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Purification of Peptides from *Bacillus* Strains with Biological Activity

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1. Introduction

One of the greatest causes of loss in the food industry is postharvest diseases of fruits and vegetables (Vero Mendez & Mondino, 1999). According to U.S.A. estimations this loss reaches 20 - 25% whereas in developing countries the losses are often more severe due to inadequate storage and transportation facilities (Sharma et al., 2009), but loss has generally been considered to be approximately 10% to 40% depending on packinghouse technology (Vero et al, 2002).

The surface of the fruit or vegetables is covered with fungal spores, bacterial cells and yeasts, which they have acquired from the air during their development on the parent plant, or which they have come in contact during picking or any of the stages of handling the harvested produced. However not every fungal spore or bacterial cell can develop and cause decay in the harvested product, even when conditions suitable for penetration and development are present.

Harvested fruit and vegetables are naturally attacked by own typical pathogens. Fungi are the principal decaying agents in fruit kept in cold storage chambers for long periods (Teixidó et al., 2001).

Fruits of tropical and subtropical origin (mango, papaya, avocado, etc.) are attacked by *Colletotrichum gloesporoides*, which cause actracnose. *Gloesporium musae* attacks banana fruits at the orchard, which becomes active only during the storage.

Diplodia natalensis, *Phomopsis citri* or *Dothiorella gregaria* invade the cut stem of tropical and subtropical fruit. *D. natalensis, Alternaria citri* and *P. citri* the causal agent of postharvest stem-end rot of citrus fruits.

During and after harvest, the citrus fruits are typically attacked by *Penicillium italicum* (blue mold), *Penicillium digitatum* (green mold), *Geotrichum candidum* (sour rot), *Alternaria citri* (black mold) and *Fusarium* sp. (Wilson & Wisniewski, 1989). *P. digitatum* is an example of a specific fungus that attacks only citrus fruits. *P. italicum* can attack other fruits and vegetables, whereas *P. expansum*, apple and pear pathogen, naturally attack citrus fruits.

P. expansum, Botrytis cinerea, Gleosporium spp. *Alternaria alternata* and *Stemphylium botryosum* are typical apple and pear pathogens. The main pathogens of peaches, apricots, nectarines and plums are *Monilia fruticola* and *Rhizopus stolonifer*.

Each harvested fruits and vegetables has its own group of characteristic pathogens to which is susceptible and for which its serves as suitable host.

Strawberries are attacked during the storage by the gray mold fungus (*B. cinerea*) and the soft watery rot fungus (*R. stolonifer*).

Alternaria alternata is the major storage decay agent of harvested tomatoes. The main causal agents of soft watery rot in harvested tomatoes are *R. stolonifer, G. candidum* and *Erwinia* spp. (Barkai Golan, 2001).

Postharvest fruit diseases are controlled with careful manipulation practices and synthetic fungicides like 2- 4 thiazalil benzimidazole and imidazole. This method is more widely used against fungal decay because of its low cost and easy application. However, it presents manifold objections since prolonged use generates resistance to synthetic fungicides by major postharvest pathogens (Wilson & Wisniewski, 1994; Fogliata et al., 2001) and increases chemical remainders in fruits with the consequent potentiality engendering iatrogenic diseases (Lingk, 1991). In addition, world trends are moving towards reduced pesticide use in fresh fruit and vegetables. Along with this trend, several physical and biological means have been evaluated as safer alternatives for the use of chemical fungicides. The use of microbial antagonists for the control of postharvest diseases received special attention, and has been extensively investigated (Droby, 2006).

Most of the reported yeast and bacteria antagonists were naturally occurring on fruit surfaces. Microbial biocontrol agents of postharvest diseases have been criticized mainly for not providing as consistent or broad-spectrum control as synthetic fungicides. The "first generation" of biological controls agents for postharvest spoilage relied on the use of single antagonists. Perhaps it is unrealistic for us to expect disease control comparable to synthetic fungicides by the use of single antagonists. It can be expected that enhancing efficacy of biocontrol agents of postharvest diseases to an acceptable level would utilize a combination of different biological and physical means (Droby, 2006).

The mechanism(s) by which microbial antagonists exert their influence on the pathogens has not yet been fully understood. It is important to understand the mode of action of the microbial antagonists because; it will help in developing some additional means and procedures for better results from the known antagonists. It will also help in selecting more effective and desirable antagonists or strains of antagonists (Wilson & Wisniewski, 1989; Wisniewski & Wilson, 1992).

Several modes of action have been suggested to explain the biocontrol activity of microbial antagonist. Still, competition for nutrient and space between the pathogen and the antagonist is considered as the major modes of action by which microbial agents control pathogens causing postharvest decay (Filonow, 1998; Ippolito et al., 2000; Jijakli et al., 2001). In addition, production of antibiotics (antibiosis), direct parasitism, and possibly induced resistance are other modes of action of the microbial antagonists by which they suppress the activity of postharvest pathogens on fruits and vegetables (Janisiewicz et al., 2000; Barkai-Golan, 2001; El-Ghaouth et al., 2004).

2. Lipopeptides produced by *Bacillus* strain

Members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogens growth, such as kanosamine or zwittermycin A from *B. cereus*. Their spore-forming ability also makes these bacteria some of the best candidates for developing efficient biopesticide products from a technological point of view. *Bacillus* spores have a high level of resistance to the dryness necessary for formulation into stable products. *Bacillus subtilis* strains are a rich source of antimicrobial peptides with a high potential for biological control applications.

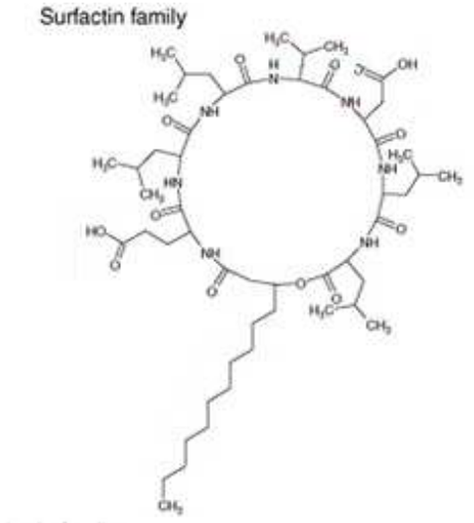
Bacillus subtilis has been used for genetic and biochemical studies for several decades, and is regarded as paradigm of Gram-positive endospore-forming bacteria (Moszer et al., 2002). Several hundred wild-type *B. subtilis* strains have been collected, with the potential to produce more than two dozen antibiotics with an amazing variety of structures. All of the genes specifying antibiotic biosynthesis combined amount to 350 kb; however, as no strain possesses them all, an average of about 4–5% of a *B. subtilis* genome is devoted to antibiotic production. Peptide antibiotics, also named lipopeptides, represent the predominant class. They exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D-amino acids and are generally resistant to hydrolysis by peptidases and proteases (Katz & Demain, 1977). Furthermore, cysteine residues are either oxidized to disulphides and/or are modified to characteristic intramolecular C–S (thioether) linkages, and consequently the peptide antibiotics are insensitive to oxidation (Stein, 2005).

Bacillus lipopepdides are synthesized non-ribosomally via large multi-enzymes (non-ribosomal peptide synthetases, NRPSs) (Kowall et al., 1998; Stein, 2005, Finking & Marahiel, 2004). These biosynthetic systems lead to a remarkable heterogeneity among the lipopeptides products generated by *Bacillus* with regards to the type and sequence of amino acid residues, the nature of the peptide cyclization and the nature, length and branching of the fatty acid chain (Ongena & Jacques, 2007).

Lipopeptides are classified into three families depending on their amino acid sequence: iturins, fengycins and surfactins (Fig. 1) (Perez García, et al., 2011). The surfactins are powerful biosurfactants, which show antibacterial activity but no marked fungitoxicity (with some exceptions) (Ongena & Jacques, 2007).

Surfactin, a cyclic lipopeptide is one of the most effective biosurfactants know so far, which was first reported in *B. subtilis* ATCC-21332. Because of its exceptional surfactant activity it is named as surfactin. Surfactin can lower the surface tension from 72 to 27.9 mN/m and have a critical micelle concentration of 0.017 g/L. The surfactin groups of compounds are shown to be a cyclic lipoheptapeptide which contain a β - hidroxy fatty acid in its side chain. Recent studies indicate that surfactin shows potent antiviral, antymicoplasma, antitumoral, anticoagulant activities as well as inhibitors of enzymes. Although, such properties of surfactins qualify them for potential applications in medicine or biotechnology, they have not been exploited extensively till date.

