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Simultaneous determination of intracellular nucleotides and coenzymes in *Yarrowia lipolytica* producing lipid and lycopene by capillary zone electrophoresis

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Highlights

- ·An optimized method was established to separate nucleotides and coenzymes.
- ·$NADP^+$ and ATP may be related to lycopene production in *Y. lipolytica*.
- ·The optimized method provides a useful approach for metabolic analysis.
- ·The optimized method paves a way for further improvement of *Y. lipolytica*.

Abstract: *Yarrowia lipolytica* is an oleaginous yeast with promise in producing terpenoids such as lycopene. Though methods for analyzing primary metabolic intermediates have been established, further work is needed to better analyze nucleotides and coenzymes. Here, we presented an optimized method for the separation

of nucleotides and coenzymes in *Y. lipolytica* using the capillary electrophoresis. The separation of twelve metabolites including four coenzymes, five nucleotides and three nucleosides was achieved within 32 min using a voltage of 15 kV and 70 mM sodium carbonate/hydrogencarbonate buffer with 1.0% β-CD at pH 10. The results show that the concentrations of adenosine triphosphate and nicotinamide adenine dinucleotide phosphate changed significantly between lycopene producing strain and the control, indicating that these two metabolites may be closely related with lycopene production. The optimized method provides a useful approach for future metabolic analysis of fermentation process as well as industrial strain improvement.

Keywords *Yarrowia lipolytica*; Capillary electrophoresis; Nucleotides; Coenzymes; Lycopene

1 Introduction

Yarrowia lipolytica is an oleaginous fungus whose genome has been sequenced. It can use a variety of carbon sources, such as glucose, fructose, sucrose, alkanes, lipids, industrial glycerol and agricultural as well as forestry waste, and can rapidly grow under low pH, relatively low temperature and high shear environment [1]. These characteristics suggested that *Y. lipolytica* is very suitable for industrial fermentation and has become a novel cell factory. In addition, *Y. lipolytica* has more advantages than other yeasts in the accumulation of terpenoids, because it contains lipid body capable of accumulating large number of hydrophobic compounds. In *Y. lipolytica*, lycopene can be accumulated by heterologous expressing a toolbox of carotenogenic (*crt*) genes. It has been reported that the engineered *Y. lipolytica* produced up to 16 mg/g (dry cell weight) lycopene, which is a relatively high yield in eukaryotes [2]. However, the mechanisms of metabolic regulations in *Y. lipolytica* and the limiting steps for lycopene production should be elucidated to facilitate further improvements.

Metabolomics analysis can directly reflect the cellular metabolism through the analysis of intracellular metabolites, which is helpful to optimizing the metabolic pathway and fermentation process for yield improvement [3]. The protocols of analyzing some metabolites such as amino acids, non-amino acids, lipids, sugars, sugar alcohols, etc. have been well developed in *Y. lipolytica*, and have been applied in studying lipid accumulation [4-6]. However, there is no special investigation on analyzing nucleotide and coenzyme in *Y. lipolytica*.

Quantitation of intracellular nucleotides and coenzymes is very important to metabolomics studies. Nucleotides are not only responsible for the synthesis of DNA and RNA molecules in cells, but also closely related to cell metabolism [7].

Coenzymes, such as NAD(P) and NAD(P)H, involve in cell's oxidation-reduction reactions including lipid synthesis and degradation, terpenoids synthesis, etc [1, 8]. In the last decades, the quantitation of nucleotides has been performed using high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). However, nucleotides can be more easily separated by capillary electrophoresis because they can be negatively charged in the range of pH 2~12, which provides the basis for nucleotide separation of capillary electrophoresis [9]. Many methods have been developed based on capillary zone electrophoresis (CZE), in which borate or carbonate is more commonly used in the buffer system [10, 11]. Addition of polyethylene glycol (PEG), *β*-cyclodextrin or other substances can significantly improve the separation.

In this study, CZE was used to detect intracellular nucleotides and coenzymes in *Y. lipolytica*. Some nucleosides were also detected to expand the scope of simultaneous analysis of metabolites. The processes of separation and sample pretreatment were optimized, and these metabolites in the engineered *Y. lipolytica* which can produce lycopene and the control were analyzed to find the key metabolites for lycopene accumulation.

2 Materials and methods

2.1 Materials

The adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), adenosine 3,5-cyclic monophosphate (cAMP), uridine monophosphate (UMP), inosine (IM), cytidine (CM), guanosine (GM), nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and nicotinamide adenine

dinucleotide phosphate reduced (NADPH) were purchased from Aladdin Industrial Corporation, China. The *β*-CD was obtained from Tokyo Chemical Industry., Ltd., Japan. Yeast extract and Tryptone were obtained from Oxoid Ltd,. Basingstoke, Hampshire, England. Sodium carbonate, sodium hydrogencarbonate and other materials were obtained from Shanghai Lingfeng Chemical Reagent Co., Ltd.China.

