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The degradation of coniferyl alcohol and the complementary production of chlorogenic acids in the growth culture of *Streptomyces albogriseolus* KF977548 isolated from decaying wood residues

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ABSTRACT

Coniferyl alcohol is one of the major precursors of lignin; the most abundant aromatic compound and a natural resource currently receiving attention because of the value-added metabolites resulting from its degradation. Growth study of *Streptomyces albogriseolus* KF977548 (strain AOB) isolated from decaying wood residues in a tropical estuarine ecosystem was carried out using coniferyl alcohol as a sole carbon source. Cell growth and metabolite production were monitored at 24 h interval by dry weight measurements and HPLC, LC–MS-DAD analyses. Biochemical and PCR assays were carried out to detect the major catabolic enzymes of interest. Strain AOB utilized coniferyl alcohol completely within 72 h ($\mu = 0.204\ h^{-1}$, $T_d = 3.4$ h). Laccase and peroxidase were released into the growth medium up to 0.099 and 98 μmol/mL respectively. Protocatechuate 3, 4-dioxygenase and demethylase were detected in the genome whilst *ortho*-adipate pathway was clearly indicated. Growth on coniferyl alcohol or caffeic acid as mono substrates resulted in the production of secondary metabolites identified by HPLC–MS as 1-caffeoylquinic and 3,4,5-tricaffeoylquinic acids, known as chlorogenic acids, in the culture medium. The microbial production of chlorogenic acids from a lignin-related substrate base by strain AOB could arouse a plausible biotechnological process.

1. Introduction

Lignin, largely responsible for the strength and rigidity of plant cells is generated from three precursors, namely; coumaryl, coniferyl and sinapyl alcohols. A typical soft wood (gymnosperm) contains building blocks originating predominantly from coniferyl alcohol [1,2]. In nature, the most abundant aromatic compound is lignin. Attention is currently drawn to lignin as a natural resource because its decomposition gives rise to value-added products that have many applications in the food, pharmaceutical, cosmetics and other chemical industries. According to Kirby [3], it is considerably difficult to investigate lignin degradation, due to its polymeric nature and intramolecular bonding pattern. Hence, the sequence of its degradation reaction is controversial. It was initially assumed that the breakdown of lignin in nature is by the activities of the white rot fungi and subsequent mineralization of the breakdown products by soil bacteria. Although some microorganisms are not capable of utilizing phenolic acids due to their toxic effects, however, a number of literature reports exist on the bacterial degradation of lignin, lignin-related aromatic compounds and lignin-derived fragments [4–6]. Lignin-degrading bacterial strains which fall into three classes (actinomycetes, α-proteobacteria and γ-proteobacteria) were identified by Bugg et al. [7].

Because of its recalcitrant nature, lignin requires many enzymes each with specific active sites for degradation. Most aromatic compounds are degraded by side chain shortening to yield protocatechuic acid or catechol which is further broken down via specific ring cleavage pathways. For example, *Pseudomonas, Acinetobacter*, *Rhodococcus* and *Streptomyces* species have been found to utilize the β-ketoadipate pathway, initiated by intradiol catechol dioxygenase and protocatechuate 3, 4-dioxygenase [8–11]. *Streptomyces* species

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are known to be capable of breaking down the chemical bonds holding lignin together because of their ability to synthesize the required degradative enzymes [3]. Ramachandra et al. [12] reported the characterization of an extracellular peroxidase produced by *Streptomyces viridosporus*, while the screening of actinomycetes for extracellular peroxidase production was reported by Mercer et al. [13]. Reports are available also on bacterial laccases which are copper-containing enzymes that require oxygen to oxidize a range of phenolic compounds. It was suggested by Sanchez [14] that the bulky nature and presence of non-phenolic subunits prohibits the action of laccases on the lignin polymer; however, these enzymes have been shown to depolymerize lignin via the oxidation of smaller molecules such as 2, 2-azo-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and hydroxybenzo-thiazoline (HBT).

Researches on natural sources of pharmaceutical and medicinal products are currently being intensified because of the adverse effects of synthetic products on the health of humans, animals and the environment [15]. Hydroxycinnamates (caffeic, *p*-coumaric, ferulic and sinapic acids) are mainly produced in plants such as vegetables, flowers, fruits, potatoes and large amounts exist in coffee beans where they have been found to conjugate with other compounds [16–18]. The conjugation of caffeic acid and quinic acid yield chlorogenic acid, a family of esters usually formed between hydroxycinnamic acids and quinic acid. Chlorogenic acids have been suggested to perform several important physiological and biological functions, most of which are considered to be of health benefits to humans including the prevention of cardiovascular disease and protection against side effects of chemotherapy and anti-osteoclastic activity [18]. They were found to have antibacterial activity by increasing the membrane permeability [19]. They have anti-viral properties [20], anti-HIV and anti-ageing activities [17]; they are anticancer agents [21] and natural antioxidants, which reduce obesity by inducing fat loss and also reduce the proliferation of new fat [22,23]. Other functions of chlorogenic acids include lowering of blood pressure and exertion of anti-diabetic effect through their action as an alpha-glucosidase inhibitor which reduces the rate or amount of carbohydrate uptake [24].

