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Research on The Proteome Response of *Acidithiobacillus ferrooxidans* to Phosphate Starvation by SELDI-Protein Chip Technologies*

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Abstract *Acidithiobacillus ferrooxidans* is a chemolithotrophic microorganism capable of using ferrous ions and sulphides as energy sources. This microorganism has an important role in the bioleaching of minerals. During this process, the bacteria are normally subjected to several stressing conditions, such as temperature changes, lack of nutrients or pH changes, which may affect the efficiency of the bacterial action. SELDI is a recent technology that allows for high-throughput proteomics studies. The Protein Chip SELDI technology was used to generate comparative protein profiles of *Acidithiobacillus ferrooxidans* grown under phosphate starvation or normal condition additionally adding Fe^{2+} as energy resource. There were 13 significantly differential expressed protein's peaks found by using SELDI Protein Chip technologies, which made a solid foundation for further isolation these low molecular proteins by adopting technologies such as HPLC *etc*.

Key words *Acidithiobacillus ferrooxidans*, phosphate starvation, proteomics, SELDI Protein Chip

Microbes display a remarkable ability to adapt to environmental change. Specific environmental changes are often detected *via* biochemical signals, and in many cases these lead to the activation of specific physiological responses that counteract the microorganism has an important role in the bioleaching of mineral [1~3]. During this process, the bacteria are normally subjected to several stressing conditions, such as temperature changes, lack of nutrients or pH changes, which may affect the efficiency of the bacterial action.

SELDI is a recent technology that afford high-throughput proteomics studies. Retentate chromatography of protein sample can be applied directly on Protein Chip Arrays(Cihergen Biosystems, Fremont, CA, USA) [4~6], which display various kinds of chemically activated surfaces which bind molecules based on established principles such as ion exchange chromatography, metal ion affinity and hydrophobic affinity. The subset of proteins binding to an array with a certain surface can be varied by changing the properties of the binding buffer used for the washing steps that remove the unbound or weakly bound molecules and salts which can interfere with the analysis. Protein detection is then accomplished by SELDI-TOF-MS. Proteins are crystallized with an excess of an energy absorbing molecule (EAM) and this mixture is desorbed and ionized by an nitrogen laser. The TOF spectrum is then acquired by the Protein Chip Reader (PBS Ⅱ, Ciphergen Biosystems). In this study, we used the Protein Chip SELDI technology to generate comparative protein profiles of *Acidithiobacillus ferrooxidans* grown under phosphate starvation and normal condition additionally adding Fe^{2+} as energy resource. This information might be

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helpful to understand the mechanisms of *Acidithiobacillus ferrooxidans* adapting to different extreme conditions.

1 Materials and methods

1.1 Strains and growth conditions

Acidithiobacillus ferrooxidans isolated from Chengmenshan Mine, China, was cultured in 9K base medium or phosphate starvation respectively additionally adding Fe^{2+} as energy resource at 30℃ [7,8]. The pH of 9K basic medium was adjusted to 2.0 with 2 mol/L H_2SO_4.The reactors were 500-ml Erlenmeyer flasks mounted to a HZQ-C constant temperature vibrator agitated at 200 r/min, and the initial liquid volume of each reactor was 200 ml. Sterility was ensured by autoclaving the flasks and liquid solutions at 121℃ for 25 min, covering the opening with cotton plugs. Bioreactors were confirmed to maintain dissolved O_2 concentrations in excess of 6 mg/L during the experimented periods. The number of cells was counted in a Helber chamber.

1.2 Preparation of protein extracts

When the culture reached the later exponential phase of growth, it was centrifuged at 5 000 *g* for 10 min, and the bacterial sediment was firstly washed three times with the same basic culture medium without ferrous ions, then (from 1 000 ml of culture) were washed three times with ice-cold water and resuspended in 50 μl of 7.5 mol/L urea, 2.5 mol/L thiourea, 1.25 mmol/L EDTA, 1.75 g/L Pepstatin A, protease inhibitor cocktail. Cells were disrupted by sonicated with a sonicater six times for 30 s with 1 min on ice in between. Samples were vortexed thoroughly, and shaken. Cell debris were removed by centrifugation at 1 0000 *g* for 10 min at 4℃, and the resulting supernatant stored at −70 ℃. Protein concentrations were measured using a Coomassie protein assay kit with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA).

