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Challenges and Solutions in D-Amino Acid Production Methods

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Abstract— *D-amino acids play a vital role in the pharmaceutical, food, and chemical industries for the manufacture of antibiotics, fertility drugs, sweeteners, and drugs against neural disorders. Examples include; they can be components in analgesics, antistress agents, antidiabetics (e.g., nateglinide), and anticoagulants. Therefore, industrial production of D amino acids is required, and owing to this, research has progressed while aiming towards improving yield and reducing the cost of production. However, to optimize large-scale production, challenges faced in currently used production methods must first be exposed for possible solutions. In this review, we provide a background of recent methods utilized, challenges in these methods, and solutions to enhance the production of D-amino acids.*

Keywords— ***D-amino acids, pharmaceutical industry, Industrial production, Production methods, Challenges and solutions***

I. INTRODUCTION

Amino acids except Glycine have a chiral center (Alpha Carbon) onto which is attached an amine group, carboxyl group, R group, and hydrogen atom. The arrangement of these four groups around the chiral center forms two enantiomers; L-amino acids and d-amino acids. L-amino acids are the most dominant and functionally common enantiomer and these universally provide building blocks for proteins made in all living organisms. Recently, Researchers have gained interest in D-amino acids. Among these, D-Alanine, and D-Glutamic acid have been identified for years as fundamental blocks in microbial physiology where they are key constituents of the peptidoglycan. (Hancock, 1960) The existence of D-amino acids in the cell wall of bacteria (Peptidoglycan) offers a protective role against destruction from some proteases since D-enantiomers would go unrecognized by the enzymes. (unnatural forms)

Bacteria, such as Vibrio Cholera and Bacillus Subtilis secrete D-amino acids other than D-Glutamate and D-Alanine; these are collectively called Non-Canonical D amino acids (NCDAAs) as they are not involved in peptidoglycan layer synthesis although some have been found to regulate its structure.

It was found by Lam et al (2009) that stationary phase supernatant fractions of wild-type *V. cholerae* consisted of four amino acids: Met, Leu, Val, and Ile D-forms, and not L-forms, of these amino acids. This shows their role in bacterial stress adaption and competing against non-D-amino acid-producing bacteria. It has also been researched that D-amino acids not only take part in the peptidoglycan layer synthesis but also regulate spore germination and biofilm dispersal in certain species. (Bucher et al., 2015)

D-Amino acids are produced following a reversible stereo-inversion of groups around the chiral carbon and such a reaction is catalyzed by a group of enzymes called racemases. Two types exist in bacteria; highly specific and

broad-spectrum racemases (Bsr). (Hernández and Cava, 2016) Bsr can catalyze racemization reactions of a wide range of amino acids although their activities may be lower than the specific racemases. It is interesting to note that most gram-negative bacteria occupying a wide range of environments contain Bsr such as soil, marine water, or animals. (Espaillat et al., 2014) This suggests a possible role of racemases in colonizing hush ecosystems by rendering bacteria the ability to utilize D-amino acids as Carbon and Nitrogen sources present in such environments. (Kubota et al., 2016)

II. APPLICATIONS OF D-AMINO ACIDS

The use of D-amino acids in antimicrobial agents is an obvious role owing to their ability to suppress bacterial growth and biofilm dispersion. Li et al (2016) found out that optimized concentration of a mixture of D-methionine, D-tyrosine, D-leucine, and D-tryptophan greatly enhanced THPS biocide treatment of two recalcitrant biofilm consortia containing sulfate-reducing bacteria (SRB), nitrate-reducing bacteria (NRB), and fermentative bacteria. In the latter case, it is noted that the D-amino acids were utilized as Biocide enhancers hence reducing bacterial biocide resistances.

D-amino acids also find significant roles in neural system health, where low or high levels would trigger disease; therefore, correcting such levels within human bodies has been seen as a potential therapy. Several reports have come out about the role of particular D-amino acids in improving emotion, memory, and cognitive ability, and as medication in neural conditions such as Parkinson's disease. An example of this is low concentrations of D-Serine (25% decrease compared to normal) in cerebrospinal fluid and significant reduction of serine racemase in the hippocampus and frontal cortex of schizophrenia patients. (Bendikov et al., 2007)

Tsai et al (1998) studying the treatment of schizophrenia found out that the symptoms improved through orally administrating D-Ser 30 mg/kg daily. At the end of the 6-week trial, a 17% reduction had occurred in the positive symptoms and a 21% reduction had occurred in the negative symptoms of the disease. D-Serine is a strong endogenous co-agonist of N-methyl-d-aspartate receptors present in the hypothalamic structure, and schizophrenia arises following hypofunction of NMDAR evident with a reduction in D-Serine concentration. It should also be noted that stimulation of NMDAR by D-amino acids (D-serine and D-Alanine) plays a role in the analgesic effect and protection of the nervous system. (Shi et al., 2022)

It has been reported by Hartman et al., (2015) that D leucine has a positive effect on seizures by attenuation of the long-term potentiation (LTP) in the hippocampus. This gives D-Leucine an advantage over other medications which completely inhibits LTP and this may impair learning and memory. (Hartman et al, 2015)

D-amino acids also find possible application in the food industry as sweeteners particularly D-valine, D-phenylalanine, and D-tryptophan owing to having a sweeter taste and flavor compared to the L-enantiomers. Of particular interest is Alitame, an artificial dipeptide sweetener containing L-aspartate and D-Alanine having over 10 times sweetening power when compared to aspartame and six times when compared to saccharin. (Chattopahyay et al., 2014)

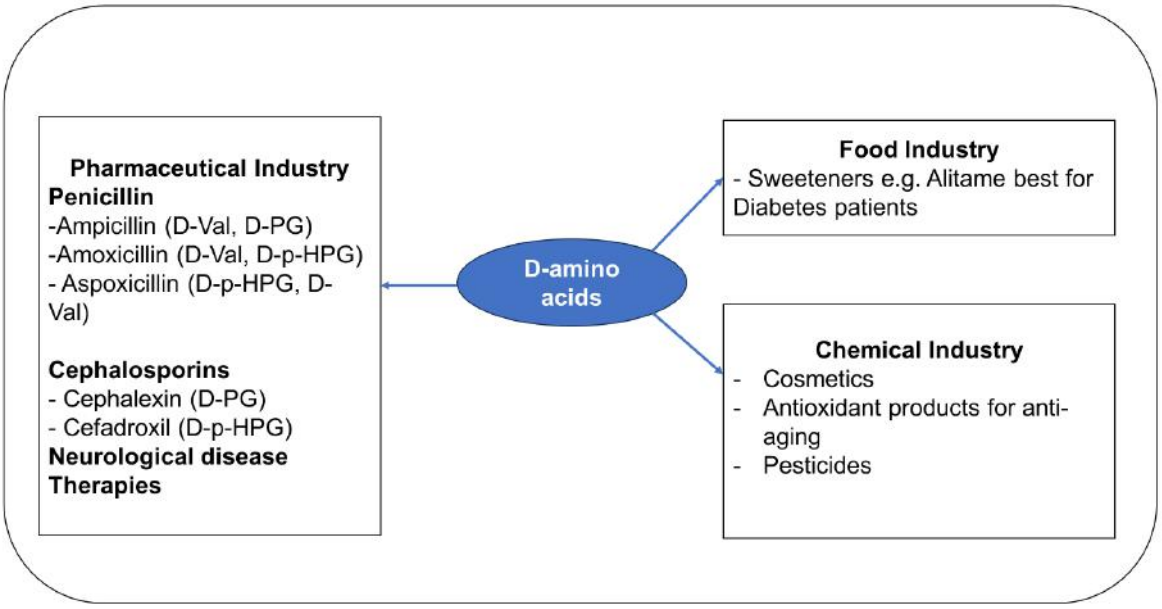

Fig 1: Examples of fields in which synthesis of D-amino acids is of great importance. In antibiotics, illustrated D-amino acids function as a core in the chemical structure.