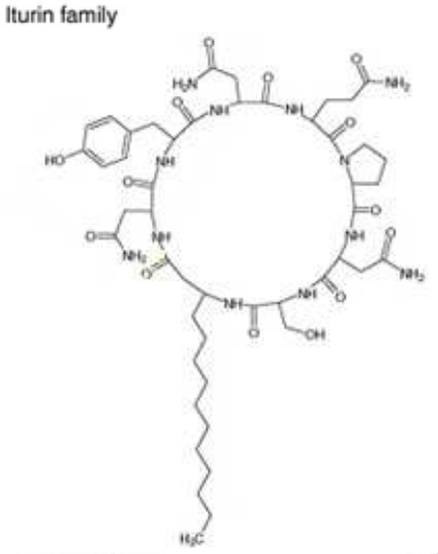
Lichenysin, produced by *Bacillus licheniformis* exhibited similar structure and physicochemical properties to that of surfactin. *B. licheniformis* also produced several other surface active agents which act synergistically and exhibit temperature, pH and salt stability. Lichenysin A produced by *Bacillus licheniformis* strain BAS50, is characterized to



Variants

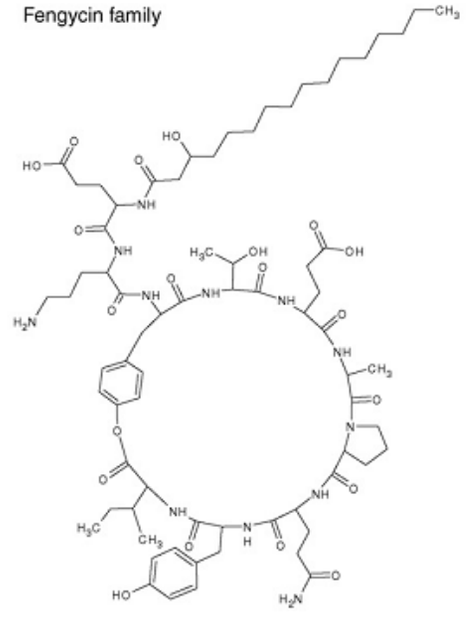
Esperin**	**L-Glu-L-Leu**-D-Leu-**L-Val**-L-Asp-D-Leu-**L-Leu-COOH**
Lichenysin***	**L-XL_1-L-XL_2**-D-Leu-**L-XL_4**-L-Asp-D-Leu-**L-XL_7**
Pumilacidin	**L-Glu-L-Leu**-D-Leu-**L-Leu**-L-Asp-D-Leu-**L-XP_7**
Surfactin	**L-Glu-L-XS_2**-D-Leu-**L-XS_4**-L-Asp-D-Leu-**L-XS_7**

** the β-carboxyl of Asp_3 is engaged in the lactone
*** or halobacillin
XL_1 = Gln or Glu ; XL_2 = Leu or Ile ; XL_4 and XL_7 = Val or Ile ;
XP_7 = Val or Ile ;
XS_2 = Val, Leu or Ile ; XS_4 = Ala, Val, Leu or Ile ; XS_7 = Val, Leu or Ile



Bacillomycin D	**L-Asn**-D-Tyr-D-Asn-**L-Pro-L-Glu-D-Ser-L-Thr**
Bacillomycin F	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Thr**
Bacillomycin L	**L-Asp**-D-Tyr-D-Asn-**L-Ser-L-Gln-D-Ser-L-Thr**
Bacillomycin LC*	**L-Asn**-D-Tyr-D-Asn-**L-Ser-L-Glu-D-Ser-L-Thr**
Iturin A	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**
Iturin A_L	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**
Iturin C	**L-Asp**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**
Mycosubtilin	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Ser-L-Asn**

* or bacillopeptin



Fengycin A**	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**L-Tyr**-L-Ile
Fengycin B**	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**L-Tyr**-L-Ile
Plipastatin A	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**D-Tyr**-L-Ile
Plipastatin B	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**D-Tyr**-L-Ile

** double bond between carbons 2-3, 3-4 or 13-14 were reported for some acyl chains

Fig. 1. Structures of representative members and diversity within the three lipopeptides families synthesized by *Bacillus* species. Boxed structural groups are those that were shown

to be particularly involved in interaction with membranes and/or are supposed to be important for biological activity in addition to the cyclic nature of the molecule (Peypoux, et al, 1999; Bonnatin et al., 2003; Dufour et al., 2005; Ongena & Jacques, 2007).

contain a long chain beta-hydroxy fatty acid molecule. Iturin A, the first compound discovered of the iturin group and its best known member, was isolated from a *Bacillus subtilis* strain taken from the soil in Ituri (Zaire) and its structure was elucidated. The subsequent isolation from other strains of *Bacillus subtilis* of five other lipopeptides such as iturin A_L, mycosubtilin, bacillomycin L, D, F and L_C (or bacillopeptin) was reported (Ongena & Jacques, 2007). All have a common pattern of chemical constitution, led to the adoption of the generic name of "iturins" for this group of lipopeptides. The iturin groups of compounds are cyclic lipoheptapeptides which contain a β-amino fatty acid in its side chain. Lipopeptides belonging to the iturin family are potent antifungal agents which can also be used as biopesticides for plant protection.

Fengycin is a lipodecapeptide containing β-hydroxy fatty acid in its side chain and comprises of C_{15} to C_{17} variants which have a characteristic Ala-Val dimorphy at position 6 in the peptide ring. Wang et al. (2004) demonstrated the identification of fengycin homologues produced by *B. subtilis* by using electrospray ionization mass spectrometry (ESI-MS) technique.

These antibiotics are either cyclopeptides (iturins) or macrolactones (fengycins and surfactins) characterized by the presence of L and D amino acids and variable hydrophobic tails. Iturins display strong antifungal action against a wide variety of yeasts and fungi but only limited antibacterial activity. Fengycins also show a strong fungitoxic activity, specifically against filamentous fungi (Ongena & Jacques, 2007). The ability of various *Bacillus* strains to control fungal soil borne, foliar and postharvest diseases has been attributed mostly to iturins and fengycins (Ongena & Jacques, 2007; Romero et al., 2007; Arrebola et al., 2010).

The surfactin family encompasses structural variants but all members are heptapeptides interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure (Peypoux et al., 1999). Because of their amphiphilic nature, surfactins can also readily associate and tightly anchor into lipid layers and can thus interfere with biological membrane integrity.

Iturin A and C, bacillomycin D, F, L and LC and mycosubtilin were described as the seven main variants within the iturin family. They are heptapeptides linked to a β-amino fatty acid chain with a length of 14 to 17 carbons. The biological activity of iturins is different to surfactins: they display a strong in vitro antifungal action against a wide variety of yeast and fungi but only limited antibacterial and no antiviral activities (Moyne et al., 2001; Phae et al., 1990). This fungitoxicity of iturins almost certainly relies on their membrane permeabilization properties (Deleu et al., 2003).

However, the underlying mechanisms based on osmotic perturbation owing to the formation of ion-conducting pores and not membrane disruption or solubilization as caused by surfactins (Aranda et al., 2005).

3. Purification and identification of lipopeptides

Chemical and structural analysis of lipopeptides is carried out using a broad range of techniques varying from simple colorimetric assays to sophisticated mass spectrometry (MS) and sequencing techniques.

After extraction, the purification procedures of lipopeptides included chromatography methods (TLC, Ion Exchange Chromatography and RP-HPLC). Each step of purification will be monitored by bioassays. The bioassays could be bioautographic methods, dual culture plate, etc.

Generally, identification of the relative percentage of the lipid and protein portions is carried out using simple colorimetric assays, such as Bradford assay for protein determination and spectroscopic methods (FTIR). The molecular mass determination of the compounds of interest may be facilitated by mass spectrometry using assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS).

This should be followed by analysis of the fatty acid portion and determination of the peptide sequence using automated Edman degradation sequencing. This combined approach would provide the necessary information required for complete structural identification.

3.1 Purification of lipopeptides

3.1.1 Extraction and Thin Layer Chromatography (TLC)

Different solvents are used for extraction of lipopeptides from cell free supernatant. The solvents used are n-hexane, ethyl acetate, petroleum ether, chloroform and methanol, to determine the best solvent for extraction of antifungal compound (Kumar et al., 2009). Yazgan et al., 2001 and Romero et al., 2007 used n-butanol for lipopeptides extraction.

The lipopeptide produced by cultures of *Bacillus mojavensis* strain ROB-2 was used to compare the efficiencies of two purification methods. Method 1 involved acid precipitation using 1 N HCl to adjust the pH of the cell-free culture fluid to 2 (Yakimov et al, 1995; Mc Keen et al., 1986) followed by TLC. Method 2 used ammonium sulfate precipitation (40%) followed by acetone extraction and TLC (Youssef et al., 2005).

Seventy-five percent of the biosurfactant activity remained in the cell-free culture fluid after cell removal. The surface-active fraction obtained from the TLC plate by method 1 had 23% ± 7% of the activity originally presents in the culture, while the surface-active fraction obtained from the TLC plate by method 2 had 63% ± 11% of the activity originally presents in the culture (Youssef et al., 2005).

In order to identify the compounds responsible for antimicrobial activity, extracts of cell-free culture filtrates *Bacillus* strains generally are separated in TLC sheets, using purified iturin A, fengycin, and surfactin as standards. Thin layer chromatography is a simple method, which can be used to detect the presence of lipopeptides while preparative TLC can be used to purify small quantities (Symmank et al., 2002).

Once the butanol layer completely evaporated in vacuum at 40^{o} C or the acid precipitation is neutralized, the residue is dissolved in methanol for further chemical analysis. The methanolic fractions are analyzed using TLC (Razafindralambo et al., 1993) with direct view developed using distilled water spraying. A white spot formed with the same Rf value when the plate was sprayed with water, indicating that the compound is lipophilic.

