Journal of Chromatography B, 879 (2011) 2746–2750

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of the antifungal lipopeptide iturin A by high performance liquid chromatography coupled with aqueous two-phase extraction

Jun Yuan, Waseem Raza, Qiwei Huang[1], Qirong Shen[*,1]

Jiangsu Key Lab for Organic Solid Waste Utilization, Nanjing Agricultural University, 210095 Nanjing, China

ARTICLE INFO

Article history:
Received 18 May 2011
Accepted 31 July 2011
Available online 6 August 2011

Keywords:
Aqueous two-phase extraction
High performance liquid chromatography
Iturin A
Quantitative detection

ABSTRACT

Iturin A, a powerful antifungal surfactant, is a kind of bacterial lipopeptide produced by *Bacillus* strains. This study addresses the use of an aqueous two-phase system (ATPS) using ethanol/ammonium sulfate to extract iturin A from *Bacillus amyloliquefaciens* NJN-6 fermentation broth and the quantification of iturin A by HPLC. Baseline separation of iturin A homologues was performed using an RP-C_{18} column with a mixture of water and acetonitrile. The results showed that the correlation coefficient between integral area and concentration was 0.9961 within the range of 20–140 mg/l. The RSD of the retention time and the peak area were 1.29% and 1.45%, respectively. The effects of some operating parameters in ATPS, e.g., pH, temperature and centrifugation time, were also studied. This method can be successfully used for the rapid quantification of iturin A.

1. Introduction

Lipopeptides are biosurfactants consist of an aliphatic chain and a peptide chain having hydrophilic and hydrophobic parts and superior surface activity. These lipopeptides are produced by a variety of microorganisms. As biosurfactants, these compounds are superior to chemically synthesized surfactants in having lower toxicity, higher biodegradability, better environmental compatibility and the capability of being produced using cheap agro-based raw materials [1]. More importantly, these lipopeptides are efficient antimicrobial agents and have been reported to be effective in antagonizing crop pathogens [2–4].

Iturin is a lipopeptide produced by *Bacillus* species which is consisted of three homologues with different lengths of carbon chain (Fig. 1 [5]). Iturin A is one of the most popular antifungal lipopeptide used for the biocontrol of fungal plant diseases. There are some methods, like measurement of emulsification [6], measurement of surface or interfacial tension [7], drop collapse tests [8] and oil spreading techniques [9], which have been used for the screening and detection of other biosurfactant producers. However, fewer studies on the quantification of antifungal lipopeptides have been reported. The development of rapid and accurate methods for estimating iturin A concentrations and yields is necessary for selecting strains presenting high production properties as well as for the routine estimation of biosurfactants produced by various strains in different media and culture conditions [10]. It can also provide an index for the construction of engineered strains and the optimization of fermentation conditions.

Various methods, such as membrane filtration [11], liquid membrane extraction [12], acid precipitation [13] and liquid–liquid extraction [13], have been used for extracting lipopeptides from fermentation broth. Among these, acid precipitation is the most popular method, whether for obtaining lipopeptides or as a pretreatment for quantification. However, this method is laborious and time consuming as the fermentation broth should stay at 4 °C for one night after pH adjustment to 2.0 then the precipitates obtained by centrifugation are dissolved in methanol after washing with water till pH 7.0. Therefore, this method is not suitable for rapid quantitative analysis, especially in industrial production. Liquid–liquid organic solvent extraction is also a pretreatment method for quantification, though it is associated with poor recovery and considerable environmental concerns. In this study, aqueous two-phase extraction (ATPE) is introduced for the preceding treatment. Aqueous two-phase systems (ATPS) are formed when two polymers or one polymer and one salt are dissolved in water above a threshold concentration [14]. This is important for bio-active biosynthesized compounds, as their active effects can be destroyed by some poisonous organic solvents and high surface tension. In ATPS, two phases are both with water content of 80% or more and show very low surface tension. So the bio-active biosynthesized compounds cannot abate [15]. In industrial production, it is used for the extraction of proteins [16], enzymes [17,18], nucleic acids [19], α-galactosidase [20], penicillin G [21] and other commercial and biosynthesized compounds [22,23].

