Biotechnology and Bioprocess Engineering 20: 988-994 (2015)
DOI 10.1007/s12257-015-0434-0

RESEARCH PAPER

Non-enzymatic PLP-dependent Oxidative Deamination of Amino Acids Induces Higher Alcohol Synthesis

Kwon-Young Choi

Received: 3 July 2015 / Revised: 12 August 2015 / Accepted: 5 October 2015

Abstract Pyridoxal phosphate (PLP) is an organic cofactor found in all transaminase enzymes. In this study PLP was used to replace the enzymatic deamination step in the Ehrlich pathway, for the oxidative conversion of amino acids into 2-keto acids. PLP functions in an enzyme-independent manner. It was further used in the synthesis of higher alcohols through a sequential enzymatic reduction *in vitro* and *in vivo*. PLP-dependent oxidation was investigated against five representative amino acids: valine, leucine, isoleucine, norvaline, and phenylalanine. *In vitro* amino acid oxidation resulted in approximately 45 ~ 75% [mole/mole] of each 2-keto acid conversion and *in vitro* ammonia formation was less than 2-keto acid formation, with 20% of conversion yields. Whole cell *E. coli* expressing reduction enzymes KivD/ADH with both single amino acid and amino acid mixture (4% yeast extract) gave the highest yield (30 ~ 55%) in the presence of the PLP-Cu complex and following enzymatic reactions.

Keywords: pyridoxal phosphate, oxidative deamination, amino acids, 2-keto acids, higher alcohols

1. Introduction

In nature, protein molecules have evolved within a given environment and adapted to function optimally over a long period of time.

Kwon-Young Choi*
Department of Environmental Engineering, College of Engineering, Ajou University, Suwon 16499, Korea
Graduate School of Environmental and Safety Engineering, Ajou University, Suwon 16499, Korea
Tel: +82-31-219-1825; Fax: +82-31-219-1825
E-mail: kychoi@ajou.ac.kr

At some point in evolutionary history, proteins began to accommodate organic cofactors, such as metal ions or vitamin derivatives [1,2]. Now, such protein-cofactor complexes are present in all known forms of organisms, playing a core roll of diverse metabolisms. Metabolism requires a vast array of chemical reactions. Most of these reactions are catalyzed by organic-inorganic cofactors [3,4]; a good example is the PLP cofactor, which is an active form of vitamin B_6 [5,6]. It serves as a prosthetic molecule in a variety of enzymes, and these enzymes generally catalyze both substitution (transaminases) and elimination reactions (deaminases) [7,8]. Enzymatic transamination, however, requires an amine acceptor, which is usually a key molecule linked to the cells central carbon-nitrogen metabolism. These heterologous transamination cycles may cause unbalanced nitrogen metabolism in cells [9]. As well as this, such correlations of amine donating/accepting are functionally coupled, so that the reaction system inevitably requires other amine accepting substrates to achieve an effective deamination reaction [10-12]. To avoid the disadvantages related to transaminase dependent reactions, we have demonstrated PLP-dependent deamination of amino acids in an enzyme-independent manner. Previous studies have indicated that the PLP cofactor itself may catalyze the conversion of amino acids to keto acids by transamination, both with and without metal ions present. It has also been observed that amino acid to keto acid conversion can occur via an oxidative deamination process in the presence of dioxygen. For example, Esmond *et al*. reported that the oxidative deamination of pyridoxamine could yield pyridoxal and ammonia, using pyridoxal with particular metal salts, such as $CuSO_4$, $KAl(SO_4)_2$, $FeCl_3$, $CoCl_2$ and $Ni(NO_3)_2$ [13]. Also various α-amino acids have been examined and were found to yield the corresponding 2-keto acids and ammonia, which resulted in the formation of equimolar

formation of 2-keto acids and ammonia. The Shanbhag *et al*. also reported the oxidative deamination of (*p*-sulfophenyl)glycine by Cu^{2+} and vitamin B_6 coenzymes, PLP or 5'-deoxypyridoxal to give (*p*-sulfophenyl)glyoxylic acid [22]. They found that the oxidative deamination reaction occurs at the optimum rate in alkaline solutions, and the reaction rates are negligible at a pH below pH 9.0. They also found that the principle Schiff base complex species involved in the rate-determining step of deamination of (sulfophenyl)glycine is the monohydroxo complex of the Cu-Schiff base chelate. The evolutionary history of cofactor chemistry inspired our hypothesis that the PLP cofactor itself could catalyze deamination reactions without incorporating itself into the polypeptide structure and, as such, bypass an enzymatic transamination reaction.