Cell free supernatant of *Bacillus subtilis* UMAF6614, UMAF6619, UMAF6639 and UMAF8561 and *Bacillus amyloliquefaciens* PPCB004 were evaluated by TLC and the spots with Rf values

similar to the standards fengycin (0.09), iturin A (0.3), and surfactin (0.7) (Romero et al., 2007 and Arrebola et al., 2010). To determine which lipopeptides were directly involved in fungal inhibition, the bioautographies were performed using the pathogens as revealing microorganism. It was found that the principal inhibitor was iturin A, which affected all fungi analyzed in this study. Fengycin was also identified as an inhibitor of *Lasiodiplodia theobromae, Botryosphaeria* sp., *C. gloeosporioides, Fusicoccumaromaticum* and *Phomopsis persea.* Surfactine was able to inhibit *L. theobromae*, although slight inhibition of *F. aromaticum* and *P. persea* was also observed (Fig. 2) (Arrebola et al., 2010).

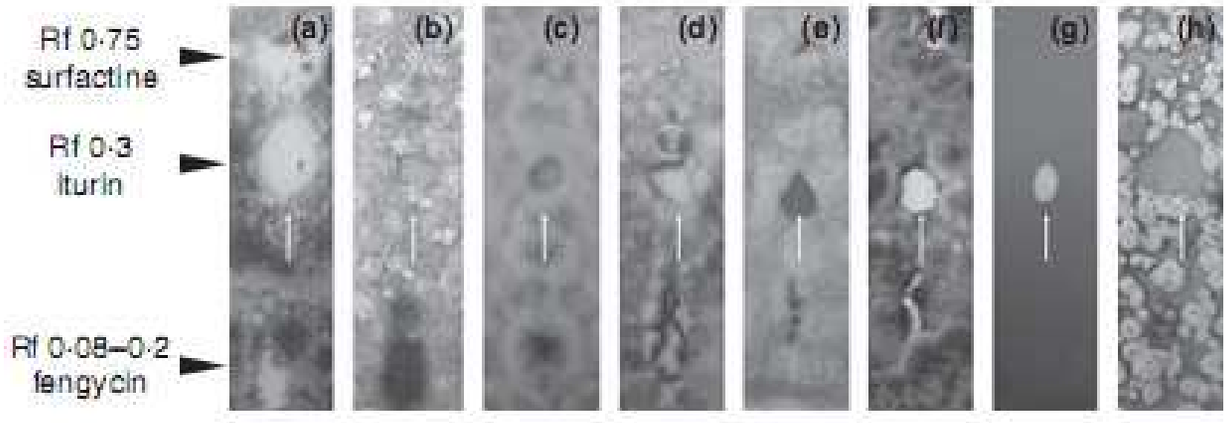


Fig. 2. Bioautographic analysis on thin-layer chromatography plates where lipopeptide extracts from *Bacillus amyloliquefaciens* PPCB004 were separated and the Rf of every lipopeptide are indicated. (a) *Lasiodiplodia theobromae*, (b) *Alternaria citri,* (c) and (d) *Botryosphaeria* sp., (e) *Colletotrichum gloeosporioides*, (f) *Fusicoccum aromaticum*, (g) *Penicillium crustosum*, (h) *Phomopsis persea*. Fungal inhibition zones produced by iturin A activity is indicated with white arrows.

The concentrated extracts from *Bacillus* MZ-7 run on silica TLC plates showed six bands under UV light, having Rf values of 0.1, 0.15, 0.26, 0.37, 0.51 and 0.57. However, a plate bioassay showed two active fractions, those with Rf values of 0.37 and 0.51. The spot with an Rf value of 0.51 was ninhydrin negative and positive to 4,4'-bis (dimehtylamino) diphenylmethane (TDM) reagent. These results indicated the absence of free amino groups and the presence of peptide. The migration and chemical properties of the compound were comparable to surfactin produced by *B. subtilis* strain ATCC 21332. The environmental isolate *B. subtilis* MZ-7 produced more surfactin (170.5 mg/L) than did *B. subtilis* ATCC 21332 (109.5 mg/L) under the same conditions (Mutaz et al., 2007).

Maldonado et al., 2009 run silica gel plates 60 F254 (Merck, 2 mm) and are carried out with a chloroform–methanol–acetic acid (40:4:1) mixture (Batrakov et al., 2003). Plates are developed under UV light at 254 and 365 nm and only one spot of Rf 0.67 is detected (Kumar et al., 2009). The inhibitory activity of the spot was confirmed after TLC by bioautographic assay. Besides the TLC plates were developed with ninhydrin and no spot was observed. Thus, a peptide without free amino groups (cyclic structure) may be presumed (Fig. 3).

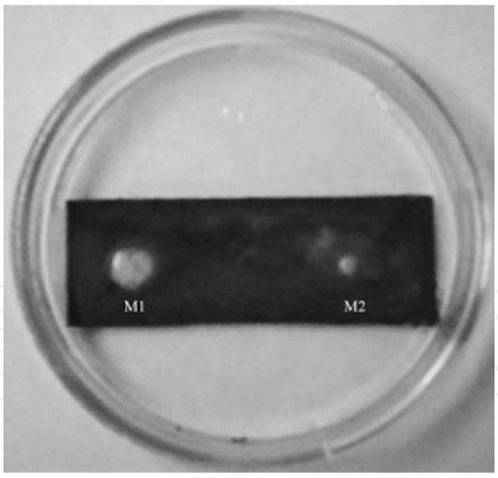


Fig. 3. Bioautographic analysis on thin-layer chromatography plates where lipopeptides extracts from *Bacillus* IBA 33 against *Geotrichum candidum*. M_1 and M_2 correspond to peaks obtained from DEAE Sephacel chromatography.

3.1.2 Ion exchange chromatography and RP-HPLC

The development of efficient HPLC methods generally needs relatively pure biosurfactant samples, which cannot be obtained without tedious isolation and purification operations.

Other technique frequently employed for the characterization and quantification of biosurfactants has been DEAE ion exchange chromatography. This analysis can provide qualitative information about biosurfactants.

The crude extract was applied to a low-pressure chromatography column (Bio-Rad Laboratories, Hercules, CA) filled with DEAE-Sephacel (Pharmacia,Uppsala, Sweden) equilibrated in 50 mM Tris-HCl, pH 7.5. A gradient of 0-0.4 M NaCl in buffer (50 mM, pH 7.5) was run through the column at 0.5 mL/min over a period of 160 min followed by straight 0.4 M NaCl in buffer (50 mM, pH 7.5) for 40 min. The eluent was monitored at 280 nm, and fractions were collected every 5 min (Bechard et al., 1998).

They demonstrated that the antibiotic was retained by DEAE-Sephacel quite strongly but eluted from the matrix in 0.4 M NaCl. Ion exchange chromatography separated the crude antibiotic sample into four peaks. Antibiotic activity corresponds with the fourth and largest peak on the chromatogram. Only 23% of the initial activity was recovered by ion exchange chromatography, and the specific activity actually decreased relative to that in previous purification step. A complete loss of activity was observed when the antibiotic was left in the ion exchange eluent overnight. For this reason the ion exchange protocol was carried out as quickly as possible at 4 °C (Bechard et al., 1998) (Fig.4).

Wang et al., 2004 loaded the crude extract on a DEAE-Sepharose Fast Flow column pre-equilibrated with buffer A (20 mmol/L Na_2HPO_4-NaH_2PO_4 pH 6.5). After washing the column with buffer A, buffer B (20 mmol/L Na_2HPO_4-NaH_2PO_4 1 mol/L $(NH_4)_2SO_4$ pH 6.5) was used to elute the fraction containing fengycin homologues and this fraction was directly loaded on a SOURCE 15 PHE hydrophobic interaction column pre-equilibrated with buffer B. Most impurities were washed by buffer A and fengycin homologues were finally eluted by Milli Q water. After adjusted to 30% acetonitrile/water by adding acetonitrile, this fengycin-containing fraction was ready for ESI mass spectrometry analysis.

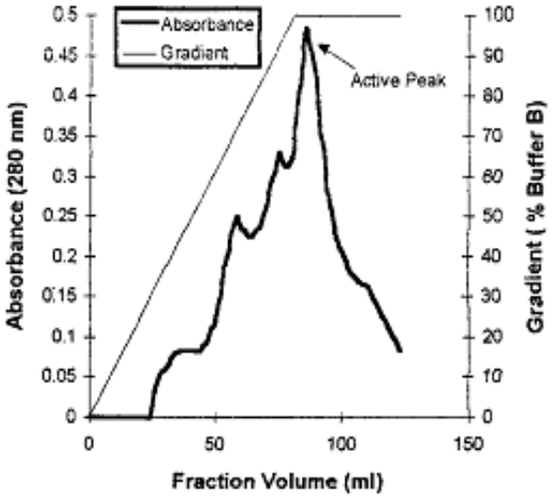


Fig. 4. Elution pattern of the HCl precipitate from DEAE-Sephacel equilibrated in 50 mM Tris buffer, pH 7.5 (buffer A). The peptide was eluted from the column with a gradient of 0.100% buffer B (50 mM tris buffer, pH 7.5, containing 0.4 M NaCl). Flow rate 0.5 mL/min.

