Effect of Polyhydroxybutyrate (PHB) storage on L-arginine production in recombinant Corynebacterium crenatum using coenzyme regulation

Meijuan Xu1, Jingru Qin1, Zhiming Rao1*, Hengyi You1, Xian Zhang1, Taowei Yang1, Xiaoyuan Wang1 and Zhenghong Xu2*

Abstract

Background: Corynebacterium crenatum SYPA 5 is the industrial strain for l-arginine production. Poly-β-hydroxybutyrate (PHB) is a kind of biopolymer stored as bacterial reserve materials for carbon and energy. The introduction of the PHB synthesis pathway into several strains can regulate the global metabolic pathway. In addition, both the pathways of PHB and l-arginine biosynthesis in the cells are NADPH-dependent. NAD kinase could upregulate the NADPH concentration in the bacteria. Thus, it is interesting to investigate how both PHB and NAD kinase affect the l-arginine biosynthesis in C. crenatum SYPA 5.

Results: C. crenatum P1 containing PHB synthesis pathway was constructed and cultivated in batch fermentation for 96 h. The enzyme activities of the key enzymes were enhanced comparing to the control strain C. crenatum SYPA 5. More PHB was found in C. crenatum P1, up to 12.7 % of the dry cell weight. Higher growth level and enhanced glucose consumptions were also observed in C. crenatum P1. With respect to the yield of l-arginine, it was 38.54 ± 0.81 g/L, increasing by 20.6 %, comparing to the control under the influence of PHB accumulation. For more NADPH supply, C. crenatum P2 was constructed with overexpression of NAD kinase based on C. crenatum P1. The NADPH concentration was increased in C. crenatum P2 comparing to the control. PHB content reached 15.7 % and 41.11 ± 1.21 g/L L-arginine was obtained in C. crenatum P2, increased by 28.6 %. The transcription levels of key l-arginine synthesis genes, argB, argC, argD and argJ in recombinant C. crenatum increased 1.9–3.0 times compared with the parent strain.

Conclusions: Accumulation of PHB by introducing PHB synthesis pathway, together with up-regulation of coenzyme level by overexpressing NAD kinase, enables the recombinant C. crenatum to serve as high-efficiency cell factories in the long-time l-arginine fermentation. Furthermore, batch cultivation of the engineered C. crenatum revealed that it could accumulate both extracellular l-arginine and intracellular PHB simultaneously. All of these have a potential biotechnological application as a strategy for high-yield l-arginine.

Keywords: l-Arginine, Poly-β-hydroxybutyrate (PHB), NAD kinase, Corynebacterium crenatum SYPA 5
Background

L-Arginine is a kind of semi-essential amino acid and plays a significant role in nitrogen metabolism and ammonia detoxification as an intermediate in the urea cycle in humans [1]. It is involved in numerous areas of application, such as food flavor, pharmacology and physiology [2, 3]. The biosynthesis of L-arginine in bacteria has become a focus of research interest for the past decades on metabolic regulation. Studies on the L-arginine production have been conducted using the mutants of Corynebacterium, Bacillus and Serratia since the 1960s [4–6]. Several elaborate strategies were designed for efficient production of L-arginine based on the pathways, regulation, and metabolic reaction of amino acids [7, 8]. Corynebacterium crenatum was successfully isolated from soil, and its mutated strain, C. crenatum SYPA 5, is an aerobic, gram-positive, non-sporulating and L-histidine auxotroph industrial bacterium [9, 10]. In our previous work, much work has been done to increase the production of L-arginine using C. crenatum SYPA 5 as the start strain by adjusting the transfer efficiency for L-arginine, increasing the dissolved oxygen in bacterial, modifying the key enzymes involved in the L-arginine synthesis pathway [5, 10–12]. Poly-β-hydroxybutyrate (PHB), stored as bacterial reserve materials for carbon and energy, is the most popular type of polyhydroxyalkanoate (PHA) that has been well studied in recent years [13]. It is an environmentally friendly biopolymer material due to its prominent properties, such as biodegradability and biocompatibility [14, 15]. PHB is intracellular while L-arginine is extracellular. It is possible to produce L-arginine and PHB simultaneously, which improves the resource utilization rate. In addition, PHB can provide the cells with carbon source, energy and reducing power, which influence intracellular metabolic flow, oxidation/reduction state and enhance stress resistance of the cells [16]. As reported, the production of several amino acids was enhanced resulting from the extra introduction of the PHB synthesis pathway. In some way, the accumulation of PHB in the cells could be seen as a strategy for amino acids and important metabolic compounds production [17–19]. More the effect of PHB accumulation in the bacterial on L-arginine yield has not been reported yet. During the PHB biosynthesis process, three key enzymes exist including PHB synthase (PhbC), β-ketothiolase (PhbA) and NADPH-dependent acetoacetyl-CoA reductase (PhbB).

Among them, PhbB is quite special and plays a significant role in PHB synthesis due to its dependence upon NADPH [20]. A high level of NADPH and/or NADPH/NADP+ ratio has a critical effect on PHB synthesis [21].

During the L-arginine biosynthesis process, the cofactor, like the NADPH concentration, is known to have an important influence on the production by microorganisms because those key enzymes involved in L-arginine biosynthesis require NADPH, such as the NADPH-dependent glutamate dehydrogenase (GdhA) and ArgC [22]. However, high-yielded L-arginine by enhancing the coenzyme level in the cells has not drawn much attention.