2.2 CE separation method

The separation was performed on PA 800 plus capillary electrophoresis system (Beckman Coulter) with diode array UV-Vis detector (DAD). Total length of fused-silica capillary was 59 cm (49 cm effective length). The inner diameter of the capillary was 50.0 μm. The running buffer, modified from Musilová et al. (2009), contained 30~80 mM sodium carbonate/hydrogencarbonate with 1.0% *β*-CD at pH 9~11. The dry sample was dissolved in 500 μL MilliQ water and the solution was injected at 0.5 psi for 15.0 s. The separations were performed under a constant voltage of 15 kV with positive polarity at 20°C. Data were detected at a wavelength of 260 nm. Before running samples, the capillary was rinsed with 1.0 M HCl (5 min), MilliQ water (5 min), 1.0 M NaOH (5 min), MilliQ water (5 min) and finally with running buffer. After each sample run, the capillary was rinsed with 1 M NaOH for 5 min, followed by MilliQ water for 2 min, and finally with running buffer for 2 min. Each peak was identified by spiking the sample with the standard and quantified by standard curve. Three replicates were prepared for each condition and each sample was run five times.

2.3 Strains and culture conditions

The *Y. lipolytica* strain Po1g (YLP) was kindly provided by Prof. Catherine Madzak (Institut National de la Recherche Agronomique/AgroParisTech, France).

The lycopene producing strain (YLEBI) was engineered from the *Y. lipolytica* strain Po1g by heterologous expression of lycopene biosynthetic genes *crtE*, *crtB* and *crtI*, and this part was completed in our previous study [12]. The strains were pre-cultivated in YPD medium containing 1% yeast extract, 2% peptone, and 2% glucose. The minimal medium used for lipid production contained (g/L) glucose 20, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 1, $K_2HPO_4 \cdot 3H_2O$ 0.16, NaCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.7, $Ca(NO_3)_2 \cdot 4H_2O$ 0.4, $CuSO_4 \cdot 5H_2O$ 0.00004, KI 0.0001, H_3BO_3 0.0005, $Na_2MoO_4 \cdot 2H_2O$ 0.0002, $MnSO_4 \cdot 4H_2O$ 0.0004, $FeCl_3 \cdot 6H_2O$ 0.006, $ZnSO_4 \cdot 7H_2O$ 0.0004, thiamine hydrochloride 0.0003. Experiments were performed in 250 mL conical flasks containing 50 mL medium inoculated with the pre-culture (initial OD_{600}= 0.01) and incubated in a rotary shaker at 220 rpm and 30°C. Cultures were prepared in triplicate.

2.4 lycopene analysis

Lycopene was extracted and analyzed according to the previous study [4].

2.5 Metabolite preparation

The metabolites were extracted according to a formerly described method with some modifications [4]. For cold methanol extraction, 2.5 mL of −40°C methanol solution (50%, v/v) was added to the cell pellet, and the mixed sample was then frozen in liquid nitrogen and subjected to three freeze-thaw cycles with 1 min of vortex mixing between each cycle. The sample was then centrifuged at 9408 g for 10 min and the supernatant was collected and stored at −40°C. For boiling ethanol extraction, 5 mL boiling ethanol (75%, v/v) at 95°C was added to the pellet, and the solutions were heated in a closed tube for 10 min at 95°C. Samples were cooled on ice for 5 min, and the cellular debris was removed by centrifugation at same condition.

The supernatant was stored at −40°C. For boiling water extraction, the biomass was resuspended in 2 mL MilliQ water and heated in boiling water for 10 min and then cooled on ice for 5 min. The cell debris was removed and the supernatant was concentrated. The supernatants in the three extractions were dried by a solvent evaporation system SpeedVac SPD131DDA (Thermo Scientific, Waltham, MA). The experiment was performed under vacuum using a pump without heating. After extraction, the cellular debris was dried at 105°C and the quantified biomass was used for further analysis.

2.6 Data processing and statistical analysis.

Standard curve was made for each nucleotide or coenzyme to quantify the metabolite. The quantified data was normalized to the dry weight of cellular debris for comparison between samples. Student's *t* test carried out with Prism 5 software (GraphPad Software, Inc., CA) was used for statistical analysis, and *P* values <0.05 were considered to be statistically significant. Heatmap and PCA analysis were used the Metaboanalyst online service (http://www.metaboanalyst.ca/faces/home.xhtml).