Bin Hu et al., 2007 collected the extract, boiled for half an hour, centrifuged at 10.000 g for 10 min and then applied to a DEAE-52 column (16 mm 15 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.05 M NaCl. Antifungal fractions were obtained from the elution with 0.5–0.7 M NaCl in the same buffer, concentrated by ultrafiltration with a PM10 membrane (Amicon) and placed onto a Sephadex G-100 gel column (15 mm 80 cm). The column was equilibrated with 10 mM ammonium acetate buffer and eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions containing antifungal active compounds were collected, concentrated and lyophilized. Through these purification steps of antifungal active compounds, fractions were determined by the absorbance at 280 nm and the anti *F. moniliforme* fractions underwent further processing.

Maldonado et al. (2009) precipitated the extracted from *Bacillus* IBA 33 with ammonium sulfate 40%, then loaded onto a DEAE-Sephacel (1.5 cm 9.15 cm) column previously equilibrated with 50 mM Tris–HCl buffer pH 7.5 (Bechard et al., 1998). After purification, the chromatogram showed two inhibition peaks in a DEAE-Sephacel column. The first one, exhibited 77% and the second peak had 64% inhibition against *G. candidum* (Fig.5).

HPLC is an excellent method for the separation of lipopeptides (Aguilar, 2004). The most commonly employed technique is reversed phase chromatography, which results in the separation of each lipopeptide structure based on polarity. The separated products are detected by UV absorbance detection and each individual peak can be collected using a fraction collector for further analysis of their structure. Coupling of HPLC with a mass spectrometer provides preliminary information on the molecular mass of each component. Purification with either HPLC-UV or HPLC-MS using different types of column chemistry is also possible.

Gueldner et al., 1988 assayed the crude material dissolved in 50:50 methanol-water, and the solution was chromatographed on a column of C-18 reversed-phase absorbent (Waters Prep Pak 500). Elution with a stepwise gradient of methanol and water (from water up to 80% methanol-water) eluted most of the lipopeptides. Further purification was achieved by

droplet counter current chromatography (DCCC) (Buchi B-670) using chloroform-methanol-water (7:13:8) in the descending mode with the chloroform layer as the mobile phase. The main peaks eluted in 300-500 mL of the chloroform layer. The active peptides started to elute from the reversed-phase adsorbent at 65-70% ethanol-water and were completely eluted with 80% methanol - 20% water. Bioassay of the C-18 column fractions with *M. fructicola* showed activity in fractions that contained several peaks as analyzed by HPLC (210 nm). Five HPLC peaks having a profile very similar to those published by Isogai et al. (1982) for *B. subtilis* metabolites and collected from an analytical C-18 column were also active in the *M. fructicola* bioassay. Peak 1 (PKl), corresponding to Iturin A-2.

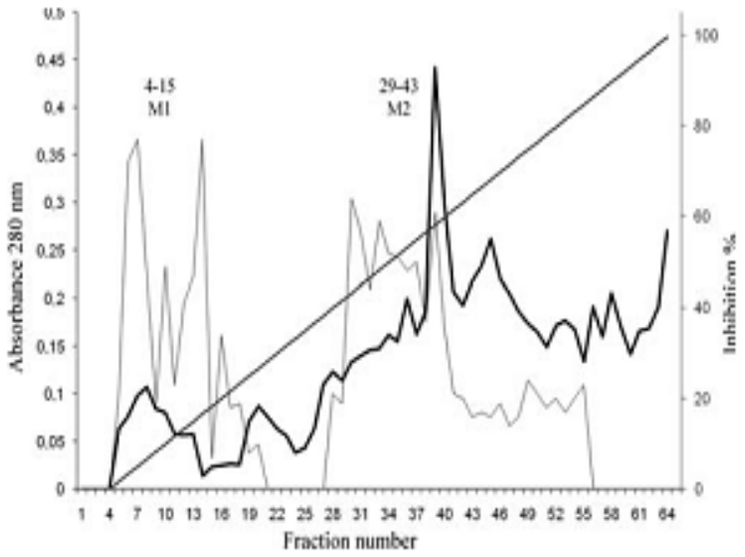


Fig. 5. *Bacillus* sp. IBA 33 antifungal active compounds (AAC) purification step by DEAE-Sephacel chromatography. Dark line: protein (absorbance at 280 nm), light line: AAC (% inhibition).

Mutaz et al., 2007 realized quantitative analysis of surfactin by HPLC active fractions from TLC were further purified by reversed-phase HPLC, using a Thermo Hypersil-Keystone ODS (particle size, 5 µm; column dimensions, 250 by 4.6 mm; Thermo Hypersil, USA). A sample was applied with eluent A (0.1% (vol/vol) trifluoroacetic acid and 20% (vol/vol) acetonitrile) and eluted with segmented gradients of eluent B (0.1% (vol/vol) trifluoroacetic acid and 80% (vol/vol) acetonitrile) as follows: 40% eluent B for 30 min and 40 to 100% eluent B for 10 min. The concentration of surfactin was determined from a calibration curve made by correlating the emulsification index (E 24) with known amounts of surfactin produced by *B. subtilis* ATCC 21332.

Romero, et al., 2007 analyzed first by TLC (Razafindralambo et al. 1993) and afterward by RP-HPLC, using an analytical Zorbax C18 column, 4.6 mm in diameter by 150 mm long (Agilent, Palo Alto, CA, U.S.A.) and solutions of 0.05% trifluoroacetic acid in acetonitrile and in water, with a flow rate of 1 ml/min. The different groups of peaks from butanolic extracts were fractionated by Flash chromatography as described earlier (Deleu et al., 1999; Razafindralambo et al., 1993), followed by (semi)-preparative RP-HPLC using a Vydak C18 column, 22 mm in diameter by 250 mm long (Separations Group, Hesperia, CA, U.S.A.) and the solutions mentioned above with a flow rate of 23 ml/min.

RP-HPLC analysis showed three main groups of peaks were observed at elution times comparable with those observed for standard lipopeptides that correspond to iturin A, fengycin and surfactin (Romero et al., 2007).

Arrebola et al., 2010 performed the analysis by reverse-phase HPLC (RP-HPLC) (Romero et al., 2007) using extracts from *Bacillus subtilis* UMAF6614 and UMAF6639 as standards of lipopeptides production.

The lipopeptide concentration was calculated using the Folin Ciocalteu's reaction (Swain & Hillis, 1959; Harborne, 1984). The results from RP-HPLC analysis showed three main groups of compounds that correspond to bacillomycin, produced by UMAF6614 and iturin A fengycin and surfactin by UMAF6639 (Fig. 6).

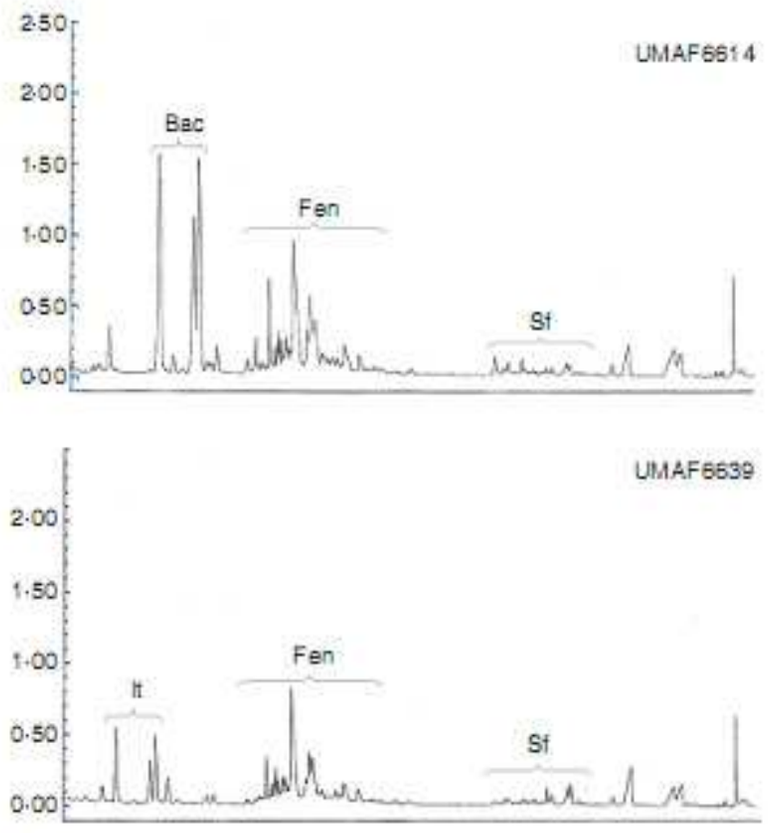


Fig. 6. Reverse-phase high-performance liquid chromatography analysis of lipopeptides produced by *Bacillus subtilis* strains UMAF6614 and UMAF6639. Peaks corresponding to iturin (It), bacillomycin (Bac), fengycin (Fen) and surfactin (Sf) are indicated. Detection is at 214 nm.

3.2 Identification of lipopeptides

3.2.1 Ultraviolet spectrum

The ultraviolet absorbance spectrum of the antibiotic was measured from the HPLC chromatogram using a Waters 990 photodiode array detector. This device allowed for the simultaneous measurement at all wavelengths from 200 to 600 nm as the antibiotic eluted from the column. The absorbance spectrum for the lipopeptide is measured in

acetonitrile/1% acetic acid (68:32) between 200 and 600 nm. The antibiotic shows absorbance maxima at 235, 278, and 285 nm (Fig.7), and there is no appreciable absorbance above 300 nm (Bechard et al., 1998).

