Sample preparation for the metabolomics investigation of poly-gamma-glutamate-producing Bacillus licheniformis by GC–MS

Xin Li a, Dan Long a, Jian Ji a, Wuming Yang a, Zhaorui Zeng b, Shaodong Guo c, Zhixia Ji a, Gaofu Qi a, Shouwen Chen a,⁎

a State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China
b Department of Chemistry, Wuhan University, Wuhan 430072, PR China
c Health Science Center, Texas A&M University, TX 77843, USA

ARTICLE INFO

Article history:
Received 26 February 2013
Received in revised form 16 April 2013
Accepted 21 April 2013
Available online 28 April 2013

Keywords:
Metabolomics
Bacillus licheniformis
Poly-γ-glutamate
Quenching
Extraction

ABSTRACT

Metabolomics aims to analyze global intracellular metabolites of organisms. To study the intracellular metabolites of poly-γ-glutamate-producing Bacillus licheniformis, the quenching and extraction conditions were assessed and optimized. It indicated that perchloric acid was an appropriate quenching solution for B. licheniformis by measuring livability of cells, leakage of ATP, energy charge and intracellular metabolites. 0.85% NaCl was an appropriate washing solvent for a sample because it did not lead to serious leakage and would not affect operation of GC–MS. Among the four different extraction methods (cold pure methanol, PM; hot ethanol/water (75:25 v/v), HE; cold methanol/water (50:50 v/v), MW; and cold chloroform/methanol/water (50:25:25 v/v), CMW), HE was superior to others on the basis of the energy charge and intracellular metabolites, which could effectively inhibit enzymatic activities and extract more intracellular metabolites. The method could obtain some metabolites which were involved in the most important synthesis pathways of poly-γ-glutamate, including glycolysis, citric acid cycle and glutamate metabolism. It is the first evaluation of a sample preparation of poly-γ-glutamate-producing bacteria, which might be used as one model for the preparation of polymer-producing samples for metabolomics analysis.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Metabolomics is the study of cells by qualitative and quantitative analysis of all or a large number of small molecular metabolites, which are under a specific physiological condition (Koek et al., 2011; Nicholson et al., 1999). As a hinge connecting biologic geno-type to phenotype, metabolomics can overcome the shortcomings of genome, transcriptome and proteomics. As it reflects more accurate physiological and metabolic information of cells, metabolomics is gradually applied to the animal, plant and microorganism (Castrillo et al., 2003; Liu et al., 2009; Maharjan and Ferenci, 2003; T’Kindt et al., 2009; Trauger et al., 2008). Two factors are major obstacles to showing the whole picture of metabolomics. First, owing to a high diversity, a different physicochemical nature and an abundance of metabolites, it is hard to cover all of the metabolites in a single equipment. So, it is essential to choose an instrument with high selectivity, sensitivity, and resolution throughout for an accurate analysis of these metabolites (Liebeke et al., 2010). Today, many accurate technologies are used in metabolomics, like gas chromatography/mass spectrometry (GC/MS) (Tian et al., 2009; Borner et al., 2007; Koek et al., 2006; T’Kindt et al., 2009), liquid chromatography/mass spectrometry (LC/MS) (T’Kindt et al., 2009; Theodoridis et al., 2008), nuclear magnetic resonance (NMR) (Boroujerdi et al., 2009; Zheng et al., 2009) and capillary electrophoresis/mass spectrometry (CE/MS) (Ramautar et al., 2009; Hasunuma et al., 2011). Second, the process of sampling is a core factor to precisely reveal the full view of bacterial metabolomics. An unsuitable protocol for sample handling caused distortion of information of metabolomics, and it was not remedied by detection technologies.

Quenching is the first step in sample preparation of microbial metabolomics. Villas-Bôas et al., 2007 indicated that the methods of quenching worked mainly through rapid changes in temperature or pH of cells, which were usually achieved by placing a biological sample in contact with a cold (−40 °C) or hot (>80 °C) solution or with an acidic (pH < 2.0) or alkaline (pH > 10) solution. The strong inorganic acid was firstly used as a bacterial quenching solution in 1976 (Cook et al., 1976). Then Larsson used perchloric acid (PCA) to stop the metabolism of yeast and Escherichia coli in 1996 (Larsson and Tornkvist, 1996). The results showed that the metabolism of glucose could be suspended efficiently when the pH was less than 3. Because extreme
phs and high temperatures would potentially degrade several metabolites, 60% (v/v) aqueous methanol at a temperature below — 40 °C was firstly developed for Saccharomyces cerevisiae quenching (Castillo et al., 2003). After that, it was widely applied to other microbial metabolomics (Faijes et al., 2007; Canellas et al., 2009; Wellerdiek et al., 2009).

