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Purification and characterization of tryptophan and indole-3-acetic acid produced by *Serratia marcescens* strain *MCB* associated with *Oscheius* sp. MCB (Nematoda: Rhabditidae) obtained from South African soil

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Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are lethal to insect pests that attack plants. These EPNs are associated symbiotically with the two enterobacteria *Xenorhabdus* and *Photorhabdus* sp., respectively. The bacteria synthesize a range of insecticidal and antimicrobial metabolites which may be useful as agricultural pest control and medical disease control. Recently, EPNs characterization and explanation have been expanded to include genera, *Oscheius*. We isolated, characterized and identified a symbiotic bacterium and the metabolites it produces. The symbiotic bacterium was isolated from a South African nematode, *Oscheius* sp. *MCB* (GenBank accession number: KF684370). The symbiotic bacterium was identified to be a *Serratia marcescens* strain *MCB* (GenBank accession number: KF793930). Two metabolites it produces are indole-3-acetic acid and tryptophan, with tryptophan as an aromatic amino acid assumed to be a precursor for indole-3-acetic acid production. From this study it is evident that *S. marcescens* strain *MCB* isolated from *Oscheius* sp. *MCB* shares similar attributes with *Xenorhabdus* and *Photorhabdus* sp., thus its host can be accepted as an EPN.

Key words: Entomopathogenic nematodes, *Serratia*, *Oscheius*, *Xenorhabdus, Photorhabdus*.

INTRODUCTION

Entomopathogenic nematodes (EPNs) have been found to be advantageous as biocontrol agents (Kaya and Stock, 1997; Poinar, 1975). The biocontrol advantages of EPNs have been well established and encouraged further surveys of EPNs on a global basis as it has been shown that EPNs are widely distributed in various soils throughout the world (Adams et al., 2006; Hominick et al., 1996; Hominick, 2002). With the biocontrol advantages

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and useful antimicrobial products produced by the symbiotic bacteria associated with EPNs (Wang et al., 2011), there exists a strong incentive to discover new EPN species associated with new species of symbiotic bacteria. Recent isolated species of *Oscheius* genus EPNs have been described (Liu et al., 2012; Ye et al., 2010). The genus *Oscheius*, shares similar attributes with two other well-known EPN genera (*Steinernema* and *Heterorhabditis*) and has all the attributes associated with these EPNs (Dillman et al., 2012; Torres-Barragan et al., 2011) and was first erected by Andrassy in 1976. Liu et al. (2012) and Ye et al. (2010) used *Galleria mellonella* larvae to test the entomopathogenicity of *Oscheius chongmingensis* and *Oscheius carolinensis* which occurred in 56 h with 35% larvae mortality and 48 h with 100% larvae mortality, respectively.

The *Oscheius* genus comprises of the following recently identified novel species of EPNs, *O. chongmingensis*, *O. rugaoensis* and *Oscheius carolinensis* (Liu et al., 2012; Torres-Barragan et al., 2011; Ye et al., 2010). The EPNs in all three genera have evolved mutualistic associations with three different genera of Gram negative, motile, rod-shaped bacteria belonging to the family Enterobactericeae, all of which are lethal pathogens to a wide range of insects. *Xenorhabdus* bacteria are located in the intestinal vesicle of the infective juveniles (IJs) of *Steinernema* (Bird and Akhurst, 1983); in contrast *Photorhabdus* bacteria are located throughout the whole intestine of *Heterorhabditis* IJs (Endo and Nickle, 1991) whereas bacteria belonging to the genus *Serratia* have been shown to be symbiotically associated with *O. chongmingensis* and are located within a pouch next to the pharyngeal bulb (Liu et al., 2012).

Infective juveniles (IJs) penetrate the insect host through natural openings such as mouth, anus and respiratory spiracles and release the bacteria in the haemocel of the host (Poinar, 1975). In this relationship, EPNs are vectors for the bacteria, which are transported into the haemolymph of insect hosts (Boemare et al., 1993). After releasing the symbiotic bacteria into the insect haemolymph, the bacteria replicates and suppress the insect host's immune response system by producing various metabolites which hamper and kill the insect host and further inhibit the growth of competing microorganism. In doing so, a monoxenic environment is maintained for the EPNs to reproduce, grow and develop (Hussien and Hanan, 2008; Wang et al., 2011). The metabolites produced by the symbiotic bacteria have shown to have antibiotic, antimycotic, insecticidal, nematicidal, antiulcer, antineoplastic and antiviral properties and may have useful medical and agricultural applications (Wang et al., 2011). Various metabolites have been reported from entomopathogenic bacteria cell cultures, and these include: indole, dithiolopyrrolones and xenocoumacins, however, very little is known about the ones secreted by *Serratia* species. In addition to the work done thus far, we strongly believe that in further accepting the *Oscheius* as an EPN genus, the metabolites produced by its symbiotic bacteria can be studied and compared with the ones produced by *Xenorhabdus* and *Photorhabdus* sp., thereby examining the similarities and differences in metabolites produced by the three bacteria. In this paper, the authors reported on metabolites produced by a new strain of *Serratia marcescens; S. marcescens* strain *MCB* isolated from *Oscheius* sp. *MCB* which was obtained from South African soil.