3 Results and discussion

3.1 Effect of buffer concentration on analysis

Firstly, samples of cell extracts (using boiling water extraction) were used to optimize the concentration of sodium carbonate/hydrogencarbonate (SCH) buffer. The starting SCH buffer concentrations of 30 to 80 mM with the 1% *β*-CD at pH 9.5 were used as the running buffer, and the separation voltage were 15 kV. Fig.1 shows the electropherograms of sample separations by 30 to 80 mM SCH buffers, in which each metabolite can be clearly separated when the buffer concentration reached 70 mM. The currents were kept stable throughout the analysis. Table 1 shows that the

current was increased with the concentration of SCH increasing, and it was 35 μA for the 70mM SCH buffer. It was reported that when the buffer concentration exceeds 100 mM, the current was more than 50 μA. Such high current always produces excessive heat in the capillary, impacting the separation seriously [13]. During the separation process, the charged ions are not only affected by the electric field force and frictional force, but also influenced by the electroosmosis [9]. The inner wall of the quartz capillary is negatively charged, and the electroosmotic flow moves toward the negative electrode because it is positively charged. However, the nucleotide metabolites must be promoted by the electroosmotic flow in order to let them move towards the negative electrode as they were negatively charged in the separation process. The buffer concentration increased, i.e. the ionic strength of the buffer increased, which affected the solution's viscosity and the separation current, as well as the thickness of the double layer between the buffer and the capillary wall. As a consequence, the flow rate of the separated ions moving to the negative electrode was reduced, which delayed the peak time and gradually separated the overlapping peaks. The addition of 1% β-CD in buffer had better effect on separation than the addition of 1% PEG, or no addition (data not shown).

3.2 Effect of pH value on analysis

Alkaline solution is widely used in the separation of nucleotides because it can provide stable electrical flow. When alkaline solution was used, wide peaks can be avoided by increasing the buffer concentration if the ionic strength of the sample is greater than that of the buffer [9]. Fig. 2 shows the migration time of each metabolite at different pH values in the buffer with a concentration of 70 mM. The migration times prolonged and the migration speeds decreased with the pH increasing. In the buffer with higher pH value, these metabolites disintegrated more anions which move

to the anode more slowly than in the buffer with lower pH value, according to Henderson-Hasselbalch equation [14]. So the increase of the pH value of the buffer caused the increase of the migration time of the detected substances. In addition, the migration time of each ion needs to be relatively stable during the sample analysis. Otherwise, it will cause difficulties in the qualitative analysis of the metabolites in the samples. Therefore, choosing a proper pH value is very important for the analysis of a variety of substances by capillary electrophoresis. Fig. 2 shows that, in addition to UMP, most of the metabolites could be stably analyzed in the pH range of 9 to 10. The pH value also had some effect on the separation current. The current at pH 11 was nearly two times of that at pH 9. Finally, the pH value of 10 was used for further analysis because pH 9 or 9.5 can't be good for separating NADPH and ATP.

3.3 Standard curves

The mixture standards of nucleotides and coenzyme were analyzed under the conditions of 70 mM SCH buffer (containing 1% β-CD) at pH 10. The electropherogram of the standard is shown in Fig. 3. In the repeated trials (n=6), the relative standard deviation reflected good reproducibility of migration times (<1%, except UMP) and peak areas (<2%, except ATP and NADPH) (Table 2). The standards were analyzed between the concentrations of 0.01~0.32 mM. For each metabolite, the correlation coefficient (R^2) was greater than 0.995, expect for NADPH 0.963. The minimum detection limits of all the standards were at the micro molar level. In addition, samples were dissolved by SCH buffer in the analysis because sample dissolved in ultra pure water produced interference peak which could not be separated from the peak of CM.

3.4 Optimization of *Y. lipolytica* (YLP) samples analysis

The common methods for metabolites extraction in yeast metabolomics including boiling water, boiling ethanol and cold methanol methods were evaluated before the analysis of the samples. Fig. 4A shows that seven metabolites can be detected for boiling water method and they had higher concentrations than those extracted by the other methods, and six metabolites can be detected for boiling ethanol method, but only three metabolites can be detected by cold methanol method. Therefore, the boiling water method was most suitable for the extraction of the nucleotides and coenzymes in *Y. lipolytica*. The three extraction methods were also compared in our previous studies for the global metabolites extracting of *Y. lipolytica.* It was suggested that cold methanol extraction was more suitable for extracting most of the metabolites rather than fatty acids in *Y. lipolytica* [4]. In this study, however, the comparison of cold and boiling ethanol showed that heat shock method rather than freezing method were suitable for extracting nucleotides or coenzymes. Meanwhile, the comparison of boiling ethanol and boiling water method showed that aqueous phase rather than organic phase were suitable for the extraction of these metabolites. NADH and NADPH can not be extracted and detected by any method used in this study. In the study of *Bacillus subtilis* and *Paracoccus denitrificans*, NADH and NADPH were also undetected, probably because of the poor stability and the low concentrations of these metabolites in the cells [11, 15]. Fig.4B shows the electropherogram of metabolites extracted by boiling water. The metabolites detected in the samples were CM, cAMP, AMP, ADP, ATP, NAD^+ and $NADP^+$, among which the concentration of CM, NAD^+, AMP and ADP were higher than others. The recoveries of metabolites were between 97.1~101.9%, indicating that the established method was efficient in separating these kinds of metabolites by capillary zone electrophoresis (Table 3). The migration time of most metabolites were extended in