* Corresponding author. Tel.: +86 025 84396291; fax: +86 025 84396824.
E-mail address: shenqirong@njau.edu.cn (Q. Shen).
[1] Qiwei Huang and Qirong Shen (Corresponding author) contributed equally to this paper.

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.07.041

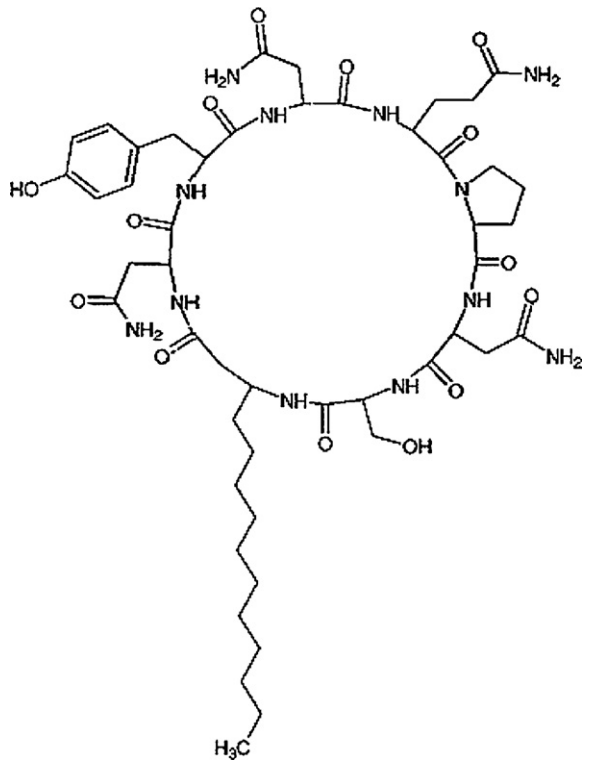

Fig. 1. The structure of iturin A. Iturin A was consisted of three homologues with different lengths of carbon chains between them. Heptapeptides: L-Asn, D-Tyr, D-Asn, L-Gln, L-Pro, D-Asn, and L-Ser; fatty acid chains: C14, C15, C16.

In the present study, the feasibility of combining aqueous two-phase systems with HPLC for the quantification of iturin A is described. In our study, a new ATPS was constructed with ethanol as extractant and ammonium sulfate as the salt for phase separation. Iturin A was pre-concentrated in an ethanol-rich phase, and the ethanol-rich phase could be directly detected by HPLC after reducing its volume in methanol. The HPLC conditions were optimized to avoid the interference of impurities. This method seems to be fast, simple and efficient for the rapid quantification of iturin A.

2. Materials and methods

2.1. Microorganisms

The iturin A producer used in this study was isolated from a soil sample by the Key Laboratory of Solid Organic Waste Utilization, Nanjing Agricultural University. This microorganism was identified as *Bacillus amyloliquefaciens* NJN-6 (NJN-6) (CGMCC accession No. 3183, China General Microbiology Culture Collection Center). The strain was grown on Luria–Bertani agar plates (LB; 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter) and re-cultured after one month.

2.2. Culture medium and conditions

Landy medium was used for the cultivation of the NJN-6 strain. The major components in the synthetic medium were (g/l): 20 g glucose, 5 g L-glutamic acid, 0.5 g $MgSO_4$, 0.5 g KCl, 1.0 g KH_2PO_4, 0.15 mg $FeSO_4$, 5.0 mg $MnSO_4$, 0.16 mg $CuSO_4$ and 2 mg L-phenylalanine, and the pH was adjusted to 7.0 with 1 M of NaOH. All fermentation was performed in 500 ml Erlenmeyer flasks with a 100 ml working volume. The cultures were incubated in a rotary incubator shaker for 72 h at 30 °C at 170 rpm.

