International Journal of Environment, Agriculture and Biotechnology
Vol-6, Issue-2; Mar-Apr, 2021
Journal Home Page Available: https://ijeab.com/
Journal DOI: 10.22161/ijeab

Current biotechnological applications of L-amino acid deaminases for production of keto acids

Abdulqader Al-Adeeb [1,a], Aqeel Sahibzada Muhammad[1,a], , Qais Al-Maqtari[2.3], Waleed AL-Ansi[2.3], Ildephonse Habimana[1], Hend Al-Jaberi[4], Ejaz Sharoon[1], Nadia Sarwar [1], Amer Ali Mahdi[3.5,*]

[a] Abdulqader Al-Adeeb and Aqeel Sahibzada Mohammed contributed equally to this work
[1] School of Biotechnology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China.
[2]School of Food Science and Technology, State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China.
[3]Department of Food Science and Technology, Faculty of Agriculture, Sana'a University, Sana'a, Yemen.
[4]Department of Clinical Laboratory, Nanjing First Hospital and Jiangsu Collaborative Innovation Centre on Cancer Personalized Medicine, Nanjing Medical University, 68 Changle Road, Nanjing 210006, Jiangsu, China
[5]School of Food and Biological Engineering, Jiangsu University, Zhenjiang, 212013, China.
*Corresponding Author

Received: 19 Nov 2020; Received in revised form: 08 Feb 2021; Accepted: 03 Mar 2021; Available online: 24 Mar 2021

Abstract— *There is a growing interest in the pharmaceutical and agrochemicals industries for the use of enantiomerically-pure amino acids. α-keto acids are commonly used in feeds, food additives, pharmaceuticals, and chemical manufacturing. Commonly, most α-keto acids are manufactured by chemical synthesis, but due to the increasing concern for environment-friendly approaches, microbial fermentation and enzymatic transformation are alternative processes for the production of keto acids. Regarding this, L-amino acid deaminase (LAAD), is a major enzyme for α-keto acids production and is only found in Proteus bacteria. In this review, we discussed the recent biological applications of the enzyme LAAD in the production of keto acids, and summarized the recent advancements in the biological production of six important α-keto acids; specifically, phenylpyruvate, α-ketoglutaric acid, pyruvate, α-ketoisocaproate, α-keto-γ-methylthiobutyric acid, and α-ketoisovaleric acid.*

Keywords— ***keto acids, LAAD, enzymatic transformation, microbial fermentation.***

I. INTRODUCTION

A variety of enzymes has been used to prepare chiral pharmaceutical and agricultural compounds containing enantiomeric amine or amino acid groups. Among these are aminotransferases (EC 2.6.4.*X*), lipases (EC 3.1.1.*X*), amine oxidases (EC 1.4.3.22), amino acid dehydrogenases (EC 1.4.1.*X*), and amino acid oxidases (EC 1.4.3.*X*) (Pollegioni & Molla, 2011; Turner, 2004).The deracemization of a racemic amino acid to obtain the L-configuration was achieved by using a stereoselective D-amino acid oxidase (DAAO, EC 1.4.3.3) followed by chemical reduction. The second step iteratively converts the amino acid produced (from the D-amino acid) back into a DL-mixture to obtain the full resolution of the racemic mixture into the L-enantiomer(Turner, 2004). This approach requires stable recombinant DAAOs possessing wide substrate specificity as well as variants engineered to act on synthetic amino acids (Pollegioni et al., 2007).

Amino acid oxidases with reverse stereoselectivity are also well known flavooxidases, mainly produced by snakes or by microorganisms. In particular, L-amino acid oxidases (LAAO, EC 1.4.3.2) catalyze the stereoselective oxidative deamination of L-amino acids into the corresponding α-keto acids and ammonia; the re-oxidation of $FADH_2$ by

dioxygen then generates H_2O_2 (Pollegioni, Motta, &Molla, 2013). These flavoenzymes catalyze an irreversible reaction (differently from aminotransferases) and do not require a specific step of cofactor regeneration, as otherwise required by the NAD-dependent dehydrogenases. However, because of the problems associated with overexpression of snake venom LAAOs in recombinant hosts and the limited substrate acceptance of the microbial counterparts, no appropriate LAAOs for biocatalysis are available (Pollegioni et al., 2013). L-Amino acid deaminases (LAADs) represent a suitable alternative to LAAOs. LAAD (first identified in the genera *Proteus*, *Providencia,* and *Morganella*) catalyzes the deamination of the L-isomer of amino acids, yielding the corresponding α-keto acids and ammonia without any evidence of H_2O_2 production. LAAD from *Proteus myxofaciens* (PmaLAAD), expressed in the *Escherichia coli* K12 strain, shows a preference for L-amino acids with aliphatic, aromatic, and sulfur-containing side chains (Pantaleone, Geller, & Taylor, 2001). This review focused on the applications of LAAD for the production of α-keto acids, and summarized the recent advancements in the biological production of six important a-keto acids; specifically, phenylpyruvate,α-ketoglutaric acid, pyruvate, α-ketoisocaproate, α-keto-γ-methylthiobutyric acid, and α-ketoisovaleric acid.

II. LAAD IN BIOCATALYSIS

2.1 EXPRESSION AND PURIFICATION OF RECOMBINANT LAADS

In order to produce LAAD in bulk for biochemical characterization and biotechnological purposes, this flavoenzyme must be expressed in different microbial hosts. Recombinant type-I L-Amino acid deaminase from *Proteus myxofaciens* (PmaLAAD) was expressed as a full-length protein in *E. coli* BL21(DE3) cells (at 28 °C), obtaining an enzyme with a specific activity of 0.6 U mg^{-1} protein on the substrate L-Phe (in the crude extract) (Motta, Molla, Pollegioni, & Nardini, 2016). Chromatographic steps were ineffective for enzyme purification due to the recombinant protein and its tight association with the bacterial membrane fragments. Thus, PmaLAAD could only be separated from soluble proteins by ultracentrifuging the crude extract Resuspension of the pellet formed by the membrane fraction (and containing the active enzyme) resulted in modest increases of a2- to 3.4-fold within specific activity (Table 1) (Motta et al., 2016; Pantaleone et al., 2001). The dual objectives of producing and preparing a pure PmaLAAD aided in designing a His-tagged fusion protein. The presence of the tag located either at the protein's N- or C-terminuses have no effect on the enzyme expression yields or its specific activity in the crude extract. A His-tagged PmaLAAD variant was purified to homogeneity by immobilized metal affinity chromatography as a soluble, yet inactive enzyme. Complete activity can only be restored (and reaching the crude extract's assayed value) after adding *E. coli* membranes to the purified enzyme sample (Motta et al., 2016). Type-I PmirLAAD in its homologous form was also produced as a His-tagged recombinant protein. In this case, the recombinant protein was expressed at 20 °C to prevent the overproduced protein from accumulating in inclusion bodies. Metal-chelating chromatology followed by solubilization with the detergent n-dodecyl-β-D-maltoside was used to purify the enzyme. Unfortunately, negatively affected enzyme activity was suboptimal; this could be due to the interaction of the latter with the bacterial membranes. The specific activity of the pure protein was 0.94 U mg^{-1} on L-Phe (Table 1).