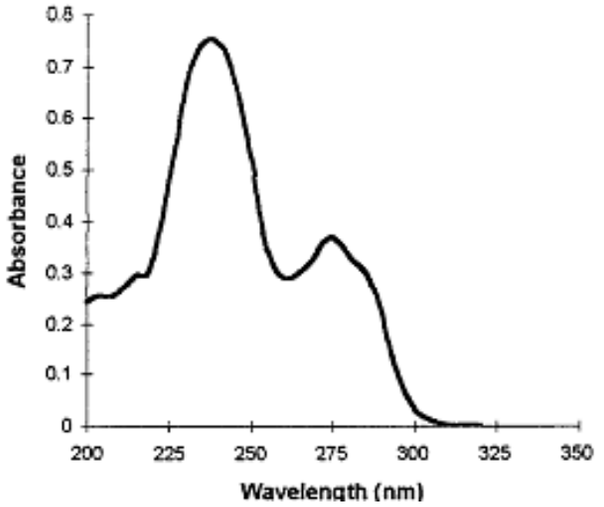


Fig. 7. Ultraviolet spectrum of the peptide antibiotic inacetonitrile/1% acetic acid (68:32).

Maldonado et al, 2009 resuspended the samples (M_1 and M_2) obtained from *Bacillus* IBA in HEPES buffer 10 mM NaCl 100 mM pH 7.1 to carry out absorption spectra between 250 and 280 nm wavelengths (Beckman DU 7500). They observed the same absorption bands for both samples. Hence, this strain might be a producer of cyclic lipopeptides with antifungal activity belonging to the iturin family. When the wavelength scanning was performed between 250 and 380 nm, they founded absorbance maximums at 280 nm for both samples (Fig. 8 a, b). Furthermore they might infered that the antifungal compounds have tyrosine or tryptophan or both in their composition (Nelson & Cox, 2006). Bechard et al., 1998 reported an absorbance maximum between 210 and 230 nm which they thought was due to the

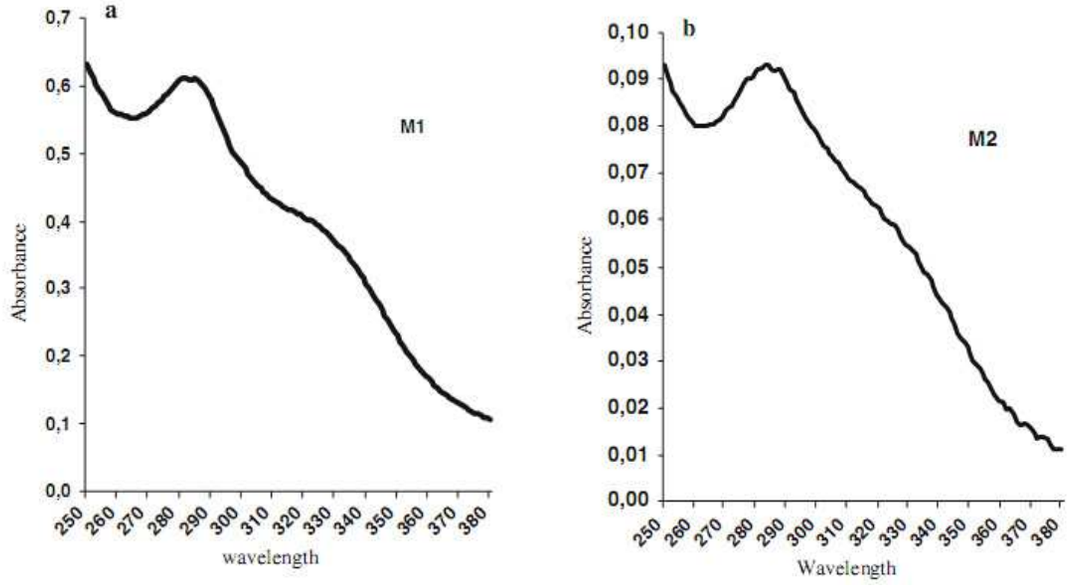


Fig. 8. UV absortion spectra between 250 and 280 nm wavelengths a: peak one, b: peak two.

presence of tyrosine. However, they later discovered that it was a residue of α-aminoacid 4 hydroxyphenilacetic which is structurally similar to tyrosine.

Kumar et al., 2009 dissolved 1 mg extract in 10 ml of methanol and the spectra were recorded at 190-600 nm range. UV spectral data of antibiotic exhibited strong absorption maxima (λ max) at 254, 255 and 277 nm and there was no appreciable absorbance above 300 nm, which was corresponding to characteristic absorption of peptide bond. It is reported that most of peptide antibiotics exhibit absorbance maxima at 210-230 and 270-280 nm (Motta & Brandelli, 2002; Kurusu & Ohba, 1987). A peptide antibiotic cerein, obtained from *Bacillus cereus*, shows UV absorbance peak at 250 and 273 nm.

3.2.2 Infrared spectrum (FTIR)

The infrared spectrum of the antibiotic was measured as a potassium bromide pellet. Approximately one-third of the KBr mixture was pressed into a pellet (8 mm diameter) using a non-evacuable Mini Press (Perkin-Elmer Norwalk, CT) and four scans of the sample were taken using a Perkin-Elmer 1600 FTIR spectrophotometer. The FTIR spectrum of the purified antibiotic is shown in (Fig.9). Characteristic absorption valleys at 1,540; 1,650, and 3,300 cm^{-1} indicate that the antibiotic contains peptide bonds. A lactone ring is suggested by the absorption at 1,740 cm^{-1} and valleys that result from C- H stretching (2,950; 2,850; 1,460 and 1,400 cm^{-1}) indicate the presence of an aliphatic chain (Bechard et al., 1998).

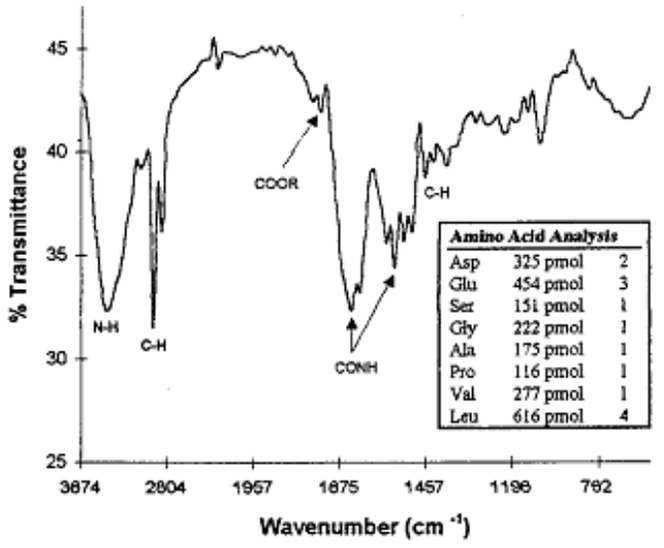


Fig. 9. Fourier transform IR spectrum (KBr pellet) and amino acid composition of the peptide antibiotic.

Romero et al, 2007 performed active extracts from *Bacillus subtilis* strains. The Fourier transform-infrared spectrum (FT-IR) analysis showed bands in the range of 1,630 to 1,680 cm^{-1}, resulting from the stretching mode of the CO-N bond (amide I band), and at 1,570 to 1,515 cm^{-1}, resulting from the deformation mode of the N-H bond combined with C-N stretching mode (amide II band), both indicating the presence of a peptide component and also bands at 2,855 to 2,960 cm^{-1}, resulting from typical CH stretching vibration in the alkyl chain. Also was observed at 1,730 cm^{-1} due to the lactone carbonyl absorption typical for surfactin and fengycin families of lipopeptides.

Maldonado et al., 2009 showed the FT-IR spectrum of the antifungal compound in D_2O, at a concentration of 1 mg/mL. Samples were placed in a liquid cell assembled with CaF_2 windows and 0.056 mm lead spacers. The spectrum was taken with a resolution of 2 cm^{-1} and three regions were observed. The one at 1,650 cm^{-1} assigned to the vibrational amida I mode which shows the peptide link; another at 1,710–1,740 cm^{-1} characteristic of carbonyl groups in ester or ketone groups and another at 2,850–2,950 cm^{-1} bands corresponding to saturated CH links assigned to long chain fatty acids (Fig.10).

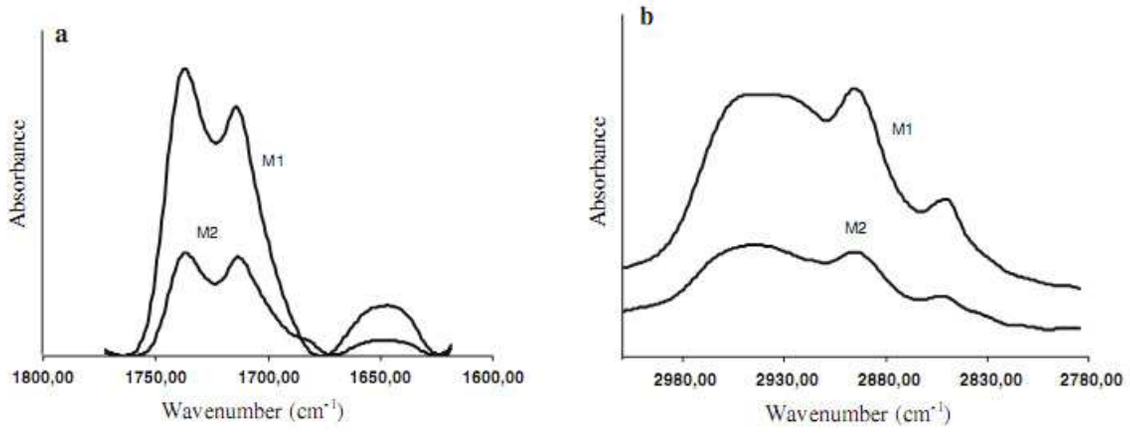


Fig. 10. a: Amida I region of FT-IR spectrum (1650 cm^{-1}) of the antifungal compound at a concentration of 1 mg/mL in D_2O. Carbonyl groups (ester or ketone groups) region (1,710–1,740 cm^{-1}) b: Saturated CH links region of FT-IR spectrum (2,850–2,950 cm^{-1}).

