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Evaluation of HPLC and MEKC methods for the analysis of lipopeptide antibiotic iturin A produced by *Bacillus amyloliquefaciens*

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Abstract: Two methods, high-performance liquid chromatography (HPLC) and micellar electrokinetic chromatography (MEKC), for analysis of multicomponent lipopeptide antibiotic iturin A are described. After conducting a series of optimizations, the HPLC separation of iturin A_2, A_3, A_4, A_5, A_6, A_7, and A_8 isomers were achieved by a NPS RP-C18 column with an isocratic elution of acetonitrile/10 mM ammonium acetate (40:60, v/v), and flow rate of 1 mL/min. On the other hand, the MEKC separation achieved at 25 °C using an applied voltage of 20 kV, and background electrolyte consisting of 20 mM boric acid buffer (pH 8.72) with 50 mM sodium dodecyl sulfate (SDS) and 10% (v/v) acetonitrile. The calibration curve was linear to 20-200 μg/mL and 50-500 μg/mL for HPLC and MEKC with R^2 = 0.9954 and R^2 = 0.9967, respectively. The detection limits of iturin A from the strain B128 cultivation medium were in the 2.5-7.0 μg/mL and 5.0-13.0 μg/mL ranges, and the reproducibility of within-day assay was 98.1 and 98.7%, for HPLC and MEKC, respectively (a UV detector was applied in the both cases). Both methods were selective, robust, and specific, allowing reliable quantification of iturin A, and could be useful for clinical and biomedical investigations of related antibiotics.

Keywords: *Bacillus amyloliquefaciens* B128; High-performance liquid chromatography; Micellar electrokinetic chromatography; Lipopeptide antibiotic; Iturin A

1. Introduction

Bacillus amyloliquefaciens is a Gram-positive, spore forming bacteria, and is closely related to *Bacillus subtilis* and has been classified as *B. subtilis* periodically [1]. *B. amyloliquefaciens* has been studied extensively as a producer of enzymes, such as α-amylase, subtilisin (a protease) and barnase (a ribonuclease), in addition to production of antibiotics like iturins [2, 3]. Iturin A, one member of the iturin group, shows a strong antibiotic activity with a broad antifungal spectrum, making it an ideal potential biological control agent with the aim of reducing the use of chemical pesticides in agriculture [4]. In addition, clinical trials on humans and animals have also shown iturin A to be a valuable drug due to its broad antifungal spectrum, low toxicity and allergic effects [5-7]. Iturin A is constituted by a cyclic lipopeptide with seven α-amino acids, and

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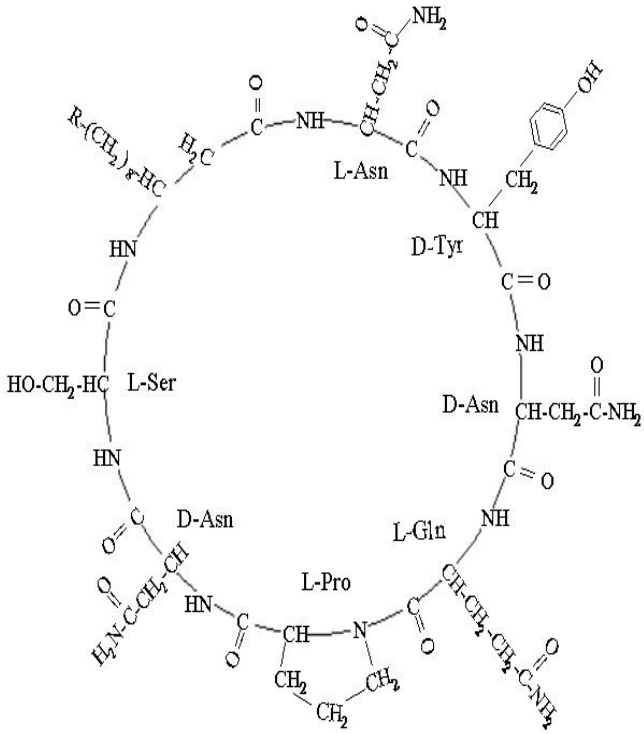
one unique β-amino fatty acid (βAA). The fatty acid chain of lipopeptide varies from 13 to 17 carbon atoms [4]. In nature, iturin A is produced as a mixture of up to eight isomers named as iturin A_1−A_8 [8]. The structure of iturin A is shown in Figure 1. Despite many advantages of iturin A over chemical agents, there have been few actual applications of iturin A until now, may be because of lack of reliable and quick purification process.