MATERIALS AND METHODS

Isolation and morphological identification of the symbiotic bacterium

The symbiotic bacteria was isolated from *Oscheius* sp. *MCB* (GenBank accession number: KF684370) obtained from soil samples collected in North West province, South Africa. The methods used for isolating the bacteria were those described by Kaya and Stock (1997). The isolated bacteria were then grown and maintained on solid media namely nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride (TTC) and 0.0025% (w/v) bromothymol blue (BTB) (NBTA) and MacConkey agar plates and sub-cultured on the same media on a monthly basis at 25°C. Long term maintenance of the bacterial cultures was done by storing the cultures in 1 mL of 30% glycerol and stored in -70°C refrigerator. Red colonies were isolated, characterized morphologically and Gram staining performed (Zhang et al., 2008; Torres-Barragan et al., 2011; Bergey et al., 1994). To deduce if the symbiotic bacteria from *Oscheius* sp. *MCB* occurs in growth phases like *Xenorhabdus* and *Photorhabdus* sp., NBTA and MacConkey agar plates were observed for the first 24 h of incubation and compared with colonies at 72-120 h. The fluorescence ability of the symbiotic bacteria was determined with Fluorescent Microscope equipped with an Olympus DP 80 video camera using the CellSens Dimension 1.9.1 software.

PCR amplification of the 16S rDNA from symbiotic bacteria

The DNA of the 16S rRNA gene of the symbiotic bacteria was amplified. Genomic DNA was isolated from the NBTA colonies using the ZR fungal/bacterial DNA MiniPrep kit (Zymo Research, catalog # D3050). The DNA extracted from the bacterial colonies was quantified using the NanoDrop ND-1000® spectrophotometer (Bio-Rad). The primers used for 16S rDNA amplification and sequencing were the 5`-AGAGTTTGATCCTGGCTCAG-3`f and 5`-AAGGAGGTGATCCAAGCCGCA-3`r (Brosius et al., 1978), corresponding to positions 8-27 and 1521- 1540 in the 16S rDNA sequence of *Escherichia coli*. The PCR products were cleaned using ExoSAP-it® and sequenced by Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

Sequence and phylogenetic relationship analysis of the symbiotic bacteria

The sequence was screened for chimeras by using DECIPHER tool (Wright et al., 2012). The NCBI-BLAST search tool was used to establish the degree of similarity of the sequence with other sequences of known bacteria taxa in the GenBank database. Bacterial species from GenBank with the highest matches after a BLAST search were selected for phylogenetic analysis. We used *Serratia nematodiphila* strain *DR186* (JQ002568), *Serratia* sp. *R26*

Table 1. HPLC determination of tryptophan and indole-3-acetic acid including the concentrations.

Parameter	Metabolites	
	Tryptophan	Indole-3-acetic acid
Retention time (min)	2.00	3.13
Equation	y = 1.9714x + 976.16	y = 25.054x - 338.8
R^2	0.995	1.000
$^{\Psi}$LOD (µg L^{-1})	46.7	7.99
^{ꭓ}LOQ (µg L^{-1})	155.7	26.6

ψLOD is limit of detectation and ꭓLOQ limit of quantification.

(2012) (JX082294), *S. marcescens WW4* (NR_102509), *S. marcescens* strain *AU1209* (AY043387), *S. marcescens* clone *HUWU3B* (KC583444), *S. marcescens* strain *DAP33* (EU302858), *Serratia* sp. *C8* (JX258135), *Serratia urelytica* (AJ854062), *Serratia rubidaea* strain *DSM 4480* (AJ233436), *S. entomophila* (NR025338), *Serratia ficaria* strain *DSM 4569* (NR_041979), *P. luminescens* subp. *luminescens* strain *Hb* (NR_037074) and *X. bovienii DSM 4766* (X82252) species to observe the relationship that the current strain (*S. marcescens* strain *MCB* KF793930) has with the other species. *Bacillus cereus* (AB334763) was used to root the tree. The sequences were aligned using MUSCLE (Edgar, 2004), with default options and the distance analysis with closest taxa were conducted in MEGA6 (Tamura et al., 2013). The evolutionary distances were computed using Jukes-Cantor model (Jukes and Cantor, 1969). Neighbour-joining tree of the homologous sequences in this study obtained from NCBI GenBank was inferred (Saitou and Nei, 1987).

