



(51) International Patent Classification:

A24B 15/20 (2006.01) C12N 1/20 (2006.01)

A24B 15/24 (2006.01)

(21) International Application Number:

PCT/GB2024/052951

(22) International Filing Date:

22 November 2024 (22.11.2024)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

2317917.9 23 November 2023 (23.11.2023) GB

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(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: USE

(57) Abstract: This invention relates to the use of *Paenarthrobacter nicotinovorans* or a combination of bacteria comprising *Paenarthrobacter nicotinovorans* for the degradation of one or more alkaloids in processed tobacco.

WO 2025/109331 A1

USE

FIELD OF THE INVENTION

- 5 The present disclosure relates to the use of *Paenarthrobacter nicotinovorans* or any combination of bacteria which comprises *Paenarthrobacter nicotinovorans* and a method of using *Paenarthrobacter nicotinovorans* or any combination of bacteria which comprises *Paenarthrobacter nicotinovorans*.

10 BACKGROUND OF THE INVENTION

- Tobacco was used by an estimated 1.3 billion people worldwide in 2020. Nicotine is the primary alkaloid in tobacco, accounting for around 98% of the total alkaloid content, while other minor tobacco alkaloids include nornicotine, anabasine, anatabine, cotinine and
15 myosmine

- At certain levels nicotine may be toxic. This may be relevant in consideration of contamination of the environment with nicotine through disposal of tobacco products such as cigarette butts or waste generated during the manufacture of tobacco products. For
20 example, nicotine may be able to leach from discarded cigarette butts and contaminate water. In many countries, such as those within the EU, waste from tobacco processing is designated as “toxic and hazardous” when the nicotine content exceeds 500 mg/kg dry weight (Novotny & Zhao (1999), *Tob Control*. 8(1):75-80) and must be disposed of through permitted means.

- 25 Furthermore, the legal limits for nicotine content in products are also strictly controlled. For example in the UK, nicotine content in e-liquids is restricted to a maximum of 20 mg/ml. There are also proposals by the United States Food and Drug Administration in the US to establish a maximum nicotine level in cigarettes and other finished tobacco products.
30 A number of methods have been employed to reduce nicotine in tobacco products. For example, chemically extracting nicotine from the tobacco leaf, adjusting the mix of tobacco leaves used, or engineering the tobacco to make low-nicotine tobacco. Biological methods have also been considered for use in the reduction of nicotine in tobacco.

- 35 It would be desirable to develop methods of reducing nicotine and/or other alkaloids in processed tobacco, for example, so that the levels of nicotine and/or other alkaloids in the

treated tobacco meets the requirements of the country in which it is to be used or disposed of.

SUMMARY OF THE INVENTION

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In one aspect, there is provided a use of *Paenarthrobacter nicotinovorans* or a combination of bacteria comprising *Paenarthrobacter nicotinovorans* for the degradation of one or more alkaloids in processed tobacco.

10 In one aspect, there is provided a method of degrading one or more alkaloids in processed tobacco, wherein the method comprises:

a) inoculating the processed tobacco with *Paenarthrobacter nicotinovorans*, or a combination of bacteria of bacteria comprising *Paenarthrobacter nicotinovorans*.

15 Suitably, the *Paenarthrobacter nicotinovorans*, or combination of bacteria comprising *Paenarthrobacter nicotinovorans* are not pre-cultured.

Suitably, the *Paenarthrobacter nicotinovorans*, or combination of bacteria comprising *Paenarthrobacter nicotinovorans* may be pre-cultured. For example the bacteria may be pre-cultured in media to grow the bacteria. Suitably, the bacteria may be pre-cultured with or without one or more alkaloids.

Suitably, the *Paenarthrobacter nicotinovorans*, or combination of bacteria comprising *Paenarthrobacter nicotinovorans* may be pre-cultured with nicotine.

25

Suitably, prior to step (a) the processed tobacco may be suspended in a liquid media substantially free from carbon and nitrogen, such that the processed tobacco provides the main carbon and nitrogen source when added to the liquid media.

30 Suitably, the method may further comprise:
incubating the inoculated processed tobacco.

Suitably, the inoculated processed tobacco may be incubated for a minimum of 18 hours.

35 Suitably, the bacteria inoculated processed tobacco may be incubated for between about 18 hours and about 120 hours.

Suitably, the method may further comprise:

drying the inoculated processed tobacco.

Suitably, the method may further comprise:

5 either

(i) further processing the inoculated processed tobacco for subsequent use;

or

(ii) disposing of the inoculated processed tobacco.

10 Suitably, the initial cell density of the pre-cultured *Paenarthrobacter nicotinovorans*, or combination of bacteria comprising *Paenarthrobacter nicotinovorans* in step (a) is between about about 9.50×10^5 CFU/ml and about 9.50×10^8 CFU/ml.

15 Suitably, the one or more alkaloids may be one or more of the group comprising nicotine, nornicotine, anabasine, anatabine, myosime, cotinine and pseudooxynicotine.

Suitably, the one or more alkaloids is a minor alkaloid, such as nornicotine, anabasine, anatabine or pseudooxynicotine.

20 Suitably, the total amount of alkaloid is reduced.

Suitably, the processed tobacco is selected from the group comprising tobacco extract, cured tobacco, reconstituted tobacco, tobacco stalks, tobacco pruning leftovers, tobacco products and tobacco dust.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the invention will now be described, by way of example only, with reference to accompanying drawings, in which:

30 **Figure 1:** shows the change in pH in tobacco extracts across time for the different cultures inoculated. Data analysis was performed by Two-way Anova (repeated measures, (GraphPad prism). Data are shown as the means + SEM . Significance ($p < 0.05$, $n = 9$) among groups is denoted by different letters (a to d).

35 **Figure 2:** shows cell growth evolution in extracts across time for the different cultures inoculated. Data analysis was performed by Two-way Anova (repeated measures,

(GraphPad prism). Data are shown as the means + SEM. Significance ($p < 0.05$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 3: shows nicotine concentration in tobacco extracts inoculated with different cultures.

- 5 Data analysis was performed by Two-way Anova (repeated measures, (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.05$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 4: shows anabasine concentration in tobacco extracts inoculated with different

- 10 cultures. Data analysis was performed by Two-way Anova (repeated measures, (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.05$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 5: shows pseudoxynicotine concentration in tobacco extracts inoculated with

- 15 different cultures. Data analysis was performed by Two-way Anova (repeated measures, (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.05$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 6: shows anatabine concentration in tobacco extracts inoculated with different

- 20 cultures. Data analysis was performed by Two-way Anova (repeated measures, (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.05$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 7: shows nicotine concentration in cured tobacco inoculated with different cultures.

- 25 Data analysis was performed by Two-way Anova (repeated measures), (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.001$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 8: shows nor nicotine concentration in cured tobacco inoculated with different

- 30 cultures. Data analysis was performed by Two-way Anova (repeated measures), (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.001$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 9: shows anabasine concentration in cured tobacco inoculated with different cultures.

- 35 Data analysis was performed by Two-way Anova (repeated measures), (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.001$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 10: shows pseudooxynicotine concentration in cured tobacco inoculated with different cultures. Data analysis was performed by Two-way Anova (repeated measures), (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.001$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 11: shows anatabine concentration in cured tobacco inoculated with different cultures. Data analysis was performed by Two-way Anova (repeated measures), (GraphPad prism). Data are shown as the means + SD. Significance ($p < 0.001$, $n = 9$) among groups is denoted by different letters (a to d).

Figure 12: Nicotine biodegradation in tobacco extract by different microbial combinations and single strains. Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data are shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the control against the rest of treatments is represented by four asterisks (****). Other significant differences are *P. putida* S16 + *P. ureafaciens* vs. *P. nicotinovorans* at 24h ($p = 0.0457$), Control vs. *P. putida* S16 + *P. ureafaciens* at 6h ($p = 0.0043$), Control vs. *P. putida* S16 at 6h ($p = 0.0045$), *P. putida* S16 + *P. ureafaciens* vs. *P. nicotinovorans* at 6h ($p = 0.0216$), PS16 + *P. ureafaciens* vs. *P. ureafaciens* ($p = 0.0106$), *P. putida* 16 vs. *P. nicotinovorans* at 6h ($p = 0.0112$), *P. putida* S16 vs. *P. ureafaciens* ($p = 0.0103$) and Control vs. *P. nicotinovorans* at 4h (0.0137).

Figure 13: Nicotine biodegradation in tobacco extract by different microbial combinations and single strains. Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data are shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the control against the rest of treatments is represented by four asterisks (****). Other significant differences are *P. putida* S16 + *P. ureafaciens* vs. *P. nicotinovorans* at 24h ($p = 0.0457$), Control vs. *P. putida* S16 + *P. ureafaciens* at 6h ($p = 0.0043$), Control vs. *P. putida* S16 at 6h ($p = 0.0045$), *P. putida* S16 + *P. ureafaciens* vs. *P. nicotinovorans* at 6h ($p = 0.0216$), PS16 + *P. ureafaciens* vs. *P. ureafaciens* ($p = 0.0106$), *P. putida* 16 vs. *P. nicotinovorans* at 6h ($p = 0.0112$), *P. putida* S16 vs. *P. ureafaciens* ($p = 0.0103$) and Control vs. *P. nicotinovorans* at 4h (0.0137).

Figure 14: Nornicotine biodegradation in tobacco extract by different microbial combinations and single strains. Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data are shown as the means + SEM.

Significance ($p < 0.001$, $n = 9$) of the control against the rest of treatments is represented by four asterisks (****).

Figure 15: Anatabine biodegradation in tobacco extract by different microbial combinations and single strains. Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data are shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the control against the rest of treatments is represented by four asterisks (****).

Figure 16: Anabasine biodegradation in tobacco extract by different microbial combinations and single strains. Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data are shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the control against the rest of treatments is represented by four asterisks (****).

Figure 17: Pseudooxynicotine biodegradation in tobacco extract by different microbial combinations and single strains. Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data are shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the control against the rest of treatments is represented by four asterisks (****).

Figure 18: Cell growth of *P. putida* S16 in Luria Broth containing no nicotine (LB -) and nicotine 4 mg/ml (LB+) compared to a positive control (H_2O_2 5%). Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data is shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the differences between the three treatments is represented by letters (a,b,c). Data compiled under the same letter does not show significant difference at 24h.

Figure 19: Nicotine degradation by *P. putida* S16 exposed to nicotine (LB+) and non-exposed (LB-) in Luria Broth containing nicotine 4 mg/ml compared to mock treatment (not inoculated). Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data is shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the differences between the three treatments is represented by letters (a,b,c). Data compiled under the same letter does not show significant difference at 24h

Figure 20: Cell growth of *P. nicotinovorans* in Luria Broth containing no nicotine (LB -) and nicotine 4 mg/ml (LB+) compared to a positive control (H_2O_2 5%). Data analysis

was performed by Two-way Anova (repeated measures, GraphPad prism). Data is shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the differences between the three treatments is represented by letters (a,b,c). Data compiled under the same letter does not show significant difference at 24h.

5 **Figure 21:** Nicotine degradation by *P. nicotinovorans* exposed to nicotine (LB+) and non-exposed (LB-) in Luria Broth containing nicotine 4 mg/ml compared to mock treatment (not inoculated). Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data is shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the differences between the three treatments is represented by letters (a,b,c). Data compiled
10 under the same letter does not show significant difference at 24h.

Figure 22: Cell growth of *P. ureafaciens* in Luria Broth containing no nicotine (LB-) and nicotine 4 mg/ml (LB+) compared to a positive control (H_2O_2 5%). Data analysis was performed by Two-way Anova (repeated measures, GraphPad prism). Data is shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the differences between the
15 three treatments is represented by letters (a,b,c). Data compiled under the same letter does not show significant difference at 24h.

Figure 23: Nicotine degradation by *P. ureafaciens* exposed to nicotine (LB+) and non-exposed (LB-) in Luria Broth containing nicotine 4 mg/ml compared to mock treatment (not inoculated). Data analysis was performed by Two-way Anova (repeated measures,
20 GraphPad prism). Data is shown as the means + SEM. Significance ($p < 0.001$, $n = 9$) of the differences between the three treatments is represented by letters (a,b,c). Data compiled under the same letter does not show significance.

DEFINITIONS

25 This disclosure is not limited by the exemplary methods and materials disclosed herein, and any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of this disclosure.

30 The headings provided herein are not limitations of the various aspects or embodiments of this disclosure which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

The term “processed tobacco” herein refers to tobacco that has undergone some form of processing following harvesting, such as curing and/or fermentation. The tobacco may be further processed by methods such as grinding or through preparation of a tobacco extract. End products, such as cigarettes, waterpipe tobacco, cigars, cigarillos, heated tobacco, roll-

your-own tobacco, pipe tobacco, bidis, kreteks, and smokeless tobacco products may also be considered “processed tobacco”. Alternatively, the “processed tobacco” may be a waste product produced during the manufacture of the aforementioned end products, such as solid waste tobacco. The tobacco plant itself and/or leaves of the tobacco plant such as green leaf tobacco are not considered processed tobacco if they are used immediately after harvesting.

Any form of aged tobacco leaves may be considered processed.

The term “inoculating” refers to the introduction of bacteria to the matter to be treated. The matter in the context of this disclosure is processed tobacco. The processed tobacco may be in a liquid or solid form. Alternatively, the processed tobacco may be a solid suspended in a liquid media. It is not necessary for the processed tobacco to be suspended in a liquid media, only that there is enough water content or humidity in the surrounding environment to enable the bacteria to degrade the alkaloids as intended. For example, the bacteria could degrade the alkaloids in processed tobacco in a similar manner to a tobacco fermentation process (i.e. damp solid processed tobacco), or alternatively, the processed tobacco provides a carbon and nitrogen source in a bacterial broth (i.e. suspended in a liquid media).

The term “bacterial extract” refers to any product that has been isolated from bacteria. For example, the product may be an enzyme extracted from bacteria. Alternatively, the bacterial extract may be a gene isolated from the genome of a bacteria which is then inserted and expressed by a different cell or organism, such as a plant, virus, or alternative bacteria.

Other definitions of terms may appear throughout the specification. Before the exemplary aspects and embodiments are described in more detail, it is important to understand that this disclosure is not limited to particular aspects or embodiments described, which as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects or embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. Thus, for

example, reference to “an alkaloid” includes a plurality of such candidate agents and equivalents thereof known to those skilled in the art, and so forth.