Iturin	R
A_2	$CH_3CH_2CH_2$-
A_3	CH_3CH_2CH-CH_3
A_4	$(CH_3)_2CHCH_2$-
A_5	$CH_3CH_2CH_2CH_2$-
A_6	$(CH_3)_2CHCH_2CH_2$-
A_7	$CH_3CH_2CH_2CH_2CH_2$-
A_8	$CH_3CH_2CHCH_2CH_2$-CH_3-

Figure 1. Chemical structures of iturin A isomers.

The analyses of antibiotics and other biomedical derivatives are involved the use of reversed phase high-performance liquid chromatography (RP-HPLC) with different detection systems [9, 10]. Earlier HPLC and other methods used for analysis of iturin A involved various pretreatment procedures include, steps of conditioned fermentation, acid precipitation, extraction with organic solvents, repeated crystallization, charcoal treatment, or ultrafiltration and solid phase extraction [11-13]. These conventional laborious pretreatment processes and are time-consuming, not specific or selective enough for rapid iturin A purification. On the other hand, a relatively simple technique named capillary electrophoresis (CE) is used extensively for analysis of antibiotics and other bioactive derivatives from biological and biomedical fields as an alternative and complementary technique to HPLC with convenient shorter time of analysis and low consumption of solvents/buffers [14-18]. Micellar electrokinetic chromatography (MEKC) is a mode of CE, and has been used for various antibiotic analyses, and their determination using sodium dodecyl sulphate (SDS) as a micelle-forming agent [19, 20]. The perspectives of MEKC in drug analysis were reviewed by Terabe [21]. However, so far no CE method for determination of iturin A has been reported. Therefore, in this study, we described RP-HPLC and MEKC to develop a simple and rapid method for the separation and determination of iturin A. Therefore, we believe the methodologies described herein with iturin A demonstrate the applicability of CE in assessing important antibiotic distributions in real-world situations.

2. Materials and Methods

2.1. Materials and reagents

Iturin A and boric acid were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH), acetonitrile, sodium dodecyl sulfate (SDS), and phosphoric acid (H_3PO_4, 85%) were supplied by Merck (Darmstadt, Germany). All solvents were of HPLC grade and/or prepared with doubly deionized ultra-high purity (18 MΩ) water, Millipore (Bedford, MA, USA).

2.2. Bacterial strain and cultivation

The strain *B. amyloliquefaciens* B128 used in this study was isolated from an infected lily plants provided by one of the author Prof. Wen-Shi Wu. The stock culture was maintained on nutrient broth (NB), (Difco, Detroit, USA) plus 10% glycerol at 4 °C and -70 °C for short term and long term storage, respectively at the Institute of Biotechnology, Chaoyang University of Technology, Wufeng, Taiwan, ROC. The strain was transferred from culture stock into a 500 mL Erlenmeyer flask containing 200 mL of medium [3% (w/v) glucose, 0.75% (w/v) bacto peptone (Difco, Detroit, USA)], and incubated for 10 h at 30 °C and 200 rpm. The standard spore suspension inoculums 2% (v/v) were used for shaker flask cultivation. Cultures were grown for 5 days at 30 °C and 200 rpm in a rotary shaker incubator (Yih-Der, LM-530R., Taipei, Taiwan), in 500 mL baffled Erlenmeyer flasks with 200 mL of production media, and no pH control was applied for all cultivations. The other culture medium components are (%, w/v); 2 of soft sugar (a sucrose sugar product with a distinctive brown color due to the presence of molasses), 1 peptone, 0.1 yeast extract, 0.01 $CaCO_3$, 0.01 NaCl, and 0.2 mM $FeSO_4.7H_2O$ (Sigma, St. Louis, MO., USA).