To assess this, we applied a PLP-dependent deamination reaction to the synthesis of higher alcohols, starting from branched-chain amino acids and phenylalanine. Higher alcohols such as isobutanol, 3-methylbutanol, 2-methylbutanol, butanol, and 2-phenylethanol, can be synthesized through a sequence of enzymatic reactions known as the Ehrlich pathway in cells: deamination of amino acids, such as valine, leucine, isoleucine, norvaline, and phenylalanine, followed by decarboxylation of the resulting 2-keto acids and final reduction of the aldehydes to alcohols [14-17]. Broad substrate-specific dehydrogenases and transaminases were utilized for the deamination reaction, and decarboxylases such as ketoisovalerate decarboxylase (KivD) were utilized for decarboxylation, and finally alcohol dehydrogenases (ADH and YqhD) were utilized for the reduction reaction [17-19]. Here, we demonstrate a conceptually new approach to the synthesis of alcohols using PLP-copper dependent oxidative deamination of model amino acids and sequential enzymatic reduction by 2-keto acid decarboxylase (KivD) and alcohol dehydrogenase (ADH).

2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were of analytical grade or higher. The amino acid substrates used (leucine, isoleucine, valine, norvaline, and phenylalanine) were purchased from Sigma-Aldrich. 2-keto acids used (KIV, KIC, KMV, PP, and KV) were also purchased from Sigma-Aldrich.

2.2. PLP-dependent oxidative deamination of each amino acid

In order to investigate the deamination of amino acids using the PLP-Cu complex, five amino acids (leucine, isoluecine, valine, norvaline, and phenylalanine) were dissolved in distilled water at 10 mg/mL. The PLP-Cu complex was prepared in a 0.1 M phosphate-buffered system (pH 7.0). The reaction was initiated by adding each amino acid substrate (10 mM) into PLP-Cu buffered solution (10 mM, 1.0 mL). The molar ratio of reactants was 1:1:1 (substrate, PLP and Cu(II), respectively) for 24 h at 37°C. After incubation, the reaction was quenched by quick cooling and the generated 2-keto acids and ammonia were prepared for quantitative analysis. The concentration of ammonia was quantified using the NeuLog Ammonium Ion-selective Sensor system.

2.3. Consecutive reactions of amino acid into higher alcohols

The reaction mixture consisted of each amino acid substrate, PLP, and Cu^{2+} at a molar ratio of 1:1:1. The reaction was initiated by adding 10 units of purified ketoisovalerate decarboxylase (KivD, *Lactococcus latis*), 10 units of alcohol dehydrogenase (ADH, *Saccharomyces cerevisiae*) enzymes, and 1 mM of NADH cofactor, into the assay mixture [18].

The assay mixture contained 10 mM of each amino acid

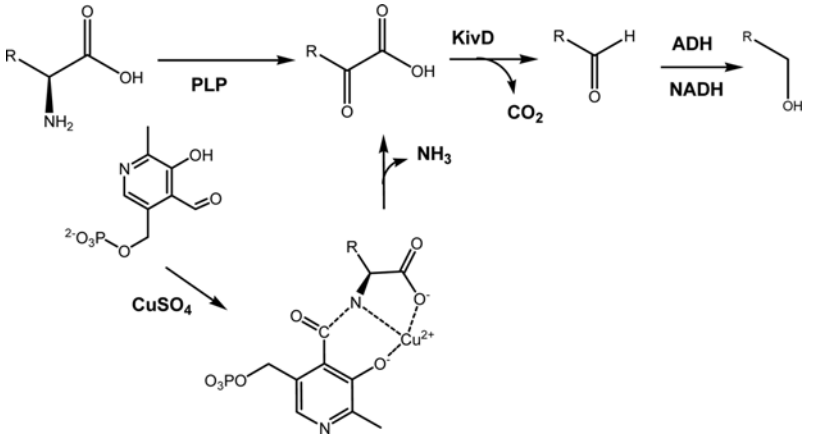


Fig. 1. Reaction schematic of amino acid conversion into higher alcohols, through PLP dependent oxidation and following enzymatic reductions.