1.3 Protein assay

The protein concentration of samples was measured by using Coomassie Protein Plus (Pierce, UK) according to the manufacturer's instructions using bovine serum albumin as the standard. Samples were modulated to a concentration of 1 g/L.

1.4 SELDI analysis and data collection

The samples were added to Protein Chips (details are shown as below) and transferred to the PBS Ⅱ reader. The Protein Chip surfaces applied in this study were NP20 (normal phase, silica), IMAC-Cu (Immobilized Metal Affinity Capture arrays), SAX2 (strong anionic exchange) and WCX2 (weak cation exchange). All chips were placed on the Protein Biological System Ⅱ mass spectrometer reader (Ciphergen Biosystems, Inc., Fremont, CA, USA), and time-of-flight spectra were generated by averaging 60 laser shots collected in the positive mode at laser intensity 250, and detector sensitivity nine[9].

1.4.1 IMAC-Cu. All incubations were performed in a humidity chamber. The IMAC surface is charged *via* incubation with a 100 mmol/L $CuSO_4$ neat solution. Spots were outlined with a Pap pen and allowed air dried. 10 μl binding buffer (100 mmol/L $CuSO_4$, 0.1% Triton X-100, 400 mmol/L NaCl, pH 7.2) added twice for 5-min incubations at room temperature, and this step repeated one time. Each spot was washed in distilled water for about 10 s to disposed redundant Cu. 2 μl sample applied to each spot and incubated for 30 min. Each spot was then washed three times in binding buffer and two times in distilled water. 1 ml of sinnapinic acidic (SPA) was applied to each spot when still moist and waited to dry before a second plus of matrix.

1.4.2 SAX2 Protein chip. All incubations were carried out in a humidity chamber. Spots were outlined by using a hydrophobic Pap pen (DAKO) and 10 μl binding buffer (0.1% Triton X-100, pH 7.5, 50 mmol/L Tris-HCl) plused twice for 5-min incubations. The buffer was disposed and 3 μl sample added to each spot, incubating for 30 min. Each spot was then washed three times in binding buffer (0.1% Triton X-100, pH 7.5, 50 mmol/L Tris-HCl) and two times in distilled water. 1 ml of sinnapinic acidic (SPA) was plused to each spot when still moist and waited to air dry prior to a second application of matrix.

1.4.3 WCX2 Protein chip. All incubations were carried out in a humidity chamber. Spots were outlined by using a Pap pen and 10 μl 10mmol/L hydrochloric acid plused two times for 5-min incubations. Each spot was washed three times with 10 μl distilled water and then incubated twice for 5 min with binding buffer (0.01 % Triton X-100, pH 5.0, 50 mmol/L ammonium acetate). The buffer was disposed and 3 μl sample added to the Protein Chip surface. After 30 min incubation, the sample was disposed and each spot washed three times in binding buffer solution and two times with distilled water. 1 ml of sinnapinic acidic (SPA) was plused to each spot when still moist and

waited to dry before a second adding of matrix.

2 Results

2.1 The measurement of growth curves for *Acidithiobacillus ferrooxidans*

To explore the growth characteristics of *Acidithiobacillus ferrooxidans* for the purpose of performing the comparative proteome analysis of this organism, we experimented the growth curves of *Acidithiobacillus ferrooxidans*. As showing in Figure 1, both growth curves followed the same lag, logarithmic, stationary and aging phase as seen in other bacteria. The logarithmic phases was from 10 to 32 h for *Acidithiobacillus ferrooxidans* cultivated with Fe^{2+} and from 20 to 60 h for *Acidithiobacillus ferooxidans* cultivated when without phosphate. When *Acidithiobacillus ferrooxidans* cultivated with Fe^{2+} for about 36 h, the number of cells would reach the highest(about $1\times10^{6.5}$ cells/ml). While *Acidithiobacillus ferrooxidans* grew under phosphate starvation, the lag phase was greatly longer than that of *Acidithiobacillus ferrooxidans* grown under normal condition additionally adding Fe^{2+} as energy resource, and the number of cells reach maximum(about $1\times10^{5.5}$ cells/ml) after 60 h cultivation.