Selection of the optimal nutrient medium for metabolite production

Five times loopful of log phase of the symbiotic bacteria obtained from NBTA/MacConkey agar plates were inoculated into each 100 mL flasks containing 20 mL of Luria-Bertani broth (LB), nutrient broth (NB), nutrient broth supplemented with canola oil and glucose and nutrient broth supplemented with canola oil only (Torre, 2003). The media were adjusted to final pH of 7.0 (Wang et al., 2011). The flasks were incubated at 28°C for 48 h in the dark on an orbital shaker at 160 rpm. At an hour interval, cell culture was centrifuged and supernatant removed, then bacterial concentration measured at 600 nm with an S-20 Boeco Spectrophotometer for 24 h. The media with high bacterial yield was deduced to be the one suitable for the bacterial growth. This experiment was carried out in triplicates.

Metabolite identification and characterization

For purification and characterization of the metabolites, all media were analyzed with an HPLC system (Model 510, waters, USA) and the metabolites were separated using a C18 reversed phase column (25 cm x 4 mm x 0.5 µm). A flow rate of 1.0 mL min^{-1} with detection of separated metabolites was achieved using a fluorescence detector at excitation and emission 280 and 435 nm, respectively. The solvent used was a mixture of acetonitrile: water with 0.3% acetic acid (50:50 v/v). After the HPLC development, the method was validated in terms of limit of detectation (LOD), limit of quantification (LOQ) and other parameters (Table 1).

Role of metabolite on the bacterial growth

Different concentrations (mg L^{-1}) of the identified metabolites were used to determine effects on bacterial growth. Luria-Bertani broths with the metabolites were inoculated with a loopful of *S. marcescens* strain *MCB*. The produced metabolites were then detected with HPLC and the concentrations determined. LB without the metabolites were used as a negative control.

RESULTS

Morphological characteristics

Colony morphology and cell morphology based on Gram staining of these bacteria were determined (Table 2). *Serratia marcescens* strain MCB was rod shaped with fluorescing ability and was located next to the pharyngeal bulb as well as throughout the intestines of adult females (Figure 1a-c).

Molecular identification

The 16S rDNA sequence product was 1152 base pairs (bp) and was deposited in GenBank and assigned the accession number KF793930. The BLAST search of this sequence showed that it belongs to the genus *Serratia,* with high sequence similarity identity with *S. marcescens* strain DAP33, *S. marcescens* strain AU1209, *S. marcescens* sp. clone HUWU3B, *S.* marcescens sp. C816, *S. marcescens* sp. R26 (2012) and *S. marcescens* WW4. Phylogenetic tree revealed the close relationship of *S. marcescens* strain *MCB* with *S. marcescens* strain DAP33 (bootstrap of 70%) as shown in Figure 2. Neighbour-joining tree indicated that *S. marcescens* strain *MCB* and *S. marcescens* strain *DAP33* form a polytomy clade with four strains of *S. marcescens* and one *S. nematodiphila* (bootstrap of 52%).

Selection of the optimal nutrient medium for antibiotic production

Distinctive colour changes were observed in all media types, that are, NB, NB with canola oil, NB with canola oil and glucose and LB inoculated with *S. marcescens* strain *MCB* (results not shown). The colour changes were a

Table 2. Morphological characteristics of symbiotic bacteria isolated from *Oscheius* sp. *MCB.*

Characteristic	Symbiotic bacteria
Host EPN	*Oscheius sp. MCB*
Symbiotic bacteria	*Serratia marcescens* strain *MCB*
Colony diameter on NBTA agar plate (mm)	1-4 mm
Colony morphology and pigmentation on NBTA agar (Phase I)	Deep red circular, with regular margins and smaller diameter
Colony morphology and pigmentation on NBTA agar (Phase II)	Brown, flat with irregular margins and larger in diameter
Pigmentation on MacConkey agar	Light pink
Pigmentation in Nutrient broth	Light Maroon
Pigmentation in Nutrient broth supplemented with canola oil	Light pink
Pigmentation in Nutrient broth supplemented with canola oil and glucose	Light pink
Pigmentation in Luria-Bertani broth	Maroon
Optimum temperature	28°C
Gram stain	Gram negative
Fluorescence ability	Present
Morphology	Rod