There have been increasing numbers of actinomycetes with biopharmaceutical potentials being isolated from marine habitats. This is probably because the environmental conditions of the ocean differ greatly from terrestrial conditions [25]. The discovery of novel marine lignin-degrading bacteria or their enzymes provide an advantage due to their ability to survive extreme environmental conditions and amenability to genetic manipulation.

Several authors reported new *Streptomyces* species shown to produce biologically-active compounds [26–28], but as suggested by mathematical modelling, well over 10^5 antibiotics are yet to be discovered from actinomycetes. According to Berdy [29], the actinomycetes are amongst the most valuable prokaryotes due to their ability to produce numerous bioactive compounds, notably antibiotics [30], Immunosuppresive agents [31] and antitumor agents [32]. One of the most dynamic and highly productive ecosystems in nature is the estuary. Microbial communities that have the abilities to produce varieties of complex enzymes may occur within this ecosystem. The estuarine microorganisms have developed unique metabolic and physiological functions that enable them to survive in extreme environmental conditions. In addition, estuarine microorganisms have the potential for the production of novel bioactive metabolites for exploitation. Moreover, estuarine microorganisms are yet to be extensively exploited as their counterparts in the terrestrial environment [33].

In view of the above, we examined the ability of *S. albogriseolus* strain AOB, a wood-digesting actinomycete isolated from the tropical estuarine environment, to breakdown coniferyl alcohol; with a view to characterizing the metabolic products of interest, and hence the detection of chlorogenic acids among other metabolites produced in growth cultures. The significance of this finding from the perspectives of pharmaceutical applications and industrial processing is hereby discussed in this paper, in view of the rarity of the possible production of chlorogenic acids from microbial sources, which is also not corroborated by any existing literature.

2. Materials and methods

2.1. Chemicals and standards

All chemicals and reagents used in this study, including coniferyl alcohol, ferulic, caffeic, vanillic, 2, 5-dihydroxybenzoic, protocatechuic, 3,4-dimethoxybenzoic,1- caffeoylquinic and 4-hydroxybenzoic acids; including HPLC-grade formic acid and acetonitrile were purchased from Sigma – Aldrich Chemical Co. (Saint Louis, MO, USA) except otherwise stated. Water for dilution (HPLC grade) was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA, USA)

2.2. Isolation of the microbial strain

Streptomyces albogriseolus strain AOB KF977548 was isolated from decomposing wood residues in a tropical estuarine ecosystem in Lagos, Nigeria. Isolation, screening and identification of this strain for ligninolytic activity have been previously described in detail [34]. After screening, this strain showed the capability to break down vanillic and veratric acids and hence, it was selected for this study. It was identified previously on the basis of morphological and biochemical characteristics, electron microscopy and 16S rRNA gene sequencing. The Phylogenetic analysis of the strain with other lignocellulolytic bacterial strains was described and discussed previously [35].

2.3. Bacterial growth studies on coniferyl alcohol and lignin-related aromatic acids

Growth study was carried out using a mineral salts medium (100 ml) in deionized water containing (gram per liter): KH_2PO_4, 2.0; K_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.05, yeast extract 0.1 g, $MnCl{\cdot}7H_2O$, 0.0003; $ZnCl{\cdot}7H_2O$, 0.0003 and KNO_3 2.0; pH 7.2, in 250 ml conical flasks supplemented with 0.1 g coniferyl alcohol as the sole carbon source. After autoclaving (121 °C,15 min) and cooling, the medium was seeded with 1.0 ml of 48 h old pure culture of *Streptomyces albogriseolus* strain AOB. Incubation was done on a rotary shaker (30 °C,150 rpm) for 7 days. Batch harvesting was done at interval (24 h). Growth of the organism was monitored by the dry weight method as described earlier [36] because of the mycelial nature of the organism. Growth experiment was carried out in triplicates and results were generated by computing the average. Similarly, to determine the metabolic pathway for the degradation of coniferyl alcohol by this strain; sequential growth studies were carried out in different conical flasks using 0.1 g each of lignin-related aromatic compounds (protocatechuic, 2,5-dihydroxybenzoic, 4-hydroxybenzoic, vanillic, *trans*-cinnamic, caffeic and ferulic acids) as sole carbon sources.

2.4. Analysis of growth kinetic data

Mean generation times (T_d) and the specific growth rate (μ) of the isolates on coniferyl alcohol was computed with non-linear regression of growth curves using graph pad software prism version 6. Briefly, an "XY 'data table was created. Time (days) was entered into "X', and response (cell biomass) into 'Y'- axis respectively. Analysis was carried out by clicking "analyse", choosing the nonlinear regression, panel of exponential equations and exponential growth respectively. The parameter Y0 (value at time

zero) was constrained to a constant value by going to the constrain tab of the nonlinear regression dialog, setting the drop down next to Y0 to "constant equal to". The values were entered. The model Y = Y0*exp(k*X) was used; where Y0 is the Y value when X (time) is zero, K is the rate constant, expressed in reciprocal of the X axis time units. Doubling time was computed as ln 2/K as explained in detail (http://www.graphpad.com/guides/prism/6/curve-fitting/index.htm?reg_exponential_growth.htm)