2.3. Aqueous two-phase system

The phase behaviors of the ethanol–salt system were studied by observing the salt concentration in a series of solutions containing different concentrations of ethanol required for the onset of turbidity. $(NH_4)_2SO_4$ was used as the phase separation salt. To characterize the ethanol–$(NH_4)_2SO_4$ systems, 1 ml of fermentation broth was added to a 2 ml centrifuge tube. Then, different volumes of ethanol and different amounts of $(NH_4)_2SO_4$ were added until a cloudy appearance was observed, and a two-phase system was produced after vortexing. The tested volumes of ethanol were 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75 and 0.80 ml, and the amount of $(NH_4)_2SO_4$ required for turbidity was measured by weight.

2.4. Aqueous two-phase extraction

For this purpose, 1 ml of fermentation broth and 0.2 ml of ethanol was added to a 2.0 ml centrifuge tube. The pH was adjusted to 9.0 with 1 M of NaOH, and 0.5 g of $(NH_4)_2SO_4$ was added to the mixture. Complete separation of the two phases was achieved by centrifugation for 5 min at 6000 × *g* following vortexing for 5 min. The upper phase was carefully removed and transferred to another 2.0 ml centrifuge tube using a pipette. The lower water phase was extracted again with 0.2 ml of ethanol. The upper phases were mixed and diluted to 1 ml with methanol. The salt was separated by centrifugation. The extract was then filtered through a 0.45-μm membrane, and iturin A was analysed by HPLC.

2.5. Optimization of HPLC conditions

The amount of iturin A in the extract was detected by HPLC (1200 series, Agilent, USA) at 230 nm using a UV detector with the flow cell size of 5 mm, 1 μL, 40 bar (G1314B). The HPLC analysis was performed using an Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm particles). Mobile phase A was water with 0.1% acetic acid, and mobile phase B was acetonitrile (A:B = 60:40). The flow rate and the temperature of the column were optimized to allow for appropriate separation of iturin A from any impurities. The tested flow rates were 0.6, 0.8 and 1.0 ml/min, and the temperatures were 20, 30, and 40 °C.

2.6. Calibration and method validation

For this purpose, 1 mg of iturin A (Sigma Chemical Company, USA, >90%) was dissolved in 1 ml methanol and diluted to obtain different concentrations (20, 40, 60, 80, 100, 120 and 140 mg/l) with methanol. Then 5 μl of the standard solutions was injected into the chromatographic system sequentially using the same chromatographic conditions described above, and the linear regression equation was obtained. A total of 5 replicates of an 80 mg/l standard solution were analysed to determine the RSD of retention time and peak area.

2.7. Application analysis

To test the accuracy and precision of the method developed in this study, fermentation broth samples were fortified with iturin A at two different concentrations (20 and 30 mg/l). The samples were treated as described above, and 10 μl of the extract was assayed in the same way as the standard solutions. Three replicates were analysed.

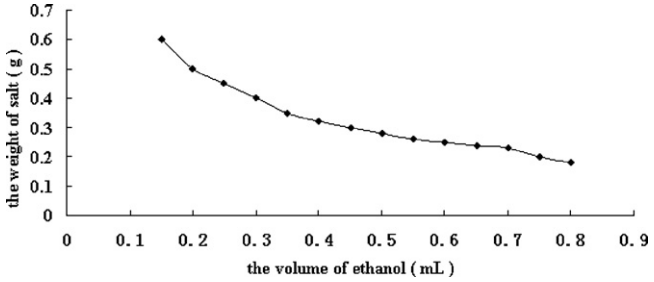

Fig. 2. Phase diagram of the aqueous-two phase system. The tested volumes of ethanol were 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75 and 0.80 ml, and the amount of $(NH_4)_2SO_4$ required for turbidity was measured by weight. The separation of the two phases was achieved by centrifugation for 5 min at 6000 × *g*.

