Phenyllactic acid: a potential antimicrobial compound in lactic acid bacteria

Abstract
3-Phenyllactic acid (PhLA) is a broad spectrum antimicrobial compound active against bacteria and fungi. PhLA exists in two chiral isomers, L-PhLA and D-PhLA which show antimicrobial activity. D-PhLA shows more antimicrobial activity than L-PhLA and hence it is receiving great attention as food and feed additive in place of antibiotics which control the microbial contamination and thereby increase the shelf life of food and food ingredients. This review summarizes the recent developments on the resources, detection and analysis of antimicrobial activity of PhLA. In addition, the article presents the recent studies of the key enzymes such as lactate dehydrogenases and pyruvate reductases involved in PhLA synthesis. The metabolic pathway and regulation of PLA synthesis in lactic acid bacteria (LAB) along with its high level production are also discussed.

Keywords: 3-phenyllactic acid, lactate dehydrogenase, antimicrobial activity, pyruvate reductase

Abbreviations: AAT, aromatic aminotransferase; GC/MS, gas chromatography/mass spectrometry; GRAS, generally accepted as safe; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; MIC, minimum inhibitory concentration; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine phosphodinucleotide; PPA, phenylpyruvic acid; PhLA, phenyllactic acid; PGDH, d-3phosphoglycerate dehydrogenase

Introduction
3-Phenyl lactic acid (2-Hydroxy-3-Phenyl propionic acid, PhLA) is an organic acid exhibiting broad spectrum antimicrobial activity against bacteria and fungi including yeast.1,2 Its molecular formula and molecular weight are C9H10O3 and 166g/mol respectively. PhLA exists in two chiral isomers namely D-PhLA and L-PhLA and both the isomers show antimicrobial activity. The antibacterial activity of PhLA was first reported by Dieuveau et al.,4 in Geotrichum candidum.4 They purified PhLA from cultural broth of Geotrichum candidum was found to show anti-Listeria activity. They also found that D-PhLA exhibited more anti-Listeria activity than L-PhLA. PhLA was also found to inhibit Gram positive and Gram negative bacteria and this effect was higher at acidic pH.5 The minimum inhibitory concentration (MIC) value of PhLA is relatively high (50-100mM at pH 4-6) when it is used against yeast.5 The MIC value against fungi at pH 4.0 is 45mM and it decreases with decrease in pH.5 Though the mechanism of the antimicrobial action is not clear, it was shown by scanning electron microscopy that it damages the cell wall when the bacteria were exposed to PhLA. It was observed that the bacteria form aggregates, secrete polysaccharides and loose cell wall rigidity leading to cell death.6

Due to the antimicrobial property of PhLA, it is used in food industry as natural antimicrobial compound to control the fungal contaminations which help in extending the shelf life of food and food ingredients. It diffuses in to the food and feed very easily due to its hydrophilic nature. PhLA has been shown to exert immune-modulatory effects in poultry feed which improves the production performance and egg quality.7 It is known to show anti-pathogenic property in the large intestine when supplemented with chick feed diets which improves the meat quality.8 Such immune-modulatory and anti-pathogenic effects were observed in growing pigs when fed with diets supplemented with PhLA.9 It is noteworthy to mention that the analogue of PhLA known as “Danshensu” used in Chinese medicine is presently used for the treatment of coronary disease.10 PhLA is marketed as skin-protecting agent, which reduce the skin wrinkles.11 PhLA and their derivatives also act as a chiral building block for the synthesis of many bio-based materials in agricultural, pharmaceutical and chemistry fields.12-14 It can be polymerized in to poly-PhLA which unlike polylactic acid showed high ultraviolet absorption ability and thermostability due to bulky aromatic side chain.15,16 The antimicrobial property, the promising applications of PhLA and its great demand sparked the great interest in the development of various strategies for its synthesis. Thus the bio-protective potential of PhLA producing LAB is high and well suited to wide applications in food safety and nutritional medicine.17,18