the sample analysis process compared to standard analysis (Fig.3). This may be because the extracts of *Y. lipolytica* cells contain complex molecules and ions which had negative effect on the whole separation process. These factors caused some difficulties for the qualitative analysis of the metabolites in the samples. In this study, the standard was added into each sample in order to ensure the accuracy of the qualitative analysis.

3.5 Analysis and comparison of YLP and YLEBI by the optimized method

The concentrations of the seven metabolites extracted from the cells of YLP and YLEBI at Day 2, Day 4 and Day 6 using boiling water were compared in a heatmap (Fig.5). The comparison in case similarity pattern shows that samples were clearly divided into two groups, YLP and YLEBI. The metabolites were also classified into two main groups based on the analysis of variable similarity patterns. The concentrations of AMP, $NADP^+$, NAD^+ and ATP were significantly lower in the YLP groups compared with YLEBI groups, while the concentrations of cAMP, CM and ADP were closely related to the culture time. These data were also explored by the widely used multivariate data analysis method-Principal Components Analysis (PCA) by dimensional reduction and data visualization [16-18]. The Biplot (Fig. 6) shows that the samples from the three days of YLP and YLEBI could be clearly divided into six groups, in which PC1 distinguished the culture days while PC 2 distinguished the yeast types. The contributions of every metabolite were also shows in this figure. It was found that ATP and $NADP^+$ were made great contribution to the separation of yeast types, YLP and YLEBI, while cAMP and CM were for the separation of culture times. The yeast changed from self growth to product(s) accumulation as culture time prolonged, and the concentration of cAMP and CM changes showed the common characteristic of these two strains during the culture. For the difference of yeast type,

YLP are naturally unable to accumulate lycopene and it was used as the control strain, but YLEBI which was engineered from YLP by heterologous expression of lycopene biosynthetic genes *crtE, crtB* and *crtI* can accumulate lycopene. The highest production of lycopene was 5.37 mg/L at Day 6 (Table 4). Table 4 also shows the lipid contents of YLP and YLEBI on Day 2, Day 4 and Day 6. The differences of lipid production of YLEBI and YLP were not significant in the preliminary stage of lycopene accumulation (Day 2). However, it became more and more significant as culture time prolonged and the lycopene accumulated. That is because the precursor for lipid accumulation is acetyl-CoA which also is the precursor for lycopene accumulation in YLEBI [2]. With the addition of the lycopene synthesis pathway, some acetyl-CoA can be converted to lycopene in YLEBI, so less acetyl-CoA converted to lipid in YLEBI compared to YLP. The changes of lycopene and lipid production may lead the changes of the energy or cofactors which are essential for lycopene or lipid production. It is clearly shown in Fig.5 and Fig.6, the addition of lycopene synthesis pathway in *Y. lipolytica* significantly increased the concentration of ATP and $NADP^+$, indicating that these substrates may relate to lycopene accumulation. Similarly, the level of ATP rising was also found in the carotenoid (the structure is similar to lycopene) production, which suggested that the accumulation of these kind of terpenoid is the process of energy requirement [19]. On the other hand, it was found that mannitol and xylitol were accumulated significantly with lycopene production, and these reduction processes consumed NAD(P)H and produced NAD(P), which could be the reason for the increased level of NAD^+ and $NADP^+$ (manuscript in press).

4 Conclusions

A CZE method for separating four coenzymes, five nucleotides, three nucleosides was established, in which NAD^+, $NADP^+$, AMP, cAMP, ADP, ATP, and CM were simultaneously detected in *Y. lipolytica*. Samples of the lycopene producing strain and the control were compared, and the data were analyzed by various statistical methods. The principal components analysis showed that ATP and $NADP^+$ may be related to the accumulation of lycopene in *Y. lipolytica*. The method provides a useful platform for simultaneously analyzing intracellular nucleotides and coenzymes contributing to current metabolomics, and the results could guide further improvement of *Y. lipolytica* for lycopene production.

Acknowledgments

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Figure Legends

Fig.1 Effect of buffer concentration on the analysis. The running buffer contained 30~80 mM sodium carbonate/hydrogencarbonate with 1.0% β-CD at pH 10.

