitation of the biosurfactant, the acidified supernatant may be stood at 4°C for a period of time to ensure precipitation is complete. The precipitate is then collected, for example, by centrifugation or filtration and resuspended in water. The pH of the suspension is adjusted to alkaline pH such as pH 8 to solubilize the precipitate. The resulting aqueous solution may be extracted with an organic solvent such as dichloromethane, ethyl acetate, chloroform, especially dichloromethane, and the organic phase evaporated to give the biosurfactant in high purity crystalline form. In some embodiments, the biosurfactant may be collected by foam distillation after culturing is complete.

The purified biosurfactant is suitable for many known uses such as detergents, emulsifiers, wetting agents, dispersants, solubilizing agents, antistatic agents, anti-clouding agents, lubricants, pipe resistance lowering agents, or may be used in cosmetics, foods, medical preparations, agricultural preparations, inks and the like as known in the art.

In another aspect of the invention there is provided a composition comprising at least one biosurfactant-producing microbe and vinasse residue, wherein the vinasse residue is formed by decomposition of vinasse by the at least one biosurfactant-producing microbe during a culturing process.

In some embodiments, the composition is the crude culture broth obtained from the method described above. In some embodiments, the composition is depleted in biosurfactant as the biosurfactant produced by the at least one biosurfactant-producing microbe is removed during the culturing process by removal of foamate comprising the biosurfactant. In these embodiments, the composition may contain trace amounts of biosurfactant not removed during the culturing process or produced by the at least one biosurfactant-producing microbe after the culturing process has been terminated. For example, in some embodiments, the amount is less than 30 μmol of biosurfactant.

This composition is useful as a fertilizer composition to stimulate plant growth. The fertilizer may have a bacterial population either from within the broth or added to the broth as a symbiotic organism for plant root adhesion.

In other embodiments, the composition further comprises biosurfactant, especially surfactin, lichenysin, iturin, fengycin or mixtures thereof. This composition may be obtained by adding a biosurfactant or mixture of biosurfactants to the composition or may be obtained as the crude culture broth from the method above from which no biosurfactant was isolated or only a portion of the biosurfactant was extracted. Typically, the amount of biosurfactant present in the composition is between 2 mg/L and 7000 mg/L, for example 50 mg/L and 7000 mg/L or 500 mg/L and 7000 mg/L, such as 500 mg/L and 3 g/L, especially 750 mg/L and 2 g/L.

In some embodiments where the composition comprises at least one biosurfactant-producing microbe, vinasse residue and biosurfactant, the composition may further comprise an added microbial food source. In some embodiments, the added food source is at least one selected from molasses, dextrose, glucose, vinasse, glycerine and other

carbohydrates. The addition of a food source may allow the microbes to grow once they are in an appropriate environment, for example, an oil well and thereby out compete other undesirable microbes and produce additional biosurfactant that may have an antimicrobial effect on undesirable microbes.

The composition comprising biosurfactant and optionally a bacterial food source is useful in tertiary oil recovery, for example, in microbial enhanced oil recovery (MEOR) processes and is particularly useful in recovering oil from depleted calcium carbonate rock reservoirs.

In some embodiments, an oil well is treated with a biosurfactant composition containing a biosurfactant such as surfactin in order to lower the surface tension of the oil and to provide rock that is water-wet. The oil well may then subsequently be treated with a composition of the invention.

In another aspect of the present invention there is provided a method of tertiary oil recovery comprising

1. treating an oil well with a composition comprising at least one biosurfactant, and
2. treating the oil well with a composition comprising at least one biosurfactant-producing microbe and vinasse residue, wherein the vinasse residue is formed by decomposition of vinasse by biosurfactant-producing microbes during a culturing process.

In some embodiments, the biosurfactant is in a composition at a concentration of 2 mg/L to 4 g/L. In some embodiments, the composition is a concentrate having about 1 g/L to 4 g/L biosurfactant, especially about 1.5 g/L to 3 g/L, for example about 2 g/L. In other embodiments, the concentrate is diluted, for example, with hydrofracking base water. For example, the concentrate is mixed with hydrofracking base water as it is pumped into the well. The concentrate may be diluted by an amount that maintains at least 2 mg/L biosurfactant. For example dilution of the biosurfactant composition may occur in the