2.3. HPLC conditions

Analytical HPLC consists of L-7100 pump; a 20 μL fixed loop, and a Model L-7400 variable-wavelength UV-Vis detector (Hitachi, Tokyo, Japan). For iturin A analysis, a Micra (Northbrook, IL, USA) NPS RP-C18 analytical column (33×4.6 mm) was employed. A 5 μL of sample was injected onto the HPLC by

an auto sampler at 25 °C. An isocratic mobile phase consisted of acetonitrile and 10 mM ammonium acetate (40:60, v/v) with flow-rate of 1 mL/min was fixed throughout the studies. The detector monitored absorption at 260 nm. Semi-preparative HPLC was performed on a 7 μm LiChrosorb RP-18 column (250 mm×10 mm) supplied by Merck (Darmstadt, Germany). The mobile phase was same as described for the analytical C_{18} analysis. A 500 μL of sample was injected, and a constant flow-rate 3 mL/min was maintained. Mobile phase buffers were filtered through 0.45 μm nylon filters and degassed by sonication prior to use.

2.4. CE conditions

All experiments were carried out using a Beckman P/ACE-2100 system (Beckman-Coulter, Fullerton, CA, USA) equipped with a UV detector set at 260 nm, a diode array detector (DAD), and a liquid-cooling device was used for electrokinetic separations. MEKC was performed in an uncoated fused-silica capillary (Polymicro Tech., Phoenix, AZ, USA) of total length 60 cm, effective length, 52 cm, 50 μm I.D.×365 μm O.D. The analytical conditions were as follows: sampling time 5 s (hydrodynamic mode; 1 psi); applied constant voltage 20 kV; column temperature 25 ºC. The running buffer was 20 mM sodium borate (pH 8.72) containing 50 mM SDS and 10% (v/v) acetonitrile. When a new capillary was used, it was rinsed by passing the following solutions: H_2O Milli-Q for 5 min, 0.1M NaOH for 5 min, H_2O Milli-Q for 5 min and finally flushed with running buffer for 15 min. Before each analysis, the capillary was flushed with NaOH for 2 min, H_2O Milli-Q for 2 min, and with running buffer for 2 min. The Beckman P/ACE-2100 Microsoft Software system was used for data processing.

2.5. Sample preparation

The standard stock solutions were prepared by dissolving 1mg of iturin A in 1mL of methanol. These solutions were stored in dark glass bottles at 4 ºC and were stable for at least 1 month. Working standard solution was freshly prepared by dissolving suitable amount of the above solution with acetonitrile and 10 mM ammonium acetate (40:60, v/v) for HPLC and with borate buffer (pH 9.0) for CE analysis, and then directly injected into the HPLC and CE systems.

The cells from *B. amyloliquefaciens* B128 cultivation medium were removed by centrifugation and filtration. The cell-free supernatant was passed through an ultrafiltration cartridge (Amicon's ultrafiltration system, Cherry Hill Drive, MA, USA) with molecular weight cutoff (MWCO) of 1000 kDa, and then concentrated with a cartridge of MWCO of 10 kDa. The concentrated fraction 100 μL was transferred into 1.5 mL polypropylene tube and mixed with 900 μL of methanol. The mixture was vortexed at room temperature for 20 min and then centrifuged at 15,000×*g* for 10 min at 4 °C. The supernatant was filtrated through 1.0 μm poly-tetra-fluoro ethylene (PTFE) membrane filter (Bedford, MA, USA) before HPLC and CE analysis.

2.6. Calibration curves

Working standard solutions, which spanned a concentration range from 20 to 200 μg/mL and from 50 to 500 μg/mL for HPLC and MEKC analyses, respectively, were prepared. Quantification determinations for iturin A by RP-HPLC or MEKC were made by utilization of external working curves. Regression analysis for RP-HPLC and CE provided a linear relationship of concentration to absorbance to over this range with an R^2 value of 0.9954, and 0.9967, respectively, was obtained. The lower limit of detection under these conditions for RP-HPLC and CE were determined to be approximately 2.5 and 5 μg/mL, respectively.