2.5. Quantitative and qualitative assessment of degradation of coniferyl alcohol and other metabolites in culture broths

The reduction in the concentration of coniferyl alcohol and other lignin-related compounds (standards) or the release of metabolic products of degradation was monitored using a Liquid Chromatograph-Mass Spectrophotometer (LC–MS), coupled with a Diode Array Detector- Electrospray Ionization (DAD-ESI); following a standardized profiling, as previously described (Lin and Harnly [37]; Plazonic et al. [38]). Standard curves were prepared from stock solutions (1.0 g/L) of coniferyl alcohol and other compounds (protocatechuic, vanillic, 4- hydroxyl benzoic, ferulic, 3,4- dimethoxybenzoic acid, 2,5- dihydroxybenzoic, caffeic, and 1- caffeoylquinic acids). Dilutions in series were made for each compound, resulting in good standard curves with regression coefficients of between 0.997 and 0.999 for each compound. At intervals (24 h), 1.0 ml of the growth medium (described earlier in 2.4 above) was withdrawn and centrifuged (10,000 x *g* for 5 min). It was filtered (2.0 μm filter, Gelman, Ann Arbor, MI, USA) into well-labeled vials. The vials were then loaded into an Agilent 5973 network mass selective detector with an Agilent Technologies 7683 B series injector (Agilent Technologies, Santa Clara, USA). The column was a 250 mm × 4.6 mm, (5 μm) symmetry C18 column (Waters Corp., Milford, MA, USA) with a 20 mm × 3.9 mm i.d., 5 μm sentry guard column. The mobile phase for elution consists of two solvents; A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile, with a flow rate of 0.8 ml/min at a column temperature of 45 °C. The HPLC–MS was used in combination with a photo DAD, electrochemical detector (Waters 2465) with a Waters 2695 separation module and waters TM 474 scanning fluorescence detector (Waters Corp., Milford, MA, USA). The DAD was set to monitor the peak intensity and full spectra (190–650 nm). Concentrations of metabolic products were calculated using the calibration curves plotted with the series of HPLC – grade phenolic compound standards individually injected into HPLC-DAD system. The MS data was collected in the total ion counting (TIC) and also the selective ionization mode using the electrospray ionization (negative and positive), at excitation energy and fragmentation voltages of 25EV and 35 KV respectively. Diode array spectra of studied chromatographic peaks were obtained and compared with those of the standards and to the reference spectra of phenolic acids in the library.

2.6. PCR-amplification of protocatechuate 3, 4-dioxygenase and demethylase genes

The catabolic gene clusters for 3, 4-dioxygenase of the strain was identified by PCR using Streptomycete-specific 16S rDNA primers. Non-degenerate forward primers; DOF (GCC GAG CAC GCG ACG TAC GAG AAG C) and reverse primers; DOR (ACG TGT CGA T GG TCG TCA TGG C), were prepared by integrated DNA technologies (IDT) based on the protocol of Iwagami et al. [39]. The PCR kit was obtained from Qiagen, Alameda, CA. The mixture contained × 10 buffer (15 mM, 2.5 μl), 25 mM $MgCl_2$ (2.5 μl), 0.5 μl 10 mM deoxynucleoside triphosphates (Biolabs Inc), 1.0 μl (46 ng) of template DNA, 0.875 μl each of DOF and DOR primers, and 0.25 μl of Taq DNA polymerase. PCR was performed in a DNA T gradient thermocycler (www.biometra.com/USA) under the following PCR conditions: The initial denaturation was 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 1 min; annealing temperature of 50 °C for 1 min. Elongation was at 72 °C for 1.5 min and a final extension for 10 min at 72 °C. Demethylase gene detection in the strain was based on the protocol of Chow et al. [40]. The forward primer was vdcC. F 20 (GGCGACGCCGCCTGAAGTCC), reverse primer was vdcC. R 20 (GGGTCGGTCGGTGTCAGACG). The PCR conditions were the same as above except for the annealing temperature (54 °C)

Electrophoresis was carried out on 200 ml agarose gel which was run for 140 min at 100 V after loading the well with 150 μl of the mix with the loading dye. The marker (ladder) used was 150–2100 bp. *Escherichia coli* DNA was used as a positive control, while Millipore water (blank) as a negative control. The amplified products were observed under the Kodak fluorescent imaging equipment, model IS 4000R (Kodak Image Station, Care Stream Molecular Imaging Health inc. Rochester, NY, USA). Sequencing was carried out using the dye terminator cycle capillary sequencing (ABI 3100 × 1) at the Molecular and Cellular Imaging Centre (MCIC), Ohio Agricultural Research Development Centre (OARDC), Ohio State University, Wooster, Ohio, USA. BLAST or BLAST X algorithm was used to identify similarity to other sequences in the GenBank.