substrate, 10 mM PLP-Cu buffered solution, and 1.0 mM NADH in assay buffer (0.1 M phosphate buffer, pH 7.0, 1 mM $MgSO_4$, 0.5 mM thiamine pyrophosphate). The enzymes, KivD and ADH, used here were prepared according to the expression and separation method described previously [14]. After a 1 h incubation, the reaction was quenched by cooling, and the alcohols generated were analyzed quantitatively by gas chromatography.

2.4. Whole cell conversion of amino acids into higher alcohols

Minimal medium for *E. coli* cell culture was prepared as described previously [15]. Each amino acid substrate (up to 10 mg/mL) and PLP-Cu complex (up to 1 mM) was added into M9 minimal media. For amino acid media (4% Yeast extract) was prepared by mixing 40 g/L BD bacto yeast extract (containing 21.64 g/L amino acids, 4.48 g/L ash, 3.05 g/L various salts, 1.24 g/L H_2O as well as 6.53 g/L carbohydrate) with M9 salts (6.3 g/L $NaHP_4$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 24 mg/L $MgSO_4$, 2.2 mg/L $CaCl_2$, and 2.0 mg/L vitamin B_1 per liter water). *E. coli* BL21 (DE3) cells expressing one or both of KivD and ADH were incubated in the M9 minimal media and amino acid media at 37°C until OD 600nm reached 0.6, and were then induced with 1 mM of IPTG. The cells were then incubated at 37°C for 4 days, and generated higher alcohols were collected every 24-h for quantitative analysis by gas chromatography (GC).

2.5. Quantitative analysis of higher alcohols, 2-keto acids, and ammonia

Butanol, isobutanol, 2-methylbutanol, 3-methylbutanol, and 2-phenylethanol were quantified using GC using the method described previously [20]. Amino acids were quantified by HPLC using a ZORBAX Eclipse AAA column and derivatization with OPA and FMOC reagents (all components and protocols from Agilent). For 2-keto acid quantification, fluorescent analysis was used after derivatization with fluorescent quinoxalines by reaction with o-phenylenediamine. The fluorescence emission was measured at 410 nm with excitation at 350 nm [21]. The fluorescence intensity obtained from each reaction sample was fitted to a calibration curve obtained with each authenticated 2-keto acid. NH_4^+ concentrations were measured with a NeuLog Ammonium Ion-Selective Sensor System.

3. Results and Discussion

3.1. Oxidative deamination of amino acids into 2-keto acids using the PLP-Cu complex

Previous studies of PLP-dependent reactions have indicated that vitamin B_6 could catalyze the conversion of amino acids to corresponding 2-keto acids in the presence of metal ions [22]. They showed that the hydrogen acceptor appeared to be the nitro group, which was reduced to an amino group. We first tested the conversion of five representative amino acids into the corresponding 2-keto acids using the PLP-Cu complex in a pH 7.0 phosphate buffered systems. Each 10 mM of amino acid was reacted with a 1:1:1 molar ratio of substrate, PLP and Cu(II), respectively, for 24 h at 37°C. Table 1 shows the susceptibility of alcohol-convertible amino acids to oxidation. Oxidative deamination of all amino acids occurred only when both PLP and Cu^{2+} ions were present simultaneously. Analysis of the final reaction mixture showed the formation of the corresponding 2-keto acids and ammonia under the reaction conditions of molar concentration ratio, pH, temperature, and reaction time.