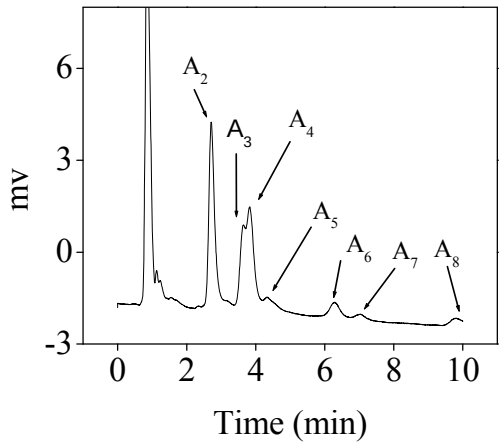
3. Results and Discussion

In this study, optimized conditions of both RP-HPLC and MEKC methods for direct determination of iturin A in *B. amyloliquefaciens* B128 cultivation medium with UV detection have been developed and compared. The UV detection has been the most common detector used for its simplicity and low cost, while the mass spectrometry has been less frequently applied due to the high instrumentation cost [3, 13]. The sample preparation procedure developed in this work, including organic solvent extraction, was optimized to eliminate time-consuming purification steps [13], entailing an ultrafiltration step as described previously [3]. Two methods have been achieved to provide optimum separations; RP-HPLC with an isocratic acetonitrile/10 mM ammonium acetate solvent and MEKC with 20 mM borate buffer (pH 8.72) that contains 10% acetonitrile and 50 mM SDS.

HPLC analysis

Different parameters for RP-HPLC analysis were varied, such as temperature, flow-rate and sample solvent. The effects of different organic solvents and acid solutions used to prepare the mobile phase were also investigated. Acetonitrile was chosen as a component of the mobile phase because a better peak symmetry was observed than after using methanol. The selectivity of the proposed method depends strongly on the percentage of acetonitrile, and the addition of 10 mM ammonium acetate to the mobile phase. Ammonium acetate added to the mobile phase shortened the retention times of standard iturin A as well as strain B128 cultivation medium sample. Other experimental parameters such as the flow-rate of mobile phase and the temperature of analytical column (in the range of 20-30 ºC) had a minor influence on separation. By referencing Rahman *et al*., [22] and optimizing the composition of the mobile phase, acetonitrile and 10 mM ammonium acetate (40:60, v/v) were finally utilized as a simpler mobile phase. Figure 2 shows the HPLC chromatograms of standard and *B. amyloliquefaciens* B128 cultivation medium sample of iturin A. Separation of the isomers of iturin A in the cultivation medium has been achieved with water-acetonitrile isocratic elution over a period of 10 min. As shown in Figure 2B, *B. amyloliquefaciens* B128 cultivation medium contains iturin A components A_2, A_3 and A_4 in highest intensity and peak area. Figure 3 shows the semi-preparative HPLC chromatogram of iturin A produced by *B. amyloliquefaciens* strain B128. The retention profile of the iturin A isomers is different from that of the analytical HPLC column. This is due to higher sample concentration (also high injection volume) which caused overlapping [23]. Chromatographic plots of purified iturin A isomers from the strain B128 cultivation medium using a isocratic elution system with a simple acetonitrile/10 mM ammonium acetate mobile phase are given in Figure 4. The individual iturin A isomers were well separated on a NPS RP-C18 analytical column. The retention times for iturin A isomers, A_2, A_3, A_4, A_5, A_6, A_7, and A_8 were 2.83, 3.71, 4.02, 4.31, 6.32, 7.08 and 9.89 min, respectively. The peaks of iturin A isomers could be assigned by the retention time of standard iturin A. The standard curve (peak-area; Y, versus concentration; X (μg/mL) was constructed in the range of 20-200 μg/mL for iturin A. The regression equations of the curve and its correlation coefficient were calculated as follows, $Y = 23109\ X + 120130$ ($R^2 = 0.9954$). The precision of the chromatographic determination for the proposed method, which is expressed as a relative standard deviation (RSD), was calculated for five replicate injections of the cultivation sample. The RSD for the iturin A was 2.9%. The concentration sum of iturin A isomers in the *B. amyloliquefaciens* B128 cultivation medium (cf. Figure 2B) was analyzed by RP-HPLC. Because this concentration is out

of the linear range of the calibration curve (20-200 μg/mL), the sample was diluted with mobile phase and confirmed that the peak area ratios and concentration are well correlated by linear regression analysis [23]. At a signal-to-noise ratio (S/N) is 3, the detection limit in the strain B128 cultivation medium for iturin A was 2.5 μg/mL, and the quantification limit (S/N = 10) is 7.0 μg/mL. Accordingly, the sum of iturin A isomers in the *B. amyloliquefaciens* B128 cultivation medium was 123.3 μg/mL. The extraction recovery tested by adding known amounts of iturin A to the cultivation medium was 98.1% (n = 3).