The cofactor pairs NADPH/NADP+ is essential for all living organisms and plays its important role, mainly in its use as donor and/or acceptor of reducing equivalents in oxidation–reduction reactions in living cells [23]. Many industrially valuable compounds require NADPH for their synthesis and there have been a variety of methods designed to (re)generate this cofactor, like chemical, electrochemical, photochemical, or enzymatic reactions [24, 25]. NADPH can be generated by phosphorylating NAD through NAD kinase. NAD kinase catalyses NAD phosphorylation using ATP and/or inorganic polyphosphate [poly(P)] as phosphoryl donors in the presence of Mg2+ [26]. NAD kinase is ubiquitously distributed from bacteria to human cells and the gene encoding NAD kinase in Escherichia coli, Saccharomyces cerevisiae, and humans have been identified and well-studied [27–30]. Changing the cofactor level by overexpressing the NAD kinase has a positive effect on many industrially valuable compounds, like L-isoleucine in C. glutamicum, isobutanol and thymidine in E. coli [31–33]. As reported, ppnK was the only NAD kinase gene in C. glutamicum and PpnK is essential in the bacteria [34].

Both synthesis pathways of extracellular L-arginine and intracellular PHB are NADPH-dependent. They compete for NADPH, to some degree. Therefore, measures must be taken to improve the cofactor level. It is interesting to see the effect of NAD kinase overexpression on the PHB and L-arginine yield in C. crenatum, together with the effect of PHB on L-arginine yield. In this study, we were intended to construct a high-yield L-arginine strain by introducing the PHB synthesis pathway using C. crenatum SYPA 5 as the start strain. Meanwhile, the NAD kinase, PpnK, was overexpressed to balance the cofactor level. Furthermore, batch cultivation of the engineered C. crenatum revealed that it was able to accumulate both extracellular L-arginine and intracellular PHB simultaneously.

Results and discussion

Construction of C. crenatum P1 and C. crenatum P2

PHB, the best-known polyhydroxyalkanoates (PHA), has been reported to influence intracellular metabolic flow, oxidation/reduction state [18] and enhance stress resistance of the host [16], as well. In this study, pDP10, containing the PHB synthesis genes, phbCAB, from Ralstonia eutropha, was introduced into C. crenatum SYPA 5 to generate C. crenatum P1 (Fig. 1). As depicted in Fig. 2, L-arginine metabolic pathway started from acetyl-CoA to α-ketoglutarate through TCA cycle, and then L-glutamate was formed with GdhA. Finally, L-arginine was produced by the catalysis of a series of enzymes encoded by the argCJBDFRJH (argC−H) cluster involved...
Fig. 1 Construction of the plasmids in this study
in l-arginine biosynthesis. On the other hand, PHB was formed from acetyl-CoA with the three key enzymes, PHB synthase (PhbC), β-ketothiolase (PhbA) and NADPH-dependent acetoacetyl-CoA reductase (PhbB).

Sources of NADPH in bacteria mainly contain the pentose phosphate pathway (PPP), isocitrate dehydrogenase in the tricarboxylic acid (TCA) cycle and the transhydrogenase system [23]. As illustrated in Fig. 2, NADPH was necessary in both the l-arginine and PHB metabolic pathways. PHB synthesis was a NADPH-dependent process for PhbB worked under the existence of NADPH and during the l-arginine metabolic pathways, GdhA and ArgC also required NADPH. In previous studies with arginine fermentation in C. crenatum SYPA 5, too much NADH can reduce the metabolic flow of the glycolytic pathway and increase the by-products (lactate and acetate) concentration [9]. As reported, in C. glutamicum, the NAD kinase (PpnK) regulated the cofactor level and could enhance the NADPH concentration [34]. Differential expression of ppnK has not been reported to date and ppnK might be essential and the only NAD kinase gene in Corynebacterium sp. [35]. Therefore, overexpressing homologous NAD kinase by cloning and amplifying the homologous ppnK gene in C. crenatum P1 deserved an attempt. We developed an approach to increase the NADPH availability in vivo through introducing NAD kinase, the key NADPH producing enzyme in C. crenatum, and were intended to see whether it had any effect on the l-arginine and PHB production or not. C. crenatum P2, containing the pDPP10, was created by introducing PpnK on the basis of C. crenatum P1.

**Enzyme activities assay of PhbC, PhbA, PhbB and PpnK**

In this study, the phbCAB cluster of *Ralstonia eutropha* was introduced into *C. crenatum* SYPA 5. In order to verify the activity of the over-expressed PhbC, PhbA and PhbB from the *C. crenatum* PHB production strains, *C. crenatum* SYPA 5, *C. crenatum* P1 and *C. crenatum* P2 were cultivated in LBG medium (LB with 0.5% glucose) for 24 h and then the crude enzyme activities of PhbC, PhbA and PhbB were detected (Table 1). The synthesis of bacterial PHB was dependent on the expression and activity of a key enzyme, PHB synthase (PhbC). Therefore, enhancing the activity of PhbC was a good way to increase PHB content [36]. To investigate the activity of PhbC, 3HB-CoA was used as the
substrate, and the release of CoA during polymerization was measured to determine the total enzyme activity [37]. The total activity of PhbC was measured using the soluble fraction of the crude extract. The total synthase activity of cell extracts containing PhbC in recombinant *C. crenatum* was about 0.28 U/mg while it was quite low, 0.02 U/mg, in *C. crenatum* SYPA 5. As the first enzyme in the PHB synthesis pathway, the regulatory role of β-ketothiolase (PhbA) has been extensively discussed. PhbA from different strains differed with respect to the effect of concentrations of acetoacetyl-CoA or NADH and NADPH on the thiolysis reaction [38]. The enzyme activities of PhbA in *C. crenatum* P1 and *C. crenatum* P2 showed a significant increase, 30-fold, compared to the *C. crenatum* SYPA 5. The effect of about 11-fold enhancement in recombinant *C. crenatum* was found in the activities of NADPH-dependent acetoacetyl-CoA reductase (PhbB). PhbB was special among the three key PHB synthesis enzymes due to its coenzyme dependency upon NADPH. Therefore, the concentration of NADPH has quiet influence on the phbB expression. The high activity of PHB synthesis related enzymes in recombinant strains could be associated with their elevated level of genes expression through introducing the exogenous plasmid pDP10 or pDPP10.