It should also be noted that as used herein and in the appended claims, that the term “about” refers to the stated value and values that can be rounded to that value. For example, 1.0 would encompass values between 0.95 and 1.04; 1 would encompass values between 0.5 and 1.4; 10 would encompass values between 9.5 and 10.4; and so on and so forth. Where a value is expressed in terms of ‘a’ x 10^y; the term about applies to ‘a’. For example, 1.0 x 10³ would encompass values between 0.95 x 10² and 1.04 x 10³.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs the use of at least one alkaloid degrading e.g. nicotine-degrading bacterial strain or species, specifically *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, and *Paenarthrobacter ureafaciens* for degrading alkaloids, in particular nicotine, in processed tobacco. During manufacture of tobacco products, liquid, solid and airborne waste is generated. For example, liquid wastes include tobacco slurries, solvents oils and greases that originate in the manufacturing processes, building services and facilities. Solid wastes include paper, wood, plastics, unusable tobacco, packaging materials and dirt that originate in the manufacturing process. Airborne wastes include odours of manufacturing, tobacco volatiles and particles and other emissions.

Further, there is a demand for low-nicotine or nicotine-free products. The present invention provides a way to reduce not only the nicotine in the processed tobacco, but also other harmful alkaloids.

Use

In one aspect, there is provided a use of one or more bacterial strains or bacterial species, or a combination thereof, for the degradation of one or more alkaloids in processed tobacco.

In one aspect there is provided a use of *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof for the degradation of one or more alkaloids in processed tobacco.

In one embodiment, there is provided a use of *Pseudomonas putida* S16 for the degradation of one or more alkaloids in processed tobacco.

In one embodiment, there is provided a use of *Paenarthrobacter nicotinovorans* for the degradation of one or more alkaloids in processed tobacco.

- 5 In one embodiment, there is provided a use of *Paenarthrobacter ureafaciens* for the degradation of one or more alkaloids in processed tobacco.

In one embodiment, there is provided a use of the combination of *Pseudomonas putida* S16 and *Paenarthrobacter nicotinovorans* for the degradation of one or more alkaloids in
10 processed tobacco.

In one embodiment, there is provided a use of the combination of *Pseudomonas putida* S16 and *Paenarthrobacter ureafaciens* for the degradation of one or more alkaloids in processed tobacco.

15 In one embodiment, there is provided a use of the combination of *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens* for the degradation of one or more alkaloids in processed tobacco.

20 In one embodiment, there is provided a use of the combination of *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens* for the degradation of one or more alkaloids in processed tobacco.

In one embodiment, there is provided use of a bacterial extract for the degradation of one or
25 more alkaloids in processed tobacco.

In a further embodiment the bacterial extract is an enzyme derived from an alkaloid-degrading bacteria (e.g. nicotine-degrading bacteria) that degrades alkaloids through the pyrrolidine or pyridine pathways. Suitably, the alkaloid-degrading bacteria may be a nicotine
30 degrading bacteria.

In yet a further embodiment, the enzyme is one or more of the group comprising or consisting of nicotine oxidoreductase (NicA), pseudooxynicotine amine oxidase (PNAO), 3-succinoylsemialdehyde pyridine dehydrogenase (SAPD), 6-hydroxy-3-succinoyl pyridine
35 hydroxylase (HspA and/or HspB), nicotine dehydrogenase (NDH), 6-hydroxy-L-nicotine oxidase (6-HLNO), 6-hydroxy-D-nicotine oxidase (6HDNO), ketone oxidase (KO), ketone dehydrogenase (KDH), 2,6-dihydroxypseudooxynicotine hydrolase (2,6-DHPONH), 2,6-

dihydropyridine-3-hydroxylase (2,6-DHPH), γ -N-methylaminobutyrate oxidase (MABO), monoamine oxidase (MAO), amine oxidase (AO), methylenetetrahydrofolate dehydrogenase/cyclohydrolase (FolD); formyltetrahydrofolate deformylase (PurU), succinic semialdehyde dehydrogenase (SsaDH), or variants thereof.

5

In one embodiment, there is provided use of a bacterial extract from *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof for the degradation of one or more alkaloids in processed tobacco.

10 In a further embodiment, the bacterial extract is an enzyme derived from *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof.

15 In yet a further embodiment, the enzyme is one or more of the group comprising or consisting of nicotine oxidoreductase (NicA2), L-6-hydroxynicotine oxidase, berberine bridge enzyme or berberine bridge enzyme-like polypeptides, and variants thereof.

In one embodiment, the one or more alkaloids are one or more of the group comprising nicotine, nornicotine, anabasine, anatabine, myosime, cotinine, and pseudooxynicotine.
20 Suitably, any combination of the following alkaloids may be degraded: nicotine, nornicotine, anabasine, anatabine, myosime, cotinine, and pseudooxynicotine.

In a further embodiment, the one or more alkaloids is nicotine. In other words, at least one alkaloid is nicotine.

25

In a further embodiment, the one or more alkaloids is nornicotine. In other words, at least one alkaloid is nornicotine.

30 In a further embodiment, the one or more alkaloids is anabasine. In other words, at least one alkaloid is anabasine.

In a further embodiment, the one or more alkaloids is anatabine. In other words, at least one alkaloid is anatabine.

35 In a further embodiment, the one or more alkaloids is myosime. In other words, at least one alkaloid is myosime.

In a further embodiment, the one or more alkaloids is cotinine. In other words, at least one alkaloid is cotinine.

5 In a further embodiment, the one or more alkaloids is pseudooxynicotine. In other words, at least one alkaloid is pseudooxynicotine.

10 In one embodiment, the one or more alkaloids are nicotine, nornicotine, anabasine, anatabine, and pseudooxynicotine. In other words, nicotine, nornicotine, anabasine, anatabine, and pseudooxynicotine are degraded.

In one embodiment, the one or more alkaloids are nicotine, nornicotine, anabasine, and anatabine. In other words, nicotine, nornicotine, anabasine, and anatabine are degraded.

15 In one embodiment, the one or more alkaloids are nicotine, nornicotine, anatabine, and pseudooxynicotine. In other words, nicotine, nornicotine, anatabine, and pseudooxynicotine are degraded.

20 In one embodiment, the one or more alkaloids are nicotine, nornicotine, and pseudooxynicotine. In other words, nicotine, nornicotine, and pseudooxynicotine are degraded.

In one embodiment, the one or more alkaloids are nicotine and anatabine. In other words, nicotine and anatabine are degraded.

25 In one embodiment the processed tobacco is selected from the group comprising tobacco extract, cured tobacco, reconstituted tobacco, tobacco stalks, tobacco pruning leftovers, tobacco products, and tobacco manufacturing waste such as tobacco dust.

30 In one embodiment, the processed tobacco may be processed by one or more of: curing, fermenting and/or pasteurising.

In a further embodiment, the cured tobacco is air cured, flue cured, sun cured, or fire cured.

Typically air curing is achieved by hanging tobacco leaf in well-ventilated barns and allowing to dry. This is usually carried out over a period of four to eight weeks. Air curing is especially
35 suitable for Burley tobacco.

Suitably the tobacco leaf may be fire cured. Fire curing is typically achieved by hanging tobacco leaf in large barns where fires of hardwoods are kept on continuous or intermittent low smoulder and usually takes between three days and ten weeks, depending on the process and the tobacco.

5

In another embodiment the tobacco leaf may be flue cured. Flue curing may comprise stringing tobacco leaves onto tobacco sticks and hanging them from tier-poles in curing barns. The barns usually have a flue which runs from externally fed fire boxes. Typically this results in tobacco that has been heat-cured without being exposed to smoke. Usually the temperature will be raised slowly over the course of the curing with the whole process taking approximately 1 week.

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Suitably the tobacco leaf may be sun cured. This method typically involves exposure of uncovered tobacco to the sun.

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Suitably the processed tobacco leaf may be processed by fermenting. Fermentation can be carried out in any manner known in the art. Typically during fermentation, the tobacco leaves are piled into stacks (a bulk) of cured tobacco covered in e.g. burlap to retain moisture. The combination of the remaining water inside the leaf and the weight of the tobacco generates a natural heat which ripens the tobacco. The temperature in the centre of the bulk is monitored daily. In some methods every week, the entire bulk is opened. The leaves are then removed to be shaken and moistened and the bulk is rotated so that the inside leaves go outside and the bottom leaves are placed on the top of the bulk. This ensures even fermentation throughout the bulk. The additional moisture on the leaves, plus the actual rotation of the leaves themselves, generates heat, releasing the tobacco's natural ammonia and reducing nicotine, while also deepening the colour and improving the tobacco's aroma. Typically the fermentation process continues for up to 6 months, depending on the variety of tobacco, stalk position on the leaf, thickness and intended use of leaf.

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Suitably the processed tobacco leaf may be processed by pasteurising. Pasteurising may be particularly preferred when the tobacco leaf will be used to make a smokeless tobacco product, most preferably snus. Tobacco leaf pasteurisation may be carried out by any method known in the art. For example, pasteurisation may be carried out as detailed in J Foulds, L Ramstrom, M Burke, K Fagerstrom. Effect of smokeless tobacco (snus) on smoking and public health in Sweden. Tobacco Control (2003) 12: 349–359, the teaching of which is incorporated herein by reference.

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During the production of snus, pasteurisation is typically carried out by a process in which the tobacco is heat treated with steam for 24–36 hours (reaching temperatures of approximately 100°C). This results in an almost sterile product and without wishing to be bound by theory one of the consequences of this is believed to be a limitation of further TSNA formation.

In one embodiment the pasteurisation may be steam pasteurisation.

The tobacco material can be derived or obtained from varieties of *Nicotiana tabacum* types, commonly known as Burley varieties, flue or bright varieties and dark varieties.

In some embodiments, the tobacco material is derived from a Burley, Virginia or a dark tobacco plant.

The tobacco plant may be selected from Burley tobacco, rare tobacco, speciality tobacco, expanded tobacco or the like.

The use of tobacco cultivars and elite tobacco cultivars is also contemplated herein. The tobacco plant for use herein may therefore be a tobacco variety or elite tobacco cultivar.

Particularly useful *Nicotiana tabacum* varieties include Flue-cured Virginia type, Burley type, and Oriental type.

In yet a further embodiment, the cured tobacco is one or more of Burley tobacco, Virginia tobacco, and Oriental tobacco.

The processed tobacco may be present in tobacco products such as cigarettes, waterpipe tobacco, cigars, cigarillos, heated tobacco, roll-your-own tobacco, pipe tobacco, bidis, kreteks, smokeless tobacco products (e.g. snus, snuff, or chewing tobacco), and hybrid vapour products.

The processed tobacco may also be a waste product from the tobacco industry, such as tobacco production waste. Waste from the tobacco industry, such as tobacco production waste may be liquid waste or solid waste. For example, liquid wastes include tobacco slurries, effluent, solvents, oils and greases that originate in the manufacturing processes, building services and facilities. Solid wastes include paper, wood, plastics, unusable tobacco, unusable cured tobacco, packaging materials and dirt that originate in the manufacturing process.

The processed tobacco may also be a waste product from consumption waste. For example, consumption waste includes cigarette butts, filters and packaging such as cartons and papers.

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Composition

In one aspect, there is provided a composition comprising two or more alkaloid-degrading bacteria e.g. nicotine-degrading bacteria. Suitably, the composition may comprise at least one bacterial strain and at least one bacterial species. Suitably, the composition may comprise at least two bacterial strains. Suitably, the composition may comprise at least two bacterial species.

In one embodiment, the alkaloid-degrading bacteria e.g. nicotine-degrading bacteria comprises two or more of *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens*.

In one embodiment, the alkaloid-degrading bacteria e.g. nicotine-degrading bacteria are *Pseudomonas putida* S16 and *Paenarthrobacter nicotinovorans*.

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In one embodiment, the alkaloid-degrading bacteria e.g. nicotine-degrading bacteria are *Pseudomonas putida* S16 and *Paenarthrobacter ureafaciens*.

In one embodiment, the alkaloid-degrading bacteria e.g. nicotine-degrading bacteria are *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens*.

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In one embodiment, the alkaloid-degrading bacteria e.g. nicotine-degrading bacteria are *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens*.

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In one embodiment, the composition is for use in the degradation of one or more alkaloids in processed tobacco.

Method

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In one aspect, there is provided a method of degrading one or more alkaloids in processed tobacco, wherein the method comprises:

a) inoculating the processed tobacco with a bacterial strain or bacterial species, or any combination thereof.

In one embodiment, the bacterial strain or bacterial species is pre-cultured with at least one alkaloid. Suitably, the bacterial strain or bacterial species is pre-cultured with nicotine.

In one aspect, there is provided a method of degrading one or more alkaloids in processed tobacco, wherein the method comprises:

a) inoculating the processed tobacco with *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof.

In one embodiment, the *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof are pre-cultured with at least one alkaloid.

In one embodiment, the *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof are pre-cultured with nicotine.

The bacteria may be pre-cultured with at least one alkaloid, such as nicotine, to promote the alkaloid-degrading (e.g. nicotine-degrading) enzymatic pathways in these bacteria. Known pathways in nicotine-degrading bacteria are the pyridine pathway and the pyrrolidine pathway, with a hybrid pathway also described.

In one embodiment, prior to step (a) the processed tobacco is suspended in a liquid media substantially free from carbon and nitrogen, such that the processed tobacco provides the main carbon and nitrogen source when added to the liquid media.

In a further alternative embodiment, prior to step (a) the processed tobacco is suspended in a liquid media substantially free from carbon and nitrogen, such that the alkaloids in the processed tobacco are degraded.

In one embodiment, prior to step (a) the processed tobacco is suspended in a liquid media substantially free from carbon and nitrogen, such that the alkaloids in the processed tobacco are preferentially degraded. Suitably, the alkaloids in the processed tobacco may be

preferentially used as a carbon and/or nitrogen source over any other carbon and/or nitrogen present.

“Substantially free from” means that the liquid media contains minimal amounts of the excluded matter, such that any source of the excluded matter that is added to the liquid media is preferentially degraded by the bacteria. For example, the liquid media disclosed herein contains minimal amounts of carbon and nitrogen, such that when the processed tobacco is added to the liquid media, the bacteria use the alkaloids in the processed tobacco as their sole source of carbon and nitrogen.

In one embodiment, substantially free from may be any concentration less than 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0.5% w/v of carbon and/or nitrogen.

In one embodiment, substantially free from may be any concentration less than 15% w/v of carbon and/or nitrogen.

In one embodiment, substantially free from may be any concentration less than 14% w/v of carbon and/or nitrogen.

In one embodiment, substantially free from may be any concentration less than 13% w/v of carbon and/or nitrogen.

In one embodiment, substantially free from may be any concentration less than 12% w/v of carbon and/or nitrogen.

In one embodiment, substantially free from may be any concentration less than 11% w/v of carbon and/or nitrogen.

In one embodiment, substantially free from may be any concentration less than 10% w/v of carbon and/or nitrogen.

In one embodiment, substantially free from may be a concentration of 0% w/v of carbon and/or nitrogen.

It is important that the liquid media is substantially free from carbon and nitrogen so that the bacteria use the alkaloids present in the processed tobacco as their carbon and nitrogen

source, thus degrading the alkaloids in the tobacco. The liquid media may contain carbon or nitrogen from sources other than the processed tobacco, such as yeast extract, providing some minimal nitrogen for the bacterial culture to establish before it begins degrading the alkaloids in the processed tobacco.