2.7. Enzyme assays

Laccase activity was assayed in the Department of Animal Science (Biotechnology and Fermentation Group) laboratory at the Ohio State University, OARDC, Wooster, Ohio, USA using culture filtrates obtained at intervals (24 h) from the culture flask containing vanillic acid. An amount (2.0 ml) was centrifuged (10,000 × *g*, at 4 °C, for 10 min). The supernatant was used as the source of enzyme. Laccase activity was measured as described earlier [41] by monitoring the oxidation of 2,2–azino-bis-[3-ethyl benzothiazoine-6-sulfonic acid] (ABTS, 500 μmol) using a UV–vis Spectrophotometer (Beckman Coulter, DUR 800, Beckman Coulter Inc. Fullerton, CA) at 420 nm for 1 min. The reaction mixture (3 ml) contained; culture supernatant (1.0 ml), ABTS (1.0 ml) and 1.0 ml phosphate buffer (0.2 M, pH 7.5). One unit of enzyme activity was defined as 1 mM of ABTS oxidized per min. (The extinction coefficient = 3.6×10^4/mol/cm).

Extracellular lignin peroxidase (Lip) activity was assayed as described earlier [42] using 2, 4-dichlorophenol (2, 4-DCP, Sigma –Aldrich Co, St. Louis, MO, USA) as the substrate. The reaction mixture (1.0 ml) contained 200 μl each of potassium phosphate buffer (0.1 M, pH 7.0), 2, 4-DCP (25 mM), 4-amino antipyrine (16 mM) and culture filtrate. The reaction was initiated by the addition of 200 μl of 50 mM hydrogen peroxide (H_2O_2) and was monitored for 1 min at a wave length of 510 nm using a spectrophotometer (Beckman Coulter, DUR 800, Beckman Coulter Inc. Fullerton, CA). An absorption coefficient of 21.647 M^{-1} cm^{-1} was used for the calculation of enzyme activity.

The presence of intradiol ring cleavage dioxygenase activity in crude cell extracts was screened using the colorimetric Rothera reaction as described earlier [43]. The presence of the *ortho* pathway intermediate (β-ketoadipate) was indicated by the development of a deep purple color.

3. Results and discussion

3.1. Growth of strain AOB on coniferyl alcohol and other lignin—related substrates

Streptomyces albogriseus (strain AOB), used in this study demonstrated the ability to utilize coniferyl alcohol completely within 72 h with specific growth rate of 0.204 h^{-1} and a doubling time

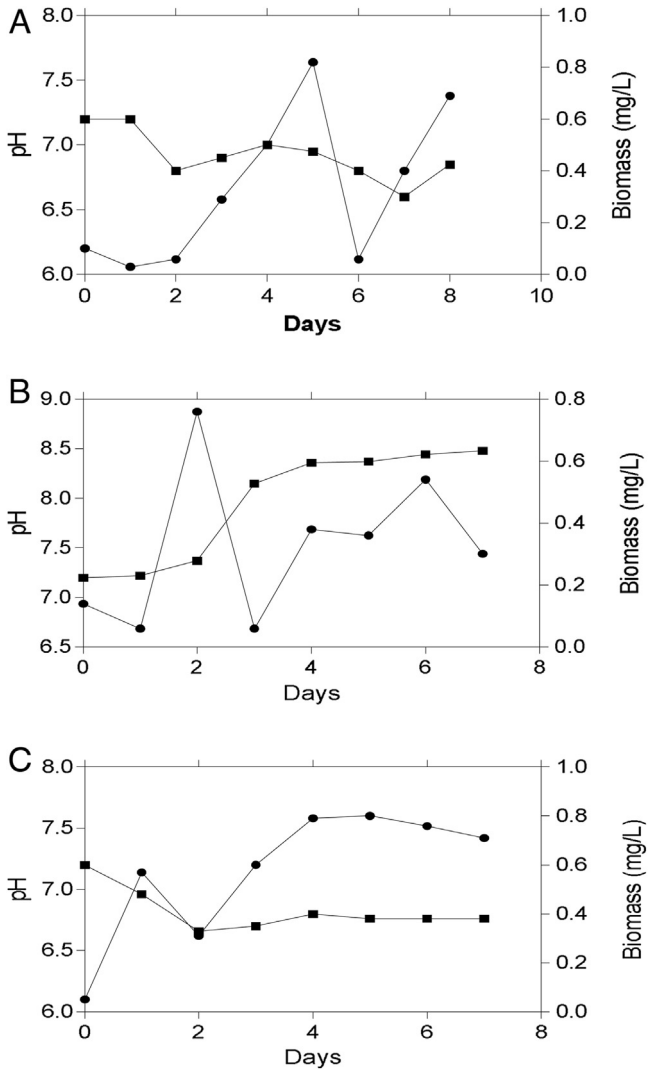

Fig. 1. Growth profiles and pH changes in the culture medium of *Streptomyces albogriseolus* strain AOB.
A = Growth on coniferyl alcohol (SD: pH = 0.170; biomass = 0.302).
B = Growth on ferulic acid (SD: pH = 1.45; biomass = 0.268.
C = Growth on vanillic acid (SD: pH = 1.14, biomass = 0.303.
SD = Standard deviation (details for the calculations can be found in supplementary Tables 2–7).
Biomass = ● pH = ■.