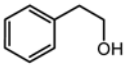
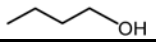
The reaction yielded approximately 45 ~ 75% conversion [mole/mole] of each 2-keto acid and *in-vitro* ammonia formation was less than 2-keto acid formation, with 20% of conversion yields. The selectivity of PLP was constant within the amino acids tested, regardless of branched chain or aromatic structure. These results suggested that PLP-dependent deamination were more effective when compared to over enzymatic deamination reactions. For example, transaminase-mediated deamination reactions require a concurrent cycle of amine-accepting reactions, which largely use 2-ketoglutarate/glutamate [7,23-25]. Accordingly, the applications of those reactions are limited by substrate specificities of the two amine-transferring cycles. Our

Table 1. Relative conversion yield of each amino acid into its corresponding 2-keto acid (2-KA)

Amino acids	2-keto acids	Conversion (%)*	
		2-keto acids	NH_3
Valine	2-ketoisovlerlate (KIV)	45	19
Leucine	2-ketoisocaproate (KIC)	73	22
Isoleucine	2-keto-3-methylvalerate (KMV)	56	20
Phenylalanine	phenylpyruvate (PP)	62	20
Norvaline	2-ketovalerate (KV)	53	21

*Conversion yields were calculated by [mM alcohols/mM amino acids].

Table 2. [PLP+Cu^{2+}]-KivD/ADH-driven advanced biofuel synthesis

Amino acids	Higher alcohols	Conversion (%)[a]	Titer (mg/L)[b]
Valine		< 5	< 25
Leucine		< 10	< 65
Isoleucine		10	85
Phenylalanine		20	170
Norvaline		< 5	< 35

[a]Conversion yields were calculated by [mM alcohols/mM amino acids].
[b]Each used 10 mM of initial substrate concentration.

results do however demonstrate versatile application of the PLP-dependent deamination of amino acids, regardless of the target amino acid.

3.2. *In vitro* higher alcohol preparation from amino acids - deamination by PLP-Cu complex followed by KivD/ADH enzymatic conversion

To verify the proposed scheme of higher alcohol synthesis in Fig. 1, an *in vitro* assay of the complete reaction was first investigated.

Table 2 summarizes the generated alcohol structures and their final conversion yields. The percent conversion from branched amino acids was less than 10%, while percent conversion from aromatic amino acid was 20%. As long as 2-keto acids were formed, the enzymatic reducing steps occurred simultaneously in the presence of reducing equivalents transferred from NADPH.

3.3. Whole cell conversion of amino acids into higher alcohols by extracellular addition of the PLP-Cu complex

The feasibility of PLP-dependent biomimetic higher alcohol synthesis *in vivo* was explored, although the final conversion yields obtained from *in vitro* reaction were relatively lower. Regarding the low conversion yields, this may be caused by a requirement for reducing enzyme equivalents, as well as the instability and inactivation of the reducing enzymes themselves.

To overcome those limitations, we next investigated [PLP-Cu] catalyst-[KivD/ADH] enzyme coupled *in vivo* synthesis of higher alcohols, using *E. coli* whole cell biotransformation. *E. coli* cells expressing KivD, ADH or both, were harvested and incubated in M9 minimal media containing [PLP-Cu] and 1 mM of each amino acid.

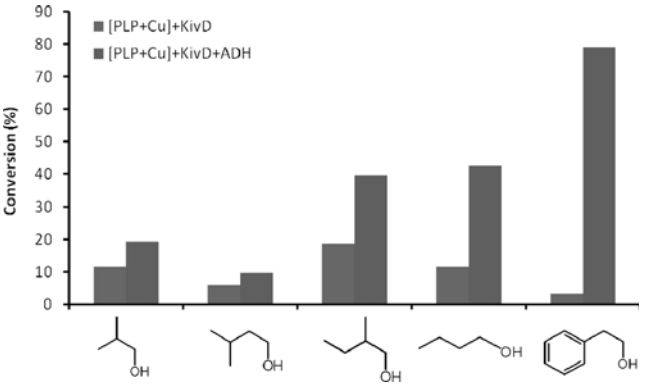


Fig. 2. *In vitro* synthesis and conversion of each higher alcohol.