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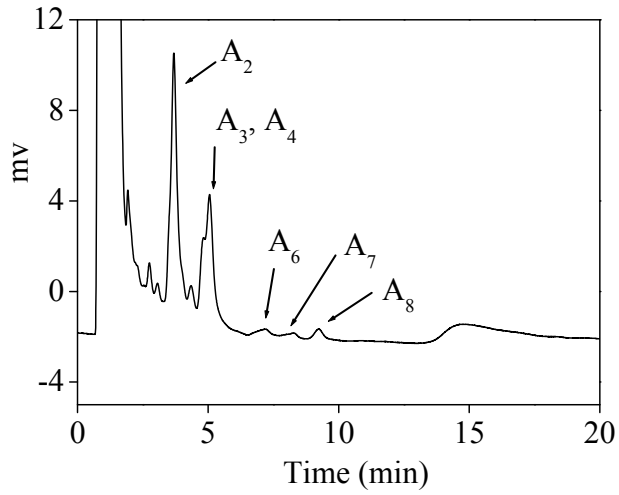


(b)

Figure 2. HPLC chromatograms of (a) iturin A reference substance, and (b) the *Bacillus amyloliquefaciens* B128 cultivation medium sample. Column: NPS RP-C18 analytical column. Eluent: acetonitrile and 10 mM ammonium acetate (40:60, v/v). Flow-rate: 1.0 mL/min. Temperature: 25 ºC. Detection: 260 nm.

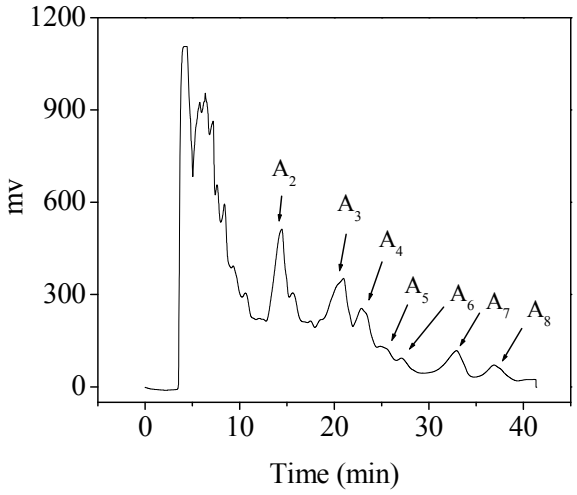
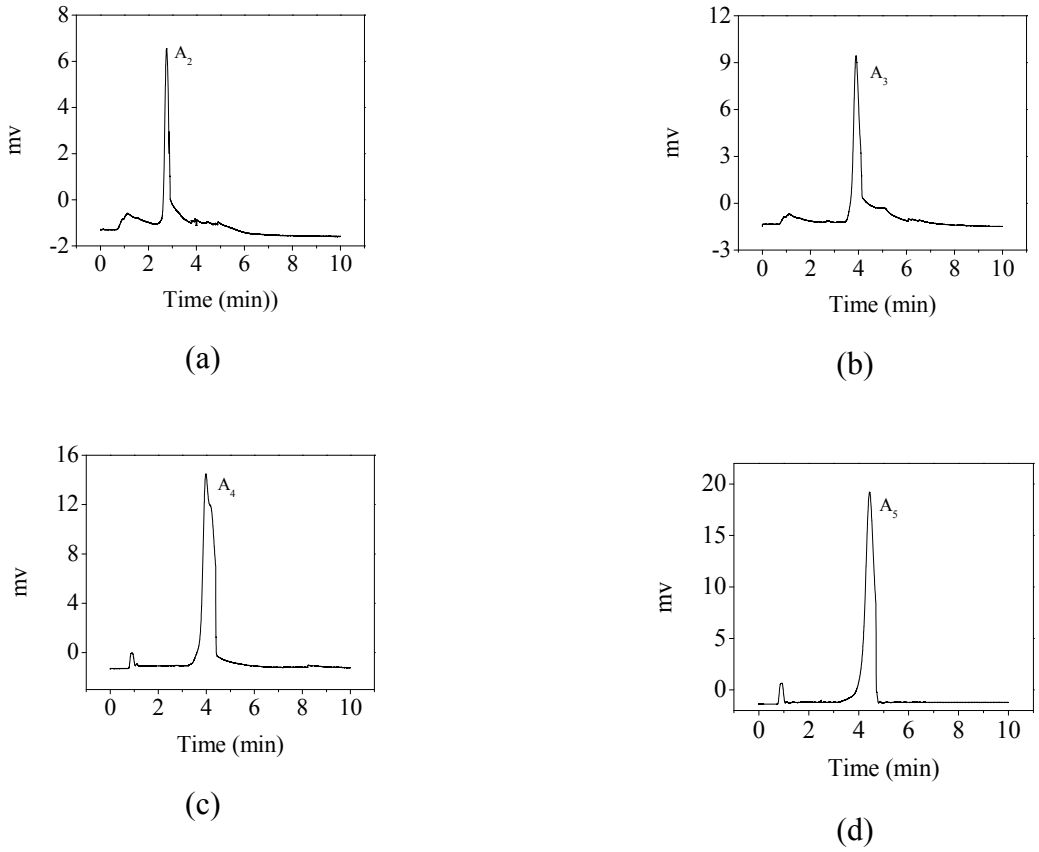


