METHODS FOR FERMENTATIVE PRODUCTION OF MASSOIA LACTONE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is related to and claim priority to U.S. provisional patent application Serial No. 62/205,996 filed 17 August 2015. Each application is incorporated herein in its entirety.

SUBMISSION OF SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577249PCTSequenceListing.txt, created on 15 August 2016 and is 294 kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0003] The present invention relates to the field of fermentation biotechnology, more particularly to methods for the fermentative production of massoia lactone by *Aureobasidium* species.

[0004] The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended bibliography.

[0005] C-10 massoia lactone [C-10 (5,6-dihydro-6-pentyl-2H-pyran-2-one)] and C-12 massoia lactone [C-12 (5,6-dihydro-6-heptyl-2H-pyran-2-one)] are the major constituent of bark oil of massoia (*Cryptocarya massoia*) [1]. Massoia lactones can also be found in cane sugar molasses, cured tobacco and the essential oil of Sweet Osmanthus (*Osmanthus fragrans*) and jasmine [2]. At 20 ppm, it has a taste described as creamy, coconut, green and slightly fruity. Massoia bark oil is used in the flavor industry as an additive in butter and milk flavors (international FEMA code 3744). Current global supply of massoia bark oil comes from Indonesia, using primitive and costly extraction process that destroy precious native forest.

[0006] Massoia lactone is the substrate for the production of saturated delta-decanolide or delta-dodecanolide, which is the key molecule for peach flavor. It can be made by biohydrogenation using a wide range of microorganisms, e.g., yeast (*Saccharomyces , Candida, Pichia*), molds (*Cladosporium*), and bacteria (*Pseudomonas, Sarcina*), [3,4].

[0007] Although methods for the chemical synthesis of massoia lactone have been available, the process is a rather complicated and requires multi-step reactions using costly raw materials and catalysts [5]. Previously, massoia lactone was found in the alkali-hydrolyzed glycolipid secreted by *Aureobasidium pullalan* A-21 that was cultured under a calcium-deficient condition. It has been reported that, in the presence of calcium, polymalic acid was produced instead [6]. The interaction between various trace elements on the production of massoia lactone has not been reported.

[0008] Certain strains of *A. pullulans* are known to produce extracellular ''heavy oils'', or polyol lipids, when $CaCO_3$ is not present in the medium. The oils have been described as 3,5-dihydroxydecanoyl and 5-hydroxy-2-decenoyl esters of arabitol and mannitol. Medium for oil production is composed of 50 g/l sucrose, 0.6 g/l (w/v) peptone, 0.4g/l yeast extract, 5 g/l K_2HPO_4, 0.4% g/l $MgSO_4*7H_2O$, and 1g/l NaCl [7]. Another reported medium uses nitrate as the sole nitrogen source with low phosphate content and is composed of 120 g/l glucose, 1.5 g/l $NaNO_3$, 1 g/l KNO_3, 0.05 g/l KH_2PO_4, 0.2g/l $MgSO_4*7H_2O$, 2 ppm $ZnSO_4*7H_2O$, and 0.2 g/l yeast extract [6].

[0009] It is desired to develop new fermentation methods for the production of massoia lactone.

SUMMARY OF THE INVENTION

[0010] The present invention relates to the field of fermentation biotechnology, more particularly to methods for the fermentative production of massoia lactone by *Aureobasidium* species.

[0011] In one aspect, the present invention provides a method for the fermentative production of massoia lactone by *Aureobasidium* species. In one embodiment, the *Aureobasidium* species is *Aureobasidium melanogenum*. In another embodiment, the *A. melanogenum* is a strain of *A. melanogenum* that does not express a functional Aureobasidin A (AbA) biosynthesis complex (aba1) gene mRNA when cultured. In one embodiment, the functional mRNA is not expressed in the culture medium described herein. In a further embodiment, the *A. melanogenum* that does not express a functional aba1 gene mRNA when cultured is the W5-2 strain of *A. melanogenum* as described herein. In one embodiment, the *Aureobasidium* species described herein is cultured in a culture medium described in further detail herein to produce a fermentation product containing massoia lactone. In one embodiment, the culturing is performed for about 4 days to about 12 days, preferably for about 5 days to about 12 days, more preferably for about 7 days to

about 10 days. In another embodiment, the culturing is performed at about 25° C to about 35°C, preferably about 28° C to about 32°C. In some embodiments, the massoia lactone is purified from the fermentation product using conventional techniques and/or as described in further detail herein.

[0012] In a second aspect, the present invention provides a culture medium for the fermentative production of massoia lactone. In one embodiment, the culture medium comprises high levels of phosphate ions, ammonium ions and calcium ions as described in further detail herein. In some embodiments, the culture medium comprises KH_2PO_4, Na_2HPO_4, $(NH4)_2SO_4$, $MgSO_4$ and $CaCl_2$. In another embodiment, the culture medium further comprises at least two trace elements as described in further detail herein. In some embodiments, the trace elements may be selected from Fe^{2+}, Cu^{2+}, Zn^{2+} and MoO_4^{2-}. In other embodiments, each trace element that may be present in the culture medium may be present in an amount from about 0.1 μM to about 1.0 mM. In a further embodiment, the culture medium comprises urea as a nitrogen source. In another embodiment, the culture medium comprises glucose, mannose, xylose or mixtures thereof as a carbon source. In one emboidment, glucose is used as the sole carbon source. In one embodiment, the culture medium has a pH from about 5.5 to about 6.5, preferably about 6.0. The fermentation method produces high levels of glycolipids. Biochemically, hydroxyl fatty acids conjugate to produce sugars and eventually produce massoia lactone. Thus, the method produces high levels of massoia lactone – a commercially desirable feature of the present invention.

[0013] In a third aspect, the present invention provides a novel strain of *A. melanogenum* designated W5-2. In some embodiments, *A. melanogenum* W5-2 does not express a functional Aureobasidin A biosynthesis complex gene mRNA when cultured. In one embodiment, a functional mRNA is not expressed in the culture medium described herein. In other embodiments, *A. melanogenum* W5-2 has been deposited with the Agricultural Research Culture Collection (NRRL), 1815 N. University Street, Peoria, Illinois 61604, USA on 28 May 2015 and assigned accession number NRRL 67063.

BRIEF DESCRIPTION OF THE FIGURES

[0014] Fig. 1 shows a phylogenetic dendrogram, based on 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene sequences and constructed from evolutionary distances, showing the position of *Aureobasidium* strain W5-2 within the radiation of members of the family Dothioraceae, order

Dothideales [36]. Numbers at branching points refer to bootstrap percentages (based on 1000 resamplings); only values above 50% are shown. GenBank accession number of each sequence is shown in parentheses.

[0015] Fig. 2 shows results of a GPD1 CDS search by BLASTn against the Whole Genome Shortgun Motifs of *Aureobasidium* species. This search identified two highly related genomic sequences from *Aureobasidium melanogenum* CBS 110374 scaffold_4_c3 and Aureobasidium pullulans AY4 contig20 (which is in fact *A. melanogenum*). *Aureobasidium namibiae* CBS , *Aureobasidium subglaciale* EXF-2481 and *Aureobasidium pullulans* EXF-150 had only partial sequences in the region. The phylogenic tree is generated Blast Tree View at NCBI using fast minimal evolution method.

[0016] Figs. 3A-3D show the characterization of Nile Red staining substance in W5-2 cells. Cell were cultured in HMDC medium with 10% glucose in shaking flasks for 5 day. Cells were stained with Nile Red and imaged by Confocal microscopy. Fig. 3A: Red channel. Fig. 3B: bright field image. Fig. 3C: Over-lay of Fig. 3A and Fig. 3B. Scale bar=10 μM. Fig. 3D: TLC image of total ethyl acetate extract. The positions for triacylglyceride (TAG) and glycolipid are indicated on the right.

[0017] Figs. 4A and 4B show GCMS analysis of *Aureobasidium* isolate W5-2. Fig. 4A: GCMS spectra of methanol-esterified products of methanol and chloroform extract of freeze-dried cell biomass. Arrows indicate the peaks for major fatty acid methyl esters and massoia lactone. Fig. 4B: Database search and the comparison of MS spectrum of the peak shown in upper panel to that of standard massoia lactone, 6-Pentyl-5,6-dihydro-2H-pyran-2-one, shown in lower panel.

[0018] Fig. 5 shows using Central Composite Design (CCD) to optimize concentration of urea, trace elements and glucose.

