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A New Exploration of Extracting and Purifying the Coenzyme F_{420} from Natural Sludge

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Abstract: Coenzyme F_{420} is one kind of special flavin cofactors which exist in some Archaea and bacteria, it is a low potential electron transfer carrier in methanogens. In this study, we designed three specific primers of F_{420}-dependent glucose-6-phos-phate dehydrogenase gene to determine which environment contains the more abundant coenzyme F_{420}, and methods for separating, extracting and purifying the coenzyme F_{420} from this environmental samples were carried out, the pure coenzyme F_{420} was obtained by some optimizing methods from the natural sludge , and the purity and concentration of coenzyme F_{420} we got is just as good as the FMN standard sample which confirmed by their fluorescence emission spectrum. These optimizing methods would save more energy and time to get the good quality coenzyme F_{420}.

Keywords : Coenzyme F_{420}; Separation and Purification; Fluorescence Spectra

Introduction

5-deazaflavin coenyme (F_{420}) was first discovered in methanogenic archaea 1972 by Chessman [1], and it was deemed only exist in the methanogenic bacteria and as a low potential electron carrier, emitting fluorescence in 420nm and 480nm[2]. Recently, however, coenzyme F_{420} has been found in some *Methanogenbacterium* sp. and Actinomycetes[3], and mostly *Streptomyces* sp. with ability to produce a wide array of bioactive secondary metabolites. The metabolites include many medically-important compounds with antibacterial, antifungal, antiviral, anti-parasitic, anticancer and immunosuppressive activities [4].

Researchs showed coenzyme F_{420} plays an irreplaceable role in the synthesis of new active substances. Essentially, coenzyme F_{420} acts as a 5-deazaflavin analog that mediates exclusively two electron transfer reactions, and has an acidic 8-hydroxy substituent that can modulate reactivity in response to its protonation state[1]. F_{420} is a flavin-like redox-active coenzyme commonly used by archaea and some eubacteria in a variety of biochemical reactions in methanogenesis, the formation of secondary metabolites, the degradation of nitroaromatic compounds, activation of nitroimidazofurans, and F_{420}-dependent photolysis in DNA repair[5].

Coenzyme F_{420} has structural features common to flavin cofactors, but more similar to nicotinamide adenine dinucleotide phosphate when participate in reactions [6]. More and more studies focused on the mechanism of the catalytic reaction, the conformation of this enzyme, the selection of special conformation protein and substrate for coenzyme F_{420}. Based on the

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phylogenetic analysis of large numbers of F_{420} genes, it was found that many of the F_{420} biosynthetic proteins in actinomycetes were F_{420} dependent enzymes[7]. Overall, F_{420} may confer an advantage to mycobacteria in anaerobic environments because it has a lower redox potential than NADP [8]. In nature most of enzymes exploit small molecules such as metals and cofactors, to enhance their catalytic properties,and these molecules can aid catalysis and expand the range of available enzymatic functions[9]. The auxiliary factor of these important and widely used, including nicotinamide adenine dinucleotide coenzyme A, pyridoxal phosphate, flavin adenine nucleotide, biotin, 5 '- deoxyadenosyl cobalamin, thiamine pyrophosphate, folic acid and coenzyme F_{420}[10]. Coenzyme F_{420} and coenzyme FMN are similar in structure(Fig 1), and they have the same effect in the reaction are used as carriers for electron transfer[11].

