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ORIGINAL ARTICLE

Characteristics of purple nonsulfur bacteria grown under *Stevia* residue extractions

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Significance and Impact of the Study: This study first reported that the purple nonsulfur bacteria could grow well and possess high quality using *Stevia* residue extractions as carbon source. Those results suggest that wastewater of *Stevia* residue can be a favourable substrate for microbial growth, which can further provide desired bioresources for the application of downstream industry.

Keywords
Cell pigment, Indole-3-acetic acid, repetitive sequence-based PCR, *Rhodopseudomonas palustris*, *Stevia* residue.

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Abstract

As a consequence of the large-scale cultivation of *Stevia* plants, releases of plant residues, the byproduct after sweetener extraction, to the environment are inevitable. *Stevia* residue and its effluent after batching up contain large amounts of organic matters with small molecular weight, which therefore are a potential pollution source. Meanwhile, they are favourite substrates for micro-organism growths. This investigation was aimed to utilize the simulated effluent of *Stevia* residue to enrich the representative purple nonsulfur bacterium (PNSB), *Rhodopseudomonas palustris* (*Rps. palustris*), which has important economic values. The growth profile and quality of *Rps. palustris* were characterized by spectrophotometry, compared to those grown in common PNSB mineral synthetic medium. Our results revealed that the simulated effluent of *Stevia* residue not only stimulated *Rps. palustris* growth to a greater extent, but also increased its physiologically active cytochrome concentrations and excreted indole-3-acetic acid (IAA) content. This variation in phenotype of *Rps. palustris* could result from the shift in its genotype, further revealed by the repetitive sequence-based PCR (rep-PCR) fingerprinting analysis. Our results showed that the effluent of *Stevia* residue was a promising substrate for microbial growth.

Introduction

Stevia rebaudiana (Bertoni), an economic plant, contains stevioside, which has manifold advantages: it is stable, it is noncalorific, it maintains good dental health by reducing the intake of sugar and it opens the possibility for use by diabetic and phenylketonuria patients and obese persons (Geuns 2003), and it is also capable of curing hypotensive, heart tonic actions (Ferri *et al.* 2006). This unique property makes stevioside in a huge demand in pharmaceutical, food and beverage industries, and further leads to the urgent demand for the large-scale production of *Stevia* plants (Puri *et al.* 2011). However, due to the continuous development of *Stevia* industry, releases of plant residues, the byproduct after sweetener extraction, to the environment are inevitable. *Stevia* residue, rich in organic matters, such as carbohydrates, amino acids, polyphenols and vitamins (WÖlwer-Rieck 2012), is the favourable substrate for micro-organism growth. Meanwhile, the water-based extraction method makes *Stevia* residue high moisture content. Therefore, the effluent with terrible odour, resulting from batching up, is a typical pollution source. The improper disposal of the effluent will definitely bring burden to environments. One of possible utilization methods is bioconversion of the wastewater into value-added biomass (Ponsano *et al.* 2008). That is to say, it is a double-edge sword. We can enrich some micro-organisms with commercial value using the effluent of *Stevia* residue.

Purple nonsulfur bacteria (PNSB) are typical purple phototrophic bacteria. They are ubiquitous distributed in oceans, rivers, lakes and soils (Kim *et al.* 2004). They

have a surprising metabolic versatility, such as photoautotrophy, photoheterotrophy and/or chemoheterotrophy under anaerobic-light or microaerobic-light conditions (Kantachote *et al.* 2005; Oda *et al.* 2008). Therefore, they can utilize various substrates especially small molecules organic compounds as sources of carbon and energy (Madukasi *et al.* 2010). Due to these properties, PNSB are frequently used to effectively treat organic wastewaters with high concentrations. During wastewater treatment, PNSB produce abundant physiologically active matters, such as pigments, proteins, vitamins, antimicrobial and therapeutic agents (Salma *et al.* 2007; Lu *et al.* 2011), all of which can be used as supplements in poultry, cultivation and medicine industry. For example, Getha *et al.* (1998) used sago-starch processing wastewater to cultivate *Rhodopseudomonas palustris* (*Rps. palustris*) with the aim of microbial biomass and carotenoid production. Lima *et al.* (2011) studied the heterotrophic metabolism of *Rubrivivax gelatinosus* in the fish industry effluent and found that the bacteria could remove organic pollutants in the wastewater. In addition, PNSB could secrete indole-3-acetic acid (IAA) in the presence of L-tryptophan (Mujahid *et al.* 2010), a typical phytohormone regulating various developmental and physiological processes in plants (Lee *et al.* 2008; Gamal-Eldin and Elbanna 2011).

