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ORIGINAL RESEARCH PAPER

Biotransformation of phenylpyruvic acid to phenyllactic acid by growing and resting cells of a *Lactobacillus* sp.

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Abstract Phenyllactic acid (PLA) is a novel antimicrobial compound derived from phenylalanine (Phe). *Lactobacillus* sp. SK007, having high PLA-producing ability, was isolated from Chinese traditional pickles. When 6.1 mM phenylpyruvic acid (PPA) was used to replace Phe as substrate at the same concentration, PLA production increased 14-fold and the fermentation time decreased from 72 h to 24 h with growing cells. With resting cells, however, 6.8 mM PLA could be obtained as optimal yield using the following conditions: 12 mM PPA, 55 mM glucose, pH 7.5, 35°C and 4 h.

Keywords Biotransformation · *Lactobacillus* sp. SK007 · Phenyllactic acid · Phenylpyruvic acid

Introduction

Phenyllactic acid (PLA) is a novel antimicrobial compound active against both Gram-positive and Gram-negative bacteria (Dieuleveux et al. 1998a, b). In contrast, nisin inhibits many Gram-positive bacteria but it has no effect on most of the Gram-negative bacteria or the yeasts or molds (Gupta and Prasad 1989). Moreover, PLA has a broad inhibitory activity against a wide range of fungi including some mycotoxigenic species (Lavermicocca et al. 2003). Although PLA was found to be less effective than nisin in controlling *Listeria monocytogenes* (Dieuleveux and Gueguen 1998), it has, however, a potential for applications as an antimicrobial agent in foods (Lavermicocca et al. 2003; Schnurer and Magnusson 2005). Furthermore, PLA being an analogue of 3,4-dihydroxyphenyllactic acid, which is a bioactive compound from Chinese medicinal herb ''Danshen'' (*Salvia miltiorrhiza*), it may have pharmaceutical applications such as inhibition of platelet aggregation and treatment of acute myocardial infarction (Yu et al. 1991).

PLA production has been reported using various microorganisms, including *Geotrichum candidum* (Dieuleveux et al. 1998a), propionibacteria (Thierry and Maillard 2002) and lactic acid bacteria (LAB) (Lavermicocca et al. 2000; Strom et al. 2002). This study has focused on the ability of LAB to produce PLA, given that LAB have GRAS (generally recognized as safe) status. A previous study showed that PLA can be produced by a wide range of LAB species but its production is strain-dependent (Valerio et al. 2004). Therefore, screening LAB with high PLA-producing

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ability from several sources is necessary. In addition, PLA is a product of Phe metabolism; in particular, Phe can be transaminated to PPA, which is further reduced to PLA by a hydroxy acid dehydrogenase (Lavermicocca et al. 2003). PLA production was improved in *L. plantarum* ITM21B using increased concentrations of Phe (Valerio et al. 2004) but in many other lactobacilli, transamination is the rate-limiting step in the formation of metabolites from amino acids. Transamination is the bottleneck in PLA formation by *L. sanfranciscensis* DSM20451^T and *L. plantarum* TMW1.468 (Vermeulen et al. 2006). However, all previous studies on PLA production by lactobacilli have used Phe rather than PPA as substrate, and the possibility to increase PLA yield by an optimized supply of substrate needs to be elucidated. Traditionally, PPA has been used to produce L-Phe which is in high demand for the production of an artificial sweetener ''aspartame'' (Matsunaga et al. 1987; Then et al. 1987). Therefore, compared with Phe, PPA can be obtained easily at a lower price.

The aim of this study was to screen LAB that have high PLA-producing ability from Chinese traditional fermented pickles. The production of PLA from PPA by growing cells and resting cells of the screened LAB was also investigated.

Materials and methods

Chemicals

DL-Phenyllactic acid and DL-phenylalanine were purchased from Sigma while phenylpyruvic acid was provided by Fluka. Other chemicals were of analytical grade and were mainly obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai).

Medium and cultivation

Lactobacillus strains were grown in de Man-Rogosa-Sharpe (MRS) medium for 3 days at 30°C without shaking (Valerio et al. 2004). Cell mass was estimated turbidometrically at 600 nm and converted to cell dry weights by a calibration curve.

Screening of PLA-producing LAB

Culture media were centrifuged (10,000 *g* for 15 min, 4°C) and the supernatant was filtered (0.45 μm pore-size membrane). PLA, PPA and Phe were determined using HPLC equipped with an Agilent Zorbax SB-C_{18} column (4.6 × 150 mm, 5 μm), eluting with methanol/0.05% trifluoroacetic acid (solvent A) and water/0.05% trifluoroacetic acid (solvent B) at 1 ml/min and A/B ratios of 10:90, 100:0, 100:0 and 10:90, with run times of 0, 20, 23 and 25 min, respectively. PLA, PPA and Phe were detected at 210 nm, according to the procedure described by Valerio et al. (2004) with minor modifications.

Identification of *Lactobacillus* sp. SK007

16S rDNA of the screened PLA-producing strain was amplified and sequenced by TaKaRa Biotechnology Co., Ltd (Dalian, China). The 16S rDNA sequences were aligned in GenBank of NCBI using BLAST.