Table 1. The production level of recombinant LAADs.

Source	Variant	Specific activity (U mg^{-1} protein on L-Phe)			Reference
		Crude extract	Ultracentrifugation step	Further purification steps	
Type-I LAAD					
P. myxofaciens	Wild-type	0.6	1.2	2.9 (thawing, dilution and further centrifugation)	(Motta et al., 2016)
	C-HisTag	0.69	2.35		(Pantaleone et al., 2001)
	N-HisTag	0.8		B.D. (HiTrap)	(Motta et al.,

Source	Variant	Specific activity (U mg^{-1} protein on L-Phe)			Reference
		Crude extract	Ultracentrifugation step	Further purification steps	
					2016)
P. mirabilis	*pel*B + C-HisTag	0.02		0.94 (detergent solubilization and HiTrap)	(Hou et al., 2015)
Type-II LAAD					
P. mirabilis	N-HisTag	ND	1.54		(Baek et al., 2011)
P. mirabilis (optimized codon usage)	*pel*B + N-HisTag (deletion variant)	ND		0.74 (refolded from inclusion bodies)	(Liu et al., 2013)
P. vulgaris[a]	N-HisTag	ND	ND	ND	(Ju et al., 2016; Takahashi, Ito, & Yoshimoto, 1999)

B.D.: below detection; ND.: not determined; HiTrap: metal-chelating chromatography. [a]No information concerning the specific activity of the recombinant enzyme has been reported.

Therefore, due to partial loss of enzymatic activity and high detergent costs, the authors concluded that biocatalytic applications are better served by using whole cells to express the recombinant protein instead (Hou et al., 2015). Similar expression yields were obtained for the His-tagged Pm1LAADtoo. through preparation of a specific activity on the L-Phe of 1.54 U mg^{-1} protein (compared to 1.2 U mg^{-1} for PmaLAAD) was achieved after the ultracentrifugation purification step (Baek et al., 2011). Interestingly, by the optimized codon usage for *E. coli* expression and the gene was fused to the *pelB* leader peptide, the recombinant chimeric protein was expressed exclusively as inclusion bodies (Liu et al., 2013). This confirms that overoptimization of codon usage could induce an actual increase in protein expression but compromise of its cellular solubility. Nonetheless, the researchers were able to solubilize (to use 8 mol urea) and refold protein from the inclusion bodies (with a yield of 40 percent), obtaining an enzyme preparation with a high degree of purity but with a reduced specific activity (0.74 U mg-1 of pure Pm1LAAD) and a very high Km for L-Phe) (31.5 mM). This result indicates that the refolding approach facilitated partial recovery of the conformation/activity of the enzyme (Table 1) (Liu et al., 2013).As a general rule, the association of the enzyme to the bacterial membranes is fundamental for the catalytic competence of type-I LAADs. Consequently, when the putative N-terminal transmembrane α-helix of PmaLAAD was removed, the resulting ΔN-LAAD deletion variant is rendered nearly inactive because of its inability to accomplish membrane interaction (in the crude extract even) (Motta et al., 2016). In contrast, with type-II LAADs, the enzymatic activity is also (partially) retained in the deletion variants lacking the transmembrane α-helix. Regarding these enzymes, the insertion module is relatively sufficient for providing the necessary interaction of the protein with the bacterial membranes, even without the N-terminal transmembrane α-helix (Ju et al., 2016). Further debates have ensued since then, focusing on using whole cells to express LAAD vs. using recombinant purified enzymes. Whole cells are employed more consistently due to their production simplicity and the low cost; some authors assert that side reactions can occur, thus accumulating into small amounts of impurities and causing adverse effects on the overall yield (Ahmed, Parmeggiani, Weise, Flitsch, & Turner, 2016; Hou et al., 2016; Hou et al., 2015). In contrast, purified enzymes are more

expensive but possess a higher specific activity and lack any side reactions. (Hou et al., 2015) stressed the following. Whole-cell transformation is an attractive alternative to the laborious and costly processes of purification, stability, external FAD addition, and recycling. With that said, when taking conversion and productivity into account, the enzyme systems showed better performance overall. Therefore, both biotransformation methods are promising and need future improvement (Molla, Melis, & Pollegioni, 2017).

2.2 ELEMENTAL SOLUTIONS OF AMINO ACIDS

In 2001, Pantaleone group was the first to propose employing LAAD in biocatalysis. The recombinant enzyme from *P. myxofaciens* oxidized the 20 natural L-amino acids, thus revealing its preference for aliphatic, aromatic, and S-containing amino acids (Pantaleone et al., 2001) . This same group used a partially purified enzyme preparation to conduct specific activity on the L-Phe of 2.35 U mg^{-1} protein to determine its pH levels (which measured at 7.5). They also collaborated with Ian Fotheringham and Nick Turner the following year, using the same *E. coli* cells, which expressed the recombinant protein and 40 equivalents of ammonia-borane. The result was a deracemization of 2.5 mmol DL-Leu (in 50 mmol ammonium formate, pH 6.7), and a continuing production of the D-enantiomer, with a 90% yield and an ee> 99% (Alexandre et al., 2002). Together, these groups achieved a conversion higher than 85% obtained through racemic solutions of norleucine, methionine, O-benzyl serine, cyclopentyl-glycine (Alexandre et al., 2002), and isoleucine (Enright et al., 2003).