III. BIOCATALYTIC METHODS FOR PRODUCTION OF D-AMINO ACIDS AND CHALLENGES INVOLVED

Several enzymes are utilized in biotransformation to form D-amino acids from a variety of substrates; D, L amino acid mixtures, N-acyl D, L amino acids, D, L-hydantoin, D, L-amides, α–keto acids and L-amino acids. Among these, L-amino acid substrates are the cheapest sources since they can be readily available from fermentation processes of engineered strains starting from cheap carbon and nitrogen sources. Biocatalysis can be both in-vivo; where whole cells are engineered to synthesize and secrete D-amino acids, or in-vitro where enzymes are extracted, purified, and modified to catalyze reactions in vessels, solutions, or surfaces.

1.) D-amino acids production from Alpha keto acids

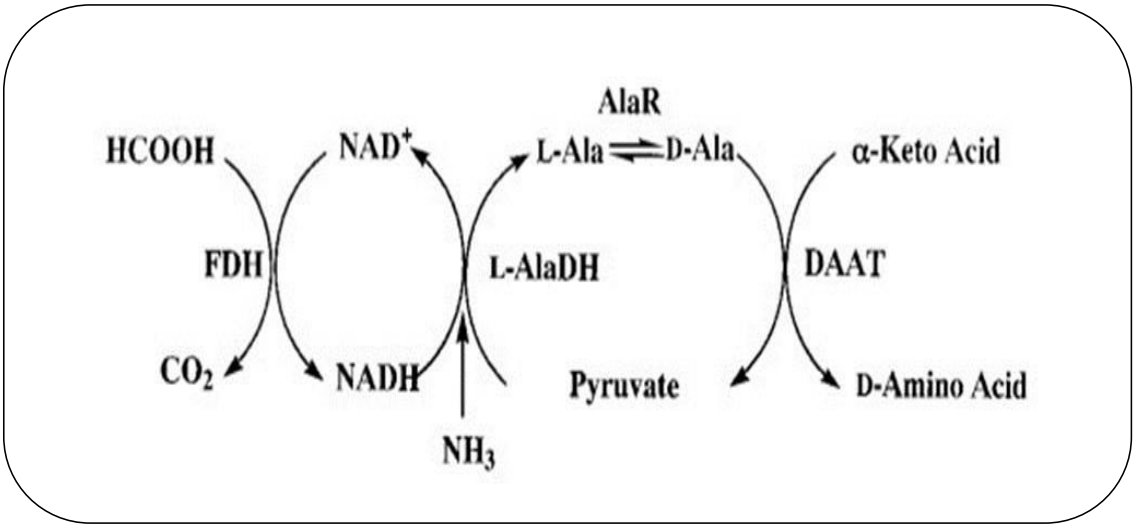

Fig 2: (Galkin et al., 1997) illustration of one of the earliest proposed methods for the production of D-amino acids particularly from corresponding alpha-keto acids.

Fig 1 demonstrates a scheme composed of Formate dehydrogenase (FDH) that generates NADH, L-Alanine Dehydrogenase which incorporates ammonia to pyruvate to generate L-Alanine, Alanine Racemase (AlaR) that catalyzes interconversion between L and D-Alanine, and lastly, D-amino acid aminotransferase (DAAT) that catalyzes transfer of amino group from D-Alanine to an alpha-keto acid to form corresponding D-amino acids. L-Alanine dehydrogenase (L-AlaDH) functions to regenerate L-Alanine from pyruvate by incorporation of ammonia. Galkin et al (1997) reported the production of D enantiomers of glutamate and leucine at high optical purities and high conversion rates obtaining a concentration of about 44g/l D-glutamate following heterologous expression of FDH, AlaDH, DAAT, and AlaR genes in E. Coli.

However, the main problem faced while using the strategy was the production of D-amino acids as racemic mixtures probably as a result of catalysis by L-amino acid aminotransferases to form L amino acids from the provided alpha-keto acids. Economically, alpha-keto acids are also expensive, limiting the application for large-scale profit production.

Such a racemic result problem could be solved by using the L-amino acid oxidase enzyme which oxidizes only L-amino acids leaving behind D-enantiomers for further purification. However, it has been investigated that L-amino acid oxidase generates hydrogen peroxide which limits the enzyme activity. Sang et al (2022) utilized Aromatic Amino acid decarboxylase (AADC) from Bacillus atrophaeus as an alternative to L-amino acid oxidase for the purification of aromatic D amino acids from racemic mixtures. This enzyme offered an advantage owing to catalytic decarboxylation of a variety of aromatic L-amino acids to respective mono-amines without the generation of Hydrogen peroxide. Results from enantioselectivity studies of AADC also showed that the enzyme offers efficient kinetic resolution without undesirable loss of D-amino acid.

2.) Production via expression of hydantoin racemase, d-hydantoinase and *N*-carbamoyl-d-amino acid amidohydrolase in E-coli (In vivo Hydantoinase process)

Hiroyuki et al (2005) utilized a system for enzymatic production of D-amino acids in whole cells by co-expression of three genes encoding for D-specific hydantoinase from Microbacterium liquefaciens AJ3912 (DHHase), *N*-carbamoyl-D-amino acid amidohydrolase (DCHase), and hydantoin racemase (HRase) from Flavobacterium sp. AJ11199 in *E. Coli*. The system works

in such a way that substituted 5-monosubstituted L-Hydantoin molecules corresponding to a particular amino acid are converted first to 5-monosubstituted D-hydantoin molecules, which are then linearized by hydrolysis catalyzed by DHHase forming an N-carbamoyl-D-amino acid derivative. The latter molecule is then hydrolyzed by DCHase forming the corresponding D-amino acid. Using this platform, D-Phe, D-Tyr, *O*-benzyl-D-Ser, D-Leu, D-norvaline, and D-norleucine proceeded efficiently, achieving a 98% molar yield after 48 h and an optical purity of more than 99% e.e. (Hiroyuki et al., 2005)

The whole-cell biocatalyst's advantage was the efficient production of a wide range of D-amino acids from corresponding 5-monosubstituted-L-hydantoins owing to the broad specificity of the three enzymes used. However, the challenge was the need for the production and purification of the substrate (Substituted Hydantoin derivatives) which could be expensive for commercial production of the D-amino acids. Also, the production of the substrate requires potassium cyanate which is a toxic chemical compound. (Suzuki et al., 2005)

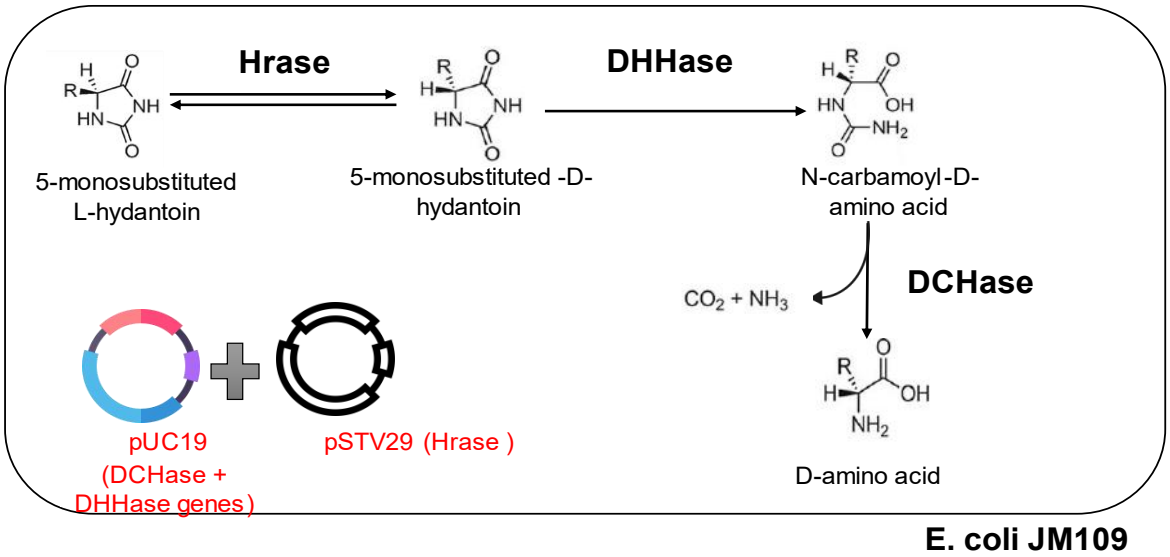

Fig 3: illustration of biotransformation platform utilized by Hiroyuki et al (2005); enzymes DCHase, and DHHase were expressed via pUC19 plasmid, while pSTV29 plasmid was used to express Hrase enzyme both utilizing trp promoter. The co-expression was carried in E. coli JM109 cells.out