NAD kinase, PpnK, was the key enzyme for the biosynthesis of NADP + and NADPH in *C. crenatum* and it was critical for the generation of NADPH [39]. It could be divided into two kinds according to the phosphoryl acceptor. The enzyme that phosphorylates only NAD + to form NADP + was termed NAD + kinase (EC 2.7.1.23), and the enzyme that phosphorylates both NAD + and NADH to form NADP + and NADPH is NADH kinase (EC 2.7.1.86) [40, 41]. So far, the NAD kinases characterized either use ATP and PolyP as phosphoryl donors or were solely active with ATP [34] and PpnK here was the former kind. In order to investigate whether the ppnK gene expressed well in *C. crenatum* P2, the NAD kinase activity was determined and compared to the control *C. crenatum* SYPA 5. In the crude extract from the ppnK-expressing strain, the ATP-dependent and PolyP-dependent NAD kinase activity increased approximately 130-fold (84.35 ± 0.41 U/g) and 16-fold (3.12 ± 0.02 U/g), respectively, compared to the control *C. crenatum* SYPA 5 (0.63 ± 0.03 U/g) and (0.18 ± 0.01 U/g) (Data showed in Additional file 1). From the above observations, these differences in enzyme activities reflected the different expression levels of the PpnK proteins in these microorganisms, which might be due to the existence of the strong tac-M promoter [42].

### The effect of PHB accumulation on l-arginine operon transcription

In order to investigate the effect of PHB accumulation on gene transcription, RT-PCR was performed. In this experiment, we selected *argB*, *argC*, *argD*, *argF*, *argG*, *argH* and *argJ* gene as the experimental target since they directly involved in the l-arginine production. We found that the transcription levels of four l-arginine operon genes, *argB*, *argC*, *argD* and *argJ* in *C. crenatum* P1 increased 1.9–3.0 times compared with the parent strain (Fig. 3). Although the transcription of other genes in *C. crenatum* P1 decreased slightly, this result at least proved that the intracellular PHB accumulation enhanced the transcription levels of several l-arginine key synthesis genes. At the same time, the *phb-CAB* overexpression in *C. crenatum* P1 caused a dramatic increase of about 11-fold enhancement in recombinant *C. crenatum* SYPA 5 (0.63 ± 0.03 U/g) and (0.18 ± 0.01 U/g) (Data showed in Additional file 1). From the above observations, these differences in enzyme activities reflected the different expression levels of the PpnK proteins in these microorganisms, which might be due to the existence of the strong tac-M promoter [42].

### Table 1 Assay of enzyme activities of crude PhbC, PhbA, PhbB and PpnK in *C. crenatum* SYPA 5, P1 (SYPA 5/PHB-CAB) and P2 (SYPA 5/PHB-CAB-PPN)

| Strains              | Specific enzyme activities | PhbC (U/mg) | PhbA (U/mg) | PhbB (U/mg) | ATP-NAD kinase (U/g) | PolyP-NAD kinase (U/g) |
|----------------------|-----------------------------|-------------|-------------|-------------|----------------------|------------------------|
| *C. crenatum* SYPA 5 | 0.02 ± 0.00                 | 0.02 ± 0.00 | 0.06 ± 0.01 | 0.63 ± 0.03 | 0.18 ± 0.01          |
| *C. crenatum* P1     | 0.27 ± 0.01                 | 0.58 ± 0.02 | 0.64 ± 0.02 | 0.67 ± 0.04 | 0.20 ± 0.01          |
| *C. crenatum* P2     | 0.29 ± 0.01                 | 0.61 ± 0.03 | 0.83 ± 0.03 | 84.35 ± 0.41 | 3.12 ± 0.02          |

Samples were taken at 24 h of the shake flask using LBG culture. ATP-NAD kinase contained ATP-NAD + kinase and ATP-NADH kinase while PolyP-NAD kinase contained PolyP-NAD + kinase and PolyP-NADH kinase. Each data represented the average value of three independent measurements.
up-regulation of ppnK transcription. In C. crenatum P2, a similar data were also obtained for the phbCAB and ppnK gene which an increase in gene expression corresponded to an increase in enzyme activities. Thus, the introduction of the PHB synthesis pathway and NAD kinase overexpression affected the transcription of key genes of the l-arginine biosynthesis pathway. Obviously, the phbC and ppnK gene under the control of the promoters PphbC and tacM caused the increased transcription of the two genes. The two genes phbB and phbA followed the same trend, but it was much less pronounced. Consequently, l-arginine yield increasing effect in recombinant C. crenatum was explained at the transcriptional level.

TEM imaging
In order to verify the expression effect of recombinant plasmid pDXW-10-phbCAB more directly, C. crenatum SYPA 5 and its recombinants were prepared for TEM analysis. The results were exhibited in Fig. 4. From Fig. 4, (a) showed C. crenatum SYPA 5 with an extremely small amount of PHB granules; (b) displayed the PHB granules in the recombinant SYPA 5/pDXW-10-phbCAB. Due to the PHB gene cluster expressing well in the strain, PHB granules could be seen obviously; thanks to NAD kinase expression in C. crenatum, the competition environment of the NADPH was eased in the process of l-arginine and PHB biosynthesis. As it is shown in (c), the PHB existed evidently in the cell, more than that of the strain with PHB synthesis operon only. Thus, we conclude that, the NAD kinase in the recombinant C. crenatum was overexpressed.