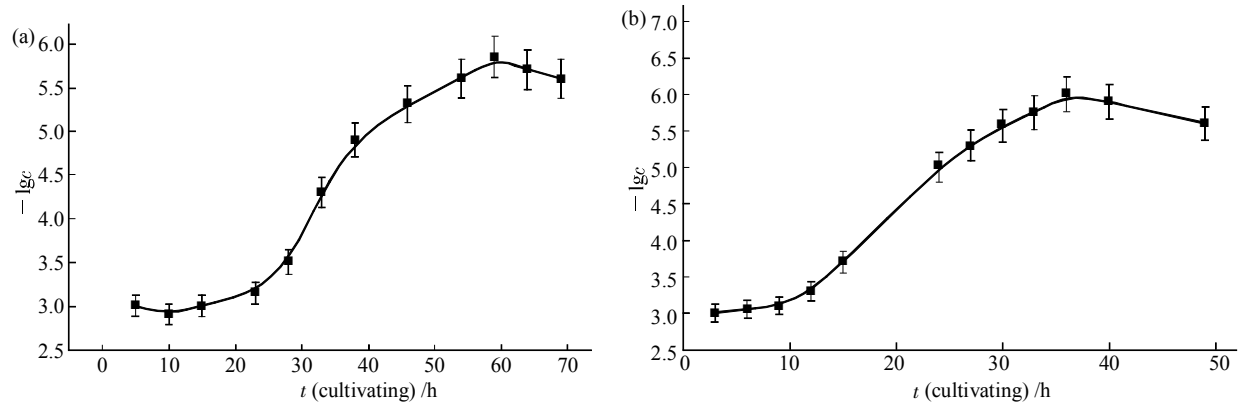

Fig. 1 Growth kinetics of *Acidithiobacillus ferrooxidans* under phosphate starvation and normal condition separately.

(a) *Acidithiobacillus ferrooxidans* bacterium grown under phosphate starvation additionally adding Fe^{2+} as energy resource; (b) *Acidithiobacillus ferrooxidans* bacterium grown under normal condition additionally adding Fe^{2+} as energy resource.

2.2 Optimal amount of protein for and reproducibility of SELDI analysis

To get an optimum amount of sample for SELDI profiling, different amounts of total proteins of *Acidithiobacillus ferrooxidans* were analyzed. A clear spectrum with 0.05 μg of total protein in 1μl of sample (Figure 2), particularly in the mass range 5 000 ～ 20 000 ku, was used in all future experiments. Small number of protein peaks were found in the higher mass range.

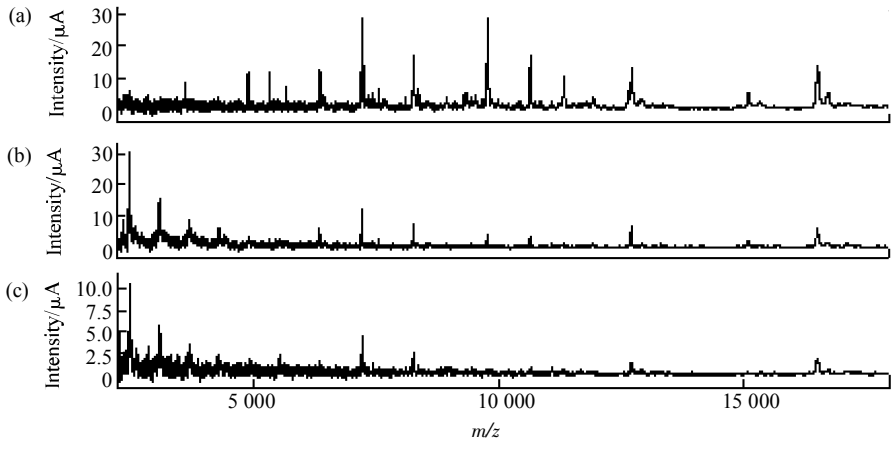

Fig. 2 Optimal amount of protein for and reproducibility of SELDI analysis

The optimal protein amount for SELDI profiles is 0.05 μg. A dilution series of a cytoplasmic/periplasmic sample was prepared and 1 μl of each analysed on an NP20 Protein Chip array using SPA as the EAM. The mass region shown is 0～20 ku. The total amount of protein analyzed was 0.05 μg, 0.2 μg and 0.3 μg respectively.(a) Fe: 0.05 g/L. (b) Fe: 0.2 g//L. (c) Fe: 0.3 g/L.

