Tropical Journal of Pharmaceutical Research December 2012; 11 (6): 909-916

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v11i6.6

Research Article

Over-expression of NAD kinase in *Corynebacterium crenatum* and its Impact on L-Arginine Biosynthesis

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Abstract

Purpose: *To improve the biosynthesis of L-arginine by overexpressing homologous NAD kinase (ppnk) in Corynebacterium crenatum SYPA5-5 and to study its impact in presence of high (HOS) and low oxygen supply (LOS).*
Methods: *A recombinant plasmid (pJC1-tac-ppnK) harboring homologous NAD kinase (ppnk) was constructed in a shuttle vector pJC1 and transferred in L-arginine producing strain Corynebacterium crenatum SYPA5-5. Furthermore, fermentation was performed by shake flask method with consecutive determination of cell growth and glucose concentration. NAD^+ kinase activity was studied by stop method and NADP(H) concentrations were determined by spectrophotometric enzymatic cycling method. To check the biosynthesis of amino acids, HPLC method was used to determine extracellular amino acid concentrations.*
Results: *In HOS condition, NAD^+ kinase activity increased by 116 %, while intracellular concentrations of $NADP^+$ and NADPH increased by 7.3 and 36.8 %, respectively. Whereas, in LOS condition , NAD^+ kinase activity increased 49 % , with intracellular 14.67 and 15 % increases in $NADP^+$ and NADPH respectively. More importantly, recombinant strain could produce 26.47 and 11.36 g/L L-arginine in HOS and LOS respectively, which is higher than control strain value of 24.29 and 7.58 g/L respectively.*
Conclusion: *These results suggest that altering the concentration of co-enzymes by NAD kinase in Corynebacterium crenatum is an effective way to increase $NADP^+$ with concurrent production of NADPH for further enhanced L-arginine biosynthesis in Corynebacterium crenatum in both conditions of high and low oxygen supply.*

Keywords: *NAD kinase, PpnK, L-arginine, Corynebacterium crenatum*

Received: 8 June 2012 *Revised accepted:* 6 November 2012

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INTRODUCTION

L-Arginine (Arg) is classified conditionally as an essential amino acid for adults and essential amino acid for birds, carnivores and young mammals. L-arginine administration reverses endothelial dysfunction, enhances wound healing, prevents the early stages of tumorigenesis, and improves cardiovascular, reproductive, pulmonary, renal, digestive, and immune functions [2].

Nicotinamide adenine dinucleotide phosphate (NADPH), an important co-enzyme during anabolic reactions plays a significant role during biosynthesis of amino acids in *C. glutamicum* [13]. L-arginine biosynthetic pathways require NADPH for reductive amination of oxoglutarate to glutamate by glutamate dehydrogenase and for the formation of N-Acetylglutamate-5 -semi-aldehyde by N-acetyl-gamma-glutamyl-phosphate reductase [19]. It has been reported that in *C. glutamicum* NADPH is generated mainly through $NADP^+$ dependent dehydrogenases such as glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH/ gnd) in oxidative Pentose phosphate pathway (PPP) and partly by NADP dependant isocitrate dehydrogenase (ICD) and NADP dependent malic enzyme (ME/ malE) [33-35]. NADP(H) could also be formed from the biosynthetic pathway of NADP(H) by Phosphorylation of NAD through NAD kinase (EC 2.7.1.23), which is a critical enzyme and expected to be essential for the regulation of NAD(H) and NADP(H) balance. It has been reported that G6PD is the rate controlling step for PPP and NADPH from oxidative part of PPP in *C .glutamicum*, seems to be essential for the higher production of Lysine biosynthesis. Moreover, kinetic studies on G6PD showed NADPH is a strong inhibitor of G6PD [14,15]. As a result, direct conversion of NADPH might affect the PPP. Presence of multiple NAD kinase genes have been reported in several microorganisms [10,32]. However, in *C. glutamicum*, only one NAD kinase gene has been reported (ppnK) and seems to be essential for this organism [13]. Very recently, the purified NAD kinase from *C. glutamicum* ssp. *lactofermentum* ATCC 13869 showed weak NADH kinase activity besides the NAD^+ kinase activity [36]. Most of the NAD kinases were found allosteric in nature and due to this fact, the NAD(H) and NADP(H) balance might be directly regulated by NAD kinase [9,10,32].