Fig.2 Effect of different pH value on the migration time in the process of metabolites separation. The running buffer contained 70 mM sodium carbonate/ hydrogencarbonate with 1.0% *β*-CD at pH 9~11.

Fig.3 Electropherogram of 12 standards (0.32 mM). The running buffer contained 70 mM sodium carbonate/hydrogencarbonate with 1.0% β-CD at pH 10. The numbers represent as below: 1 CM, 2 NAD^+, 3GM, 4 cAMP, 5 IM, 6 NADH, 7 AMP, 8 $NADP^+$, 9 ADP, 10 NADPH, 11 ATP, 12 UMP.

Fig.4 (A) Comparison of the three extraction methods in intracellular metabolites' concentration from *Y. lipolytica.* (B) Electropherogram of adenine nucleotides and coenzymes in *Y. lipolytica.*

Fig.5 Clustering of adenine nucleotides, cytidine, NAD^+ and $NADP^+$ profiles during the cultivation of YLP and YLEBI on Day 2, 4 and 6. Similarity assessment for clustering was based on the Euclidean distance measure and Ward clustering algorithm. Each column and each row represent a culture sample and an individual metabolite, respectively.

Fig.6 Biplot of principal components analysis. PC1 and PC2 represented 68.1% and 22.4% of the data, respectively.

Figr-1

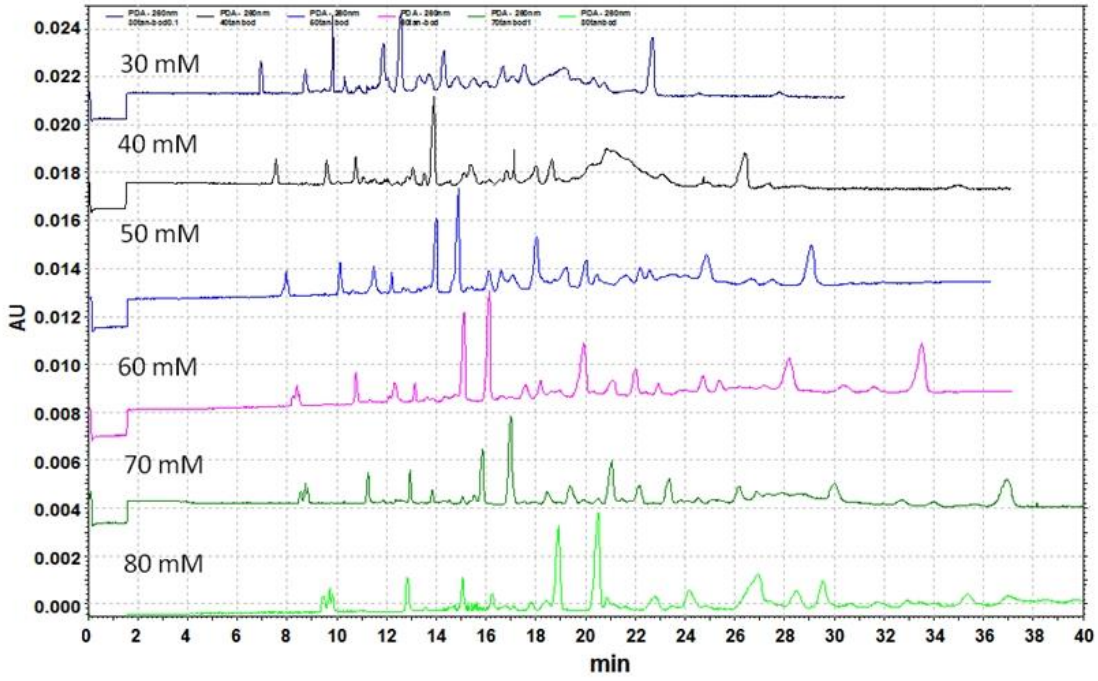
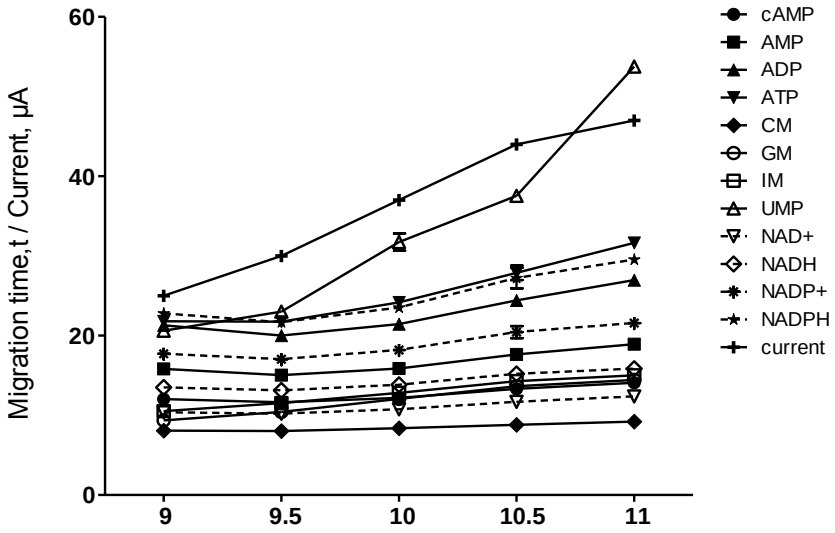