range of 1:100 to 1:2000, especially 1:1000 biosurfactant to diluent. A typical concentrate comprises 10-40% w/v culture broth comprising microbes, vinasse residue and crude biosurfactant, water and minerals, 10 to 20% w/v aqueous biosurfactant composition comprising 0.01 to 1% w/v biosurfactant, 10 to 30% surfactant composition comprising 10 to 25% w/v surfactant and water, and the balance of the concentrate being water. Upon dilution at entry into an oil well, the composition may contain 94.9 to 98.98% w/v water, 1-5% w/v surfactant, 0.01 to 1% w/v biosurfactant and 0.01 to 0.1% w/v microorganisms. In some embodiments, the composition further comprises a microbial food source, for example, a carbohydrate, such as molasses, glucose, dextrose, vinasse and the like. The food source may replace water in the concentrate up to about 30% w/v. For example, a concentrate comprising a food source may comprise 10-40% w/v culture broth comprising microbes, vinasse residue and crude biosurfactant, water and minerals, 10 to 20% w/v aqueous biosurfactant composition comprising 0.01 to 1% w/v biosurfactant, 10 to 30% surfactant composition comprising 10 to 25% w/v surfactant and water, 10 % to 25% molasses optionally containing up to 1% vinasse, and the balance of the concentrate being water.

The composition comprising the at least one biosurfactant-producing microbe and vinasse residue is as described above. In some embodiments, at least a portion of the microbes are in spore form. In some embodiments, all of the microbes are in spore form. In some embodiments, the spores are present in an amount of 10^2 cfu/mL to 10^{10} cfu/mL, especially 10^4 cfu/mL to 10^8 cfu/mL.

In some embodiments, steps 1 and 2 are performed separately. In other embodiments, steps 1 and 2 are performed simultaneously with a composition comprising at least one biosurfactant-producing microbe and vinasse residue and at least one biosurfactant.

The composition comprising biosurfactant-producing bacteria, vinasse residue and biosurfactant, is also useful as a biocide in high salt content compositions, for example, 7% salt solution. Such compositions are used as hydraulic fracturing (hydrofracking) compositions in natural gas high pressure well processing. The initial hydrofracking

composition may not include high salt but during use may solubilize salts from the rocks it contacts increasing its salt concentration from between 0 and 12%.

The composition may be added to hydrofracking compositions to prevent the formation of biofilms of unwanted bacteria such as sulfate and iron reducing bacteria, on the inner surfaces of pipes used in the natural gas processing. Steel pipes used in such processing often suffer from corrosion by hydrogen sulfide producing bacteria, or blockage or resistance on the inside of piping by biofilms of other types of bacteria such as salt tolerant bacteria. Problem bacteria include *Acidithiobacillus ferrooxidans* and *Desulfotomaculum halophilum*.

Without wishing to be bound by theory, the composition of the invention contains and produces biosurfactant and disrupts biofilm formation by bacteria or disperses biofilms that have already formed thereby reducing or preventing hydrogen sulphide production and blockage or sludge forming on pipes. The biosurfactant may also disrupt the cell walls of the unwanted bacteria forming micelles and disrupting cell cytoplasm, resulting in biocidal activity.

In some embodiments, particularly for use in hydrofracking water, the biosurfactant-producing bacteria present are a combination of *B subtilis* and *B. licheniformis* and the biosurfactants present are surfactin and lichenysin.

The invention will now be described with reference to the following examples which illustrate some preferred aspects of the invention. However, it is to be understood that the particularity of the following description of the invention is not to supersede the generality of the preceding description.

Brief Description of the Figure

Figure 1 is a graphical representation showing the growth of biosurfactant-producing microorganisms over time with no sulfate ions or varying amounts of sulfate ions.

Example 1: Production of Surfactin

Bacillus subtilis NRRL B-3383 strain (originally obtained from the United States Department of Agriculture) from bacterial culture on nutrient agar plates was transferred at a 2% volume by volume inoculum into 4 L shake flasks containing 2.5 L of 10% vinasse based MMS broth. The vinasse based MMS broth containing:

component	quantity
Vinasse	100 mL
Ammonium nitrate	4.1 g
sodium phosphate dibasic	5.68 g
tetrasodium tetrahydrate EDTA	1.8 mg
Manganese sulfate	6.8 mg
autoclaved deionized water	to 1 L

The flasks were placed on orbital shakers (SKC 6100, Jeio Tech) at 150 rpm while incubating at 30°C (MCO-801C Incubator, Sanyo). After 72 hours, flasks were removed from the incubator and the biomass removed from the crude culture broth by centrifugation at 8,500 rpm for 20 min at 4°C (Sorvall Evolution RC).