Effect of PHB accumulation on l-arginine production by C. crenatum P1 in 5–l fermentor
To find the effect of PHB accumulation on l-arginine production, the growth of the strains C. crenatum SYPA 5 and C. crenatum P1 were compared under batch cultivation condition. From Fig. 5, it was easy to find that the PHB content in C. crenatum SYPA 5 was low, varied below 3.0 %, while in C. crenatum P1, the PHB content increased to 12.7 %, about fourfold, at maximum and more PHB accumulated in the C. crenatum P1. The recombinant C. crenatum P1 demonstrated a superior ability in growth compared to that of the control C. crenatum SYPA 5 with the final OD₅₆₂ reaching 81 at 96 h. However, the glucose consumption of the recombinant strain was more than that of the control strain. Besides these differences, the recombinant strain exhibited similar l-arginine production with the control strain in the early stage of the culture. Notably, the large gap in l-arginine production appeared after 32 h comparing to the control. The final concentration of l-arginine of C. crenatum P1 was 38.54 ± 0.81 g/L, increasing by 20.6 % comparing to C. crenatum SYPA 5 (31.95 ± 0.68 g/L). Meanwhile, the metabolic intermediate and by-product, α-ketoglutarate, acetate and lactic acid (Table 2) and some other amino acids (Table 3) in batch cultivation were also analyzed to investigate the effect of PHB accumulation on C. crenatum metabolism. These data show that, during fermentation, the formation of some other amino acids except for l-arginine and l-glycine in C. crenatum P1 were apparently less than that in the C. crenatum SYPA 5. However, the concentration of acetate and lactic acid increased.

As reported, the expression of PHB synthesis genes, which increased L-glutamate production with 39–68 % in shake flask and 23 % in fermentor, had a positive effect on glutamate production in C. glutamicum [18]. PHB also had a global effect on the host in l-tryptophan producing strain and upregulated the transcription of a tryptophan operon, leading to improvement of the l-tryptophan production [17]. Given these results, it was assumed that the introduction of the PHB synthesis pathway into the cells would affect microbial global metabolism, leading to a difference
in the formation of some products, such as some kind of amino acids. In this study, due to the exogenous plasmid pDP10 harboring the PHB synthesis genes, \textit{phb\textit{CAB}}, in \textit{C. crenatum P1}, the recombinant strain grew faster even though the glucose metabolism was enhanced. At the same time, the significant metabolite intermediate for \textit{l}-arginine production, \textit{α-ketoglutarate} (from 0.64 ± 0.03 g/L to 0.96 ± 0.02 g/L), had a slight increase flux in the recombinant strain. More bacterial and more metabolic precursor might result in more \textit{l}-arginine production than that in the parent strain. Moreover, the existence of PHB in the cells might enhance stress resistance of the host and protect the cells, to some degree. Still, more glucose was consumed and conserved in the cell at the cost. It was obvious that the accumulation of PHB affecting the productivity of the long \textit{l}-arginine fermentation. This might be a major reason why overexpressed \textit{phb\textit{CAB}} could evolve significant \textit{l}-arginine yield.

PHB accumulation in the bacteria influenced and regulated the global pathways, including the co-factor level. The NADPH pool in the cells had a slight increase, from 35 ± 2 pmol/OD$_{562}$ to 39 ± 2 pmol/OD$_{562}$, in \textit{C. crenatum P1} (Table 4), this was likely why more \textit{l}-arginine was yielded. However, considering that the competitive relationship between PHB and \textit{l}-arginine production on NADPH, it is interesting to take measures to regulate the cofactor level in the cells in order to see whether any further positive effect on the \textit{l}-arginine yield exists.
Table 2 Concentrations of acetate and lactic acid in batch cultivation of *C. crenatum* SYPA 5, P1 (SYPA 5/phbCAB) and P2 (SYPA 5/phbCAB-ppnK)

| By-products | Concentration (g/L) |
|-------------|-------------------|
|             | 24 h   | 48 h   | 60 h   | 84 h   | 96 h   |
| Acetate     |        |        |        |        |        |
| *C. crenatum* SYPA 5 | 2.02 ± 0.03 | 1.74 ± 0.02 | 1.50 ± 0.01 | 0.82 ± 0.02 | 0.74 ± 0.01 |
| *C. crenatum* P1   | 2.13 ± 0.03 | 1.81 ± 0.02 | 1.69 ± 0.02 | 1.37 ± 0.02 | 1.18 ± 0.01 |
| *C. crenatum* P2   | 2.08 ± 0.04 | 1.88 ± 0.02 | 1.46 ± 0.02 | 1.01 ± 0.02 | 0.94 ± 0.02 |
| Lactic acid    |        |        |        |        |        |
| *C. crenatum* SYPA 5 | 2.26 ± 0.03 | 1.80 ± 0.02 | 1.35 ± 0.02 | 1.07 ± 0.02 | 0.86 ± 0.01 |
| *C. crenatum* P1   | 2.37 ± 0.03 | 1.97 ± 0.03 | 1.65 ± 0.02 | 1.76 ± 0.02 | 1.95 ± 0.02 |
| *C. crenatum* P2   | 2.21 ± 0.01 | 1.78 ± 0.02 | 1.29 ± 0.02 | 1.37 ± 0.02 | 1.50 ± 0.02 |

Each data represented the average value of three independent measurements.