[0019] Fig. 6 shows glycolipid profiles of various media. Medium composition is shown in Table 2. Seed culture was prepared by inoculating 1.5 ml of frozen 7% DMSO culture into 100 ml of the original HDCM in a 250ml flask and cultured for 48 hrs at 30° C. The cells were harvested by centrifugation, re-suspended in water and inoculated at 2% into each medium in 250 ml flasks. Cells were cultured at 30° C with 200 rpm agitation. Samples shown are taken from the 6th day.

[0020] Figs. 7A and 7B show the effects of trace elements. Media were inoculated with water-washed cell stock cultured in HMDC medium to 0.1 OD_{600} and cultured at 30° C with 200 rpm shaking for 8 days. Fig. 7A: Factorial Design was used to evaluate the effects of FeSO4,

MnSO4, ZnSO4, CuCl2 and AlCl3. Run #6 (T2) and Run #12 (T3) were selected for further optimization. Fig. 7B: Factorial Design was used to evaluate the effects of H3BO4, CoCl2 and NaMoO4 based on Medium 2 of step 5.

[0021] Fig. 8 shows production of massoia lactone with various carbon sources. W5-2 was cultured in T2 medium with glucose, mannose, xylose or glycerol as carbon sole source for 4 days. Each data set derived from 3 biological replicates. Error bars indicates SD.

[0022] Figs. 9A-9C show a comparison of massoia lactone yields in 4 media. The symbols for the media are shown in Fig. 9C. AM refers to a modified A-21M medium (MA-21M) containing 120 g/l glucose, 1.5 g/l $NaNO_3$, 1.0 g/l KNO_3, 0.05 g/l KH_2PO_4, 0.2 g/l $MgSO_4$, 0.0056 g/l $FeSO_4$, 0.2 g/l Yeast Extract (pH 5.5). Seed culture was made in HMDC medium with complete trace elements. Fig 9A: Massoia lactone. Fig. 9B: Dry biomass yield. Fig. 9C: Residual NH_4^+ in the medium.

[0023] Fig. 10 shows a comparison of cells cultured in T0, T2 and T3 media. Cells were cultured in 2L fermentor for 9 days.

[0024] Fig. 11 shows the effect of medium pH on massoia lactone yield. T2 medium was adjusted to various pH from pH 4 to pH 6.5. Cells were cultured for 5 days.

[0025] Figs. 12A-12C show production of massoia lactone. Fig. 12A: GCMS chromatograph of W5-2 Sample cultured in R15 Medium in shake flask. Fig. 12B: GCMS chromatograph of W5-2 Sample cultured in T3 medium in 2L fermenter. Fig. 12C: database search of ML massoia lactone peak of (Fig. 12B).

DETAILED DESCRIPTION OF THE INVENTION

[0026] The present invention relates to the field of fermentation biotechnology, more particularly to methods for the fermentative production of massoia lactone by *Aureobasidium* species.

[0027] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

[0028] The term “about” or “approximately” means within a statistically meaningful range of a value. Such a range can be within an order of magnitude, preferably within 50%, more preferably within 20%, more preferably still within 10%, and even more preferably within 5% of a given value or range. The allowable variation encompassed by the term “about” or “approximately” depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art.

[0029] The term "as described in further detail herein" means the description of the embodiments set forth in the detailed description of the invention and in the Examples. In this context, the Examples comprise additional details of the general nature of the invention, as well as certain specific aspects not found elsewhere in the specification.

[0030] The term "massoia lactone" as used herein means C-10 massoia lactone [C-10 (5,6-dihydro-6-pentyl-2H-pyran-2-one)] and/or C-12 massoia lactone [C-12 (5,6-dihydro-6-heptyl-2H-pyran-2-one)].

[0031] The term "*Aureobasidium melanogenum* W5-2" or "*A. melanogenum* W5-2" or "W5-2" refers to a novel strain of *A. melanogenum* isolated and characterized as described herein and a culture of which has been deposited as described herein.

[0032] In one aspect, the present invention provides a method for the fermentative production of massoia lactone by *Aureobasidium* species. In one embodiment, the *Aureobasidium* species is *Aureobasidium melanogenum*. In another embodiment, the *A. melanogenum* is a strain of *A. melanogenum* that does not express a functional Aureobasidin A (AbA) biosynthesis complex (aba1) gene mRNA when cultured. In one embodiment, the functional mRNA is not expressed in the culture medium described herein. The sequence of a functional aba1 gene is set forth in Genbank Accession No. EU886741 (SEQ ID NO:5). In a further embodiment, the *A. melanogenum* that does not express a functional aba1 gene mRNA when cultured is the W5-2 strain of *A. melanogenum* as described herein. In one embodiment, the *Aureobasidium* species described herein is cultured in a culture medium described in further detail herein to produce a fermentation product containing massoia lactone. In one embodiment, the culturing is performed for about 4 days to about 12 days, preferably for about 5 days to about 12 days, preferably for about 5 days to about 11 days, preferably for about 6 days to about 11 days, more preferably for about 7 days to about 10 days. In another embodiment, the culturing is performed at about 25° C to about 35° C, preferably at about 27° C to about 32° C., more preferably at about 28° C to about 32° C. In a further embodiment, the culturing is performed in shake flasks agitated with a speed from about 175 rpm to about 225 rpm, preferably about 200 rpm. In some embodiments, the massoia lactone is purified from the fermentation product using conventional techniques and/or as described in further detail herein.

[0033] In some embodiments, the massoia lactone is purified from the fermentation product using conventional techniques, e.g., by alkaline hydrolysis and solvent extraction [6]. In other embodiments, a strong inorganic acid is added to the fermentation product to hydrolyze the fermentation product. In one embodiment, the strong organic acid is sulfuric acid or

hydrochloric acid. In a further embodiment, the massoia lactone is purified by solvent extraction or distillation. In some embodiments, the solvent is ethyl acetate or hexane.

[0034] In a second aspect, the present invention provides a culture medium for the fermentative production of massoia lactone. In one embodiment, the culture medium comprises high levels of phosphate ions, ammonium ions and calcium ions as described in further detail herein. In some embodiments, the culture medium comprises KH_2PO_4, Na_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4$ and $CaCl_2$. In some embodiments, the culture medium comprises about 10.0 g/l to about 15 g/l, preferably about 12.5 g/l KH_2PO_4, about 0.5 g/l to about 2.0 g/l, preferably about 1.0 g/l Na_2HPO_4, about 3.5 g/l to about 6.5 g/l, preferably about 5.0 g/l $(NH_4)_2SO_4$, about 1.0 g/l to about 4.0 g/l, preferably about 2.5 g/l $MgSO_4 \cdot 7H_2O$ and about 0.10 g/l to about 0.40 g/l, preferably about 0.25 g/l $CaCl_2 \cdot 2H_2O$. In another embodiment, the culture medium further comprises at least two trace elements. In a further embodiment, the culture medium comprises at least three trace elements. In an additional embodiment, the culture medium comprises four trace elements. In some embodiments, the trace elements may be selected from Fe^{2+}, Cu^{2+}, Zn^{2+} and MoO_4^{2-}. In other embodiments, each trace element that may be present in the culture medium may be present in an amount from about 0.1 μM to about 1.0 mM, from about 1.0 μM to about 1.0 mM, from about 10.0 μM to about 1.0 mM,, or from about 100 μM to about 1.0 mM. In a further embodiment, the culture medium comprises urea or ammonium as a nitrogen source. In some embodiments, the urea is present in the culture medium in an amount from about 1.5 g/l to about 2.5 g/l, preferably from about 1.8 g/l to about 2.2 g/l, more preferably about 2 g/l. In some embodiments, ammonium is present in the culture medium in an amount to provide the same amount of nitrogen as provided by the noted urea amounts. In another embodiment, the culture medium comprises glucose, mannose, xylose or mixtures thereof as a carbon source. In some embodiments, the carbon source is present in the culture medium in an amount from about 4% to about 12%, preferably from about 5% to about 12%, preferably from about 5% to about 11%, more preferably from about 5% to about 10%. In some embodiments, glucose is the sole carbon source. In one embodiment, the culture medium has a pH from about 5.5 to about 6.5, preferably about 6.0.

[0035] In a third aspect, the present invention provides a novel strain of *A. melanogenum* designated W5-2, including a pure culture of the novel strain or the isolated novel strain or the isolated and biologically pure culture of the novel strain. In some embodiments, *A. melanogenum* W5-2 does not express a functional Aureobasidin A synthase gene mRNA when cultured. In one embodiment, the functional mRNA is not expressed in the culture medium

described herein. In other embodiments, *A. melanogenum* W5-2 was deposited on 28 May 2015 under terms of the Budapest Treating with the Agricultural Research Culture Collection (NRRL) located at 1815 N. University Street, Peoria, Illinois 61604, USA and assigned Accession Number NRRL 67063.