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In a further embodiment, the liquid media is adjusted to between pH 6.0 and pH 8.0.

In a further embodiment, the liquid media is adjusted to between pH 6.1 and pH 7.9.

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In a further embodiment, the liquid media is adjusted to between pH 6.2 and pH 7.8.

In a further embodiment, the liquid media is adjusted to between pH 6.3 and pH 7.7.

In a further embodiment, the liquid media is adjusted to between pH 6.4 and pH 7.6.

15

In a further embodiment, the liquid media is adjusted to between pH 6.4 and pH 7.5.

In a further embodiment, the liquid media is adjusted to between pH 6.5 and pH 7.5.

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In a further embodiment, the liquid media is adjusted to between pH 6.6 and pH 7.4.

In a further embodiment, the liquid media is adjusted to between pH 6.7 and pH 7.3.

In a further embodiment, the liquid media is adjusted to between pH 6.8 and pH 7.2.

25

In a further embodiment, the liquid media is adjusted to between pH 6.9 and pH 7.1.

In a further embodiment, the liquid media is adjusted to pH 7.0.

30

In one embodiment, the method further comprises:

b) incubating the inoculated processed tobacco.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 10 hours.

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In one embodiment, the inoculated processed tobacco is incubated for a minimum of 11 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 12 hours.

- 5 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 13 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 14 hours.

10

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 15 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 16 hours.

15

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 17 hours.

- 20 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 18 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 19 hours.

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In one embodiment, the inoculated processed tobacco is incubated for a minimum of 20 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 21 hours.

30

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 22 hours.

- 35 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 23 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 24 hours.

5 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 25 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 26 hours.

10 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 27 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 28 hours.

15 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 29 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 30 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 36 hours.

25 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 42 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 48 hours.

30 In one embodiment, the inoculated processed tobacco is incubated for a minimum of 72 hours.

In one embodiment, the inoculated processed tobacco is incubated for a minimum of 96 hours.

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In one embodiment, the inoculated processed tobacco is incubated for a minimum of 120 hours.

5 In one embodiment, the inoculated processed tobacco is incubated for between about 18 hours and about 120 hours.

In one embodiment, the inoculated processed tobacco is incubated for between about 18 hours and about 144 hours.

10 In one embodiment, the inoculated processed tobacco is incubated for between about 18 hours and about 168 hours.

In one embodiment, the inoculated processed tobacco is incubated for between about 24 hours and about 120 hours.

15 In one embodiment, the inoculated processed tobacco is incubated for between about 24 hours and about 144 hours.

20 In one embodiment, the inoculated processed tobacco is incubated for between about 24 hours and about 168 hours.

In one embodiment, the inoculated processed tobacco is incubated for between about 48 hours and about 120 hours.

25 In one embodiment, the inoculated processed tobacco is incubated for between about 48 hours and about 144 hours.

In one embodiment, the inoculated processed tobacco is incubated for between about 48 hours and about 168 hours.

30 In a further embodiment, the inoculated processed tobacco is incubated at between 10°C and 45°C.

35 In a further embodiment, the inoculated processed tobacco is incubated at between 15°C and 40 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 15°C and 39 °C.

5 In a further embodiment, the inoculated processed tobacco is incubated at between 15°C and 38 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 15°C and 37 °C.

10 In a further embodiment, the inoculated processed tobacco is incubated at between 16°C and 37 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 17°C and 37 °C.

15 In a further embodiment, the inoculated processed tobacco is incubated at between 18°C and 37 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 19°C and 37 °C.

20 In a further embodiment, the inoculated processed tobacco is incubated at between 20°C and 37 °C.

25 In a further embodiment, the inoculated processed tobacco is incubated at between 21°C and 37 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 22°C and 37 °C.

30 In a further embodiment, the inoculated processed tobacco is incubated at between 23°C and 37 °C.

35 In a further embodiment, the inoculated processed tobacco is incubated at between 24°C and 36 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 25°C and 35 °C.

5 In a further embodiment, the inoculated processed tobacco is incubated at between 26°C and 34 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 27°C and 33 °C.

10 In a further embodiment, the inoculated processed tobacco is incubated at between 28°C and 32 °C.

In a further embodiment, the inoculated processed tobacco is incubated at between 29°C and 31 °C.

15

In a further embodiment, the inoculated processed tobacco is incubated at a minimum of 15 °C, 16 °C, 17 °C, 18 °C, 19 °C, 20°C, 21 °C, 22 °C, 24 °C, 25 °C, 26 °C, 27 °C, 28 °C, 29 °C, 30 °C, 31 °C, 32 °C, 33 °C, 34 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, or 40 °C.

20 In a further embodiment, the inoculated processed tobacco is incubated at a maximum of 45 °C , 44 °C , 43°C , 42 °C , 41 °C, 40 °C, 39 °C, 38 °C, 37 °C, 36 °C, 35 °C, 34 °C, 33 °C, 32 °C, 31 °C, 30 °C, 29 °C, 28 °C, 27 °C, 26 °C, 25 °C, 24 °C, 23 °C, 22 °C, 21 °C, 20 °C, 19 °C, 18 °C, 17 °C, 16 °C, or 15 °C.

25 In a further embodiment, the inoculated processed tobacco is incubated at 15 °C, 16 °C, 17 °C, 18 °C, 19 °C, 20 °C, 21 °C, 22 °C, 23 °C, 24 °C, 25 °C, 26 °C, 27 °C, 28 °C, 29 °C, 30 °C, 31 °C, 32 °C, 33 °C, 34 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, 40 °C, 41 °C, 42 °C, 43 °C, 44 °C, or 45 °C.

30 In a further embodiment, the inoculated processed tobacco is incubated at 26 °C.

In a further embodiment, the inoculated processed tobacco is incubated at 27 °C.

In a further embodiment, the inoculated processed tobacco is incubated at 28 °C.

35

In a further embodiment, the inoculated processed tobacco is incubated at 29 °C.

In a further embodiment, the inoculated processed tobacco is incubated at 30 °C.

In a further embodiment, the inoculated processed tobacco is incubated at 31 °C.

5

In a further embodiment, the inoculated processed tobacco is incubated at 32 °C.

In a further embodiment, the inoculated processed tobacco is incubated at 33 °C.

10 In a further embodiment, the inoculated processed tobacco is incubated at 34 °C.

In a further embodiment, the inoculated processed tobacco is shaken whilst it is incubated.

In yet a further embodiment, the inoculated processed tobacco is shaken at between

15 100 rpm to 450 rpm.

In yet a further embodiment, the inoculated processed tobacco is shaken at between

150 rpm to 400 rpm.

20 In yet a further embodiment, the inoculated processed tobacco is shaken at between

150 rpm to 350 rpm.

In yet a further embodiment, the inoculated processed tobacco is shaken at between

150 rpm to 300 rpm.

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In yet a further embodiment, the inoculated processed tobacco is shaken at between

150 rpm to 250 rpm.

In yet a further embodiment, the inoculated processed tobacco is shaken at between

30 150 rpm to 200 rpm.

In yet a further embodiment, the inoculated processed tobacco is shaken at 150 rpm.

In yet a further embodiment, the inoculated processed tobacco is shaken at 160 rpm.

35

In yet a further embodiment, the inoculated processed tobacco is shaken at 170 rpm.

In yet a further embodiment, the inoculated processed tobacco is shaken at 100 rpm, 110 rpm, 120 rpm, 130 rpm, 140 rpm, 150 rpm, 160 rpm, 170 rpm, 180 rpm, 190 rpm, 200 rpm, 210 rpm, 220 rpm, 230 rpm, 240 rpm, or 250 rpm.

5 In one embodiment, the method further comprises:

c) drying the inoculated processed tobacco.

In one embodiment, the method further comprises:

e) either

- 10 (i) further processing the inoculated processed tobacco for subsequent use;
or
(ii) disposing of the inoculated processed tobacco.

The skilled person will understand that “subsequent use” may include the manufacture of
15 tobacco containing products, such as cigarettes, waterpipe tobacco, cigars, cigarillos, heated tobacco, roll-your-own tobacco, pipe tobacco, bidis, kreteks, smokeless tobacco products (e.g. snus, snuff, or chewing tobacco), and hybrid vapour products.

In one embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16,
20 *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 9.50×10^5 CFU/ml and about 9.50×10^8 CFU/ml.

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof
25 in step (a) is between about 9.50×10^5 CFU/ml and about 9.40×10^8 CFU/ml.

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof
in step (a) is between about 9.60×10^5 CFU/ml and about 9.40×10^8 CFU/ml.
30

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof
in step (a) is between about 9.70×10^5 CFU/ml and about 9.40×10^8 CFU/ml.

35 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 9.75×10^5 CFU/ml and about 9.35×10^8 CFU/ml.

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 9.77×10^5 CFU/ml and about 9.34×10^8 CFU/ml.

5

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 9.80×10^5 CFU/ml and about 9.30×10^8 CFU/ml.

10 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is a between about 9.5×10^5 CFU/ml and about 3.5×10^6 CFU/ml.

15 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is a between about 9.5×10^5 CFU/ml and about 1.5×10^6 CFU/ml.

20 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is a between about 2.5×10^6 CFU/ml and about 3.5×10^6 CFU/ml.

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 1.0×10^7 CFU/ml and about 9.0×10^7 CFU/ml.

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In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 1.5×10^7 CFU/ml and about 2.5×10^7 CFU/ml.

30 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 8.0×10^7 CFU/ml and about 9.0×10^7 CFU/ml.

35 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 4.5×10^8 CFU/ml and about 1.0×10^9 CFU/ml.

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 4.5×10^8 CFU/ml and about 5.5×10^8 CFU/ml.

- 5 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about 9.0×10^8 CFU/ml and about 1.0×10^9 CFU/ml.

- 10 In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16 in step (a) is a minimum of about 9.8×10^5 CFU/ml.

In a further embodiment, the initial cell density of the pre-cultured *Pseudomonas putida* S16 in step (a) is a minimum of about 2.9×10^6 CFU/ml.

- 15 In a further embodiment, the initial cell density of the pre-cultured *Paenarthrobacter nicotinovorans* in step (a) is a minimum of about 5.2×10^8 CFU/ml.

In a further embodiment, the initial cell density of the pre-cultured *Paenarthrobacter nicotinovorans* in step (a) is a minimum of about 9.3×10^8 CFU/ml.

20

In a further embodiment, the initial cell density of the pre-cultured *Paenarthrobacter ureafaciens* in step (a) is a minimum of about 2.1×10^7 CFU/ml.

- 25 In a further embodiment, the initial cell density of the pre-cultured *Paenarthrobacter ureafaciens* in step (a) is a minimum of about 8.3×10^7 CFU/ml.

As used herein, “initial cell density” refers to the cell density of the bacteria before application to the processed tobacco, for example the cell density in the pre-culture.

- 30 In one embodiment, the one or more alkaloids are reduced to a level suitable for safe disposal.

- “Suitable for safe disposal” is considered to be the level of alkaloids at which the alkaloids will cause minimal harm to the environment or people. This level may not be a complete elimination of the alkaloids and local regulations for what is considered suitable for safe disposal may differ between locations or jurisdictions. The parameters of the method, such as temperature, cell density and incubation time, can be tailored to alter the final
- 35

concentration of the alkaloids, such that they result in a level which is considered "suitable for safe disposal".

In one embodiment, the level of nicotine suitable for safe disposal is less than or equal to 0.25 % w/v.

In one aspect the processed tobacco is inoculated with *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, or *Paenarthrobacter ureafaciens*, or any combination thereof.

In one embodiment, the processed tobacco is inoculated with *Pseudomonas putida* S16.

In one embodiment, the processed tobacco is inoculated with *Paenarthrobacter nicotinovorans*.

In one embodiment, the processed tobacco is inoculated with *Paenarthrobacter ureafaciens*.

In one embodiment, the processed tobacco is inoculated with a combination of *Pseudomonas putida* S16 and *Paenarthrobacter nicotinovorans*.

In one embodiment, the processed tobacco is inoculated with a combination of *Pseudomonas putida* S16 and *Paenarthrobacter ureafaciens*.

In one embodiment, the processed tobacco is inoculated with a combination of *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens*.

In one embodiment, the processed tobacco is inoculated with a combination of *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens*.

In one embodiment, the processed tobacco is inoculated with a bacterial extract from *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, or *Paenarthrobacter ureafaciens*, or any combination thereof.

In a further embodiment, the bacterial extract is one or more enzymes derived from *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, or *Paenarthrobacter ureafaciens*, or any combination thereof.

In yet a further embodiment, the enzyme is one or more of the group comprising or consisting of nicotine oxidoreductase (NicA2), L-6-hydroxynicotine oxidase, berberine bridge enzyme or berberine bridge enzyme-like polypeptides, and variants thereof.

5

In one embodiment, the one or more alkaloids are one or more of the group comprising nicotine, nornicotine, anabasine, anatabine, myosime, cotinine and pseudooxynicotine.

10 In a further embodiment, the one or more alkaloids is nicotine. In other words, at least one alkaloid is nicotine.

In a further embodiment, the one or more alkaloids is nornicotine. In other words, at least one alkaloid is nornicotine.

15 In a further embodiment, the one or more alkaloids is anabasine. In other words, at least one alkaloid is anabasine.

In a further embodiment, the one or more alkaloids is anatabine. In other words, at least one alkaloid is anatabine.

20

In a further embodiment, the one or more alkaloids is myosime. In other words, at least one alkaloid is myosime.

25 In a further embodiment, the one or more alkaloids is cotinine. In other words, at least one alkaloid is cotinine.

In a further embodiment, the one or more alkaloids is pseudooxynicotine. In other words, at least one alkaloid is pseudooxynicotine.

30 In one embodiment, the one or more alkaloids are nicotine, nornicotine, anabasine, anatabine, and pseudooxynicotine. In other words, nicotine, nornicotine, anabasine, anatabine, and pseudooxynicotine are degraded.

35 In one embodiment, the one or more alkaloids are nicotine, nornicotine, anabasine, and anatabine. In other words, nicotine, nornicotine, anabasine, and anatabine are degraded.

In one embodiment, the one or more alkaloids are nicotine, nornicotine, anatabine, and pseudooxynicotine. In other words, nicotine, nornicotine, anatabine, and pseudooxynicotine are degraded.

- 5 In one embodiment, the one or more alkaloids are nicotine, nornicotine, and pseudooxynicotine. In other words, nicotine, nornicotine, and pseudooxynicotine are degraded.

- 10 In one embodiment, the one or more alkaloids are nicotine and anatabine. In other words, nicotine and anatabine are degraded.

- 15 In one embodiment the processed tobacco is selected from the group comprising tobacco extract, cured tobacco, reconstituted tobacco, tobacco stalks, tobacco pruning leftovers, tobacco products and tobacco manufacturing waste such as tobacco dust.

In a further embodiment, the cured tobacco is air cured, flue cured, sun cured, or fire cured.