Figure 3. Semi-preparative reversed-phase HPLC chromatogram of iturin A produced by *Bacillus amyloliquefaciens* strain B128. Column: 7 μm LiChrosorb RP-18 column (250 mm × 10 mm). Mobile phase: isocratic elution with acetonitrile and 10 mM ammonium acetate (40:60, v/v). The flow rate was 3 mL/min and detection at 260 nm.



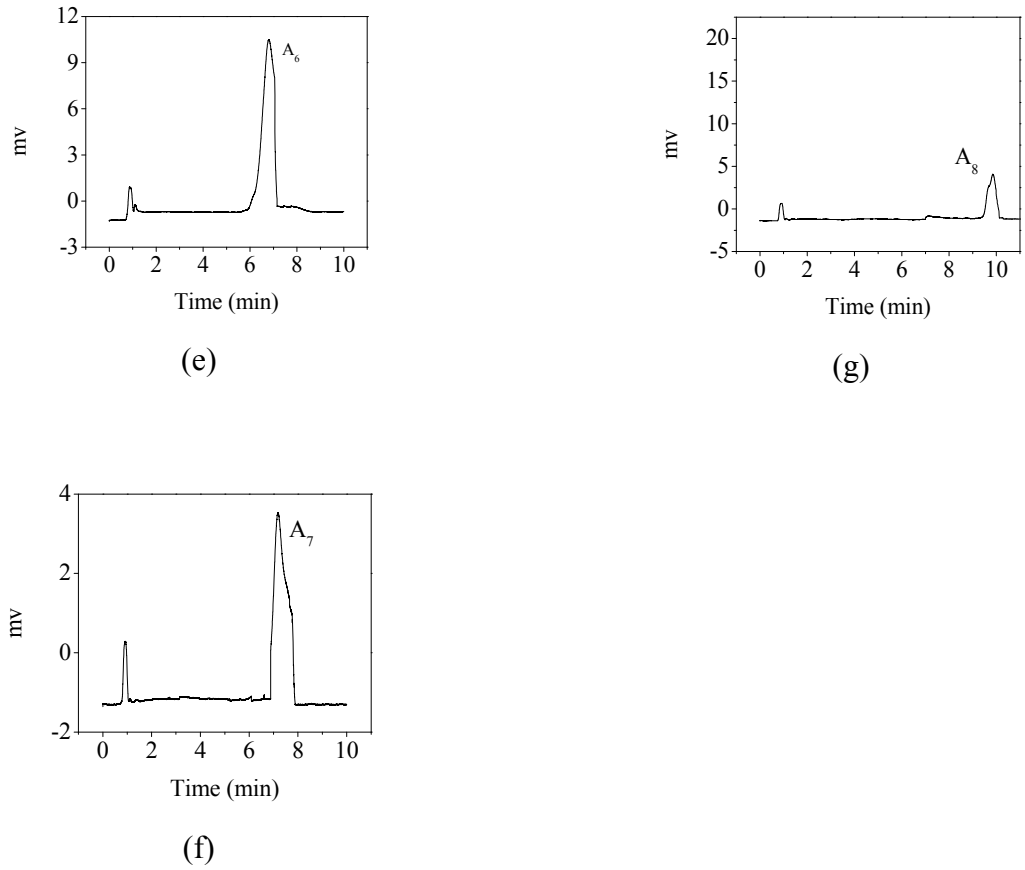


Figure 4. HPLC analysis of *Bacillus amyloliquefaciens* produced iturin A isomers; A_2 (a), A_3 (b), A_4 (c), A_5 (d), A_6 (e), A_7 (f), and A_8 (g) from semi-preparative analysis. Chromatographic conditions, column: NPS RP-C18 analytical column. Eluent: acetonitrile and 10 mM ammonium acetate (40:60, v/v). Flow-rate: 1.0 mL/min. Temperature: 25 °C. Detection: 260 nm.

CE analysis

A large number of individual components are present in *B. amyloliquefaciens* B128 cultivation medium that can modify the chromatographic column, leading to difficulty in resolving the target iturin A. There has been considerable interest in performing single-step analyses with direct injection of cultivation medium in CE. This is quite often feasible in CE because the open capillary columns are less prone to irreversible modification by sample components [24]. In order to select the best conditions that could influence the separation of the iturin A using the MEKC methodology, several screening experiments were performed to study the influence of pH and concentration of the running buffer and micellar medium, the separation voltage and the capillary temperature. CE using a buffer solution of borate with different pH values as the carrier was employed in our preliminary trials. The pH of the run buffer largely influences the electroosmotic flow (EOF) as well as the degree of ionization of analytes [25]. Therefore, a careful control of pH is necessary. The pH-dependence of the migration time of iturin A was examined with 30 mM borate buffer with 30 mM SDS, at in a pH range 8-11. The migration time became longer as the pH value increased, as is known because of the increased EOF. Optimum pH separation conditions were established at pH 8.72 because of the good separation/migration-time