of 3.4 h to attain a maximum biomass of 9.0 mg/ L. The typical diauxic growth patterns with an observed lag phase of about 24 h was exhibited by the organism while utilizing coniferyl alcohol and ferulic acid as growth substrates (Fig. 1A and B respectively); suggesting the abililty of the organism to break down the two compounds into smaller fragments. The sharp drop in biomass by day 6 and 3 for coniferyl alcohol and ferulic acid respectively, is most likely to be an indication that new substrates are released into the medium requiring physiological adaptation. This suggestion is further strengthened by a pronounced drop in pH by day 7 in the case of coniferyl alcohol and a progressive increase in the pH of the medium as observed with ferulic acid. A sequential growth study of strain AOB on other lignin-related aromatic metabolites showed that the organism utilized 100, 38, 100, 100, and 28% of protocatechuic, 2, 5-dihydroxybenzoic, vanillic, 4-hydroxybenzoic and ferulic acids respectively. *Streptomyces albogriseolus* strain AOB completely utilized coniferyl alcohol as a substrate, but the sequential growth study on other lignin-related phenolic compounds showed that the strain preferentially utilized different substrates.

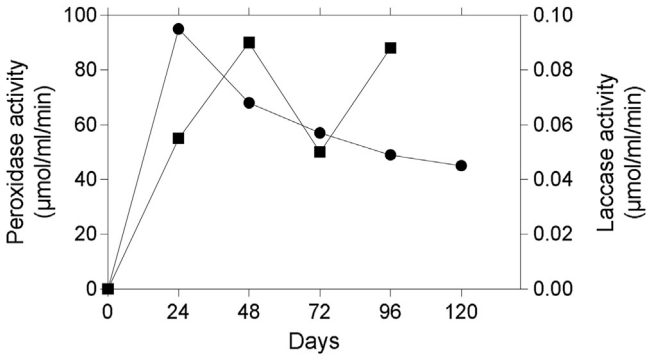

Fig. 2. Time-course of Laccase (●) and peroxidase (■) activities of *Streptomyces albogriseolus* strain AOB grown on vanillic acid.
SD: Laccase = 0.037; peroxidase = 36.58.
SD = Standard deviation (details for the calculations can be found in supplementary Tables 8 and 9).

The reasons for varied preferences for different substrates by different microorganisms have been well established by Kirby [3] and Nishimura et al. [11]. The authors opined that most isolates will preferentially metabolize the less complex compounds within a mixture of substrates. However, strain AOB showed greater preference for vanillic acid than 2, 5-dihydroxybenzoic acid even though the former is more complex than the latter. A plausible reason is that strain AOB was able to produce the requisite enzymes for metabolizing the compound. The incubation period and culture conditions such as pH and temperature could be other reasons responsible for substrate preferences by microorganisms. Strain AOB completely utilized protocatechuic acid, a counterpart of 2, 5-dihydroxybenzoic acid which is usually metabolized via the β-Ketoadipate route, leading to the TCA cycle. This might be an indication that strain AOB prefers to go through the protocatechuic acid pathway rather than the gentisic acid (2, 5-dihydroxybenzoic acid) pathway. The organism did not exhibit any visible lag phase during its growth on vanillic acid. There appeared to be a slight drop in biomass from 0.58 to 0.32 mg/L by day 2, reaching maximal biomass level of 0.78 mg/L within four days after which the growth appeared to have reached its declining phase as shown in Fig. 1C.

3.2. Enzyme detection and assay

The presumption that strain AOB used in this study utilized the protocatechuate pathway for aromatic acid metabolism is further strengthened by the detection of protocatechuate 3, 4- dioxygenase gene cluster in its genome. Gel electrophoresis of amplified PCR products of target genes in the organism revealed the presence of protocatechuate 3, 4-dioxygenase and demethylase gene clusters which correspond to 800 and 1480 bp respectively (supplementary Fig. 1A and B). Sequencing of amplified DNA fragments and the subsequent analysis using the BLAST software showed that *Streptomyces albogriseolus* strain AOB possessed the protocatechuic acid catabolic gene cluster (protocatechuate 3, 4-dioxygenase) with 88% similarity to that of *Streptomyces* sp. Strain 2065 with the GenBank accession number AF-10938662. The demethylase gene sequence had 80% identity with *Streptomyces coelicolor* A_3 (2) with the reference number AL-939111.1; most likely to be a branched chain amino acid binding protein. Furthermore, strain AOB showed the ability to produce laccase and peroxidase activities. As shown in Fig. 2, the highest laccase activity (0.099 µmol/mL/min) occurred within 24 h after which there was a gradual drop of activity till the termination of the assay. The peroxidase activity fluctuated throughout the assay period, reaching the highest value of 98 µmol/mL/min within 24 h. In his study on the degradation of lignin by actinomycetes, Kirby [3] reported that the two major