Without overexpression of KivD, no alcohol was detected during a resting cell assay regardless of [PLP-Cu] complex presence, suggesting that KivD is the key enzyme in alcohol production. Overexpression of KivD in the presence of [PLP-Cu] could convert each amino acid into their corresponding alcohols with approximately 10 ~ 20% conversion yields, while no alcohol was produced in the absence of PLP-Cu (Fig. 2). These results suggest that the PLP-dependent 2-keto acid conversion is also important for the synthesis of higher alcohols, and the existing alcohol dehydrogenases in *E. coli* host genome could alternate ADH enzyme so that alcohols were observed even in the absence of ADH.

To increase higher alcohol production, we examined *E. coli* co-expressing both ADH and KivD. The resulting strain could obtain approximately two times higher production yields of higher alcohols than without ADH overexpression. In particular, 2-phenylethanol production increased dramatically, reaching a conversion yield of over 80%.

According to previous findings of improved higher alcohol production by increased carbon flux to 2-keto acids, supplying additional 2-keto acids into the KivD-expressing *E. coli* culture medium showed higher conversion yields [14]. These titers are promising as pools of 2-keto acids are a key factor in higher alcohol synthesis through the Ehrlich pathway. In order to investigate the effect of PLP-Cu complex dependent oxidation of amino acid mixture on higher alcohol production, the direct synthesis of higher alcohols from amino acid mixture of 4% yeast extract media (YEM, 20 g/L) was investigated (Fig. 3). The media prepared consists of each amino acid mixture (1.20 g/L of Ile, 1.64 g/L of Leu, 1.04 g/L of Phe, and 1.40 g/L of Valine, respectively), 2.40 g/L of amino nitrogen, and trace elements approximately (Manufacturers' information).

In most cases, *E. coli* cells co-expressing KivD and ADH in the presence of PLP-Cu resulted in greatly increased higher alcohol production, when compared to

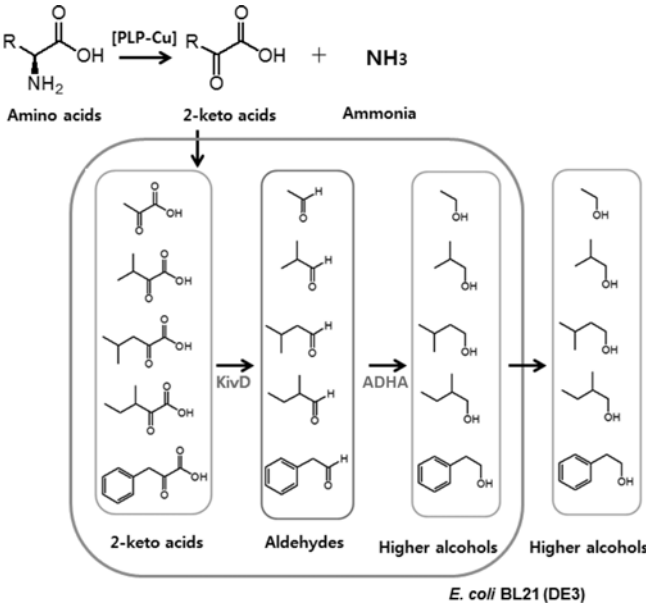


Fig. 3. Schematic of *in-vivo* application of extracellular PLP-Cu to induce higher alcohol production.