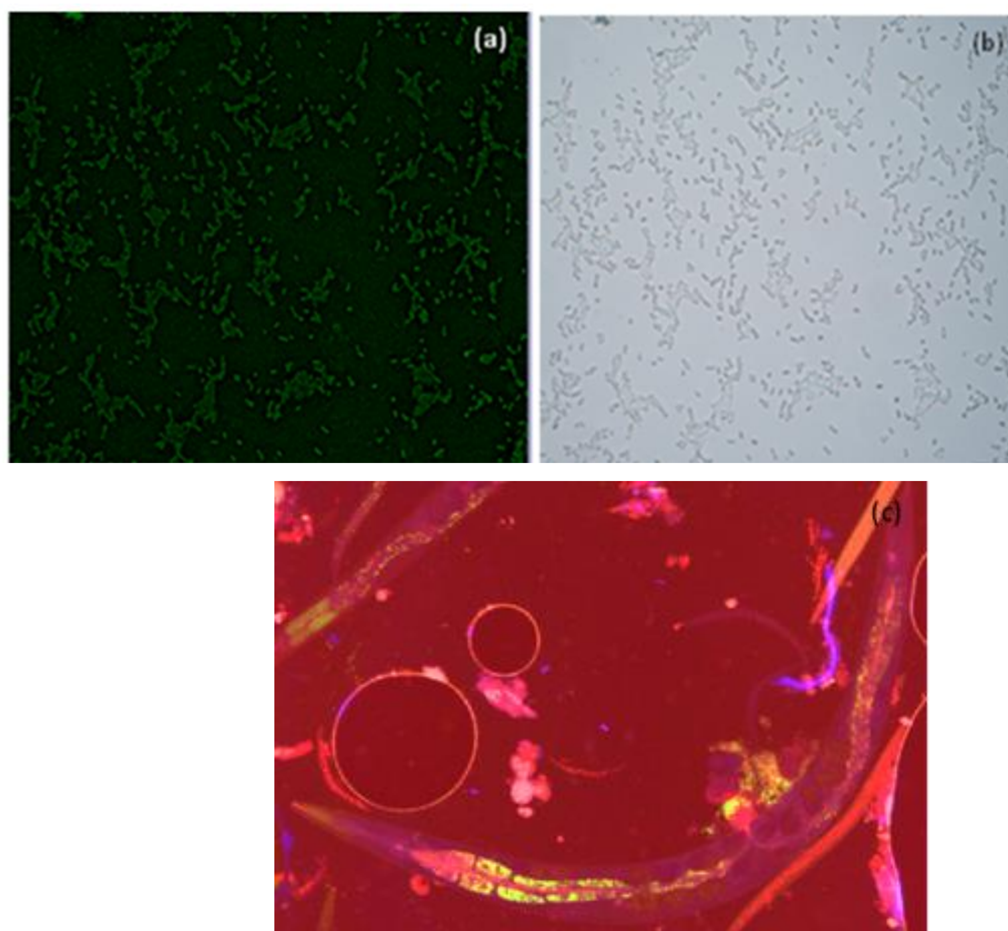

Figure 1. *S. marcescens* strain *MCB* under the light microscope (a) fluorescing rod shaped bacteria, (b) bacteria without fluorescence and (c) fluorescing bacteria within the *Oscheius* sp. *MCB* adult nematode.

