Chapter 6

Characterization of fengycin homologues produced by

*Bacillus subtilis* K1 by MALDI-TOF tandem mass spectrometry (MALDI TOF MS/MS)
6.1 Introduction:
The fengycin is a family of biologically active cyclic lipodepsipeptides produced by various species of *Bacilli*, such as *B. subtilis*, *B. cereus* and *B. amyloliquefaciens*, *B. globigii* (Vanittanakom et al., 1986; Nishikiori et al., 1986; Schneider et al., 1999; Volpon et al., 2000; Vater et al., 2002; Williams et al., 2002; Sun et al., 2006; Romero et al., 2007; Hu et al., 2007; Bie et al., 2009; Pyoung et al., 2010). The peptide portion of fengycin molecule consists of a decapeptide chain, of which eight amino acids are involved in the formation of peptide ring via lactone linkage between side chain phenolic –OH group of Tyr^3^ and –COOH group of Ile^{10} and the lipid portion consists of a β-hydroxy fatty acid (β-OH FA), with its –COOH group amide bonded to α-NH$_2$ of Glu^{1} (Fig 6.1).

![Figure 6.1: Primary structure of fengycin homologues](image)

The members of fengycin family exhibit heterogeneity at 6th position in peptide moiety as well as in chain length of β-OH FA which varies from 14-18 carbons. On the basis of variation at single amino acid at position 6 in peptide ring, fengycins have been classified in two classes viz. fengycin A/ plipastatin A and fengycin B/ plipastatin B. Fengycin A contains Ala at position 6 which is replaced by Val in case of fengycin B (Vanittanakom et al., 1986; Nishikiori et al., 1986; Schneider et al., 1999). The structure of fengycin A produced by *B. subtilis* F-29-3 was elucidated by Vanittanakom et al., using $^{13}$C-NMR, GC-MS,
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CD and IR spectroscopy while fengycin A and fengycin B structures have been described by Schneider et al. (1999). The two classes of fengycin and their fatty acid isomers have also been characterized by using tandem mass spectrometric techniques such as, ESI-MS/MS, MALDI-PSD MS, Atmospheric pressure MALDI (AP-MALDI), GC-MS (Vater et al., 2002; Williams et al., 2002; Medonna et al., 2003; Wang et al., 2004; Bie et al., 2009, Pyoung et al., 2010). The plipastatins produced by B. cereus BMG302-Ff67 exhibited very close structural similarities with fengycins varying in stereochemistry of Tyr residues (Nishikiori et al., 1986). The solution structure of plipastatins, was investigated using 2D NMR spectroscopy. In solution, the structures of plipastatins A and B were found to be stabilized by type I β-turn comprising of residues 6 to 9 and several other specific hydrogen bonds. The plipastatins are reported for their inhibitory action on phospholipase A2, an enzyme involved in various important physiological cellular functions viz. inflammation, acute hypersensitivity and blood platelet aggregation (Umezawa, et al., 1986). The fengycins are also known to exhibit strong fungitoxic activity specifically against filamentous fungi viz. Ascodesmis sphaerospora, Botrytis cinerea, Colletotrichum acutatum, Monilinia fluecticola, Mucor hientalis, M. hiemalis, Mycosphaerella pinodes, Paecilomyces variotii, Pleospora herbarunt, Pyricularia oryzae, Rhizoctonia solani, Schizophyllum commune, Stempylium sp., Thielaviopsis basicola, Fusarium moniliforme Sheldon ATCC38932, Colletotrichum gloeosporioides, and Podosphaera fusca (Vanittanakom et al., 1986; Sun et al., 2006; Romero et al., 2007; Hu et al., 2007; Bie et al., 2009; Pyoung et al., 2010) but they are ineffective against yeasts and bacteria. Inspite of strong antifungal activity, fungycins are 40 and 70 times less hemolytic incomparison to surfactins and iturins, respectively (Vanittanakom et al., 1986). The fengycins induce morphological changes in the several fungi such as bulging, curling or emptying of the hyphae. It also has been reported to form complex with sterols, which suggest the ability of fengycin to interact with membrane lipids. The difference in the behavior of filamentous and budding colonies of Mycotypha africana in presence of fengycin has been correlated to difference in the sterol concentration of membranes in corresponding growth types of the fungus (Vanittanakom et al., 1986). The mode of action of fengycin is less understood, however like other
natural antifungal peptides, bioactivity of fengycin seems to be driven by its amphiphilic character and affinity for lipid bilayers which facilitates its interaction with lipid bilayers in a dose dependent manner and to some extent it is known to alter cell membrane structure as well as permeability (Eeman et al., 2005; Deleu, et al., 2005; 2008). The antifungal activity of fengycin gets enhanced in presence of surfactin (Sun et al., 2006; Ongena et al., 2007) and iturin (Romero et al., 2007). This synergism observed between three different families of cyclic lipopeptides also suggests that they act via different mechanisms.