[0036] In some embodiments, *Aureobasidium melanogenum* and/or *Aureobasidium melanogenum* strain W5-2 is characterized by the sequence of its genome. In one embodiment, the *Aureobasidium melanogenum GDP1* genomic sequence shares at least 97.5% identity over at least 98.5% of SEQ ID NO:2, preferably 99%-100% identity to over at least 98% of SEQ ID NO:2. In another embodiment, the *Aureobasidium melanogenum TEF1A* genomic sequence shares at least 98% identity over at least 94% of SEQ ID NO:8, preferably 99%-100% identity to over at least 99% of SEQ ID NO:8. In an additional embodiment, the *Aureobasidium melanogenum RBP1* genomic sequence shares at least 91% identity over at least 92% of SEQ ID NO:10, preferably 96%-100% identity to over at least 98% of SEQ ID NO:10. In a further embodiment, the *Aureobasidium melanogenum GDP1* genomic sequence shares at least 97.5% identity over at least 98.5% of SEQ ID NO:2, preferably 99%-100% identity to over at least 98% of SEQ ID NO:2, the *Aureobasidium melanogenum TEF1A* genomic sequence shares at least 98% identity over at least 94% of SEQ ID NO:8, preferably 99%-100% identity to over at least 99% of SEQ ID NO:8 and the *Aureobasidium melanogenum RBP1* genomic sequence shares at least 91% identity over at least 92% of SEQ ID NO:10, preferably 96%-100% identity to over at least 98% of SEQ ID NO:10.

[0037] In other embodiments, the *Aureobasidium melanogenum* and/or *Aureobasidium melanogenum* strain W5-2 is characterized by the amount of fatty acids stored in the fungal cells. In one embodiment, the *Aureobasidium melanogenum* and/or *Aureobasidium melanogenum* strain W5-2 fungal cells can store fatty acids at about 40% of its dry weight.

[0038] In accordance with the present invention, it has surprisingly been found that the fermentation of *A. melanogenum* W5-2 in the culture medium that contains all of the components described herein for the culture medium produces a very high yield of massoia lactone. For example, as shown in the Examples, batch fermentation of *A. melanogenum* W5-2 in this culture medium produced massoia lactone at a 7 day peak of 10.268 g/l in a 2 L bioreactor, with a volume productivity of 61.11 mg/hr/l. Thus, in one embodiment, the batch fermentation of *A. melanogenum* W5-2 in this culture medium yields 10.268 g/l of crude massoia lactone. In another embodiment, the batch fermentation of *A. melanogenum* W5-2 in this culture medium yields more than 10 g/l of crude massoia lactone. In some embodiments,

the yield of crude massoia lactone is at least 11 g/l, or at least 12 g/l, or at least 13 g/l, or at least 14 g/l or at least 15 g/l. In other embodiments, the yield of crude massoia lactone is from about 10 g/l to about 25 g/l, or from about 10 g/l to about 24 g/l, or from about 10 g/l to about 23 g/l, or from about 10 g/l to about 22 g/l, or from about 10 g/l to about 21 g/l, or from about 10 g/l to about 20 g/l, or from about 10 g/l to about 19 g/l, or from about 10 g/l to about 18 g/l, or from about 10 g/l to about 17 g/l, or from about 10 g/l to about 16 g/l, or from about 10 g/l to about 15 g/l, or from about 10 g/l to about 14 g/l.

[0039] In some embodiments, the yield of purified massoia lactone from the crude extract is at least 50%. In other embodiments, the yield of purified massoia lactone from the crude extract is more than 5 g/l. In some embodiments, the yield of purified massoia lactone from the crude extract is at least 5.5 g/l, or at least 6 g/l, or at least 6.5 g/l, or at least 7 g/l or at least 7.5 g/l. In other embodiments, the yield of purified massoia lactone from the crude extract from about 5 g/l to about 12.5 g/l, or from about 5 g/l to about 12 g/l, or from about 5 g/l to about 11.5 g/l, or from about 5 g/l to about 11 g/l, or from about 5 g/l to about 10.5 g/l, or from about 5 g/l to about 10 g/l, or from about 5 g/l to about 9.5 g/l, or from about 5 g/l to about 9 g/l, or from about 5 g/l to about 8.5 g/l, or from about 5 g/l to about 8 g/l, or from about 5 g/l to about 7.5 g/l, or from about 10 g/l to about 7 g/l.

[0040] It has surprisingly been found that the fermentation method using strain W5-2, as described herein, has several advantages.

[0041] The method produces high levels of glycolipids. Biochemically hydroxyl fatty acids conjugate to produce sugars and eventually produce massoia lactone. Thus, the method produces high levels of massoia lactone – a commercially desirable feature of the invention.

[0042] Massoia lactone is exuded into the medium and about 80% of the supernatant contains massoia lactone – reflecting the ability to obtain high amounts of massoia lactone.

[0043] The method can produce more than 10 g/l massoia lactone within 5 days at lab scale using glucose as a sole carbon source.

[0044] The crude extract has more than 10 g/l of massoia lactone. Upon purification the yield is more than 5 g/l of the crude extract, which is at least about 50% distillation recovery.

[0045] Use of 2 liter bioreactor yields high amounts of massoia lactone.

[0046] The method is highly efficient – the crude extract has less of impurities, including negligible or minimum intermediates. The method is highly time and cost efficient.

[0047] The massoia lactone is capable of producing multiple odors and/or flavorsflavors, e.g., coconut, waxy, oily aroma, creamy, green and slightly fruity flavors and/or odors. The

massoia lactone can be converted into delta-decanolide or delta-dodecanolide to produce a peachy flavor and/or odor.

[0048] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire et al., *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley–VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0049] The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Materials and Methods

[0050] *Isolation of microbial strains*: Microbial samples were collected from various regions in Singapore. Samples were mixed with 100 ml autoclaved sea water and incubated overnight at 28° C. A 100 μl of the overnight culture was withdrawn and streaked on modified seawater-YPD (Yeast Peptone Dextrose) medium (containing 1 g/l peptone, 2 g/l yeast extract, 4 g/l glucose, and 8 g/l agar, pH 7), which was further supplemented with 0.5 μg/ml Nile red and an antibiotic selected from ampicillin (25 μg/ml), kanamycin (25 μg/ml) and streptomycin (100 μg/ml). Potato dextrose agar (PDA) was also used for the initial screening. The plates were incubated at 28° C for 48 hr and the strongly Nile red staining strains were identified by examining the plates under a Nikon C-DSS230 stereomicroscope microscope (Nikon, Japan) equipped with Digital Sight DS-L1 camera. Candidate strains were purified by 2 rounds of single colony isolation on seawater-YPD plates followed by verification of Nile red staining in small scale liquid cultures. Florescence was measured using the TECAN infinite M200 fluorometer with the excitation wave-length and emission wave-length set at of 530 nm and 575 nm respectively (TECAN, USA).

[0051] *Phylogenetic analysis of the microbial strains by ITS sequencing*: Yeast isolates were cultured in YPD medium at 30° C. Genomic DNAs were extracted from 2 ml of 48 hr cultures using the MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, USA) according to the manufacturer's instructions. PCR amplification reactions were performed in 40 μl 1X buffer with 2.5 mM dNTP, 50 μM each primer, 50 ng of total DNA and 3 units of Taq DNA polymerase (i-DNA Biotechnology, Singapore). PCR cycling conditions were as followed: 95°C/10min, 30 cycles of 95° C/1 min, 61.8° C/1 min and 72° C/1 min and final extension for 5 min/72° C. The ITS1(5'-tccgtaggtgaacctgcgg; SEQ ID NO:3) and ITS4 (5'-tcctccgcttattgatatgc SEQ ID NO:4) [8] primers were used to amplify the ITS region of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. [9]. Gel-purified PCR products were sequenced with the Big-Dye sequencing method in Applied Biosystems 3730xl

DNA Analyzer (Life technologies, USA). Sequences were analyzed by BLAST against the NCBI database and aligned using the CLUSTAL W tool in MEGA version 5.05 [10]. Phylogenetic analyses were performed by the Neighbour-Joining [11], Maximum-Likelihood [12] and Maximum-Parsimony methods [13] using the MEGA version 5.05 with the bootstrap values set at 1000 replications.