Table 3 Production of other related amino acids by *C. crenatum* SYPA 5, P1 (SYPA 5/phbCAB) and P2 (SYPA 5/phbCAB-ppnK)

| Amino acids | Concentration (g/L) |
|-------------|-------------------|
|             | SYPA 5 | P1 (SYPA 5/CAB) | P2 (SYPA 5/phbCAB) |
| Ile         | 2.62 ± 0.00 | 2.34 ± 0.00 | 3.60 ± 0.01 |
| Lys         | 4.39 ± 0.01 | 4.09 ± 0.01 | 5.04 ± 0.02 |
| Glu         | 1.03 ± 0.02 | 0.73 ± 0.00 | 0.51 ± 0.00 |
| Gly         | 0.30 ± 0.00 | 0.45 ± 0.00 | 0.23 ± 0.00 |
| Thr         | 0.29 ± 0.00 | 0.19 ± 0.00 | 0.17 ± 0.00 |
| Val         | 0.56 ± 0.00 | 0.42 ± 0.00 | 0.38 ± 0.00 |
| Orn         | 0.52 ± 0.00 | 0.26 ± 0.00 | 0.26 ± 0.00 |
| Ser         | 0.21 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 |

The samples were taken at 96 h of the batch fermentation in 5-L fermentor. Each data represented the average value of three independent measurements.

Effect of PpnK introduction on l-arginine production

As reported, the impact of *ppnK* overexpression on lysine production was positive in *C. glutamicum* [34], so was isoleucine production in *C. glutamicum* [39] and thymidine production in *E. coli* [33]. In this study, we found that PHB accumulated in recombinant *C. crenatum* P1 and that l-arginine production increased by 20.6 % due to the accumulation of PHB (Fig. 5). Furthermore, NADPH pool played an important role in PHB and l-arginine production. Therefore, it was interesting to see how the extra introduction of NAD kinase into *C. crenatum* P1 affected PHB and l-arginine biosynthesis. In the bacteria, the reducing power [H] was mainly generated by EMP, HMP and TCA cycle while the formation of PHB and l-arginine consumed [H], which keep the oxidation/reduction state balanced. The overexpression of PpnK increased the (NADP⁺ + NADPH)/(NAD⁺ + NADH) rate by fourfold in *C. crenatum* P2 comparing to that in *C. crenatum* SYPA 5. As consequence, there was more NADPH supply for PHB synthesis, accounting for 15.7 % of increased PHB at max while 12.7 % in *C. crenatum* P1 (Fig. 5). Excess amount of NAD kinase could be inhibited by the regulation mechanism, like NADP⁺ [43]. In our investigation, two more exogenous genes existed in the cells. Both of these could contribute to the decrease in OD₅₆₂ in *C. crenatum* P2 comparing to that in *C. crenatum* P1. However, the OD₅₆₂ in *C. crenatum* P2 was still above that in *C. crenatum* SYPA 5 and this might be due to the PHB synthesis genes existed (Fig. 5). The concentration of α-ketoglutarate exhibited no much difference between *C. crenatum* SYPA 5 and *C. crenatum* P2 in the early stage but a slight increase in *C. crenatum* P2.

Table 4 Concentrations of intracellular NAD⁺, NADH, NADP⁺ and NADPH in *C. crenatum* SYPA 5, P1 (SYPA 5/phbCAB) and P2 (SYPA 5/phbCAB-ppnK)

| Strains                        | Concentration (pmol/OD₅₆₂) |
|-------------------------------|---------------------------|
|                              | NAD⁺          | NADH          | NADP⁺          | NADPH         | (NADP⁺ + NADPH)/(NAD⁺ + NADH) |
| *C. crenatum* SYPA 5          | 571 ± 42       | 61 ± 4        | 161 ± 9        | 35 ± 2        | 0.31                        |
| *C. crenatum* P1              | 366 ± 27       | 70 ± 5        | 127 ± 8        | 39 ± 2        | 0.38                        |
| *C. crenatum* P2              | 163 ± 10       | 65 ± 4        | 205 ± 14       | 71 ± 5        | 1.21                        |

The samples were taken at 96 h of the batch fermentation in 5-L fermentor. Each data represented the average value of three independent measurements.
the enhancement of the ratio of (NADP+ / NADPH) in C. crenatum P2 were lower than those in P1 because of production of L-arginine (Table 3). With respect to the P1, represently, also contributed to the high and increased by 53.8 and 23.2% comparing to that of P2 but Ile and Lys, which were quiet NADPH-dependent less production of some other amino acids in C. crenatum (Table 2). All of these could also explain why more L-arginine fermentation. Meanwhile, the high-yield fermentation. Therefore, it was optimistic that the accumulation of PHB in the cells might enhance stress resistance of the host and protect the cells. Obviously, by employing the PHB synthesis pathway, the L-arginine productivity was increased during the late stage of high-yield L-arginine fermentation. Moreover, the high-yield L-arginine recombinant C. crenatum P2 was constructed by overexpressing the NAD kinase encoding gene ppnK into C. crenatum P1. Overexpressing the NAD kinase also enhanced these effects. Furthermore, batch cultivation of the engineered C. crenatum revealed that it could accumulate both extracellular L-arginine and intracellular PHB simultaneously. With those strategies, the recombinant C. crenatum with PHB accumulation and NAD kinase overexpression could increase the concentration of NADPH in the coenzyme pool of the cell and serve as high-efficiency cell factories for L-arginine production.