The fengycin is synthesized nonribosomally by peptide synthetases. The biosynthesis of the fengycin requires five fengycin synthetases encoded by fenC, fenD, fenE, fenA and fenB. These enzymes typically contain one or several amino acid activation modules approximately 1,000 amino acids long. In each peptide synthetase module, an adenylation domain recognizes and adenylates a specific amino acid, which forms a thioester bond with the cofactor 4’phosphopantetheine at the thiolation domain. A transpeptidation process then transfers the activated amino acids in the initiation module to the activated amino acid in the thiolation domain in the next module, ultimately forming a peptide. This process continues from one module to another till the entire molecule is constructed (Lin et al., 1998; 1999; Shu et al., 2002; Wu et al., 2006).

Fengycins due to their potential antifungal activity are promising biocontrol agents. The protective effect of fengycins against damping-off disease of bean seedlings caused by *Pythium ultimum* as well as against grey mold post harvest disease of apple has been demonstrated by Ongena et al. (2005). The same research group has also demonstrated the role of fengycin as elicitor of induced systemic resistance in *Phaseolus vulgaris* against fungal infections. They proposed that fengycin induced systemic resistance by stimulating the secretion of phenolic compounds derived from the phenyl propanoid metabolism. In potato tuber disc assay they demonstrated the production of tyrosine, chlorogenic acids, ferulic acid and cinnamic acids, known for their role as antimicrobial plant defence agents, in response to fengycin treatment (Ongena et al., 2005; 2007). Thus fengycins can be regarded as potential biocontrol agents as direct fungal
antagonists as well as elicitors for induced systemic resistance against plant pathogens. In order to investigate antifungal compounds produced by Banyan endophyte, *Bacillus subtilis* K1, we attempted the purification of antifungal compounds followed by their identification using MALDI-TOF mass spectrometry (described in chapter 2). The compounds separated in peaks no. 8-19 could be identified as fengycin homologues on the basis of literature and this chapter describes the sequence determination of fengycin homologues produced by *B. subtilis* K1.

### 6.2 Materials and Methods

#### 6.2.1 Mass spectrometry

The MALDI MS data were acquired on *Ultraflex* TOF/TOF spectrometer (Bruker Daltonics, Billerica, MA, USA and Bremen, Germany) equipped with 50 Hz pulsed N₂ laser (337nm) operated in positive ion reflectron mode using 90 ns delay time and 25 kV accelerating voltage. Samples were prepared by mixing equal amount of purified HPLC fractions of antifungal compounds with α-cyano-4-hydroxy-cinnamic acid or 2, 5 Dihydroxy benzoic acid saturated in acetonitrile:water (1:1 v/v) with 0.1% (v/v) trifluoroacetic acid and applied on the MALDI sample plate. The sample spots on MALDI plate were allowed to air dry. The MS/MS data were recorded using the LIFT option of Ultraflex II TOF/TOF by selecting appropriate precursor ion (± 3Da window). An acquisition run was the sum of at least 5 series with 10 total added shots to obtain high quality MS/MS spectra. The data were processed using Flexanalysis software (Bruker Daltonics, version 3.2).