[0052] *Small scale culture and fed-batch fermentation*: *Aureobasidium* strain W5-2 was cultured in 100 ml liquid medium in 250 ml shake flasks, agitated with a speed of 200 rpm and constant temperature of 30°C. The High Density Culture Medium (HDCM) developed for *Rhodotorula glutinis* [14] was used as the basic culture medium. It contains 90 g/l glucose, 12.5 g/l KH_2PO_4, 1.0 g/l Na_2HPO_4, 5.0 g/l $(NH4)_2SO_4$, 1.9 g/l yeast extract, 2.5 g/l $MgSO_4{\cdot}7H_2O$, 0.25 g/l $CaCl_2{\cdot}2H_2O$ and 0.25ml/l trace element mix (pH 5.5). Trace elements mix was made in 5N HCl and contains 40 g/l $FeSO_4.7H20$, 40 g/l $CaCl_2.2H_20$, 10 g/l $MnSO_4.7H_20$, 10g/l $AlCl_3.6H_20$, 4 g/l $CoCl_2$, 2 g/l $ZnSO_4.7H_20$, 2 g/l Na_2MoO_4, 1 g/l $CuCl_2{\cdot}2H_20$ and 0.5 g/l H_3BO_3. Where indicated, levels of trace elements and various nitrogen sources were varied. For comparison purpose, the strain was also cultured in the A-21M medium reported previously [6], which contains 120 g/l glucose, 1.5 g/l $NaNO_3$, 1.0 g/l KNO_3, 0.05 g/l KH_2PO_4, 0.2 g/l $MgSO_4.7H_20$, 2 ppm $ZnSO_4$, 0.2 g/l yeast extract (pH 5.5).

[0053] Fed-batch fermentation was carried out in a 2L Biostat B plus bioreactor (Sartorius Stedim Biotech, Germany). Dissolved oxygen level (pO_2) and air flow was maintained at 30% and 1.5 vvm, respectively. 25 ml samples were taken daily to monitor glucose, ammonium, NO^{2-} and NO^{3-} levels.

[0054] *Optimization of massaio lactone production by Design of Experiment (DOE)*: Optimization of massaio lactone production by Design of Experiment (DOE) was aided with the Design-Expert® V8 Software (Stat-Ease, USA) using both the Optimised Factorial Design and Central Composite Design (CCD). Seed cultures prepared in the HDCM medium were harvested by centrifugation, washed with sterile distilled water and suspended in sterile distilled water. A fraction of which (2 ml) was inoculated into 100 ml of the respective designed medium in a 250 ml shake flask, which was cultured in a 30° C shaking platform agitated at 200 rpm. Culture samples (15 ml) were taken daily for the analyses of cell biomass, OD_{600} and metabolites.

[0055] *Extraction and quantification of glycolipids*: Ethyl acetate was added to cell culture at a volumetric ratio of 1:1 in a 15 ml Falcon tube. The mixture was vortexed vigorously for 20 seconds; centrifuged at 3,500g for 10 minutes and 1 ml of the upper phase was transferred to a 1.5 ml Eppendorf tube and left to air dry overnight at room temperature in an exhaust hood. The

resultant dried residue was weighed with a microbalance before being added with 10 µl menthol in methanol (10% w/v) (as the internal standard) and 300 µl of 2M NaOH. The suspended mixture was allowed to hydrolyze overnight in a shaking platform at room temperature. After mixing with 150 µl of 5M H_2SO_4, 450 µl ethyl acetate was added and vortexed vigorously. After centrifugation, the upper phase was used for GCMS analysis directly. Alternatively, glycolipids were extracted directly from wet cell biomass collected by centrifugation from 10 ml culture. 2 ml of 72% H_2SO_4 was added to the cell pellet, mixed well and allowed to stand for half an hour and then mixed with 4 ml water. After boiling for one hour, 4 ml of the mixture was added with equal volume of ethyl acetate; vortexed vigorously for 20 seconds; centrifuged at 3,500g for 10 minutes and 1 ml of the upper phase was analyzed by GCMS or thin-layer chromatography (TLC). Conditions used TLC were as described previously [15]. Hexane can be used in place of ethyl acetate in this procedure. HCl can be used in place of H_2SO_4 in this procedure.

[0056] *Preparation and quantification of massoia lactone*: Equal volume of H_2SO_4 was added to 2 ml of cell culture in a 15 ml Falcon tube. Samples were vortexed briefly and left in room temperature for 30 mins. After adding 4 ml of water, Falcon tubes were boiled in a water-bath for 60 min; cooled for 10 mins at room temperature, and then 4ml of ethyl acetate was added into the tubes, which were mixed vigorously with a vortex for 20 seconds. Samples were centrifuged for 10 mins at 3,500 rcf and 1 ml of the top organic layer was transferred to a 2 ml glass vial containing 50 mg of Na_2SO_4 and 10 µl menthol solution in methanol (10%). Massoia lactone was quantified using GCMS using menthol as the internal standard. HCl can be used in place of H_2SO_4 in this procedure.

[0057] *Quantification of fatty acid*: Cell biomass was collected from 20 ml culture by centrifugation and dried in a 60° C oven until constant weight is reached. The resultant dried pellets were frozen in liquid nitrogen and grinded to a fine powder using mortar and pestle. A 250 mg sample was transferred to a 15 ml Falcon tube and then mixed with 2 ml of 72% H_2SO_4 and 10 µl of 10% (w/v) pentadeconoic acid in methanol (as the internal standard). The samples were hydrolyzed at room temperature for 30 mins and then mixed with 4 ml water. After boiling for 1 hour, a 500 µl fraction was transferred to a 2 ml Eppendorf tube and fatty acids were extracted by mixing with 1 ml methanol/chloroform mixture (1:1 v/v). After centrifugation, the bottom layer was collected by pipetting and washed once with PBS buffer in an Eppendorf tube. The methanol/chloroform bottom layer was collected after centrifugation and left to dry at room temperature in an exhaust hood. 300 µl petroleum ester (Fisher Chemicals, CAS: 64742-49-0)/benzene (QREC Asia SDN BDH, CAS 71-43-2) mixture (1:1 v/v) and 300 µl 0.4 M KOH in

methanol was added to solubilize the dried residues. Esterification was performed at room temperature for 3 hours. After separation by centrifugation, 50 µl of the top layer was diluted with 450 µl of methanol and subjected to analysis by GC-MS.

[0058] *GC-MS analysis*: GC-MS analysis was performed using GCMS-QP2010 Ultra (Shimadzu Corporation, Japan). Samples (1µl) were injected into a HP-88 column (30m × 0.25mm ID × 0.20µm) (Agilent Technologies, USA) and run with helium as the carrier gas maintained at 10 psi. A split-less injection time of 0.5 min was used. The GC started at an initial temperature of 50°C for 1 minute, ramped at 15° C per minute up to 150° C and 3° C per minute to a final temperature 240° C. The spectrometer was scanned from 41–400 amu. The compounds were identified by searching against the NIST 08 mass spectral library. Quantification of fatty acid ester and massoia lactone was done by comparing the peak area between the target compounds and the respective internal standards.

[0059] *Other quantification methods*: Glucose levels were quantified using a Shimadzu Prominence UFLC (Shimadzu, Japan). Samples were run through an Aminex HPX-87H column (Bio-rad, USA) maintained at 50° C. 5 mM H_2SO_4 was used as the mobile phase and run at a flow rate of 0.7 ml/min. Total nitrates, nitrites and ammonia levels were determined using the method as described [16, 17].

[0060] *RNA sampling and RNA-sequencing*: Cell cultures (1 ml) were collected at day 1, 2, 3 and RNAs were immediately extracted with RiboPure™ RNA Purification Kit, yeast (Life Technologies, USA). After determination of the RNA quantity and quality by Nanodrop and agarose gel electrophoresis, the RNA samples were sent to Macrogen Inc. (Korea) for cDNA shotgun library construction and sequencing using Illumina Hiseq 2000.

[0061] *Computational analysis*: Computational analysis was performed in the Galaxy platform (http://galaxyproject.org/) installed locally [18]. Raw reads (100 bp paired-end) were analyzed with NGS QC toolkit [19] for their quality. Then, Tophat and Cufflinks [20-22] were used to identify differential gene/transcript expression based on published genome sequences as the references. de novo assembly was performed with Trinity [23, 24], which produced ~18k isoforms or transcripts. Differentially expressed (DE) transcripts and expression profile clusters were done with Bioconductor with rsem and edgeR packages [25, 26].

EXAMPLE 2

Identification of Massoia Lactone-Producing Microbial Strains

[0062] Nile red is an uncharged hydrophobic molecule whose fluorescence is strongly influenced by the polarity of its environment and it is often used as a marker for hydrophobic substances, such as lipid, glycolipids and hydrophobic proteins [27-29]. By screening water and soil samples obtained from local costal environment using Nile red as a marker, 32 candidates were identified that showed significant red fluorescence, among which strain W5-2 was identified as fungus closely related to *Aureobasidium species* based on the sequence comparison of the rDNA ITS region (SEQ ID NO.1) (Fig. 1). W5-2 colonies turned black after 7 days of culture on solid medium (not shown).