- 20 In one embodiment, the processed tobacco may be processed by one or more of: curing, fermenting and/or pasteurising.

In yet a further embodiment, the cured tobacco is one or more of Burley tobacco, Virginia tobacco or Oriental tobacco.

- 25 The processed tobacco may be present in tobacco products such as cigarettes, waterpipe tobacco, cigars, cigarillos, heated tobacco, roll-your-own tobacco, pipe tobacco, bidis, kreteks, smokeless tobacco products (e.g. snus, snuff, or chewing tobacco), and hybrid vapour products.

- 30 The processed tobacco may also be a waste product from the tobacco industry, such as tobacco production waste. Waste from the tobacco industry, such as tobacco production waste may be liquid waste or solid waste. For example, liquid wastes include tobacco slurries, effluent, solvents, oils and greases that originate in the manufacturing processes, building services and facilities. Solid wastes include paper, wood, plastics, unusable tobacco, unusable cured tobacco, packaging materials and dirt that originate in the
35 manufacturing process.

The processed tobacco may also be a waste product from consumption waste. For example, consumption waste includes cigarette butts, filters and packaging such as cartons and papers.

- 5 The various aspects and/or embodiments described herein are presented only to assist in understanding and teaching the claimed features. These aspects and/or embodiments are provided as a representative sample of aspects and/or embodiments only, and are not exhaustive and/or exclusive. It is to be understood that advantages, embodiments, examples, functions, features, structures, and/or other aspects described herein are not to be considered limitations on the scope of the invention as defined by the claims or limitations on equivalents to the claims, and that other embodiments may be utilised and modifications may be made without departing from the scope of the claimed invention. Various embodiments of the invention may suitably comprise, consist of, or consist essentially of, appropriate combinations of the disclosed elements, components, features, parts, steps, means, etc., other than those specifically described herein. In addition, this disclosure may include other inventions not presently claimed, but which may be claimed in future.

The invention will now be described with reference to the following non-limiting examples.

20 EXAMPLES

Example 1: Pre-culturing bacteria

- Three different bacteria strains were selected and used in this experiment. These microorganisms were chosen due their ability to degrade nicotine through different metabolic pathways. Therefore, these bacteria could use nicotine as the main carbon and nitrogen source.

- *Pseudomonas putida* S16 (BAA 2546) (pyrrolidine pathway)
- 30 • *Paenarthrobacter nicotinovorans* (DSM420) (pyridine pathway)
- *Paenarthrobacter ureafaciens* (DSM419) (pyridine pathway)

- From glycerol stocks, the different strains were streaked in Inorganic Salt (IS) + 4g/L Nicotine Agar plates. Petri plates were sealed with parafilm and incubated at 30°C. After 3 days single colonies appeared in the media.

One single colony per strain was then selected and inoculated into 10 ml IS+ Nicotine 4g/L Liquid Media. Inoculums were incubated at 235 rpm and 30°C (50 ml falcon covered by aluminium film) for 24 h.

- 5 Cell growth was confirmed prior to further use of the inoculums.

Preparation of the nicotine pre-culture media

- 10 The media was comprised of the components listed in Table 1, excluding nicotine. The final pH of the resultant media was pH 4.2 and was not adjusted. The prepared media was then sterilised by autoclaving.

The nicotine was filtered by syringe filters and the desired amount was measured using a sterile microcentrifuge tube under sterile conditions, by measuring weight on a balance.

- 15 Following autoclaving of the media, the filtered and weighed nicotine was transferred to the sterile media using a micropipette to take all the liquid. The final concentration of the nicotine in the media was 4 g/L.

- 20 Following addition of the nicotine to the media, the pH was corrected to pH 7.0 using filter sterilised NaOH 1 M and HCl 1 M.

Control media was prepared in the same way as above, excluding the addition of nicotine.

Table 1: Components of the Inorganic Salt Media with Nicotine

Nicotine	4 g
FeSO ₄	0.025 g
KH ₂ PO ₄	2 g
KCl	5 g
MgSO ₄	0.25 g
Yeast Extract	0.1 g
Agar	15 g
DI Water	to 1 L

Example 2: Degrading alkaloids in tobacco extractTobacco Extract preparation

5 Tobacco extracts were made by mixing 5 grams of cured Tobacco, Burley TN90 Tobacco-K Control Upper Leaves with RO water up to a volume of 245ml. Once well mixed, the solution was filtered 2 times with laboratory paper filter to discard suspended particles in the resultant extract. Once filtered, pH was adjusted to 7.0 using NaOH 1 M. After this, the volume was adjusted in a measuring cylinder using RO water up to 250 ml. The final mix was then filter
10 sterilised using vacuum filtering (0.2 uM filter) and stored at 5 °C.

Inoculation and sampling

The inoculums were prepared as outlined in Example 1.

15

After the inoculums were ready and cell growth was confirmed, 15 ml of Tobacco Extract was inoculated with 1 ml of the initial inoculum. Initial concentrations of cultures were *Pseudomonas putida* S16 9.77×10^5 CFU/ml; *Paenarthrobacter nicotinovorans* 5.24×10^8 ; *Paenarthrobacter ureafaciens* 2.08×10^7 .

20

Samples were taken for each replicate at 0 h, 24h, 48 h and 120h. pH was measured using 0.5 ml of the sample, 0.5 ml was centrifuged and re-suspended in MgCl₂ 10 mM to measure O.D 600nm and 0.5 ml of sample was frozen to -80C for posterior nicotine content analysis by HPLC- LCMS.

25

Results are shown in Figures 1-6.

Example 3: Degrading alkaloids in solid cured tobacco30 Tobacco Extract preparation

Dry, cured Burley TN90 Tobacco-K Control (Upper Leaves) was ground resulting in particles between 30-50 mm and no significant tobacco powder. 10 ml tubes with 1 g of tobacco added to each was prepared.

35

The pH of the tobacco was measured by re-suspending the dry particles in RO water and mixing well. The pH of this material was pH 5.2 - 5.3.

Preparation of inoculum

Bacteria pre-cultures were prepared as in Example 1, by isolating single colonies from

- 5 Inorganic Salt (IS) + 4 g/L nicotine agar plates, and incubating them in liquid IS + 4g/L Nicotine for one day. In this example, 1 single colony from 10 day old plates stored at 5 °C, was inoculated in 5 ml of liquid IS + 4g/L nicotine, and at the end of the same day, 2.5 ml of this pre-inoculum was then inoculated in two x 12.5 ml of liquid IS+ 4g/L nicotine media.

- 10 Next day, the samples were combined, centrifuged at 3700 RPM for 15 min, and re-suspended in Na_3PO_4 0.05 M pH 7.0 for a final O.D 600nm of 1.0. This equates to 2.9×10^6 CFU/ml for *P. putida* S16, 9.34×10^8 CFU/ml for *P. nicotinovorans*, and 8.28×10^7 CFU/ml for *P. Ureafaciens*.

15 Inoculation and sampling

Immediately after the cultures were re-suspended in Na_3PO_4 (0.05 M, pH 7) for a final O.D 600nm of 1.0 (see above for CFU/ml values), the cured tobacco was treated. 5ml of Na_3PO_4 (0.05 M, pH 12.0) was mixed with 1 g of tobacco in each tube, to increase the tobacco pH in
20 order to make it more suitable for these microorganisms. After 10 minutes, 2.5 ml of every inoculum (1.0 O.D) was then inoculated in every tube using a sterile micro loop and mixing well. The tubes containing the inoculated tobacco were then incubated and mixed at room temperature on a shaker for 30 minutes. After this, the whole contents of each tube was poured into mini petri dishes. The mini petri dishes were then incubated at 30 °C and 150 rpm, with
25 the lid on and covered by aluminium foil.

- Samples were taken for each replicate at 0 h, 24h, 48 h and 120 h. pH was measured by re-suspending 0.10 g of the tobacco in RO Water. In addition, about 3 samples / replicate of between 0.15 and 0.20 g were taken to -80 °C in microcentrifuge tubes to freeze dry them
30 and then weighed so that there was the same amount of dried sample for every tube. At the 48 hour sampling point (3rd day), the aluminium foil was removed to dry the mixture. At the 120 h sampling point (day 5) the mixtures were completely dry. Samples were freeze dried for 3 days. After being freeze dried, 0.01 g (10 mg) was weighed from each of the samples and ground along with 1 ml of Alk. Extraction Buffer. Samples were kept at -80 °C for
35 subsequent nicotine content analysis by HPLC- LCMS.

Results are shown in Figures 7-11.

Example 4: Concentrated Bacterial Strains Combinations for Nicotine Biodegradation in Tobacco Extracts

Different combinations of concentrated microbial strains were prepared and differences between treatments were measured by quantifying nicotine content (mg/ml) in all treated samples during incubation time after inoculation.

Material and Methods:

Tobacco extract was prepared by grinding 10 grams of cured Tobacco (Burley TN90 Tobacco-K Control Upper Leaves (Harvested 2019-08-05, Packed: 2019-10-01, Stored: Dark at 5°C). Tobacco was mixed with deionized water up to a volume of 500 ml in a laboratory baker. Once well mixed, the result was filtered 2 times using laboratory paper filter. In order to discard suspended particles in the resultant extract, the final mix was then filter sterilised using vacuum filtering (0.2 µm filter) and stored at 5°C. Once filtered, pH was adjusted to 7.0 using NaOH 1 M.

Pre-cultures were prepared in advance growing three different microbial strains separately: *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens*. On day one, glycerol stocks were used for inoculating Luria Broth Agar plates (pH 7.0). Plates were incubated for 56h at 30°C in dark. After this, single colonies were picked and used to inoculate 3 ml of Luria Broth liquid (pH 7.0) for each of the strains and incubated overnight 30°C and shaking at 200 rpm. Next day, liquid cultures would be scaled up by inoculating these 3 ml into a falcon tube with 22ml of liquid Luria Broth for its incubation overnight until the next day at 30°C and shaking at 200 rpm. After this, the total of 25 ml of every culture was centrifuged in falcon tubes at 3,750 rpm at room temperature for 15 minutes. Supernatant was discarded and cell pellet was washed by pouring 30 ml of x1 PBS (Phosphate Buffer) in each falcon and centrifuged again at 3,750 rpm at room temperature for 15 minutes. After this, the supernatant was discarded, and cell pellet was resuspended for each of the cultures in PBS to 5.0 OD_{600 nm}.

Single strains treatments were prepared at 5.0 OD_{600 nm}. After this, 50 µL of each of the resuspended cultures were inoculated into 200 µL of tobacco extract for 9 different replicates (n=9) in a sterile 96 well plate (300 µL wells). Pair combinations were prepared in advance by mixing the same volumes of prepared cultures at 5.0 OD_{600 nm}, while all strains

combinations were made by three equal thirds of the different strains at 5.0 OD_{600 nm}. For combinations, 50 µL of the resultant mix was inoculated into 200 µL of tobacco extract for 9 different replicates per treatment (n=9) in a sterile 96 well plate (300 µL wells). Mock was included and inoculated with plain PBS as reference to compare the efficacy of the different treatments for reducing nicotine in tobacco extracts.

Samples were taken for each replicate at 0 h, 4h, 6 h, 24h and 48h and frozen to -80°C for posterior nicotine content analysis by Liquid Chromatography Mass Spectrophotometer (3 replicates per strain).

Results

As shown in Figure 12 and Figure 13, nicotine biodegradation seems to occur between 24 and 48h, with no major differences between treatments. The data shown in Figure X show no difference between all treatments beyond 24h, showing a significant nicotine biodegradation ($p < 0.001$) compared to the mock, with a reduction of 99% in nicotine content. These results show nor negative or positive effect on nicotine biodegradation when combining these three strains together.

As shown in Figure 14, results show a significant decrease of nor nicotine at 42 h of all combinations treatments compared to the mock treatment, being reduced down to below 0.0002 for most treatments. However, combination treatment *P. putida* S16 + *P. ureafaciens* does not show the same decrease, being equal to individual treatments of *P. putida* S16 and *P. ureafaciens*, affecting somehow the efficiency of *P. ureafaciens*. Additionally, the graph shows combinations including *P. nicotovorans* are the most effective treatment, being no difference between these and *P. nicotovorans* individual treatment.

As shown in Figure 15, results show a significant decrease of anatabine at 42 h of all combinations treatments compared to the mock treatment, being reduced down to below 0.0005 for most treatments. However, combination treatment *P. putida* S16 + *P. ureafaciens* does not show the same decrease, being similar to the individual treatment of *P. putida* S16, not affecting the efficiency of *P. putida* S16. Additionally, the graph shows combinations including *P. nicotovorans* are effective treatment, being no difference between these and *P. nicotovorans* individual treatment.

Again, as shown in Figure 16, results show a significant decrease of anatabine at 42 h of all combinations treatments compared to the mock treatment, being reduced down to below 0.00005 for most treatments. However, combination treatment *P. putida* S16 + *P. ureafaciens* does not show the same decrease, being similar to the individual treatment of *P. putida* S16, not affecting the efficiency of *P. putida* S16. Additionally, the graph shows combinations including *P. nicotinovorans* are effective treatment, being no difference between these and *P. nicotinovorans* individual treatment.

In the case of Pseudooxynicotine, as shown in Figure 17, results show a significant decrease at 42 h of all combinations treatments compared to the mock treatment, being reduced down to below 0.0002 for most treatments. However, results show a huge increase in the concentration from 0 to 24h of all treatments when compared to the control, being the initial levels of these treatments significantly different to the control. The increase in pseudooxynicotine may be due to degradation pathways. For example, the biodegradation of nicotine into pseudooxynicotine has previously been described in literature on the pyrrolidine pathways of nicotine degradation followed by *Pseudomonas* sp., such as *Pseudomonas putida* S16. Gurusamy, R., & Natarajan, S. (2013). Current status on biochemistry and molecular biology of microbial degradation of nicotine. The Scientific World Journal, 2013(1), 125385.

In conclusion, for all minor alkaloids analysed in this experiment, results show a huge decrease at 42 h for *P. nicotinovorans* individual treatment when compared to the control, being the most effective treatment for reducing minor alkaloids content. This translates to any combination including *P. nicotinovorans*, being its effectiveness not compromised by any other tested strain.

Example 5: Nicotine exposure biodegradation

Results from previous experiments show nicotine biodegradation from non-exposed to nicotine strains. We next assessed the potential effect of nicotine exposure to three tested nicotine to understand the degradation speed at early times in the biodegradation process to determine if nicotine presence is needed for the nicotine biodegradation at early times.

Nicotine-exposed and non-nicotine exposed pre-cultures were produced and we assessed their efficiency to degrade nicotine in Luria Broth containing nicotine 4 mg/ml, taking sample at early times from 0 h to 4 h.

The nicotine content mg/ml, OD_{600nm} was measured.

5 Material and Methods:

Pre-cultures were prepared in advance growing three different microbial strains separately: *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans* and *Paenarthrobacter ureafaciens*.