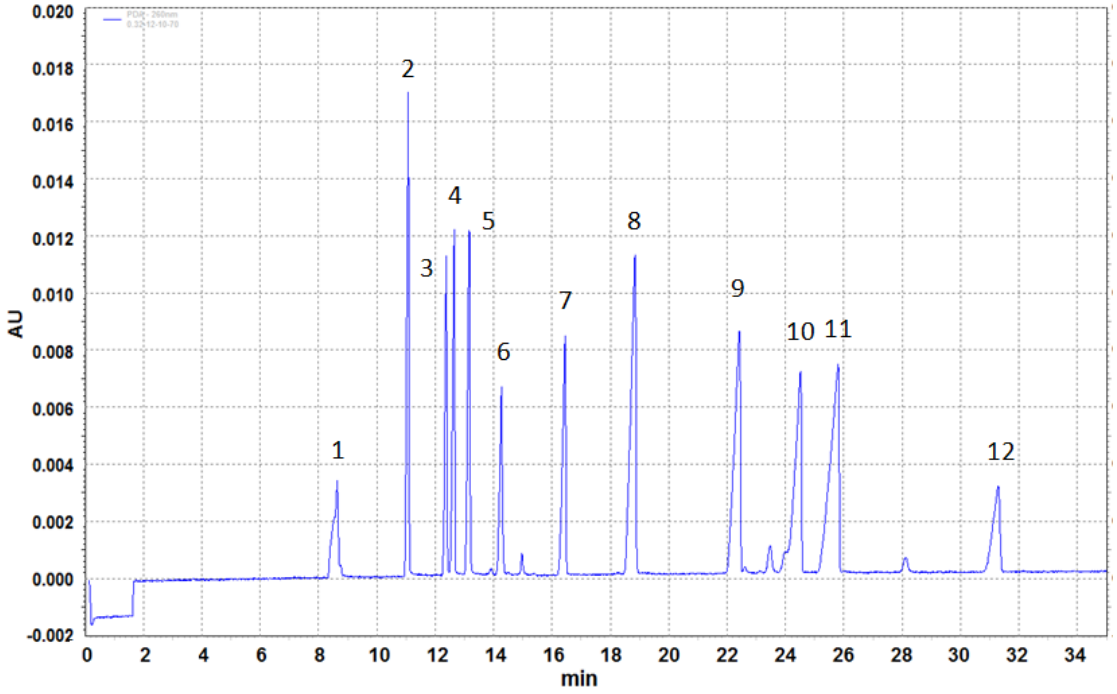
Fig.1



Figr-2

Fig.2

Figr-3



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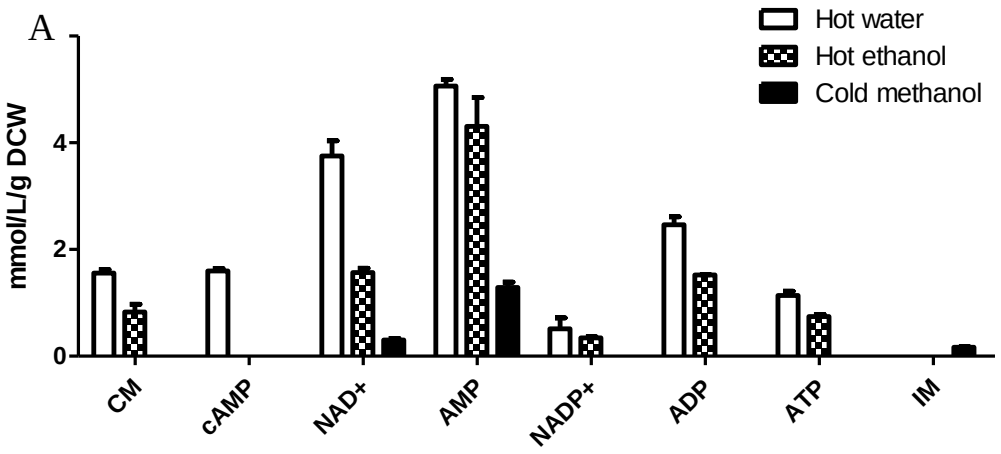