The pH of the resulting supernatant was brought to a pH of 2.0 using HCl which resulted in precipitation of surfactin and the supernatant stored overnight at 4°C to ensure complete precipitation. The precipitate was collected by centrifugation at 8,500 rpm for 20 minutes at 4°C. Approximately 2.5g/L of crude material was collected in the pellet. The pellet was suspended in deionized water and the pH adjusted to 8.0 using 1 M NaOH. The aqueous solution was extracted with an equal volume of dichloromethane. The dichloromethane layer was separated and allowed to evaporate to provide purified crystalline surfactin in an amount of 50 mg/L to 750 mg/L.

The samples of crystalline surfactin were examined for purity against a standard composition of pure surfactin (Sigma Aldrich, 98% pure). Analysis of the standard

composition by LC-MS showed peaks with retention times at 1.03, 1.23, 1.61, 1.74, 2.15 and 2.93 minutes. Purity was calculated based on peak area.

Four samples tested for purity using the above method were found to be 80%, 56%, 58% and 61% pure.

Example 2: Production of blends of surfactin and lichenysin

Bacillus subtilis and *Bacillus licheniformis* were used to inoculate 4 L shake flasks containing 10% molasses based MMS broth. The molasses based MMS broth containing:

component	quantity
molasses	100 mL
Ammonium nitrate	4.1 g
sodium phosphate dibasic	5.68 g
tetrasodium tetrahydrate EDTA	1.8 mg
Manganese sulfate	6.8 mg
autoclaved deionized water	to 1 L

The flasks were placed on orbital shakers (SKC 6100, Jeio Tech) at 150 rpm while incubating at 30°C (MCO-801C Incubator, Sanyo). After 72 hours, flasks were removed from the incubator and the biomass removed from the culture broth by centrifugation at 8,500 rpm for 20 min at 4°C (Sorvall Evolution RC).

The crude products were labelled MEGR102, MEGR103 and MEGR104, each being blends of varying concentrations of surfactin and lichenysin.

Example 3: Antibiotic properties of MEGR102, MEGR103 and MEGR104

Each composition MEGR102, MEGR103 and MEGR104 was tested for antibiotic properties against *E. coli*, *Desulfotomaculum halophilum* and *Acidithiobacillus ferrooxidans*.

Each composition was tested by adding the composition to 7% salt water containing 54,356 mg/L NaCl, 16,151 mg/L $CaCl_2$, 2,383 mg/L $MgCl_2$ and 535 mg/L KCl (to simulate hydrofracking water) in amounts of 1 mg/L, 3 mg/L and 5 mg/L. The salt water compositions of each concentration were then inoculated with *E. Coli* (10^8cfu), *Desulfotomaculum halophilum* (10^8 cfu) and *Acidithiobacillus ferrooxidans* (10^8 cfu).

The controls were inoculation of the 7% salt water with each bacteria in equal amounts to the test samples without the addition of MEGR composition.

The compositions were cultured at room temperature. At time intervals, samples were taken and were analysed for culture growth on agar plate to determine visual count of cfu.

The results are shown in the following Tables:

Table 1: MEGR102 / *E. coli*

Sample	Time 0	2 hours	4 hours	6 hours	72 hours
Control	Growth	Growth	Growth	Growth	No Growth
1 mg/L	Growth	Growth	No Growth	No Growth	No Growth
3 mg/L	Growth	Growth	No Growth	No Growth	No Growth
5 mg/L	Growth	Growth	No Growth	No Growth	No Growth

Table 2: MEGR102 / *D. halophilum*

Sample	Time 0	2 hours	4 hours	6 hours	72 hours
Control	Growth	Growth	Growth	Growth	Growth
1 mg/L	Growth	Growth	No Growth	No Growth	No Growth
3 mg/L	No Growth	No Growth	No Growth	No Growth	No Growth
5 mg/L	Growth	No Growth	No Growth	No Growth	No Growth

Table 3: MEGR102 / *A. ferrooxidans*

Sample	Time 0	2 hours	4 hours	6 hours	72 hours

Control	Growth	Growth	Growth	Growth	Growth
1 mg/L	Growth	Growth	Growth	Growth	No Growth
3 mg/L	Growth	Growth	Growth	No Growth	No Growth
5 mg/L	Growth	Growth	No Growth	No Growth	No Growth