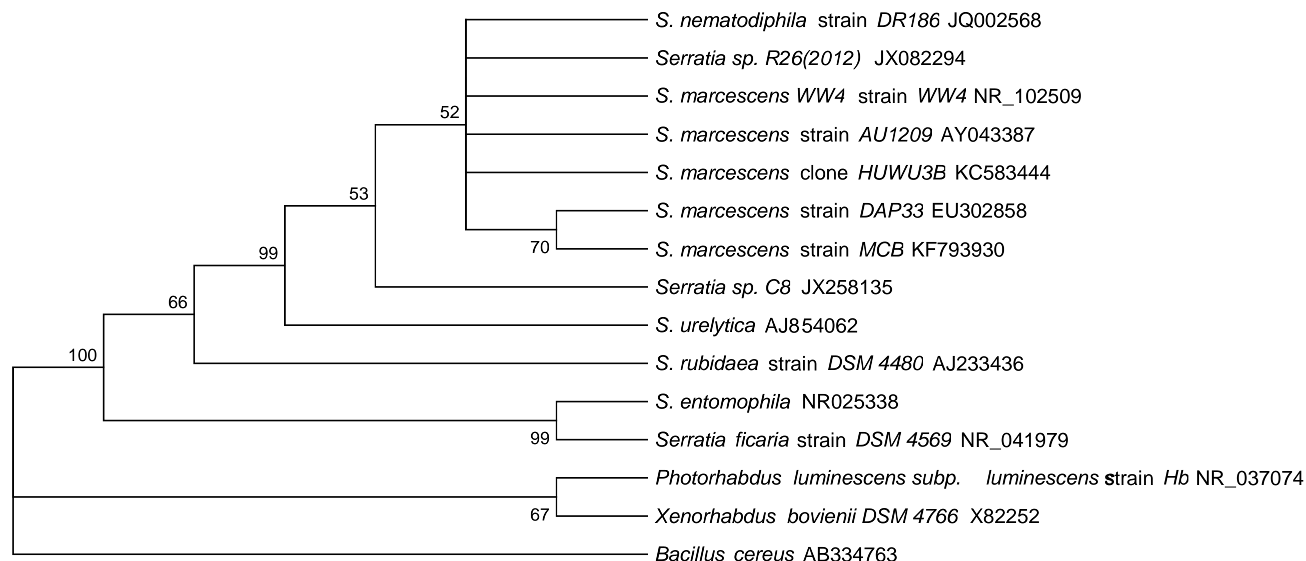

Figure 2. Neighbour-joining tree of *S. marcescens* strain *MCB* with closely related bacteria inferred by homologous sequences based on 16S rRNA gene sequence data. The nodal supports are bootstrap values from 1000 replications.

result of metabolites secreted by the bacteria as it proliferates and some metabolites have antibiotic activity against opportunistic bacterial and fungi that may be in the media. The colour changes in the media thus serve as a method to visually verify purity of the bacterial culture.

The different growth media had significant effects on bacterial growth and metabolite production. Luria-Bertani broth (LB) showed a maximum bacterial yield, thus it increases bacterial growth and metabolism, increased bacterial yield results in an even increased antibiotic production due to nutrients available and a highly active metabolism and most antibiotics or metabolites get secreted in the stationery phase of bacterial growth (Thomashow, 1996). LB had a higher bacterial yield with an optical density of 1.5, followed by NB with an optical density of 0.9 at the 24th h and for NB supplemented with canola oil had the highest optical density of 0.6 at 19th h and NB supplemented with canola oil and glucose had optical density of 0.4 at the 16th h (Figure 3a and b).

Metabolite identification and characterization

Metabolites were analyzed from all the four different media used. Three metabolites; tryptophan, indole-3-acetic acid (IAA) and indole were expected to be produced; in this study only two were identified, tryptophan and IAA produced by *S. marcescens* strain *MCB* (Figure 4a to e). Tryptophan was detected first with high quantity concentration as compared to IAA (Table 3) and it was only produced in all the media whilst IAA was produced in LB and NB + canola oil.

Role of metabolite on the bacterial growth

The bacterial growth increased in with an increment in tryptophan concentration (results not shown). It was assumed that the aromatic amino acid tryptophan was the precursor for IAA production (Dewick, 2002), because from the metabolite analysis, the IAA production increased with an increase in tryptophan, however there was no relationship between IAA in the broth and tryptophan production (Tables 4 and 5).

DISCUSSION

A new species of *Oscheius* genus of entomopathogenic nematodes (EPNs) (Ye et al., 2010) had been accepted as a genus displaying characteristic EPN adaptation which have been acquired through convergent evolution (Dillman et al., 2012); however more research needs to be done to support its acceptance as an EPN genus. One of the criteria for EPNs, is for their symbiotic bacteria to produce metabolites which not only kill the insect host but also other competitive microorganisms, thus, we strongly believe that analyzing metabolites produced by symbiotic bacteria from *Oscheius* species, will shed light as to whether *Oscheius* symbiotic bacteria is similar to *Xenorhabdus* and *Photorhabdus* sp. In this paper, we managed to identify two compounds produced by *S. marcescens* strain *MCB*, namely tryptophan and IAA. Indole derivative compounds produced by *Xenorhabdus* and *Photorhabdus* sp. have been identified and shown to inhibit growth of insect larvae and other microorganisms (Sundar and Chang, 1993; Hu and Webster, 2000; Paul et al., 1981). Tryptophan was recently added to growth