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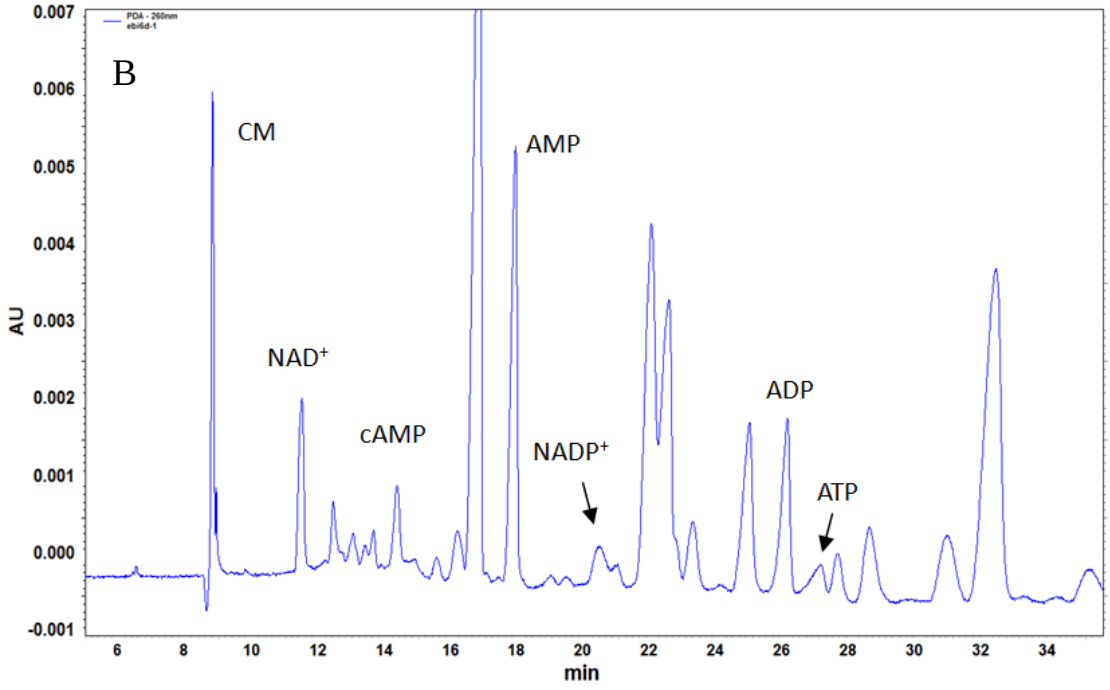


Fig.4

Figr-5

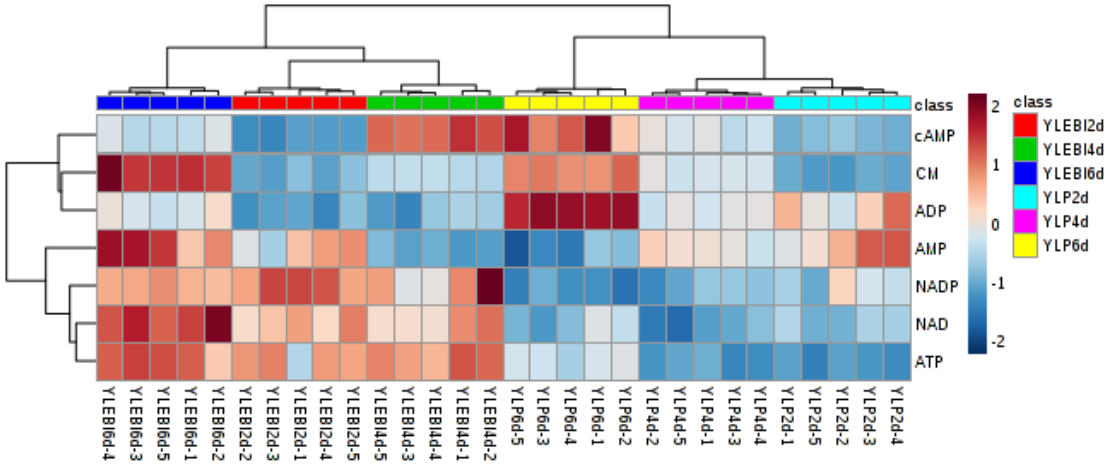
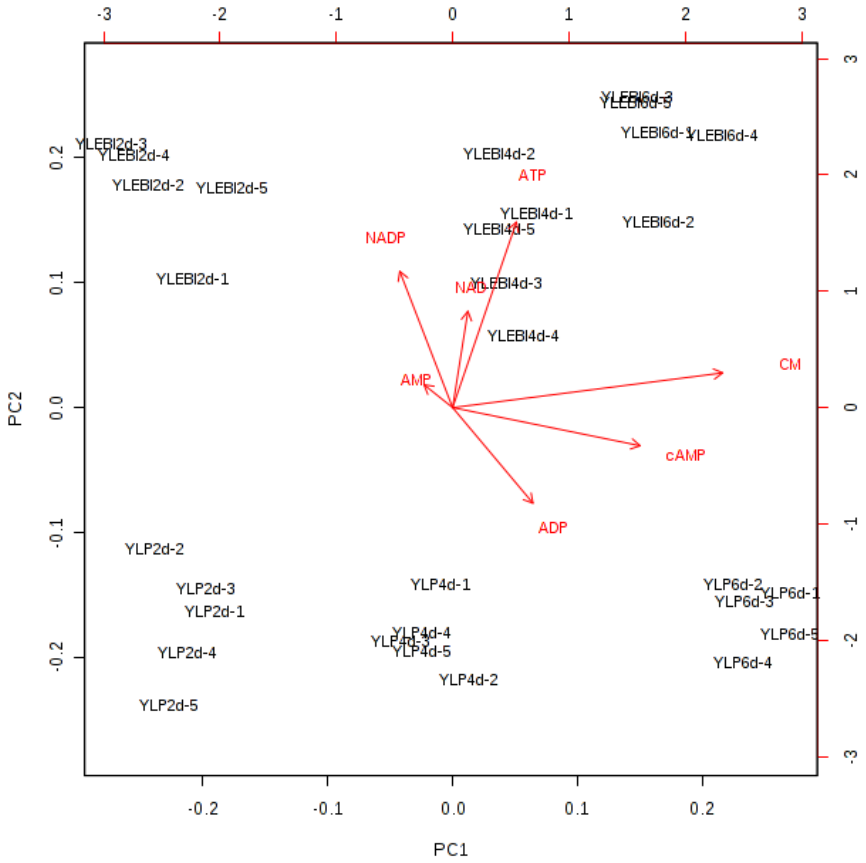