In view of the properties of PNSB and the disposal problems of *Stevia* residue wastewater, two kinds of extractions of *Stevia* residue were used as the culture media in this study. One was extracted under 25°C for 4 h (RT), which was aimed to simulate the effluent of *Stevia* residue from batching up. The other was extracted under 95°C for 1 h (HT), which was conducted with the purpose of increasing the economic value of the *Stevia* residue. A purple nonsulfur bacterium, *Rps. palustris*, was enriched under RT or HT. Its growth profile and quality were characterized under RT and HT in comparison with those under the chemical synthetic medium. Repetitive sequence-based PCR (rep-PCR), which has been widely applied to classify and analyse the genetic distinctions of species within a genus, was further used to determine the genomic shifts of *Rps. palustris* under different treatments. This knowledge of the characteristics of PNSB grown under *Stevia* residue extractions would open a new avenue for the utilization of *Stevia* residue and the solution of the potential pollution source, which meanwhile is in favour of the downstream industry of PNSB application.

Results and discussion

Properties of *Stevia* residue extractions

Physicochemical data revealed that *Stevia* residue extractions had the abundance in organic matters and demonstrated their potentials for a biotechnological purpose (Table 1). Pairwise comparison revealed that concentrations of total organic carbon (TOC) and a majority of organic acids in HT were approximately twofolds of those in RT, whereas the contents of four-carbon-based organic acids (C_4) in RT, such as tartaric acid and malic acid, were much higher than those in HT. Meantime, it was noted that the contents of total N (TN) in RT and HT were extremely low, which could not satisfy microbial growth due to the improper ratio of carbon to nitrogen (Madukasi *et al.* 2010). Therefore, nitrogen source was added to the extractions with the aim to stimulate bacterial growth.

Bacterial growth

Purple nonsulfur bacterium could utilize organic components in wastewater as carbon sources in the presence of light (Chiemchaisri *et al.* 2007). The composition and amount of carbon sources available have a remarkable influence on the bacterial growth. In our investigation, the growth rates of *Rps. palustris* under *Stevia* residue extractions and the synthetic medium were shown in Fig. 1a, based on the optical density measurements at 660 nm. For all treatments, no lag phases were observed, and the absorption values significantly increased immediately. The bacterial growth rates under HT and RT were faster than that under the synthetic medium, and this variation was kept to the stationary phase. In detail, the stationary phase began on the 4th day with a mean absorption value around 0·6 under the synthetic medium and 1·2 under RT and HT. Although the contents of the TOC in HT or RT were close to that in synthetic medium (1 g l^{-1}), the compositions of carbon sources in *Stevia* residue extractions were substantially different from those of the synthetic medium. Specifically, a variety of low-molecular weight organic carbon sources were present in

Table 1 Properties of *Stevia* residue extractions (mg l^{-1})

Treatments	RT	HT
TOC	605·4 ± 208·2	1290·1 ± 112·5
TN	20·5 ± 2·8	31·0 ± 5·2
Reduced sugar	314·4 ± 30·5	319·2 ± 52·8
Organic acid		
Formic acid	5·0 ± 2·1	27·2 ± 6·2
Acetic acid	14·4 ± 2·4	20·2 ± 1·7
Oxalic acid	38·6 ± 8·8	54·3 ± 5·5
Tartaric acid	52·7 ± 6·7	29·5 ± 7·0
Malic acid	18·5 ± 2·9	8·5 ± 3·1
Ascorbic acid	10·7 ± 3·3	18·0 ± 2·0
Citric acid	4·4 ± 1·8	7·5 ± 1·2

Data represented mean ± standard deviation of three independent experiments.