Sivapathasekaran et al., 2009 in order to reveal the chemical nature of the biosurfactant, the HPLC purified isoforms from marine *Bacillus* strain (A, B, C and D) were performed by Fourier transform-infrared spectrophotometry (FTIR) analysis. The purified samples were dispersed in spectral-grade KBr (Merck, Darmstadt, Germany) and made into pellets by applying pressure. The spectrum was generated in the range of 400 to 4,000 cm^{-1} with a resolution of 4 cm^{-1}. The spectral measurement of the compound was carried out in transmittance mode with average of 32 data scans over the entire range of wave numbers (Das & Mukherjee, 2005; Pueyo, 2009).

The IR absorption pattern for fractions A, B, C, and D (Fig. 11) revealed the presence of peptide and carboxyl groups that indicated their lipopeptide nature (Desai & Bannat, 1997; Thaniyavaran et al. 2003; Das & Mukherjee, 2005; Pueyo, 2009). The antimicrobial isoform (fraction A) showed a transmittance valley at 3,274 cm^{-1} as a result of N–H stretching indicating the peptide groups. Aliphatic chain was indicated by C–H weak stretching vibration observed in the range 2,930 cm^{-1}. The transmittance occurred in 1,658 cm^{-1} due to the amide I band frequency (C–O stretching in the peptide bond) and transmittance at 1,531 cm^{-1} range showed C═O bonds. The transmittance at 1,390 cm^{-1} range may be due to the aliphatic chain of C–H group. Similarly in fraction B, the N–H stretching was observed at 3,477 cm^{-1} with a broad valley of transmittance. The transmittance at 1,670 cm^{-1} range resulted in the stretching mode of C–O bond and at 1,143 cm^{-1} resulted in C–N stretch. The transmittance at 1,203 cm^{-1} corresponding to C–N stretch and at 1,440 cm^{-1} and 2,364 cm^{-1} due to the presence of aliphatic chain (C–H stretching mode) was observed. The absorption at 1,024 cm^{-1} indicated a C–O stretch. In fraction C, IR spectrum showed absorption at 3,109

cm^{-1}, 1,623 cm^{-1}, and 1,132 cm^{-1} corresponding to strong absorption band of peptides, resulted from the stretching mode of N–H and C–O bonds, respectively. The presence of CH stretching at 1,398 cm^{-1} indicated the presence of aliphatic chain. The absorption at 1,198 cm^{-1} indicated a C–N stretching. The IR spectrum for fraction D revealed absorption corresponding to the presence of peptides at 3,087 cm^{-1}, 1,667 cm^{-1}, and 1,198 cm^{-1} respectively. The C–H stretching at 1,439 cm^{-1} and 1,398 cm^{-1} indicated the aliphatic chain presence. In all the fractions (A–D), the peaks observed at the 800–600 cm^{-1} range revealed the presence of C–H bend aliphatic chain (Sivapathasekaran et al., 2009).

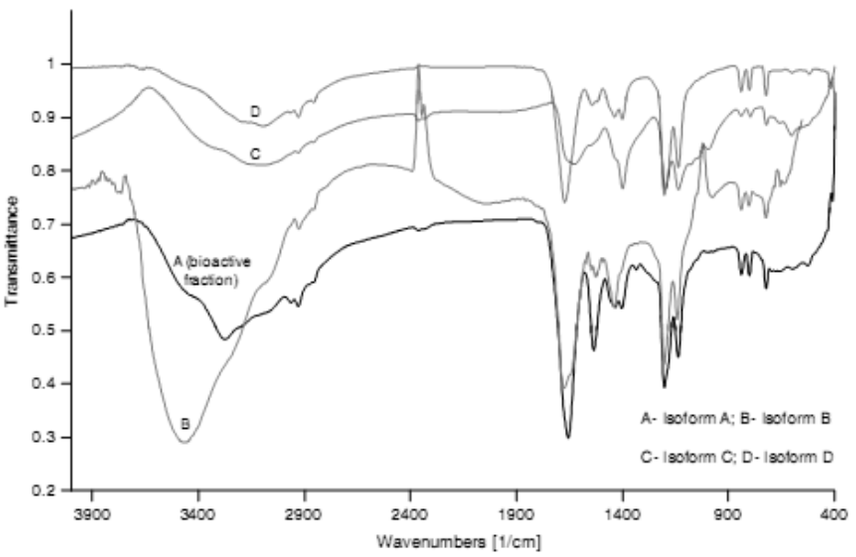


Fig. 11. IR spectrum of HPLC purified surface-active lipopeptide isoforms produced by the marine *Bacillus circulans* DMS-2 (MTCC 8281).

FTIR spectra of antifungal compound had a broad band centering around 3,421.5 cm^{-1} indicated an amino and hydroxyl group of amino acids (Kumar et al., 2009). Analysis of the spectrum also shows typical absorption bands (1,670.5 and 1,539.8 cm^{-1}) corresponding to N-H stretching of proteins and peptides bonds (Maquelin et al., 2002). Additional absorption valleys 1,418.4 and 1,488.6 cm^{-1} indicating (C-H) aliphatic side chain may be related with predominance of hydrophobic amino acids such as Val, Leu and Ile or its contains fatty acids in their structure (Bizani et al., 2005).

3.2.3 MALDI-TOF mass spectrometry

The lipopeptide molecules are detected, in their protonated form or as Na^+ or K^+ adducts, by MALDI-TOF mass spectrometry in the m/z range of 1,400–1,550 Da (Deleu et al., 2008). Several literature reports are available that highlight the analysis and purification of lipopeptide biosurfactants (Desai & Banat, 1997; Maneerat & Phetrong, 2007; Mukherjee et al., 2009; Sen & Swaminathan, 2005). Although methods like ion exchange chromatography (Mukherjee et al., 2006), thin layer chromatography (Desai & Banat, 1997), gel permeation chromatography (Mukherjee et al., 2009) and ultrafiltration (Sen & Swaminathan, 2005; Lin et al., 1998) have been used for the purification of lipopeptide biosurfactants, these techniques have a serious limitation as they do not separate individual isoforms present in the crude lipopeptide mixture.

Mutaz et al., 2007 studied the fractions correlated with surfactin from TLC and reverse phase HPLC using MALDI-TOF-MS. The samples were mixed on the target plate with the matrix solution (α-hydroxycinnaminic acid in acetonitrile-methanol-water, 1:1:1). MALDI-TOF-MS spectra was recorded by using a 337-nm nitrogen laser for desorption and ionization. The mass spectrometer was operated in the refraction mode at an accelerating voltage of 18 kV with an ion flight path that of 0.7 m. The delay time was 375 ns. Matrix-suppression was also used and the mass spectra were averaged over 50 to 100 individual laser shots. The laser intensity was set just above the threshold for ion production. Surfactin isomers were anticipated to have an m/z range of 500–1500. The variance of the m/z of ± 0.8 Da was considered acceptable.

Running MALDI-TOF-MS in refractron mode, was observed a cluster of peaks with mass/charge (m/z) ratios between 1,036 and 1,058, which could be attributed to protonated surfactin isoforms (Fig.12). The peak with a m/z ratio 1,045.86 corresponds to the mass of $[M+Na]^+$ ion of surfactin with a fatty acid chain length of 14 carbon atoms (Huszcza & Burczyk, 2006).

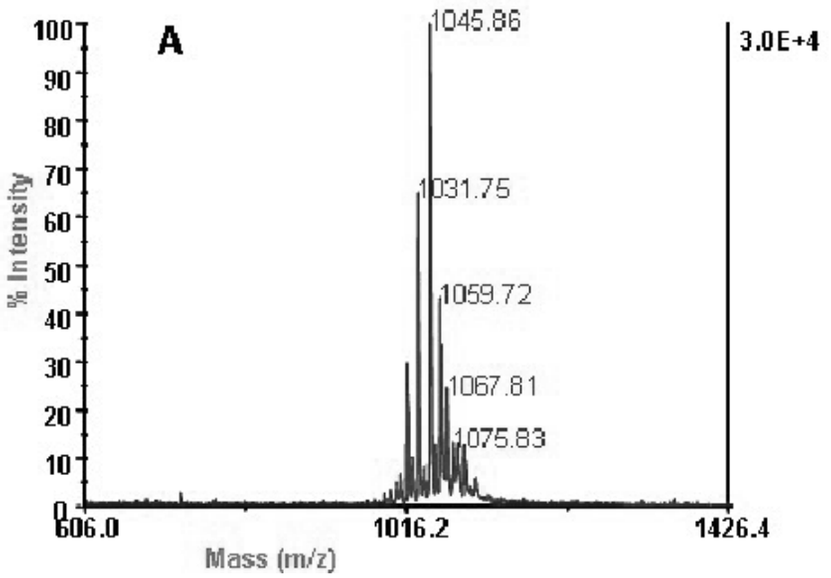


Fig. 12. Surfactin cluster + Na^+obtained by MALDI-TOF-MS.