#### 6.2.2 Chemical modification for structural determination

##### 6.2.2.1 Saponification

The purified or partially purified HPLC fractions were mixed in equal proportion with 2M sodium hydroxide (NaOH) and allowed to react overnight at room temperature. The reaction was terminated by addition of few drops of 100 mM glacial acetic acid to the reaction mixture so as to lower the pH to 2. The saponified peptides were extracted with equal volume of ethyl acetate, thrice. All ethyl acetate fractions were pooled and solvent was evaporated using a SpeedVac. The residue was solubilized in methanol and excess of NaOH was
removed by passing the solution thorough a Zip Tip C18 (Millipore, India). The trapped sample was washed twice with 0.1% TFA, and then eluted from the cartridge with 50% (v/v) of MeOH in 0.1% TFA. For mass spectrometry analysis TFA was removed using SpeedVac and the sample was reconstituted with required amount of MeOH:H₂O:acetic acid (50:50:2, v/v/v).

6.2.2.2 Acetylation
Acetylation was carried out by incubating HPLC fraction with NH₄HCO₃ (200mM) and acetic anhydride (AC₂O) mixture for 3-4 hours at 37°C. The sample was dried using SpeedVac.

6.2.2.3 Esterification
The esterification of peptide was carried out by incubating the extracted cyclic lipopeptides with methanolic HCl for 1 h at ambient temperature (The methanolic HCl was made by the drop wise addition of 160μl of acetyl chloride to 1ml of methanol). Then esterification reaction mix was dried using a SpeedVac and solid residue thus obtained was suspended in MeOH: H₂O: Acetic acid (70:30:0.1, v/v/v).

6.3 Results and Discussion
The metabolites produced by B. subtilis K1 were separated by reverse phase HPLC and the peaks labeled 8-19 were collected as separate fractions. The protonated precursor ions with m/z in the range of 1421 to 1521 were detected when these fractions were analyzed by MALDI-TOF MS (Figure 6.2). On the basis of mass values, these extracellular antifungal metabolites were putatively assigned as fengycins (Vater et al., 2002; Williams et al., 2002; Wang et al., 2004; Hu et al., 2007; Bie et al., 2009; Pyoung et al., 2010).

Fengycin A
In order to further characterize the fengycin homologues produced by B. subtilis K1, the MALDI LIFT MS/MS analysis of protonated precursor ions at m/z 1435.8, 1447.9, 1449.8, 1463.8, 1478.0, 1491.0 and 1505.8 were carried out. The MS/MS spectra of all these precursor ions were found to contain characteristic product ion peaks m/z 1080 and 966, corresponding to y₈ and y₉ fragment ions of fengycin homologues (Fig. 6.3 (a-b)) (Table 6.1). The y₈ will result due to cleavage at peptide bond between Orn² and Glu¹ while y₉ ion will form upon
cleavage between Tyr\(^3\) and Orn\(^2\) (Medonna et al., 2003).

The product ion peak \(m/z\) 1417.7 in MS/MS spectrum of the precursor ion \(m/z\) 1435.8 could be due loss of water molecule from the parent ion. Similar product ions formed due to loss of water molecules (\(m/z\) 1429.9, 1431.7, 1445.7, 1460.2, 1473.2 and 1487.8) were observed in MS/MS spectra of respective precursor ions (\(m/z\) 1447.9, 1449.8, 1464.0, 1478.0, 1491.0 and 1505.8). The presence of product ion at \(m/z\) 1393.8 in MS/MS spectrum of precursor ion at \(m/z\) 1421.7 with a mass difference of 28 Da may be attributed to a loss of CO\(^+\) ion. Similar loss of CO\(^+\) ion was found in spectra of above mentioned precursor ions suggesting the cyclic depsipeptide nature of these precursor ions (Sabreesh et al., 2007). The b type fragment ion signals were absent in all the MS/MS spectra of intact fengycin ions. This may be due to the highly stabilized charged octapeptide lactone ring system thus suppressing collision induced dissociation (CID) along the peptide backbone (Medonna et al., 2003).