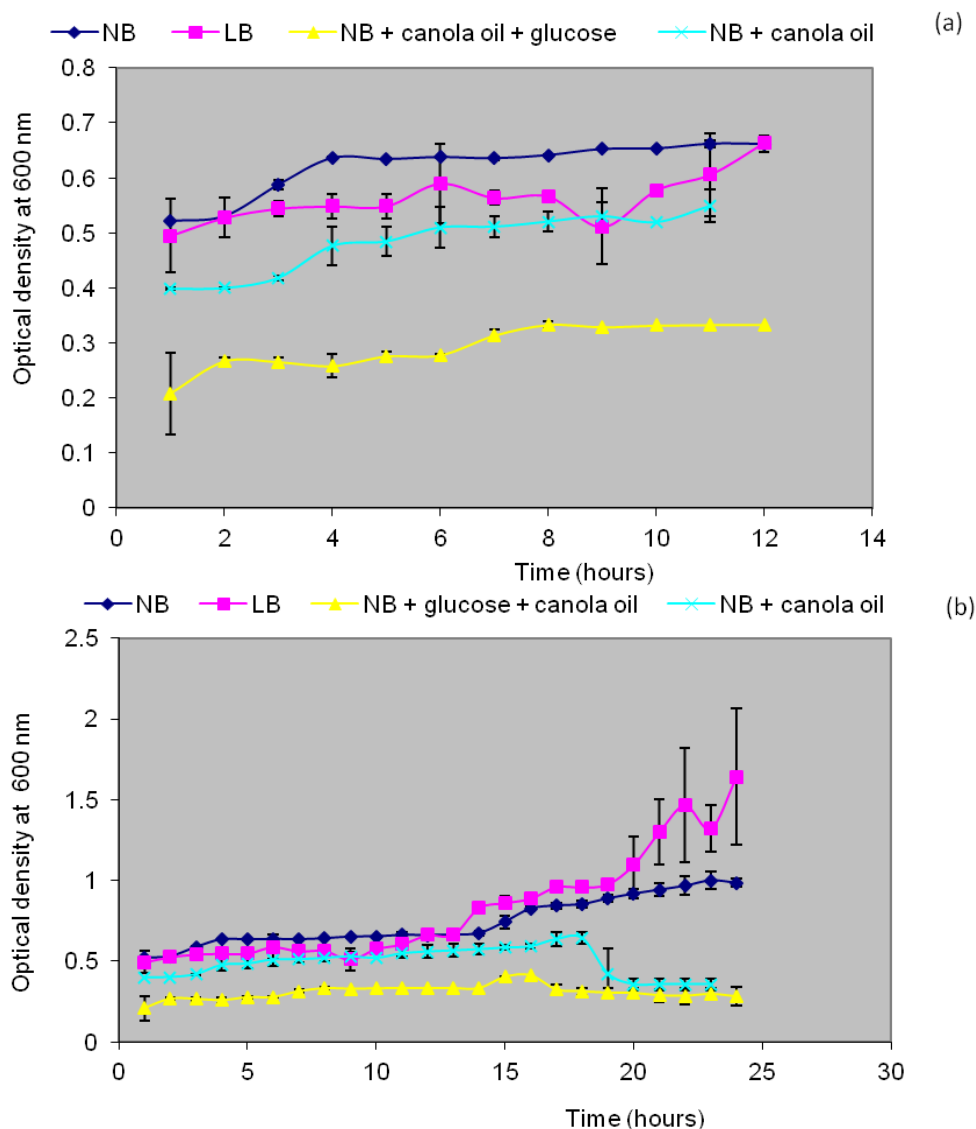

Figure 3. Absorbance over time at 600 nm of *S. marcescens* strain *MCB* in Luria-Bertani broth (LB), nutrient broth (NB), nutrient broth supplemented with canola oil and nutrient broth supplemented with glucose and canola oil at approximately 25°C on a shaker at 160 rpm for (a) 12 h and (b) 24 h. Each point represents the average of three replicates. Bars indicate size exclusion.

medium for bacteria to induce production of other antimicrobial metabolites (Sundar and Chang, 1993), in this paper we identified tryptophan as one of the metabolites produced and we strongly believe that tryptophan is a precursor for IAA production and other compounds (Sundar and Chang, 1993; Manulis et al., 1991; Paul et al., 1981; Martino et al., 2003). Previous studies indicate that both *Xenorhabdus* and *Photorhabdus* sp. are biochemically and physiologically suitable as symbionts of entomopathogenic nematodes (Thaler et al., 1998; Thomas and Poinar, 1979; Akhurst, 1980; 1982), due to production of these metabolites. The metabolites produced by these bacteria also contribute towards the symbiotic relationship with the entomopathogenic nematodes in providing a monoxenic growth environment and nutrients for the nematodes which in turn act as vectors.