Bacterial fermentation from natural carbon sources is currently the major approach to industrial scale production of L-arginine [1,3,4]. *Corynebacterium crenatum* SYPA5-5 is an aerobic, Gram-positive, non-sporulating, and L-histidine auxotroph industrial bacterium was isolated from soil and mutated by UV in our previous work [7]. As an aerobic bacterium, *C. crenatum* requires a large amount of dissolved oxygen (DO) for L-arginine production and plays an important role for L-arginine production [7].

Altering the level of co-enzyme(s) involved in L-arginine production pathway could be one of the possible approaches for further enhancing L-arginine production. In this present studies we have successfully constructed the homologous NAD kinase in shuttle vector pJC1 (pJC1-tac-*ppn*K) , overexpressed in *C. crenarum* to further increase the $NADP^+$ that might stimulate PPP with concurrent production of NADPH and precursor metabolites. Moreover, the impact of NAD Kinase over-expression on L-arginine fermentation in the presence of high oxygen supply (HOS) and low oxygen supply (LOS) was investigated.

EXPERIMENTAL

Reagents, Strains, plasmids and oligonucleotide primers

Plasmids were constructed in *E. coli* JM109 from PCR-generated fragments (ExTaq DNA Polymerase, TaKaRa) using *C. crenatum* SYPA5-5 DNA as a template prepared according to Eikmanns *et al*. [25].

In order to construct pMD19-T-ppnK and pJC1-tac-ppnK, the ppnK gene was amplified by PCR using the upstream primer ppnK F Xba I: 5'-GCTCTAGA**ATG**ACTGCACCCAC GAACG-3'; the introduction of a Xba I restriction site was underlined and start codon is highlighted in bold. The downstream primer ppnK R Sal I: 5'-GCGTCGAC **TTA**CCCCGCTGACCTGG-3'; the introduce-tion of a Sal I restriction site was underlined; the stop codon is highlighted in bold. The PCR fragment was cloned into the pMD19-T vector (TaKaRa) , the obtained plasmid pMD19-T-ppnK was confirmed by digestion with Xba I and SalI, and the Xba I /Sal I fragment of ppnK from pMD19-T-ppnK was then inserted into the corresponding sites of pJC1-tac [8], resulting in plasmid pJC1-tac-ppnK. *C. crenatum* SYPA5-5 with an empty vector was used as control. $NADP^+$, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, alcohol dehydrogenase, thiazolyl blue were purchased from Sigma (St. Louis, MO, USA). phenazine ethosulphate was from Santa Cruz Biotech(California, USA) .NAD^+, NADPH, ATP, glucose-6- phosphate were from Sangon(Shanghai, China).

Transformation and Screening

The ligation mixture was used to transform into *Escherichia coli* by the calcium chloride method [26].Transformation in *C. crenatum* was performed by using electroporation method and freshly transformed culture was incubated at 46°C water bath for 6 min with an additional shaking at 200 rpm,30°C. Finally, transformed culture was incubated on solid LB containing 9.1% sorbitol at 30°C until the colonies appeared [7,8]. When necessary, ampicillin (100 μg mL^{-1}) or kanamycin (50 μg mL^{-1}) was added at final concentration for transformant selections.

Growth medium and conditions for L-arginine production

Bacteria were cultivated in rich Luria–Bertani (LB) medium, standard for *E. coli*. *Corynebacterium s*p were cultivated in LB medium with 0.5% glucose and for *C. crenatum* competent cell medium, LB including 3% glycine and 0.1%Tween was used [7,26]. Slant medium, seed medium, and shake flask fermentation medium of *C. crenatum* prepared according to Xu [7,27].

For L-arginine batch fermentation, *C. crenatum* strains were activated in slant medium. After 24 h, the seed was inoculated from agar slants and cultured at 30°C for about 15 h in shake flasks. Furthermore, the shake flask seed cultures were transferred into 250 ml flask to final OD_{562} =1; the shake flask feed batch fermentation was performed at 30°C for 96 h. For shake flask fermentation, 15% glucose was added in two steps. Initially 10% and an additional 5% glucose were added after 36 h to the feed batch shake flask fermentation culture. 20 ml fermentation culture in 250 ml flask and 60 ml fermentation culture in 250 ml flask were classified as high oxygen supply (HOS) and low oxygen supply (LOS) respectively [28]. Strain harboring the recombinant plasmid pJC1-tac-ppnK was cultivated with kanamycin (30 μg mL^{-1}) before being transferred into the shake flask fermentation medium [27].