Table 4: MEGR103 / *E. coli*

Sample	**Time 0**	**2 hours**	**4 hours**	**6 hours**	**72 hours**
Control	Growth	Growth	Growth	Growth	
1 mg/L	Growth	Growth	Growth	No Growth	No Growth
3 mg/L	Growth	Growth	No Growth	No Growth	No Growth
5 mg/L	Growth	Growth	No Growth	No Growth	No Growth

Table 5: MEGR103 / *D. halophilum*

Sample	**Time 0**	**2 hours**	**4 hours**	**6 hours**	**72 hours**
Control	Growth	Growth	Growth	Growth	Growth
1 mg/L	Growth	Growth	Growth	Growth	Growth
3 mg/L	Growth	No Growth	No Growth	No Growth	No Growth
5 mg/L	Growth	No Growth	No Growth	No Growth	No Growth

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Table 6: MEGR103 / *A. ferrooxidans*

Sample	**Time 0**	**2 hours**	**4 hours**	**6 hours**	**72 hours**
Control	Growth	Growth	Growth	Growth	Growth
1 mg/L	Growth	Growth	Growth	Growth	No Growth
3 mg/L	Growth	Growth	Growth	No Growth	No Growth
5 mg/L	Growth	Growth	No Growth	No Growth	No Growth

Table 7: MEGR104 / *E. coli*

Sample	**Time 0**	**2 hours**	**4 hours**	**6 hours**	**72 hours**
Control	Growth	Growth	Growth	Growth	No Growth
1 mg/L	Growth	Growth	No Growth	No Growth	No Growth

3 mg/L	Growth	Growth	No Growth	No Growth	No Growth
5 mg/L	Growth	No Growth	No Growth	No Growth	No Growth

Table 8: MEGR104 / *D. halophilum*

Sample	Time 0	2 hours	4 hours	6 hours	72 hours
Control	Growth	Growth	Growth	Growth	Growth
1 mg/L	Growth	Growth	No Growth	No Growth	Growth
3 mg/L	Growth	No Growth	No Growth	No Growth	Growth
5 mg/L	No Growth	No Growth	No Growth	No Growth	No Growth

Table 9: MEGR104 / *A. ferrooxidans*

Sample	Time 0	2 hours	4 hours	6 hours	72 hours
Control	Growth	Growth	Growth	Growth	Growth
1 mg/L	Growth	Growth	Growth	Growth	Growth
3 mg/L	Growth	Growth	Growth	No Growth	Growth
5 mg/L	Growth	Growth	Growth	No Growth	No Growth

Example 4: Reduction in interfacial tension of oil

Using test method ASTM D1331-89 to measure interfacial tension, the interfacial tension of 99.065 g of used bearing grease was found to be 7510 dynes/cm.

The bearing grease was mixed with 25 mL of a composition containing water 95.4%, surfactants 3.5%, dodecylbenzenesulfonic acid > 0.9% (DBSA), dextrose > 0.5%, sodium hydroxide > 0.2%, *Bacillus* spores 10^5 cfu/L and surfactin 5000 ppm, and allowed to stand.

At 624 hours, the interfacial tension of the bearing grease had reduced to 630 dynes/cm and at 696 hours, the interfacial tension had reduced further to 420 dynes/cm.

The reduction in interfacial tension indicated the breakdown of the bearing grease matrix to lower order hydrocarbons. This process indicates the product is suitable for use in tertiary oil recovery of heavy oil in carbonated rock formations.

Example 5: The effect of sulfate ions at different concentrations on culture

The effect of sulfate on culture broth was tested by removing all sources of sulfate from the media and replacing them with chloride salts. The culture broth contained monopotassium phosphate/dipotassium phosphate buffer adjusted to pH 7 with potassium hydroxide. Samples were then spiked with varying cocentrations of sodium sulfate (1.8 M) at 1 mL/L, 0.8 mL/L, 0.6 mL/L, 0.4 mL/L and 0.2 mL/L. Every half hour the optical density, pH and surface tension was evaluated. This test was done with the RSA-203 *B. subtilis*.

The results are shown in Figure 1. The results show that the microorganisms grow equally well with chloride salts as they do with sulfate salts. In all samples, the surface tension had dropped and stabilized around 27 dynes by 5 hours.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of producing biosurfactant comprising culturing at least one biosurfactant-producing microbe in a liquid culture medium comprising vinasse as a carbon source, wherein the culturing occurs at pH 6 to 8 and a temperature of 25°C to 40°C.