Methods
Bacterial strains and plasmids
All the bacterial and plasmids used in the study are listed in Table 5.

Construction of plasmids
The pbhCAB operon was obtained by double-digesting the plasmid pHBR68 with EcoRI and BamHI. It was then ligated into the E. coli-C. crenatum shuttle expression vector, pDXW-10, to generate the recombinant plasmid pDP10. The native promoter of the pbhC gene was deleted and the native ribosome binding site (RBS) was changed with a consensus RBS sequence AAA-GGAGGGAATC of highly expressed genes. To construct pDP10, two steps were done. Firstly, ppnK gene from C. crenatum SYPA 5 was amplified by primers (5′- ATTTGGGCGCGC AAAAGGGAATC ATGACTGCACCCAGAA-3′) and (5′- CCAAGCTTTTACCCCGCTGACCTGG-3′) using C. crenatum SYPA 5 genome as a template (the two primers above was designed using the ppnK gene of C. glutamicum ATCC 13032). Then the PCR product was digested by NotI and HindIII and inserted into NotI-HindIII sites of pDXW-10. All of these resulted in the pDP01. Secondly, the pDP01 was used as the template and the tacM-ppnK site was amplified by primer 5′- CCGCCGCTTACCCCGGTGACGGCATG-3′ and 5′- CCAAGCTTTCGGAAAGCT GTTGATAGC-3′. The PCR product was inserted into pDP10 with the HindIII digestion site after dephosphorylation using CIAP. Both of the pDP10 and pDP10 were transferred into C. crenatum SYPA 5, generating C. crenatum P1 and C. crenatum P2.

Cultivation medium and conditions
LBG medium (LB with 0.5% glucose) was used for seed culture preparation supplemented with appropriate antibiotic (kanamycin, 25 mg/L). The fermentation medium contained the following (g/L): glucose 170, (NH4)2SO4 20, yeast extract 12, MgSO4·7H2O 0.5, KCl 1, KH2PO4 1.5, FeSO4·7H2O 0.02, MnSO4·H2O 0.02 (pH 7.0).

The seed cultures were incubated at 30°C in LBG for 24 h at 160 rpm on a rotary shaker and then 125 ml seed culture was incubated into the fermentation medium (inoculated with 5% v/v seed culture) for the 5–1
fermentor (BIOTECH-5BG, Baoxing Co., China) with a working volume of 2.5–l. The culture condition was set at 30 °C and pH 7.0 under the 600 rpm agitation speed. The pH was controlled automatically by addition of 50 % ammonia water. Temperature was also adjusted automatically by the fermentor.

RNA preparation and transcriptional analysis

Samples for RNA preparation were cultivated for 24 h in LBG in shake flask at 30 °C. Total cellular RNA was extracted using the RNA simple Total RNA Kit (TIAN GEN, China). Reverse transcription was carried out with the PrimeScript™ RT reagent Kit (TaKaRa, China) according to the instructions of the manufacturer. The mRNA levels were determined by semi-quantitative reverse transcription (q) RT-PCR using SYBR green PCR master mix (ABI 7000; Applied Biosystems, CA). The 16S rRNA gene was used as an endogenous control. For qRT-PCR, 1/20 of each RT-PCR product was used as the template for DNA amplification, using specific primer pairs for each gene. The results of the reactions were processed using specific software (ABI Prism 7000 SDS software). The RT-PCR measurement was repeated three times for each sample. The argB, argC, argD, argF, argG, argH, argJ, phbC, phbA, phbB and pnpK gene transcript primers were listed in Table 6.

Sample preparation for TEM imaging

For all different samples for TEM imaging were prepared as reported [45]. Bacteria were fixed with glutaraldehyde 2 % and paraformaldehyde 2 % in a buffer of sodium cacodylate 0.1 mol/L (pH 7.4) and then post-fixed in 1 % buffered osmium tetroxide. The bacteria was then completely dehydrated with ethanol at room temperature and then embedded in epoxy resin (polymerization at 60 °C for 48 h). Ultrathin sections of 50 nm thicknesses were cut with a diamond knife, deposited on copper grids.

---

Table 5 Strains and plasmids used in this study

| Strains and plasmids | Relevant characteristics | References |
|----------------------|--------------------------|------------|
| Corynebacterium crenatum SYPA 5 | l-Arginine production bacterium | [12] |
| C. crenatum P1 | Derived from C. crenatum SYPA 5, Harboring pDP10 | This study |
| C. crenatum P2 | Derived from C. crenatum SYPA 5, Harboring pDP10 | This study |
| pBH68 | pBluescript SK-derivative, containing the entire phbCAB operon of Ralstonia eutropha H16 | [44] |
| pDXW-10 | E. coli–C. crenatum shuttle expression vector, Km’ | [42] |
| pDP10 | Derived from pDXW-10, harboring phbCAB operon | This study |
| pDPP10 | Derived from pDP10, harboring phbCAB operon and pnpK gene | This study |
| pDP01 | Derived from pDXW-10, harboring pnpK gene | This study |