In this study, various liquid media for *S. marcescens* strain *MCB* were used and it was found that LB resulted in higher bacterial yield which in turn lead to an increased metabolite synthesis and production (Figures 3a and b). Increased bacterial yield results in an increased level of metabolite production due to nutrient availability and a highly active metabolism (Thomashow, 1996). Luria-Bertani broth contains sodium chloride, yeast extract and tryptone, whereas nutrient broth contains meat extract,

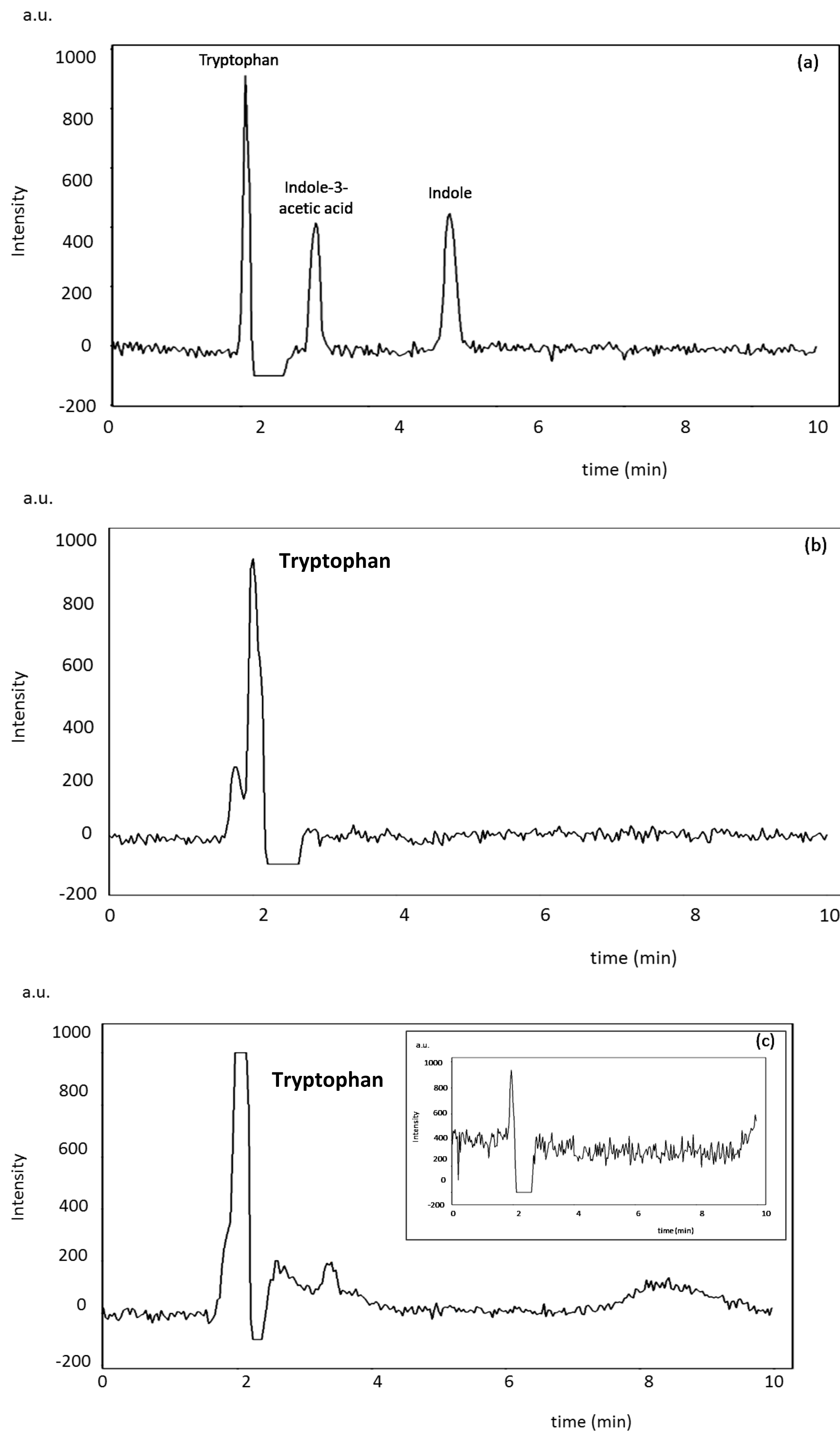

Figure 4. Chromatograms showing: (a) retention times of tryptophan, indole-3-acetic acid and indole standards, (b) 250X diluted NB sample, (c) 100X diluted NB + canola oil + glucose sample with 200X diluted, (d) 100X diluted LB sample and (e) 150X diluted NB + canola oil sample with 250X diluted.

