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Coenzyme precursor-assisted cooperative overexpression of an active pyridoxine 4-oxidase from *Microbacterium luteolum*

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Abstract

The overexpression system of the active pyridoxine 4-oxidase from *Microbacterium luteolum* was developed. When chaperonin GroEL/ES genes in plasmid pKY206 were coexpressed, the pyridoxine 4-oxidase gene cloned in the vector pTrc99A was overexpressed in *Escherichia coli* JM109 cultured in LB medium containing 50 μM riboflavin, the precursor of coenzyme (FAD) of the enzyme, under the cold stress at 23 °C. The crude extract from the cotransformant cells showed 88-fold higher specific activity than that from *M. luteolum*. The chaperonins, cold stress, and the riboflavin cooperatively served to increase the soluble form of the enzyme. A significant correlation between the specific activity and percentage of the soluble form in the total expressed enzyme was found. The overexpressed pyridoxine 4-oxidase was easily purified to homogeneity with two steps of the conventional column chromatography.
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Keywords: Pyridoxine 4-oxidase; Overexpression; Chaeronin; Cold shock; *Microbacterium luteolum*; Riboflavin; FAD; IPTG

Pyridoxine 4-oxidase (EC 1.1.3.12) is a FAD[1]-dependent enzyme and catalyzes the oxidation of pyridoxine to pyridoxal. This reaction is the first step of the pyridoxine degradation pathway-I found in *Pseudomonas* MA-1 [1] and *Microbacterium luteolum* [2], both of which grow in the synthetic medium containing pyridoxine. We have purified the enzyme to homogeneity from *M. luteolum*, characterized its enzymatic properties, cloned the gene encoding the enzyme, and determined the nucleotide sequence of the gene [2]. Pyridoxine 4-oxidase from *M. luteolum* shows the sequence similarity with GMC oxidoreductase family proteins. The enzyme has the shortest polypeptide chain in the protein family and contains only one (GSLESPALLMRSGIG) of the two significant peptide sequences at 254–268. Thus, the enzyme may have a novel conformation to be elucidated among GMC oxidoreductases.

Recently, we have developed a highly sensitive assay for pyridoxal [3] that is intended to mainly analyze its contents in foods and vegetables containing a very low amount of pyridoxal or the other free forms of vitamin B_6. The method can be adapted for an assay of pyridoxine if pyridoxine 4-oxidase is coupled to oxidize pyridoxine to pyridoxal. Pyridoxine 4-oxidase from *M. luteolum* shows very high substrate specificity [2] and is fit to be used for the purpose if the enzyme is available in a large amount. Although the expression system of the enzyme has been established [2], the specific activity of the crude extract from the recombinant *Escherichia coli* cells is only 5-fold higher than that from *M. luteolum*, and it is not easy to purify a sufficient amount of the enzyme from the recombinant *Escherichia coli* cells.

Here, we have established a high expression system of pyridoxine 4-oxidase from *M. luteolum* and its purification method. The high expression system utilized the cooperative expression of the enzyme and the chaperonins GroEL/ES under the cold stress and the assistance

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[1] *Abbreviations used:* GMC oxidoreductase, glucose-methanol-choline oxidoreductase; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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of the precursor of the coenzyme, riboflavin. The crude extract from the transformed *E. coli* cells under the present system showed 88-fold higher specific activity than that from *M. luteolum* cells, and the enzyme was efficiently purified and could be used for the crystallization and the pyridoxine assay.

Materials and methods

Bacteria, plasmids, and cultivation

Escherichia coli JM109 and BL21(DE3) were purchased from TaKaRa (Shiga, Japan) and Novagen (Madison, WI), respectively. The plasmids, p3T, pET21a, and pTrc99A, were obtained from MoBiTec (Goettingen, Germany), Novagen, and Amersham Biosciences (Piscataway, NJ), respectively. The plasmid pKY206 was prepared according to the method by Mizobata et al. [4]. The plasmid p3T was used for cloning of the pyridoxine 4-oxidase gene. The plasmids, pET21a and pTrc99A, were used for expression of the enzyme. The plasmid pKY206 derived from pACYC184 can be harbored in the host bacterium together with pETPNO1 or pTPNO3 which was prepared in this study as described below. *E. coli* cells were cultured in the LB medium containing an adequate antibiotic or combination of antibiotics at various temperatures. *M. luteolum* (ATCC 16738) was aerobically cultured in the synthetic pyridoxine medium as described previously [5].