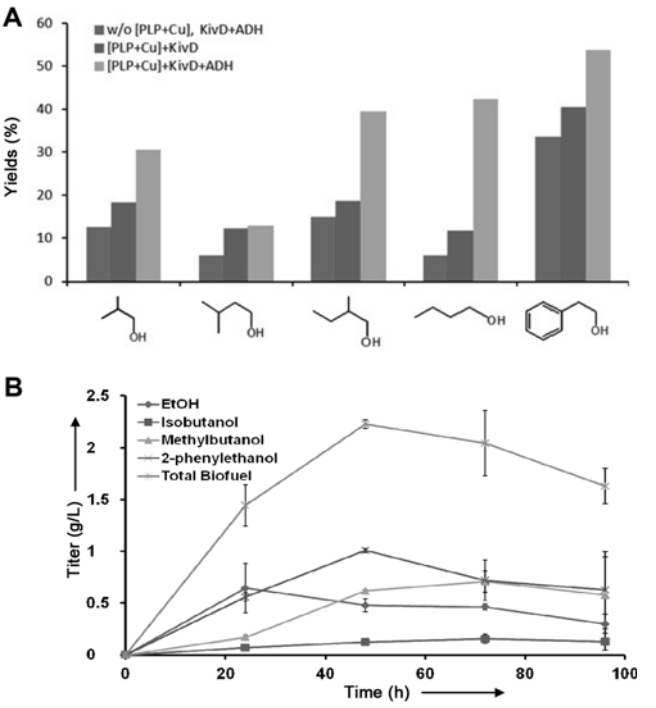


Fig. 4. Production of higher alcohols from 4% yeast extracts fermentation using PLP-Cu dependent oxidation and KivD/ADH dependent enzymatic reduction.

cells lacking such co-expression.

The conversion of phenylalanine especially was the highest with a 55% conversion (1.0 g/L) similar to its conversion ranking in our previous results (Fig. 4). These results suggested that an extracellular supply of the PLP-Cu complex could be effective in the synthesis of higher alcohols from the already present amino acid containing biomass.

3.4. Understanding PLP-dependent deamination and its applications

We investigated PLP-Cu dependent oxidative deamination for higher alcohols synthesis followed by subsequent enzymatic oxidation reactions. First, PLP-Cu dependent deamination was evaluated against our target five amino acids and resulted in 45 ~ 73% yields. Simultaneous KivD-ADH enzymatic reactions converted each amino acid into its corresponding higher alcohol. From these, the synthesis of 2-phenylethanol was the highest. Several mechanisms of PLP-catalyzed oxidative deamination have been proposed; however, the full mechanism has not been fully elucidated to date [22]. One suggestion, by Hamilton and Revesz, involves the coordination of an oxygen atom with the metal ion of the Schiff base complex, and the subsequent transfer of a pair of electrons from an amino acid, through the coenzyme to the oxygen molecule, via the metal ion [26].

Enzymatic transamination in nature requires an amine acceptor. This amine acceptor is usually a key molecule linked to central carbon-nitrogen metabolism, indicating heterologous transamination cycles, which may in turn cause unbalanced nitrogen metabolism when extratransaminases are introduced.

As well as this, transamination coupling reactions are quite specific between amine donors and amine acceptors, which may indicate a narrow range of target deamination [17].

The novel and fundamental finding of this study is the use of the chemical cofactor PLP in the deamination reaction of amino acids, followed by step-wise enzymatic reductions, and finally the synthesis of higher alcohols. This process does not require any amine accepting partner and does not cause any cellular nitrogen imbalance. We also showed that an exogenous supply of PLP and copper in a whole cell reaction in the presence of amino acids aids higher alcohol synthesis.

From these findings, the cofactors used in the biological systems seem to play the same role in the absence of a polypeptide structure. Biochemically, cofactors have been regarded as the 'helper molecule' for protein functionality, usually as enzymes [27]. Organic cofactors such as ATP, coenzyme A, FAD, and NAD share the same nucleotide adenosine monophosphate (AMP) in their chemical structures and function, and this AMP containing cofactors have been working key roles in the mechanisms of diverse enzymes [28].

4. Conclusion

Here, we have demonstrated the application of the organic cofactor PLP as an alternate enzymatic deamination step in the Ehrlich pathway. The PLP-Cu complex catalyzed oxidative deamination of the five amino acids tested: valine, leucine, isoleucine, norvaline, and phenylalanine, into their corresponding 2-keto acids with 45 ~ 75% of conversion ranges. This was further investigated through the examination of higher alcohol synthesis through sequential enzymatic reduction *in vitro* and *in vivo*. Among the examined amino acids, *in vitro* conversion of phenylalanine into 2-phenylethanol was the highest with 20% conversion, while the conversions of branched amino acids were less than 10%. Finally, whole cell reaction of *E. coli* expressing reduction enzymes KivD and ADH, in both single amino acid and amino acid mixture (4% yeast extract) have been investigated and resulted in 30-55% conversions to higher alcohol in the presence of PLP-Cu complex and following enzymatic reactions. From an evolutionary and functional point of view, a PLP-Cu complex is surely an attractive organic cofactor, regardless of its high cost of synthesis, and especially as no amine accepting molecules are required throughout the reaction.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant, funded by the Korean government (MEST) (NRF-2015R1A2A2A04006014). This work was partially supported by the new faculty research fund of Ajou University.