After rapid metabolic inactivation, the next step is to extract metabolites from the cell pellets using organic solvent. Recently, the commonly used reagents of extraction were cold methanol (Sellick et al., 2009; Faijes et al., 2007) and hot ethanol (Taymaz-Nikerel et al., 2009; Canellas et al., 2008; Faijes et al., 2007; Meyer et al., 2010). Dietmair compared twelve different extraction methods with CHO cells, which almost contained bacterial extraction methods in the present studies (Dietmair et al., 2010).

Bacillus licheniformis, a gram-positive forming-spore bacterium, has been widely used as a poly-γ-glutamate (γ-PGA) producing organism (Manocha and Margaritis, 2010; Bajaj and Singhal, 2010; Wang et al., 2011; Wei et al., 2010). γ-PGA is a homopolymer composed of γ- and l-glutamic acid units. Owning to its excellent characteristics including water-solubility, biodegradability, edibility, biocompatibility, metal-binding qualities, and non-toxicity toward humans and the environment, it has broad applications in the fields of medicine, foods, agriculture, chemical industry, etc. (Shih and Van, 2001). In order to understand how B. licheniformis efficiently synthesise γ-PGA, the metabolite characterization of this bacterium is indispensable to make sure that the metabolic network can work efficiently for the collection of cells from a high viscosity sample. Based on the results of the livability of a cell, leakage, energy charge (EC) and activity for the collection of cells from a high viscosity sample, perchloric acid was chosen as a quenching solution. Furthermore, four different extraction methods were evaluated and hot ethanol was a suitable method. The total process set up in this study could satisfy demand on the metabolomics investigation of poly-γ-glutamate-producing B. licheniformis.

2. Materials and methods

2.1. Strain, media, and culture condition

All pure standards were purchased from Sigma-Aldrich (American). B. licheniformis WX-02 (China Center for Type Culture Collection, CCTCC M208065) was grown at 37 °C on Luria–Bertani (LB) medium (per liter: tryptone 10 g, yeast extract 5 g, NaCl 10 g, pH 7.2). A single colony of B. licheniformis was inoculated into 10 mL LB broth and shaken at 180 rev/min and 37 °C for 12 h. Then the inoculum (1.5 mL) was transferred into a 250 mL flask containing 50 mL modified E medium (Wei et al., 2010) (gram per liter: glucose 20, l-glutamate 20, citrate 12, NH₄Cl 7, K₂HPO₄ 0.5, MgSO₄ · 7H₂O 0.5, FeCl₃ · 6H₂O 0.04, CaCl₂ · 2H₂O 0.15, MnSO₄ · H₂O 0.104, pH 6.5), cultured at 37 °C and 180 rev/min for 10 h until the mid-exponential phase.

2.2. Quenching solution and method

These quenching solutions included: (i) 0.25 mol/L HCl, (ii) 0.25 mol/L H₂PO₄, and (iii) 0.25 mol/L PCA. A 10 mL volume of culture in the exponential growth phase (OD₆₀₀ = 2.5) was cast into the precooled quenching solution at a ratio of 1: 1 and maintained at — 10 °C for 10 min. The mixture was centrifuged (9410 × g, 5 min) and the pellet was washed twice with sterile deionized water. Before determining the optimal extraction conditions, a common extraction method (boiling ethanol) was used for all quenching samples (Winder et al., 2008).

2.3. Washing of cell

In order to clear away residual acid and extracellular metabolites, the cells were washed twice with 10 mL of water, 0.85% NaCl, 2.3% NaCl and 2.5% (NH₄)₂CO₃ at — 4 °C respectively. Meanwhile the supernatant was collected, and an ATP assay kit (Beiytime, China) was used to detect the leakage of ATP of the samples.

2.4. Extraction of metabolites

Four different extraction protocols selected from the literature were tested in this work. Every extraction method was performed in triplicate on fermentation broth.

2.4.1. Pure-methanol extraction (PM)

After quenching with 0.25 mol/L PCA, the pellets were resuspended in 2.5 mL of pure methanol (— 40 °C), followed by vigorous mixing. Then the samples were incubated at — 40 °C for 10 min and vortexed several times during this period. Next, the samples were centrifuged, and the pellets were extracted again. After centrifugation, the supernatant was combined and stored at — 80 °C for further analysis.