Romero et al., 2007 confirmed the identification of the antifungal compounds (Lp-a, Lp-b and Lp-c) by scoring the mass spectra contained in the purified fractions using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, U.S.A.) operated in positive ion mode. The samples were prepared according to Williams et al. (2002). Mass spectra were recorded by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF-MS). The mass spectra of LP-a showed a series of mass number of m/z = 1,030 to 1,074; those of LP-b, m/z = 1,034 to 1,095; and those of LP-c, m/z = 1,435 o 1,499 for UMAF6639 strain (Fig. 13).

Sivapathasekaran et al., 2009 analyzed the HPLC purified isoforms by matrix-assisted laser desorption/ionization time-of-flight analysis (MALDI-TOF) for molecular mass determination. The matrix used for co-crystallization was 2,5-dihydroxybenzoic acid (Sigma, USA). A matrix stock solution was prepared in acetonitrile, methanol, TFA (5:4:1). Equal volume of the purified sample and the matrix were mixed vigorously. After proper mixing,

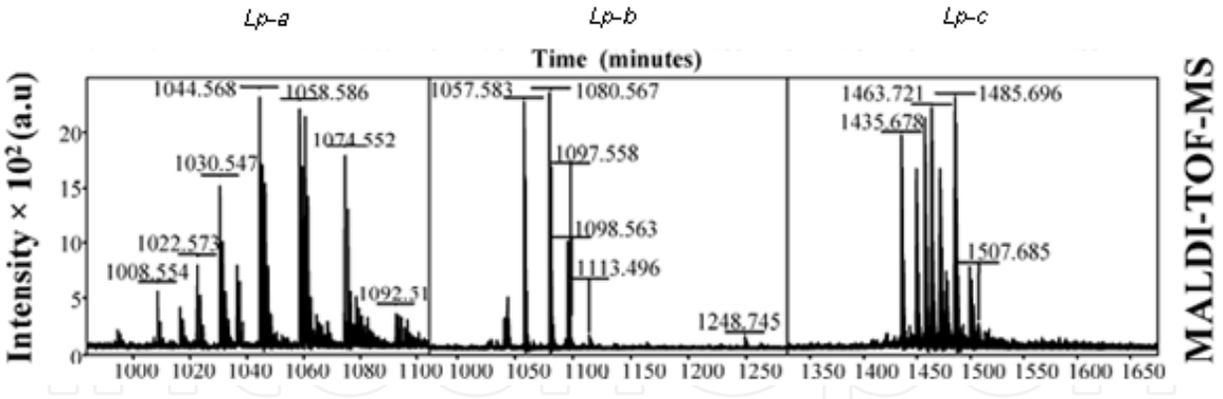


Fig. 13. Mass spectra scored for the purified fractions LP-a, LP-b, and LP-c from *Bacillus subtilis* UMAF6639 strain.

the sample was spotted on the target plate, dried, and was placed inside the sample cabinet of Voyager DE-Pro MALDI-TOF spectrometer (Applied Biosystems Inc, CA, USA). The nitrogen UV laser (337 nm) was used for desorption and ionization and a voltage of 20 kV was maintained to accelerate the molecules. The molecules were separated according to their mass and were detected by the ion detector set in reflector mode (Maneerat & Phetrong, 2007; Leenders et al., 1999). The mass spectral analysis showed that all purified isoforms (fractions A–D) were lipopeptides and belonged to fengycin family (Vater et al., 2002).

The mass spectra of fraction A revealed the presence of a major peak of C16 lipopeptides (1,482 Da and 1,484 Da) in their Na^+ adduct form. Similarly, in fraction B showed the presence of a major peak corresponding to protonated C16 lipopeptides (1,464 Da and 1,466 Da). In the surface-active fraction C, C16 lipopeptides were revealed as a major peak in their H^+ (1,492 Da) and Na^+ (1,514 Da) adduct form, respectively. In the similar manner mass spectra of fraction D, C15 isoform was detected in its protonated form (1,448 Da) and K^+adduct (1,480 Da) (Sivapathasekaran et al., 2009).

3.2.4 Electrospray ionization/collision induced dissociation (ESI/CID) mass spectrometry (MS)

Fengycin homologues produced by *Bacillus subtilis* JA were analyzed. When each homologue was subjected to ESI/CID analysis, ions representing characteristic fragmentations were detected. These ions can help to identify the homologues; even homologues of the same nominal mass can be discriminated by their ESI/CID spectra. Based on the CID results, fengycin homologues can be correctly assigned (Wang et al., 2004).

They showed the ESI mass spectrum of a group of fengycin homologues purified from the culture of *B. subtilis* JA. Peaks of m/z 1,435.7; 1,449.9; 1463.9; 1477.9; 1,491.8 and 1,506.0 represent different fengycin homologues (Fig.14). Each of these ions was selected as precursor ion for further CID analysis. The results showed the appearance of productions of these precursor ions had regularities: product ions of m/z 966 and 1,080 were found in the CID spectra of precursor ions of m/z 1,435.7; 1,449.9 and 1,463.9; product ions of m/z 994 and 1,108 were found in CID spectra of precursor ions of m/z 1,491.8 and 1,506.0. Product ions at m/z 1,080 and 966 can be explained as neutral loses of (fatty acid –Glu) and (fatty acid –Glu–Orn), respectively, from the N-terminus segment of fengycin A (Fig.15). Ions at m/z 1,063 and 949 found in the spectrum corresponded to neutral loses of ammonia

(−17 Da) from m/z 1,080 and 966. Similarly, product ions with neutral loses of (fatty acid –Glu) and (fatty acid –Glu–Orn) were also found in the CID spectra of precursor ions of m/z 1,491.8 and 1,506.0. But they appeared at m/z 1,108 and 994, exactly 28 Da higher than the corresponding ions of fengycin A (Fig.16). The observation of mass difference reflected the substitution of Ala for Val in the lactone ring and so homologues of m/z 1,491.8 and 1,506.0 belonged to fengycin B. In fact, product ions of m/z 1,209 and 1,237 representing the neutral loses of the fatty acid chain were also detected respectively from the CID spectra of fengycins A and B. But the abundance of these two ions was very low, so sometimes we could not easily find them in the spectra.

An interesting phenomenon was found in the CID result of precursor ion at m/z 1,477.9 (Fig. 17) shows part of the entire CID spectrum). Product ions of m/z 966; 994; 1,080 and 1,108 were all detected. This indicated both fengycins A and B contributed the peak of m/z 1,477.9.

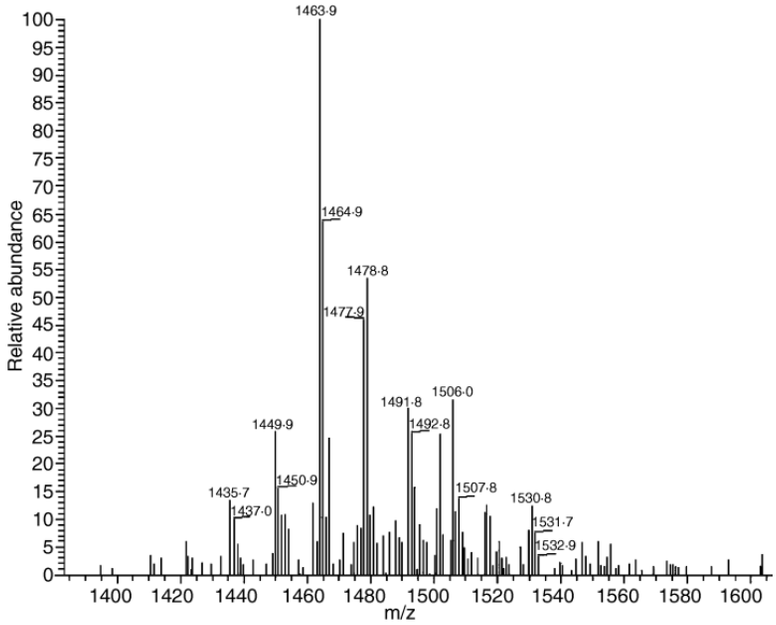


Fig. 14. ESI mass spectrum of the fengycins produced by *B. subtilis*JA.

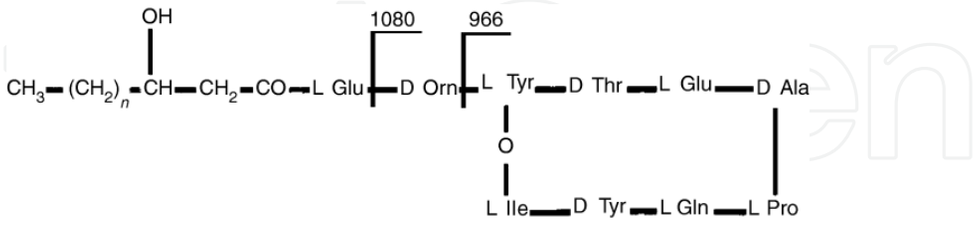


Fig. 15. Structure of fengycin A.

