Isolation and characterization of chemical constituents from *B. amyloliquefaciens* and their nematicidal activity

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Abstract
Secondary metabolites from cell free culture filtrate of *B. amyloliquefaciens* (Ba-14.5) showed strong nematicidal activity against root-knot nematode in preliminary tests. In the present study, chemical constituents present in secondary metabolites of *B. amyloliquefaciens* were investigated. Methanol fractions obtained from silica gel chromatography were analysed by using GC-MS. Thirteen compounds were revealed by GC-MS analysis and these constituents include stigmast-3,5-dien-7-one (9.82%), Pregna-5, 16-dien-20-one, 3-(acetylxyloxy)-(3.beta)-(5.12%), Benzenacetaldehyde (3.96%), N-acetyl-3-methyl-1, 4-diaza bicyclo[4.3.0]nonan-2,5-dione (3.65%), Pyrrolo (1,2-1) pyrazine-1,4-dione, hexahydro-(39.57%), 2,4,6-Tetramethyldec-1-ol (0.54%), Pyrrolo (1,2-1) pyrazine-1,4-dione, hexahydro-3,2- methyldihydropyran (4.96%), Hexadecanoic acid, methyl ester (0.35%), 5, 10-Diethoxy-2,3,7,8-tetrahydro-1H, 6H-dipyrolo[1,2-a,1.35%), n-Octadecanoic acid methyl ester (0.39%), 3,6-Disobutyl-2,5-Piperazinedione (4.21%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (9.7%), and Octadecanoic acid, 2,3-dihydroxypropyl ester (21.8%). Among thirteen compounds, three compounds like Benzenacetaldehyde, 2-hydroxy-1-(hydroxymethyl) ethyl ester and Octadecanoic acid, 2,3-dihydroxypropyl ester possess nematicidal activity based on previous studies. Other compounds possess antimicrobial, antifungal, antioxidant, anticancer and insecticidal properties and are yet to be confirmed for their nematicidal activity. Our findings help to find potential compounds/metabolites from microbial source to develop nematicicides for the management of root-knot nematode in horticultural crops.

Keywords: *B. amyloliquefaciens*, culture filtrate, secondary metabolites, GC-MS, nematicidal activity

1. Introduction
Plant parasitic nematodes are hidden enemies of crops and are recognised as one of the greatest threats to crops throughout the world [29]. Among the plant parasitic nematodes, root knot nematode is the major nematode causing damage to most of the economically important crops [28]. They are obligate parasites and have a wide host range including vegetables, cereals and fruit crops [1, 15]. It causes an estimated $118b annual losses to world crops [5]. Nematode infected plants exhibit symptoms like stunted growth, chlorosis, wilting and presence of galls on roots. The management of nematodes is more difficult than that of other pests because nematodes mostly inhabit the soil and usually attack the underground parts of the plants [44]. Although the application of chemical nematicides are effective but in long term usage and wide scale caused environmental pollution and enhancement of resistance in nematodes [16, 40]. Therefore, the search for novel, environmentally friendly alternative means instead of chemicals becomes emergency demand for management of root-knot nematodes [32, 49]. Nowadays, microorganisms and their metabolites have attracted the most attention as potential nematode biocontrol agents. Several antagonistic microbial strains such as * Bacillus*, *Pasteuria* and *Pseudomonas* have been developed into commercial formulations and are successfully used to control nematodes in agricultural fields [8, 19, 26, 31, 43]. Among bacterial bioagents, *Bacillus* spp. are dominant within the rhizosphere and are directly associated with plants and the soil environment, the inhibition of phytopathogens is a fundamental function of the bioactive molecules they produce [15, 47]. Number of investigations showed that nematode pathogenic bacteria like *Bacillus* kill nematodes by different mechanisms, including parasitism, antibiotic production of toxins, indirectly by interfering with the recognition of host plants inducing systemic resistance and by improving plant health [47]. They can synthesize various molecules that are toxic to nematodes [11, 36]. For example, *B.
thuringiensis shows nematicidal activity towards M. incognita and Heterodera glycines by producing crystal inclusions, a family of toxic proteins to a wide range of insect species including nematodes [47]. A number of studies showed that the secondary metabolites of certain Bacillus spp. that are responsible for their nematicidal activity exhibit a manner of chemical structure. These unknown variables lead to questionable the efficacy and reproductibility of biochemical products, compounded by the lack of standards for such products. Bacillus amyloliquefaciens (Ba-14.5) was an isolate from the rhizosphere area of yams in the fields of CTCRI. The preliminary results have shown that the crude extract of the culture filtrate of B. amyloliquefaciens had shown strong nematicidal activity against root knot nematode. In this present investigation, the potential nematicidal compounds were investigated from the fore-said bacterial secondary metabolites.