V_{max} (µmol min^{-1} mg^{-1} protein)	**K_m (mM)**	**Reference**	**Note**
Recombinant *P. myxofaciens* LAAD			
0.26	2.28	(Pantaleone et al., 2001)	Crude extract (PmaLAAD)

Table 2. Apparent kinetic parameters on l-Phe of various L-AAD preparations

Recombinant *P. mirabilis* LAAD:			
0.73	31.5	(Liu et al., 2013)	Refolded ΔN-L-AAD variant (Pm1LAAD)
0.34	–	(Hossain, Li, Shin, Chen, et al., 2014)	Membrane preparation from *B. subtilis* cells (Pm1LAAD)
1.64	26.2	(Hossain et al., 2016; Hou et al., 2015)	Purified *P. mirabilis* KCTC2566 L-AAD (PmirLAAD)
2.63	22.0	(Hou et al., 2016)	Purified D165K/F263M/L336M variant of *P. mirabilis* KCTC2566 L-AAD (PmirLAAD)

2.3 PHENYLPYRUVIC ACIDPRODUCTION

In recent years, phenylpyruvic acid (PPA) has been utilized as a product reference of L-AAD catalyzed L-Phe conversion (Table 2). PPA is a limiting raw material for the sweetener aspartame (production > 10,000 tons year-1) and employed primarily for patients with kidney diseases to reduce urea accumulation in their diets PPA producers were subsequently screened and selected using a variety of microorganisms such as *P. Vulgaris*, which was the most effective strain (Coban, Demirci, Patterson, & Elias, 2014). Reaching ~ 1 g L^{-1} of PPA. Pantaleone et al experimented even more with PPA production by using the *Proteus Vulgaris* B-123 strain, which reached ~ 3g L^{-1} of PPA in fed-batch fermentation (adding 4 g L-Phe at 30 hours of fermentation), and 104 and 259 mg L -1h^{-1} by conducting fed-batch and continuous fermentation, respectively (Coban, Demirci, Patterson, & Elias, 2016). *P. mirabilis* KCTC 2566 L-AAD (type-II) was overproduced in *E. coli* cells, which can be used as a whole-cell biocatalyst or as a purified enzyme preparation (Hou et al., 2015). The maximal PPA production was 2.6 and 3.3 g L^{-1} of PPA (86.7 and 82.5% conversion rate) beginning with 3 and 4 g L^{-1} L-Phe for the pure enzyme (0.2 mg mL^{-1} at pH 7.4 and 35 °C) and the whole-cell system (1.2 g cell L^{-1} at pH 7.4 and 40 °C), respectively. PPA generation through *E. coli* cells that overproduce L-AAD from *P. mirabilis* KCTC 2566 is often hampered by two critical factors: product degradation and a low substrate preference of this flavoenzyme(Hou et al., 2016). Thus, they achieved a moderate increase in PPA level (from 3.3 to 3.9 g L^{-1}) by eliminating three genes encoding aminotransferases. This led to increased PPA production on a higher scale (up to 10.0 g L^{-1} corresponding and reaching full substrate conversion) by utilizing a L-AAD variant that harbors the D165K/F263M/L336M substitutions, generated by error-prone PCR (Hou et al., 2016). The evolved L-AAD variant shows a 1.6-fold increase in maximal activity (2.63 μmol min-1 mg^{-1} protein), see (Table 2). Under optimized fed-batch conditions (i.e., feeding 4 g L^{-1} of L-Phe per hour), the maximal PPA production reached 21 g L^{-1}. However, PPA's current chemical synthesis methods have relatively low yield and result in unwanted environmental pollution. In this regard, biotransformation processes seem unsuitable as industrial alternatives.

2.4 PRODUCTION OF A-KETOGLUTARIC ACID

The Jian Chen group used L-AAD from *P. mirabilis* KCTC to transform L-glutamic acid into 2- ketoglutaric acid (α-KG), which can be utilized in various applications such as pharmaceuticals, fine chemical, and the food and animal feed industries. It is believed that the biocatalytic process bypasses the multi-step chemical process regarding succinic acid and oxalic acid diethyl esters, thereby employing toxic compounds, generates toxic waste, and showing a much lower product yield in comparison. The ΔN-L-AAD variant enzyme was used after refolding (Liu et al., 2013). This preparation of enzymes showed the optimal activity at pH 8.0 and 45 °C and an apparent Vmax = 0.73 U mg^{-1} protein and Km for L-Phe of 31.5 mmol (Table 2 at air saturation). Studies have shown that 12.6% of α-KG can be produced in 6 hours, which is considered to be optimal conditions (12 g L^{-1} L-Glu, 0.1 g L^{-1} ΔN-LAAD, 5 mmol MgCl2, pH 8.0 and 40 °C) (Liu et al., 2013). Notably, the conversion was hampered by-product inhibition (Ki = 12.6 mmol for α-KG). Notably, product inhibition (Ki = 12.6 mmol for α-KG) significantly hampered the conversion process. Later on, the wild-type L-AAD from *P. mirabilis* KCTC 2566 was overproduced in *Bacillus subtilis 168* cells (Table 2) and used as membrane fraction for the transformation of

L-Glu. Within optimal parameters (15g L^{-1} L-Glu, 20g L^{-1} cells, 5 mmol MgCl2, pH 8.0 and 40 °C) 31% of α-KG can be produced in as little as 2 hours (Hossain, Li, Shin, Chen, et al., 2014). The maximal volumetric yield reached 4.65 g α-KG L^{-1} In order to further improve α-KG production, a protein engineering study was conducted by Che's group using Pm1L-AAD (Hossain, Li, Shin, Liu, et al., 2014). By employing EP-PCR and site-saturation mutagenesis at six positions they identified the pm1-3-3 variant harboring the F110I/A255T/E349D/R228C/T249S/I352A substitutions. This variant enzyme shows a 2.4-fold higher Vmax and a 2.1-fold lower Km for L-Glu in contrast to the wild-type Pm1L-AAD does. When this variant of enzyme expression in a *B. subtilis 168* strain lacking the *sucA* gene encoding for α-ketoglutarate dehydrogenase, the α-KG titer reached 12.2 g L^{-1} (after 24 h of transformation using 15 g L^{-1} L-Glu, 20 g L^{-1} whole-cell biocatalyst, pH 8.0) (Hossain, Li, Shin, Chen, et al., 2014). After multiple rounds of error-prone PCR of *P. mirabilis* KCTC 2566 L-AAD followed by DNA shuffling with the gene coding for *pv*LAAD, they managed to isolate pm1338g4, a variant of enzyme-containing substitutions at 34 positions and further possessing maximal activity at a 4.5-fold increase and a subsequent decreased Km for L-Glu (i.e.6.6 mmol) by ~ 4-fold (Hossain, Li, Shin, Du, et al., 2014). This system produced 53.7 g α-KG L^{-1} when 100 g of monosodium glutamate was used and up to 89.1 g α-KG $L^{-1}$when the substrate was continuously fed at a constant rate of 6 g $L^{-1}h^{-1}$ with an initial concentration of 50 g L^{-1}. Regardless of the advantage of reducing environmental pollution and the significant α-KG production reached by L-AAD biocatalysis, this bioconversion system is not ready to be turned into an industrial level.