Construction of plasmids carrying the pyridoxine 4-oxidase gene

The chromosomal DNA of *M. luteolum* used as a template was prepared by the method of Saito and Miura [6]. The pyridoxine 4-oxidase gene was amplified by PCR using the two oligonucleotides: 5′-GCG**CAT ATG**GCTCAGTATGATGTGGCG-3′ (primer PNO-F, sense) and 5′-GCG**GGATCC**TAATTTGAATCCTGTT GTG 3′ (primer PNO-R, antisense) to introduce *Nde*I (boldfaced in primer PNO-F) and *Bam*HI (boldfaced in primer PNO-R) sites, respectively. The reaction mixture (50 μl) for PCR consisted of Ex Taq buffer, 20 nmol dNTP, 2.5 U Ex Taq polymerase (TaKaRa), 0.5 μg of the template DNA, and 50 pmol of each primer. The PCR condition was: heating to 96 °C for 5 min; 30 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 3 min, followed by a final extension for 10 min.

The PCR product was ligated into the p3T vector for TA cloning, to construct p3PNO. After no mutations were verified by DNA sequencing of cloned pyridoxine 4-oxidase gene with ABI PRISM 3100 genetic sequencer (Applied Biosystems, Foester City, CA), the p3PNO was digested with *Nde*I and *Bam*HI and re-cloned into *Nde*I/*Bam*HI sites of pET21a. The constructed plasmid was designated as pETPNO1. The pETPNO1 was digested with *Xba*I and *Hin*dIII and then ligated into *Xba*I/*Hin*dIII sites of pTrc99A, to construct pTPNO3.

Expression of pyridoxine 4-oxidase and preparation of crude extracts

The *E. coli* BL21(DE3)/pETPNO1 and *E. coli* JM109/pTPNO3 were cotransformed with the plasmid pKY206 carrying GroEL/ES genes. The cotransformants were grown in LB broth containing ampicillin (50 μg/ml), tetracycline (25 μg/ml), and different concentrations of riboflavin at different temperatures. The expression of the proteins was induced by the addition of different concentrations of IPTG to the culture, when their optical density at 600 nm reached 0.6–0.8 under cultivation on a rotary shaker set at 100 rpm. The cells were harvested by centrifugation, washed with 50 mM potassium phosphate buffer, pH 8.0, and stored at −20 °C.

The stored cells were thawed and resuspended in buffer A (20 mM potassium phosphate buffer, pH 8.0, containing 5 μM FAD, 1% of 2-mercaptoethanol, and 10% glycerol) supplemented with 1 mM EDTA and 1 mM PMSF. The cell suspension was thoroughly sonicated at 4–15 °C with a Heat systems-Ultrasonics sonicator W-220 (Farmingdale, NY). Cell debris and insoluble proteins were precipitated by centrifugation at 10,000*g* for 10 min at 4 °C and the supernatant was used as the soluble fraction. The precipitate was mixed with buffer A containing 1 mM EDTA and 6 M urea and kept on ice for 2 h. Insoluble materials were removed by centrifugation and the supernatant containing the dissolved inclusion proteins was used as the dissolved fraction. The proteins in both soluble and dissolved fractions were assayed for pyridoxine 4-oxidase activity as described below and then analyzed by SDS–PAGE [7]. The densities of protein bands on the strained gels were analyzed with the software, NIH image.

Enzyme and protein assay

Pyridoxine 4-oxidase activity was assayed as described previously (2) or by a simpler method as follows. The reaction mixture (1 ml) consisted of 0.1 M Tris–HCl, pH 8.0, 5 μM FAD, and 5 mM pyridoxine, and the enzyme was incubated aerobically at 30 °C for 10 min. The yellow color development produced by the Schiff base between Tris base and pyridoxal was measured by the absorbance at 415 nm. One unit of enzyme was defined as the amount of protein that catalyzes the formation of 1 μmol of pyridoxal per min.