2. Materials and Methods
2.1. Culturing of B. amyloliquefaciens
Fresh culture of B. amyloliquefaciens was prepared in slants by inoculating culture from mother culture and incubated at room temperature for 24 h. The fresh cultures from slants were inoculated to one litre broth (5 g of Peptone, 5 g of Sodium Chloride, 1.5 g of meat extract and 1.5 g of yeast extract) and were incubated in gyro-rotatory shaker at 30 °C for three days. The cultures were grown as batch culture in 500 ml conical flasks containing 100 ml of the nutrient broth.

2.2. Extraction of bacterial metabolites from B. amyloliquefaciens
The culture media were then centrifuged (10000 g, 20 min, 4 °C) followed by filtration through a 0.45 µm Whatman filter paper to obtain cell-free culture filtrate. 1000ml of that filtrate was concentrated to 5 ml at 30 °C using a rotary flash evaporator. The extract was further eluted using silica gel column chromatography with hexane and ethyl acetate solvents. No fractions obtained with both hexane and ethyl acetate solvents. After elution with non-polar solvents the column was subjected to polar solvents such as methanol powder were lyophilised, so that methanol solvent got evaporated leaving behind the compounds in powder form. This powdered form compounds were dissolved in water and tested against nematodes and water as control.

2.3. Nematicidal activity assay
The sample of 2ml each methanol fractions (F31–F34) were added to petri dish containing ~20 second juveniles of root knot nematodes. Each treatment was replicated thrice with sterile water as control at 25 °C. The number of live and dead where calculated after incubation of 8, 12 and 24 hours. The nematodes (J2s) were considered as dead when they are straight and are not responding to physical stimuli (touch with needle) while observing under a light microscope [9, 32]. Mortality values were calculated according to Abbott’s formula [2],

\[ \text{Mortality percentage} = \frac{\text{Mortality percentage in treatment} - \text{Mortality percentage in control}}{\text{100} - \text{Mortality percentage in control}} \times 100 \]

2.4. GC - MS analysis
Based on nematicidal bioassay study, potential methanol fractions were selected and extracted by using rotary evaporator to obtained viscous semi solid material. The semi dry methanol crude extract was suspended in water and sent outsourcing for GC-MS analysis. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology having more than 62,000 patterns. The spectrum of the known components demonstrated to understand the number of compounds present in each fraction. Based on TLC profile, methanol fractions 1–3, 4–6, 7–10 and 11–16, were then pooled, respectively, into four fractions (F31–F34).

3. Results and Discussion:
3.1. Effect of methanol fractions of B. amyloliquefaciens
Four fractions i.e. F31, F32, F33, F34 were tested against root knot nematode under lab conditions. Solvent methanol is highly toxic to nematodes. So, all four methanol fractions were lyophilised, so that methanol solvent got evaporated leaving behind the compounds in powder form. This powdered form compounds were dissolved in water and tested against nematodes and water as control.

Table 1: Effects of the cell-free culture filtrate of B. amyloliquefaciens on mortality of M. incognita in vitro

<table>
<thead>
<tr>
<th>Mortality %</th>
<th>8h</th>
<th>12h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.E (Mortality %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F31</td>
<td>12.00 ± 0.57 (60%)</td>
<td>17.00 ± 0.88 (85.00%)</td>
<td>20 ± 0 (100%)</td>
</tr>
<tr>
<td>F32</td>
<td>1.33 ± 0.33 (6.65%)</td>
<td>4.66 ± 0.33 (23.33%)</td>
<td>6.33 ± 0.88 (31.65%)</td>
</tr>
<tr>
<td>F33</td>
<td>7.66 ± 0.33 (38.30%)</td>
<td>11.66 ± 1.2 (58.3%)</td>
<td>17.66 ± 0.88 (88.33%)</td>
</tr>
<tr>
<td>F34</td>
<td>12.66 ± 0.33 (63.33%)</td>
<td>17.33 ± 0.88 (86.65%)</td>
<td>20 ± 0 (100%)</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C.D</td>
<td>1.18</td>
<td>2.24</td>
<td>2.06</td>
</tr>
</tbody>
</table>