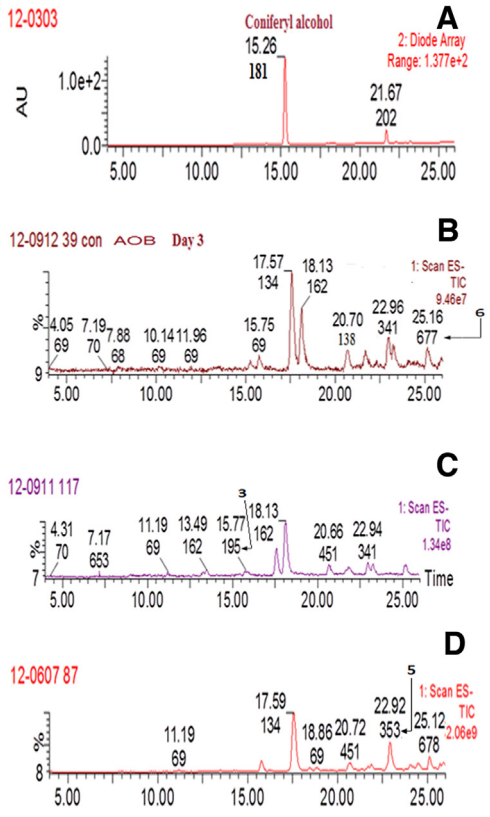

Fig. 3. Chromatograms showing the metabolites in the culture medium of *S. albogriseolus* strain AOB, during the degradation of coniferyl alcohol. Day 0 (A), Day 3 (B, C, and D).
Arrows: 3 (ferulic acid), 5 (1-caffeoylquinic acid), 6 (3, 4, 5-Tricaffeoylquinic acid).

groups of enzymes involved with lignin degradation are peroxidases and laccases. Undoubtedly, strain AOB exhibited enzymatic degradation of coniferyl alcohol by oxidizing this substrate. In their study on the degradation and detoxification of pulp paper mill effluent (PPME) containing lignin by mixed bacterial strains, Chandra et al. [44] noted that degradation of the compound and induction of the enzymes involved with its degradation is a simultaneous process which supports each other. Several authors also reported the presence of these enzymes; capable of degrading aromatic compounds in bacterial species [39,11,45].

3.3. Identification of metabolic products of degradation

Chromatograms for the metabolites detected in the culture fluid of *Streptomyces. albogriseolus* strain AOB using the total ionization counting (TIC) and the selective ionization (ESI^- & ESI^+) mode are shown in Fig. 3. Most of the identified peaks were achieved in the TIC and ESI^- mode. The retention time, molecular weight, chemical formula and wavelength of some of the major peaks identified are listed in Supplementary Table 1A. Positive identification of the compounds in Table 1A was made based on a comparison of retention times, ESI, UV/Vis and mass spectra of standards; except for 3,4,5-tricaffeoyiquinic acid which was confirmed from the reference HPLC–MS library and/literature [17,46–48]. At day 0 (Fig. 3A), The major peak which appeared with an *m*/*z* of 181 at a retention time of 15.26 min, detected by a diode array is coniferyl alcohol, the growth substrate, when compared with the *m*/*z*, UV/Vis absorption wavelength of the standard, although the retention time of the standard was 15.30. By day 3, the peak corresponding to coniferyl alcohol had disappeared, suggesting that it has been completely broken down by the organism into other fragments (Fig. 3B). From the chromatograms, 3, 4, 5- Tricaffeoylquinic acid (*m*/*z* = 677/678) was identified as one of the products released into the growth medium. Two of the major peaks (162 and 341) with retention times of 18.13 and 22.96, UV/Vis absorptions at 300 and 325 nm; which were observed to be persistent in the replicate chromatograms could not be identified. However, it has been surmised that radical coupling reaction of coniferyl alcohol may also occur since laccase or peroxidase activities were detected and it is well known that enzymatic actions by laccase or peroxidase on coniferyl alcohol produce phenoxy radical that couples each other forming dimers or oligomers. Hence, higher molecular weight intermediate (*m*/*z* 341) may be a fragment from a dimer following a loss of water and *m*/*z* 162 also maybe a dehydrate fragment of coniferyl alcohol. Since triplicate samples were used, another sample, which was harvested by day 3 showed a compound with an *m*/*z* of 195. Based on the retention time, ESI, UV/Vis absorption of the standard and the LC–MS library, it was identified as ferulic acid; a major degradation product of coniferyl alcohol with a known molecular mass of 194 (Fig. 3C). Another compound with an *m*/*z* of 353 and a retention time of 22.92 (Fig. 3D) was identified in the third sample as 1-caffeoylquinic acid. It was observed that some of the metabolites were consistently detected in the triplicate culture samples harvested on day 3 of the growth studies.

Although coniferyl alcohol was rapidly degraded and completely disappeared from the medium in less than 72 h, leading to the generation of ferulic acid as mentioned earlier, coniferylaldehyde, the generally known immediate metabolic product of coniferyl alcohol degradation was not observed in any of the chromatograms; but it was detected by the mass spectra as one of the aglycones or diagnostic fragments of coniferyl alcohol (data not shown). It has an *m*/*z* of 177 with a retention time of 18.07 min and UV/Vis absorption of 340, ca, 300 sh, 236. However, from literature, the molecular weight of coniferylaldehyde is 178.185 g/mol). Also, vanillic acid, a key intermediate product in the conversion of ferulic acid to protocatechuic acid was not detected in the culture fluid; this is consistent with the findings of Nishimura et al. [11], the formation of vanillic acid is most likely to be transient. However, during the sequential growth study when vanillic acid was used as the growth substrate; it was mineralized to yield protocatechuic acid. Similar results have been reported by Nishimura et al. [49]. Generally, vanillic acid is either metabolized by demethylation to yield the protocatechuic acid or by decarboxylation to yield guaiacol [40].