2.4.2. Ethanol/water (75:25, v/v) extraction (HE)

After quenching with 0.25 mol/L PCA, the pellets were resuspended in 5.0 mL ethanol/water (75:25, v/v), followed by vigorous mixing. Then the samples were incubated at 90 °C for 10 min and subsequent incubation was done at — 40 °C for 5 min. Finally, the supernatant was collected after centrifugation, and stored at — 80 °C for further analysis.

2.4.3. Methanol/water (50:50, v/v) extraction (MW)

After quenching with 0.25 mol/L PCA, the pellets were resuspended in 2.5 mL methanol/water (50:50, v/v) (— 40 °C), followed by vigorous mixing. Then the samples were incubated at — 40 °C for 10 min and vortexed several times during this period. Then, the samples were centrifuged, and the pellets were extracted again. After centrifugation, the supernatant was combined and stored at — 80 °C for further analysis.

2.4.4. Chloroform/methanol/water (50:25:25, v/v/v) extraction (CMW)

After quenching with 0.25 mol/L PCA, the pellets were resuspended in 2.5 mL methanol/water (50:50, v/v) (— 20 °C). Then 2.5 mL chloroform (— 20 °C) was immediately added into the above sample. After vigorous mixing, the samples were incubated at — 20 °C for 10 min and vortexed several times during this period. Then, the samples were centrifuged and the pellets were extracted again. After centrifugation, the supernatant was combined and stored at — 80 °C for further analysis.

2.5. Derivatization of metabolites

Cell extracts and standard solutions were freeze-dried (Thermo Heto PowerDry LL1500) at — 110 °C in sampler vials. The dry extracts were oxidized with 50 μl pyridine with 20 mg/mL methoxamine hydrochloride solution at 37 °C for 90 min. Before derivatization, 10 μl of 0.50% phenylenyl acetate was added to the samples and used as internal standards. Subsequently, they were silylated at 37 °C for 60 min with 50 μl Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Shin et al., 2010; Koek et al., 2006). The samples used were measured by GC–MS.
2.6. Metabolism measurement and identification

The derived extracts were analyzed with an Agilent 7890 gas chromatograph coupled with an Agilent 5975C inert XL MSD with Triple-Axis detector. A 1 μL aliquot of the derivatives was injected into a HP-5MS capillary column (30 m × 250 μm i.d., 0.25 μm film thickness) with a splitless injection mode. The initial GC oven temperature was 70 °C, 2 min after injection the GC oven temperature was increased with 5 °C/min to 280 °C and kept at 280 °C for 5 min. Helium was used as a carrier gas and the helium flow was kept constantly at a flow rate of 1.0 mL/min. Detection was achieved using an MS detector in an electron impact mode and a full scan monitoring mode (m/z 30–600). The temperature of the ion source and the quadrupole were set at 230 °C and at 150 °C respectively.

Identification of metabolite was finished by retention time and by mass spectrometry (MS) data. Comparing between the total ion chromatogram of the sample and the standard was the first step to the identification of the known metabolite within relative retention time. For the other unknown peaks in the sample, the determination of the metabolite depended on the degree of matching between the sample and the mass spectral library by automated mass spectral deconvolution and identification system software (AMDIS; http://chemdata.nist.gov/mass-spc/amdis/). Rationality of the result from software was analyzed based on the metabolite characterization of Gram-positive bacterium. An absolute quantification of each metabolite detected in our samples is an involute and unnecessary procedure for a comparative study. All comparisons of metabolite levels were based on data from samples quenched from the same culture flask at the same growth phase. All standards were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.7. Detection of adenine nucleotide (ATP, ADP, AMP)

ATP in suspension in quenching solution and washing solution were directly measured from the luminescence produced in the luciferin-luciferase reaction using the ATP assay kit (Beyotime, China).

Intracellular ATP, ADP and AMP were detected by HPLC. HPLC analysis was performed using a Waters 1525 Binary Pump with a 2489 UV/Visible Detector, a 2707 Autosampler, and an Agilent TC-C18 column (5 μm, 250 × 4.6 mm, Agilent). The 1.0 L mobile phase contained 13.6 g KH2PO4, 0.608 g NaOH, 0.767 mL formic acid, 50 mL methanol and 950 mL water. The methanol was chromatographic pure, and others were of analytical grade. The flow rate was 1.0 mL/min and the detection wavelength was 210 nm. Injecting volume was 20 μL and temperature of the column was 30 °C.