[0063] Because *Aureobasidium* species are identified by phylogenetic analysis of their whole genome sequences as well as certain phylogenetically important genes, such as house-keeping genes encoding Actin, β-tubulin, calmodulin, chytin synthase, NAD-dependent glycerol-3-phosphate dehydrogenase and translation elongation factor 1α (TEF1A), the whole transcriptome of W5-2 was chosen to compare those of the type *Aureobasidium* strains [30].

[0064] The overall alignment rates of raw reads to the 4 reference genomes of *Aurebasidium* species [31] were low, ~55.7% to *A. melanogenum* and ~15-19 % to the rest *Aureobasidium* species (Table 1). Therefore, *de novo* assembly of RNA-seq data was performed using the Trinity program [32], producing a sequence library of ~18k isoforms/transcripts. The overall alignment rate of raw reads to this local reference was increased to above 95%.

TABLE 1

Mapping Rates to Reference Genomes of *Aurebasidium* Species

	T0-1d	T0-2d	T0-3d	T2-1d	T2-2d	T2-3d	Average
A. melanogenum	60.47%	52.76%	52.10%	58.24%	56.73%	54.06%	55.72%
A. pullulans	21.68%	15.71%	15.81%	16.80%	15.06%	13.86%	16.49%
A. subglaciale	19.80%	14.23%	14.41%	15.46%	13.48%	12.41%	14.97%
A. namibiae	24.90%	18.11%	18.23%	20.08%	18.04%	16.32%	19.28%

Note: Raw reads were mapped to reference genome using TopHat software[33]. RNA samples were extracted from W5-2 cell culture in Medium T0 and Medium T2 at Day, 1, 2 and 3 respectively.

[0065] DNA sequence divergence in *GPD1* gene is used as an important indicator for phylogenetic analysis in *Aureobasidium* species [30]. *GPD1* CDS sequence (SEQ ID NO:2) was

searched by BLASTn against the Whole Genome Shortgun sequences of *Aureobasidium* species at NCBI. Six highly related sequences were identified. Phylogenic tree generated Blast Tree View uisng fast minimal evolution method is shown in Fig. 2, which clearly places the W5-2 *GPD1* sequence between *A. melanogenum* CBS 110374/*A. pullulans* AY4, which has been re-classified as *A. melanogenum* [30], and *A. namibiae* CBS 147.97/*A. subglaciale* EXF-2481. W5-2 showed highest divergence from *A. pullulans* EXF-150 and lowest divergence to *A. melanogenum*.

[0066] *A. pullulans* is known to produce antibiotic aureobasidin A [34], which is made by a huge polyketide synthase Aba1[35]. A search of *ABA1* CDS (Genbank no. EU886741) against the W5-2 whole transcriptome failed to identify any homologs in the genome. Therefore, strain W5-2 does not encode a functional *ABA1* gene and is not likely to produce any antibiotics.

[0067] Confocal imaging confirmed that Nile red stained the cytoplasm intensely with little signal in the cytoplasmic membrane, suggesting the high accumulation of hydrophobic substance in the cells (Fig. 3C). Fatty acid profiling by GCMS revealed that the total ethyl acetate extract contained 52.28% oleic acid, 33.9% palmitic acid, 3.98% linoleic acid, 1.31% palmatolic acid and about 1.3% of long chain (C24 and above) fatty acids (Table 2). Thin layer chromatography (TLC) confirmed the production of triacylglyceride (TAG) and glycolipids (Fig. 3D). Unexpectedly, the GCMS profile showed a significant peak with a retention time of approximately 25.7 min, which had >93% similarity to massoia lactone, i.e., 6-pentyl-5,6-dihydro-2H-pyran-2-one (Figs. 4A and 4B). Sensory test of the dried cell pellet confirmed the presence a strong coconut-like aroma.

TABLE 2

Fatty Acid Profile of W5-2

	C16	C16:1	C18	C18:1	C18:2	C24
W5-2	33.91%	1.31%	7.24%	52.28%	3.98%	1.29%

EXAMPLE 3

Medium Optimization Using Central Composite Designs (CCD)

[0068] To improve massoia lactone production, the *Rhodotorula glutinis* high density (HMDC) medium [14] was modified by changing the levels of nitrogen source (urea), carbon source (glucose) and trace element mix, which contains $FeSO_4.7H_2O$, $CaCl_2 \cdot 2H_2O$, $MnSO_4$,

$AlCl_3 \cdot 6H_2O$, $CoCl_2$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$ and H_3BO_4. Medium compositions are shown in Table 3. Massoia lactone production was low, ranging from 0.05 mg/l (Run 6) to 1.61 mg/l (Run 16) (Fig. 5). While there were obvious differences amongst the runs, none of the parameters appeared to significantly influence the production by ANOVA analysis ($p<0.05$) (Table 4). Since massoia lactone produced in *A. pullulans* has been reported to derive from glycolipid [6], we monitored glycolipid levels by TLC. Again, CCD was employed to optimize the nitrogen source and carbon source in HMDC medium, with urea and yeast extract set as category factors while glucose level was set at 7.5, 20, 50, 80, 92.4 g/l respectively. In addition, the trace element mix was set at 0.034, 0.2, 0.6, 1.0, 1.17 ml per litre medium respectively (Table 5). High variations in glycolipid profiles were observed: Run No. 15 appeared to be the best combination for glycolipid production (Fig. 6), with 285 mg/l massoia lactone present in the acid hydrolyzed glycolipid products. Therefore, we chose a medium containing high level of glucose (~100 g/l), 2 g/l urea as the sole nitrogen source and 0.6 ml original HMDC trace element mix for subsequent medium optimizations. For convenience, this medium is referred as the Run 15 (R15) medium.

TABLE 3

Optimization of Nitrogen source, Carbon Source and Trace Elements by CCD

Run No.	Urea (g/L)	Glucose (g/l)	Trace (ml/l)
1	2.89	36.21	0.95
2	1.11	83.78	0.24
3	2	60	1.2
4	2	60	0.6
5	0.5	60	0.6
6	2	60	0
7	2.89	36.21	0.24
8	2	60	0.6
9	2	60	0.6
10	2	60	0.6
11	3.5	60	0.6
12	1.11	83.78	0.957
13	1.11	36.21	0.96

14	2	20	0.6
15	2	60	0.6
16	2	60	1.00
17	2.89	83.78	0.24
18	1.11	36.21	0.24
19	2	100	0.6
20	2.89	83.78	0.96

TABLE 4
Anova Analysis of Table 3

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Remarks
Model	0.75	3.00	0.25	1.98	0.1580	not significant
A-Trace elements	0.23	1.00	0.23	1.86	0.1919	
B-Urea	0.00	1.00	0.00	0.02	0.8813	
C-Glucose	0.51	1.00	0.51	4.05	0.0612	
Residual	2.02	16.00	0.13			
Lack of Fit	1.87	11.00	0.17	5.95	0.0308	significant
Pure Error	0.14	5.00	0.03			
Cor Total	2.77	19.00				
Std. Dev.	0.36		R-Squared	0.27		
Mean	0.59		Adj R-Squared	0.13		
C.V. %	60.42		Pred R-Squared	-0.30		
PRESS	3.60		Adeq Precision	4.27		

TABLE 5

Medium Compositions of Central Composite Design 1 (CCD1)

Run	Nitrogen source	Glucose (g/l)	Trace element mix (ml/l)	lactone titre (mg/l)
1	Urea	7.57	0.6	0.256
2	Yeast extract	50	0.03	4.192
3	Yeast extract	50	0.6	2.384
4	Yeast extract	7.57	0.6	1.386
5	Yeast extract	80	0.2	2.594
6	Yeast extract	20	0.2	3.257
7	Yeast extract	20	1	4.130
8	Urea	80	0.2	3.082
9	Urea	50	1.17	4.978
10	Yeast extract	50	0.6	3.100
11	Yeast extract	80	1	3.366
12	Urea	20	1	1.853
13	Urea	20	0.2	4.103
14	Urea	80	1	4.935
15	Urea	92.43	0.6	6.731
16	Urea	50	0.6	2.300
17	Urea	50	0.6	5.272
18	Urea	50	0.6	3.011
19	Yeast extract	50	0.6	4.325
20	Yeast extract	50	1.17	2.510
21	Yeast extract	50	0.6	2.651
22	Urea	50	0.6	5.715
23	Urea	50	0.03	4.990
24	Yeast extract	92.43	0.6	0.934
25	Urea	50	0.6	3.700
26	Yeast extract	50	0.6	4.855

Note: urea and yeast extract were both set at 1.9 g/l.