2.5 PYRUVATE PRODUCTION

Currently, the production of pyruvate by the biotechnological process has become a fast-growing trend, and fermentation processes have been extensively established in this regard. Through metabolic engineering strategies, microbes such as *E. coli* (Yihui Zhu, Eiteman, Altman, & Altman, 2008), *Torulopsisglabrata* (Yang, Chen, Xu, Liu, & Chen, 2014) *Corynebacterium glutamicum* (Wieschalka, Blombach, & Eikmanns, 2012) and *Saccharomyces cerevisiae* (Van Maris et al., 2004). have been constructed to achieve high production of pyruvate. Overall, the engineering methods target on accelerating the rate of glycolysis and on limiting cellular growth (Yihui Zhu et al., 2008). Hossain et al. attempted a production of pyruvate from DL-alanine which utilized a whole-cells system engineering of an *E. coli* cell strain (Hossain et al., 2016). The genes encoding of these two alanine uptake transporters and one pyruvate uptake transporter were eliminated to minimize their usage by the cell system. These cells also succeeded in overproduction of both the wild-type and an isolated variant (30,000 clones generated by error-prone PCR) of Pm1LAAD. Pm1ep3, in its best form, contains 9 substitutions overall (at a great distance of > 15 Å from the active site) and displaying a 1.8-fold increase in maximal activity (3.1 μmol min-1 mg^{-1} protein) with a ~ 2-fold decrease in Km for L-Ala (23.3 mmol) (Hossain et al., 2016). Interestingly, the enzyme can also be active on D-Ala (Vmax = 0.81 μmol min^{-1} mg^{-1} protein, Km= 31.5 mM), allowing further usage of the racemic amino acid to produce pyruvic acid. LAAD's ability to use the D-isomer of the amino acids as substrate require a more detailed evaluation.

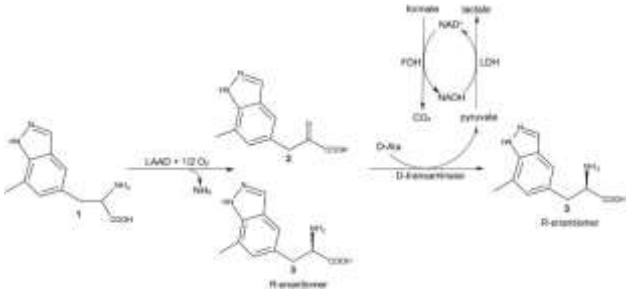

Fig.1. Enzymatic reactions by LAAD and D-transaminase used to convert a racemic amino acid (1) solution into the corresponding R-enantiomer (3), with coupling for pyruvate removal (Hanson et al., 2008). (2) S-enantiomer; LDH, lactate dehydrogenase; FDH, formate dehydrogenase.

This is due to the fact that the four-location model for substrate binding discriminates the enantiomers for their ability for hydride transferal from their αC-H to the N (5) flavin position, see above. Under ideal conditions (i.e., 50 g L^{-1} DL-Ala, 20 g L^{-1} whole-cells expressing pm1ep3, pH 8.0, at 40 °C and 220 rpm) 14.6 g L^{-1} of pyruvate was achieved, corresponding to a biotransformation ratio of ~ 29% of in just 36 hours (Hossain et al., 2016).

2.5.1 *T. GLABRATA* FOR IMPROVING PYRUVATE PRODUCTION

The earliest microbial stain used for industrial-scale pyruvate production was the multi-vitamin auxotroph *T. glabrata*. The highest pyruvate production by *T. glabrata* reached 94.3 g/L within 82 h, and the yield on glucose substrate was about 0.635 g/g by a NaCl-tolerant strain (Lütke-Eversloh, Santos, & Stephanopoulos, 2007). Further investigation was focused on controlling the NADH level to enhance the rate of glycolysis. Two different NADH re-oxidation pathways were introduced to reduce NADH in the cytoplasm and in mitochondria. The

highest pyruvate yield and productivity were increased to 38% and 21%, respectively (Qin, Johnson, Liu, & Chen, 2011). An optimal nutrient environment is also crucial to achieving a high pyruvate yield. With nitrogen optimization, the pyruvate concentration reached 85.9 g/L within 72 h with the optimal addition of thiamine, nicotinic acid, pyridoxine, biotin, and riboflavin (Yang et al., 2014).

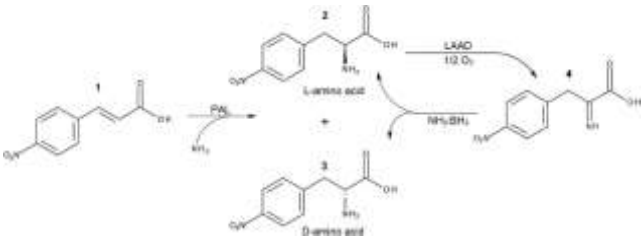

Fig.2. Amination/deracemization cascade by phenylalanine ammonia lyase (PAL) and LAAD to produce optically pure p-nitro-D-Phe (3) from p-nitrocinnamic acid (1) (Parmeggiani et al., 2015). (2) p-Nitro-L-amino acid; (4) p-nitro-amino acid.

2.5.2 CONSTRUCTING *C. GLUTAMICUM* FOR THE PRODUCTION OF PYRUVATE

C. glutamicum is well-established organic acid producer, it has been established as a microbial cell factory for pyruvate production. *C. glutamicum* strain was engineered by inactivating its pyruvate dehydrogenase complex, NAD^+-dependent l-lactate dehydrogenase, quinone oxidoreductase, and alanine transaminases AlaT and AvtA, and a reduced variant of acetohydroxyacid synthase was presented. Under low oxygen tension and in fed-batch fermentations, the strain formed 45 g/L of pyruvate, with a yield of 0.97 mol/ mol of glucose (Wieschalka et al., 2012; Zou, Hang, Chu, Zhuang, & Zhang, 2009). Even though this yield of pyruvate was minor than that of *T. glabrata*, the conversion rate from glucose was higher with *C. glutamicum*. Furthermore, *C. glutamicum* is more applicable for medicinal-grade pyruvate production owing to its generally recognized as safe (GRAS) status (Meiswinkel, Rittmann, Lindner, & Wendisch, 2013).