The concentration of adenine nucleotide was defined as the ratio of adenine nucleotide content to dry weight. The energy charge was defined as follows:

\[
\text{Energy charge (EC)} = \frac{[\text{ATP}] + 0.5 \times [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]
\]

2.8. Statistical analyses

All experiments were carried out in triplicate and the mean values were given. Data analysis was performed using SPSS 16.0 software package by ANOVA analysis.

3. Results and discussion

3.1. Choice of quenching solution

Methanol is used as a standard quenching solution in most literatures of metabolomics research. Because of its stronger permeability, it can immediately pass through the cell membrane and stop enzymatic activity. At the same time, the low freezing point of methanol can ensure that the mixture does not freeze in low temperature (−40 °C). However, if cells are not easily collected from the broth, all of the advantages of methanol are not valuable for metabolomics research. Unlike most microbial samples, γ-PGA broth is a viscous solution in which the cells were packaged by γ-PGA, it was difficult to collect the cells from γ-PGA broth by centrifugation (see supplemental material Fig. 1). Therefore, obtaining cells was as important as the quenching in the first step of sample handling for γ-PGA broth. The effects of perchloric acid (PCA) and methanol on centrifugation of γ-PGA broth were shown in supplemental material Fig. 1. When the methanol/water (3:2, v/v) mixture was mixed with the γ-PGA broth, γ-PGA enclosed the cells as a colloid and the cells were not being efficiently separated from γ-PGA. The collection of cells was also not easy even when pure methanol was used. Conversely, when PCA was used as a quenching agent, the broth’s pH value decreased from 6.2 to 2.8, the broth turned into a nonviscous fluid and the cells were efficiently gained by centrifugation, so that a bigger cell pellet emerged at the bottom of the centrifuge tube (see supplemental material Fig. 1). Other inorganic acids (H3PO4 and HCl) had similar effects as PCA and dimethyl sulfoxide had the same performance as methanol for the γ-PGA broth (data not showed). In addition, fast filtration also could not be used as the quenching method due to the weak filtration capability of these samples containing γ-PGA (data not showed).

The addition of acid in the sample will change the pH and cause the splitting of the cell. The effects of different concentrations of PCA on cell livability in γ-PGA fermentation broth were tested (see supplemental material Table 1). As the concentration of PCA increased, the number of the living cells and survival rate decreased from 3.7 ± 0.81 × 10⁸ CFU/mL and 100% (not added PCA) to 0.35 ± 0.01 × 10⁸ CFU/mL and 9.3% (added 1.0 mol/L PCA). The optimized concentration of PCA was 0.25 mol/L, and it hardly affected the survival rate of cells (115%) and effectively reduced the sample viscosity. These results suggested that low concentration PCA was a selectable solvent for isolating cells from the γ-PGA broth before extraction. It meant that acidic quenching might be an alternatively method for high viscosity samples in metabolomics.

3.2. Effect of acid quenching on cellular charge and leakage

To develop a suitable acidic quenching method for representative sampling of the intracellular metabolite pool of γ-PGA producer B. licheniformis, different acid solutions were first evaluated at the same concentration according to the following four indexes: energy charge (EC), ATP leakage and the concentration of special intracellular metabolites.

The EC, defined as ([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP]), is an important physiological indictor which shows cellular metabolic activity. In general, the EC is maintained in the range of 0.80 to 0.95 in bacteria during the log-growth phase and is dropped to a lower level in the stationary phase (Sellick et al., 2009). As shown in Fig. 1A, the EC values of all quenched cells were more than 0.80. The result showed that the three inorganic acids could effectively inhibit intracellular enzymes and stop the cellular metabolism immediately.

Almost all literatures showed that the cellular leakage existed during quenching and it was unavoidable. In E. coli, the percentage of ATP leakage could rise to about 70% when methanol was used as a quenching solution (Link et al., 2008). The concentration of ATP from the leakage of cell was determined by ATP assay kit which is more sensitive than HPLC method. As shown in Fig. 1B, the percentage of ATP leakage, defined as extracellular [ATP] / (extracellular [ATP] + intracellular [ATP]), and extracellular ATP including the leakage of quenching and washing, was about 24% by quenching with PCA, which was less than that of H3PO4 (30%) and HCl (60%). These results indicated that PCA might be the most suitable quenching agent for B. licheniformis.
3.3. Effect of acid quenching on intercellular metabolite