On day one, glycerol stocks were used for inoculating Luria Broth Agar with 4 mg/ml nicotine (LB+) and without nicotine plates (LB-) (pH 7.0). Plates were incubated for 56h at 30°C in dark. After this, single colonies were picked and used to inoculate 3 ml of liquid Luria Broth (pH 7.0) LB + and LB - and incubated overnight 30°C and shaking at 200 rpm. Next day, liquid cultures would be scaled up by inoculating these 3 ml into a falcon tube with 22ml of liquid Luria Broth LB+ and LB - for its incubation overnight until the next day at 30°C and shaking at 200 rpm. After this, the total of 25 ml of every culture was centrifuged in falcon tubes at 3,750 rpm at room temperature for 15 minutes. Supernatant was discarded and cell pellet was washed by pouring 30 ml of x1 PBS (Phosphate Buffer) in each falcon and centrifuged again at 3,750 rpm at room temperature for 15 minutes. After this, the supernatant was discarded, and cell pellet was resuspended for each of the cultures in PBS to 1.0 OD_{600 nm}.

A volume of 25 µL of each of the resuspended cultures were inoculated into 225 µL of LB (4 mg/ml)+ (Nicotine 4three replicates per treatment and dilution (n=3) in a sterile 96 well plate (300 µL wells). 225 µL of 5% H₂O₂ was used as positive control, inoculated with the same volume of bacteria as other treatments. Mock was included and inoculated with plain PBS as reference to be used as a blank for absorbance at OD_{600 nm}.

Samples in a 96 well plate were incubated in a FLUOstar BMG LABTECH for 27h at 30°C in dark. During the incubation time, the absorbance at 600 nm of every sample was measured every 5 minutes. Liquid samples were taken for each replicate at 0 h, 1h, 2 h and 4h. Samples were frozen at -80°C for posterior nicotine content analysis by Liquid Chromatography Mass Spectrophotometer (3 replicates per strain).

Results – shown in Figures 18-23.

For *P. putida* S16, bacterial cell growth for this strain is similar in both liquid medias, Luria Broth – and Luria Broth containing nicotine 4 mg/ml reaching about OD_{600nm} 1.0.

Surprisingly, nicotine drops within 2 and 4h for LB + precultured strains. This means that for quick treatments it would be advisory to pre-culture this strain in nicotine containing media. However, we have probed in previous experiments that nicotine degradation occurs despite of the absence of nicotine in the pre-culturing media.

Similarly, we see similar cell growth for *P. nicotinovorans*. However, cell growth seems to be higher at the experiment (24h) in Luria Broth containing nicotine 4 mg/ml reaching about OD_{600nm} 1.0 and about 0.6 mg/ml. However, no significant decrease in the nicotine content occurs within 4 h for any of the treatments, indicating that this strain would be better for longer incubation times, as it has been proven do biodegrade nicotine and other minor alkaloids efficiently in previous experiments.

By last, show less cell growth for all liquids medias tested, being a significant difference between liquid media LB+ and LB - and being higher in this last liquid media at 24h. Additionally, no nicotine decrease within 4 h was observed for any of the treatments, indicating that this strain would be better for longer incubation times. Moreover, results from previous experiments show this strain in is less efficient that *P. nicotinovorans* when degrading alkaloids, being advisory to prioritise the use of *P. nicotinovorans* in first place, which also shows a higher cell growth in the tested medias.

The invention is further described by the following numbered paragraphs:

1. Use of *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof for the degradation of one or more alkaloids in processed tobacco.

2. A method of degrading one or more alkaloids in processed tobacco, wherein the method comprises:

a) inoculating the processed tobacco with *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof.

3. The method of paragraph 2, wherein the *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof are pre-cultured with one or more alkaloids.
- 5 4. The method of paragraph 3, wherein the *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof are pre-cultured with nicotine.
- 10 5. The method of paragraph 2 or paragraph 3, wherein prior to step (a) the processed tobacco is suspended in a liquid media substantially free from carbon and nitrogen, such that the processed tobacco provides the main carbon and nitrogen source when added to the liquid media.
- 15 6. The method of any one of paragraphs 2-5, wherein the method further comprises:
b) incubating the inoculated processed tobacco.
7. The method according to paragraph 6, wherein the inoculated processed tobacco is incubated for a minimum of 18 hours.
- 20 8. The method according to paragraph 6 or paragraph 7, wherein the bacteria inoculated processed tobacco is incubated for between about 18 hours and about 120 hours.
- 25 9. The method of any one of paragraphs 2-8, wherein the method further comprises:
c) drying the inoculated processed tobacco.
10. The method of any one of paragraphs 2-9, wherein the method further comprises:
e) either
(i) further processing the inoculated processed tobacco for subsequent use;
30 or
(ii) disposing of the inoculated processed tobacco.
11. The method according to any one of paragraphs 2-10, wherein the initial cell density of the pre-cultured *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*,
35 *Paenarthrobacter ureafaciens*, or any combination thereof in step (a) is between about about 9.50×10^5 CFU/ml and about 9.50×10^8 CFU/ml.

12. The use of paragraph 1 or the method of any one of paragraphs 2-11, wherein the *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof is *Pseudomonas putida* S16.
- 5 13. The use of paragraph 1 or the method of any one of paragraphs 2-11, wherein the *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof is *Paenarthrobacter nicotinovorans*.
- 10 14. The use of paragraph 1 or the method of any one of paragraphs 2-11, wherein the *Pseudomonas putida* S16, *Paenarthrobacter nicotinovorans*, *Paenarthrobacter ureafaciens*, or any combination thereof is *Paenarthrobacter ureafaciens*.
- 15 15. The use of any one of paragraphs 1 or 12-14, or the method according to any one of claims 2-14, wherein the one or more alkaloids are one or more of the group comprising nicotine, nornicotine, anabasine, anatabine, myosime, cotinine and pseudooxynicotine.
16. The use or the method of paragraph 15, wherein the one or more alkaloids is nicotine.
- 20 17. The use of any one of paragraphs 1 or 12-16, or the method according to any one of paragraphs 2-17, wherein the processed tobacco is selected from the group comprising tobacco extract, cured tobacco, reconstituted tobacco, tobacco stalks, tobacco pruning leftovers, tobacco products and tobacco dust.

CLAIMS

1. Use of *Paenarthrobacter nicotinovorans* or a combination of bacteria comprising *Paenarthrobacter nicotinovorans* for the degradation of one or more alkaloids in processed tobacco.
2. A method of degrading one or more alkaloids in processed tobacco, wherein the method comprises:
- a) inoculating the processed tobacco with *Paenarthrobacter nicotinovorans*, or a combination of bacteria of bacteria comprising *Paenarthrobacter nicotinovorans*.
3. The method of claim 2, wherein the *Paenarthrobacter nicotinovorans*, or combination of bacteria comprising *Paenarthrobacter nicotinovorans* thereof are pre-cultured, with or without one or more alkaloids.
4. The method of claim 3, wherein the *Paenarthrobacter nicotinovorans*, or combination of bacteria comprising *Paenarthrobacter nicotinovorans* are pre-cultured with nicotine.
5. The method of claim 2 or claim 3, wherein prior to step (a) the processed tobacco is suspended in a liquid media substantially free from carbon and nitrogen, such that the processed tobacco provides the main carbon and nitrogen source when added to the liquid media.
6. The method of any one of claims 2-5, wherein the method further comprises:
- b) incubating the inoculated processed tobacco.
7. The method according to claim 6, wherein the inoculated processed tobacco is incubated for a minimum of 18 hours.
8. The method according to claim 6 or claim 7, wherein the bacteria inoculated processed tobacco is incubated for between about 18 hours and about 120 hours.
9. The method of any one of claims 2-8, wherein the method further comprises:
- c) drying the inoculated processed tobacco.
10. The method of any one of claims 2-9, wherein the method further comprises:
- e) either

- (i) further processing the inoculated processed tobacco for subsequent use;
or
- (ii) disposing of the inoculated processed tobacco.

5 11. The method according to any one of claims 2-10, wherein the initial cell density of the pre-cultured *Paenarthrobacter nicotinovorans*, or combination of bacteria comprising *Paenarthrobacter nicotinovorans* in step (a) is between about about 9.50×10^5 CFU/ml and about 9.50×10^8 CFU/ml.

10

12. The use of claim 1, or the method according to any one of claims 2-11, wherein the one or more alkaloids are one or more of the group comprising nicotine, normicotine, anabasine, anatabine, myosime, cotinine and pseudooxynicotine.

15 13. The use or the method of claim 12, wherein the one or more alkaloids is a minor alkaloid, such as normicotine, anabasine, anatabine or pseudooxynicotine.

14. The use of claim 1 or the method according to any one of claims 2-11, wherein the total amount of alkaloid is reduced.

20

15. The use of any one of claims 1 or 12-14, or the method according to any one of claims 2-14, wherein the processed tobacco is selected from the group comprising tobacco extract, cured tobacco, reconstituted tobacco, tobacco stalks, tobacco pruning leftovers, tobacco products and tobacco dust.