Assay of cell concentration, glucose, L-arginine and other amino acids

Initial cell concentration was monitored at 562 nm, and the dry cell weight (DCW) was determined by a pre-calibrated relationship (1 OD = 0.375 gL^{-1} DCW) [7]. Glucose concentration in the media was measured by using anthrone method (35). Concentrations of L-arginine and other amino acids were measured using a reversed-phase high-pressure liquid chromatography [7]. For the quantification of product formation and glucose concentration, a 200~300 μ L sample was removed from the culture and was centrifuged at 12,000 rpm for 10 min, further the supernatant was used to determine the amino acid concentration in the culture fluid. All of the measurements, particularly the

most important state variables, such as the concentrations of cells, L-arginine, and glucose, were measured in triplicate.

Assay of NAD^+ kinase activity

Crude cell extracts were prepared from cells grown in fermentation medium as described above. Cells were harvested during the exponential phase by centrifugation (12,000 rpm for 5 min, 4°C), washed with disruption buffer (100mM Tris/HCl, pH 8.0, 10mM MgCl2 and 0.75mM DTT) and disrupted by sonication at 4°C. Cell debris was removed by centrifugation (12,000 rpm for 15 min at 4°C). The obtained clear supernatant was used as a crude enzyme for determination of NAD^+ kinase activity. NAD^+ kinase activities were assayed at 30°C using a stop method according to F. shi et al. [16]. In brief, the formation of NADPH was measured at 340 nm in a reaction mixture (1.0 mL) consisting of 5.0 mM NAD, 5.0 mM ATP, 5.0 mM MgCl2, 100 mM Tris／HCl (pH 8.0) and an appropriate amount of enzyme. Crude enzyme solution of less than 100 μ L was added to the reaction mixture to initiate the reaction. The reaction was terminated by immersing the tube in boiling water for 5 min, followed by addition of 0.1 mL of 50 mm glucose-6-phosphate to the mixture, and the amount of NADP formed was determined enzymatically with 0.5 U glucose-6-phosphate dehydrogenase. Negative controls were carried out without crude enzyme or without ATP, respectively. Enzyme activity (one unit) (U) was defined as 1.0 μmol $NADP^+$ produced for 1 min at 30°C in the initial mixture (1.0 ml), and the specific activity was expressed as U/mg of protein. Bradford method was used to determine protein concentrations with BSA as a standard [24].

Determination of the intracellular NADP(H) concentrations

The intracellular NADP(H) concentrations were determined according to the method of Shi F et al [36], Crude cell extracts were prepared from cells grown in the fermentation medium of *Corynebacterium crenatum* and frozen in liquid nitrogen. Cells were washed with cold 100 mM PBS (pH 7.5) and pellet ~10^5 cells for each sample were maintained. $NADP^+$ and NADPH were extracted using HCl and NaOH, respectively [37]. The amounts of $NADP^+$ and NADPH in each extract were quantified using a spectrophotometric enzymatic cycling method with an $NADP^+$-specific glucose-6-phosphate dehydrogenase (G6PDH) and an NAD^+-specific yeast alcohol dehydrogenase (ADH). The reactions were carried out at 25°C and were initiated by the addition of the cell extract. The absorbance at 570 nm was followed for 5 min to determine the $NADP^+$ and NADPH concentrations. The concentrations were calculated using external $NADP^+$ and NADPH standards and with the intracellular volume of 1.6 μ L/ mg dry weight.

RESULTS

Effects of homologues NAD kinase overexpression on Cell growth and glucose consumption

NAD kinase had slight negative effect on cell growth during the overall 108 h fermentation period (Figs 1A and 2) In case of LOS condition the control and recombinant strain produced 21 and 17 g/L, respectively (Fig 2A) which is significantly lower when compared with HOS where the control and recombinant strain produced 32 and 27 g/L, respectively (Fig 1A). After 36 h of fermentation 40 % glucose was consumed by recombinant stain which is lower than control strain where 50 % glucose was consumed.

Enzymatic activity of NAD^+ kinase and intracellular NADP (H) concentration

Enzymatic assay illustrates that NAD^+ kinase activities in the recombinant strain harboring homologous NAD kinase gene increased in the both HOS and LOS conditions by 116 and 49 %, respectively, when compared to the control strain. (Fig 3). At HOS,

concentrations increased 7.3 and 36.8 % in $NADP^+$ and NADPH respectively, whereas, at LOS, concentration were increased 14.7 and 15.0 % in $NADP^+$ and NADPH, respectively (Table 1).