The opening of the cyclic peptide ring is essential in order to deduce the sequence information using MS analysis, which was facilitated by mild alkali hydrolysis of lactone bond between Tyr\(^3\) and Ile\(^{10}\). The cleavage of lactone bond was accompanied by addition of \(\text{H}^+\) and \(\text{OH}^-\) to tyrosine\(^3\) and isoleucine10 resulting in gain of mass by 18 Da. However, the linear product ions thus obtained appeared in mass spectra with a gain of 19 Da corresponding to their precursor ions. The additional one mass unit may be attributed to the conversion of Glu to Gln as demonstrated by Williams et al. (2002). In order to determine the number of carboxyl groups, global esterification of intact fengycins was done and mass of esterified precursor ions were checked by MALDI-TOF MS. Figure 6.4 shows expanded view of control and esterified fengycin precursor ions. Upon esterification, the mass of fengycin precursor ions were found to increase by 28 Da thus suggesting presence of two esterification sites in fengycin homologs produced by \textit{B. subtilis} K1. In intact fengycin homologues, the C terminal end of Ile\(^{10}\) is blocked due to formation of lactone bond with Tyr\(^3\) and therefore the increment of 28 Da suggests the presence of two glutamic acids.
Figure 6.2: MALDI-TOF-MS spectra of HPLC peaks no 8-19 separated through reverse phase C-18 column (150 x 4.6 mm; 4 μm particle size) using MeOH:H₂O:TFA (0.1%) gradient (80 to 95% MeOH for 50', 95% MeOH for 5') pumped at a flow rate of 1mL/min.
Figure 6.3(a): MALDI TOF MS/MS of [M+H]^+ at m/z 1435.8(P8), 1447.9(P19), 14449.8(P13) and 1463.8(P13)
Figure 6.3(b): MALDI-TOF-MS/MS of [M+H]^+ at m/z 1478.0 (P16), 1491.0 (P17) and 1505.8 (P19)
Figure 6.4 MALDI TOF MS of esterified and unesterified methanolic cyclic lipopeptide extract obtained from liquid broth of B. subtilis K1

The β- and γ- series of fragment ions could be easily assigned in the MALDI LIFT MS/MS spectra of linear protonated precursor ions \( m/z \) 1454.6, 1467.1, 1483.0 (corresponding to intact precursor ions \( m/z \) 1435.8, 1447.9 and 1464.0 intact precursor ions) (Figure 6.5(a-c)). Figure 6.6 shows the assignment of fragment ions formed from precursor ions, \( m/z \) 1454.7, \( m/z \) 1467.1 & \( m/z \) 1483.0 and sequence determination. The deduced sequence based on the fragmentation pattern is: β-OH FA-Glu<sup>1</sup>-Orn<sup>2</sup>-Tyr<sup>3</sup>-Thr<sup>4</sup>-Glu<sup>5</sup>-Ala<sup>6</sup>-Pro<sup>7</sup>-Gln<sup>8</sup>-Tyr<sup>9</sup>-Ile<sup>10</sup>. On the basis of the deduced sequence, the parent ions \( m/z \) 1435.8, 1447.9 & 1464.0 were assigned to be homologues of fengycin A with variation in the length of fatty acid (Vater et al., 2002; Wang et al., 2004; Hu et al., 2007). The \( b \)-ion series of corresponding precursor ions differed from each other by multiple of 14 Da while the \( y \)-ion series of all the corresponding precursor ions were found to be same. This variation in mass by multiple of 14 Da could be attributed to the difference in number of CH<sub>2</sub> residues in β-OH FA.
Figure 6.5(a): MALDI-TOF-MS/MS of linear [M+H]^+ ion at m/z 1454.7 obtained through saponification of cyclic peptide [M+H]^+ ion at m/z 1435.8 (P8)
Figure 6.5(b) : MALDI-TOF- MS/MS of linear [M+H]^+ ion at m/z 1467.1 obtained through saponification of cyclic peptide [M+H]^+ ion at m/z 1447.9 (P19)
Figure 6.5 (c) : MALDI-TOF-MS/MS of linear [M+H]⁺ ion at m/z 1483.0 obtained through saponification of cyclic peptide [M+H]⁺ ion at m/z 1463.8 (P13)
Figure 6.6: Sequence assignments of linear [M+H]$^+$ fengycin A homologues at \( m/z \) 1454.6, 1483.0 and 1467.1
Thus, the precursor ions $m/z$ 1435.7, 1447.9, 1464 may be assigned as Fengycin A homologues with 14, 15, and 16-C β-OH FA chain length, respectively. Similarly the [M+H]$^+$ ions at $m/z$ 1478.0, 1491.0 and 1505.8 fragmented into $m/z$ 966 and 1080 product ions (Fig.6.3b), characteristic of fengycin A family (Madonna et al., 2002; Wang et al., 2004) and thus may be assigned as fengycin A isoforms with 17, 18 and 19-C β-OH FA chains, respectively. The characteristic fengycin A product ions $m/z$ 966 and 1080 were also observed upon fragmentation of precursor ion $m/z$ 1449.9, however it differs from fengycin A homolog $m/z$ 1447.9 by just 2 Da, which could be explained by the presence of one double bond in the β-OH FA chain. However we could not determine the position of desaturation in the fatty acid chain in this homolog on the basis of mass spectrometric analysis. Table.6.2 shows the sequence summary of protonated precursor ions belonging to the class of fengycin A.