Protein concentration was measured by the dye-binding method [8] with bovine serum albumin as a standard.

Purification of pyridoxine 4-oxidase

Pyridoxine 4-oxidase was purified by three steps as follows. The harvested cells (2.8 g) were suspended in 15 ml buffer B (buffer A with 0.01% Tween 20) supplemented with 1 mM EDTA and 1 mM PMSF. The suspension was sonicated on ice for 6 min with the Heat systems-Ultrasonics sonicator W-220. The supernatant (15 ml) obtained by centrifugation at 10,000*g* for 10 min at 4 °C was used as the crude extract. The crude extract was fractionated with ammonium sulfate, and the precipitate of 35–75% saturation collected by centrifugation at 10,000*g* for 30 min was dissolved in 3 ml buffer B. The solution was thoroughly dialyzed against buffer B and applied to a QA 52 column (1.8 × 10 cm, Whatman, Clifton, NJ) equilibrated with buffer B. The column was washed with buffer B until the absorption of the eluate at 280 nm was below 0.1. Bound proteins were eluted with a linear gradient (0–0.3 M NaCl) in buffer B and pyridoxine 4-oxidase activity was detected in 0.1 M NaCl fractions. The eluted enzyme solution (32 ml) was concentrated to 2.2 ml with ammonium sulfate precipitation (75% saturation at a final concentration) and thoroughly dialyzed against buffer C (5 mM potassium phosphate buffer, pH 8.0, containing 5 μM FAD, 1% of 2-mercaptoethanol, 10% glycerol, and 0.01% Tween 20). After centrifugation to remove precipitates, the supernatant (2.7 ml) was applied to a hydroxyapatite column (1.4 × 4.8 cm, Wako Pure Chemicals, Osaka, Japan) equilibrated with buffer C. The column was washed with buffer C as described above and bound proteins were eluted with a linear gradient of potassium phosphate from 5 to 50 mM in buffer C. Pyridoxine 4-oxidase was eluted with a concentration of 20 mM potassium phosphate.

Results and discussion

Expression and distribution of active and inactive (insoluble) pyridoxine 4-oxidase proteins in the transformants

Two *E. coli* transformants, JM109/pTPNO3 and BL21(DE3)/pETPNO1, expressed active pyridoxine 4-oxidase (Fig. 1). On the whole, JM109/pTPNO3 cells showed the higher specific activity than BL21(DE3)/pETPNO1 cells (Fig. 1, columns 1–6 vs. columns 7–12). The treatment with IPTG caused no increase in the specific activities of the enzyme in the crude extracts from both transformant cells. The T7 promoter [9] on pETPNO1 is basically stronger than the *trc* promoter [10] on pTPNO3, so we expected that the *E. coli* transformants containing pETPNO1 would show the higher pyridoxine 4-oxidase activity than those with pTPNO3. The result was the other way and could be attributable to the formation of inclusion bodies in the extremely high-level expression system found in the pET system [11]. The protein was expressed too high to fold into the proper conformation, resulting in the formation of inactive inclusion bodies. While the inclusion bodies, in this study, were formed in cells containing both pETPNO1 and pTPNO3, the expression level itself was lower in T7-based pETPNO1 than in *trc*-based pTPNO3 (Fig. 2, cf. lanes 1D and 7D). It is well known that when the gene product is toxic to *E. coli* cells, they easily lose the gene on the plasmid or the plasmid itself [12]. Although we have not checked the stability of pETPNO1 in BL21(DE3) during the cultivation, it might be unstable and partly lost, and thus the expression level of pyridoxine 4-oxidase became low.