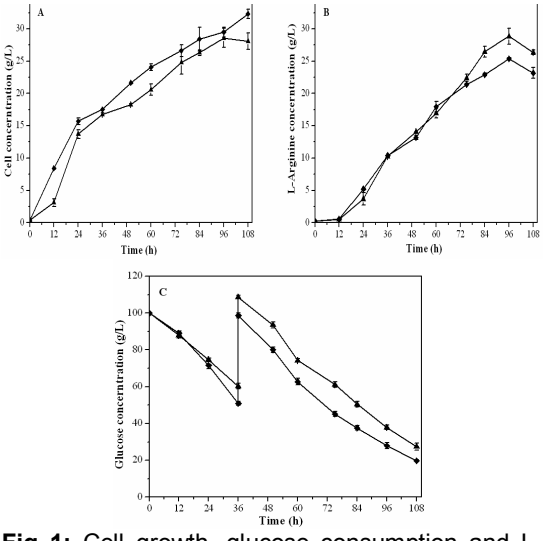

Fig 1: Cell growth, glucose consumption and L-arginine concentration from fermentation medium of *C. crenatum* under HOS condition. (A) Cell concerntration; (B) L-Arginine concerntration; (C) glucose concerntration; (▲) Recombinant strain; (♦) control strain

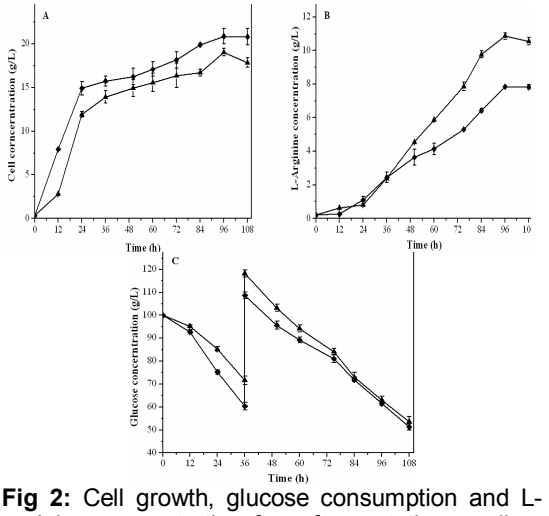

Fig 2: Cell growth, glucose consumption and L-arginine concentration from fermentation medium of *C. crenatum* under LOS condition. (A) Cell concerntration; (B) L-arginine concerntration; (C) glucose concerntration. (▲) Recombinant strain; (♦) Control strain

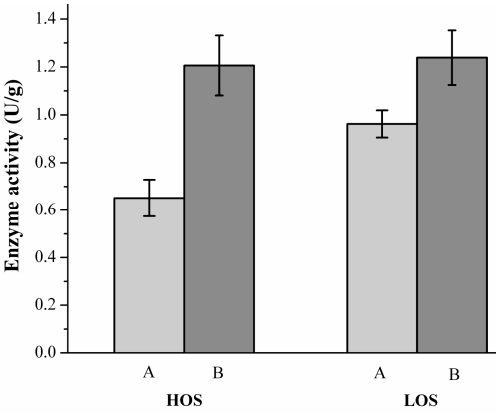

Fig 3: Comparison of NAD^+ kinase activities under HOS and LOS conditions. (A) Control strain. (B) Recombinant strain.

Table 1: Intracellular concentration (μM) of $NADP^+$ and NADPH in *C. crenatum*

	$NADP^+$		NADPH	
	HOS	***LOS***	***HOS***	***LOS***
SYPA 5-5	739±17	545±13	19±2	20±1
SYPA5-5 / NADK	793±18	627±28	26±2	21±1