In-vitro Hydantoinase process for production of D-amino acids

Similar to the whole cell (in-vivo) hydantoinase process employed by Hiroyuki et al (2005) optically pure D-amino acids can also be produced by hydrolysis of their respective L-5-monosubstituted hydantoins in a similar classical three-enzyme cascade reaction, (dynamic kinetic resolution cascade process) carried out in-vitro as illustrated in figure 2. (Yafei et al., 2017) This method offers no need for intermediate removal and purification since it offers 100% theoretical conversion. (Yamaguchi et al., 2007) Although theoretically strong, 100% bioconversion cannot be achieved owing to the thermal instability of enzymes mainly D-Carbamoylase, Insolubility of enzymes, and sparingly soluble Hydantoins owing to these challenges, several research efforts towards searching for highly compatible and thermostable enzymes have been ongoing. Yafei et al (2017) identified a d-carbamoylase from Arthrobacter *crystallopoietes* (*Ac*HyuC) through genome mining and screening of Carbamoylases with pH compatibility, high activity, enantioselectivity, and solubility to establish a DKR cascade for efficient production of d-Trptophan from l-indolylmethylhydantoin. With this enzyme used in the cascade, 80 mM L-indoylmethylhydantion could be completely converted into d-tryptophan within 12 h in a 0.5-L system, reaching a yield of 99% and productivity of 36.6 g L^{-1} d^{-1}.

Chemical methods combined with biocatalysis have been widely used for the synthesis of D-amino acids; however, such methods are so expensive requiring high starting material, low specificity, and high need for purification from toxic chemicals. To solve this problem, microbial production of D-amino acids is seen as a potential solution through stereoisomeric conversion of L-amino acids to D-amino acids. L-amino acids are mostly generated by fermentation from inexpensive and renewable natural sources, therefore providing a cheaper way.

3.) Preparation of D-amino acids from DL mixtures via Selective L-amino acid Degradation

Recent studies have shown that microorganisms use mainly L-amino acids although they may also have the ability to utilize D-amino acids as Carbon and Nitrogen sources. Some strains showed asymmetric degrading activity against DL-amino acids and degraded only the L-enantiomer. (Takahashi et al., 1997) Zhang et al (2015) isolated and characterized Candida maltose DLPU-zpb which could degrade L-valine effectively but not D-valine, and based on this, a preparation method for D-valine was suggested from DL-valine mixture using the yeast strain, Candida maltose DLPU-zpb as a biocatalyst.

Zhang et al (2015) noted that DL-valine is produced commercially at a low cost via racemization of L-valine and therefore suggested that Candida maltose DLPU-zpb biocatalyst provides a cheap option for industrial production of D-valine.

However, we noted that although the L-isomer was completely degraded within 72 h under the conditions of 30°C and at pH 6, the final yield was dependent on the D-amino acid concentration in DL-amino acid mixtures. Therefore, this method only purifies but does not change the initial concentration of D-amino acids as produced by upstream racemization procedures. L-enantiomers' degradation leads to a high loss of resources since several methodologies utilize L-amino acids as starting raw materials for D amino acid synthesis. Therefore, we suggest that such a method requires a recycling process instead of only degradation to minimize the loss of useful L-enantiomers.

To prevent wasteful loss of L-amino acids, acetylation of the DL-amino acid mixture can be utilized together with *N*-acyl-D-amino acid amidohydrolase which selectively removes the acetyl group from only N-acyl-D-amino acids to form a corresponding D-amino acid. (Wakayama and Moriguchi, 2001) The un-converted N-acyl-L-amino acid can then be racemized to N-acyl-DL-amino acid for continued reaction.

4.) Biosynthesis via in-vitro dynamic kinetic resolution of N-succinyl amino acids

D-Phenyl alanine, D-Tryptophan, and D-Valine can be easily synthesized from corresponding N-Succinyl-L-amino acids or racemic mixtures of N-Succinyl-DL-amino acids via in-vitro catalysis by purified N-Succinyl amino acid racemase (NSAR) and D-Succinylase (DSA) (Sumida et al., 2018) In the suggested one-pot dynamic kinetic resolution, NSAR catalyzes the interconversion between D and L forms of N-Succinyl amino acids while DSA catalyzes the removal of Succinyl group from N-succinyl-D-amino acids to form an unconjugated D-amino acid form. Using this strategy, Sumida et al (2018) were able to convert 100 mM *N*-succinyl-DL-tryptophan, N-Succinyl-DL Phenylalanine, and N-Succinyl-DL-Valine to corresponding D-amino acids at 81.8% yield with 94.7% ee under optimized concentrations of DSA and NSAR

However, it is reported that the chiral purity of the D- amino acids is dependent on the ratios of DSA and NSAR, and adjusting such a concentration ratio is not feasible for industrial applications. Therefore, despite the promising industrial application, genetic engineering of DSA is required to improve the optical purity of the D-amino acids by minimizing side chain reactions with N-Succinyl-L-amino acids that would lead to the formation of L amino acids.

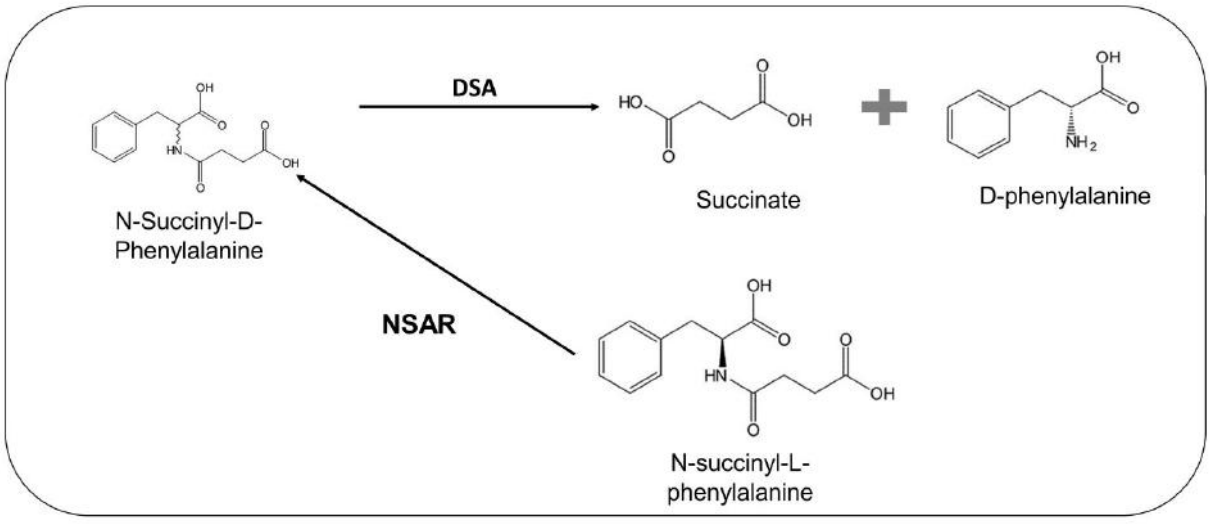

Fig 4: illustration of bioconversion of N-succinyl-D-phenylalanine to D-phenylalanine by DSA enzyme. NSAR functions to generate the D-enantiomer from N-succinyl-L-phenylalanine for further formation of D-phenylalanine.

5.) D-Tryptophan synthesis via engineered D-amino acid aminotransferase in a one-pot biocatalytic system

It has been investigated that complete stereo inversion of the L-Phenyl alanine could be achieved by combining oxidative deamination of L-amino acids using Proteus mirabilis LAAD (PmirLAAD) with transamination of D-amino acids using an engineered DAAT from Bacillus sp. YM-1 (DAAT-T242G) (Walton et al., 2018) Using this background, Parmeggiani et al (2019) combined mutations; V33G, S240G, and T242G with the idea that these will further increase the size of the binding pocket of DAAT. Out of the three combinations, DAAT-V33G/T242G) was the most active variant showing a 35-fold improvement to kcat/KM against D-tryptophan compared with the wild-type towards stereo inversion of L-Tryptophan to D-Tryptophan.