2.3 SELDI profiles of protein extracts obtained from *Acidithiobacillus ferrooxidans* cultivated with normal condition and phosphate starvation separately

The reproducibility of SELDI spectra, i.e. mass location and intensity from array to array on a single chip (intra-assay) and between chips (inter-assay), was determined using the pooled normal control sample. Three peaks in the range of 5 000～15 000 u observed on spectra randomly selected over the course of the study were used to calculate the coefficient of variance. The intra- and inter-assay coefficient of variance for normalized intensity (peak height or relative concentration) was 10% and 15% respectively.

We successfully established protein profilings of 2 normal condition-cultivated *Acidithiobacillus ferrooxidans*' cases and phosphate starvation-cultivated *Acidithiobacillus ferrooxidans'* cases by using three types of Protein Chips, among which about 130 peaks were detected in each spectrum for Protein Chip WCX2, about 76 peaks for Protein Chip IMAC-Cu and about 115 peaks for Protein Chip SAX2. To reduce the

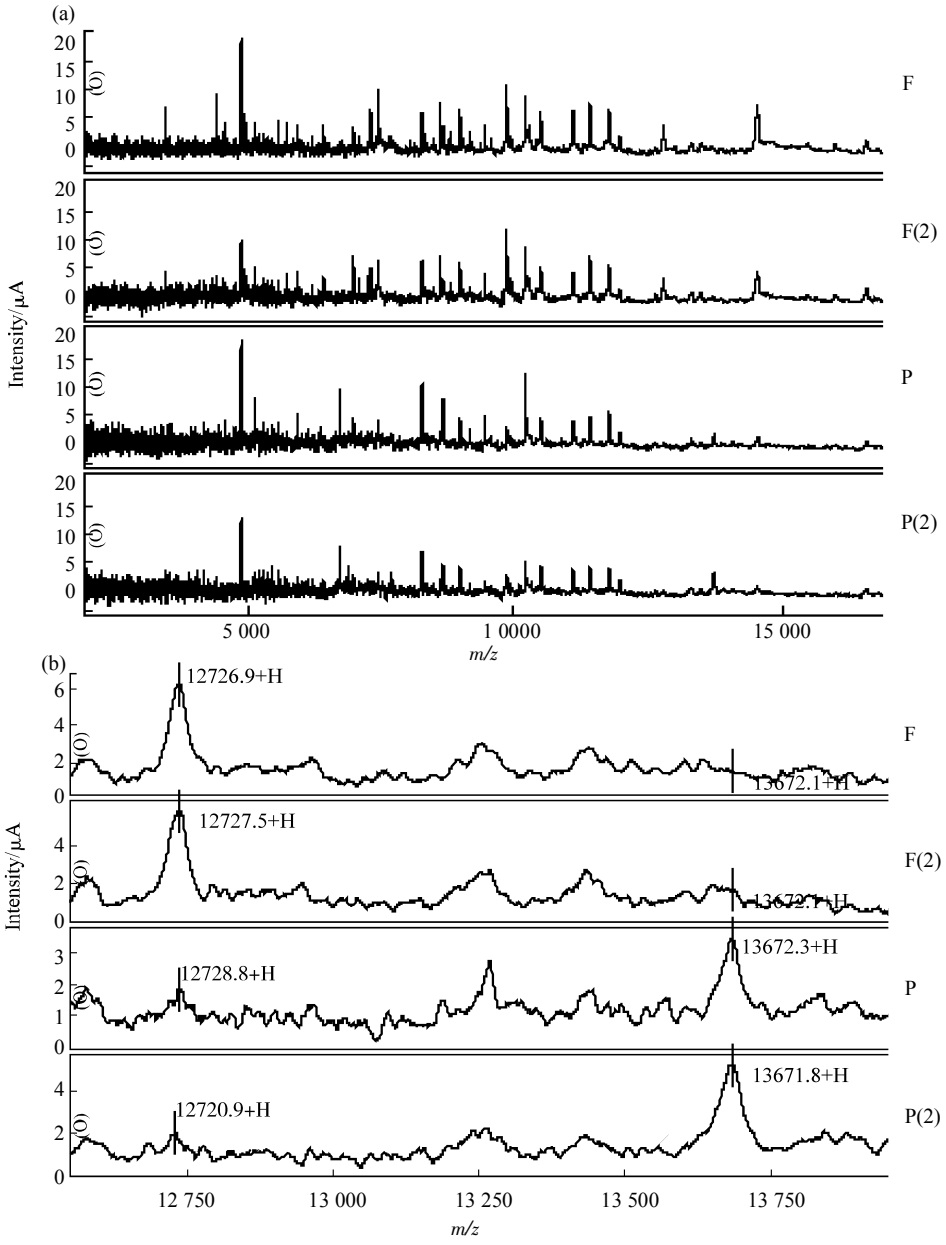

Fig. 3　Representative views of the spectra of proteins retained on the IMAC-Cu protein chips by SELDI-TOF-MS analysis

(a) Total View of WCX-2 spectra of proteins. (b) Among the scope of 12.5 to 14.0 ku, a 13.672 ku protein peaks was upregulated under phosphate starvation, while a 12.726 u protein peak was downregulated, taking Fe^{2+} as energy resource. (F), (F2): *Acidithiobacillus ferrooxidans* grown under normal condition additionally adding Fe^{2+} as energy resource; (P), (P2): *Acidithiobacillus ferrooxidans* grown under phosphate starvation additionally adding Fe^{2+} as energy resource.