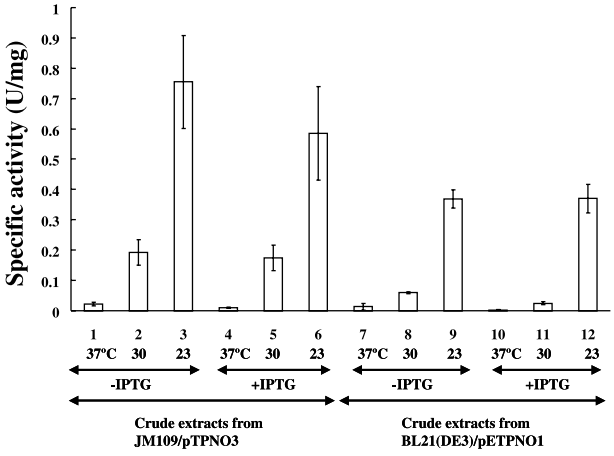

Fig. 1. Effects of temperature and IPTG on the specific activity of pyridoxine 4-oxidase in crude extracts from transformants. The crude extracts (1, 2, and 3) were prepared from JM109/pTPNO3 cells cultivated in the LB medium containing ampicillin at 37, 30, and 23 °C, respectively. The crude extracts (4, 5, and 6) were prepared from the same cells cultivated and subjected to the IPTG (1 mM) induction at 37, 30, and 23 °C, respectively. The crude extracts (7, 8, and 9) were prepared from BL21(DE3)/pETPNO1 cultivated in the same medium at 37, 30, and 23 °C, respectively. The crude extracts (10, 11, and 12) were from the same cells cultivated and subjected to the IPTG induction at 37, 30, and 23 °C, respectively.

The cold stress significantly increased the activity of pyridoxine 4-oxidase in the crude extracts. The crude extract prepared from cells cultivated at 23 °C showed the highest enzyme activities on both transformants. The crude extract from JM109/pTPNO3 cells cultivated at 23 °C showed 34-fold and 8.7-fold higher specific activity than those from the cells cultivated at 37 and 30 °C, respectively (Fig. 1, columns 1–3). The crude extract from BL21(DE3)/pETPNO1 cultivated at 23 °C showed 26-fold and 4.2-fold higher specific activity than those from the cells cultivated at 37 and 30 °C, respectively (Fig. 1, columns 7–9).

Then, the crude extracts, and the dissolved fractions, which contained the insoluble form of pyridoxine 4-oxidase protein solubilized by urea, were subjected to SDS–PAGE. The protein bands with a molecular weight

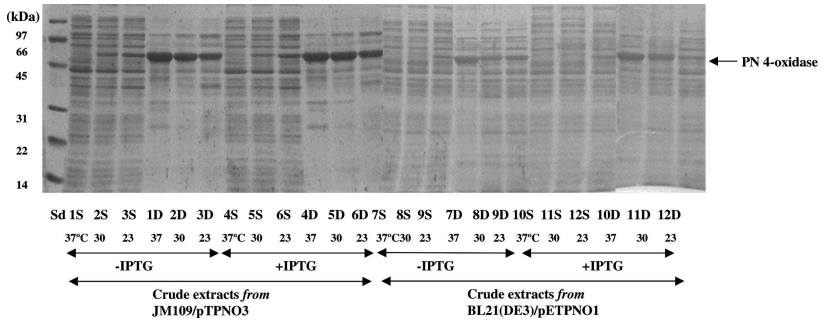

Fig. 2. SDS–PAGE patterns of the crude extracts and the dissolved fractions from the transformants. Twelve crude extracts (the soluble fractions, each 10 μg of proteins) prepared from the transformants described in the legend of Fig. 1 were subjected to SDS–PAGE. They are shown as numbers with letter S. The corresponding dissolved fractions (each 10 μg of proteins) prepared from the transformants as described under Materials and methods were also subjected to SDS–PAGE. They are shown as numbers with letter D. SD shows molecular weight markers: rabbit muscle phosphorylase ($M_r = 97{,}400$), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400).

of 54,000, corresponding to pyridoxine 4-oxidase enzyme, were found in the crude extracts prepared from cells of both JM109/pTPNO3 and BL21(DE3)/pETPNO1 cultivated at 23 °C (Fig. 2, lanes 3D and 9D, respectively). These two crude extracts showed the highest specific activities (Fig. 1, columns 3 and 9, respectively). The comparison of the densities of the protein bands indicated that the increase in the specific activity reflected the increase in the soluble forms and the decrease in the insoluble forms of pyridoxine 4-oxidase (Fig. 2, lanes 1S and 1D vs. lanes 3S and 3D). The correlation between the specific activity and percentage of the soluble form in the total pyridoxine 4-oxidase (the soluble and insoluble forms), which was calculated based on the densities of the protein bands on the SDS–PAGE gel, was examined. There was a significant correlation between them (R^2 was 0.70). These results demonstrated that the amount of soluble form of pyridoxine 4-oxidase protein rather than total expression level should be increased in order to get the high specific activity.