Results revealed that 100% mortality of second stage juveniles (J2) was observed with two methanol fractions (F31 and F34) within 24 h incubation time (Table 1). Whereas, 88.3 % and 31.6% mortality observed with methanol fractions F31 and F32 respectively. The effect of nematicidal activity of this fractions was in this order F31>F34>F33>F32. F31 and F34 showed high nematicidal activity followed by F33. But fraction F32 showed very less nematicidal activity.

3.2. GC-MS analysis of the secondary metabolites of B. amyloliquefaciens
Two methanol fractions F31 and F34 were selected to identify compounds/metabolites present in culture filtrate of B. amyloliquefaciens. Chromatogram GC-MS analysis of the two methanol fractions of B. amyloliquefaciens showed the presence of thirteen major peaks at respective temperature (Figure 1 & 2). The peak areas (or percentage compositions of the metabolites shown in the brackets) are relative to other constituents within the crude extracts and whose match factors were greater than 700.

Compounds that are identified through GC-MS analysis were tabulated in tables 1 and 2. The compound prediction is based on National Institute Standard and Technology Database. The results revealed the presence of stigmasta-3,5-dien-7-one (9.82%), Regina-5, 16-dien-20-one, 3-(acetyloxy)-, (3.96%), Benzeneacetaldehyde (3.96%), N-acetyl-3-methyl-
1,4-diazabicyclo[4.3.0]nonan-2,5-dione (3.65%), Pyrrolo(1,2-1)pyrazine-1,4-dione, hexahydro-(39.57%), 2,4,6,8-Tetramethyldecan-1-ol (0.54%), Pyrrolo(1,2-1)pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-(4.96%), Hexadecanoic acid, methyl ester (0.35%), 5, 10-Diethoxy-2,3,7,8-tetrahydro-1H, 6H-dipyrrrolo(1,2-a (1.35%), n-Octadecanoic acid methyl ester (0.39%), 3,6-Disobutyl-2,5-Piperazinedione (4.21%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (9.79%) and Octadecanoic acid, 2,3-dihydroxypropyl ester (21.48%).

In the present study, the GC-MS analysis of the methanolic fractions of B. amyloliquefaciens showed the presence of thirteen compounds. Nematicidal activity are shown by Benzenecetaldehyde, 2-hydroxy-1-(hydroxymethyl) ethyl ester and Octadecanoic acid, 2,3-

![Fig 1: Chromatogram of GC-MS analysis of methanol fraction (Fm-1) of secondary metabolites produced by B. amyloliquefaciens](image1)

![Fig 2: Chromatogram of GC-MS analysis of methanol fraction (Fm-4) of secondary metabolites produced by B. amyloliquefaciens](image2)

### Table 2: Compounds identified after GC-MS analysis of methanol fraction (Fm-1) extracted from secondary metabolites produced by Bacillus amyloliquefaciens

<table>
<thead>
<tr>
<th>S. No</th>
<th>Retention time</th>
<th>Area %</th>
<th>Compound</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>24.46, 25.88, 27.07, 29.22, 29.73, 30.04, 31.58, 32.03, 34.10, 33.09, 33.70, 38.57</td>
<td>9.82</td>
<td>stigmasta-3,5-dien-7-one</td>
<td>C_{29}H_{40}O</td>
<td>410</td>
<td>free radical scavenging, anti-diabetic, anticancer, free radical scavenging, anti-inflammatory</td>
<td>[9, 14, 36]</td>
</tr>
<tr>
<td>2.</td>
<td>41.896</td>
<td>5.12</td>
<td>Pregna-5, 16-dien-20-one, 3-(acetoxy)-, (3.beta.-)</td>
<td>C_{25}H_{34}O_{3}</td>
<td>356.49</td>
<td>anticancer agents</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3: Compounds identified after GC-MS analysis of methanol fraction (Fm-4) extracted from secondary metabolites produced by Bacillus amyloliquefaciens