2.5.3 CONSTRUCTING *S. CEREVISIAE* FOR THE PRODUCTION OF PYRUVATE

S. cerevisiae does not usually produce organic acids in large scale, its tolerance to pH makes it a suitable producer of pyruvate.(Van Maris et al., 2004) reported that the highest production of pyruvate (135 g/L) was obtained by a pyruvate decarboxylase-deficient mutant of *S. cerevisiae* TAM. Yet, supplementation of a C_2 compound was required for the growth of this mutant because of the pyruvate decarboxylase deficiency. In Wang et al., (2012) study, by using rational cofactor engineering and adaptive evolution, a pyruvate concentration of 75.1 g/L with a yield of 0.63 g pyruvate/g glucose was obtained. Furthermore, by regulating the thiamine biosynthesis genes, which are related to unstable cellular growth on sole carbon sources, a pyruvate amount of 8.21 ± 0.30 g/L was achieved after 96 h of cultivation (Xu, Hua, Duan, Liu, & Chen, 2012). fermentation conditions were also optimized by focusing on the carbon and nitrogen substrates, pH, osmotic pressure, and hydrogen peroxide (H_2O_2) level for the improvement of pyruvate production by different strains (Song et al., 2016)

2.5.3 BIOCONVERSION APPROACH OF PYRUVATE PRODUCTION ENHANCEMENT

In current years, whole-cell and enzyme bioconversion methods have been developed. E.g., lactate dehydrogenase, glycolate oxidase, and lactate oxidase were used to convert lactate into pyruvate. In a previous study they used glycolate oxidase in *Pichia* pastoris to catalyze the conversion of pyruvate from L-lactate by the whole-cell transformation process (Payne et al., 1995). Acetaldehyde and carbon dioxide were converted into pyruvate by*Rhodotorulagracilis* d-amino acid oxidase (d-AAO), giving a 90% conversion rate (Miyazaki, Shibue, Ogino, Nakamura, & Maeda, 2001). In another study, a high pyruvate quantity of 111.9 g/L was obtained by expressing both glycolate oxidase and catalase together (Eisenberg et al., 1997). The catalase was needed to remove the effect of H_2O_2, which could convert the pyruvate to acetate.

2.6 PRODUCTION OF A-KETOISOCAPROATE

ketoisocaproate Essential branched-chain amino acids L-Val, L-Ile and L-Leu, alongside the corresponding keto acids, have different applications in the feed, food and pharmaceutical sector: they are produced in quantities of up to 5,000 tons year-1 (Becker & Wittmann, 2012). In a previous study, in order to establish a successful fermentation mechanism, a recombinant *Corynebacterium glutamicum* strain was designed by metabolic engineering, and the maximal α-ketoisocaproate titter was reached 9.23 g/L (Bückle-Vallant, Krause, Messerschmidt, &Eikmanns, 2014). However, with the exception of the poor yield of alpha-ketoisocaproate, an auxotroph for branch-chained amino acids is still a barrier to industrial development owing to the deletion of ilvE. Another study has fabricated a plasmid-free *C. glutamicum* to produce 6.1 g/L α-ketoisocaproate (Vogt, Haas, Polen, van Ooyen, & Bott, 2015). Yet, the production of α-ketoisocaproate of *C. glutamicum* by metabolic engineering is still narrow by the growth reliant on the L-isoleucine. The whole-cell biosynthesis mechanism offers a bright path to the low-cost development process of α-ketoisocaproate. In a study, α-ketoisocaproate was prepared using the whole-cell

transformation technique of *Rhodococcusopacus* DSM 43250, and α-ketoisocaproate titers reached 1275 mg/L (Yuhong Zhu et al., 2011). In another research, for the development of α-ketoisocaproate from leucine, an *Escherichia coli* BL21 (DE3) was constructed by whole-cell biocatalyst with membrane-bound L-amino acid deaminase (LAAD) from *Proteus vulgaris*. The highest titter was reached 69.1 gL^{-1}(Song et al., 2015). In another study conducted, an even higher α-ketoisocaproate production of 86.55 gL^{-1} and a higher L-leucine conversion rate of 94.25 percent were achieved via three engineering strategies; altering the plasmid origin with various copy numbers, modulating the mRNA composition downstream of the initiation codon, and designing the ribosome binding-site synthesis sequences (Song et al., 2017).

2.7 PRODUCTION OF A-KETO-Γ-METHYLTHIOBUTYRIC ACID

E. coli cells overproducing pvLAAD managed to convert L-Met into α-keto-γ-methylthiobutyric acid, which is an indirect inhibitor of tumor cell growth and a methionine supplement in livestock feed. By using previously optimized conditions (Hossain, Li, Shin, Chen, et al., 2014). 70 g L^{-1} L-Met was converted by 20 g L^{-1} whole-cell biocatalyst into α-keto-γ-methylthiobutyric acid with a 71.2% yield. The LAAD variant harboring the K104R/A337S substitutions, generated by error-prone PCR, possesses a lower Km for L-Met (decreased from 305 to 238 mM), and 63.6 g L^{-1} of α-keto-γ-methylthiobutyric acid could be produced in 24 hours. This single-step enzymatic process for production of α-keto-γmethylthiobutyric acid has a great potential at the industrial level compared with the traditional multi-step chemical systems.

2.8 A-KETOISOVALERIC ACID

α-ketoisovaleric acid (α-KIV) is as a precursor in leucine and valine synthesis. It also serves as an initial compound in vitamin B5 biosynthesis (Chassagnole, Diano, Létisse, & Lindley, 2003). α-KIV is mainly synthesized via a multistep chemical method (i.e. the hydrolysis of azlactones and the Grignard reagents with diethyloxamates) (Cooper, Ginos, & Meister, 1983; Waters, 1947). These chemical processes are costly and complex which restrain the high industrial production of α-KIV. i et al. (2017) studied an alternative biotechnological way by expressing the L-AAD from *Proteus myxofaciens* ATCC 19,692 in *E. coli BL21* (DE3) as a whole-cell biocatalyst system. Under the optimized conditions, the α-ketoisovaleric acid production with the wild type L-AAD was 2.014 g/L. Using the 3D structural model of L-AAD from *P. myxofaciens* and the simulation results when docking with the L-valine, key amino acid residues (N100, Q276, R316, and F318) were identified as potential target for site-saturation mutagenesis. The evolved L-AAD improved the biotransformation to 8.197 g/L after combining the mutated sites (Li et al., 2017). the rational molecular engineering of the L-AAD using site-saturation mutagenesis improved the efficiency of the biocatalysis.

2.9 2,5-DIKETO-D-GLUCONATE

In one study, 50 g of d-glucose was converted to 2,5-DKG with a 92% conversion rate in 150 h by a mixed culture of two newly isolated strains, *Flavimonasoryzihabitans* and *Pseudomonas cepacian* (Sulo, Hudecová, Properová, Bašnák, & Sedláček, 2001) . The metabolic engineering of bacteria has also been described for the production of 2,5-DKG from glucose. *Gluconobacteroxydans* was used for the oxidation of 2-ketogluconate to 2,5-DKG by engineering the overexpression of 2-ketogluconate dehydrogenase (Kataoka, Matsutani, Yakushi, & Matsushita, 2015). 2,5-DKG was also prepared in high yield and in high broth concentration by a newly isolated strain of genus *Erwinia* cultivated in an aqueous nutrient medium in the presence of d-glucose, where the conversion of d-glucose to 2,5-DKG reached 90% yield within 31 h (Sonoyama, Yagi, Kageyama, & Tanimoto, 1984).