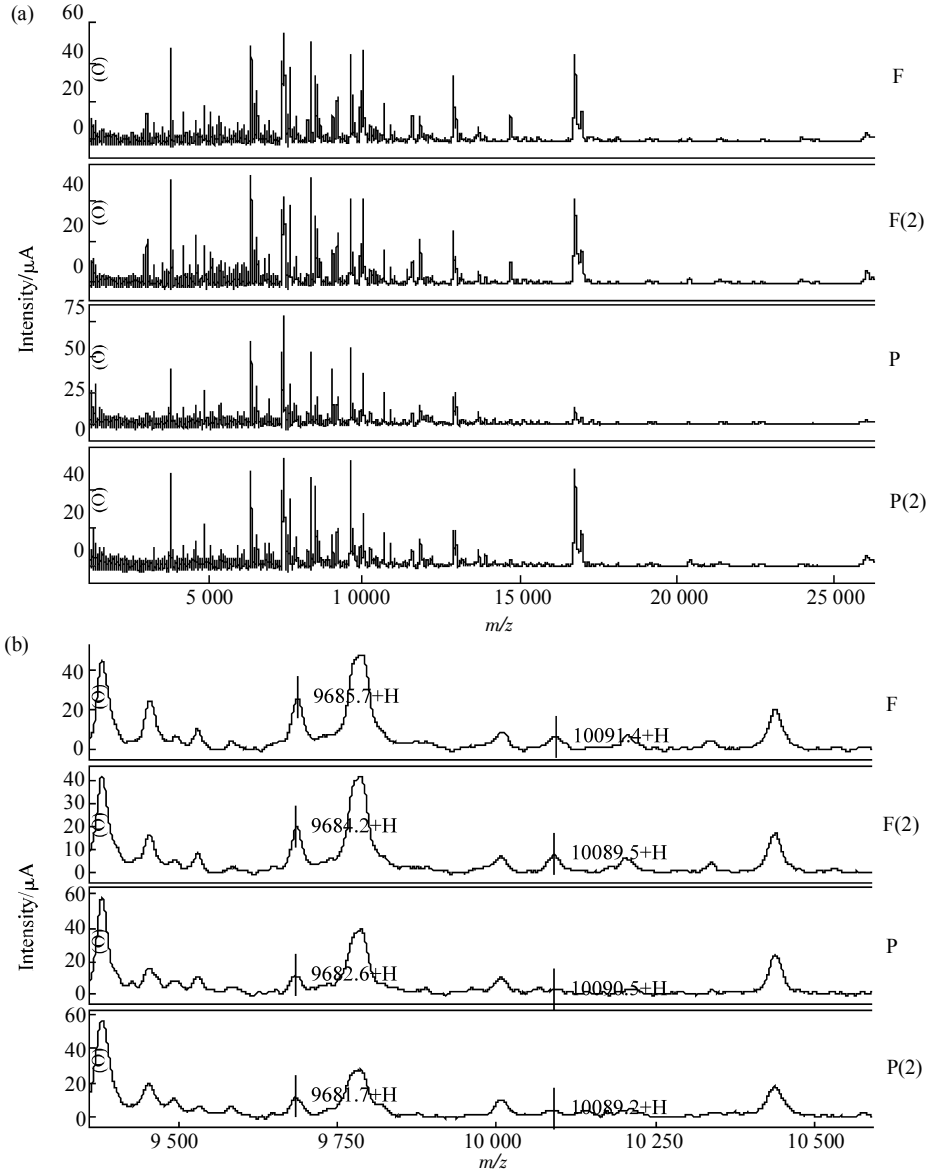

Fig. 4 Representative views of the spectra of proteins retained on theWCX-2 protein chips by SELDI-TOF-MS analysis

The protein profiling spectra were displayed according to their mass to charge ratio (*m*/*z*). (a) Total view of WCX-2 spectra of proteins. (b) Among the scope of 9 250～10 750 u, the 9.68 ku and 10.08 ku protein peaks were both downregulated under phosphate starvation taking Fe^{2+} as energy resource. (F), (F2): *Acidithiobacillus ferrooxidans* grown under normal condition additionally adding Fe^{2+} as energy resource; (P), (P2): *Acidithiobacillus ferrooxidans* grown under phosphate starvation additionally adding Fe^{2+} as energy resource.

deviation of masses and compensate slight shifts in mass caused by imperfections on the protein Chip Array surface, the protein profiles were normalized against a common peak (5.75 u for SAX2 Protein Chip; 16.527 ku for WCX2 ProteinChip; 16.5 ku for IMAC-Cu Protein Chip) used as common calibration.

After the optimized condition in which reproducible SELDI profiles were acquired have been established, differentially expressed protein were analyzed for protein extract from *Acidithiobacillus ferrooxidans* grown under phosphate starvation. A summary of the molecular masses of the differentially regulated proteins found on WCX2, IMAC-Cu and SAX2 are shown in Table 1. The cultivation of *Acidithiobacillus ferrooxidans* under phosphate starvation was related with the increase in relative peak intensity (8 780.82 u, 15 659.51 u,WCX2; SAX; 6 666.7 u, 13 672.08 u, IMAC-Cu) and decrease in relative of peaks (6 360.18 u, 10 087.07 u, 12941.04 u, SAX; 9 685.72 u, 11 185.53 u, WCX2；7 237.49 u, 8 546.39 u, 12 726.93 u, 14 483.85 u IMAC-Cu) (Figure 3～5).