**Fengycin B**

The MALDI LIFT MS/MS spectra of all the precursor ions $m/z$ 1463.8, 1477.8, 1491.8, 1505.5 and 1520.1 showed characteristic product ion peaks $m/z$ 994 and 1108 (Figures.6.3(b-P17, P19) and 6.7) which result due to neutral loss of β-OH FA-Glu-1 ($y_9$) and β-OH FA-Glu –Orn ($y_8$) from the N-terminal end of lipopeptide, respectively. On the basis of characteristic product ion pairs, these precursors were putatively assigned as homologues of fengycin B (Wang et al., 2004). The product ions at $m/z$ 1474 and 1464 with 18 and 28 Da mass difference with respect to their parent ion ($m/z$ 1491.8) must have resulted due to loss of H$_2$O and CO$^+$ from the protonated precursor ion (M+H$^+$), thus suggesting the cyclic depsipeptide nature of the peptide. Similar product ions resulting from the loss of CO$^+$ and H$_2$O ions were also detected in CID spectra of precursor ions at $m/z$ 1463.8, 1477.8, 1491.8, 1505.5 and 1520.1(Figures.6.3(b-P17,P19) and 6.7). The ring cleavage due to breakdown of lactone bond resulted in 19 Da increase in the mass of precursor ions (upon saponification) $m/z$ 1477.8, $m/z$ 1491.8 , $m/z$ 1505.5 as observed for fengycin A homologues discussed above.
Figure 6.7: MALDI–TOF- MS/MS of [M+H]$^+$ at m/z 1463.8 (P16), 1477.8 (P17) and 1520.1 (P19)
Figure 6.8(a) : MALDI-TOF- MS/MS of linear [M+H]+ ion at m/z 1497 obtained through saponification of cyclic peptide [M+H]+ ion at m/z1477.9(P19)
Figure 6.8(b): MALDI-TOF- MS/MS of linear [M+H]+ ion at m/z 1511.2 obtained through saponification of cyclic peptide [M+H]+ ion at m/z 1491(P17)
Figure 6.8(b): MALDI-TOF-MS/MS of linear [M+H]$^+$ ion at m/z 1524.0 obtained through saponification of cyclic peptide [M+H]$^+$ ion at m/z 1505.8 (P17)
Figure 6.9: Sequence assignments of linear [M+H]^+ fengycin B homologues at m/z 14597, 1511.2, 1524.0
The b- and y-ions in the CID spectra of saponified linear precursor ions m/z 1497.0, 1511.2 and 1524.0 (corresponding to the intact precursor ions m/z 1477.8, 1491.8 and 1505.5) (Figure 6.8(a-c)) could be assigned so as to build a characteristic sequence of fengycin B: β-OH FA-Glu\(^1\)-Orn\(^2\)-Tyr\(^3\)-Thr\(^4\)-Glu\(^5\)-Val\(^6\)-Pro\(^7\)-Gln\(^8\)-Tyr\(^9\)-Ile\(^{10}\). The b-ion series of corresponding precursor ions differed from each other by multiple of 14 Da while the y-ion series of all the corresponding precursor ions were found to be same, suggesting the heterogeneity in the chain length of β-OH FA(6.9). Thus, precursor ions m/z 1463.8, 1477.8, 1491.8, 1505.5 and 1520.1 may be considered to be fengycin B homologues with β-OH FA chain length of 14, 15, 16, 17 and 18–CH\(_2\) units, respectively (Table-6.1).