Over 200 standards and 360 plant materials which provide chromatographic and spectra data for some food phenolic compounds have been collected by Lin and Hanly [37,50]. 1-caffeoylquinic acid and 3, 4, 5-tricaffeoylquinic acids among other hydroxycinnamoylquinic acids were identified by Lin and Hanly [17], when studying the phenolic components of chrysanthemum flower. The properties of the two hydroxycinnamoylquinic acids identified by the above authors are similar to the ones detected in this study except in terms of their retention times (6.6 and 48.4 min) as against (22.92 and 25.16 min) respectively. Similarly, Jaiswal et al. [46] detected and characterized some triacyl quinic acids from green Robusta coffee beans; one of them was 3, 4, 5-tricaffeoylquinic acid with an *m*/*z* of 677.1512, similar to the one produced by strain AOB in this study (*m*/*z* 677).

It is apparent from the combination of metabolite identification involving the LC–MS- DAD data analyses and the detection of key aromatic-cleaving enzymes in strain AOB that the degradation of coniferyl alcohol by *Streptomyces albogriseolus* strain AOB involved an initial oxidative attack via coniferylaldehyde to yield ferulic acid which was metabolized through divergent pathways; either undergoing demethylation to yield caffeic acid or β-oxidation of the side chain to yield vanillic acid. Vanillic acid could have been demethylated to yield protocatechuate which then undergoes ring fission