To further expand such an improved activity, a one-pot biocatalytic system utilizing Salmonella Tryptophan synthase (TrpS), LAAD from Proteus myxofaciens, and the engineered DAAT-V33G/T242G was developed in which D-Tryptophan was synthesized from indoles. With optimized substrate and reaction conditions, around 5g/l of D-Tryptophan was obtained with a percentage yield of 66%

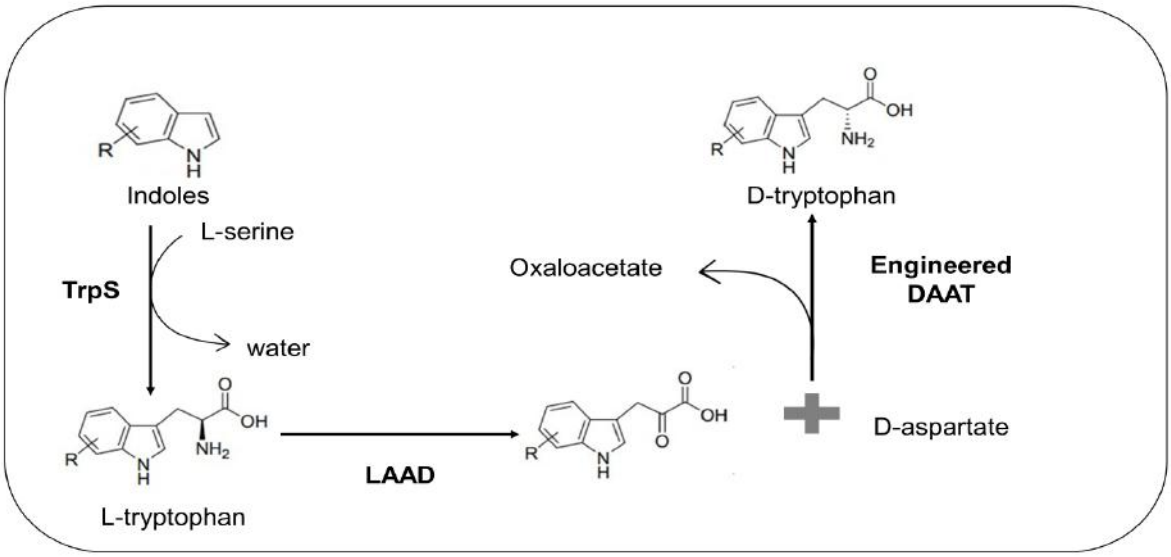

Fig 5: illustration of D-Tryptophan synthesis from indoles. It should be noted that R in the illustration represents Hydrogen atom in the case of Tryptophan. However more substituted D-tryptophans can be synthesized using the platform and for this case, R can be Fluoride, Chloride, Methyl, or Methoxy groups.

There are a few challenges with the one-pot biosynthesis of D Tryptophan from indoles; the DAAT enzyme requires a D-amino acid as an amino donor, the best substrate being D-glutamate which is expensive. Although the enzyme can also utilize D-Aspartate which is cheaper, a lower activity was reported as compared to using D-glutamate. This problem has been solved by the utilization of *meso*-diaminopimelate dehydrogenases which catalyze reductive amination of 2-keto acids such as pyruvic acid to generate d-amino acids in up to 99% conversion and 99% enantiomeric excess using inorganic ammonium ions. (Zhang et al., 2019; Gao et al., 2012) The second problem noted in this platform that may contribute to a reduction in yield is the utilization of separate enzyme systems; LAAD and DAAT are expressed in separate E. coli BL21(DE3), and TrpS is expressed in vitro. This means the conversion rate will be dependent and limited by the translocation of reaction intermediates between the media and the cells. The third challenge is that the production of L-Tryptophan intermediate from indoles is dependent on the presence of L-Seine in the reaction media, which therefore has to be provided. Scaling up such a platform to an industrial level would be economically challenging owing to the need for both L-Serine and D-Aspartate as mixture components.

6.) One Pot Biocatalytic stereo inversion cascade (In-vivo and in-vitro biocatalytic components)

Zhang et al (2019) utilized recombinant Escherichia *coli* BL21(DE3) cells for expression of L-Amino acid deaminase from *Proteus mirabilis* (PmLAAD) and purified *meso*-diaminopimelate dehydrogenases (DAPDHs) in a cascade route for the synthesis of D-amino acids from L-amino acids. This model was used to efficiently catalyze the transformation of L-phenylalanine into D-phenylalanine.

Studies showed that pmLAAD enzyme is membrane-bound and so a whole-cell biocatalyst with intact membranes

produces higher reaction than when utilized within in-vitro reactions. (Hou et al., 2016) Through optimization procedures, pmLAAD (whole cell biocatalyst) was found to have no activity on D-phenylalanine and Phenylphyruvic acid, however, with the ability to convert L-phenylalanine to Phenylpyruvic acid PPA at a yield of 58.7% 100 mM of substrate. Another advantage of pmLAAD enzyme is the ability to catalyze deamination without the generation of Hydrogen Peroxide, which would be toxic to the cells.

Screening results from various DAPDH enzymes showed StDAPDH/H227V mutant to have the highest activity towards the conversion of Phenylpyruvic acid to over 99% optically pure D-Phenylalanine, with no activity on D and L phenylalanine which means that the direction of reaction (equilibrium) is towards the formation of D-Phenylpyruvate. Since DAPDH/H227V is dependent on NADPH, *Burkholderia stabilis* formate dehydrogenase (BsFDH) produced and purified from recombinant E. coli was included in the cascade to generate the required co-factor. PmLAAD whole-cell biocatalyst (100 mg mL^{-1}), StDAPDH/H227V (4 mg mL^{-1}), and BsFDH (0.35 mg mL^{-1}, 1 U) were assembled to construct the one-pot stereo inverting cascade. Subsequently, the D-Phe was obtained in a yield of 76.2% by the addition of 30 mM L-Phe, 90 mM NH_4Cl, 60 mM sodium formate, and 3 mM $NADP^+$ to the 50 mM Tris–HCl (pH 9.0) reaction buffer during the cascade reaction at 45 °C (Zhang et al., 2019)

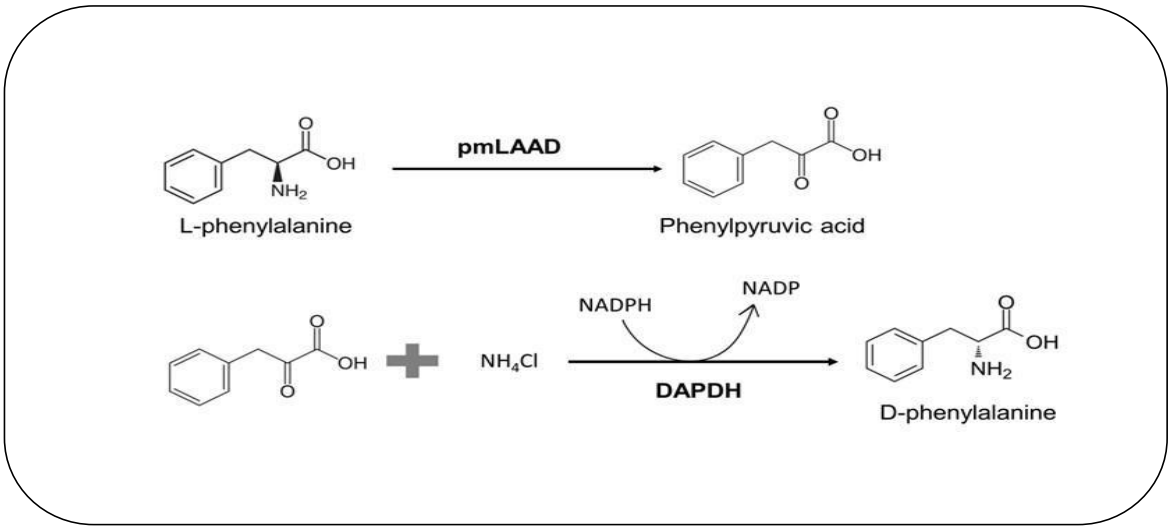

Fig 6: illustration of a two-step stereo inversion proposed by Zhang et al., NADPH is regenerated from NADP by BsFDH enzyme in the presence of Formate ions.