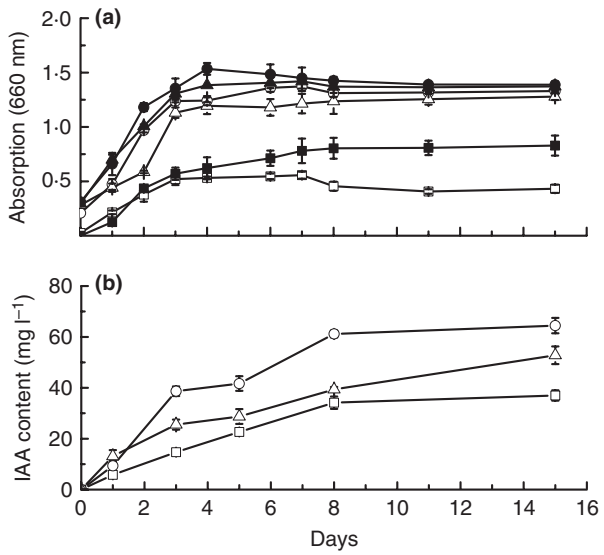

Figure 1 Growth characteristics (a) and IAA productions (b) of *Rhodopseudomonas palustris* under different substrates. (□) Syn + Trp, (○) RT + Trp, (△) HT + Trp, (■) Syn + NH_4Cl, (▲) RT + NH_4Cl, (●) HT + NH_4Cl.

Stevia residue extractions, whereas the single carbon source was present (L-malic acid) in the synthetic medium. Therefore, diverse carbon sources could promote *Rps. palustris* growth (Madigan and Jung 2009). In addition, the growth rate of *Rps. palustris* in RT was significantly faster than that in HT with the same nitrogen source throughout the growth periods, which might be due to the higher contents of C_4 compounds in RT than those in HT. As reported, C_4 compounds are more preferred by PNSB, and stimulate cell growth (Lee *et al.* 2011). Collectively, the *Stevia* residue extractions, especially RT, facilitated *Rps. palustris* growth.

Production of IAA by PNSB

Stevia residue extractions not only stimulated *Rps. palustris* growth, but also enhanced their capability of producing physiologically active compounds. Indole-3-acetic acid (IAA) is one of the major derivatives of L-tryptophan catabolism by PNSB, and its production is influenced by growth conditions (Rajasekhar *et al.* 1999). As shown in Fig. 1b, for all treatments, the increments of IAA contents were quite evident during initial 8 days of bacterial growth and obscure afterwards. The IAA productions under different culturing conditions varied from 37·0 mg l^{-1} to 64·4 mg l^{-1} during the whole incubation course, which was in the range of former literature at the same concentration of tryptophan (Koh and Song 2007).

Significant differences of IAA contents were observed among different treatments. In the stationary phase, RT + Trp ranked first followed by HT + Trp, and Syn + Trp was the last, which meant *Stevia* residue extractions could enhance the IAA production. Those could be explained by the fact that higher IAA contents could be achieved in the presence of other substrates such as glycine, glutamate and glucose (Rajasekhar *et al.* 1999). *Stevia* leaf contains abundant components, such as lipids, minerals and amino acids (Abou-Arab *et al.* 2010; Lemus-Mondaca *et al.* 2012), a portion of which may be dissolved in the *Stevia* residue extractions. Those components resulted in stimulated IAA production in *Stevia* residue extractions.

It is well known that tryptophan serves as a physiological precursor for IAA biosynthesis in plants and microorganisms (Prasanna *et al.* 2010). However, tryptophan is an expensive compound used in pharmaceutical formulations and feed stock modifications. Approaches should be explored to reduce the cost for IAA production as much as possible. In our investigation, the addition of tryptophan to *Stevia* residue extractions could enhance IAA production, which opens up a new avenue for higher IAA production, and therefore maximize the benefits.