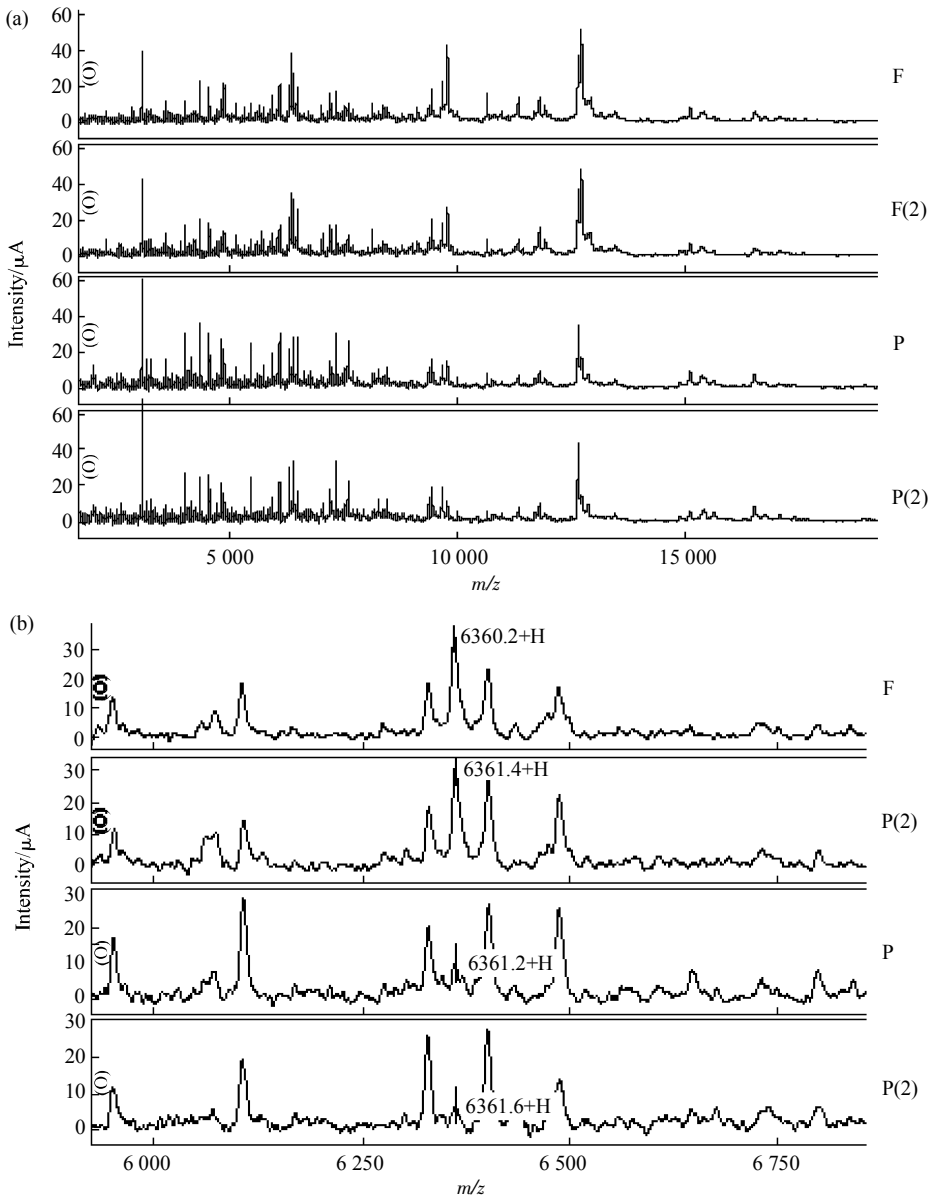

Fig. 5 Representative views of the spectra of proteins retained on the SAX-2 Protein chips by SELDI-TOF-MS analysis

(a) Total view of SAX-2 spectra of proteins. (b) Among the scope of 5.75 ku to 7.0 ku, a 6.36 ku protein peak was downregulated under phosphate starvation taking Fe^{2+} as energy resource. (F), (F2): *Acidithiobacillus ferrooxidans* grown under normal condition additionally adding Fe^{2+} as energy resource; (P), (P2): *Acidithiobacillus ferrooxidans* grown under phosphate starvation additionally adding Fe^{2+} as energy resource.

Table 1 The molecular masses of the differentially regulated proteins found on WCX2, IMAC-Cu and SAX2

m/ku	Average intensity ratio (P versus N)	*m*/ku	Average intensity ratio (P versus N)
6 360.18	0.314 173	11 185.53	0.342 190
6 666.70	6.088 083	12 726.93	0.285 033
7 237.49	0.255 374	12 941.04	0.269 164
8 546.39	0.368 709	13 672.08	3.950 701
8 780.82	13.871 191	14 483.85	0.291 940
9 685.72	0.489 122	15 659.51	3.004 933
10 087.07	0.464 078		

P: *Acidithiobacillus ferrooxidans* cultivated under phosphate starvation; N: *Acidithiobacillus ferrooxidans* cultivated under normal condition with Fe^{2+} as energy resource.