The biocatalytic stereo-inversion cascade also showed biotransformation efficiencies of 70%, 100%, 75%, 45%, 100%, 100%, 100%, and 100% for L-leucine, L-Glutamic acid, L-Norvaline, L-Tyrosine, L-Phenylalanine, L-Homophenylalanine, 2-Chloro-L-phenylalanine, 3-Chloro-L-phenylalanine and 4-Chloro-L-phenylalanine showing a reasonable versatility. Possible challenges with the cascade include the need for purification steps of BsFDH and StDAPDH/H227V enzymes and proper optimization of the reaction conditions, considering all three biocatalysts.

Also, the Phenylpyruvic acid intermediate from pmLAAD needs to be transferred through the cell membrane for the connection between the two necessary steps of the stereo-inversion reaction, and this would affect the conversion efficiency of the entire biocatalytic system. This challenge has been solved by incorporation of the three enzymes in a single cell for whole-cell biocatalysis as described by Zhang et al (2021)

7.) Whole-cell in-vivo Stereoinversion (pmLAAD, DAPDH, and BsFDH co-expression in E. coli)

To modify the previous one-pot stereo inversion, Zhang et al (2021) developed an in vivo cascade cell factory by co-expression of the three enzymes; L-amino acid deaminase from *Proteus mirabilis* (LAAD), *meso*-diaminopimelate dehydrogenase from *Symbiobacterium thermophilum* (DAPDH), *and* formate dehydrogenase from *Burkholderia stabilis* (fdh) into *E. Coli*. By using the obtained *E. coli* pET-21b-MBP-*laad*/pET-28a-*dapdh-fdh* whole-cell biocatalyst under optimized conditions, L-Phenyl alanine was stereo-inverted to D-Phenyl alanine with high conversion efficiency and optical purity.

Appling the whole cell (*E. coli* pET-21b-MBP-*laad*/pET-28a-*dapdh-fdh*) biocatalyst for the transformation of a variety of aliphatic L-amino acids as substrates, only L-Leu, L-norvaline, L-Glu, and L-Met were transformed to

corresponding D-amino acids obtained in quantitative conversion. (Zhang et al., 2021)

However, it has been reported in recent studies by Yanqi et al (2023) that high concentrations of some D-Amino acids inhibit bacterial growth. This was significantly observed with D-arginine, D-Leucine, D-methionine, D-valine, D-Cysteine, and D-glutamate. Therefore, this approach is likely to be limited to the production of low concentrations as the whole-cell biocatalyst would seem to be inhibited at high D-amino acid concentrations. Since Zhang et al (2023) reported high conversion efficiency, the yield can be improved by utilization of the acetylation strategy described in the next section as reported by Yanqi et al (2023)

IV. CURRENT STRATEGIES UTILISED FOR ENHANCED D-AMINO ACID PRODUCTION

a) Reduction of toxicity via D-amino acid production in E-Coli as Acetylated forms

For microbial cells to be used successfully as D-amino acids production factories, the toxicity of D-amino acids towards the host cells has to be overcome. Yanqi et al (2023) used an acetylation strategy by expressing both a racemase and D amino acid N-acetyltransferase enzymes into E-coli. The use of acetyltransferase enzyme presented the importance of driving the reaction equilibrium between L and D amino acids towards the formation of the D type and also rendering D-amino acids unreactive hence reducing toxicity towards the development of the host cells.

To demonstrate the role of acetylation of D-amino in the reduction of toxicity to cells, Yanqi et al (2023) cultured BW25113 (wild type, WT) E-Coli cells in an M9 liquid medium containing nineteen natural D-amino acids at 10 g/L, and results showed significant growth inhibition from 15 D-Amino acids, however, it is D-Asp, D-Glu, D-Pro, and D-His, did not cause growth defects at this concentration. When a similar procedure was repeated with acetylated forms of D-Arginine, D-Leucine, D-methionine, D-phenylalanine, D-Valine, and D-Serine included in the growth medium, there was a general increase in OD at 600nm showing that acetylation of D-amino acids offers a protective role against D-amino acid toxicity.

It was found that Yeast cells can continue growth even in the presence of D-amino acids. Yow et al (2006) found out that the enzyme; D-amino acid-*N*-acetyltransferase (DNT) in Yeast was essential in the detoxification of D-amino acids via acetylation. To reach this conclusion, results showed that a yeast mutant with the DNT gene (HPA3) knocked out was far less tolerant to D-amino acids as compared to the wild type, while over-expression of the DNT gene via p426Gal1 plasmid reversed the first result with over 100 times activity of DNT as compared to the wild type. It was also found that the production of N-acetyl D-amino acids was intracellular and then secreted to the medium. This suggests that Yeast cells are incapable of utilizing the acetylated form of D-amino acids as a nitrogen source. (Yow et al., 2006)

It is on this basis that Yanqi et al (2023) selected the heterologous expression of HPA3 from S. cerevisiae into E-Coli strain BW25113 for the production of D-amino acid transferase enzyme, an enzyme reported for the selective acetylation of a wide range of D-amino acids in vitro. (Yow et al., 2004) First, an HPA3 bioconversion assay was performed on nineteen D-amino acids to test its efficiency in E. coli. Plasmid pZElac-*hpa3* was introduced into strain BW25113 to create strain RN1 along with a negative control which were later cultured with 10g/l D-amino acids. Results showed bioconversion to acetylated D forms for 12 amino acids; Ala, Asp, Cys, Glu, Gln, Ile, Leu, Met, Phe, Ser, Thr, and Val with Ser, Glu, and Phe having the highest bioconversion yields.

A variety of Racemases are available for inter-conversion between L and D-amino acids. In E-Coli, two Ala racemases (*alr* and *dadX*), one Glu racemase (*murI*), and an Asp/Glu racemase (*ygeA*) are present and among these, YGEA has broad-spectrum catalytic activity with the ability to produce non-canonical D-amino acids other than D-Ala and D-Glu. A broad specific racemase also exists in Bacillus Subtilis encoded by the RacX gene and has a similar function to ygeA both having low catalytic activity. (Miyamoto et al., 2017) The isoleucine 2-epimerase (ILEP) from *Lactobacillus buchneri* has been previously characterized as a pyridoxal 5′-phosphate (PLP)-dependent racemase and can epimerize nonpolar L-amino acids into their D-forms (Mutaguchi et al., 2013, 2018). Additionally, the amino acid racemase BSRV from *V. cholerae* enables the bacteria to synthesize noncanonical D-amino acids (Espaillat et al., 2014). To test the efficiency of the broad specific racemases, Yanqi et al (2023) transformed Plasmids pZElac-*hpa3-alr*, pZElac-*hpa3-ygeA*, pZElac-*hpa3-bsrV*, and pZElac-*hpa3*-ILEP into strain BW25113 and results showed ILEP transformants had the ability of racemization of a wide range of amino acids; L-Ala, L-Cys, L-Gln, L-Ile, L-Leu, L-Met, L-Phe, L-Ser, L-Thr, L-Val, L-Phenylglycine, L-Norleucine, L-2-Aminobutyrate giving rise to a considerable measurable (g/L) of corresponding D-Acetyl-D-amino acids

From these results, it can be noted that some L-amino acids such as Try, Arg, and Lys are poor substrates for the ILEP enzyme. Therefore, screening of other Racemases for increased activity and directed evolution should solve this problem. Also, despite that N-acetyl transferase expression

saves E-Coli cells from D-amino acid toxicity, over-expression of the enzyme causes growth inhibition owing to acetylation effects of other proteins that could be essential in cellular activities.

b) Screening of Strains that tolerate D-amino acid toxicity

D-amino acids are toxic to E-coli cells as reported by Yanqi et al (2023) and it is on this basis that screening of other bacterial strains would improve their production without the need for acetylation strategies. It has been observed that toxicity depends on the form of D-amino acid, and the host strain. An example of this is that although the majority of D-amino acids inhibit the growth of E-coli, D-His, D-Asp, D-Glu, and D-Pro do not affect growth, while D-Leu, D-Arg, D-Met, D-Val, and D-Cys cause the highest growth inhibition. D-Ala, D-Asn, D-Ile, D-Lys, D-Phy, D-Ser, D-Thr, and D-Trp moderately inhibit growth in E-coli.

However, it was found by Stäbler et al (2011) that Corynebacterium glutamicum tolerates D-Thr, D-Arg, D-Lys, D-Ser, and D-Ala although D-Asn and D-Met inhibited the rate of growth in all cases, maximal growth (at OD 600) was attained. This was different from results observed with E-coli as maximal growth could not be attained in the presence of D-Ser, D-Val, D-Met, D-Phe, D-Leu, and D-Arg.