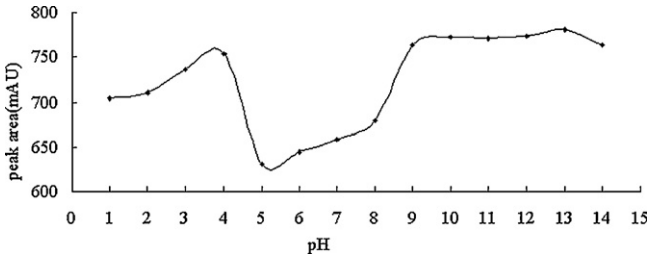

Fig. 3. The effect of pH of the solution used in the extraction process on the recovery of iturin A. The samples were analysed by HPLC, and the peak areas were used for the evaluation of recovery. ATPE conditions: 0.2 ml ethanol, 0.5 g $(NH_4)_2SO_4$, extracted twice at room temperature.

3. Results and discussion

3.1. Phase diagrams

The phase diagram (Fig. 2) shows the plots of the concentrations of the $(NH_4)_2SO_4$ solution at which the two coexisting isotropic phases convert to a single isotropic solution of ethanol, as examined at 25 °C. The two-phase system was the area above the curve, and the single-phase system was below the binodal curve. The data on phase separation showed that with the increase of the concentrations of $(NH_4)_2SO_4$, the volume of the coacervate phase was decreased, and the viscosity of the coacervate phase was increased, so that it was difficult to handle. Different volumes of ethanol and different amounts of $(NH_4)_2SO_4$ were added until a cloudy appearance was observed, and a two-phase system was produced after vortexing. Therefore, 0.2 ml of ethanol and 0.5 g $(NH_4)_2SO_4$ were selected for use in the following experiments.

3.2. ATPE procedure

3.2.1. Effects of temperature and centrifugation time on extraction efficiency

The effects of temperature and centrifugation time on the recovery of iturin A were studied. It was found that the temperature and centrifugation time did not exert any notable effect on the recovery of iturin A. Therefore, all extraction experiments were processed at room temperature with centrifugation at 6000 × *g* for 5 min. These results were in agreement with findings from a previous study [24] in which isoniazid was quantified using an ATPE coupled with HPLC method.

3.2.2. Effect of pH on extraction efficiency

To evaluate the effects of the pH of the solution used in the extraction process, the recovery of iturin A was determined at different pH levels (1–14). As shown in Fig. 3, the extraction efficiency was decreased when the pH was in the range from 5 to 8. The efficiency was lowest at a pH of 5 to 6. Below pH 4 and above pH 9, the highest recovery level was observed.

3.3. Optimization of HPLC conditions

The temperature of the column is an important factor considered in the HPLC analysis. The results presented in Fig. 4 shows that iturin A could be separated from impurities better at low temperature than at high temperature. Iturin A separation was performed at 20, 30 and 40 °C. However, the maximum purity was obtained at 20 °C. Therefore, 20 °C was chosen as the temperature of the column. Three flow rates (0.6, 0.8 and 1.0 ml/min) were chosen and best separation of iturin A was obtained at a flow rate of 0.6 ml/min.

3.4. Application analysis

3.4.1. Method calibration

In this step, 5 μL of the standard solutions was injected into the chromatographic system using the chromatographic conditions described above. The obtained linear regression equation was $Y = 2.8345x + 12.614$ (x is mg/l and Y is peak area of the chromatogram), correlation coefficient ($R2$) was 0.996, relative standard deviation of retention time was 1.29% and relative standard deviation of peak area was 1.45%.

3.4.2. Method validation

The proposed method was applied for the determination of the concentration of iturin A in a fermentation sample. The chromatograms of the extracted solution and the standard samples are shown in Fig. 5. The retention times of three homologues of iturin A were 8.61, 11.61 and 12.17. The concentration of 33.86 mg/l of iturin A was determined by the regression equation obtained from the chromatogram of peak areas and concentrations of standard solutions. To test the accuracy and precision of the method, fermentation samples were fortified with iturin A at two different concentrations (20 and 30 mg/l), and 3 replicates per sample were analysed. The mean of the obtained results were 53.1 and 62.6 mg/l of iturin A. The recovery values were varied from 96 to 97%. The results showed that the pretreatment and the determination of iturin A in the fermentation broth were successfully accomplished using the ATPE-HPLC method.