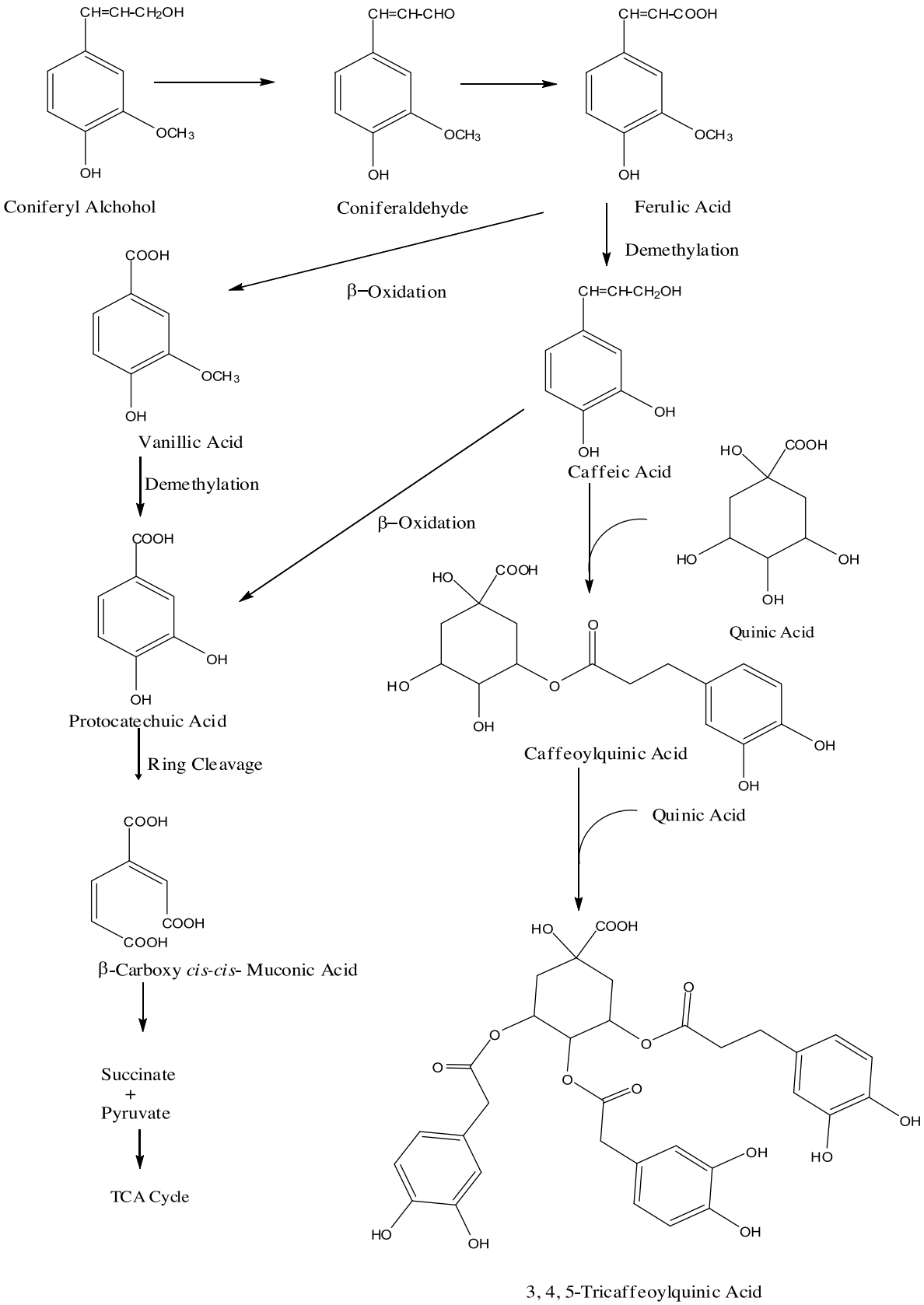

Fig. 4. Proposed pathway for metabolic transformation of coniferyl alcohol and production of chlorogenic acids by *Streptomyces albogriseolus* strain AOB.

by the action of protocatechuate 3, 4-dioxygenase through the β-ketoadipate pathway to enter into the TCA cycle. The conversion of ferulic acid to vanillic acid and subsequent demethylation to yield protocatechuate is similar to the findings of several authors. The degradation of coniferyl alcohol by Nocardia sp. DSM 1069 has been studied by Trojanowski et al. [51], who reported the formation

of coniferylaldehyde, ferulic acid and quantitative accumulation of vanillic acid in cell extracts, it was shown to cleave protocatechuic acid by *ortho* fission. Mechanism of coniferyl alcohol degradation involving an initial attack from either the methoxyl group or the side chain by Nocardia sp. DSM 1069 was also reported by Pometto III and Crawford [52]. However, Crawford [53] reported an alternative mechanism for vanillic acid catabolism by *Streptomyces setonii*, which yields guaiacol and catechol. The opinion of Chow et al. and Crawford [40,53] is that vanillic acid was catalyzed via an alternative pathway involving non-oxidative decarboxylation to yield guaiacol with further catabolism via demethylation and mineralization. However, the degradation of vanillic acid by *Streptomyces* spp via both routes (catechol and protocatechuate) was demonstrated by Pometto III et al. [54]. In the alternate pathway of strain AOB, caffeic acid is most likely to have undergone further degradation through β-oxidation to form protocatechuic acid. Conversion of ferulic acid to caffeic acid had been reported by de la Torres [55]. Landete et al. [56] reported that the most frequently observed metabolic pathway of caffeic acid is by decarboxylation and further reduction to yield 4-ethylcatechol.

An interesting observation in this study however, was the detection of secondary metabolites as metabolic products of coniferyl alcohol by *Streptomyces albogriseolus* strain AOB, an actinomycete isolated from the tropical estuarine environment. These metabolites were identified as 1-caffeoylquinic acid and 3,4,5-tricaffeoylquinic acids which belong to a generic group known as chlorogenic acids, that are conjugation (esterification) products of quinic acid and caffeic acid as shown in the proposed metabolic scheme for coniferyl alcohol (Fig. 4). Whilst the caffeic acid molecule is supplied from demethylation of ferulic acid, the quinic acid component could have plausibly been supplied from the robust biosynthetic machinery of the actinomycete. Quinic acid is a product of microbial metabolism, an intermediate in the biosynthesis of aromatic amino acids such as tryptophan, phenylalanine and tyrosine in living systems via the shikimic acid pathway [57,58]. A couple of authors have expressed their opinion about the formation of chlorogenic acids. According to Levy and Zucker [59], chlorogenic acid was synthesized through 5-o-cinnamoylquinic acid and 5-o-coumaroylquinic acid in potato tubers, as a result of conjugation of t-cinnamic acid and quinic acid. However, Kojima and Uritani [60] are of the opinion that chlorogenic acid is a conjugate of *trans*-cinamic acid and some sugars different from quinic or shikimic acid. They then suggested that since the structure of chlorogenic acids has been proposed to be 5-o-caffeoylquinic acid, therefore, the non-aromatic moiety of chlorogenic compound would have to be replaced with quinic acid in a later step of chlorogenic acid biosynthesis. However, we are of the opinion that *Streptomyces albogriseolus* under consideration plausibly possesses the requisite enzymes to conjugate caffeic acid and quinic acid to form caffeoylquninic acid in a way similar to the conjugation of various acyl substituents with 6-aminopenicillanic acid (β-lactan ring) in the production of semi-synthetic penicillins, a subject of further study in our laboratory. Actinomycetes are undoubtedly the largest producers of bacterial secondary metabolites. Although the production of chlorogenic acids from certain plants appear to be a common phenomenon, but the production of chlorogenic acid through the microbial metabolic route observed in this study is not common and could therefore be a plausible industry in pharmaceutical processes because of the numerous physiological and biological applications mentioned earlier.

4. Conclusions

The production of a chlorogenic acid through the robust metabolic pathway of an estuarine *Streptomyces* sp reported herein may open up a new frontier of knowledge for a further pursuit. This report also offers additional information to the existing knowledge on the bacterial degradation of coniferyl alcohol in the tropical estuarine ecosystem. Estuarine or marine bacteria especially the actinomycetes are genomically and metabolically diverse and worth exploring for the discovery of novel secondary metabolites of commercial importance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.10.015.

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