Comparing the susceptibility effects of E-coli and Corynebacterium glutamicum shows that the latter could tolerate the accumulation of D amino acids. It is expected that screening will unveil the existence of more strains with the ability to tolerate toxicity associated with the accumulation of D-amino acids and hence their possible use as hosts in D-amino acid production.

c) Enhanced transfer rates of substrates across the cell wall

Some precursors such as Hydantoins utilized in the synthesis of D-amino acids have low solubilities which thereby reduces biotransformation efficiencies following an increased mass transfer resistance across cell walls. Overexpression of D-carboxypeptidases A and B is reported to have a disruption on the peptidoglycan structure without affecting growth. This strategy was utilized by Yang et al (2019) by co-expression of the D-hydantoinase (Hase), N-carbamoyl D-amino acid amidohydrolase (Case), and D-carboxypeptidases A and B. Optimisation of such a system resulted in the production of 23.4 g/L of D -p - hydroxyphenyl glycine (D-p-HPG) in 32 h from DL p-Hydroxyphenyl hydantoin.

d) Enzyme engineering for improved catalytic properties

A number of enzymes involved in production of D-amino acids have been engineered for improved thermostability, broadened substrate specificity, improved catalytic activity, changing of the reaction site, and few reports on enantioselectivity. Here we have discussed a few engineering strategies identified in recent research aimed at improving D amino acid production.

i) Engineering of N-carbamoyl D-amino acid amidohydrolase enzyme for improved thermostability

Although Yang et al (2019) reported the production of 23.4 g/L of D-p-Hydroxyphenylglycine (D-p-HPG) in 32 h with 100% conversion, a low space-time yield (STY) of 0.7 g/(L·h) was obtained probably because of the low thermostability of the Case enzyme. Zhang et al (2024) utilized salt bridge engineering to improve AkDCase (Agrobacterium sp. strain KNK712) thermostability and to demonstrate this, a complex crystal structure of AkDCase with N-carbamoyl-D-p-hydroxyphenylglycine (Cp-HPG) was constructed in which 87 salt bridges were found. Out of these bridges, three were not conserved; D30-K34, E87-K84, and E135-K134. Following the identification of these three non-conserved salt bridges, D30, E87, and E135 were replaced by neutral (A) or basic residues (K/R) via virtual mutation. Studies of these mutants revealed the D30A and E87R mutation as the best variants showing the highest thermal stability. Of the two, D30A was taken as the combination of the two mutations results in negative steric effects against each other. Comparison RMSD from Molecular dynamics simulation showed that AkDCaseD30A was stable and lower than that of WT at 40 and 60 °C. Co-expression of AkDCaseD30A with Hase from Geobacillus stearothermophilus in a single Escherichia coli cell, 29.53 g/L D-p-HPG within 12 h, was produced with a 97% conversion and a 2.46 g/(L·h) space-time yield which was higher than that previously reported by Yang et al (2019).

ii) Engineered DAPDH in the synthesis of D-p-HPG

Xu et al (2021) expressed a highly stereoselective DAPDH variant from Corynebacterium glutamicum ($CgDAPDH^{BC621}$ containing five mutations: R196M/T170I/H245N/Q151L/D155G) in E. Coli together with Laad, Hmas (4-hydroxy mandelate synthase), and MDH after successful invitro biotransformation. However, biotransformation results showed that conversion of HPGA to D-P-HPG was the rate-limiting step and low activity of $CgDAPDH^{BC621}$ would result in low HPG with accumulation of 4-hydroxyphenylglyoxalate. To increase the activity of $CgDAPDH^{BC621}$, a directed evolution strategy was employed to reduce the hydride transfer distance during

the enzyme catalytic reaction. Three mutations were introduced in the CgDAPDH^{BC621} enzyme and when combined into CgDAPDH$^{BC621/D120S/W144S/I169P}$, a specific activity of 5.32±0.85 U·mg−1 ·protein which was 37-fold higher than that of CgDAPDH^{BC621} was obtained and therefore, CgDAPDH$^{BC621/D120S/W144S/I169P}$ replaced CgDAPDH^{BC621} in the biosynthetic cascade. To regulate the four enzyme expressions and activity, Tac and T7 promoters were utilized in pETDuet-1 plasmid to control the expression of PaMDH and CgDAPDH$^{BC621/D120S/W144S/I169P}$ respectively. For pmL-AAD and SambHmaS, T7 promoters were used in the pACYCDuet-1 plasmid. Both plasmids were then co-expressed in E. coli BL21 (DE3) and under the optimal induction and transformation conditions (0.7-mM NADP+, 0.5-mM CoSO4, 20-mM Tris– HCl buffer (pH 8.5) and 30 °C), 42.69-g/L D-HPG was obtained in 3-L fermentation using 20 g/L (wet cell) of resultant E. coli from 50-g/L L-tyrosine in 20 h with 92.5% conversion and>99% ee.

Tan et al (2023) engineered DAPDH from Prevotella timonensis by broadening the size of the active site to form PtDAPDH^{M4} (W121V/H227I/R181T/S72D/S160R) that exhibited a catalytic efficiency of 26.75-fold higher than the wild type toward 4-hydroxyphenyl glyoxylate to form D-p-HPG. PtDAPDH^{M4} was co-expressed with E. coli aromatic amino acid aminotransferase and Bacillus megaterium glucose dehydrogenase catalysing transamination to form 4-hydroxyphenyl glyoxylate (HPGA) and regeneration of NADPH respectively. Under optimum conditions, conversion reached 98.8% with a D-HPG titer of 19.76 g/L in 10 h from 20 g/L L-HPG conducted at a scale of 100mL.

iii) Engineering of Alanine Racemase (Alr) for D-Arginine synthesis

Willies et al (2012) utilized error-prone PCR to construct a mutant library that included three significant mutations; I195T, N223D, and I374N. All of these mutations were identified in a strain that was characterized to have a 20-fold reduction in catalytic efficiency and a 5-fold increase in K_m towards racemization of L to D Alanine. When the mutant was tested for racemic activity on L-Lys, L-Arg, and L-Leu, it exhibited a 1300% increment in activity towards the formation of D-Arg from L-Arg. However, the strain had no activity on L Lysine and Leucine. For the latter, a reason was obtained from docking studies which showed that the point mutation of I374N existed in the active site and thereby changed the polarity from hydrophobic to hydrophilic, thereby favoring attachment of polar substrates such as L-Arg and not L-Leucine. Based on this reason it was expected that L-Lys would also exhibit an increment in activity, however, this was not the case, hence suggesting more mutations that would favor L-Lys binding.

When the mutant was tested on other amino acids, it showed a 107% increment in activity towards the racemization of L-Ornithine and a +14% increment towards the racemization of L-Glu. However, L-Ala, L-His, and L-Ile activities significantly decreased. These results show the use of directed evolution of racemases towards broadening substrate activities although native activities may be lost. (Willies et al., 2012)

iv) Engineering via signal peptide deletion of Pseudomonas taetrolens Arginine Racemase for increased production of D-Lys, D-Ser, D-Orn, and D-Arg

Arginine Racemase is a broad substrate racemase containing a signal peptide of 23 amino acids t the 5’ end of its gene. Matsui et al., 2009 characterized Arginine racemase to have a high racemic activity on L-Lys among other substrates of L-Arg, L-Orn, and L-Ala. Sequencing of the amino-terminal end of ArgR revealed a possibility of processing of the enzyme with its signal peptide cleaved off. Results obtained by Stäbler et al (2011) through heterologous expression of Arginine Racemase showed that the racemase with signal peptide (ArgR-sp) was absent in the cell extract of Corynebacterium glutamicum while expression with signal peptide cleaved off (ArgR) revealed the presence of the racemase in the cell extract. These results are in agreement with the fact that ArgR-sp is localized in the periplasm and therefore, since racemization takes place in the cytosol, engineering by cleaving off the N-terminal signal peptide improves enzymatic catalysis in host cells.