25

Figure 1

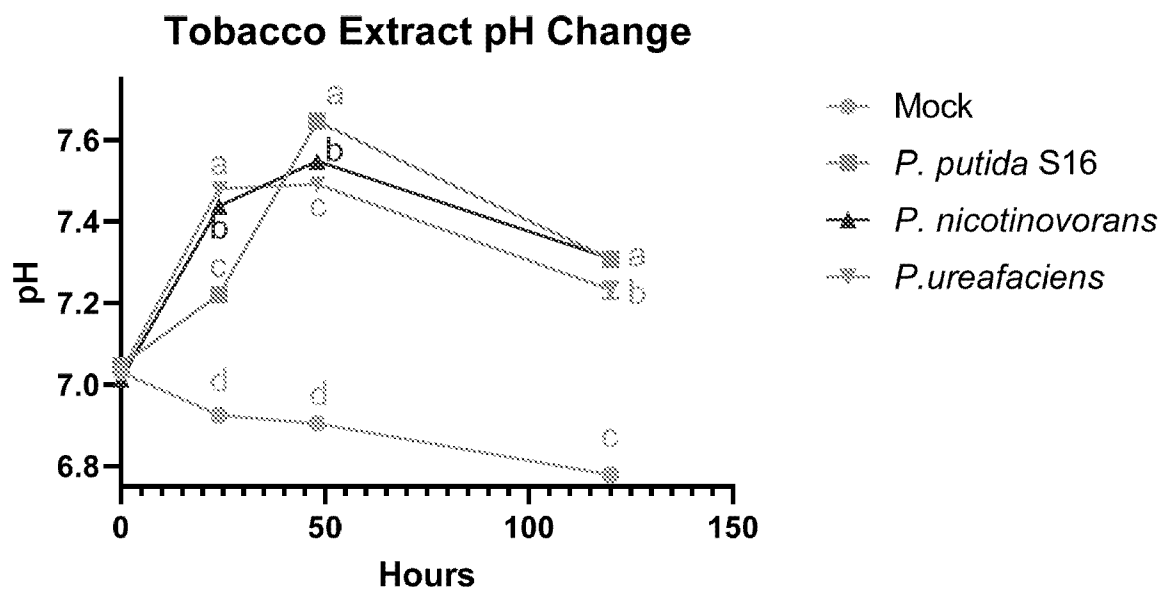


Figure 2

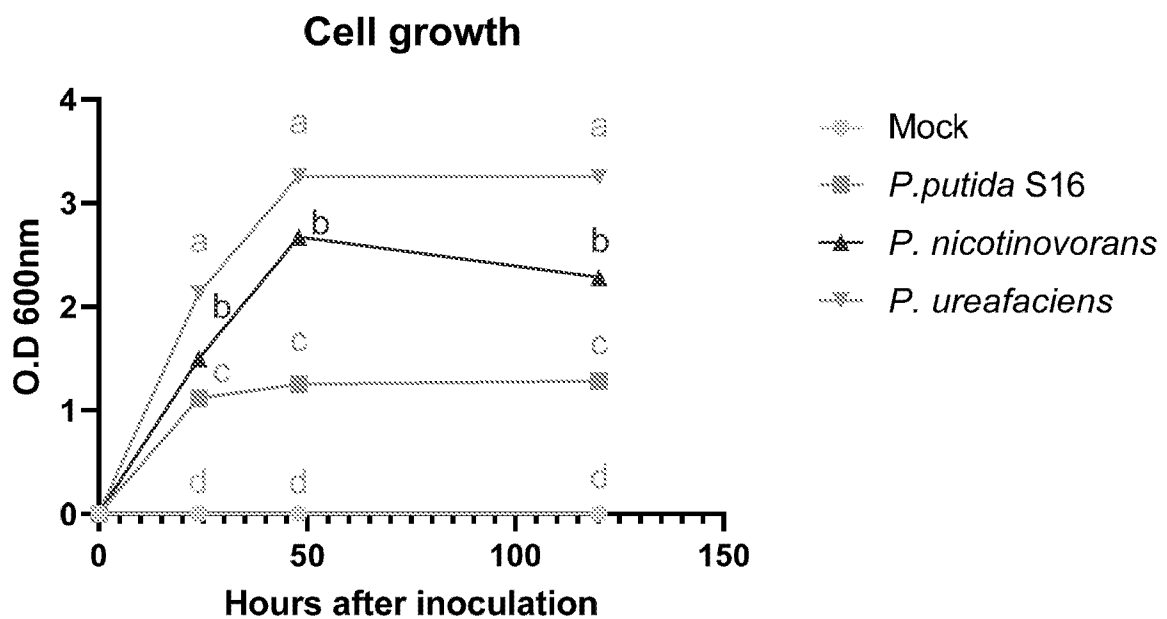


Figure 3

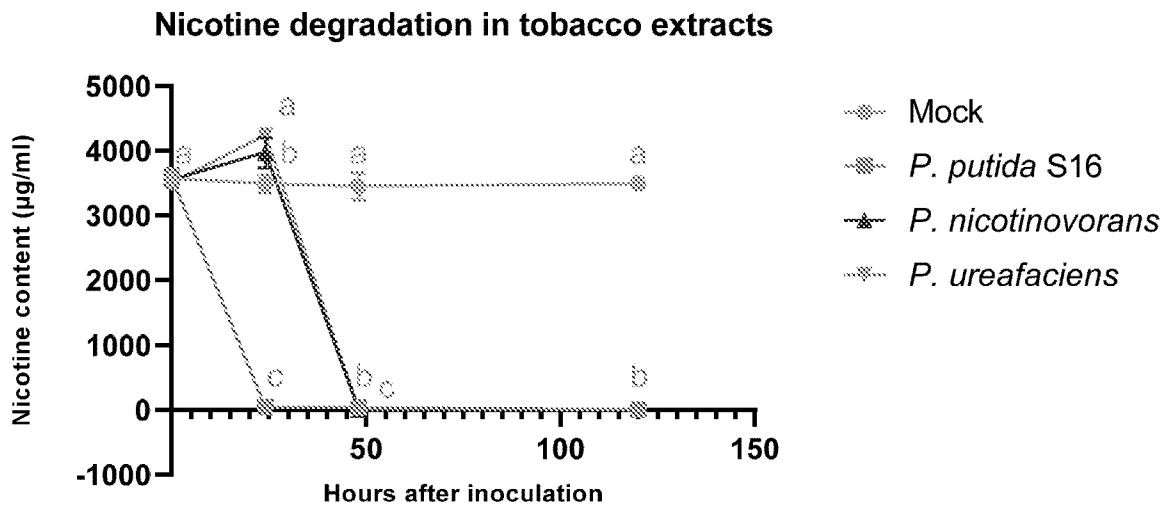


Figure 4

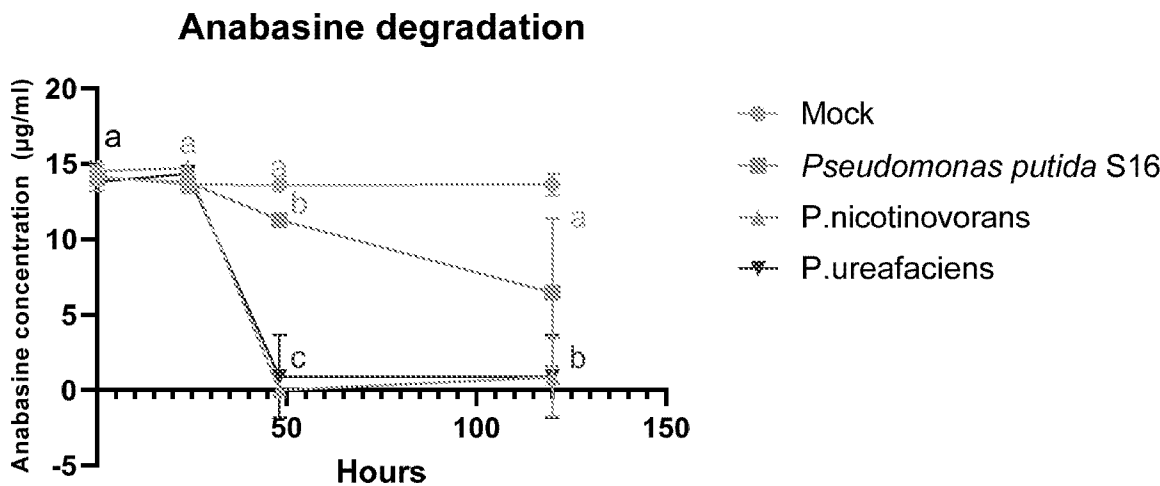


Figure 5

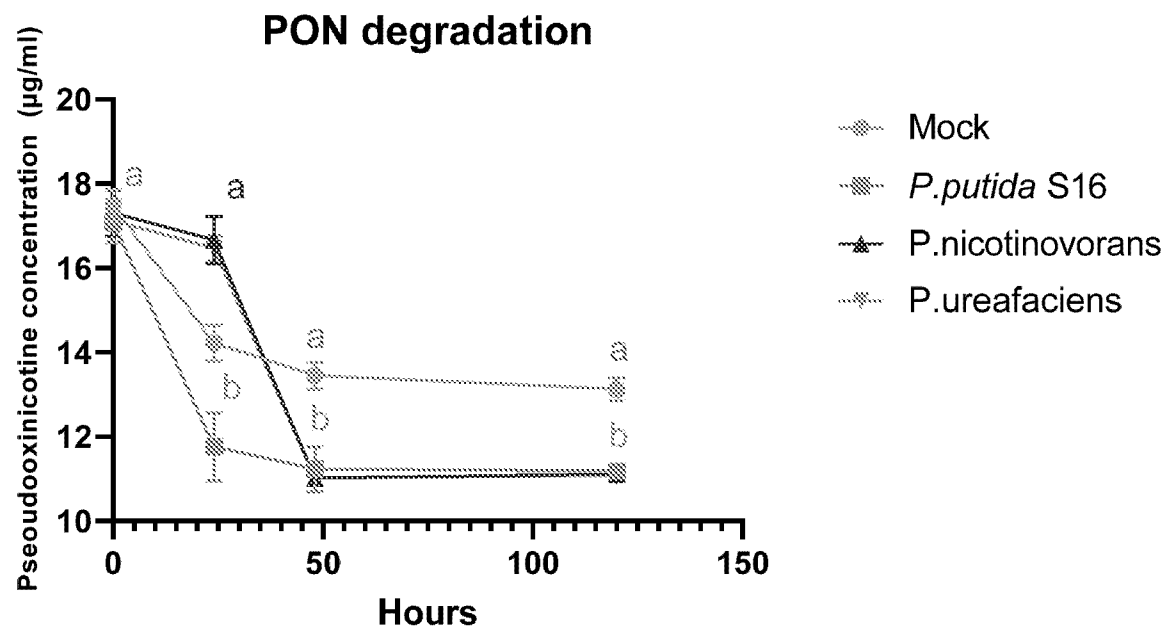


Figure 6

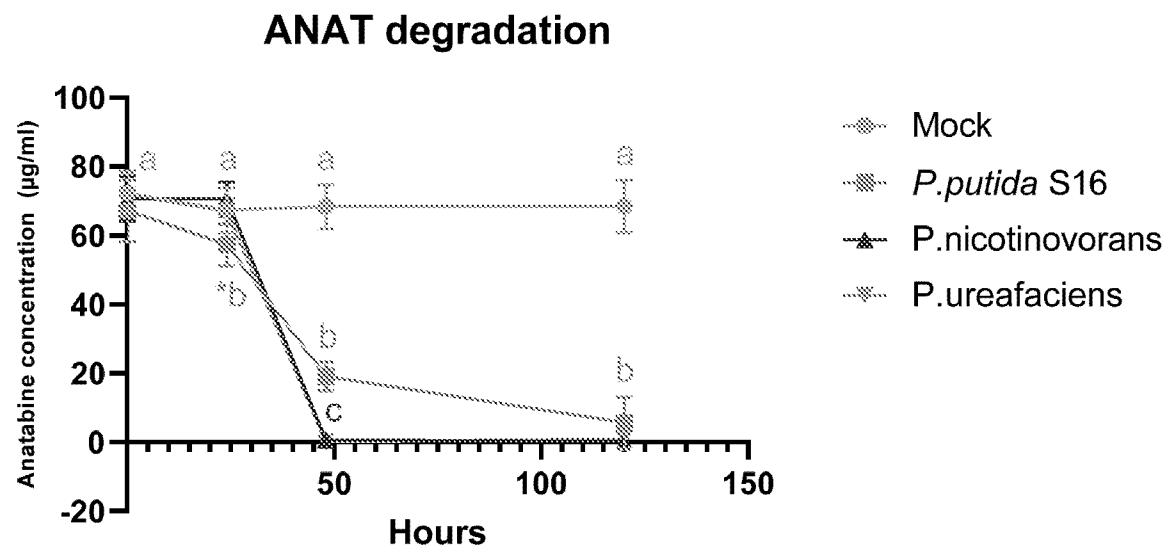


Figure 7

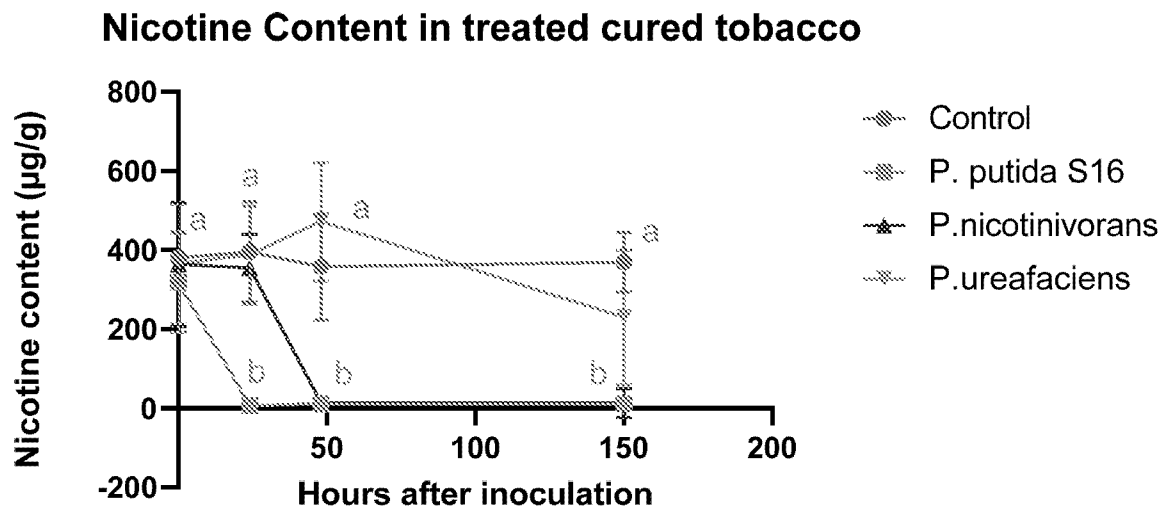


Figure 8

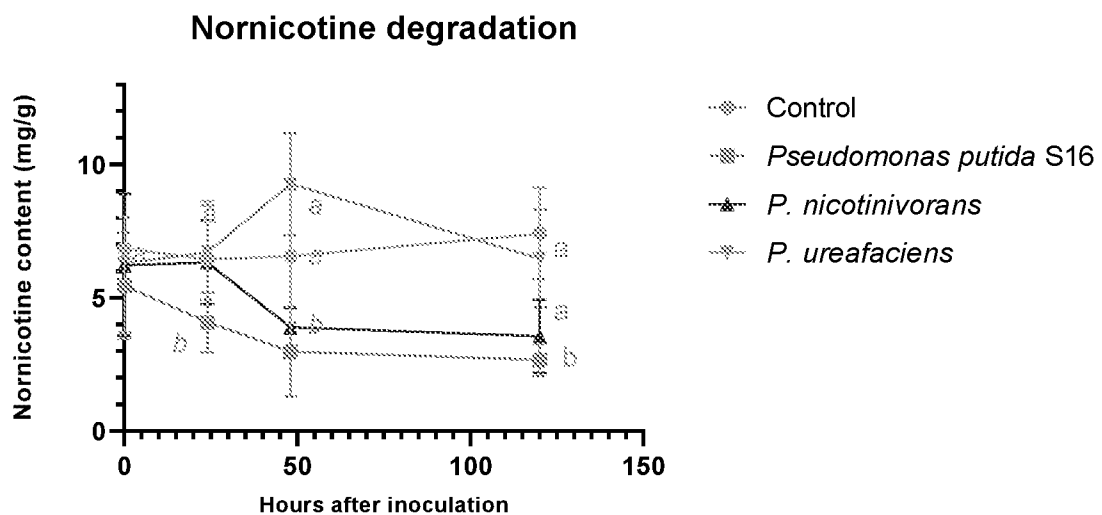


Figure 9

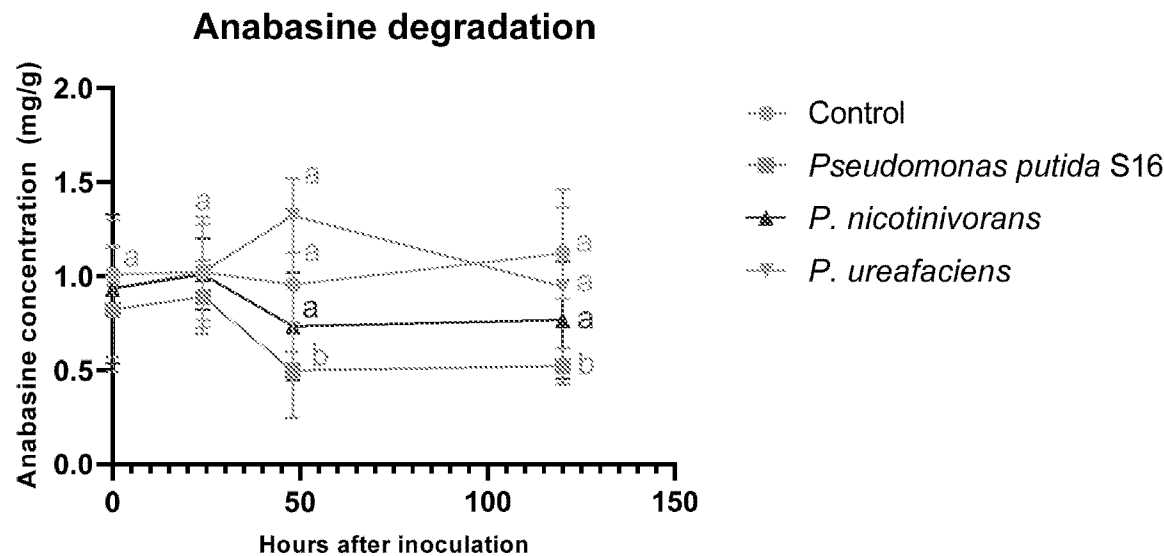


Figure 10

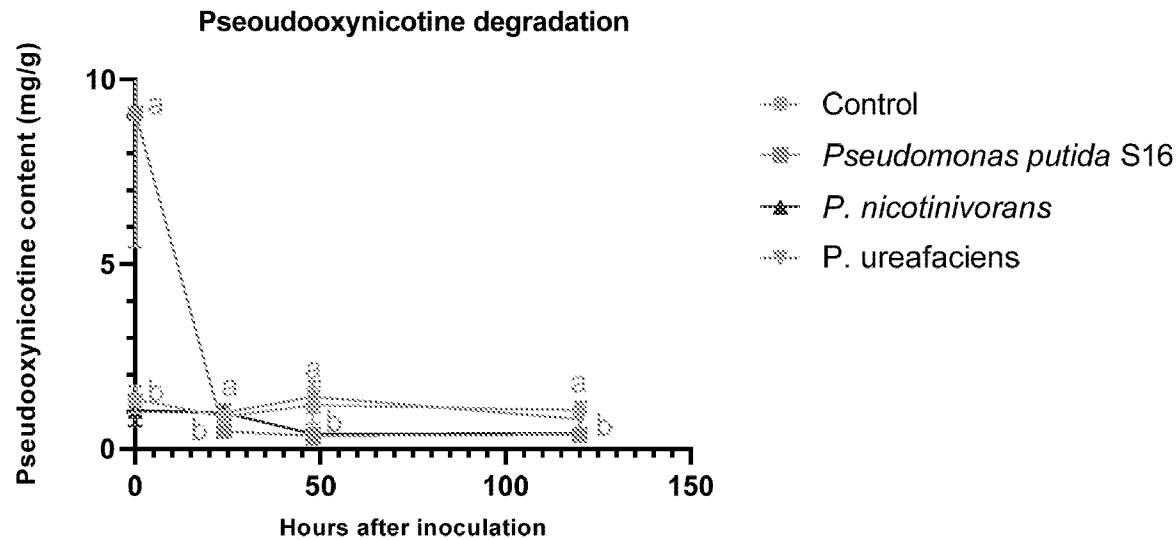


Figure 11

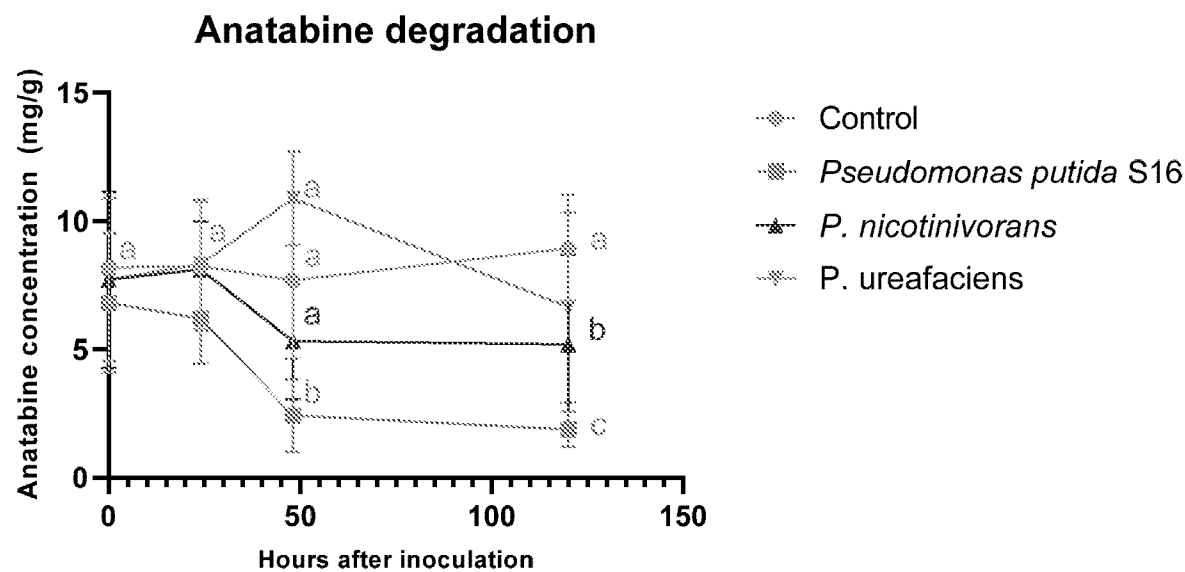


Figure 12

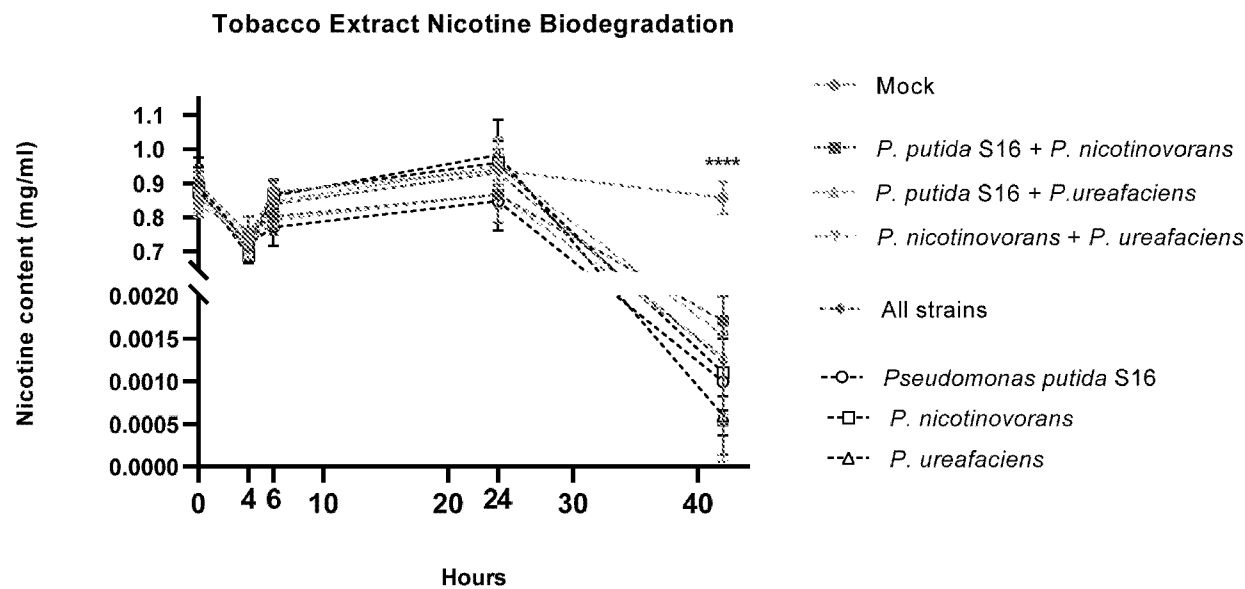


Figure 13

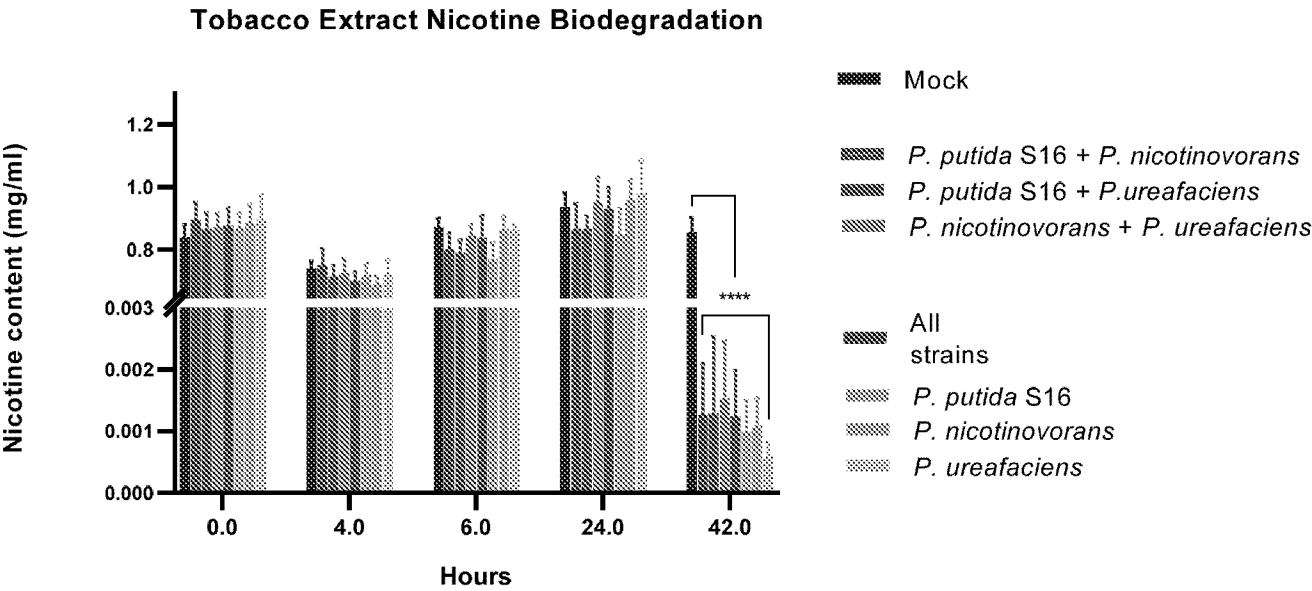


Figure 14

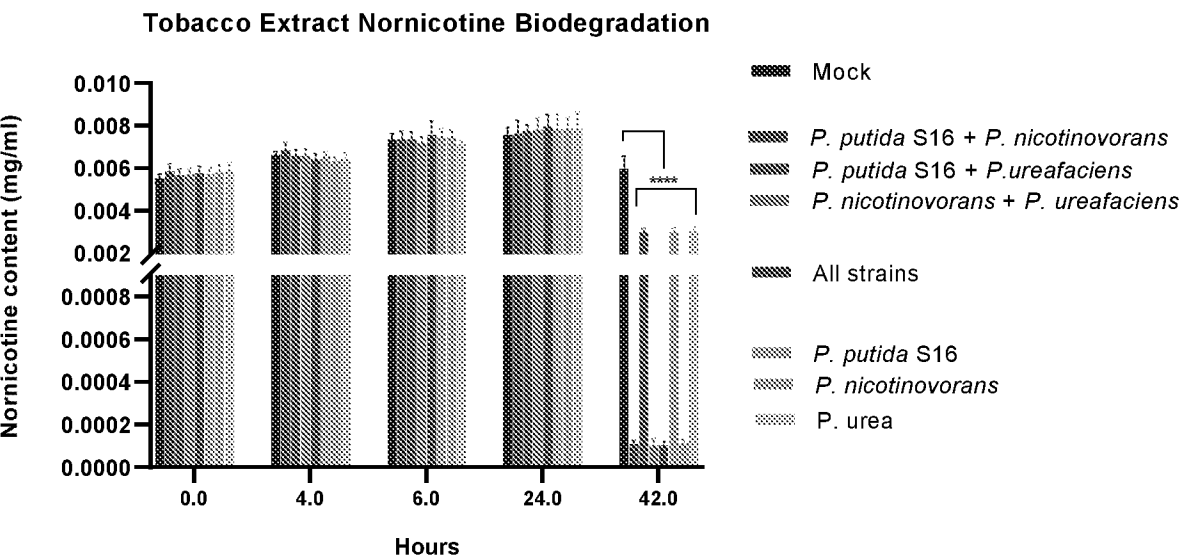


Figure 15

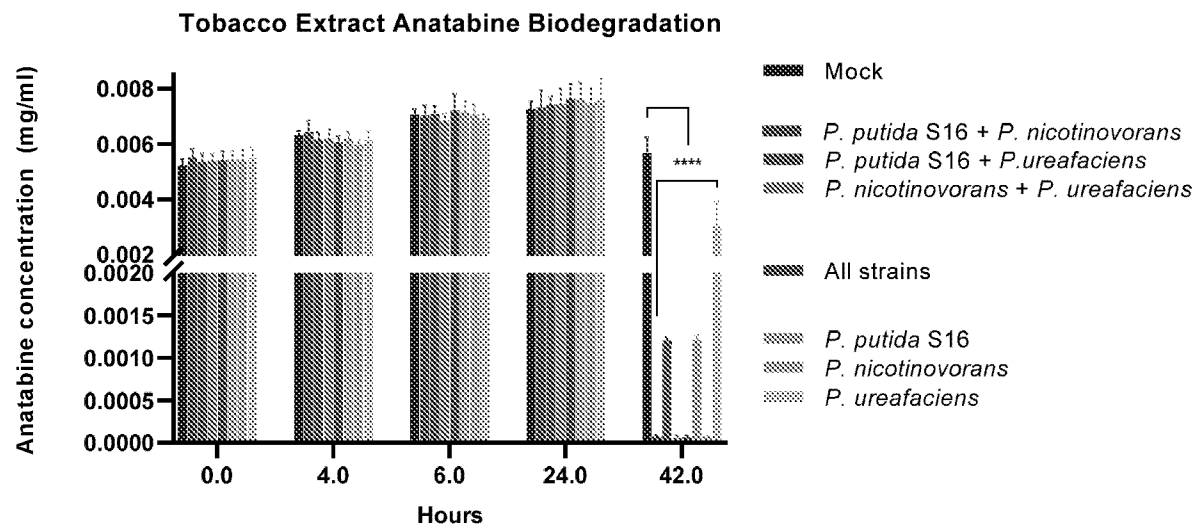


Figure 16

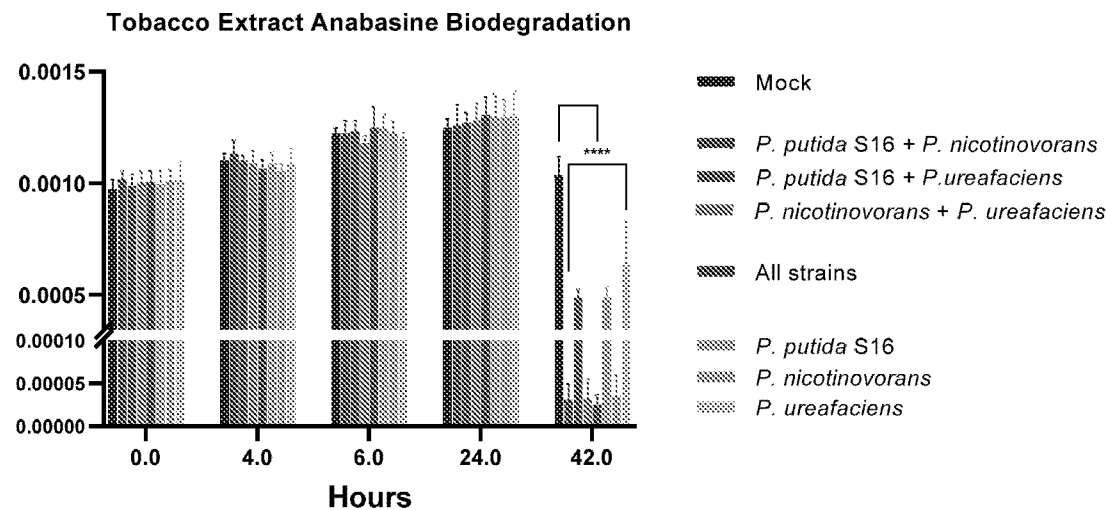


Figure 17

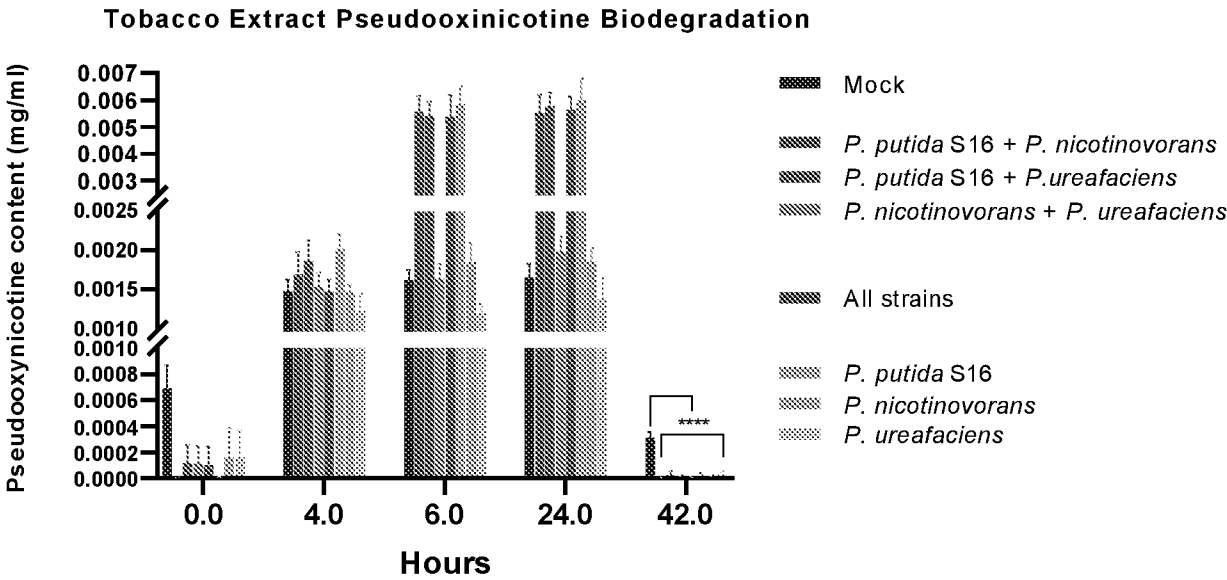


Figure 18

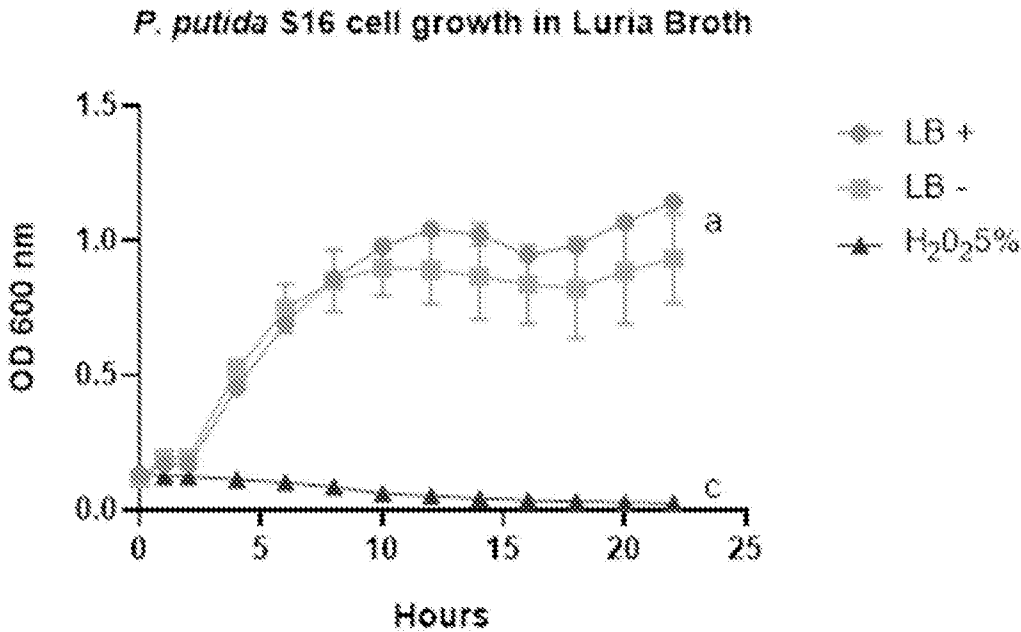


Figure 19

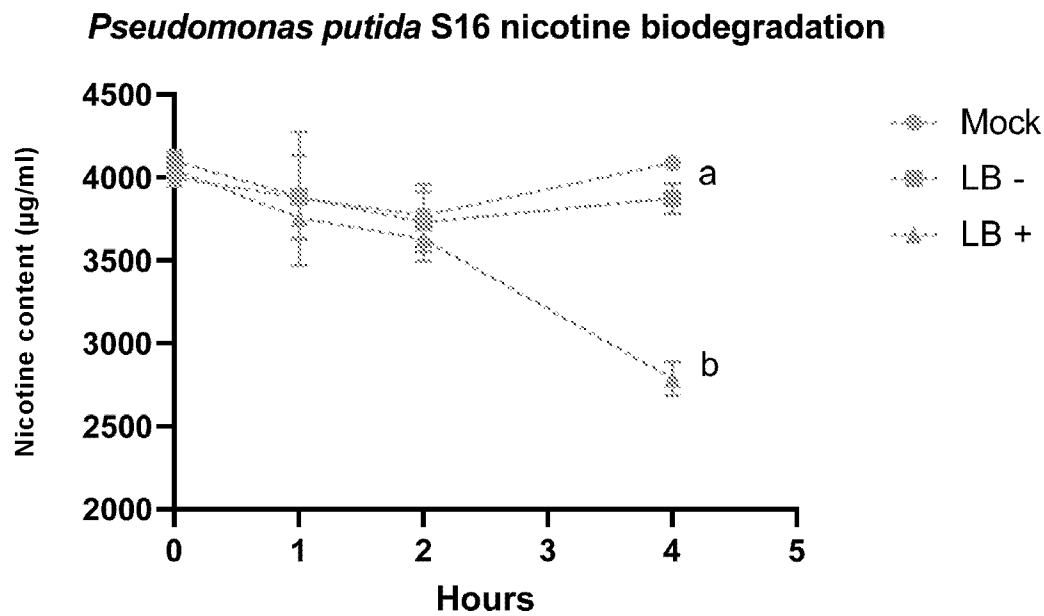