The intermediate metabolites, like glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), were regarded as the faster transfer rate substances in bacterium (Canelas et al., 2008; Taymaz-Nikerel et al., 2009). The substrate and key metabolites in carbon catabolism, such as glucose, citrate and glutamate, were carbon metabolic regulators and PGA precursors. If quenching was incomplete, these metabolites would be transformed or consumed. Therefore, intracellular G6P, F6P, glucose, citrate and L-glutamate were used as objective indicators to evaluate the quenching effect. The peak areas of the five metabolites with a different quenching solution were showed in Fig. 1C. Their relative concentration (peak area) in quenched groups was higher than CK. Especially, the highest relative concentration was obtained in a sample processed with PCA, excluding citrate. These results indicated that inorganic acid as a quenching solution was a feasible method for γ-PGA broth in a quenching procedure and that PCA was able to stop the metabolism more effectively. The conclusion was inconsistent with the result of the EC (Fig. 1A) and ATP leakage (Fig. 1B).

3.4. Effect of washing solutions on cellular leakage

The washing of a cell pellet was an indispensable process for removing broth residue from the pellet. Different washing agents brought different levels of leakage of intracellular metabolites (Dai et al., 2004). The effects of four different washing solutions (sterile water, 0.85% NaCl, 2.3% NaCl and 2.5% (NH₄)₂CO₃) on the leakage of ATP were investigated.

Table 1 revealed that the leakage of ATP decreased with the increase of salt ions in the washing solutions. And washing them with pure water led to serious leakage of ATP compared with other washing agents which contained salt ions. The leakage of ATP washed with 2.5% (NH₄)₂CO₃ was less than other washing solutions. However, in this sample, G6P and F6P were not detected by GC–MS (data were not). When washed with 2.3% NaCl, there was too much precipitation in the following derivation step, which was harmful to the GC–MS analysis.

![Fig. 1. Energy charge (EC) (A), ATP concentrations (B) and relative concentration of intracellular metabolites (C) in different quenching solutions. The concentrations of quenching solutions used in this experiment were both 0.25 mol/L. The concentration of F6P (fructose-6-phosphate) and G6P (glucose-6-phosphate) were respectively amplified 100 and 10 times. The content of glutamate was shrunk 10 times. (Each data point represents the average of three independent experiments with the error bar indicating the standard deviation.)](image)

<table>
<thead>
<tr>
<th>Washing solution</th>
<th>First washing (µmol/g)</th>
<th>Second washing (µmol/g)</th>
<th>Total (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>6.1 × 10⁻¹</td>
<td>1.6 × 10⁻¹</td>
<td>1.6 × 10⁻¹</td>
</tr>
<tr>
<td>0.85% NaCl</td>
<td>1.2 × 10⁻¹</td>
<td>1.9 × 10⁻¹</td>
<td>3.1 × 10⁻¹</td>
</tr>
<tr>
<td>2.3% NaCl</td>
<td>1.3 × 10⁻¹</td>
<td>9.1 × 10⁻³</td>
<td>1.3 × 10⁻¹</td>
</tr>
<tr>
<td>2.5% (NH₄)₂CO₃</td>
<td>8.8 × 10⁻²</td>
<td>2.0 × 10⁻³</td>
<td>9.8 × 10⁻²</td>
</tr>
</tbody>
</table>
instrument. So, 0.85% NaCl was selected as the washing solution because of less leakage of ATP than pure water and less precipitation than 2.3% NaCl.

3.5. Comparison of four kinds of extraction methods

Extraction is a key process for obtaining abundant intracellular information in metabolomics. So, the kinds of extraction methods were evaluated for the \( \gamma \)-PGA fermentation broth. Before extraction, all of the samples were quenched with 0.25 mol/L PCA and washed with 0.85% NaCl. Enzymes should still remain inactive to prevent substance transformation during the extraction process (Faijes et al., 2007). Then, the cellular EC was used as the first indicator to evaluate extraction methods. Fig. 2 showed the cellular ECs of cells treated with different extraction methods. Among the four extraction methods, only the HE method could assure that the cellular EC of cells was higher than 0.80 and was kept at pace with the quenching process (0.81 in quenching, 0.83 in extraction). The result indicated that HE might be the most suitable extraction method for \( B. licheniformis \).