**Fengycin A2:**

The MS/MS spectra of precursor ions m/z 1421.7, 1435, 1450 and 1464 also showed another pair of characteristic fragment ions m/z 1066 and 952 (Figures 6.3(a-P8,P13), 6.10) and thus represents a variant of fengycin A. The fragment ions m/z 1066 and 952 have a mass difference from corresponding characteristic marker ion pair of fengycin A (m/z 1080 and 966, respectively) by 14 Da suggesting a difference in amino acid sequence of octapeptide ring part of fengycin. Since the site of heterogeneity in peptide ring of fengycin family has been known to be at position 6 only between Ala (fengycin A) and Val (fengycin B), it tempted us to believe that the fengycin homologues with product ion pairs m/z 1066 and 952 probably contain Gly at position 6 in place of Ala/Val and constitute a novel fengycin class.

In order to confirm the sequence of proposed new fengycin class linearized precursor ions m/z 1440.7, 1454.6, 1469 and 1483 (corresponding to intact precursor ions m/z 1421.7, 1435.8, 1449.9 and 1464.0) obtained upon saponification were subjected to further fragmentation. The b’ and y’ ions in CID spectra of above mentioned linear ions were assigned and b’ ion series of corresponding precursor ions were found to vary by multiple of 14 Da while y’ ion series remained same (Figures 6.5(a and c), 6.11(a,b)). This further suggests that all these precursor ions represent same class of fengycin with variation in their β-OH FA chain length.
Figure 6.10 MALDI-TOF-MS/MS of [M+H]+ ion at 1421.7(P8)
Figure 6.11(a) : MALDI-TOF- MS/MS of linear \([\text{M+H}]^+\) ion at \(m/z\) 1440.7 obtained through saponification of cyclic peptide \([\text{M+H}]^+\) ion at \(m/z\) 1421.7 (P8)
Figure 6.11(b): MALDI-TOF- MS/MS of linear [M+H]^+ ion at m/z 1469.0 obtained through saponification of cyclic peptide [M+H]^+ ion at m/z 1449.9 (P14)
Figure 6.12: Sequence assignments of linear [M+H]⁺ fengycin A2 homologues at m/z 1440.7, 1454.6, 1469.0 and 1483.0
The sequence based on the assigned b’ and y’ ions could be determined as, β-OH FA-Glu^1^-Orn^2^-Tyr^3^-Thr^4^-Glu^5^-Ala^6^-Pro^7^-Gln^8^-Tyr^9^-Val^10 (Figure 6.12). To our surprise, our earlier assumption that fengycin homologues with characteristic product ion pairs \( m/z \) 1066 and 952 may contain Gly in place of Ala/Val at position 6 was not true. Rather, the sequence determination of this proposed new class of fengycin (designated as class A2 henceforth) showed an additional site for heterogeneity i.e at position 10. Thus fengycin A2 seems to be a variant of fengycin A with substitution of Ile^{10} with Val^{10}. The heterogeneity at 10th position amongst fengycins has been reported by Pueyo et al. (2009). The precursor ions as well as b’ ions of fengycin A_{2} homologs differed in mass by multiple of 14 Da suggesting a variation in β-OH FA chain length. The β-OH FA chains of \( m/z \) 1421.7, 1435.8, 1449.9 and 1464 consists 14, 15, 16 and 17 -CH\_2 units, respectively(Table 6.1).