PhLA occurrence and its detection
PhLA was found in honey in higher amounts than other phenolic acids. PhLA content in thistle unifloral honey was around 100-800mg/kg19 but the high concentrations of PhLA were found in heather (820mg/kg), ling heather (875mg/kg) and manuka honeys (243mg/kg).20 Very recently, anti-staphylococcal activity against methicillin resistant Staphylococcus aureus was reported in manuka honey which contained high levels of acetic and lactic acids.21 It was found that LAB produced PhLA as a reaction product of amino acid metabolism.22,23 Its detection by reverse-phase HPLC is simple and widely adopted method for quantitative measurement of PhLA present in fermentation broth,24 honey,19 and rumen fluid.25 GC/MS and capillary electrophoresis were also used for quantitative measurement of PhLA. However, these methods could not detect the PhLA chiral isomers. PhLA enantiomers, L- and D-PhLA were determined by chiral HPLC method which involves the use of Chiracel OJ-RH column to separate PLA enantiomers and a UV detector at 205nm.26

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The use of chiral additives such as modified cyclodextrins during chromatography and capillary electrophoresis methods could separate both the PhLA enantiomers. \(^{27}\)

**Metabolic pathways and enzymes involved in pla synthesis**

PLA synthesis in LAB results from amino acid metabolism and the acids involved in PLA synthesis are phenylalanine and a-ketoglutarate. The phenylalanine is first transaminated to phenylpyruvic acid (PPA) and PPA is further reduced to PhLA. \(^{24}-^{30}\) The transamination reaction is mediated by aromatic amino acid transferase (AAT) which has broad substrate specificity including leucine, tyrosine and methionine. \(^{31}\) The enzyme AAT transfer the amino acid group from phenyl alanine to any suitable a-keto-acid preferably a-ketoglutarate in LAB. Hence a-ketoglutarate is an important that effects the both catabolism of phenylalanine and regulation of PhLA synthesis indirectly. \(^{32}\) This regulatory effect was observed in L. plantarum resulting in enhanced production of PLA in presence a-ketoglutarate. \(^{26,38}\) In presence of substrates like glucose, citric acid and fructose acting as electron acceptor, glutamate dehydrogenase activity is enhanced leading to formation of a-ketoglutarate which in turn upregulate the PhLA synthesis. \(^{32}\) The other precursor is PPA which can be reduced to PhLA with help of enzymes such as reductases or dehydrogenases present in LAB. When the Lactobacillus strains were grown in media supplemented with PPA in place of phenylalanine, PLA production was enhanced several fold indicating that PPA serves as better precursor than phenylalanine. \(^{24}\) The transamination of phenylalanine was a limiting factor which is a bottle-neck in PhLA synthesis. Hence it was suggested that the use of PPA could overcome this bottle-neck leading to increased PhLA synthesis. \(^{31,34}\)