The aim of extraction was to pick up intracellular metabolites. An ideal extraction agent should obtain as many intracellular metabolites as possible. So, five intracellular metabolites (F6P, G6P, glucose and citrate, glutamate) and three adenine nucleotides (ATP, ADP and AMP) were chosen as indices. As shown in Fig. 3, the concentrations of all of the intracellular metabolites in the HE method were the highest among the other three methods. The result revealed that the HE method could extract more metabolites than the other three methods. Combined with the results of energy charge, HE method was an excellent extraction method for \( B. licheniformis \).

3.6. Optimization of HE method for extraction

Based on the above experimental results, the concentration of ethanol and the time of heat treatment were optimized. Under the same experimental conditions, the effect of different concentrations of ethanol on extraction was shown in Fig. 4. Pure ethanol could not extract F6P and G6P, which might be related to its polarity. 75% ethanol was remarkably superior to 50% ethanol and pure ethanol. The results of the concentrations of adenine nucleotides and intracellular metabolite concentrations at different extraction times were shown in Fig. 5. The

![Fig. 2. Effect of different extraction methods on EC of \( B. licheniformis \) WX-02. PM, HE, CMW and MW represented pure-methanol extraction, ethanol/water (75:25, v/v) extraction, methanol/water (50:50, v/v) extraction and chloroform/methanol/water (50:25:25, v/v/v) extraction, respectively. (Each data point represents the average of three independent experiments with the error bar indicating the standard deviation.)](image)

![Fig. 3. Comparison of the relative concentration of intracellular metabolites of a cell extracted by different extraction methods. PM, HE, CMW and MW represented pure-methanol extraction, ethanol/water (75:25, v/v) extraction, methanol/water (50:50, v/v) extraction and chloroform/methanol/water (50:25:25, v/v/v) extraction, respectively. The concentrations of F6P (fructose-6-phosphate) and G6P (glucose-6-phosphate) were respectively amplified 100 and 10 times. (Each data point represents the average of three independent experiments with the error bar indicating the standard deviation.)](image)

![Fig. 4. Effect of ethanol concentration on the relative concentration of intracellular metabolites of \( B. licheniformis \) WX-02 quenched by PCA. The concentrations of F6P (fructose-6-phosphate), G6P (glucose-6-phosphate) and Glu (glutamate) were shrunk 100, 10 and 10 times, respectively. (Each data point represents the average of three independent experiments with the error bar indicating the standard deviation.)](image)
concentrations of all of the metabolites were the highest when cells were extracted for 10 min by 75% ethanol. The result showed that these intracellular metabolites were efficiently extracted for 10 min. Consequently, the optimal extraction condition of intracellular metabolites for *B. licheniformis* was incubation with 75% ethanol in a boiling water bath for 10 min.

3.7. Basic evaluation of the optimized protocol of γ-PGA broth for metabolomics analysis

By using the above optimized quenching, washing and extraction methods, 118 kinds of peaks from the intracellular sample of *B. licheniformis* were shown in the total ion chromatogram (as in Fig. 6). Among them, 34 metabolites were picked up and were valuable for understanding the metabolites of poly-γ-glutamate-producing *B. licheniformis*. These intracellular metabolites of *B. licheniformis* were constituted mainly of amino acids, organic acids, and monosaccharides and its derivatives (see supplemental material Fig. 2). The proportions of these metabolites were 41%, 21% and 21% respectively. Importantly, these metabolites were involved in the most important metabolite pathways of γ-PGA biosynthesis, including glycolysis, citric acid cycle and glutamate metabolism.

4. Conclusion

Metabolomics is an important part of system biology. The pretreatment protocol of a sample is different between the different source and the characterization of the sample. The conditions of quenching and washing and the method of extracting γ-PGA producing *B. licheniformis* were optimized. The suitable quenching agent was PCA, which was mixed with an equal volume of samples and held for 10 min at −10 °C. 0.80% NaCl was the optimal washing solvent for quenched cells of *B. licheniformis* because it can reduce the leakage of intracellular metabolites and the effect of salt on MS equipment at the same time. Hot 75% ethanol at a boiling water bath for 10 min was the optimal extraction solution from four different extraction solutions. The metabolites that were obtained employing this method were involved in the most important metabolite pathway of γ-PGA synthesis. The new pretreatment protocol for viscous polymer samples will serve researchers in microbial metabolomics.

Acknowledgments

This work was kindly supported by the National Natural Science Foundation of China (Grant No. 31170046), Key Science & Technology Specific Projects in Wuhan and the State Key Laboratory of Agricultural Microbiology in Wuhan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2013.04.006.

References