Cell pigments

Typical absorption spectra for *Rps. palustris* grown under various culture substrates were presented in Fig. 2. In all cases, several typical absorption peaks around 375, 500, 590, 805 and 865 nm were observed. Those peaks occurred due to the blend of alternative spirilloxanthin series carotenoids and/or bacteriochlorophyll *a* (BChl *a*), the main photosynthetic pigments produced by phototrophic bacteria (Madigan *et al.* 2000). Those cell pigments can be directly or indirectly affected by variations in temperature, illumination, chemical compounds, metal ions, salts and solvents (Bhosale 2004; Ponsano *et al.* 2008). BChl *a* is the light-harvesting pigment and a component of the reaction centre complex in phototrophic

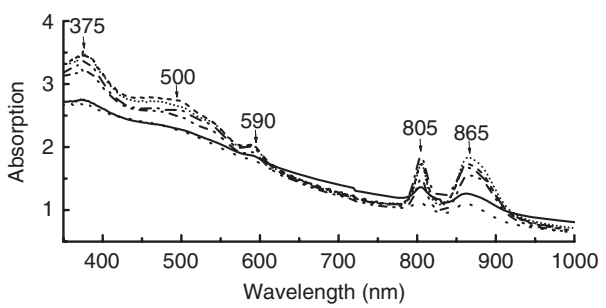

Figure 2 Absorption spectra of *Rhodopseudomonas palustris* under different substrates. (Solid line) Syn + Trp, (short dash line) RT + Trp, (dot line) HT + Trp, (dash line) Syn + NH_4Cl, (dash dot line) RT + NH_4Cl, (dash dot dot line) HT + NH_4Cl.

bacteria (Kolber *et al.* 2001). Carotenoids are produced with the aim of capturing excess of light and so making photosynthesis more effective and still protecting cells from injury (Lima *et al.* 2011). In our investigation, *Stevia* residue extractions could increase the productions of BChl *a* and carotenoids of *Rps. palustris* in comparison with the synthetic media. The enhanced cell pigments of *Rps. palustris* make the photoheterotrophic metabolism more vigorous, which may be the underlying mechanism for the increments of the cell biomass of *Rps. palustris* and IAA production in RT or HT.

Genotypic variability of *Rps. palustris* under different culture substrates

The variations in the phenotype might derive from their genomic shifts. Microbial genomes contain a variety of repetitive DNA sequences, accounting for up to 5% of the genome (Ussery *et al.* 2004). The rep-PCR technique uses primers targeting several of these repetitive elements to generate unique DNA profiles of individual microbe at the strain or isolate level (Ishii and Sadowsky 2009). Therefore, rep-PCR has been used to discriminate the shifts in *Rps. palustris* genome in response to different niches based on their high reproducibility of fragmentation patterns (Oda *et al.* 2002). For the same reason, rep-PCR was conducted in our investigation in order to elucidate the genomic variation of *Rps. palustris* under different substrates (Fig. 3). The detected bands number ranged from 10 to 14. The molecular sizes of those bands varied from 100 to 1000 base pairs, which was consistent with the former report (Larimer *et al.* 2004). Computer-assisted cluster analysis further revealed that *Rps. palustris* with different nitrogen sources exhibited the lowest *r* value of 0·59, suggesting that nitrogen sources had a predominant influence on the genotype of the cultures. Different carbon sources also resulted in genotypic variations of *Rps. palustris*. For instance, the genotypic profiles of *Rps. palustris* under RT + Trp differed markedly from those under Syn + Trp and HT + Trp with *r* value of 0·66, while the obvious discrimination with *r* value of 0·68 was observed between the synthetic media and residue extractions supplemented with NH_4Cl. As reported, genomic fingerprints having *r* values of over 0·8 were considered to be the same genotype (Oda *et al.* 2003). Therefore, the genotypes of *Rps. palustris* were different under different carbon or nitrogen sources. Although DNA fingerprint patterns of rep-PCR are thought to be stable over many generations of microbial growth, they are susceptible to change over time by polymorphisms, rearrangement of indigenous plasmids, recombination between repeated sequences and introgression of genomic DNA via horizontal gene transfer (Ishii and Sadowsky 2009). In our investigation, the differences in genomic fingerprint patterns among genotypes were accompanied by differences in phenotypic characteristics such as bacterial growth, cell pigments and IAA production, which implied that the shift in genomic structure of *Rps. palustris* in response to different substrates might result in the variations of its phenotypes.