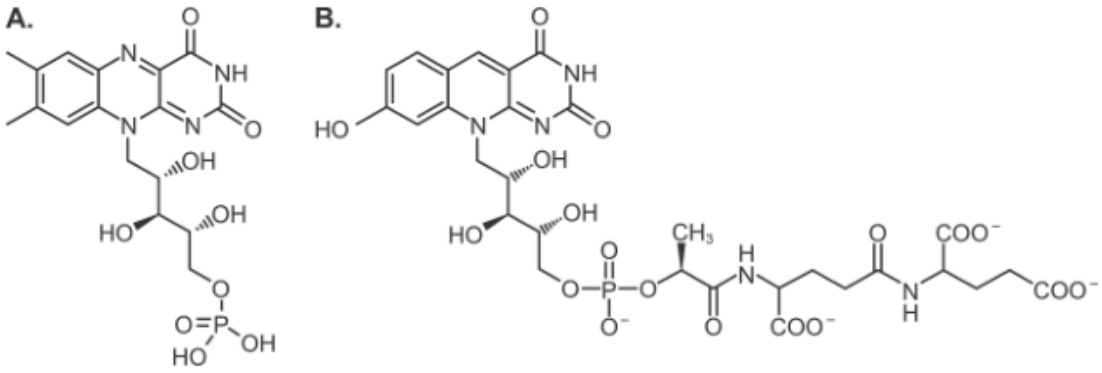

Fig.1. Flavonoid cofactor structures. (A) FMN. (B) Coenzyme F_{420} . F_{420} is a deazaflavin while FMN is riboflavin based. All cofactors contain a ribityl moiety, which is followed by a phosphate in FMN, as well as a phospholactate polyglutamate tail in F_{420}.

Coenzymes F_{420} can have two to seven glutamate residues ligated to the coenzyme ribitol side chain through a phospholactate. The reduction of F_{420} at the C_5 atom (labelled) gives F_{420} –H_2 (Fig.2)[12].

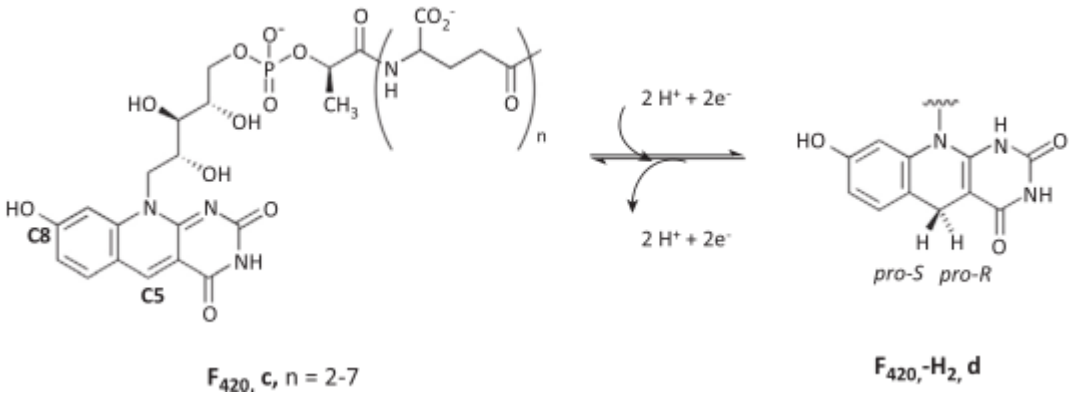

Fig.2. the 8-hydroxy deazaflavin coenzyme F_{420} in both (c) oxidized and (d) reduced forms.

At present, the direction of researches on coenzyme F_{420} dependent enzymes is becoming increasingly diverse, and it has been studied in different application fields. For example in the field of Medicine. Each year more than 10 million people are affected by TB and nearly 1/3 of the patients to death by this disease. There are many resistance and side effects of drugs are now being used, and the study on this issue of coenzyme F_{420} dependent enzymes in the process, drugs aimed at F_{420}-related targets would act by mechanisms