EXAMPLE 4

Optimization of Trace Elements using Factorial Designs

[0069] $FeSO_4$, $MnSO_4$, $ZnSO_4$, $CuCl_2$, $AlCl_3$ were supplemented to the R15 medium using Factorial Optimal Design at the same concentration [14], either individually or in combinations (Table 6). Results showed that Run #12 which was supplemented with Fe^{2+}, Zn^{2+} and Cu^{2+} (named as T3 medium hereafter) produced the highest level of massoia lactone, reaching 433 mg/l on day 8. Run #6 in which Mn^{2+} and Zn^{2+} were both supplemented (named as T2 medium hereafter) ranked 2nd, yielding 218 mg/l massoia lactone (Fig. 7A; Table 6). In contrast, Basal medium (Run 2, referred to as T0 medium) with $CoCl_2$, HBO_3 and $NaMoO_4$ only produced only 58 mg/l massoia lactone, more than 14 folds lower than with T3 medium (Fig. 7A, Table 6). Anova analysis showed that $ZnSO_4$, $CuCl_2$, $FeSO_4$, $ZnSO_4$, $FeSO_4$-$CuCl_2$, $FeSO_4$-$AlCl_3$, and $CuCl_2$-$AlCl_3$ significantly affected massoia lactone production (Tables 6 and 7).

TABLE 6

Trace Elements Part 1

Run #	**$FeSO_4$**	**$MnSO_4$**	**$ZnSO_4$**	**$CuCl_2$**	**$AlCl_3$**
1	Y	N	Y	Y	Y
2	N	N	N	N	N
3	Y	Y	N	Y	Y
4	N	Y	N	N	Y
5	N	N	N	N	Y
6	N	Y	Y	N	N
7	N	N	Y	N	Y
8	Y	N	Y	N	N
9	N	N	Y	Y	N
10	Y	N	N	N	Y
11	Y	N	N	Y	N
12	Y	N	Y	Y	N
13	Y	N	N	N	N
14	N	Y	Y	Y	Y
15	Y	N	N	Y	N

16	Y	Y	N	N	Y
17	Y	Y	N	N	N
18	Y	Y	Y	Y	Y
19	N	Y	N	Y	N
20	Y	Y	Y	Y	N
21	N	N	N	Y	Y

Note: Basal medium (T0 medium) contained 100 g/l glucose, 12.5 g/l KH_2PO_4, 1.0 g/l Na_2HPO_4, 5.0 g/l $(NH4)_2SO_4$, 2g/l urea, 2.5 g/l $MgSO_4 \cdot 7H2O$, 0.25 g/l $CaCl_2 \cdot 2H2O$, 2.4 mg/l $CoCl_2$, 0.3 mg/l HBO_3 and 1.2 mg/l $NaMoO_4$, pH 5.5. 0.6 ml of trace element mix was added to each litre of medium. Where indicated by N, the trace element was omitted in the trace element mix. The final medium contained various combinations of $FeSO_4 7H_2O$ (24 mg/l), $MnSO_4$ (6 mg/l), $ZnSO_4$ (1.2 mg/l) and CuCl2 (0.6 mg/l).

TABLE 7

Anova Analysis of Table 6

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Remarks
Model	469.03	15.00	31.27	17.67	0.0025	significant
A-$FeSO_4$	6.58	1.00	6.58	3.72	0.1118	
B-$MnSO_4$	5.67	1.00	5.67	3.20	0.1335	
C-$ZnSO_4$	305.29	1.00	305.29	172.50	**< 0.0001**	significant
D-$CuCl_2$	22.61	1.00	22.61	12.78	**0.0160**	significant
E- $AlCl_3$	3.46	1.00	3.46	1.95	0.2211	
AB	0.87	1.00	0.87	0.49	0.5151	
AC	69.62	1.00	69.62	39.34	**0.0015**	significant
AD	22.14	1.00	22.14	12.51	**0.0166**	significant
AE	16.67	1.00	16.67	9.42	**0.0278**	significant
BC	2.88	1.00	2.88	1.63	0.2579	
BD	0.44	1.00	0.44	0.25	0.6408	
BE	5.87	1.00	5.87	3.32	0.1282	
CD	0.18	1.00	0.18	0.10	0.7598	
CE	0.29	1.00	0.29	0.16	0.7043	
DE	19.93	1.00	19.93	11.26	**0.0202**	significant

Residual	8.85	5.00	1.77			
Cor Total	477.88	20.00				
Std. Dev.	1.33		R-Squared	0.98		
Mean	21.66		Adj R-Squared	0.93		
C.V. %	6.14		Pred R-Squared	0.29		
PRESS	339.46		Adeq Precision	16.34		

[0070] In another experiment, the effects of the remaining trace elements, i.e.., $CoCl_2$, HBO_3 and $NaMoO_4$.were tested. Results showed that $CoCl_2$ (2.4 mg/l) strongly inhibited massoia lactone production ($p<0.0001$) while $NaMoO_4$ (1.2 mg/l) significantly improved the production ($p<0.05$). HBO_3 (0.3 mg/l) did not appear to have significant effect (Tables 8 and 9). The best medium was Run 12, with a massoia lactone yield of 1300 mg/l (Fig. 7B). Notably, this medium contains high level of $CaCl_2$ (0.25 g/l $CaCl_2 \cdot 2H_2O$).

TABLE 8

Trace Elements Part 2

Run #	$CoCl_2$	$NaMoO_4$	$H3BO_4$
1	N	Y	Y
2	Y	Y	N
3	N	N	N
4	N	Y	N
5	N	N	N
6	Y	N	Y
7	N	Y	Y
8	N	N	Y
9	Y	Y	Y
10	Y	N	N
11	N	N	Y

12	N	Y	N
13	Y	Y	Y

Note: Media were inoculated to 0.1 OD_{600} with water-washed cell stock cultured in HMDC medium and cultured at 30°C with 200 rpm shaking for 8 days. Basal medium was T0 medium (Table 6) with various combinations of $CoCl_2$ (2.4 mg/l), HBO_3 (0.3 mg/l) and $NaMoO_4$ (1.2 mg/l).

TABLE 9

Anova Analysis of Table 8

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Remarks
Model	2702668.48	6.00	450444.75	47.99	< 0.0001	significant
A-$CoCl_2$	2431273.40	1.00	2431273.40	259.01	**< 0.0001**	significant
B-$NaMoO_4$	60353.96	1.00	60353.96	6.43	**0.0443**	significant
C-H_3BO_4	769.68	1.00	769.68	0.08	0.7842	
AB	21411.70	1.00	21411.70	2.28	0.1817	
AC	320.50	1.00	320.50	0.03	0.8595	
BC	18462.23	1.00	18462.23	1.97	0.2103	
Residual	56320.37	6.00	9386.73			
Lack of Fit	3671.82	1.00	3671.82	0.35	0.5805	not significant
Pure Error	52648.56	5.00	10529.71			
Cor Total	2758988.86	12.00				
Std. Dev.	96.89	R-Squared	0.98			
Mean	741.48	Adj R-Squared	0.96			
C.V. %	13.07	Pred R-Squared	0.91			
PRESS	243152.35	Adeq Precision	15.75			

EXAMPLE 5

Utilization of Various Carbon Sources by *A. melanogenum* W5-2

[0071] To see if strain W5-2 is able to utilize other carbon sources for the production of massoia lactone, glucose in T2 medium was replaced with the same concentrations of D-(+)-mannose, D-(+)-xylose and glycerol. Mannose was essentially as efficiently utilized as glucose. The strain also efficiently converted xylose to massoia lactone although the yield was about 20% lower than with glucose under the conditions tested. Glycerol was a poor carbon source (Fig. 8).

EXAMPLE 6

Fed-Batch Fermentation

[0072] To verify the performance of the optimized media, fed-batch fermentation was performed in 2L bioreactors using T0, T2 and T3 media. As the original A-21M medium barely supports the growth of W-52, a modified A-21M medium (MA-21M) was used as a reference. MA-21M contained 120 g/l glucose, 1.5 g/l $NaNO_3$, 1.0 g/l KNO_3, 0.05 g/l KH_2PO_4, 0.2 g/l $MgSO_4$, 0.0056 g/l $FeSO_4$, 0.2 g/l Yeast Extract (pH 5.5). Indeed, both T2 and T3 media showed drastically improved massoia lactone production compared to the T0 medium (Fig. 9A). Best result was observed with T3 medium, in which massoia lactone level peaked at Day 7, reaching 10.268 g/l, with a volumetric productivity of 61.11 mg/hr/l. In T2 medium, massoia lactone production was significantly lower than T3 medium throughout the time course. Maximal production was delayed at least 3 days with a titre of 6.924 g/l and volumetric productivity of 28.85 mg/hr/l. In contrast, maximal titre for T0 medium was only 0.441 g/l observed at Day 10, with a volumetric productivity of 1.84 mg/hr/l. The MA-21M medium performed better than T0 medium, peaking Day 9 with a titre of 1.777 g/l and volumetric productivity of 8.23 mg/hr/l. Thus, the volumetric productivity in T3 and T2 medium were 7.43-fold and 3.51-fold higher than that of A-21M medium, respectively.