Table 6 Primers of RT-PCR used in this study

| Primers | Nucleotide sequence (5′–3′) |
|---------|--------------------------|
| argB    | TCGTGTTGCTGGAGCTTT        |
| -F      | TCCCCATCCTTCTCTGCTTT      |
| -R      | AGTCCCTTCTACTCGCAATC      |
| argC    | CTGCCCTCCTCATCAAAACCA     |
| -F      | CTTAGTGTGCGCTGCTGTT      |
| -R      | AGCCGGTTTCTGCTTATC       |
| argD    | GCCTGCTGATTCTCTGCTT      |
| -F      | ACCACACCTTCTGTTCTTACC    |
| -R      | AGGCCGTTTCTGCTTATC       |
| argF    | TGACATCTCACAAGCTCTG      |
| -F      | AATCATTGCGGAGAAGAAAA     |
| -R      | GTCAAGGTCGAAAACCAACCA    |
| argG    | AGCGGTTTCTGCTTATC       |
| -F      | GCGTTACTCTCGTCAATG       |
| -R      | GATGTGCGCAGAGAAGTT      |
| argH    | GCGTGTATGCGACTGAGAAGAACA |
| -F      | GTGAAAGGTGAGCGAAGAAGAAC |
| -R      | ACCATTACAGCTAGATCCATTACC |
| phbC    | GCGTTACTCTCGTCAATG       |
| -F      | CGAACTCGTGGTGCTGACTG     |
| -R      | GACAGATGTAGCAGATGC       |
| phbA    | GACAGATGTAGCAGATGC       |
| -F      | GCAACTCAGTAGCAGAAGGC    |
| -R      | GCAACCAAGGGAAGCAAC      |
Additional file

**Additional file 1.** Assay of enzyme activities of crude PpnK. Samples were taken at 24 h of the shake flask using LBG culture. NAD kinase encoding by pppK in *C. crenatum* contained ATP-NAD⁺ kinase, ATP-NADH kinase, PolyP-NAD⁺ kinase and PolyP-NADH kinase. Each data represented the average value of three independent measurements.

**Abbreviations**

PhB: Poly-β-hydroxybutyrate; Phbc: PHB synthase; PhbA: β-Ketothiolase; PhbB: NADPH-dependent acetoacetyl-CoA reductase; ArgC: N-acetylglutamate 5-semialdehyde dehydrogenase; PpnK: NAD kinase; LBG: LB with 0.5% glucose; TEM: Transmission electron microscope; DCW: Dry cell weight.

**Authors’ contributions**

MX and ZK participated in the design of the study and performed the statistical analysis. MX and JQ carried out the construction of the strains, enzyme activities determination, participated in the fermentation research and drafted the manuscript. HY participated in detecting the by-products. YG and ZK gave several useful suggestions. XW provided pOXW-10 and p8HR68 for the study. All authors read and approved the final manuscript.

**Acknowledgements**

This work was supported by the National Basic Research Program of China (973 Program) (2012CB725202), the High-tech Research and Development Programs of China (2012AA022102, 2015AA021004), the National Natural Science Foundation of China (31300028), the Research Project of Chinese Ministry of Education (113033A), the Jiangsu Provincial National Basic Research Program (BK20130137), the Fundamental Research Funds for the Central Universities (USRPS1306A), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (No. 111-2-06), and the Jiangsu province “Collaborative Innovation Centre for Advanced Industrial Fermentation” industry development program.

**Competing interests**

The authors declare that they have no competing interests.

**Received:** 25 October 2015  **Accepted:** 8 January 2016

**Published online:** 19 January 2016

**References**

1. Granik VG. Metabolism of l-arginine. Pharm Chem J. 2003;37;111–27.
2. Elias DB, Barbosa MC, Rocha LB, Dutra LL, Silva HF, Martins AM, Gonçalves RF. l-arginine as an adjuvant drug in the treatment of sickle cell anaemia. Br J Haematol. 2013;160:410–2.
3. Hristina K, Langerholc T, Trapecar M. Novel metabolic roles of l-arginine in body energy metabolism and possible clinical applications. J Nutr Health Aging. 2014;18:213–8.
4. Glansdorff N, Xu Y. Microbial arginine biosynthesis: pathway, regulation and industrial production. In: Wendsch V, editor. Amino acid biosynthesis--pathways, regulation and metabolic engineering, vol. 5. Berlin, Heidelberg: Springer; 2007. p. 219–57. (Microbiology Monographs).
5. Xu M, Rao Z, Yang J, Dou W, Xu Z. The effect of a LYSE exporter overexpression on l-arginine production in *Corynebacterium crenatum*. Curr Microbiol. 2013;67:271–8.
6. Schneider J, Niermann K, Wendsch V. Production of the amino acids l-glutamate, l-lysine, l-orotidine and l-arginine from arabinose by recombinant *Corynebacterium glutamicum*. J Biotechnol. 2011;154:191–8.
7. Ikeda M, Mituhashi S, Tanaka K, Hayashi M. Reengineering of a *Corynebacterium glutamicum* l-arginine and l-L-citrulline producer. Appl Environ Microbiol. 2009;75:1635–41.
8. Caldara M, Dupont G, Leroy F, Goldbeter A, De Vyust L, Cunin R. Arginine biosynthesis in *Escherichia coli*: experimental perturbation and mathematical modelling. J Biol Chem. 2008;283:6347–58.
9. Xu H, Dou W, Xu H, Zhang X, Rao Z, Shi Z, Xu Z, A two-stage oxygen supply strategy for enhanced l-arginine production by *Corynebacterium crenatum* based on metabolic fluxes analysis. Biochem Eng J. 2009;43:41–51.
10. Xu M, Rao Z, Xu H, Lan C, Dou W, Zhang X, Jin J, Xu Z. Enhanced production of l-arginine by expression of *Vitreoscilla* hemoglobin using a novel expression system in *Corynebacterium crenatum*. Appl Biochem Biotechnol. 2011;163:707–19.
11. Xu M, Rao Z, Yang J, Xia H, Dou W, Jin J, Xu Z. Heterologous and homologous expression of the arginine biosynthetic argC–H cluster from *Corynebacterium crenatum* for improvement of l-arginine production. J Ind Microbiol Biotechnol. 2012;39:495–502.
12. Xu M, Rao Z, Dou W, Yang J, Jin J, Xu Z. Site-directed mutagenesis and feedback-resistant N-acetyl-l-glutamate kinase (NAGK) increase *Corynebacterium crenatum* l-arginine production. Amino Acids. 2012;43:255–66.
13. Brauneck G, Lefebvre G, Genser KF. Polyhydroxyalkanoates, biopolymers from renewable resources: physiological and engineering aspects. J Biotechnol. 1998;65(2–3); 127–61.
14. Lee YS. Plastic bacteria: Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol. 1996;14:431–8.
15. Harding KG, Dennis JS, von Blottnitz H, Harrison ST. Environmental analysis of plastic production processes: comparing petroleum-based polylpolyene and polylethylene with biologically-based poly-beta-hydroxybutyric acid using life cycle analysis. J Biotechnol. 2007;130:57–66.
16. Wang Q, Yu H, Xia Y, Kang Z, Qi Q. Complete PHB mobilization in Escherichia coli enhances the stress tolerance: a potential biotechnological application. Microb Cell Fact. 2009;8:9.