2.9 OTHER APPLICATIONS INVOLVING LAAD

P. mirabilis LAAD's reaction (whole *E. coli* recombinant cells) and a commercially available D-transaminase aided in resolving a racemic mixture of 2-amino-3(7-methyl-1-H-indazol-5-yl) propanoic acid (Hanson et al., 2008). This resulted in an intermediate synthesis of antagonists of calcitonin gene-related peptide receptors. By making use of a 1 L batch scale and 20 g of racemic substrate, 40 g D-Ala (as amino donor for D-transaminase), 100 g *E. coli* cells that express LAAD (27 U g^{-1} cell wet weight), 200 mg D-transaminase (Biocatalytics, 4.4 U mg^{-1} protein), at pH 7.5, with 1 L min^{-1} aeration and following downstream processing, a final yield of 68% with ee> 99% was reached (Hanson et al., 2008). By coupling this two-step reaction with formate dehydrogenase and lactate dehydrogenase to remove pyruvate (Fig. 1) reaction rate and product yield can be increased. Substituted D-phenylalanines are present in natural products (such as macrolide antibiotics) and active pharmaceutical ingredients and are employed as chiral structural fragments or peptide components. Nick Turner's group paired a phenylalanine ammonia lyase (PAL, EC 4.3.1.24) with *P. mirabilis* LAAD and converted p-nitrocinnamic acid into p-nitro-D-phenylalanine. The outcome was a 71% conversion and a 96% ee rate (Fig. 2) (Parmeggiani, Lovelock, Weise, Ahmed, & Turner, 2015). By using the H359Y PAL variant containing a 3.5-fold increase in D-formation

activity, p-nitro-D-Phe'see value increased to > 99% with a rate of 78% conversion (Parmeggiani et al., 2015). Reaction conditions were: 5 mM substrate, 5 M NH4OH, 25 mg mL^{-1}*E. coli* cells expressing PAL, 35 mg mL^{-1}*E. coli* cells expressing LAAD, and 40 equivalent ammonia-borane, at pH 9.6 and 37 °C.

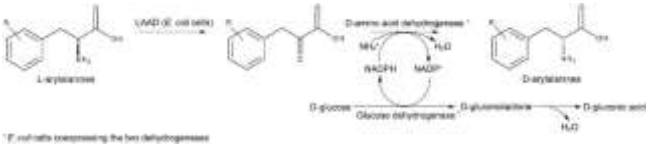

Fig.3. Enzymatic cascade by LAAD, D-amino acid dehydrogenase and glucose dehydrogenase for the synthesis of D-arylalanines by deracemization or stereo inversion (Parmeggiani et al., 2016).

This amination/deracemization cascade began at an efficient pace by utilizing numerous electron-deficient cinnamic acids that produced D-phenylglycine derivatives with sufficient optical purity. This led to increased L-isomer production when DAAO replaced LAAD. The same group paired the deamination of L-arylalanines by LAAD with the consumption of the produced keto acid by an engineered D-amino acid dehydrogenase to generate the corresponding D-amino acid (to achieve NADP+ recycling through glucose dehydrogenase, (Fig. 3) (Parmeggiani et al., 2016).

This could be a more beneficial way of generating the D-enantiomer when the corresponding L- or the DL-amino acid is commercially available in the future. Lysed *E. coli* cells expressing PmaLAAD converted natural L-amino acids into conforming α-keto acids, then asymmetrically reduced by L- or D-isocaproate reductases into (R)- or (S)-2-hydroxy acids (Fig. 4) (Busto, Richter, Grischek, & Kroutil, 2014).

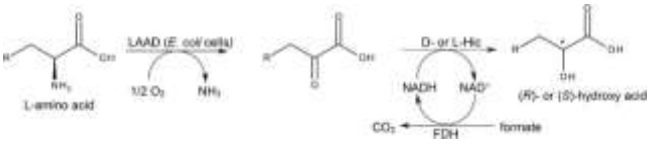

Fig.4. One-pot cascade enzymatic system based on LAAD and isocaproate reductases for the synthesis of enantiomerically pure (R)- or (S)-2-hydroxy acids starting from L-amino acids (Busto et al., 2014). D- or L-Hic, D- or L-isocaproate reductases; FDH, formate dehydrogenase.

Under 1 bar O2 pressure, 50 – 200 mM L-amino acids were fully converted into (S)- or (R)-hydroxy acids with a > 99% ee L-Tyrosine was transformed at 200 mM concentration (a value higher than its solubility) and 100 mg scale, and up to 0.5 g of L-isoleucine was converted (Busto et al., 2014). Among the production of 2-hydroxy acids, (R)- and (S)-4 hydroxy lactic acids are used to prepare various biologically active compounds such as Saroglitazar (Lypaglyn), which can treat type II diabetes.

III. CONCLUSION

Regardless, the fermentation method yielded higher amounts of pyruvate and a-KG, other a-keto acids yielded lower amounts. As a result, the genome-scale metabolic model of amino acid synthesis pathways should be investigated in order to accumulate more a-keto acids. Furthermore, because of its high conversion rate and simple and cost-effective separation process, the enzymatic bioconversion method may be a more effective approach for a-keto acid production. Enzymes with high substrate affinity and high conversion efficiency are expected to be developed in the future as new enzyme discovery tools and genetics information become available.Lastly, advances in metabolic engineering of whole-cell biocatalysts are propelling biocatalyst use to new heights. The ability to improve the overall performance of biocatalytic processes has been demonstrated by combining enzyme engineering with the modification of recombinant strain mechanism pathways. However, some work remains to be done, such as a better understanding of enzymes.

REFERENCES

[1] Ahmed, S. T., Parmeggiani, F., Weise, N. J., Flitsch, S. L., & Turner, N. J. (2016). Synthesis of enantiomerically pure ring-substituted l-pyridylalanines by biocatalytic hydroamination. *Organic letters*, *18*(21), 5468-5471.

[2] Alexandre, F.-R., Pantaleone, D. P., Taylor, P. P., Fotheringham, I. G., Ager, D. J., & Turner, N. J. (2002). Amine–boranes: effective reducing agents for the deracemisation of DL-amino acids using L-amino acid oxidase from Proteus myxofaciens. *Tetrahedron Letters*, *43*(4), 707-710.

[3] Baek, J. O., Seo, J. W., Kwon, O., Seong, S. I., Kim, I. H., & Kim, C. H. (2011). Expression and characterization of a second L-amino acid deaminase isolated from Proteus mirabilis in Escherichia coli. *Journal of basic microbiology*, *51*(2), 129-135.

[4] Becker, J., & Wittmann, C. (2012). Systems and synthetic metabolic engineering for amino acid production–the heartbeat of industrial strain development. *Current opinion in biotechnology*, *23*(5), 718-726.