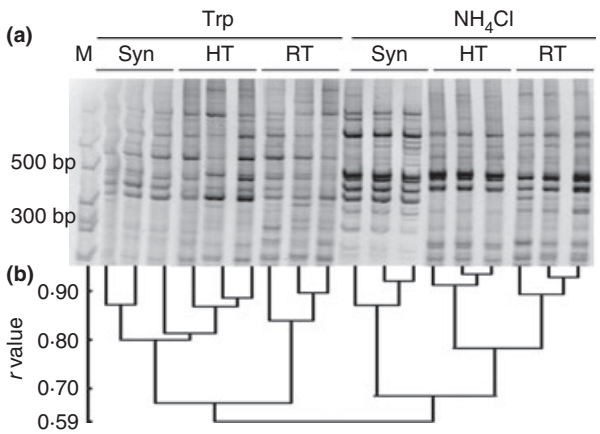

Figure 3 (a) Rep-PCR fingerprinting profiles of *Rhodopseudomonas palustris* grown under different culture media. Sample designations were indicated above each rep-PCR lane. M, 100-bp marker and (b) Cluster analysis of rep-PCR fingerprinting profiles.

Biotechnologically, the use of *Stevia* residue extractions or its effluent as substrates for micro-organism growth is a feasible and economic method to explore the potential advantages of the wastewater. Compared to HT, RT costs lower energy, but has more positive effects on PNSB growth and phytohormone production. These phenomena may result from variations in *Rps. palustris* genotype in response to different nutrients in *Stevia* residue extractions, due to its metabolic diversity. Based on these promising results, further investigations are needed for applying PNSB grown under *Stevia* residue extractions to poultry or farming in field scale to investigate its effects on the downstream industry. In short, the research presented here not only opens a new avenue for the utilization of *Stevia* residue, but also is in favour of the downstream part of PNSB application.

Materials and methods

Test strain

Rhodopseudomonas palustris, a purple nonsulfur bacterium, was isolated by our laboratory and used in the subsequent culture.

Water extractions of *Stevia* residue

Stevia residue, a waste after sweeteners extraction process using a water-based system, was donated by Zhucheng

Haotian Pharm. Co., Ltd. (Shandong, China). After air-dried, *Stevia* residue was cut into particles of size 1–2 mm for liquid extraction.

Two extracting methods, either under room temperature (RT) or hot water (HT), were conducted to simulate the effluent from *Stevia* residue. Specifically, *Stevia* residue was rinsed in deionized water at a ratio of 1:20 and shaken at 200 rpm at 25°C for 4 h (RT) or bathed in boiling water at 95°C for 1 h (HT), respectively. The filtered fluids were autoclaved at 115°C for 20 min and then cooled for further use.

The total nitrogen and total organic carbon were determined using a TOC-TN analyzer (Skalar, the Netherlands). The low-molecular-weight organic acids were analysed according to Mato *et al.* (2005) using a high-performance liquid chromatography (Shimadzu Co., Kyoto, Japan).

Culture media

To evaluate the growth characteristics of *Rps. palustris* under different conditions, six treatments were conducted. When 7 mmol l^{-1} of ammonium chloride (NH_4Cl) was served as a nitrogen source, three treatments were carried out as follows: (i) chemical synthetic medium with NH_4Cl (Syn + NH_4Cl), (ii) the extraction of *Stevia* residue under room temperature supplemented with NH_4Cl (RT + NH_4Cl) and (iii) the extraction of *Stevia* residue under 95°C supplemented with NH_4Cl (HT + NH_4Cl). For 3 mmol l^{-1} tryptophan was served as the nitrogen source, three treatments were carried out as below: (i) chemical synthetic medium with tryptophan (Syn + Trp), (ii) the extraction of *Stevia* residue under room temperature supplemented with tryptophan (RT + Trp) and (iii) the extraction of *Stevia* residue under 95°C supplemented with tryptophan (HT + Trp). The chemical synthetic medium is comprised of 4 g D, L-malic acid, 1 g NH_4Cl, 7·5 ml phosphate buffer (0·2 mol l^{-1}, pH 7·0), 0·2 g $MgSO_4{\cdot}7H_2O$, 0·075 g $CaCl_2{\cdot}2H_2O$, 0·0118 g $FeSO_4{\cdot}7H_2O$, 0·04 g EDTA, 1 ml trace element solution (g l^{-1}, $MnSO_4{\cdot}4H_2O$ 2·1, H_3BO_3 2·8, $Cu(NO_3)_2{\cdot}7H_2O$ 0·04, $ZnSO_4{\cdot}7H_2O$ 0·24, $Na_2MoO_4{\cdot}2H_2O$ 0·75), 1 ml vitamin solution (g l^{-1}, nicotinic acid 10, vitamin B_1 5, biotin 0·1) per litre (Li *et al.* 2011). The pH of the medium was adjusted to 6·8 before autoclaving at 115°C for 20 min. All treatments were conducted in three triplicates.