completely different from those of the more widely used drugs[13]. On the other hand, the application of F_{420} in the field of chemical electrodes has also made great progress, and the advantages of coenzyme F_{420} as an electron transfer vector are not negligible. Using the elementary step thermodynamic parameter of coenzyme F_{420} mode releasing hydride ions in acetonitrile. The information disclosed in this work can not only fill the blank of chemical thermodynamics of coenzyme F_{420} models as a kind of important organic source electron, hydride ions, hydrogen atoms and protons, but also strongly promote the fast development of the chemistry and applications of coenzyme F_{420} [6]. However, the most popular research field of coenzyme F_{420} is the special role which plays in the synthesis of new compounds. Some steps in tetracycline[14] and lincomycin [15] biosynthesis by *Streptomyces* species require coenzyme F_{420} . Recently studies of prokaryotic organisms have revealed important insights into the remarkable chemistry and mechanisms of biosynthesis of cofactors such as coenzyme F_{420}[16]. Several assays have been used to quantify coenzyme F_{420}[17] [18]. It was first discovered in methanogenic archaea and it is involved in several reactions in methane biosynthesis[19]. At least five methanogenic enzymes are F_{420} dependent: F_{420}-dependent methylene-tetrahydromethanopterin dehydrogenase[20].

By using a fluorimetric assay originally developed by Delafontaine et al., a positive correlation has been found for a number of digestion systems between the coenzyme F_{420} content and the specific methanogenic activity [21][22]. Recently, methanogenic cofactor assays based on reversedphase high-performance liquid chromatography (HPLC) were introduced by van Beelen et al[23]. It has been suggested that the reduced F_{420} ($F_{420}H_2$) produced by the action of the F_{420} -dependent glucose-6-phos-phate dehydrogenase(FGD)[24]. Therefore, we can detect the FGD gene to identify whether the bacteria can produce coenzyme F_{420}. Here, we tried to extract coenzyme F_{420} from the mud samples with rich methane content. Before the experiment, we make the verification of the existence of FGD gene in the bacteria, and then carried out the extraction experiment.

The coenzyme F_{420} is extracted from some anaerobic bacteria usually, but the method and condition for anaerobic bacteria cultivation is very complex and strict. So it is difficult to culture these bacteria in the general laboratory. In this paper, an idea of separation, extraction and purification of coenzyme F_{420} from anaerobic sludge was proposed, obtained the pure content successfully through series optimized experiments which make it easier to get coenzyme F_{420} in the general laboratory.

MATERIAL AND METHOD

1. PCR verifies the presence of F_{420} -dependent glucose-6-phosphate dehydrogenase(FGD)

Research shows hat although the Fo precursor to F_{420} originated in methanogens, F_{420} itself was first synthesized in an ancestral actinobacterium.F_{420} biosynthesis genes were then disseminated horizontally to archaea and other bacteria. And suggest that the cofactor is more significant in aerobic bacterial metabolism and soil ecosystem composition than previously thought[25] . A homolog of FGD which has a very high similarity to the methanogenic archaea . Multiple alignment of FGD with the hypothetical proteins also revealed portions of the same conserved sequences.

Three pairs of primers were designed based on the F_{420} -dependent glucose-6-phosphate dehydrogenase(FGD) gene in this study[13]. The corresponding pairs of primers as listed in Table.1. We selected samples from methane digesters, ponds and rivers, and extracted the genomic DNA using the PowerSoil DNA kit of Sigma , and then amplified the gene by PCR with different primers.

Table 1:List of primers used in this study

Primer name	Forward primers (5′–3′)	Reverse primer (5′–3′)
FGD-1011	GTGGCTGAACTGAAGCTAGG	GGTTGCGGCGACTTGGCTGA
FGD-301	CCGTCAGCGACCATTTTC	TGAATCGGTGGGGCTAAT
FGD-392	CTTCGGTGCTGACCCCCA	GGCAAGGGCGAGGAGCTC
FGD-546	TGAATCGGTGGGGCTAAT	ATTTCACGCACCAGGACA

2. Coenzyme extraction

Samples will be taken from the biogas production, ponds and rivers, named F420-1 F420-2, F420-3 respectively. Took 10 grams of three samples each, washed with distilled water for a period of time, centrifuged at 500 rpm/min to separate the supernatant fluid. Then soaked in saline for half an hour, centrifuged supernatant fluid, washed three times with pure water. The mud samples were frozen for 30 minutes at -80℃, added pure water to 30 ml, stirred and boiled in 95℃ water bath for 30 minutes, then centrifugal separated at the speed of 13600 rpm/min. The supernatant fluid was added into 2 times the volume of isopropyl alcohol, then mixed and precipitated for 2h, then supernatant fluid was centrifuged at 13600 rpm/min, and the absorbance value of coenzyme F_{420} was determined by fluorescence spectrophotometer. The fluorescence intensity was measured by fluorescence spectrophotometer at the excitation wavelength of 420 nm. Prepared 200 μM FMN standard solution respectively, diluted 10 times, detected absorption spectra at the the fluorescence emission spectra.