17. Gu P, Kang J, Yang F, Wang Q, Liang Q, Qingsheng Q. The improved l-tryptophan production in recombinant Escherichia coli by expressing the polyhydroxybutyrate synthesis pathway. Appl Microbiol Biotechnol. 2013;97:4121–7.

18. Liu Q, Ouyang SP, Kim J, Chen GQ. The impact of PHB accumulation on l-glutamate production by recombinant Corynebacterium glutamicum. J Biotechnol. 2007;12:273–9.

19. Kang Z, Gao C, Wang Q, Liu H, Qi Q. A novel strategy for succinate and polyhydroxybutyrate co-production in Escherichia coli. Bioprocess Technol. 2010;101:7675–8.

20. Senior PJ, Dawes EA. The regulation of poly-P-hydroxybutyrate metabolism in Azotobacter beijerinckii. Biochem. J. 1973;134:225–38.

21. Li ZJ, Cai L, Wu Q, Chen GQ. Overexpression of NAD kinase in recombinant Escherichia coli harboring the phcAB operon improves poly-(3-hydroxybutyrate) production. Appl Microbiol Biotechnol. 2009;83:939–47.

22. Glansdorff N, Xu Y. Microbial Arginine Biosynthesis patheway. Regulation and Industrial Production. Microbiol Monogr. 2006;5:219–57.

23. Wang Y, San KY, Bennett GN. Improvement of NADPH bioavailability as a gene encoding an NAD kinase into Saccharomyces cerevisiae of UTR1 of a yeast. FEMS Microbiol Lett. 2001;200:181–4.

24. Hk C, Gm W. Regeneration of nicotinamide cofactors for use in organic synthesis. Appl Biochem Biotechnol. 2000;6:5:219–57.

25. Kang Z, Mori S, Mukai T. Molecular characterization of Escherichia coli NAD kinase. Eur J Biochem. 2001;268(15):4359–65.

26. Garavaglia S, Raffaelli N, Finaurini L, Magni G, Rizzi M. A novel fold revealed by mycobacterium tuberculosis NAD kinase a key allosteric enzyme in NADP biosynthesis. JBC Papers in Press. 1993;279(13):40980–6.

27. Shi F, Huan X, Wang X, Ning J. Overexpression of NAD kinases improves the l-isoleucine biosynthesis in Corynebacterium glutamicum spp. lactofermentum. Enzyme Microb Technol. 2012;51:73–80.

28. Shigeyuki K, Kousaku M. Structure and function of NAD kinase and NADP phosphatase: key enzymes that regulate the intracellular balance of NAD(P)(H). Biosci Biotechnol Biochem. 2008;72:919–30.

29. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

30. Nishimura T, Sato T, Tomita K. Purification and properties of β-ketothiolase from Zoogloea ramigera. Arch Microbiol. 1978;116:21–7.

31. Shi F, Huan X, Wang X, Ning J. Overexpression of NAD kinases improves the l-isoleucine biosynthesis in Corynebacterium glutamicum spp. lactofermentum. Enzyme Microb Technol. 2012;51:73–80.

32. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

33. Lindner SN, Niederholmeyer H, Schmitz K, Schoberth SM, Wendsich VF. Polyphosphate/ATP-dependent NAD kinase of Corynebacterium glutamicum: biochemical properties and impact of ppnK overexpression on lysine production. Appl Microbiol Biotechnol. 2010;87:5383–93.

34. Shi F, Li K, Huan X, Wang X. Expression of NADH kinase and glucose-6-phosphate dehydrogenase improve NADPH supply and l-isoleucine biosynthesis in Corynebacterium glutamicum spp. lactofermentum. Appl Microbiol Biotechnol. 2013;171:504–21.

35. Shubhanal K, Chua JA, Shouzi F, Brigham CJ, Taguchi S, Sinskey AJ, Rha C, Sudesh K. Characterization of the highly active polyhydroxyalkanoate synthase of Chromobacterium sp. strain USM2. Appl Environ Microbiol. 2011;77:2926–33.

36. Braunegg G, Sonnleimer B, Lafferty R. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in recombinant Corynebacterium glutamicum ssp. strain USM2. Appl Biochem Biotechnol. 2009;31:1929–36.

37. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

38. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

39. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

40. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

41. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

42. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

43. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

44. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

45. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

46. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

47. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

48. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

49. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

50. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.

51. Steinbuchel PSA, Schlegel DHG. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol. 1988;170:5837–47.