**Fengycin B\_2**

The MS/MS spectra of precursor ions \( m/z \) 1449.7, 1461.9, 1464, 1478, 1492 and 1505.5 also showed a pair of characteristic fragment ions \( m/z \) 1094 and 980 (Figures (6.2(a), 6.13) apart from characteristic fragment ion pairs of fengycin A (\( m/z \) 1080 and 966), fengycin B (\( m/z \) 1108 and 994). These pair of characteristic product ions have been reported by Hu et al.(2007) suggesting their precursor to be a novel fengycin type containing Abu in place of Ala/Val at 6th position. The fragment ions \( m/z \) 1094 and 980 differ in mass from characteristic product ion pair of fengycin A (\( m/z \) 1080 and 966, respectively) by 14 Da suggesting a difference in amino acid sequence of octapeptide ring part of fengycin. The linearized precursor ions \( m/z \) 1481.1, 1483,1497 and 1511.1 (for corresponding intact fengycin ions \( m/z \) 1461.9, 1464.0,1478.0 and 1492) were obtained by lactone bond cleavage (Figure 6.14(a-d)). The b” & y” ion series were assigned in the CID spectra of aforementioned linearized precursor ions and the sequence deduced is: β-OH FA-Glu^1^-Orn^2^-Tyr^3^-Thr^4^- Glu^5^- Val^6^- Pro^7^-Gln^8^-Tyr^9^-Val^10 (Figure 6.15) representing a variant of fengycin B with heterogeneity at position 10 (Val in place of Ile).
Figure 6.13: MALDI–TOF MS/MS of [M+H]^+ at m/z 1461.9 (P19), 1464 (P14) and 1492.0 (P18)
Figure 6.14(a): MALDI-TOF-MS/MS of linear \([\text{M+H}]^+\) ion at \(m/z\) 1481.1 obtained through saponification of cyclic peptide \([\text{M+H}]^+\) ion at \(m/z\) 1461.9 (P19)

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Figure 6.14(b) : MALDI-TOF- MS/MS of linear [M+H]+ ion at m/z 1483 obtained through saponification of cyclic peptide [M+H]+ ion at m/z 1464.0 (P15)
Figure 6.14(c): MALDI-TOF-MS/MS of linear [M+H]^+ ion at m/z 1497 obtained through saponification of cyclic peptide [M+H]^+ ion at m/z 1478.0 (P16)
Figure 6.14(d): MALDI-TOF MS/MS of linear [M+H]$^+$ ion at m/z 1511.1 obtained through saponification of cyclic peptide [M+H]$^+$ ion at m/z 1492(P18)
Figure 6.15: Sequence assignments of linear [M+H]\(^{+}\) fengycin B2 homologues at m/z 1481.1, 1483, 1497 and 1511.1
This sub-class of fengycin B variants has been referred to as fengycin B2 henceforth in this chapter and consists of Val at position 6 as well as 10. The precursor ions as well as b" ions of fengycin B2 homologs differed in mass by multiple of 14 Da suggesting a variation in β-OH FA chain length. The fengycin B2 homologs \( m/z \) 1449.9, 1461.9, 1464, 1478.0, 1492.0 and 1505.5 consists β-OH FA chain with 14, 15, 15, 16, 17 and 18 –CH₂ units, respectively. The mass difference of 2 Da observed between precursor ions \( m/z \) 1461.9 and 1463.0 may be explained by presence of one double bond (-C=C-) in β-OH FA backbone of 1461.9 \( m/z \) (Table 6.1).