Inoculum and culture condition

A 5% (v/v) culture was used as inoculum, which was aseptically transferred to the 100 ml of *Stevia* residue extractions or chemical synthetic medium inside sterilized flasks. *Rps. palustris* were cultured anaerobically at 30°C under continuous illumination with incandescent lamps at a light intensity of about 2000 lux for 4 weeks under different culture substrates.

Bacterial growth and cell pigments

The growth of *Rps. palustris* was measured by monitoring the optical density at 660 nm with a spectrophotometer during the whole incubations.

Bacteriochlorophyll *a* (BChl *a*) and carotenoids was spectrophotometrically assayed on the 8th day of the incubation. Two millilitres of the phototrophic bacterial culture were centrifuged at 10 000 ***g*** for 15 min. The cell pellets were resuspended in 60% sucrose (Pfennig 1969). Scans were performed on a Shimadzu UV-3600 UV-VIS spectrophotometer (Shimadzu Co.).

Production of indole 3-acetic acid (IAA)

Indole-3-acetic acid (IAA) contents in the culture supernatants were determined with the Salkowski's reagent (Glickmann and Dessaux 1995). Bacterial cells in samples collected were removed from the culture medium by centrifugation at 10 000 ***g*** for 15 min. A 1-ml aliquot of supernatant was mixed with 4 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7·5 ml of 0·5 mol l^{-1} $FeCl_3{\cdot}6H_2O$) and incubated at 25°C for 20 min. The red colour resulting from the chemical reaction was quantified at 535 nm. A standard curve was prepared from serial dilution of a 100 mg l^{-1} IAA (Sigma-Aldrich Co., St. Louis, MO, USA) stock solution in ethanol.

DNA isolation and rep-PCR fingerprinting

The bacterial cells after 4 weeks culture in culture medium were collected by centrifuging at 10 000 ***g*** for 15 min. DNA was isolated using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-Tek, Frederick, CO, USA) following the manufacturer's instruction. Rep-PCR reactions were performed according to Versalovic *et al.* (1994) with the primer set of BOX A1R (5′-CTACGGCAAGGCGACGCTGACG-3′). Amplification was performed in a 25 μl reaction volume, typically containing 50 ng genomic DNA template, 2·5 μl 10× PCR buffer (Mg^{2+} plus), 2 μl dNTPs mixture (each 2·5 mmol l^{-1}), 1 μl BOX A1R primer (20 μmol l^{-1}), 0·125 μl *Taq* HS (5 U μl^{-1}, TaKaRa) and 0·5 μl Bovine Serum Albumin (20 g l^{-1}). The PCR cycling conditions were as follows: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 94°C for 3 s, annealing at 50°C for 1 min and extension at 65°C for 8 min; and a final extension step at 65°C for 8 min. The molecular sizes of the amplified DNA

fragments were estimated by comparison to a 100-bp DNA ladder. PCR products were separated by electrophoresis at 50 V on a 2% (w/v) agarose.

Statistical analysis

The gel was visualized and photographed under Gel Doc™ EQ Imager (Bio-Rad, Hercules, CA, USA). Gel image obtained from rep-PCR was analysed using Quantity One software (Bio-Rad). Computer-assisted analysis of the genomic fingerprints was performed by using Quantity One software (Bio-Rad). Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages. Rep-PCR fingerprint patterns having *r* values of more than 0·8 were considered to be the same genotype (Oda *et al.* 2003).

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