2. A method of claim 1 wherein the at least one biosurfactant-producing microbe is selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus popilliae*, *RSA-203* and mixtures thereof.

3. A method of claim 2 wherein the at least one biosurfactant-producing microbe is selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis* and mixtures thereof.

4. A method of either of claims 2 or 3 wherein the *Bacillus subtilis* is *Bacillus subtilis* NRRL B-3383, *Bacillus subtilis* RSA-203 or mixtures thereof.

5. A method of any one of claims 2 and 4 wherein the at least one biosurfactant-producing microbe comprises a mixture of *Bacillus subtilis* and *Bacillus licheniformis*.

6. A method of any one of claims 1 to 5 wherein the vinasse is sugar cane vinasse.

7. A method of any one of claims 1 to 6 wherein the vinasse is present in the liquid culture medium in an amount of from 3 to 10% w/v.

8. A method of any one of claims 1 to 7 wherein the biosurfactant is selected from the group consisting of surfactin, iturin, lichenysin, fengycin and mixtures thereof.

9. A method of claim 8 wherein the biosurfactant is surfactin.

10. A method of any one of claims 1 to 9 wherein the temperature is 30°C to 35°C.

11. A method of any one of claims 1 to 10 wherein the pH is 6.5 to 7.2.

12. A method according to any one of claims 1 to 11 wherein the liquid culture medium is inoculated with an inoculum of at least one biosurfactant-producing microbe to provide an initial optical density at 600_{nm} of 1.2 to 1.4

13. A method of any one of claims 1 to 12 wherein the liquid culture medium further comprises a catabolizable nitrogen source.

14. A method of claim 13 wherein the catabolizable nitrogen source is an ammonium salt.

15. A method of claim 14 wherein the ammonium salt is ammonium nitrate.

16. A method of any one of claims 1 to 15 wherein the liquid culture medium further comprises at least one inorganic salt.

17. A method of claim 16 wherein the at least one inorganic salt is a sulfate, chloride or phosphate of manganese, sodium or iron, or mixtures of said salts.

18. A method of any one of claims 1 to 17 wherein the culturing further comprises aerating the liquid culture medium during culturing and collecting foamate produced during aeration.

19. A method of claim 18 wherein aeration is begun before the culturing is begun.

20. Biosurfactant produced by the method of any one of claims 1 to 19.

21. A composition comprising at least one biosurfactant-producing microbe and vinasse residue, wherein the vinasse residue is formed by decomposition of vinasse by biosurfactant-producing microbes during a culturing process.

22. A composition of claim 21 further comprising at least one biosurfactant.

23. A composition of claim 22 wherein the biosurfactant is selected from surfactin, lichenysin, iturin, fengycin or mixtures thereof.

24. A composition of claim 23 wherein the biosurfactant is surfactin.

25. A composition of any one of claims 21 to 24 further comprising a microbial food source.

26. Use of a composition of any one of claims 21 to 25 in tertiary oil recovery.

27. Use of a composition of any one of claims 21 to 25 as an antibacterial composition to protect equipment from corrosion during tertiary oil recovery or natural gas high pressure well processing.