[5] Bückle-Vallant, V., Krause, F. S., Messerschmidt, S., & Eikmanns, B. J. (2014). Metabolic engineering of Corynebacterium glutamicum for 2-ketoisocaproate production. *Applied microbiology and biotechnology*, *98*(1), 297-311.

[6] Busto, E., Richter, N., Grischek, B., & Kroutil, W. (2014). Biocontrolled Formal Inversion or Retention of L-α-Amino Acids to Enantiopure (R)-or (S)-Hydroxyacids. *Chemistry–A European Journal*, *20*(35), 11225-11228.

[7] Chassagnole, C., Diano, A., Létisse, F., & Lindley, N. D. (2003). Metabolic network analysis during fed-batch cultivation of Corynebacterium glutamicum for pantothenic acid production: first quantitative data and analysis of by-product formation. *Journal of biotechnology*, *104*(1-3), 261-272.

[8] Coban, H. B., Demirci, A., Patterson, P. H., & Elias, R. J. (2014). Screening of phenylpyruvic acid producers and optimization of culture conditions in bench scale bioreactors. *Bioprocess and biosystems engineering*, *37*(11), 2343-2352.

[9] Coban, H. B., Demirci, A., Patterson, P. H., & Elias, R. J. (2016). Enhanced phenylpyruvic acid production with Proteus vulgaris in fed-batch and continuous fermentation. *Preparative biochemistry & biotechnology*, *46*(2), 157-160.

[10] Cooper, A. J., Ginos, J. Z., & Meister, A. (1983). Synthesis and Properties of the. alpha.-Keto Acids. *Chemical Reviews*, *83*(3), 321-358.

[11] Eisenberg, A., Seip, J. E., Gavagan, J. E., Payne, M. S., Anton, D. L., & DiCosimo, R. (1997). Pyruvic acid production using methylotrophic yeast transformants as catalyst. *Journal of Molecular Catalysis B: Enzymatic*, *2*(4-5), 223-232.

[12] Enright, A., Alexandre, F.-R., Roff, G., Fotheringham, I. G., Dawson, M. J., & Turner, N. J. (2003). Stereoinversion of β-and γ-substituted α-amino acids using a chemo-enzymatic oxidation–reduction procedure. *Chemical communications*(20), 2636-2637.

[13] Hanson, R. L., Davis, B. L., Goldberg, S. L., Johnston, R. M., Parker, W. L., Tully, T. P., . . . Patel, R. N. (2008). Enzymatic preparation of a D-amino acid from a racemic amino acid or keto acid. *Organic Process Research & Development*, *12*(6), 1119-1129.

[14] Hossain, G. S., Li, J., Shin, H.-d., Chen, R. R., Du, G., Liu, L., & Chen, J. (2014). Bioconversion of l-glutamic acid to α-ketoglutaric acid by an immobilized whole-cell biocatalyst expressing l-amino acid deaminase from Proteus mirabilis. *Journal of biotechnology*, *169*, 112-120.

[15] Hossain, G. S., Li, J., Shin, H.-d., Du, G., Wang, M., Liu, L., & Chen, J. (2014). One-step biosynthesis of α-keto-γ-methylthiobutyric acid from L-methionine by an Escherichia coli whole-cell biocatalyst expressing an engineered L-amino acid deaminase from proteus vulgaris. *PloS one*, *9*(12), e114291.

[16] Hossain, G. S., Li, J., Shin, H.-d., Liu, L., Wang, M., Du, G., & Chen, J. (2014). Improved production of α-ketoglutaric acid (α-KG) by a Bacillus subtilis whole-cell biocatalyst via engineering of L-amino acid deaminase and deletion of the α-KG utilization pathway. *Journal of biotechnology*, *187*, 71-77.

[17] Hossain, G. S., Shin, H.-d., Li, J., Du, G., Chen, J., & Liu, L. (2016). Transporter engineering and enzyme evolution for pyruvate production from D/L-alanine with a whole-cell biocatalyst expressing L-amino acid deaminase from Proteus mirabilis. *RSC advances*, *6*(86), 82676-82684.

[18] Hou, Y., Hossain, G. S., Li, J., Shin, H.-d., Du, G., & Liu, L. (2016). Combination of phenylpyruvic acid (PPA) pathway engineering and molecular engineering of l-amino acid deaminase improves PPA production with an Escherichia coli whole-cell biocatalyst. *Applied microbiology and biotechnology*, *100*(5), 2183-2191.

[19] Hou, Y., Hossain, G. S., Li, J., Shin, H.-d., Liu, L., & Du, G. (2015). Production of phenylpyruvic acid from L-phenylalanine using an L-amino acid deaminase from Proteus mirabilis: comparison of enzymatic and whole-cell biotransformation approaches. *Applied microbiology and biotechnology*, *99*(20), 8391-8402.

[20] Ju, Y., Tong, S., Gao, Y., Zhao, W., Liu, Q., Gu, Q., . . . Zhou, H. (2016). Crystal structure of a membrane-bound L-amino acid deaminase from Proteus vulgaris. *Journal of Structural Biology*, *195*(3), 306-315.

[21] Kataoka, N., Matsutani, M., Yakushi, T., & Matsushita, K. (2015). Efficient production of 2, 5-diketo-D-gluconate via heterologous expression of 2-ketogluconate dehydrogenase in Gluconobacter japonicus. *Applied and environmental microbiology*, *81*(10), 3552-3560.

[22] Li, R., Sakir, H. G., Li, J., Shin, H.-d., Du, G., Chen, J., & Liu, L. (2017). Rational molecular engineering of L-amino acid deaminase for production of α-ketoisovaleric acid from L-valine by Escherichia coli. *RSC advances*, *7*(11), 6615-6621.

[23] Liu, L., Hossain, G. S., Shin, H.-d., Li, J., Du, G., & Chen, J. (2013). One-step production of α-ketoglutaric acid from glutamic acid with an engineered l-amino acid deaminase from Proteus mirabilis. *Journal of biotechnology*, *164*(1), 97-104.

[24] Lütke-Eversloh, T., Santos, C. N. S., & Stephanopoulos, G. (2007). Perspectives of biotechnological production of L-tyrosine and its applications. *Applied microbiology and biotechnology*, *77*(4), 751-762.

[25] Meiswinkel, T. M., Rittmann, D., Lindner, S. N., & Wendisch, V. F. (2013). Crude glycerol-based production of amino acids and putrescine by Corynebacterium glutamicum. *Bioresource technology*, *145*, 254-258.

[26] Miyazaki, M., Shibue, M., Ogino, K., Nakamura, H., & Maeda, H. (2001). Enzymatic synthesis of pyruvic acid from acetaldehyde and carbon dioxide. *Chemical communications*(18), 1800-1801.