[0073] In T0 and T2 medium, cells produced much higher biomass at the cost of the desired metabolite (Fig. 9B). Nitrogen source in both T2 and T3 media were rapidly consumed and became depleted after Day 2. In contrast, NH^{4+} level remained high in T0 medium until Day 8 (Fig. 9C). The cells showed very different colors: cells contained strong black pigments, presumably melanin, in T2 and T3 media. In stark contrast with previous report [6], the result suggests that the production of black pigment was associated with high massoia lactone production.

EXAMPLE 7

Effect of Medium pH

[0074] W5-2 cells cultured in YPD medium was cultured in T2 medium adjusted to various pH values ranging from pH 4 to pH 6.5. The maximal titre was observed with pH6.0. Slight reduction of yield was seen for pH 5.5 and 6.5 (Fig. 11).

EXAMPLE 8

Purity of Massoia Lactone

[0075] Fed-batch fermentation was performed in 2L bioreactors using T3 media. The total fermentation broth was hydrolyzed with H_2SO_4. GCMS analysis of the showed the high production of a single peak of massoia lactone (Figs. 12A and 12B). This is in contrast to earlier work with *Aureobasidium pullalan* A-21M, which produced 3-hydroxyl delta-decalactone at ratio of about 1:1.7 [6].

EXAMPLE 9

Comparison of House-Keeping Genes

[0076] The coding sequences (CDS) of five house-keeping genes of W5-2 strain were identified using known protein sequences of *Ustilago maydis* as the query to search against the W5-2 whole transcriptome database. SEQ ID NOs:2, 7, 8, 9 and 10 are the CDS sequence for *GPD1, Actin*, *TEF1A*, *Tubulin1* and *RPB1* (RNA polymerase 2, the largest subunit), respectively. There were high variations in the levels of sequence identity to homologs of different *Aureobasidium* species (Tables 10-14). Note that *A. pullulans* AY4 has been re-classified as *A. melanogenum* AY4. For example, *GDP1* genomic sequence shares at least 99% identity to homologs of *A. melanogenum* species at least over 98% of SEQ ID NO:2 while the inter-species homology for *GPD1* CDS is below 97% over 98% of SEQ ID NO:2 (Table 10). Similarly, *TEFA* genomic sequence shares at least 99% identity to homologs of *A. melanogenum* species over 99% of SEQ ID NO:8 while the inter-species homology for *TEF1A* CDS is below 98% over 94% of SEQ ID NO:8 (Table 12). *RPB1 (*SEQ ID NO:10) sequence is the most divergent.

[0077] *RPB1* genomic sequence shares at least 96% identity to homologs of *A. melanogenum* species over 98% of SEQ ID NO:10 while the inter-species homology for *RPB1* CDS is below 90% over 92% of SEQ ID NO:10 (Table 14).

TABLE 10

BLASTn Search Results of *GPD1* (SEQ ID NO:2)

Description	**Max score**	**Total score**	**Query cover**	**E value**	**Ident**	**Accession**
Aureobasidium melanogenum CBS 110374 scaffold_4_c3, whole genome shotgun sequence	1459	1812	98%	0.0	99%	AYEN01000011.1
Aureobasidium pullulans AY4 contig20, whole genome shotgun sequence	1448	1795	98%	0.0	99%	AMCU01000020.1
Aureobasidium namibiae CBS 147.97 scaffold_2_c1, whole genome shotgun sequence	1371	1692	98%	0.0	97%	AYEM01000004.1
Aureobasidium pullulans isolate Santander contig_514, whole genome shotgun sequence	1321	1635	98%	0.0	96%	LVWM01000514.1
Aureobasidium subglaciale EXF-2481 scaffold_2_c1, whole genome shotgun sequence	1315	1636	98%	0.0	96%	AYYB01000004.1
Aureobasidium pullulans EXF-150 scaffold_6_c1, whole genome shotgun sequence	1293	1605	98%	0.0	95%	AYEO01000008.1

TABLE 11

BLASTn Search Results of *Actin* (SEQ ID NO:7)

Description	**Max score**	**Total score**	**Query cover**	**E value**	**Ident**	**Accession**
Aureobasidium pullulans AY4 contig120, whole genome shotgun sequence	1216	2960	99%	0.0	92%	AMCU01000120.1
Aureobasidium melanogenum CBS 110374 scaffold_3_c1, whole genome shotgun sequence	1210	2960	99%	0.0	92%	AYEN01000004.1

Aureobasidium namibiae CBS 147.97 scaffold_18_c1, whole genome shotgun sequence	917	1016	44%	0.0	86%	AYEM01000024.1
Aureobasidium pullulans isolate Santander contig_225, whole genome shotgun sequence	828	1993	96%	0.0	84%	LVWM01000225.1
Aureobasidium pullulans EXF-150 scaffold_2_c1, whole genome shotgun sequence	811	1872	93%	0.0	84%	AYEO01000002.1
Aureobasidium subglaciale EXF-2481 scaffold_19_c1, whole genome shotgun sequence	614	735	39%	4e-174	82%	AYYB01000022.1

TABLE 12

BLASTn Search Results of *TEF1A* (SEQ ID NO:8)

Description	**Max score**	**Total score**	**Query cover**	**E value**	**Ident**	**Accession**
Aureobasidium melanogenum CBS 110374 scaffold_11_c1, whole genome shotgun sequence	2041	2472	99%	0.0	99%	AYEN01000021.1
Aureobasidium pullulans AY4 contig64, whole genome shotgun sequence	2041	2472	99%	0.0	99%	AMCU01000064.1
Aureobasidium namibiae CBS 147.97 scaffold_4_c1, whole genome shotgun sequence	2015	2368	94%	0.0	98%	AYEM01000007.1
Aureobasidium pullulans EXF-150 scaffold_17_c1, whole genome shotgun sequence	1881	2112	92%	0.0	96%	AYEO01000027.1

Aureobasidium subglaciale EXF-2481 scaffold_23_c1, whole genome shotgun sequence	1753	1985	93%	0.0	94%	AYYB01000027.1

TABLE 13

BLASTn Search Results of *Tubulin1* (SEQ ID NO:9)

Description	**Max score**	**Total score**	**Query cover**	**E value**	**Ident**	**Accession**
Aureobasidium melanogenum CBS 110374 scaffold_23_c2, whole genome shotgun sequence	1284	2316	97%	0.0	98%	AYEN01000040.1
Aureobasidium pullulans AY4 contig32, whole genome shotgun sequence	1284	2305	97%	0.0	98%	AMCU01000032.1
Aureobasidium namibiae CBS 147.97 scaffold_17_c1, whole genome shotgun sequence	1194	2187	97%	0.0	96%	AYEM01000023.1
Aureobasidium pullulans isolate Santander contig_153, whole genome shotgun sequence	1160	1981	92%	0.0	95%	LVWM01000153.1
Aureobasidium pullulans EXF-150 scaffold_3_c1, whole genome shotgun sequence	1158	1984	92%	0.0	95%	AYEO01000003.1
Aureobasidium subglaciale EXF-2481 scaffold_0_c1, whole genome shotgun sequence	1140	2033	97%	0.0	95%	AYYB01000001.1

TABLE 14

BLASTn Search Results of *RPB1* (SEQ ID NO:10)

Description	**Max score**	**Total score**	**Query cover**	**E value**	**Ident**	**Accession**
Aureobasidium melanogenum CBS 110374 scaffold_1_c2, whole genome shotgun sequence	7915	9706	98%	0.0	96%	AYEN01000002.1

Aureobasidium pullulans AY4 contig81, whole genome shotgun sequence	7866	9631	99%	0.0	96%	AMCU01000081.1
Aureobasidium namibiae CBS 147.97 scaffold_1_c2, whole genome shotgun sequence	6032	6855	91%	0.0	89%	AYEM01000002.1
Aureobasidium subglaciale EXF-2481 scaffold_15_c1, whole genome shotgun sequence	5389	6038	91%	0.0	87%	AYYB01000017.1
Aureobasidium pullulans contig_319, whole genome shotgun sequence	5334	5999	92%	0.0	86%	LVWM01000319.1
Aureobasidium pullulans contig_233, whole genome shotgun sequence	5334	5999	92%	0.0	86%	LVWM01000233.1
Aureobasidium pullulans contig_45, whole genome shotgun sequence	5334	5999	92%	0.0	86%	LVWM01000045.1
Aureobasidium pullulans EXF-150 scaffold_18_c1, whole genome shotgun sequence	5323	5993	92%	0.0	86%	AYEO01000028.1

[0078] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0079] Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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WHAT IS CLAIMED IS:

1. A pure culture of *Aureobasidium melanogenum* that expresses no functional Aureobasidin A biosynthesis complex gene mRNA when cultured.