Different kinds of dehydrogenases have been reported to convert PPA to PhLA and the most reports suggest that lactate dehydrogenases (LDHs) are mainly responsible for PPA to PhLA. The other dehydrogenases especially, D-type dehydrogenases such as D-mandelate dehydrogenase (D-ManDH) from Enterococcus faecalis\(^ {35}\) and D-hydroxyisocaproate dehydrogenase (D-HicDH) from Lactobacillus casei\(^ {36}\) are also to involve in catalytic dehydrogenation. These D-type of enzymes exhibit broad substrate specificity including PPA. \(^ {37}\) Fuji et al.,\(^ {37}\) discovered a novel fungal strain, Wickerhamia fluorescens TK1 which produced novel enzyme known as PPA reductase that reduced PPA to D-PhLA using either NADPH or NADH. \(^ {37}\) This enzyme has more preference for NADPH than NADH. They demonstrated that this PPA reductase belongs to D-isomer specific 2-hydroxyacid dehydrogenase family. Very recently, novel NADH dependent PPA reductase was identified from Lactobacillus spp. CGMCC 9967 which belongs to D-phosphoglycerate dehydrogenase (PGDH) subfamily of 2-hydroxy acid dehydrogenase superfamily. \(^ {38}\) It is noteworthy to state that these PPA reductases prefer aromatic a-keto acids than aliphatic keto acids and PPA was reduced more efficiently than other aromatic keto acids. In the synthesis of PhLA in LAB, PPA is converted to PhLA by LDH. There are two types of LDH existing in LAB based on catalytic specificity. PPA is converted to either L-PLA by L-LDH (EC.1.1.1.27) or D-PLA by D-LDH (EC.1.1.1.28). These enzymes are optimally active at pH 5-7 and temperature ranging from 30°C to 45°C. The LAB are mesophilic and acidophilic in nature and therefore the LDH in them exhibited less thermostability. LDH reported from B. coagulans and T. ethanolicus showed optimal activity and stability at higher temperatures. \(^ {39,40}\) The possible pathway for PLA synthesis and enzymes involved in it are given in Figure 1.

**Figure 1 Possible pathways and enzymes involved in Phenylactic acid production.**

**Microorganisms producing pla and their improvement**

PhLA is produced by wide range microorganisms including LAB, non-LABs and fungi such as Aspergillus, Geotrichum. \(^ {30,31}\) Table 1 summarizes the production of PhLA by some of the promising bacteria which produced significant amounts of PhLA. It was found that PhLA is produced by various LAB species such as Lactobacillus, Leuconostoc and Enterococcus. Among the Lactobacillus species, L. plantarum, L. acidophilus, L. paracasei are reported to produce PhLA in significant quantities. We identified the L. lactis NCIM 5449 which produces high levels of D-lactic acid and it was also used for D-lactic acid production from sugarcane bagasse derived cellulose. \(^ {41}\) The strain was found to produce 18g/L of D-PhLA from 25g/L of PPA within 2h in biotransformation reaction (unpublished data). Arena et al.,\(^ {42}\) evaluated 79 wine derived L. plantarum strains for their antimicrobial activity against food borne pathogens. \(^ {42}\) They found that several L. plantarum strains showed significant ability to contrast various pathogenic bacteria which contaminate the food. Apart from bacteriocins produced by LAB that are effective against Gram-positive bacteria, these strains are known to produce organic acids including PhLA and hydroxyl-PhLA which exhibit a broad spectrum of antimicrobial action. Among the non-LABs, Bacillus coagulans and Brevibacterium lactofermentum produced high amounts of PhLA. In general, PPA is converted to PhLA by LDHs present in LAB and therefore the studies were carried out on cloning and expression of native or site-mutated LDHs to enhance the PhLA productivity (Table 1). However, the attempts were not successful since there was no much increase in PhLA production. This could be attributed to low activity of native LDHs towards PPA due to its bulkier structure. An attempt was made to engineer D-LDH744 of Sporolactobacillus inulinus which resulted in increased enzymatic activities towards PPA using protein structure-guided modeling analysis. \(^ {43}\) When this engineered D-LDH7407 was expressed in E.coli, the strain produced 21.43 g/L of D-PhLA. This increase was due to enhanced enzymatic activity of the engineered enzyme towards bulkier PPA. Most of the studies on engineering D-LDH of L. pentosus dealt with the replacement of
Tyr52 with such as Leucine, Val and Ala. Zhu et al. also replaced Tyr52 of D-LDH of L. pentosus with small hydrophobic residues to enhance the PhLA synthesis activity. Zheng et al. selected Tyr52 and Phe299 of D-LDH of L. pentosus for site directed mutagenesis to reduce steric exclusion effect. However, only Y52L mutant of D-LDH gave good performance and showed elevated activities towards unnatural substrates with large substituents at C-3 position. The gene responsible for PPA reductase from Lactobacillus sp.