<table>
<thead>
<tr>
<th>S. No</th>
<th>Retention time</th>
<th>Area %</th>
<th>Compound</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>8.675</td>
<td>3.96</td>
<td>Benzene acetaldehyde</td>
<td>C_{6}H_{4}O</td>
<td>120.1485</td>
<td>Nematicidal activity</td>
<td>[21, 23]</td>
</tr>
<tr>
<td>2.</td>
<td>26.065</td>
<td>3.65</td>
<td>N-acetyl-3-methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione</td>
<td>C_{18}H_{13}N_{2}O_{3}</td>
<td>210.22</td>
<td>No activity reported</td>
<td>[41]</td>
</tr>
<tr>
<td>3.</td>
<td>27.085</td>
<td>39.57</td>
<td>Pyrrolo(1,2-1)pyrazine-1,4-dione, hexahydro-</td>
<td>C_{18}H_{10}N_{2}O_{3}</td>
<td>154.1665</td>
<td>antioxidiant activity, antibiotic activity, antifungal compound</td>
<td>[20, 28, 39]</td>
</tr>
<tr>
<td>4.</td>
<td>27.455</td>
<td>0.54</td>
<td>2,4,6,8-Tetramethyldecan-1-ol</td>
<td>C_{14}H_{22}O</td>
<td>206.328</td>
<td>Volatile compound in sex pheromone of Margarodes prieskaensis</td>
<td>[7]</td>
</tr>
<tr>
<td>5.</td>
<td>28.162</td>
<td>4.96</td>
<td>Pyrrolo (1,2-1) pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-</td>
<td>C_{11}H_{10}N_{2}O_{3}</td>
<td>210</td>
<td>Antifungal activity</td>
<td>[12]</td>
</tr>
<tr>
<td>6.</td>
<td>30.335</td>
<td>0.35</td>
<td>Hexadecanoic acid, methyl ester</td>
<td>C_{16}H_{31}O</td>
<td>270</td>
<td>Antibacterial, antifungal, Antioxidant, nematicide, insecticide, lubricant, antianadrogenic, haemolytic</td>
<td>[4]</td>
</tr>
<tr>
<td>7.</td>
<td>30.641</td>
<td>1.35</td>
<td>5, 10-Diethoxy-2,3,7,8-tetrahydro-1H, 6H-dipyrrrolo (1,2-a</td>
<td>C_{14}H_{22}N_{2}O_{2}</td>
<td>250</td>
<td>Antimicrobial</td>
<td>[27]</td>
</tr>
<tr>
<td>8.</td>
<td>35.287</td>
<td>0.39</td>
<td>n-Octadecanoic acid methyl ester</td>
<td>C_{18}H_{31}O_{2}</td>
<td>284</td>
<td>Antimicrobial activity</td>
<td>[37]</td>
</tr>
<tr>
<td>9.</td>
<td>36.636</td>
<td>4.21</td>
<td>3,6-Diobutyl-2,5-Piperazinedione</td>
<td>C_{20}H_{31}N_{2}O_{2}</td>
<td>184.24</td>
<td>Antifungal activity</td>
<td>[4]</td>
</tr>
<tr>
<td>10.</td>
<td>44.613</td>
<td>9.79</td>
<td>Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester</td>
<td>C_{16}H_{31}O_{2}</td>
<td>330</td>
<td>Haemolytic, pesticide, flavour, antioxidant, nematocide, Insecticide</td>
<td>[4]</td>
</tr>
<tr>
<td>11.</td>
<td>49.203</td>
<td>21.48</td>
<td>Octadecanoic acid, 2,3-dihydroxypropyl ester</td>
<td>C_{18}H_{31}O_{3}</td>
<td>358.5558</td>
<td>Antimicrobial, Anticancer, nematicide, hepatoprotective, Anti-arthritis, anti-asthma, diuretic</td>
<td>[37]</td>
</tr>
</tbody>
</table>


20. Gopi M, Dhayanithi NB, Devi KN, Kuma R TTA. Marine natural product, *Pyrolo* [1,2-α] pyrazine-1,4-