References

1. Chen, X., N. Li, and A. D. Ellington (2007) Ribozyme catalysis of metabolism in the RNA world. *Chem. Biodivers*. 4: 633-655.
2. Ouzounis, C. and N. Kyrpides (1996) The emergence of major cellular processes in evolution. *FEBS Lett*. 390: 119-123.
3. Schipp, C. J., E. Marco-Urrea, A. Kublik, J. Seifert, and L. Adrian (2013) Organic cofactors in the metabolism of *Dehalococcoides mccartyi* strains. *Philos. Trans. Royal Soc. Lond. B, Biol. Sci*. 368: 20120321.
4. Richter, M. (2013) Functional diversity of organic molecule enzyme cofactors. *Nat. Prod. Rep*. 30: 1324-1345.
5. Richard, J. P., T. L. Amyes, J. Crugeiras, and A. Rios (2011) The PLP cofactor: Lessons from studies on model reactions. *Biochimica et Biophysica Acta* 1814: 1419-1425.
6. Vanderlinde, R. E. (1986) Review of pyridoxal phosphate and the transaminases in liver disease. *Ann. Clin. Lab. Sci*. 16: 79-93.
7. Eliot, A. C. and J. F. Kirsch (2004) Pyridoxal phosphate enzymes: Mechanistic, structural, and evolutionary considerations. *Ann. Rev. Biochem*. 73: 383-415.
8. Phillips, R. S. (2015) Chemistry and diversity of Pyridoxal-5'-phosphate dependent enzymes. *Biochimica et Biophysica Acta* 1854: 1167-1174.
9. Griswold, W. R. and M. D. Toney (2011) Role of the pyridine nitrogen in pyridoxal 5'-phosphate catalysis: Activity of three classes of PLP enzymes reconstituted with deazapyridoxal 5'-phosphate. *J. Am. Chem.l Soc*. 133: 14823-14830.
10. Shin, J. S. and B. G. Kim (2009) Transaminase-catalyzed asymmetric synthesis of L-2-aminobutyric acid from achiral reactants. *Biotechnol. Lett*. 31: 1595-1599.
11. Yun, H. and B. G. Kim (2008) Asymmetric synthesis of (S)-alpha-methylbenzylamine by recombinant *Escherichia coli* co-expressing omega-transaminase and acetolactate synthase. *Biosci. Biotechnol. Biochem*. 72: 3030-3033.
12. Yun, H., S. Lim, B. K. Cho, and B. G. Kim (2004) omega-Amino acid:pyruvate transaminase from Alcaligenes denitrificans Y2k-2: A new catalyst for kinetic resolution of beta-amino acids and amines. *Appl. Environ. Microbiol*. 70: 2529-2534.
13. Miyoshi Ikawa, E. E. S. (1954) Oxidative deamination of amino acids by pyridoxal and metal salts. *J. Am. Chem. Soc.* 76: 4900-4902.
14. Atsumi, S., T. Hanai, and J. C. Liao (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451: 86-89.
15. Huo, Y. X., K. M. Cho, J. G. Rivera, E. Monte, C. R. Shen, Y. Yan, and J. C. Liao (2011) Conversion of proteins into biofuels by engineering nitrogen flux. *Nat. Biotechnol*. 29: 346-351.
16. Choi, K. Y., D. G. Wernick, C. A. Tat, and J. C. Liao (2014) Consolidated conversion of protein waste into biofuels and ammonia using *Bacillus subtilis. Metabol. Eng*. 23: 53-61.
17. Hazelwood, L. A., J. M. Daran, A. J. van Maris, J. T. Pronk, and J. R. Dickinson (2008) The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. *Appl. Environ. Microbiol*. 74: 2259-2266.
18. Li, S., J. Wen, and X. Jia (2011) Engineering Bacillus subtilis for isobutanol production by heterologous Ehrlich pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression. *Appl. Microbiol. Biotechnol*. 91: 577-589.
19. Kim, B., B. R. Cho, and J. S. Hahn (2014) Metabolic engineering of *Saccharomyces cerevisiae* for the production of 2-phenylethanol *via* Ehrlich pathway. *Biotechnol. Bioeng*. 111: 115-124.
20. Lan, E. I. and J. C. Liao (2013) Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources. *Bioresour. Technol*. 135: 339-349.
21. Koike, K. and M. Koike (1984) Fluorescent analysis of alpha-keto acids in serum and urine by high-performance liquid chromatography. *Anal. Biochem*. 141: 481-487.
22. Shanbhag, V. M. and A. E. Martell (1991) Oxidative deamination of amino acids by molecular oxygne with pyridoxal derivatives and metal ions as catalysts. *J. Am. Chem. Soc*. 113: 6479-6487.
23. Cooper, A. J. (1985) Glutamate-branched-chain amino acid transaminase. *Meth. Enzymol*. 113: 71-73.
24. Goto, M., I. Miyahara, H. Hayashi, H. Kagamiyama, and K. Hirotsu (2003) Crystal structures of branched-chain amino acid aminotransferase complexed with glutamate and glutarate: True reaction intermediate and double substrate recognition of the enzyme. *Biochem*. 42: 3725-3733.
25. Viljoen, A. J., C. J. Kirsten, B. Baker, P. D. van Helden, and I. J. Wiid (2013) The role of glutamine oxoglutarate aminotransferase and glutamate dehydrogenase in nitrogen metabolism in Mycobacterium bovis BCG. *PloS one*. 8: e84452.
26. Gordon A. Hamilton, and A. Revesz (1966) Oxidation by molecular oxygen. IV. A possible model reaction for some amine oxidases. *J. Am. Chem. Soc*. 88: 2069-2070.
27. Toohey, J. I. (2006) Vitamin B12 and methionine synthesis: A critical review. Is nature's most beautiful cofactor misunderstood? *BioFactors*. 26: 45-57.