Improved L-arginine biosynthesis through NAD kinase overexpression

The *C. crenatum* recombinants and control strains were fermented to produce L-arginine and extracellular amino acid concentrations were detected from the fermentation medium, according to the procedure described in material and methods section. For HOS condition, till 72 h of fermentation, L-arginine biosynthesis of recombinant strain was almost same with control strain. However, during 72 to 108 h the L-arginine biosynthesis was increased for recombinant strain (Fig 1 B). In contrast, in LOS condition, after 36 h of fermentation, the L-arginine biosynthesis of recombinant strain was increased significantly compared to control strain (Fig 2 B). The results demonstrated that presence of recombinant plasmid pJC1-tac-ppnK in C*. crenatum* SYPA5-5 could enhance L-arginine production, despite the fact that the NAD kinase gene had a negative effect on cell

growth in presence of HOS. Recombinant strain synthesis 26.47 g/L L-arginine compared to control strain 24.29 g/L. Besides L-arginine, other byproducts amino acids L-lysine and L-isoleucine yield were also improved. It has been reported that NADPH plays a significant role for L-lysine and L-isoleucine biosynthesis [13,36]. However, for LOS, recombinant stain could yield 11.36 g/L compared to control strain 7.58 g/L, which is severely lowered than HOS condition (Table 2).

NAD kinase overexpression enhanced the intracellular concentration of $NADP^+$ and altered the co-enzymes in the cell and this might cause the negative cell growth for recombinant strain. Glucose was used as a sole carbon source for the fermentation. As the glucose was introduced into two steps, initially at starting of the fermentation and after 36 h of the fermentation, an increased peak at 36 h is observed in the graph of glucose consumption. Both cases of HOS and LOS showed the lower consumption rate of glucose for recombinant stain compared with control. However, during the 72 to 108 h of fermentation, the glucose consumption rate for recombinant and control was almost the same for LOS conditions. In all, the glucose consumption rate in the recombinant stains was retarded during HOS and LOS fermentation that could be due to the decreased cell mass formation.

$NADP^+$ and NADPH concentrations increased in recombinant strain harboring NAD kinase gene. It has been reported that NADK regulation can easily allow an organism to switch between a reductive state and an oxidative state [5]. NADPH quantification may vary due to the variations of state of cells [34]. NADPH is required in many pathways including fighting against stressed conditions [20]. It has been reported that the respiratory chain in *C. glutamicum* could also be a potential route for NADPH consumption [38].

L-arginine and other by products amino acids (L-lysine and L-isoleucine) production was increased in presence of HOS. In contrary, L-arginine production was perturbed severely in presence of LOS.The interactions between the metabolic reactions and by product formation depend on dissolved oxygen (DO) levels [11]. The low yield of L-arginine in oxygen limiting condition could be due to the suppression of the genes involved in L-arginine production. Interestingly, homologous NAD kinase expression could increase amino acid yield in oxygen limiting condition. It seems likely that enhanced $NADP^+$ and NADPH concentration could play a significant role in L-arginine production.

CONCLUSION

Direct pathway engineering and mutageneses are common practices for amino acid biosynthesis. However, recently several researchers have reported improved biosynthesis of amino acids and metabolites by co-factor engineering [13,36]. Until now, metabolic networks that produce and

Table 2: Production of L-arginine and other amino acids by *C. crenatum* under different conditions of oxygen supply. Samples were taken from fermentation medium at 96 h.

Concentration (g/L)	HOS		LOS	
	SYPA 5-5	***SYPA 5-5/NADK***	***SYPA 5-5***	***SYPA 5-5/NADK***
Arg	24.29±0.40	26.47±0.37	7.58±0.790	11.36±0.14
Ile	1.38±0.00	1.55±0.04	0.89±0.13	1.50±0.020
Lys	1.65±0.01	1.82±0.05	1.44±0.12	2.60±0.06

maintain the balance of NAD^+, NADH, $NADP^+$, NADPH is still not clear in *Corynebacterium sp*. Several factors including oxygen supply, NADPH availability influence the L-arginine production. Despite the fact that altering the balance of co-enzymes may affect the central metabolic pathways [36], in this paper, we have demonstrated that overexpression of homologous ppnK gene in *Corynebacterium crenatum* SYPA5-5 has increased the NAD^+ kinase activity, intracellular $NADP^+$ and NADPH concentrations along with increased L-arginine biosynthesis in both conditions of high oxygen supply and low oxygen supply.

ACKNOWLEDGMENT

This work was supported by Programs for New Century Excellent Talents in University (no. NCET-07-0380, NCET-10-0459), National Basic Research Program (973 Program) (no. 2007CB707804), and the National High-Tech Programs of China (no. 2006AA020104, 2006AA020301, 2007AA02Z 207).

Contribution note

ZQQ contributed to the work as much as the first-named author and has the status of co-first author

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