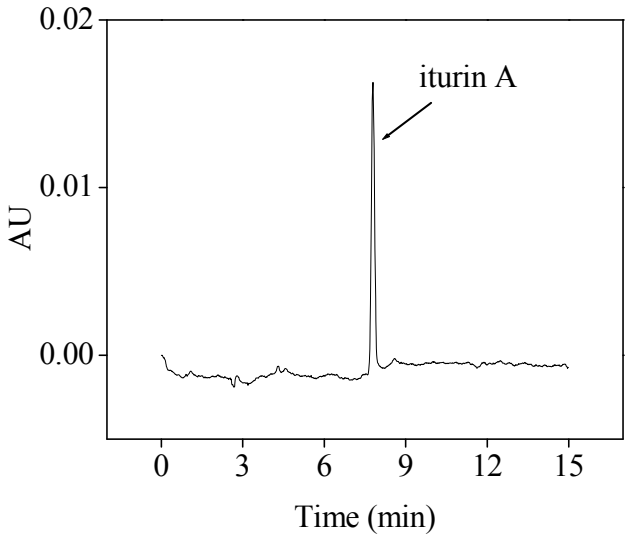
ratio.

Next, we tried to separate the iturin A using CE by changing the buffer solutions. It is well known that when the concentration of the buffer increases, the EOF decreases, which increases the migration times [21]. In order to predict the optimal buffer solution for carry-out the separation of iturin A in these pH ranges, it was necessary to select concentration and composition of buffer at which the differences between the mobility of iturin A was greatest. Buffer solutions such as borate, ammonium acetate, sodium carbonate, citrate and phosphate were proved, selecting borate buffer for giving the best results. Borate buffer concentration was modified between 10 and 50 mM in order to obtain the better resolutions of iturin A and adequate current, avoiding the Joule effect [26]. MEKC of the standard iturin A under the 10-50 mM boric buffer with 30 mM SDS can give good separation efficiency and resolution, and a shorter migration time is obtained in 10 mM boric buffer. To prevent the generation of too much Joule heating, 20 mM boric buffer was chosen. The boric buffer concentrations for iturin A determination are all set at 20 mM.

The effect of temperature in the migration time of antibiotics was quite significant [15]. Remarkable decreases in migration times were observed upon increasing capillary temperature (from 15 to 30 ºC). These were primarily due to decreases in buffer viscosity, although higher temperatures resulted in lower separation efficiency. Low temperatures resulted in poor reproducibility of migration times and in moisture condensation on the optical window, thus increasing noise and decreasing sensitivity [19]. Optimum separation temperature was established at 25 ºC due to good separation/migration-time ratio.