3 Discussion

As a necessary nutrient element for *Acidithiobacillus ferrooxidans*' growth, the starvation of phosphate will greatly influence the bioleaching activity of *Acidithiobacillus ferrooxidans*. Jerez *et al.*[10,11] found an out-membrane protein Omp40 was differentially expressed under phosphate starvation. Vera *et al.*[12] found the expression of PstS was markably increased when *Acidithiobacillus ferrooxidans* grown under phosphate starvation and anchored its position. There were two PstS-analogous genes in *Acidithiobacillus ferrooxidans*, each of which

had a putative signal peptide, indicating that PstS-encoded protein might be located at cytoplasm.

The convenience of use of SELDI-TOF makes its application possible even by individuals with minimal MS experience. This technique not only allows the discovery of proteins of interest in a sample, but it also suggests chromatographic supports useful in purification. Another important superiority of the Protein Chip Arrays lies in its applicability when only small amounts of sample are available for analyses, especially compared to other proteomic techniques. In our experiments, we found that the maximal concentration of *Acidithiobacillus ferrooxidans* when grown on Fe^{2+} was only about $1\times10^{6.5}$ cells/L. We also found that the *Acidithiobacillus ferrooxidans* genomic sequence in hand contains many proteins of a predicted molecular mass < 20 ku. So the SELDI technique is very suitable for *Acidithiobacillus ferrooxidans*' proteome research. Many papers have described the use of SELDI for the discovery of biomarkers for various diseases [13～18], but up to date, only a few showed the application of SELDI-TOF technology to bacterial proteomics. These papers mainly describe the use of the Protein Chip Arrays with purified proteins [19～22], or extracted surface proteins [23～26]. Several reports are available in the literature in which this technology is used with protein extracts[27]. In our study, IMAC-Cu, SAX2 and WCX2 SELDI Protein Chips were selected because types of ion exchange resin and buffer conditions for purification of the recognized peaks could be based on Protein Chip affinity condition during SELDI analysis. There were 13 significantly differential expressed protein's speak found in this study by using SELDI Protein Chip technologies, which made a solid foundation for further isolation these low molecular proteins by adopting technologies such as HPLC.

As the results we get from upon, we found that SELDI is an applicable tool for the analysis of cellular fraction of *Acidithiobacillus ferrooxidans* by compensating existing techonologies. Its use has the potential to promote proteomics research of *Acidithiobacillus ferrooxidans*.

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采用 SELDI 蛋白质芯片技术研究氧化亚铁硫杆菌对磷酸盐缺失的反应 *

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摘要 氧化亚铁硫杆菌(*At.f*)是能够利用 Fe^{2+} 和硫化矿来获取能量的一种化能自养菌．这种细菌在金属硫化矿的生物浸出中起着重要的作用．在硫化矿的生物浸出过程中，浸矿细菌通常会遇到多种胁迫条件，如温度的变化、营养成分的缺失和 pH 值的变化等，这些因素会影响到细菌的活性．因此对在胁迫条件下这类细菌的应急反应生理机制的研究具有重要的意义．SELDI 蛋白质芯片技术是近年一种高通量的蛋白质组学研究技术．测定了以 Fe^{2+} 为能源正常条件培养的 *At.f* 和磷酸盐缺失培养 *At.f* 的生长情况，绘制了相应的生长曲线；采用 NP20 蛋白质芯片，对 *At.f* 总蛋白的蛋白质芯片上样量进行了优化．在此基础上，采用 IMAC-Cu、SAX2、WCX2 三种特异性 SELDI 蛋白质芯片技术，获取了磷酸盐缺失培养 *At.f* 与正常条件培养的 *At.f* 的比较蛋白质图谱，采用软件对比较蛋白质图谱进行分析，发现了磷酸盐缺失培养 *At.f* 的 13 个明显差异表达的蛋白质分子，为进一步分离鉴定这些差异表达蛋白质奠定了基础．

关键词 氧化亚铁硫杆菌，磷酸盐缺失，蛋白质组学，生物浸出，SELDI 蛋白质芯片技术

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