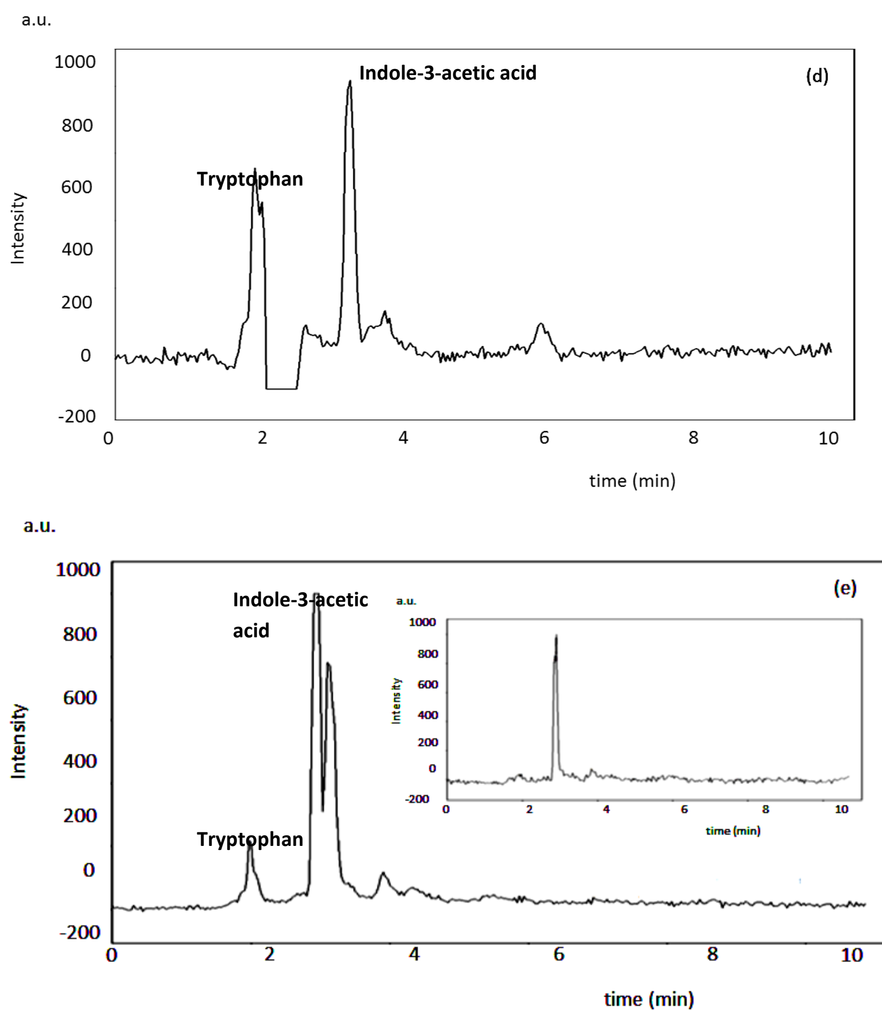

Figure 4. Contd.

Table 3. Concentrations of the metabolite from each media.

Media name	Concentration (mg L^{-1})		
	Tryptophan	Indole-3-acetic acid	Indole
LB	380	32	-
NB+ canola oil + glucose	42	-	-
NB	1194	-	-
NB+ canola oil	148	54	-

Table 4. Effects of tryptophan concentrations on tryptophan and indole-3-acetic acid production.

Concentration of tryptophan in LB (mg L^{-1})	Metabolites (mg L^{-1})	
	Tryptophan	Indole-3-acetic acid
0	1123	45
10	1342	78
20	1402	83
30	1409	94

Table 5. Effects of indole-3-acetic acid concentrations on tryptophan and indole-3-acetic acid production.

Concentration of indole-3-acetic acid in LB (mg L^{-1})	Metabolites (mg L^{-1})	
	Tryptophan	Indole-3-acetic acid
0	1124	45
10	1124	51
20	1134	55
30	1123	69

yeast extract, peptone and sodium chloride. Sodium chloride increases metabolite production due to its osmolarity and peptone, tryptone as well as yeast extract are good nitrogen sources (Wang et al., 2011), which explains the high bacterial growth which resulted in high metabolite quantity and concentrations in both media (Table 3). Peptone has been found to be a better nitrogen source as compared to tryptone (Wang et al., 2011), this is validated by the high concentration of IAA produced in NB as compared to LB. Results reported here are similar to those reported in previous studies which indicate that both NB and LB are suitable for metabolite production of *S. marcescens* strain *MCB* due to presence of NaCl, peptone, tryptone and yeast extract which are good sources of nitrogen and also increase metabolite production (Wang et al., 2011).

Conclusions

In this study, we showed that media composition affects bacterial yield and quality and quantity of metabolite production. Tryptophan is a precursor of IAA which is produced by *S. marcescens* strain *MCB*. *S. marcescens* strain *MCB* isolated from *Oscheius* sp. *MCB* has similar attributes with *Xenorhabdus* and *Photorhabdus* sp., thus its host can be accepted as an EPN. Future studies may be focused on determining the genetic characteristics, mechanism and the pathway that are responsible for the production of metabolites in the symbiotic bacteria.

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