CGMCC 9967 was co-expressed with glucose dehydrogenase from Bacillus megaterium in E. coli for efficient D-PhLA production with internal NADPH regeneration system. The newly constructed E. coli converted 100g/L of PPA to PhLA with 91.3% isolation yield and 99% ee and the productivity was about 10g/Lh. To our knowledge, no traditional microbial strain improvement strategies such as mutations are reported which give the enhanced yields and productivity of PLA.

### Table 1 Production of PLA by some promising organisms including genetically engineered strains

| Strains                          | Production process     | Chirality | Pla (g/l) | Yield (%) | Reference                      |
|---------------------------------|------------------------|-----------|-----------|-----------|--------------------------------|
| Lactobacillus plantarum IMAU 10124 | Fermentation           | Racemic   | 2.9       | 96        | 48                            |
| Lactobacillus sp. SK007          | Biotransformation      | Racemic   | 1.13      | 56.7      | 28                            |
| Lactobacillus sp. SK007          | Fed-batch fermentation | Racemic   | 17.38     | 51.1      | 33                            |
| Lactobacillus lactis NCIM 5449   | Biotransformation      | D-PLA     | 18.5      | 74        | Our studies (unpublished data) |
| Bacillus coagulans               | Biotransformation      | Racemic   | 37.3      | 70        | Zheng et al.                  |
| Leuconostoc mesenteroides ATCC 8293 | Biotransformation    | D-PLA     | 5.82      | 75.2      | 49                            |
| Brevisbacterium lactofermentum   | Fermentation           | D-PLA     | 1.92      | --        | 50                            |
| E. Coli BL21 (DE3)               | Biotransformation      | D-PLA     | 15.6      | 77        | 46                            |
| E. Coli JM109 (DE3)              | Biotransformation      | D-PLA     | 21.4      | 82.3      | 43                            |
| E. Coli BL21 (DE3) with pET28-gdh-T7-ppr | Biotransformation | D-PLA     | 91.3      | 91.3      | 38                            |

### Future perspective

To our knowledge, there is limited information available on biological production of PhLA although natural foods such as honey and LAB fermented foods contain PLA in significant amounts. However, the use of PhLA as a pure compound is not permitted in food since no studies carried out on its metabolism in the human body and toxicity effects. The generation of this data is essential to make PhLA GRAS cleared. Also No literature is available on its recovery from the cultural or biotransformation broth which is the prerequisite for total integrated process for PhLA synthesis followed by its recovery in pure form. PhLA was produced by adding phenylalanine or PPA in the fermentation medium. The fermentation broth contains several compounds which make the recovery of pure PhLA cumbersome. The alternative approach would be the biotransformation in which whole cells are used to convert PPA to PhLA. Biotransformation is usually carried out in presence of glucose and fructose needed for cofactor (NADH/NADPH) regeneration. The biotransformation broth does not contain much of the impurities and hence the downstream processing for recovery of PhLA would be simpler and easy. Lactobacillus strains are fastidious and hence they need rich medium for their growth which may not be cost effective. We suggest that the Lactobacillus cells generated during D-lactic acid production may be used to convert PPA to PhLA using biotransformation approach. This will save the cost of growing the Lactobacillus strains separately.

### Acknowledgements

None.

### Conflict of interest

The author declares no conflict of interest.

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### Citation

Chaudhari SS, Gokhale DV. Phenyllactic acid: a potential antimicrobial compound in lactic acid bacteria. J Bacterial Mycol Open Access. 2016;2(5):121–125. DOI: 10.15406/jbmoa.2016.02.00037
Phenyllactic acid: a potential antimicrobial compound in lactic acid bacteria

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Citation: Chaudhari SS, Gokhale DV. Phenyllactic acid: a potential antimicrobial compound in lactic acid bacteria. J Bacterial Mycol Open Access. 2016;2(5):121–125. DOI: 10.15406/jbmona.2016.02.00037
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