Figure 20

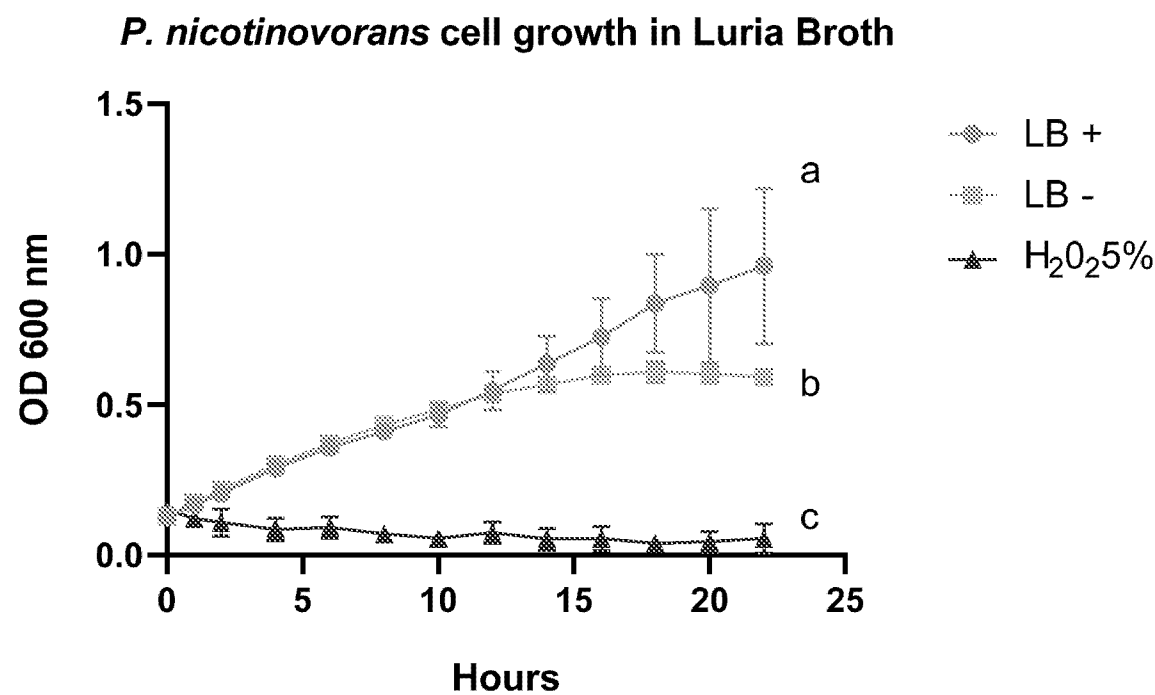


Figure 21

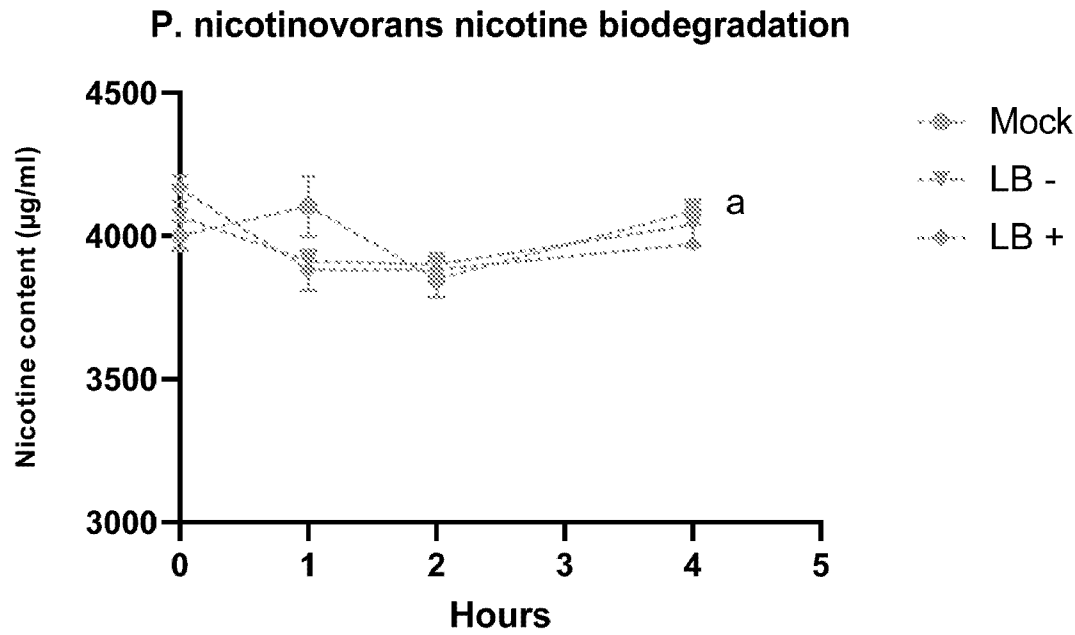


Figure 22

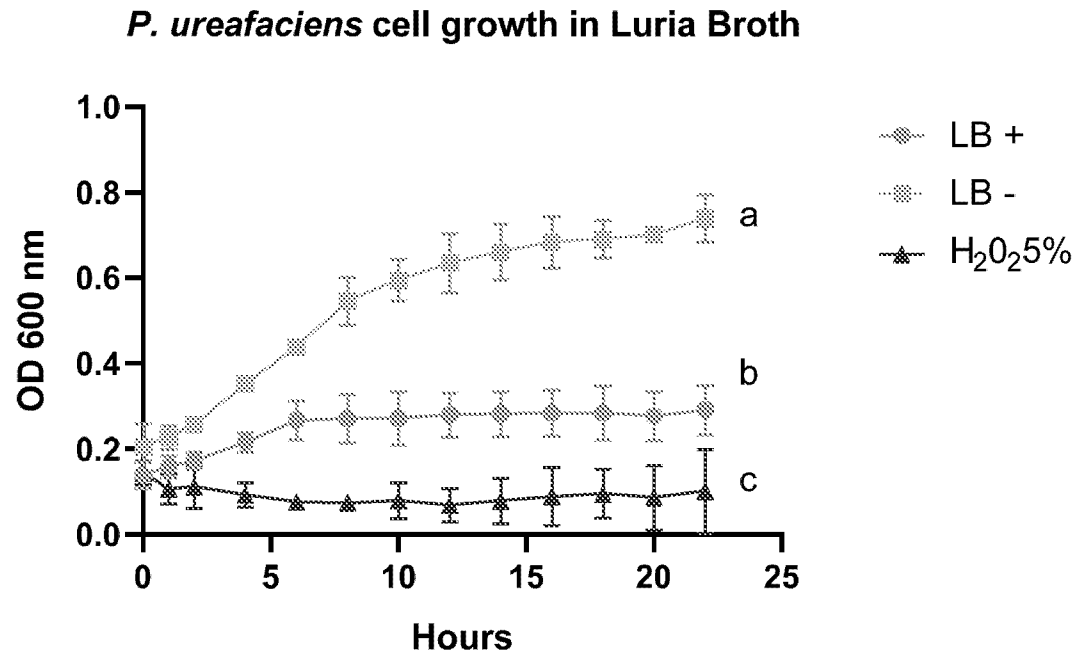
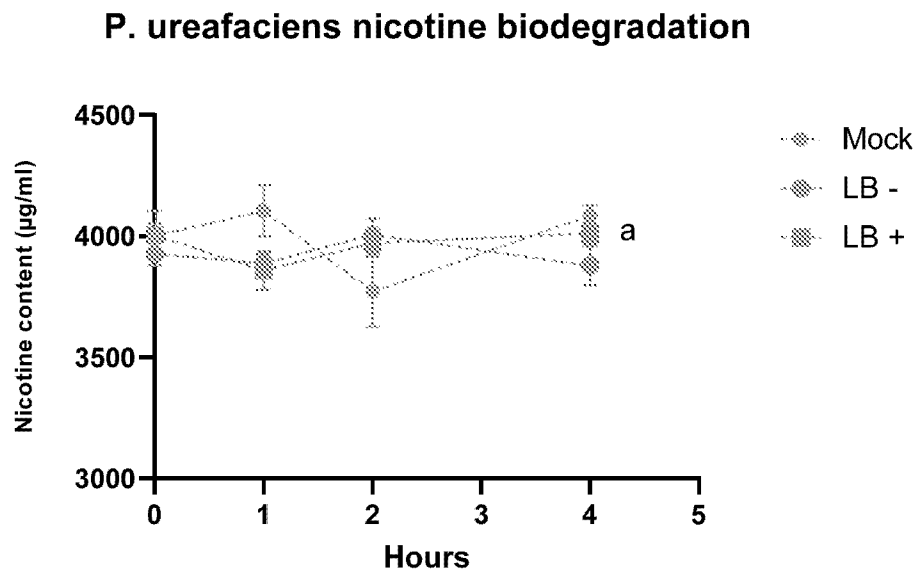


Figure 23



INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2024/052951

A. CLASSIFICATION OF SUBJECT MATTER INV. A24B15/20 A24B15/24 C12N1/20 ADD.				
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) A24B C12R C12N 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) EPO-Internal				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	ZHANG ZELING ET AL: "Nicotine metabolism pathway in bacteria: mechanism, modification, and application", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER BERLIN HEIDELBERG, BERLIN/HEIDELBERG, vol. 106, no. 3, 24 January 2022 (2022-01-24), pages 889-904, XP037684012, ISSN: 0175-7598, DOI: 10.1007/S00253-022-11763-Y [retrieved on 2022-01-24] page 890 page 892 table 1 page 900, line 1 - line 4 ----- -/-	1 - 15		
<table><tr><td>☒ Further documents are listed in the continuation of Box C.</td><td>☒ See patent family annex.</td></tr></table>			☒ Further documents are listed in the continuation of Box C.	☒ See patent family annex.
☒ Further documents are listed in the continuation of Box C.	☒ See patent family annex.			
<table><tr><td>* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "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</td><td>"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</td></tr></table>			* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "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
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Date of the actual completion of the international search 9 February 2025		Date of mailing of the international search report 19/02/2025		
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Dimoula, Kerasina		

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2024/052951

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