When ArgR-sp and ArgR were separately expressed in Corynebacterium glutamicum strains engineered for accumulation of L-Lys, L-Ser, L-Arg, and L-Orn, no corresponding D-enantiomers were detected in strains carrying ArgR-sp plasmids. However, strains carrying ArgR were able to accumulate D enantiomers in the extracellular medium. The only challenge was the presence of both L and D enantiomers at equimolar concentrations, therefore more research is required toward uni-directional racemization. Finally, these results showed how the location of an enzyme within a cell could affect its catalytic activity, and therefore engineering of localization signals could provide a way to improve enzyme catalytic properties. (Stäbler et al., 2011)

e) Reduction of Degrative pathways

Some bacteria, mainly gram-negative strains can utilize both L and D amino acids as sources of Carbon and Nitrogen and it appears that two distinct degradative pathways for L and D-amino acids exist which means that racemases play a key role in linking the two pathways by interconversion of L and D forms. In Bacteria, an enzyme

called D-amino acid dehydrogenase (DAD) exists which mediates the oxidation of free neutral D-amino acids to their corresponding α-keto acids leading to energetic catabolism. It was found by Naganuma et al (2018) that a strain A25 isolated from an ordinary river exhibited a higher growth rate with D-Glutamate than with L-Glutamate. Gene expression studies by reverse-transcription quantitative PCR (RT-qPCR) for target genes involved in Glutamate metabolism; encoding D-amino acid dehydrogenase (DAD; EC 1.4.99.1), glutamate racemase (EC 5.1.1.3), D-glutamate oxidase (EC 1.4.3.7 or EC 1.4.3.15), and UDP-N-acetyl-α-D-muramoyl-L-alanyl-D-glutamate ligase (EC 6.3.2.9) showed enhanced expression for DAD gene confirming its degradative role in D-amino acids. (Naganuma et al 2018)

Therefore, attention to D-Amino acid metabolism in host bacterial cells is crucial for the heterologous expression of interested synthetic pathways. Two main D-Amino acids are essential in E. coli; D-Alanine and D-Glutamate. For the former, two types of Alanine Racemases exist; anabolic alanine racemase (Alr), and catabolic alanine racemase (dadX). Alr appears to be expressed constitutively and functions in the production of D-Alanine primarily for cell wall synthesis. However, dadX is only activated by a high L-Alanine concentration in the growth medium, and functions to produce D-Alanine from L-Alanine for further breakdown into pyruvate by D-Amino acid dehydrogenase (dadA). It should be noted that even though dadA is most active on Alanine, it can act on several other D-amino acids such as D-methionine, D-serine, and D-proline but to a lesser extent. In Bacillus Subtilis, D amino acid transferase (DAT) occurs and this appears to catalyze the transfer of an amino group from D-amino acids of alanine, leucine, aspartate, glutamate, aminobutyrate, norvaline, and asparagine to alpha-keto acids generating mainly D-Alanine and D-Glutamate. It is on this basis that two main degradative enzymes may interfere with the yield of some D-amino acids if synthesis by heterologous expression is to be utilized; dadA, and DAT, and therefore depending on the D-amino acid of interest, there would be a need for downregulation of these two genes in host cells.

f) Broad-specificity Racemases in D amino acid production

Several racemases have been characterized to have racemic activity towards amino acids however, there are limited studies and research about the utilisation of racemases in the production of D-amino acids yet they can catalyze a one-step stereo inversion of L to D enantiomers.

The main reason for few reports can be explained by the low enantioselectivity of Racemases and therefore engineering of native racemases towards the improvement of the stereospecificity for D-amino acids production is seen as the only solution. It is also reported that racemases can be engineered for broad substrate activity. In both cases, the available knowledge dictates the directed evolution method to be utilized; either random mutagenesis or the use of structural and bioinformatics-supported considerations.

There is limited information present on substrate-specific racemases catalyzing the interconversion of some amino acids, particularly Tryptophan, Arginine, and Lysine, therefore, screening of broad-specific racemases that would have racemic activity including the three amino acids would be a solution to diversify biotransformation. Below we have identified a few racemases with possible application towards D amino acid production.

Broad specificity amino acid racemase from Pseudomonas Putida (bar)

This racemase enzyme catalyzes the interconversion of L-lysine and D-lysine, and L-arginine and D-arginine at optimum conditions of 37^{o}c and pH of 9.0. Kino et al (2007) found a low activity of the enzyme for racemization between L-Tryptophan and D-Tryptophan. To increase the latter activity, random mutagenesis was carried out and it was found that the substitutions at Y396H and I384M increased the tryptophan-specific racemization activity and the racemization activity for overall amino acids**.** It was reported that mutant I384M resulted in a yield of 1.8g/l of D-Tryptophan and although it was lower compared to when the Hydantoin process is used, L-Tryptophan as a substrate is cheaper hence affordable production. This demonstrates how the directed evolution of enzymes could be a solution for enhancing the catalytic activity of broad-specific racemases towards bioconversion of L to D amino acids.

Broad specificity amino acid racemase from Vibrio Cholerae (bsrV)

bsrV amino-acid racemase is able to utilize a broad range of mainly basic amino acid substrates. It reversibly racemizes ten of the 19 natural chiral amino acids known, including both non-beta-branched aliphatic amino acids (Ala, Leu, Met, Ser, Cys, Gln and Asn) and positively charged amino acids (His, Lys and Arg). Among these substrates, bsrV racemizes lysine and arginine best. It is also able to catalyze the racemization of several amino acids that are not typically incorporated into proteins such as ornithine and norleucine. It Is not active on negatively charged (Glu and Asp) or aromatic (Tyr, Trp and Phe) amino acids and displays minimal activity towards beta-branched aliphatic (Ile, Val, and Thr) substrates.

Vibrio Cholerae accumulates D-Arginine using bsrV: D-Arginine is one of the most dominant Non-canonical D-amino acids (NCDAAs) synthesized by Vibrio cholerae during the stationary phase and it functions to display

toxicity towards competing bacterial species meaning that D-Arginine limits the growth of several bacterial species. (Alvarez et al., 2018) It was found that no D-Arginine can be produced by V. Cholerae mutants without bsrV enzyme thereby confirming that the enzyme is responsible for the accumulation of D-Arginine in the extracellular environment. (Alvarez et al., 2018)

Broad specificity amino-acid racemase RacX from Bacillus Subtilis

Amino-acid racemase can utilize a broad range of substrates. Preferentially catalyzes the epimerization of LL-diaminopimelate, as well as the racemization of D-lysine, L-arginine, L-ornithine, L-lysine, and D-arginine. Has lower activity against D-ornithine, L-histidine, L-alanine, L-tyrosine, L-phenylalanine, L-serine, L-glutamine, L-methionine, L-asparagine and L-homoserine. Has weak activity against L-norleucine, L-aminobutyric acid, and L-norvaline. Has no activity toward nine L-amino acids (Thr, Glu, Asp, Val, Leu, Ile, Trp, Cit, and Aad) (Miyamoto et al., 2017)

Broad-spectrum amino acid racemase (Alr) of *Pseudomonas putida* KT2440

Previous studies on Pseudomonas putida showed its high efficiency in utilizing D-amino acids as sole sources of Carbon and Nitrogen. Radkov and Moe (2013) identified a putative biosynthetic alanine racemase (Alr) in Pseudomonas and invitro enzyme assays showed that the enzyme has broad substrate specificity, exhibiting measurable racemase activity with 9 of the 19 chiral amino acids. Among these amino acids, activity was the highest with lysine. Radkov and Moe (2018) reported Alr enzyme to be a link between the independent pathways that utilize L and D lysine by enabling the bacteria to convert L form to D form for further breakdown. This was confirmed by results obtained when Δ*alr* strains accumulated no D-Lysine in the media as compared to wild-type strains when the two were cultured on LB medium containing L-Lysine. Also, through analysis of the media supernatant, it was found that Δ*alr* strains accumulated L-Lysine in the growth medium despite the high concentrations of L-Lysine present as a substrate. This further shows that Alr is crucial in the catabolism of L-Lysine by first converting it to D-Lysine which is further broken down through the L-pipecolate pathway. (Radkov & Moe, 2018)

Broad specific Isoleucine-2-epimerase (ILEP)

ILEP Racemase enzyme from *Lactobacillus buchneri* was utilized by Yanqi et al (2023) and compared to Alr, ygeA, and bsrV, it showed the highest D-amino acid titer (g/l) and was able to racemize a great deal of L amino acids. Awad et al (2017) characterized the structure of ILEP enzyme and found out that it catalyzes the pyridoxal 5'-phosphate (PLP)-dependent racemization and epimerization of a broad spectrum of nonpolar amino acids from L- to D-form and vice versa, in particular isoleucine. This enzyme was the first epimerase to be identified from Lactobacillus and is responsible for the accumulation of D-leucine, D-allo-leucine, and D-valine in the culture medium of Lactobacillus. (Mutaguchi et al., 2013)

It should be noted that the overall 3D structure among fold-type I PLP-dependent enzymes is conserved although residues in the binding pocket must be different to offer distinctive topologies to accommodate specific substrates. Among conserved residues is Arginine at position 408 which is responsible for the formation of a salt bridge with the amino acid Cα carboxylate. Awad et al (2017) characterized potential residues in the active site that could be responsible for specific binding and orientation of the branched nonpolar amino acid substrates. These were Ala54, Tyr142, Met159, Tyr84, Leu307, and Thr309 and they provided hydrophobic patches within the active site. Following substrate recruitment, the ILEP enzyme utilizes conserved catalytic residues that may be accountable for its amino acid racemization reactivity, notably the lysine at position 280, and the tyrosine 142 pair in deprotonation/protonation reactions.