In this study, tandem mass spectrometric analysis of fengycin isomers revealed four distinct types of paired product ions which may be useful as marker ions for identification of fengycin isoforms. The two marker ions \( m/z \) 966-1080 & 994-1108 have been used for typing of fengycin A and Fengycin B (Wang et al., 2004). The additional product ions \( m/z \) 952-1066 & 980-1094 observed in this study were characterized to be variants of fengycin A and fengycin B, respectively. The occurrence of fengycin homologues with product ions \( m/z \) 980-1094 were also reported by Madonna et al., (2003) and Hu et al., 2007. However Madonna et al (2003) could not describe the sequence of these homologs of fengycin while Hu et al (2007) proposed it to be a new form of fengycin consisting of amino butyric acid at position 6. In the present study, fengycin homologs which fragmented into \( m/z \) 952-1066 marker ions contained Ala⁶ and Val¹⁰, which seems to be the variant of fengycin A (due to substitution of Ile¹⁰ to Val¹⁰) while fengycin homologues, which fragmented into \( m/z \) 980-1094 marker ions contained Val⁶ and Val¹⁰ may be considered as a variant of fengycin B (due to substitution at position 10). These variants of fengycin A and B have also been proposed by Pueyo et al. (2009) in culture supernatants of B. megaterium. The sequences of all four fengycin groups produced by B. subtilis K1 is summarized in Table 6.1 and figure 6.16.
Table 6.1 Summary of fengycin homologues obtained in this study

<table>
<thead>
<tr>
<th>Mass</th>
<th>Typical product ions (Intact ms/ms)</th>
<th>Sequence (obtained through saponification)</th>
<th>Type of compound</th>
</tr>
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<tbody>
<tr>
<td>1421.0</td>
<td>952,1066</td>
<td>$R'_{(C14)}$EOrnYTEA$\Delta$PEYV-OH ($R'$-228)</td>
<td>(C14) Fengycin A2</td>
</tr>
<tr>
<td>1435.0</td>
<td>952,1066,966,1080</td>
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<td>(C15) Fengycin A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$R'_{(C14)}$EOrnYTEA$\Delta$PEYI-OH ($R'$-228)</td>
<td>(C14) Fengycin A</td>
</tr>
<tr>
<td>1447.9</td>
<td>966,1080</td>
<td>$R'_{(C-15)}$EOrnYTEA$\Delta$PEYI-OH ($R'$-240)</td>
<td>(C15) Fengycin A</td>
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<td></td>
<td></td>
<td>Single(=) one double bond at BOH FA</td>
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<td>952,1066</td>
<td>$R'_{(C16)}$EOrnYTEA$\Delta$PEYV-OH ($R'$-256)</td>
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<td>1519.8</td>
<td>994,1108</td>
<td>$R'_{(C-18)}$EOrnYTEVPEYI-OH ($R'$-284)</td>
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</table>
The variation of Ala to Val or Val to Ile could be possible as all three have similar chemical properties, i.e., all of them are non polar, hydrophobic and possess a short side chain. Furthermore, many peptide synthetases, including those for surfactin (Baumgart, 1999), cyclosporine (Traber et al., 1989), lycenisin (Konz et al. 1999) synthesis, exhibit relaxed substrate specificity and are capable of activating amino acids having similar chemical characteristics, thus resulting in microheterogeneity amongst these peptides.

Figure 6.16: Characteristic fragmentation pattern represents the four different types of marker ions under tandem mass spectrometry of four classes of fengycin homologues.
6.4 Summary

*Bacillus subtilis* K1 was found to produce mixture of fengycin homologues, which could be assigned to four different classes, viz., fengycin A, A2, B and B2, on the basis of aminoacid variation at 6th and 10th position in the peptide moiety of fengycin molecule. The sequences derived by MALDI-TOF-MS/MS are summarized in table 6.1 and figure 6.16. The heterogeneity within a particular class of fengycin was due to variation in fatty acid chain length. An interesting observation made during this study was that two different fengycin homologues varying in fatty acid chain by one –CH₂ unit, may still have same molecular mass as a result of substitution of ala to val at position 6 or ile to leu at position 10 of peptide ring.

6.5 References:


Hu, LB., Shi, ZO., Zhang, T., Yang, ZM. (2007) Fengycin antibiotics isolated from B-FSO1 culture inhibit the growth of Fusarium moniliforme Sheldon ATCC38932. FEMS Microbiology Letters, 272, 91-98.