Coexpression of GroEL/ES

Because it has been reported that the coexpression of the *E. coli* chaperonins GroEL/ES increases the soluble protein of D-carbamoylase in a transformant [13], we examined the effect of coexpression of *E. coli* chaperonin proteins on pyridoxine 4-oxidase activity. The specific activity of pyridoxine 4-oxidase in JM109/pTPNO3/pKY206 cells, which were cultivated with IPTG at 23 °C, was 3.0 ± 0.81 U/mg, the 3.9-fold higher value than that in JM109/pTPNO3 cells. Densitometric analysis revealed that the crude extract from JM109/pTPNO3/pKY206 cells contained 1.9-fold higher amount of soluble form of pyridoxine 4-oxidase than that from JM109/pTPNO3 cells without the chaperonins, although the insoluble forms of pyridoxine 4-oxidases were still found in the dissolved fraction (Fig. 3, inset). The coexpression of the chaperonins did not increase the soluble form of pyridoxine 4-oxidase when the JM109/pTPNO3/pKY206 cells were cultivated at 37 °C (data not shown). The bacterial chaperonins facilitate the correct folding of many recombinant proteins by preventing the formation of the inclusion bodies [14]. Moreover, several proteins, including D-carbamoylase [13], require the cold stress together with the expression of the chaperonins to increase their soluble forms. The recombinant pyridoxine 4-oxidase protein also needed both the bacterial chaperonins and the cold stress.

Supplement of coenzyme or its precursor

Because pyridoxine 4-oxidase from *M. luteolum* easily releases its coenzyme FAD and then changes to the inactive form [2], FAD or its precursor riboflavin was added to the cultivation medium to examine their effects on the pyridoxine 4-oxidase specific activity in the recombinant cells. As shown in Fig. 3, riboflavin increased the specific activity in a dose-dependent manner, while FAD was not effective. The specific activity reached the maximum at 50 μM of riboflavin, with 2.1-fold increase. Densitometric analysis revealed that the soluble form of pyridoxine 4-oxidase increased 1.5-fold in the presence of riboflavin, while the solubility of GroEL was constant (Fig. 3, inset, lanes 0S and 10S). Riboflavin is transported into the bacterial cells by a carrier-mediated process and concentrated as the phosphorylated coenzymes, FMN and FAD, in *Bacillus subtilis* [15]. So far, the transport of FAD into bacterial cells has not been reported. Thus, the *E. coli* cells could transport only riboflavin and change riboflavin to FAD in the cells. FAD could increase the active form of the enzyme. The difference between the increase in the activity and in percentage of the soluble form suggests that not all of the soluble protein was active.