Insilco studies show a possibility of Racemase engineering toward stereospecificity

There are few reports about the engineering of Racemases towards L enantiomer specificity and it is on this basis that we further used molecular docking and molecular dynamics simulation on an already characterized structure of Isoleucine-2-epimerase enzyme for racemization of L and D Leucine to suggest residues responsible for specific enantiomer substrate binding and thus demonstrate a possibility of enzyme engineering towards enantiospecificity thereby exploring the findings by Awad et al (2017)

There were two suggested approaches towards active site characterization; the first was the docking of L and D Leucine with PLP complexed ILEP (5LL3), and the second was the docking of external PLP aldimines of both L and D Leucine with Apo ILEP enzyme (5LL2) (Awad et al., 2017). We tested the two approaches using Autodock Vina (Trott & Olson, 2010). However, to our surprise, we found no docking poses showing signs of substrates interacting with the PLP for the first approach.

We then constructed external PLP-Leucine aldimines for both enantiomers using MolView software (Smith, 1995), and using Apo ILEP, we performed docking. From docking, the first poses with the lowest energy were considered for Molecular dynamics simulation using GROMACS, and results were analyzed using VMD and PyMOL software

tools. Here we assumed that the enzyme should change conformation following the formation of an internal aldimine with the PLP of the Holoenzyme. This meant that docking with an external aldimine would make the enzyme achieve similar conformation as the case of real substrate binding to the PLP-bound Holoenzyme.

Analysis of MD simulation results showed Lys280 and Tyr142 residues to be associated with both L and D PLP-L/D Leucine aldimines throughout the simulation as illustrated in Fig 7 and 8. This is in line with previous research carried out by Awad et al (2017) and suggests that racemization of both L and D Leucine utilizes Lys280 and Tyr142 catalytic pair. Of the potential residues responsible for stereospecificity, (Ala54, Tyr142, Met159, Tyr84, Leu307, and Thr309) Awad et al (2017), Tyr84 was associated with PLP L-Leucine aldimine, while Met159 was associated with PLP D-Leucine aldimine. In addition to Met159, Ser145 was found to make polar contacts as illustrated in Fig 8. This probably suggests that the binding of L and D Leucine causes two different conformational changes of the enzyme although the position of the catalytic pair relative to the substrate is conserved. The residues Tyr84, and Met159, and Ser145 could be responsible for the orientation of L-Leucine and D-Leucine respectively in the active site of ILEP. It is at this point that such results provide an example of residues that could be mutated to disable the proper binding and orientation of one of the enantiomers to favor stereo inversion in a particular direction.

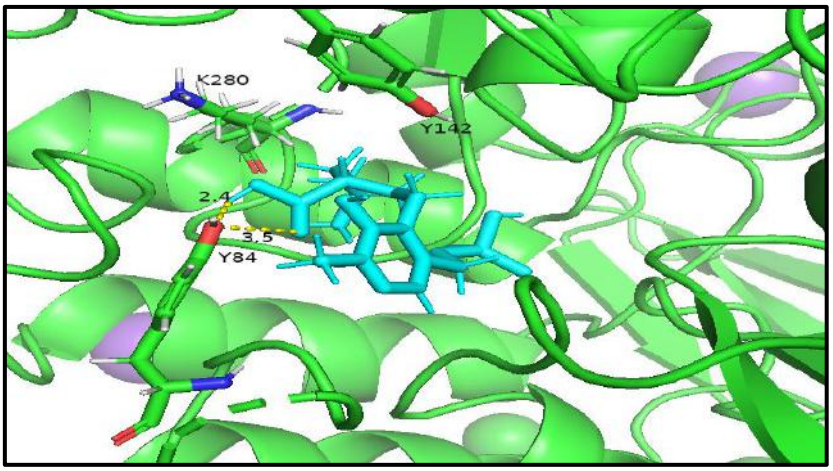

Fig 7: Molecular dynamics simulation of ILEP with an external PLP-L-leucine aldimine shows Tyr 84 forming polar contacts with L-leucine possibly suggesting a role in substrate stabilization in addition to Tyr 142 and Lys 280 catalytic pair.

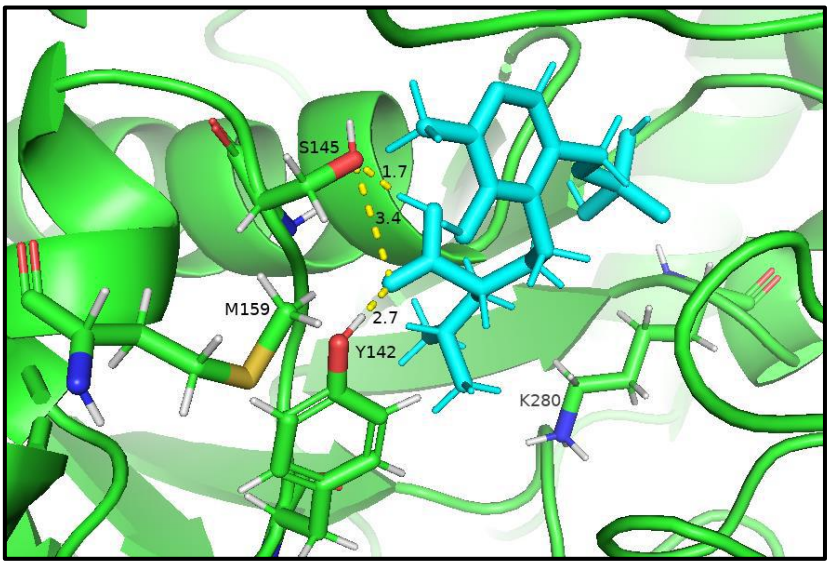

Fig 8: Molecular dynamics simulation of ILEP with an external PLP-D-leucine aldimine shows Met 159 and Ser 145 with the later forming polar contacts with D-Leucine.

V. CONCLUSION

This review summaries recent methods and challenges in the synthesis of D amino acids. These challenges identified mainly include the high cost of some raw materials such as alpha-keto acids, toxicity of D amino acids to host cells, and low yield probably coming as a result of degradation of the D amino acids within the host cells and reaction intermediates translocation. We have tried to identify solutions utilised to these challenges primarily focusing on toxicity through acetylation and screening of host cells, enhancement of yield via reduction of degradative pathways and engineering of enzymes involved in D amino acid production. Furthermore, since racemases catalyze stereo inversion of both L and D amino acids, we suggest that enzyme engineering towards enantiomer specificity could solve the problem. To show the applicability of this solution, we utilized the PDB structure of alloisoleucine-2-epimerase and external PLP-L/D-leucine aldimines in docking and molecular dynamics simulation. From simulation results, we suggest that out of Ala54, Tyr142, Met159, Tyr84, Leu307, and Thr309 residues identified by Awad et al (2017), Tyr84 and Met159 could be involved in substrate binding and orientation of L and D Leucine respectively hence highlighting the two possible residues that could be utilized in ILEP engineering. The later findings provide opportunities for using site-directed mutagenesis of Racemases toward one direction L to D stereo-inversion in a one-step reaction with an overall increase in yield.

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