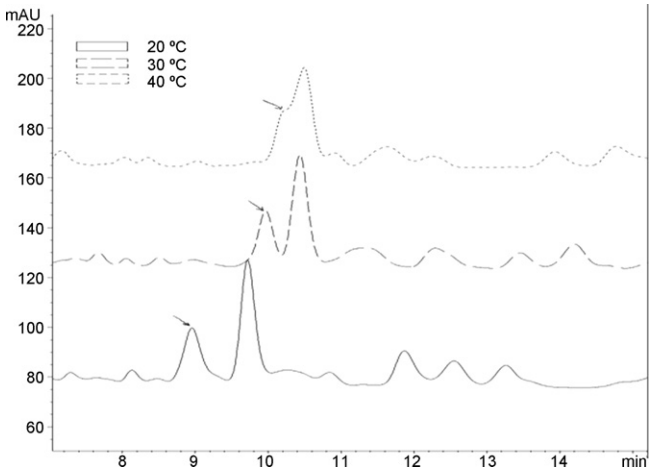

Fig. 4. Chromatogram of iturin A extracted sample obtained at different temperatures of the column. The tested temperatures were 20, 30, and 40 °C. HPLC conditions were 0.6 ml/min and 230 nm.

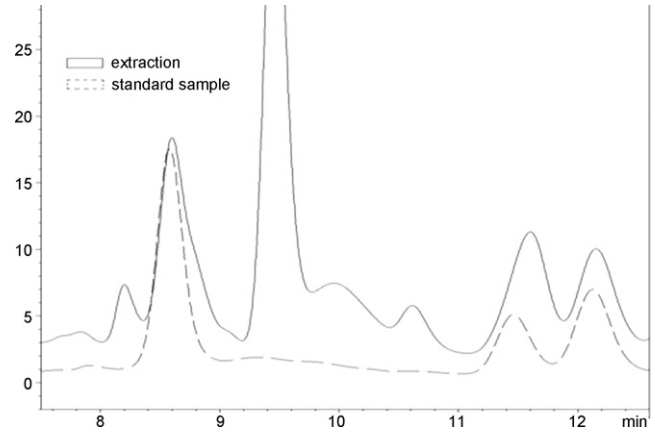

Fig. 5. Chromatogram of the extract and the standard sample of iturin A. HPLC conditions: 0.6 ml/min, 20 °C, 230 nm.

Iturin A contains a peptide region, and thus, it is expected that its structure should be dependent on pH. The peptide should be precipitated at its isoelectric point, and it could not be extracted efficiently. Iturin A is a cyclolipopeptide containing seven residues of α-amino acids (L-Asn, D-Tyr, D-Asn, L-Gln, L-Pro, D-Asn, and L-Ser) and one residue of a β-amino acid [25]. The p*I* values of all of the five kinds of α-amino acids, except L-Pro, are at 5.0–6.0, so the p*I* value for iturin A may be 5.0–6.0. In the present study, the recovery was found to be lowest at pH of 5.0–6.0. The structure of iturin A can affect its antibiotic activity [9], and its structure can be affected by pH. Therefore, the pH used in the precipitation step was neutralized to 7.0 with 6 N NaOH for subsequent study [26]. Extraction was performed at pH 9 in our experiments, and the structure and antibiotic activity of iturin A were not affected.Several methods were performed to analyse some antibiotics from liquid or solid feed premixes. Colistin [27,28] and sulfamethazine [29,30] were analysed by HPLC method, while oxytetracycline [31,32] was analysed by spectrofluorimetry method and some parameters were also studied. In our experiment, acetonitrile and water containing 0.1% TFA are the mobile phase most commonly used for lipopeptide separation [9]. However, we used acetic acid in our study instead of TFA as acetic acid is more safe and facile. An impurity associated with the approximate retention time of iturin A was found, and the change of the proportion of acetonitrile and water was not efficient. Thus, optimization of the temperature appeared to be necessary. We optimized the procedure by decreasing the temperature and flow rate used for HPLC detection. The RSD of the retention time and the peak area were below 2.0%, which showed that HPLC detection was an authentic method for the quantification of iturin A. HPLC is one of the most popular method used for the determination of antibiotics. Kinsella [33] quantified the iturin A and surfactin in the rhizosphere soil using ultrasonic and solid-phase extraction (SPE) with HPLC and mass spectroscopy (MS). This method was much more burdensome than the method used in our experiments, as they extracted rhizosphere samples ultrasonically by acetonitrile: water containing 0.1% trifluoroacetic acid. After ultrasonic agitation, extracts were evaporated with dry nitrogen and dissolved in methanol. Using SPE columns of octadecyl unendcapped bonded silica, iturin A were eluted with methanol, dried, redissolved in methanol, and refiltered for subsequent HPLC injection and analyses. Furthermore, the HPLC conditions used in their study were not sufficiently optimized to separate iturin A and surfactin from impurities.