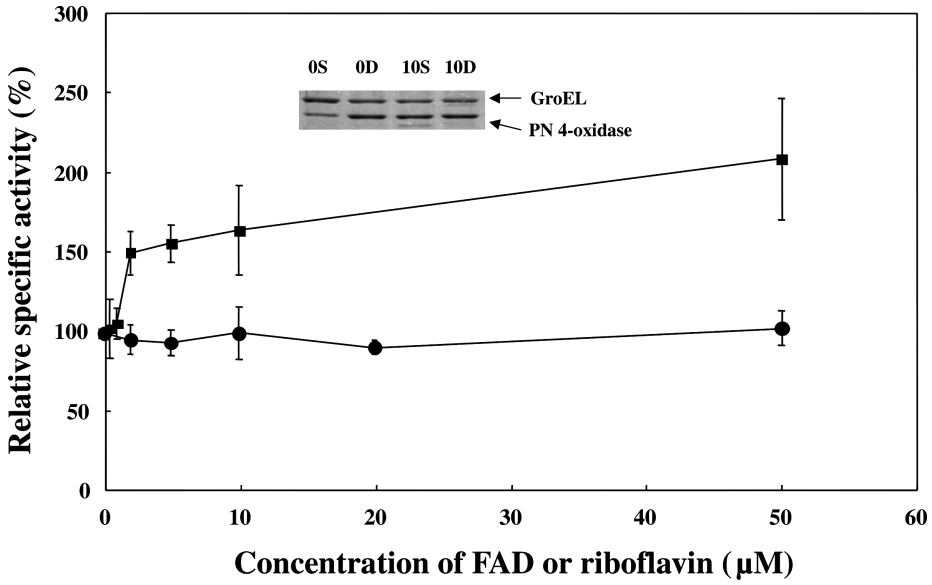

Fig. 3. Effects of FAD and riboflavin on the specific activity of pyridoxine 4-oxidase in the transformant. The crude extracts were prepared from JM109/pTPNO3/pKY206 cells cultivated in LB medium containing IPTG, ampicillin, tetracycline, and various concentrations of FAD (closed circles) or riboflavin (closed squares) at 23 °C. The relative specific activity of pyridoxine 4-oxidase in the crude extract at different concentrations of FAD or riboflavin was calculated by setting the enzyme activity without FAD or riboflavin as 100%. The inset shows the enzyme and GroEL protein bands on the section of SDS–PAGE gel. 0S and 10S, and 0D and 10D show the proteins in the crude extracts and the dissolved fractions from the cells cultured without riboflavin and with 10 μM riboflavin, respectively.

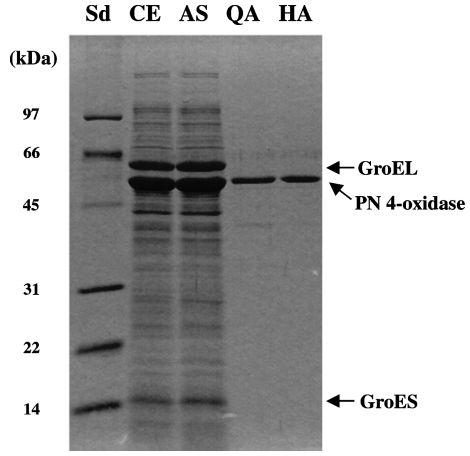

Fig. 4. SDS–PAGE analysis of the purification of pyridoxine 4-oxidase. To lane CE, crude extract (10 μg of protein) was applied; lane AS, ammonium sulfate fraction (10 μg); lane QA, QA52 fraction (2 μg) and; and lane HA, hydroxyapatite fraction (1 μg). Lane SD shows the molecular weight markers.

Purification of recombinant pyridoxine 4-oxidase

The recombinant enzyme was purified to homogeneity by only two conventional column chromatography steps, QA52 cation-exchange and hydroxyapatite column chromatographies (Fig. 4). From 1.0 g (wet weight) of the recombinant cells of JM109/pTPNO3/pKY206, 8.8 mg of purified pyridoxine 4-oxidase was obtained with the yield of 42% (Table 1). The recombinant enzyme showed the same specific activity and kinetic properties as those of the native enzyme (data not shown) and could be crystallized at 4 °C with a commercial crystal screen kit.

In conclusion, here, we showed the high expression system of active pyridoxine 4-oxidase: the active enzyme protein and the bacterial chaperonins GroEL/ES were cooperatively coexpressed under the cold stress at 23 °C with the assistance of the precursor of the coenzyme, riboflavin. To our knowledge, this is the first report of a

Table 1
Purification of pyridoxine 4-oxidase from *E. coli* JM109/pTPNO3/pKY206 cells

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	330	1400	4.1	100	1.0
Ammonium sulfate	270	1300	7.4	92	1.1
QA 52	58	780	14	57	3.3
Hydroxyapatite	25	570	23	42	5.6

Wet cells (2.8 g) was used.

coenzyme precursor-assisted cooperative overexpression system. This type of system may be applicable for other enzymes that require coenzymes or cofactors.

Acknowledgments

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