[27] Molla, G., Melis, R., & Pollegioni, L. (2017). Breaking the mirror: L-amino acid deaminase, a novel stereoselective biocatalyst. *Biotechnology Advances*, *35*(6), 657-668.

[28] Motta, P., Molla, G., Pollegioni, L., & Nardini, M. (2016). Structure-function relationships in L-amino acid deaminase, a flavoprotein belonging to a novel class of biotechnologically relevant enzymes. *Journal of Biological Chemistry*, *291*(20), 10457-10475.

[29] Pantaleone, D. P., Geller, A. M., & Taylor, P. P. (2001). Purification and characterization of an L-amino acid deaminase used to prepare unnatural amino acids. *Journal of Molecular Catalysis B: Enzymatic*, *11*(4-6), 795-803.

[30] Parmeggiani, F., Ahmed, S. T., Thompson, M. P., Weise, N. J., Galman, J. L., Gahloth, D., . . . Turner, N. J. (2016). Single-biocatalyst synthesis of enantiopure D-arylalanines exploiting an engineered D-amino acid dehydrogenase. *Advanced Synthesis & Catalysis*, *358*(20), 3298-3306.

[31] Parmeggiani, F., Lovelock, S. L., Weise, N. J., Ahmed, S. T., & Turner, N. J. (2015). Synthesis of D-and l-phenylalanine derivatives by phenylalanine ammonia lyases: a multienzymatic cascade process. *Angewandte Chemie*, *127*(15), 4691-4694.

[32] Payne, M. S., Petrillo, K. L., Gavagan, J. E., Wagner, L. W., DiCosimo, R., & Anton, D. L. (1995). High-level production of spinach glycolate oxidase in the methylotrophic yeast Pichia pastoris: engineering a biocatalyst. *Gene*, *167*(1-2), 215-219.

[33] Pollegioni, L., & Molla, G. (2011). New biotech applications from evolved D-amino acid oxidases. *Trends in biotechnology*, *29*(6), 276-283.

[34] Pollegioni, L., Motta, P., & Molla, G. (2013). l-Amino acid oxidase as biocatalyst: a dream too far? *Applied microbiology and biotechnology*, *97*(21), 9323-9341.

[35] Pollegioni, L., Sacchi, S., Caldinelli, L., Boselli, A., Pilone, M. S., Piubelli, L., & Molla, G. (2007). Engineering the properties of D-amino acid oxidases by a rational and a directed evolution approach. *Current Protein and Peptide Science*, *8*(6), 600-618.

[36] Qin, Y., Johnson, C. H., Liu, L., & Chen, J. (2011). Introduction of heterogeneous NADH reoxidation pathways into Torulopsis glabrata significantly increases pyruvate production efficiency. *Korean Journal of Chemical Engineering*, *28*(4), 1078-1084.

[37] Song, Y., Li, J., Shin, H.-d., Du, G., Liu, L., & Chen, J. (2015). One-step biosynthesis of α-ketoisocaproate from l-leucine by an Escherichia coli whole-cell biocatalyst expressing an l-amino acid deaminase from Proteus vulgaris. *Scientific reports*, *5*, 12614.

[38] Song, Y., Li, J., Shin, H.-d., Liu, L., Du, G., & Chen, J. (2016). Biotechnological production of alpha-keto acids: current status and perspectives. *Bioresource technology*, *219*, 716-724.

[39] Song, Y., Li, J., Shin, H.-d., Liu, L., Du, G., & Chen, J. (2017). Tuning the transcription and translation of L-amino acid deaminase in Escherichia coli improves α-ketoisocaproate production from L-leucine. *PloS one*, *12*(6), e0179229.

[40] Sonoyama, T., Yagi, S., Kageyama, B., & Tanimoto, M. (1984). Method for preparing 2, 5-diketo-d-gluconic acid. In: Google Patents.

[41] Sulo, P., Hudecová, D., Properová, A., Bašnák, I., & Sedláček, I. (2001). 2, 5-Diketo-D-gluconate production by a mixed culture of two newly-isolated strains: Flavimonas oryzihabitans and Pseudomonas cepacia. *Biotechnology letters*, *23*(9), 693-696.

[42] Takahashi, E., Ito, K., & Yoshimoto, T. (1999). Cloning of L-amino acid deaminase gene from Proteus vulgaris. *Bioscience, biotechnology, and biochemistry*, *63*(12), 2244-2247.

[43] Turner, N. J. (2004). Enzyme catalysed deracemisation and dynamic kinetic resolution reactions. *Current opinion in chemical biology*, *8*(2), 114-119.

[44] Van Maris, A. J., Geertman, J.-M. A., Vermeulen, A., Groothuizen, M. K., Winkler, A. A., Piper, M. D., . . . Pronk, J. T. (2004). Directed evolution of pyruvate decarboxylase-negative Saccharomyces cerevisiae, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. *Applied and environmental microbiology*, *70*(1), 159-166.

[45] Vogt, M., Haas, S., Polen, T., van Ooyen, J., & Bott, M. (2015). Production of 2-ketoisocaproate with C orynebacterium glutamicum strains devoid of plasmids and heterologous genes. *Microbial biotechnology*, *8*(2), 351-360.

[46] Waters, K. L. (1947). The a-Keto Acids. *Chemical Reviews*, *41*(3), 585-598.

[47] Wieschalka, S., Blombach, B., & Eikmanns, B. J. (2012). Engineering Corynebacterium glutamicum for the production of pyruvate. *Applied microbiology and biotechnology*, *94*(2), 449-459.

[48] Xu, G., Hua, Q., Duan, N., Liu, L., & Chen, J. (2012). Regulation of thiamine synthesis in Saccharomyces cerevisiae for improved pyruvate production. *Yeast*, *29*(6), 209-217.

[49] Yang, S., Chen, X., Xu, N., Liu, L., & Chen, J. (2014). Urea enhances cell growth and pyruvate production in Torulopsis glabrata. *Biotechnology progress*, *30*(1), 19-27.

[50] Zhu, Y., Eiteman, M. A., Altman, R., & Altman, E. (2008). High glycolytic flux improves pyruvate production by a metabolically engineered Escherichia coli strain. *Applied and environmental microbiology*, *74*(21), 6649-6655.

[51] Zhu, Y., Liu, L., Zhou, J., Fang, J., Li, J., Du, G., & Chen, J. (2011). Medium optimization for biosynthesis of alpha-ketoisocaproate by Rhodococcus opacus DSM 43250. *Chinese Journal of Bioprocess Engineering*, *9*(6), 6-10.

[52] Zou, X., Hang, H.-f., Chu, J., Zhuang, Y.-p., & Zhang, S.-l. (2009). Oxygen uptake rate optimization with nitrogen regulation for erythromycin production and scale-up from 50 L to 372 m3 scale. *Bioresource technology*, *100*(3), 1406-1412.