Results and analysis

1.PCR results

By continuously improving the PCR condition, and finally using the PrimeSTAR GXL DNA Polymerase under the condition of adding GC-buffer, we obtained the corresponding size of the gene segments from the samples which collected from the biogas digester and pond(Fig.3). Therefore, we believe that these places where the bacteria can produce coenzyme F_{420}.

According the result, we found the FGD genes of samples from biogas pools and ponds are much easier amplified, but there is hardly no amplified bands in the river samples, which indicates that there is more coenzyme F_{420} in the digester and the pond then the river, so we can get the coenzyme F_{420} much easier from the mud of digester and pond. Moreover, the amplification efficiency of primers FGD-1011 and FGD-546 was high relatively, while the other two pairs of primers did not amplify the corresponding size of the target band.

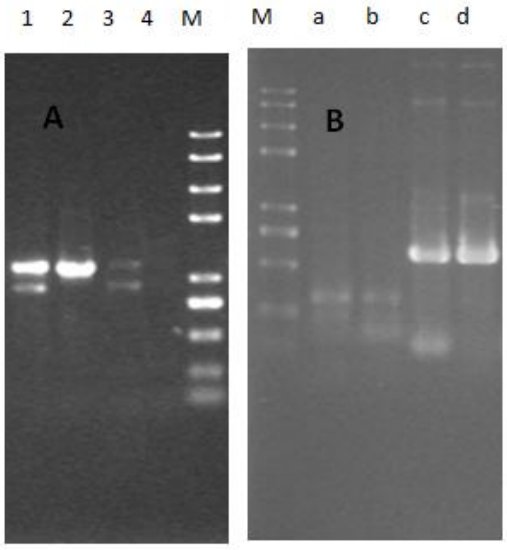

Fig.3. PCR results

M：Trans 2K Plus II DNA Marker（From top to bottom：8000bp、5000bp、3000bp、2000bp、1000bp、750bp、500bp、250bp、100bp）； A: primer FGD-1011 PCR amplification product; B :primer FGD-546 amplified product of PCR 1,d:F_{420}-1; 2,c:F_{420}-2; 3,b:F_{420}-3; 4,a:Blank control

2. Fluorescence emission spectra and UV-Visible spectra of coenzyme F_{420}

The UV-visible spectra of the samples were obtained by using a Molecular Devices SpectraMax M2

Multi-mode Microplate reader and an Agilent Cary 60 UV-vis spectrophotometer[26]. Coenzyme F_{420} is photolabile aerobically in neutral and basic solutions, whereas the acid stable chromophore is not photolabile under these conditions[27]. Under the condition of pH value of 7, coenzyme F_{420} has the largest absorption value at 420 nm. HITACHI-F4500 and North Leary-UV1801 were used to measure the fluorescence emission spectra and UV-Vis absorption spectra.

Figure A shows the spectra of crude extract product with isopropyl alcohol, we can find that there are many twists and turns in the fluorescence spectra through the emission spectra of excitation light at 420nm and 480nm, fluorescence emission spectrum displays purity of the sample is not high, which needs further purification. Figure B shows that fluorescence emission spectra of the crude extract product through distillation for 2 times, the purity has been greatly improved, and the fluorescence emission spectra of samples which collected from methane digesters was significantly higher than ponds and rivers samples. In the Figure C, compare fluorescent emission spectra of 0.1μm FMN standard sample with the diluted sample 1's fluorescent emission spectra which after purified, we can find that the quality of purified coenzyme F_{420} is good as standard sample. Figure D is a UV-Vis absorption spectrum of coenzyme F_{420} extracted from sample 1.