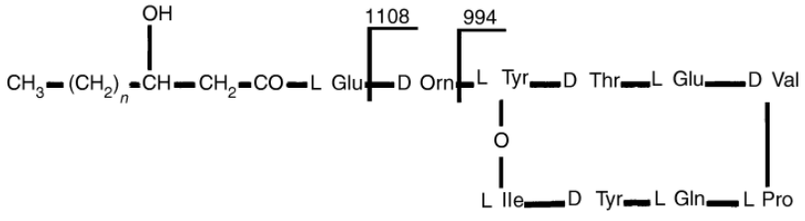


Fig. 16. Structure of fengycin B.

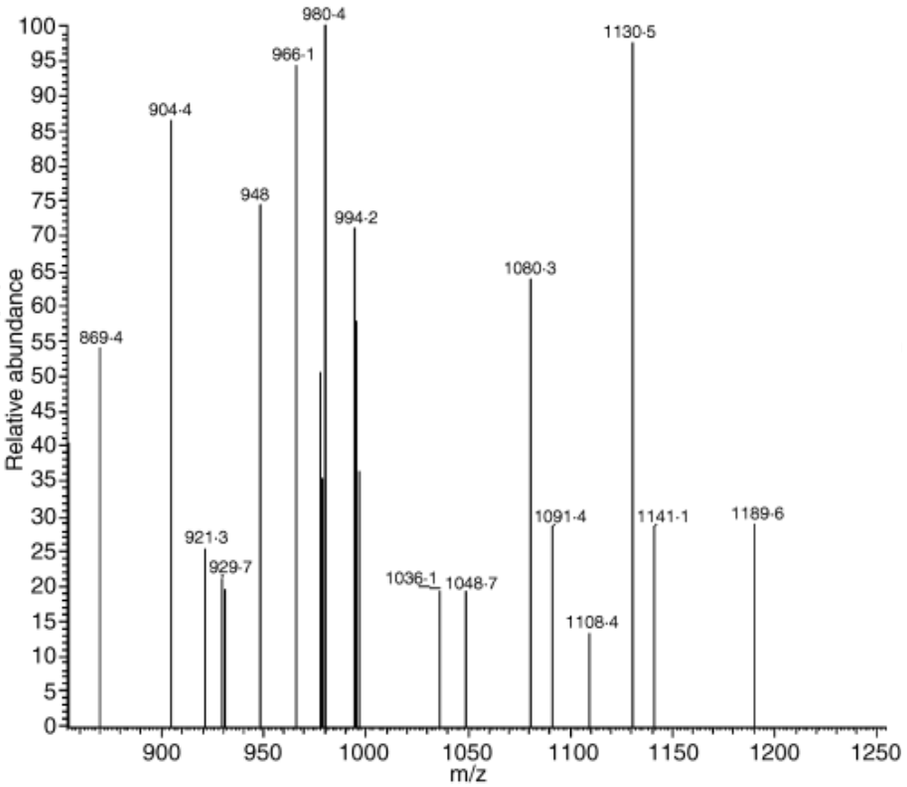


Fig. 17. CID spectrum of precursor ion of m/z 1477.9.

Romero et al, 2007 determined by ESI-MS-MS the amino acid sequences for the peptide moiety. Samples of ring-opened lipopeptides by cleavage of the lactone bond (Williams et al. 2002) were analyzed on an Esquire 3000 Plus ion trap mass spectrometer (Bruker Daltonics) and sequences deduced by comparing the fragmentation spectra with the available databases. When the amino acid compositions were determined, it was found that the fraction LP-a contained Asp, Glu, Val, and Leu in a ratio of 1:1:1:4; fraction LP-b comprised Asp, Ser, Glu, Pro, and Tyr in a ratio of 3:1:1:1:1; and fraction LP-c was composed of Thr, Glu, Pro, and Ala or Val, Ile, Tyr, and Orn in a ratio of 1:3:1:1:1:2:1, corresponding to strain UMAF6639 (Fig. 18).

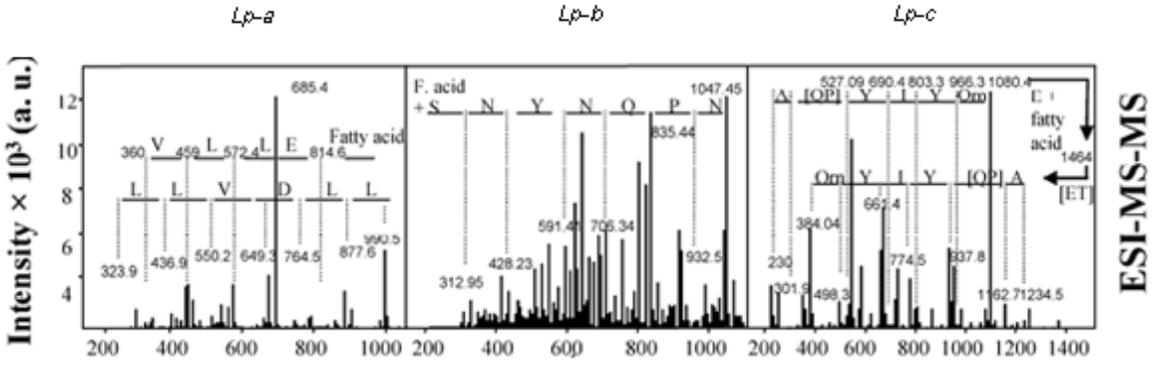


Fig. 18. Mass spectra scored for the purified fractions LP-a, LP-b, and LP-c.
Fragmentation spectra observed by electrospray ionization ion trap mass spectrometry (ESI-MS-MS) and amino acid sequences and fatty acid compositions deduced from the parental peaks 1,008.7 (LP-a, surfactin), 1,065.4 (LP-b, iturin A), and 1,464 (LP-c, fengycin).

3.2.5 Edman degradation

Edman degradation is the classic technique for sequencing peptides using chemical methods (Zachara & Gooley, 2000). The method provides an assignment for each residue in the peptide, unlike amino acid analysis which only provides an indication of the ratio of amino acids in the peptide. Edman experiments take place in an oxygen-free environment and involve the modification of the N-terminal residue with phenylisothiocyanate to provide a cleaved phenylthiohydantoin (PTH) amino acid. All of the chemical processes take place on automated sequencers and are followed by a chromatographic step where the retention time of the cleaved PTH amino acid is compared with the retention times of a series of PTH modified amino acid standards to ascertain its identity. The method is relatively slow taking approximately 45 min for each residue, however the amino acid assignment is called with a high degree of confidence and less subject to interpretation, as can be the case with MS/MS methods. The quality of sequence information obtained by the Edman method is subject to the amount of starting material and its purity. For successful sequencing to take place, peptides and proteins must be purified to near homogeneity by chromatographic methods to prevent mixed sequencing during Edman experiments. Lipopeptides needs to be in the open ring form for this type of analysis, which is carried out using mild alkaline hydrolysis.

4. Perspectives and conclusions

A great deal of research has been carried out the properly methods for fully characterization of cyclic lipopeptides and their structures.

This review highlights the competitive advantage of efficient purification of surfactin, fengycin and iturin with structural elucidation. Within each family, some structural homologues are seemingly more active than others. In this regard, reverse-phase high-performance liquid chromatography (RP-HPLC) could be extensively used because it is efficient in separation and purification of isoforms (Lin et al., 1998; Thaniyavaran et al., 2003). However the HPLC method, if not optimized, may lead to improper resolution of peaks and longer elution times. Thus, an efficient high-resolution HPLC method is a prerequisite for the purification up to the individual isoform level, required for subsequent commercialization of a particular lipopeptide isoform as a potential therapeutic agent. The products obtained with these methods shows a greater level of purity and profound biological activity (Sivapathasekaran et al., 2009).

Mass spectrometry methods developed to rapidly characterize the lipopeptides nature.

The lipopeptides and moreover the particular isoforms could be exploited as a powerful tools for the selection of useful strains in the context of biocontrol.

5. References

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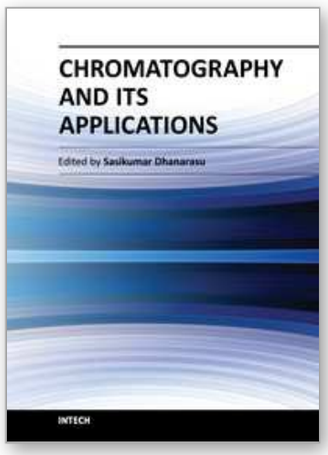

Chromatography and Its Applications

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Chromatography is a powerful separation tool that is used in all branches of science, and is often the only means of separating components from complex mixtures. The Russian botanist Mikhail Tswett coined the term chromatography in 1906. The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures. A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties. Many types of chromatography have been developed. These include Column chromatography, High performance liquid chromatography (HPLC), Gas chromatography, Size exclusion chromatography, Ion exchange chromatography etc. In this book contains more details about the applications of chromatography by various research findings. Each and every topics of this book have included lists of references at the end to provide students and researchers with starting points for independent chromatography explorations. I welcome comments, criticisms, and suggestions from students, faculty and researchers.

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