28. Denessiouk, K. A., V. V. Rantanen, and M. S. Johnson (2001) Adenine recognition: A motif present in ATP-, CoA-, NAD-, NADP-, and FAD-dependent proteins. *Proteins* 44: 282-291.
29. Sniekers, M., V. Foulon, G. P. Mannaerts, L. Van Maldergem, H. Mandel, B. D. Gelb, M. Casteels, and P. P. Van Veldhoven (2006) Thiamine pyrophosphate: An essential cofactor for the alpha-oxidation in mammals--implications for thiamine deficiencies? *Cell. Mol. Life Sci.* 63: 1553-1563.
30. Foulon, V., M. Casteels, G. P. Mannaerts, B. D. Gelb, and P. P. Van Veldhoven (2003) Thiamine pyrophosphate: An essential cofactor in the mammalian metabolism of 3-methyl-branched fatty acids--implications for thiamine deficiencies? *Adv. Exp. Med. Biol*. 544: 305-306.
31. Frank, R. A., F. J. Leeper, and B. F. Luisi (2007) Structure, mechanism and catalytic duality of thiamine-dependent enzymes. *Cell. Mol. Life Sci.* 64: 892-905.
32. Bolander, F. F. (2006) Vitamins: Not just for enzymes. *Curr. Opin. Investig. Drugs* 7: 912-915.
33. Fischer, J. D., G. L. Holliday, S. A. Rahman, and J. M. Thornton (2010) The structures and physicochemical properties of organic cofactors in biocatalysis. *J. Mol. Biol*. 403: 803-824.
34. Rotello, V. M. and R. P. Swenson (2001) Organic redox cofactors. *Antioxid. Redox Signal*. 3: 721-722.
35. Fischer, J. D., G. L. Holliday, and J. M. Thornton (2010) The CoFactor database: Organic cofactors in enzyme catalysis. *Bioinformat*. 26: 2496-2497.