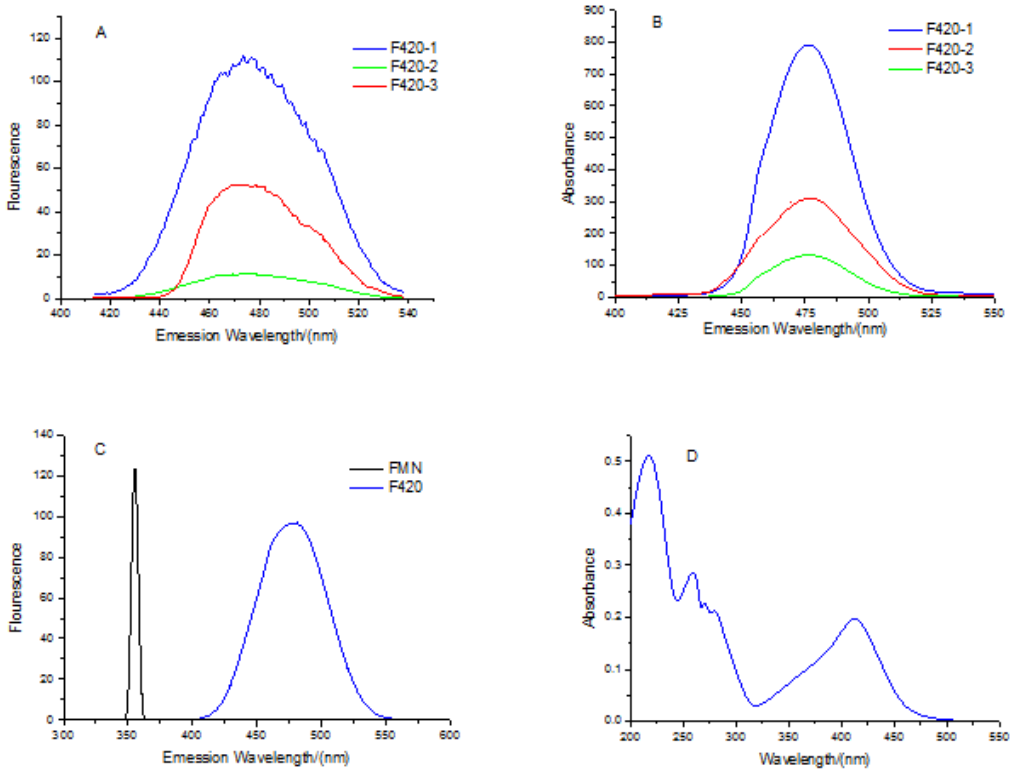

Fig.3. Fluorescence emission spectra and UV-Visible spectra

A:Fluorescence emission spectra of crude extracts; B:Fluorescence emission spectra of samples purified by distillation; C:Comparison of fluorescence emission spectra of FMN and F_{420}; D:UV-Visiable spectra of F_{420}

Conclusion and Discussion

In this study, coenzyme F_{420} was extracted and purified from the natural sludge of biogas digesters, ponds and rivers. It found that the coenzyme F_{420} in biogas digesters are more abundant than ponds and rivers by PCR testing for F_{420}-dependent glucose-6-phosphate dehydrogenase(FGD) gene.

The results of the experiments show that the coenzyme

F_{420} which extracted from the sludge samples has good purity which confirmed by the fluorescence emission spectrum and UV-Vis absorption spectrum. What's more, the quality of purified coenzyme F_{420} is much better then we expect, and it could be used to make other further research. Therefore, it is feasible to extract and purify coenzyme F_{420} from sludge of biogas digesters in the future which could avoid spending much energy, money and time to cultivate the methanogenic bacteria in the laboratory.

In the future, more research will be made on the function of coenzyme F_{420} which extracted from the sludge of biogas digesters, aim at verifying whether the coenzyme F_{420} extracted from natural is just as good as the product extracted from anaerobic bacteria which cultivated in the lab.

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