The concentration of SDS in the buffer was also investigated. It was varied between 10 and 70 mM, keeping the borate concentration at 20 mM and the pH at 8.72. The higher the SDS concentration decreased the interference for the tested iturin A separation [20]. With concentrations of SDS 50 mM in boric buffer, a baseline resolution with high separation efficiency of iturin A can be achieved.

The next parameter to be investigated was the applied voltage. The separation voltage was modified between 15 and 30 kV. An increase in voltage resulted in increased EOF due to higher electric field strength which led to poor separation of iturin A; 20 kV was selected as to obtain a compromise between good separation and analysis time. Next, the addition of organic modifiers to the buffer system was examined. If methanol was added instead of acetonitrile the running time was almost tripled. This may be due to effects of EOF flow, which is largely suppressed by the alcohols [14]. Additionally, with a relatively large organic composition in the running buffer, SDS micelles may be less tortured in acetonitrile than in methanol [15]. Furthermore, the addition of 10% (v/v) acetonitrile was found useful in reducing the solute-wall interactions and thus enhanced the efficiency of the separation Figure 5A is an electropherogram, showing the separation of standard iturin A. Under the optimum conditions, iturin A in the strain B128 cultivation medium was determined. A typical electropherogram of cultivation medium is shown in Figure 5B. For quantitative analyses, correlations between the peak area and the sample concentration were determined. The limit of detection and quantification was 5 and 13.0 µg/mL, respectively. Accordingly, the concentration sum of iturin A isomers in the cultivation medium determined by MEKC is 124.1 mg/mL, which is nearly similar to the RP-HPLC determinations. The recovery has been determined by comparing the peak area of iturin A obtained from the *B. amyloliquefaciens* B128 cultivation medium spiked samples with the iturin A peak area obtained from the calibration standards. The level of recovery was 98.7% for iturin A ($n = 5$). The reproducibility, expressed as the RSD, was calculated on the basis of the migration time over five replicate injections and was 2.39%.



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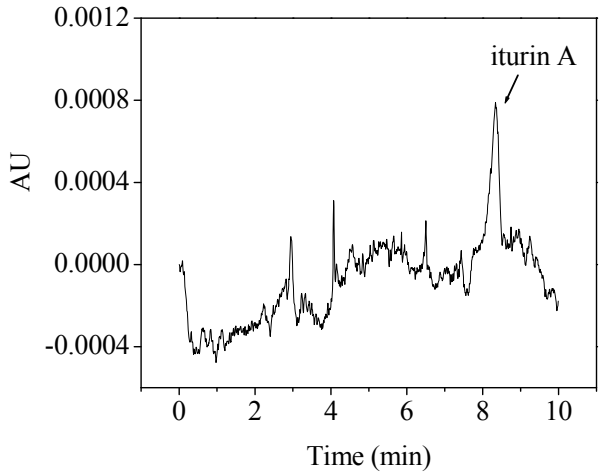

(b)

Figure 5. Typical electropherograms of standard iturin A (a) and *Bacillus amyloliquefaciens* B128 cultivation medium sample (b). CE conditions: capillary, 50×365 μm uncoated fused silica, 60 cm (52 cm effective length); separation buffer, 20 mM sodium borate (pH 8.72) containing 50 mM SDS and 10% (v/v) acetonitrile; voltage, 20 kV; temperature, 25 ºC.

4. Conclusions

In this study iturin A is separated and determined in 10 min in the *B. amyloliquefaciens* B128 cultivation medium using either the HPLC or MEKC method. In the RP-HPLC method, iturin A isomers are well separated. The MEKC method may improve in the future to encompass the separation of these isomers. Though both RP-HPLC and CE pro-

vide acceptable analysis times, the detection limit of RP-HPLC is lower than MEKC (HPLC: 2.5-7.0 μg/mL, MEKC: 5.0-13.0 μg/mL). However, MEKC offers advantage over RP-HPLC in terms of lower cost, quick separation, less sample requirements, and alleviating irreversible column damage commonly associated with RP-HPLC analysis of antibiotics.

Acknowledgements

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