In this study, a new rapid, safe and time-saving ATPE method was developed. Successful extraction of iturin A was achieved using 0.2 ml of ethanol and 0.5 g of $(NH_4)_2SO_4$. It was found that the separate two phases did not appear when the volume of ethanol was under 0.1 ml in 1 ml of fermentation broth; this was also the case for $(NH_4)_2SO_4$ under 0.2 g. When two aqueous solutions of certain incompatible substances, such as polyethylene glycol (PEG) and glucose or PEG and salt, are mixed above a critical concentration, liquid–liquid phase separation occurs [34]. There is a limitation related to each substance, in that two phases will not form, no matter how high the concentration of one substance, when the concentration of the other substance is below a certain limit.

The ATPS using ethanol/$(NH_4)_2SO_4$ presented a high recovery rate of iturin A from the NJN-6 strain fermentation broth. Employing this method could reduce the time required for the extraction operation compared to the acid precipitation method [26,35], which requires 12 h for acid precipitation and 8 h for redissolution in methanol, whereas half an hour was sufficient to complete the ATPE-HPLC method. The method described here is suitable for industrial detection of iturin A, especially for real-time monitoring, considering its celerity, high efficiency and safety.

4. Conclusion

In this study, the antifungal substance iturin A produced by the NJN-6 *B. amyloliquefaciens* strain was extracted by ATPE and quantified by HPLC. Our results showed that the ATPE-HPLC method performed favorably in the extraction and quantification of iturin A and thus has potential to be used in the rapid quantification of iturin A. Additionally, ATPE-HPLC quantification of iturin A has the following distinct advantages: (1) compared with traditional liquid–liquid extraction or acid precipitation, the ATPE technique is simple, rapid and more environment friendly; and (2) using HPLC as the identification method results in accurate quantification.

Acknowledgements

This research was financially supported by the Agricultural Ministry of China (201103004), Ministry of Science and Technology of China (2010AA10Z401) and the Nature Science Foundation of China (31070462).