Fig.5



Figr-6

Fig.6

Table 1 Current values of different buffer concentration. The running buffer contained 30~80 mM sodium carbonate/hydrogencarbonate with 1.0% β-CD at pH 10.

Carbonate concentration/mM	Current/μA
30	16.1
40	23.3
50	27.2
60	30.1
70	35.0
80	39.5

Table 2 Repeatability, linearity and sensitivity of developed CE method for nucleosides, nucleotides and coenzymes.

	Migration time	Migration time RSD(n=6)%	Peak area RSD(n=6)%	Correlation	R^2	LOD (S/N = 3) μM
CM	8.3	0.177	0.889	y = 9E-06x + 0.005	0.997	6.85
NAD^+	10.75	0.146	1.454	y = 4E-06x + 0.008	0.995	5.33
GM	12.05	0.580	1.417	y = 6E-06x - 0.003	0.998	6.85
CAMP	12.2	0.391	0.644	y = 5E-06x + 0.002	0.999	2.79
IM	12.8	0.451	1.217	y = 5E-06x - 0.000	0.999	6.85
NADH	13.84	0.427	1.589	y = 5E-06x + 0.004	0.997	2.68
AMP	15.89	0.427	1.174	y = 4E-06x + 0.003	0.999	3.71
$NADP^+$	18.18	0.606	1.245	y = 2E-06x + 0.008	0.996	2.14
ADP	21.45	0.474	1.254	y = 3E-06x + 0.004	0.999	3.16
NADPH	23.5	0.681	2.851	y = 4E-06x + 0.013	0.963	3.69
ATP	24.19	0.681	2.096	y = 2E-06x + 0.013	0.998	2.19
UMP	31.74	1.015	1.670	y = 4E-06x + 0.012	0.995	5.33

Table 3 Recovery of adenine nucleotides, cytidine, NAD^+ and $NADP^+$. The recovery was performed by adding spike standard compounds into the cells extract. Recovery=(Peak area$_{(standard+cells\ extract)}$- Peak area$_{(cells\ extract)}$)×100%/ Peak area$_{(standard)}$.

	CM	NAD^+	cAMP	AMP	$NADP^+$	ADP	ATP
Recovery (%)	97.6±2.5	100.6±3.9	97.1±3.7	98.0±4.4	99.8±1.3	101.9±3.5	97.9±4.0

Table 4 Lipid contents (g/g DCW) and lycopene production on Day 2, 4 and 6. *T*-tests were conducted to evaluate statistical significance between YLP and YLEBI at $p<0.01$(**) and $p<0.001$(***).

	YLP Lipid, %	YLEBI Lipid, %	YLEBI Lycopene, mg/L
Day 2	19.20±0.75	19.91±0.05	0.48±0.12***
Day 4	24.62±0.20	21.84±0.26**	3.26±0.18***
Day 6	26.83±0.25	22.48±0.47***	5.37±0.31***