2. The pure culture of claim 1, wherein the *Aureobasidium melanogenum* strain is *Aureobasidium melanogenum* strain W5-2.

3. The pure culture of claim 2, wherein *Aureobasidium melanogenum* W5-2 is deposited with Agricultural Research Culture Collection (NRRL) and assigned Accession Number NRRL 67063.

4. The pure culture of any one of claims 1-3, wherein the *Aureobasidium melanogenum GDP1* genomic sequence shares at least 97.5% identity over at least 98.5% of SEQ ID NO:2, preferably 99%-100% identity to over at least 98% of SEQ ID NO:2.

5. The pure culture of any one of claims 1-3, wherein the *Aureobasidium melanogenum TEF1A* genomic sequence shares at least 98% identity over at least 94% of SEQ ID NO:8, preferably 99%-100% identity to over at least 99% of SEQ ID NO:8.

6. The pure culture of any one of claims 1-3, wherein the *Aureobasidium melanogenum RBP1* genomic sequence shares at least 91% identity over at least 92% of SEQ ID NO:10, preferably 96%-100% identity to over at least 98% of SEQ ID NO:10.

7. The pure culture of any one of claims 1-3, wherein the *Aureobasidium melanogenum GDP1* genomic sequence shares at least 97.5% identity over at least 98.5% of SEQ ID NO:2, preferably 99%-100% identity to over at least 98% of SEQ ID NO:2, wherein the *Aureobasidium melanogenum TEF1A* genomic sequence shares at least 98% identity over at least 94% of SEQ ID NO:8, preferably 99%-100% identity to over at least 99% of SEQ ID NO:8 and wherein the *Aureobasidium melanogenum RBP1* genomic sequence shares at least 91% identity over at least 92% of SEQ ID NO:10, preferably 96%-100% identity to over at least 98% of SEQ ID NO:10.

8. Use of the pure culture of any one of claims 1-7 for the fermentative production of massoia lactone.

9. A method of fermentative production of massoia lactone comprising culturing an *Aureobasidium melanogenum* species in a culture medium comprising high levels of phosphate ions, ammonium ions, calcium ions, at least two trace elements selected from the group consisting of Fe^{2+}, Cu^{2+}, Zn^{2+} and MoO_4^{2-}, urea or ammonium as the nitrogen source and a carbon source selected from the group consisting of glucose, mannose, xylose and mixtures thereof for a sufficient period of time to produce massoia lactone in a fermentation product.

10. The method of claim 9, wherein the pH of the culture medium is from about 5.5 to about 6.5, preferably 6.0

11. The method of claim 9 or 11, wherein the amount of urea present in the culture medium is from about 1.5 g/l to about 2.5 g/l, preferably from about 1.8 g/l to about 2.2 g/l, more preferably about 2 g/l.

12. The method of any one of claims 9-11, wherein the carbon source present in the culture medium is from about 4% to about 12%, preferably from about 5% to about 12%, preferably from about 5% to about 11%, more preferably from about 5% to about 10%.

13. The method of any one of claim 9-12, wherein each trace element in the culture medium is present in an amount from about 0.1 µM to about 1.0 mM, from about 1.0 µM to about 1.0 mM, from about 10.0 µM to about 1.0 mM,, or from about 100 µM to about 1.0 mM.

14. The method of any one of claims 9-13, wherein the culture comprises about 10.0 g/l to about 15 g/l, preferably about 12.5 g/l KH_2PO_4, about 0.5 g/l to about 2.0 g/l, preferably about 1.0 g/l Na_2HPO_4, about 3.5 g/l to about 6.5 g/l, preferably about 5.0 g/l $(NH_4)_2SO_4$, about 1.0 g/l to about 4.0 g/l, preferably about 2.5 g/l $MgSO_4 \cdot 7H_2O$ and about 0.10 g/l to about 0.40 g/l, preferably about 0.25 g/l $CaCl_2 \cdot 2H_2O$.

15. The method of claim 14, wherein the culture medium further comprises about 2 g/l urea, about 5% to about 10% carbon source and at least two of the trace elements.

16. The method of any one of claims 9-15, wherein the period of time is for about 4 days to about 12 days, preferably for about 5 days to about 12 days, preferably for about 5 days to about 11 days, preferably for about 6 days to about 11 days, more preferably for about 7 days to about 10 days.

17. The method of any one of claims 9-16, wherein the *Aureobasidium melanogenum* is a strain that expresses no functional Aureobasidin A synthase gene mRNA when cultured.

18. The method of claim 17, wherein the *Aureobasidium melanogenum* strain is *Aureobasidium melanogenum* W5-2.

19. The method of claim 18, wherein *Aureobasidium melanogenum* W5-2 is deposited with Agricultural Research Culture Collection (NRRL) and assigned Accession Number NRRL 67063.

20. The method of any one of claims 9-19, which further comprises hydrolyzing the fermentation product by a strong inorganic acid and purifying the massoia lactone from the fermentation product.

21. The method of any one of claims 9-20, wherein the fermentation product is substantially free of contaminant 3-hydroxyl delta-decalactone (3-hydroxydecan-5-olide).

22. A culture medium for the fermentative production of massoia lactone by *Aureobasidium melanogenum* species, the culture medium comprising high levels of phosphate ions, ammonium ions, calcium ions, at least two trace elements selected from the group consisting of Fe^{2+}, Cu^{2+}, Zn^{2+} and MoO_4^{2-}, urea or ammonium as nitrogen source and a carbon source selected from the group consisting of glucose, mannose, xylose and mixtures thereof.

23. The culture medium of claim 22, wherein the pH of the culture medium is from about 5.5 to about 6.5, preferably 6.0

24. The culture medium of claim 22 or 23, wherein the amount of urea present in the culture medium is from about 1.5 g/l to about 2.5 g/l, preferably from about 1.8 g/l to about 2.2 g/l, more preferably about 2 g/l.

25. The culture medium of any one of claims 22-24, wherein the carbon source present in the culture medium is from about 4% to about 12%, preferably from about 5% to about 12%, preferably from about 5% to about 11%, more preferably from about 5% to about 10%.

26. The culture medium of any one of claim 22-25, wherein each trace element present in the culture medium is present in an amount from about 0.1 μM to about 1.0 mM, from about 1.0 μM to about 1.0 mM, from about 10.0 μM to about 1.0 mM,, or from about 100 μM to about 1.0 mM.

27. The culture medium of any one of claims 22-26, wherein the culture medium comprises about 10.0 g/l to about 15 g/l, preferably about 12.5 g/l KH_2PO_4, about 0.5 g/l to about 2.0 g/l, preferably about 1.0 g/l Na_2HPO_4, about 3.5 g/l to about 6.5 g/l, preferably about 5.0 g/l $(NH_4)_2SO_4$, about 1.0 g/l to about 4.0 g/l, preferably about 2.5 g/l $MgSO_4 \cdot 7H_2O$ and about 0.10 g/l to about 0.40 g/l, preferably about 0.25 g/l $CaCl_2 \cdot 2H_2O$.

28. The culture medium of claim 27, wherein the culture medium further comprises about 2 g/l urea, about 5% to about 10% carbon source and at least two of the trace elements.

29. The culture medium of any one of claims 22-28, wherein the culture medium further comprises an *Aureobasidium melanogenum* species.

30. The culture medium of claim 29, wherein the *Aureobasidium melanogenum* is a strain that expresses no functional Aureobasidin A synthase gene mRNA when cultured.

31. The culture medium of claim 30, wherein the *Aureobasidium melanogenum* strain is *Aureobasidium melanogenum* W5-2.

32. The culture medium of claim 31, wherein *Aureobasidium melanogenum* W5-2 is deposited with Agricultural Research Culture Collection (NRRL) and assigned Accession Number NRRL 67063.

33. The culture medium of any one of claims 22-32 further comprising more than 10 g/l of massoia lactone.