References

[1] S. Mukherjee, P. Das, R. Sen, Trends Biotechnol. 24 (2006) 509.
[2] Z. Zhao, Q. Wang, K. Wang, K. Brian, C. Liu, Y. Gu, Bioresour. Technol. 101 (2010) 292.
[3] H.-L. Chen, Y.-S. Chen, R.-S. Juang, J. Membr. Sci. 299 (2007) 114.
[4] G.Y. Yu, J.B. Sinclair, G.L. Hartman, B.L. Bertagnolli, Soil Biol. Biochem. 34 (2002) 955.
[5] M. Ongena, P. Jacques, Trends Microbiol. 16 (2008) 115.
[6] D.G. Cooper, B.G. Goldenberg, Appl. Environ. Microb. 53 (1987) 224.
[7] M.E. Mercade, L. Monleon, C. deAndres, I. Rodon, E. Martinez, M.J. Espuny, A. Manresa, J. Appl. Bacteriol. 81 (1996) 161.
[8] A.A. Bodour, R.M. Miller-Maier, J. Microbiol. Methods 32 (1998) 273.
[9] M. Morikawa, Y. Hirata, T. Imanaka, BBA: Mol. Cell Biol. L. 1488 (2000) 211.
[10] S. Mukherjee, P. Das, R. Sen, J. Microbiol. Methods 76 (2009) 38.
[11] M.H.M. Isa, R.A. Frazier, P. Jauregi, Sep. Purif. Technol. 64 (2008) 176.
[12] K. Dimitrov, F. Gancel, L. Montastruc, I. Nikov, Biochem. Eng. J. 42 (2008) 248.
[13] H.L. Chen, R.S. Juang, Biochem. Eng. J. 38 (2008) 39.
[14] P.A. Albertsson, Anal. Biochem. 161 (1987) 227.
[15] M. Kabiri-Badr, H. Cabezas, Fluid Phase Equilibr. 115 (1996) 39.
[16] C.L. Liu, D.T. Kamei, J.A. King, D.I.C. Wang, D. Blankschtein, J. Chromatogr. B 711 (1998) 127.
[17] M.C. Madhusudhan, K. Raghavarao, S. Nene, Biochem. Eng. J. 38 (2008) 414.
[18] S. Nitsawang, R. Hatti-Kaul, P. Kanasawuda, Enzyme Microb. Technol. 39 (2006) 1103.
[19] F. Luechau, T.C. Ling, A. Lyddiatt, Sep. Purif. Technol. 68 (2009) 114.
[20] K. Naganagouda, V.H. Mulimani, Process Biochem. 43 (2008) 1293.
[21] P.-y. Bi, D.-q. Li, H.-r. Dong, Sep. Purif. Technol. 69 (2009) 205.
[22] A.F. Jozala, A.M. Lopes, P.G. Mazzola, P.O. Magalhdes, T.C.V. Penna, A. Pessoa, Enzyme Microb. Technol. 42 (2008) 107.
[23] Z.G. Li, H. Teng, Z.L. Xiu, Process Biochem. 45 (2010) 731.

[24] Z.M. Zhou, D.Y. Zhao, J. Wang, W.J. Zhao, M.M. Yang, J. Chromatogr. A 1216 (2009) 30.
[25] A. Grau, A. Ortiz, A. de Godos, J.C. Gomez-Fernandez, Arch. Biochem. Biophys. 377 (2000) 315.
[26] X. Bie, Z. Lu, F. Lu, J. Microbiol. Methods 79 (2009) 272.
[27] B.C. Grande, M.S.G. Falcon, C. Perez-Lamela, M.R. Comesana, J.S. Gandara, Chromatographia 53 (2001) S-460.
[28] B. Cancho-Grande, M. Rodriguez-Comesana, J. Simal-Gandara, Chromatographia 54 (2001) 481.
[29] B. Cancho Grande, M.S. Garcia Falcon, M. Rodriguez Comesana, J. Simal Gandara, J. Agric. Food Chem. 49 (2001) 3145.
[30] F.d. Zayas-Blanco, M.S. Garcia-Falcon, J. Simal-Gandara, Food Control 15 (2004) 375.
[31] M. Arias, M.S. Garcia-Falcon, L. Garcia-Rio, J.C. Mejuto, R. Rial-Otero, J. Simal-Gandara, J. Food Eng. 78 (2007) 69.
[32] R. Fernandez-Gonzalez, M.S. Garcia-Falcon, J. Simal-Gandara, Anal. Chim. Acta 455 (2002) 143.
[33] K. Kinsella, C.P. Schulthess, T.F. Morris, J.D. Stuart, Soil Biol. Biochem. 41 (2009) 374.
[34] M. Goldberg, Biochimie 68 (1986) 1312.
[35] H.-S. Kim, B.-D. Yoon, C.-H. Lee, H.-H. Suh, H.-M. Oh, T. Katsuragi, Y. Tani, J. Ferment. Bioeng. 84 (1997) 41.