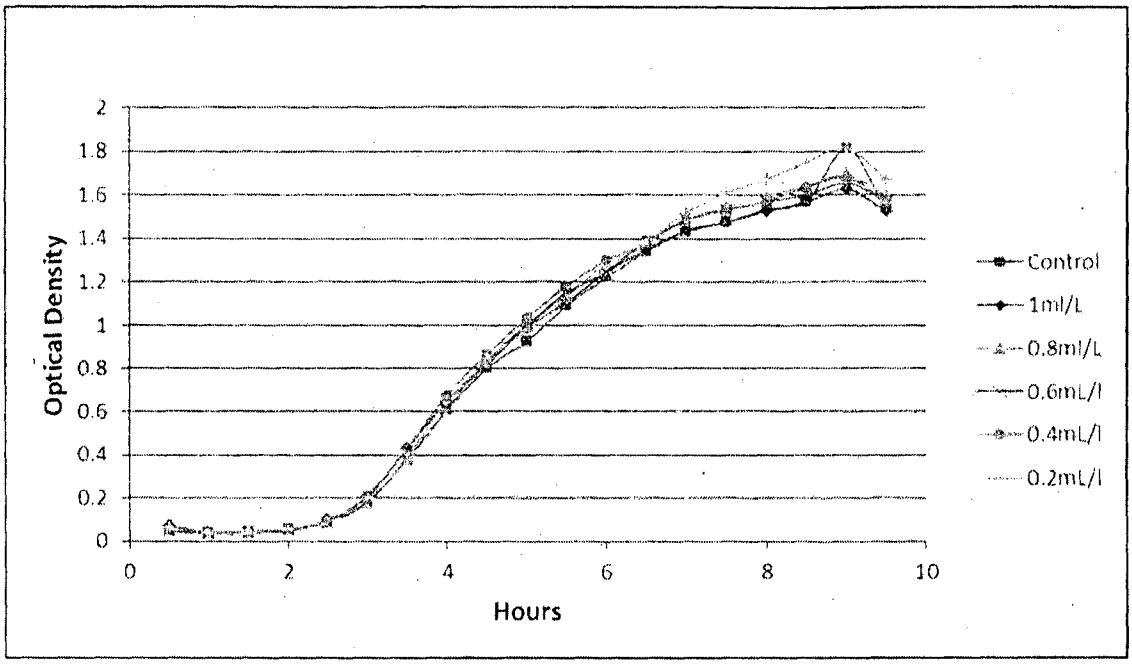
28. Use according to claim 27 wherein the natural gas high pressure well processing is water fracking.

29. Use of a composition of any one of claims 21 to 25 as a fertilizer.

30. A method of tertiary oil recovery comprising

1. treating an oil well with a composition comprising a biosurfactant, and
2. treating the oil well with a composition according to any one of claims 21 to 25.

Figure 1


Optical Density
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Hours
Control
1ml/L
0.8ml/L
0.6mL/l
0.4mL/l
0.2mL/l

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2013/000059

A. CLASSIFICATION OF SUBJECT MATTER

C12N 1/00 (2006.01) C12N 1/34 (2006.01) C05F 5/00 (2006.01) C09K 8/582 (2006.01) C09K 8/584 (2006.01) C07K 14/195 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: MEDLINE AGRICOLA 1MOBILITY TRIBO UFORDAT BIOSIS BIOTECHNO CEABA-VTB HCAPLUS NTIS EMBASE. KEYWORDS: VINASSE, SUGAR, WASTE, BIOSURFACTANT, SURFACTANT, SURFACTIN and like terms
EPOQUE: WPI, WPIDS. KEYWORDS: VINASSE, SUGAR, WASTE, BIOSURFACTANT, SURFACTANT, SURFACTIN and like terms
PATENTLENS: KEYWORDS: (MICROBIAL ENHANCED OIL RECOVERY), SURFACTANT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 2013	01 March 2013

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2013/000059**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN Accession number: 2006: 921433. & IN 189459 A1 [COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH, INDIA] 23 September 1994 Abstract	1-30
X	PLAZA, GA. ET AL. Agroindustrial wastes as unconventional substrates for growing of Bacillus strains and production of biosurfactant. Environment Protection Engineering. 2011, 37(3): 63-71. Abstract; paragraphs 3-6 p65; Figure 1; Table 2; 2nd last paragraph p69; last paragraph, p65; Conclusion p70;	1-30
X	DUBEY, K. ET AL. Distillery and curd whey wastes as viable alternative sources for biosurfactant production. World Journal of Microbiology and Biotechnology. 2001, 17: 61-69. p66 col 1; Table 3; p65 col 2; col 1 p66; col 2 p62; Biosurfactant recovery, p63; col 2 p67-col 1 p68;	1-30
X	RODRIGUEZ, N. ET AL. Use of waste materials for Lactococcus lactis development. Journal of the Science of Food and Agriculture. 2010, 90: 1726-1734. Abstract; Table 4; 2nd column p1732; 2nd column p1727; col 2, p1726;	1-30
A	MALIK, MA. ET AL. Anti-corrosion ability of surfactants: A review. International Journal of Electrochemical Science. 2011, 6:1927-1948. 3rd paragraph, p1928	26-30
A	KOSARIC, N. Biosurfactants in industry. Pure and Applied Chemistry. 1992, 64(11):1731-1737. Abstract; 1st paragraph, p1734	26-30
A	US 4,905,761 A (BRYANT, RS) 06 March 1990 col 2, lines 30-51; col 9, lines 22-50	26; 30;

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2013/000059**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 4905761 A	06 Mar 1990	US 4905761 A	06 Mar 1990

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.