Preparation of *Lactobacillus* sp. SK007 growing cells

Lactobacillus sp. SK007 was grown in MRS medium containing either Phe or PPA at 6.1 mM. PPA was first dissolved with 2 M NaOH and was then added into MRS broth. After addition of PPA, the pH was adjusted to 6.5.

Preparation of *Lactobacillus* sp. SK007 resting cells

The strain was grown in MRS medium at 30°C for 12 h, the culture was centrifuged (10,000 *g* for 15 min, 4°C) and the cell pellets washed twice with 50 mM Tris/HCl buffer (pH 7.1) and resuspended in the same buffer at 22 g dry cell wt l^{-1}.

Results and discussion

Screening and identification of PLA-producing LAB

Up to 112 LAB strains were isolated, from which 60 strains produced 0.10–0.37 mM PLA and 10

strains could produce even more than 0.40 mM PLA. The strain SK007 showed the highest PLA-producing ability (0.55 mM), which is closer to the highest level of PLA (0.57 mM) produced by *L. mesenteroides* subsp. *mesenteroides* ITMY30 grown in MRS broth (Valerio et al. 2004).

The screened strain was identified as a *Lactobacillus* by 16S rDNA sequence (1483 bp), and showed 99% similarity with *Lactobacillus* sp. MR-2 (AF516755), *Lactobacillus plantarum* WCFS1 (AL935258) and *L. pentosus* (D79211). The 16S rDNA sequence of *Lactobacillus* sp. SK007 has been deposited in GenBank under the accession number DQ534529.

Bioconversion of PPA to PLA using growing cells of *Lactobacillus* sp. SK007

PLA production from Phe by *Lactobacillus* sp. SK007 grown in MRS broth is shown in Fig. 1. PLA formation reached a maximum level after 72 h. However, 94% of Phe remained, while PPA concentration stayed below the detection level and only 6% of the Phe added was converted to PLA after 72 h. These results, indicating that transamination of Phe to PPA is a rate-limiting step in PLA production by *Lactobacillus* sp. SK007, were found to be in agreement with the recent report by Vermeulen et al. (2006).

Therefore, it was hypothesized that PPA might be the preferred substrate for PLA production and an investigation of whether PLA formation could be improved using PPA instead of Phe as substrate was carried out. As expected, with PPA addition, PLA yield increased about 14-fold and the fermentation time decreased from 72 h to 24 h (Fig. 2). Results showed that PLA formation was in relation with the growth of cells. After 2 h, small amounts of PLA were already formed, and PLA levels increased exponentially until 12 h to reach an average of 5.17 mM after 24 h. However, the production of PLA from Phe started after 6 h, reaching a maximum level after 72 h. These findings indicated that reduction of PPA to PLA starts with the lag phase, whereas transamination of Phe to PPA begins at the exponential growth phase. Small amounts of Phe were also produced from PPA during PLA formation. This may be due to some PPA being converted into Phe by transamination utilizing diammonium citrate of MRS medium as the amino group donor.

The effect of PPA concentration on PLA production by *Lactobacillus* sp. SK007 growing cells is shown in Fig. 3. The PLA yield increased with increasing PPA concentration. However, above 18.3 mM PPA, the cell growth was strongly inhibited.

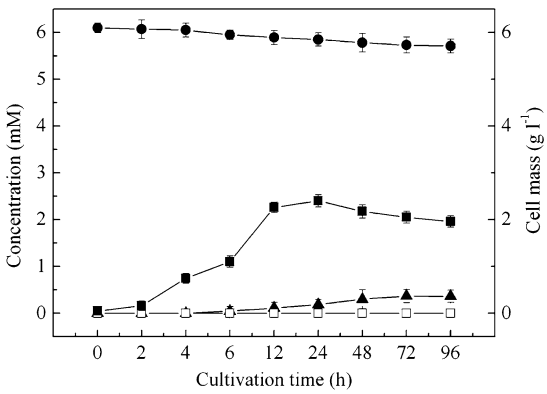

Fig. 1 PLA production from Phe during *Lactobacillus* sp. SK007 cell growth. Phe (●), PLA (▲), PPA (□) and cell mass (■) were measured when *Lactobacillus* sp. SK007 was grown in MRS broth containing 6.1 mM Phe over 72 h at 30°C. Data were expressed as mean ± SD from three independent experiments

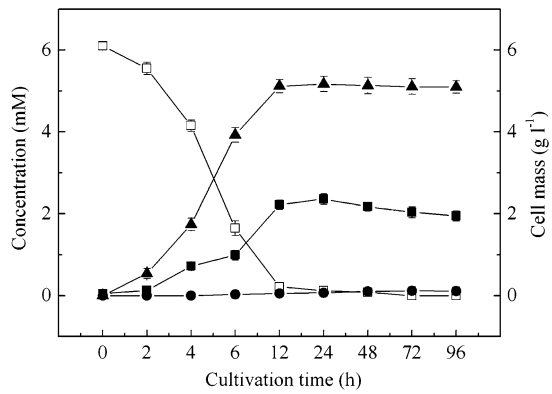

Fig. 2 PLA production from PPA during *Lactobacillus* sp. SK007 cell growth. PPA (□), PLA (▲), Phe (•) and cell mass (■) were measured when *Lactobacillus* sp. SK007 was grown in MRS broth containing 6.1 mM PPA over 72 h at 30°C. Data were expressed as mean ± SD from three independent experiments

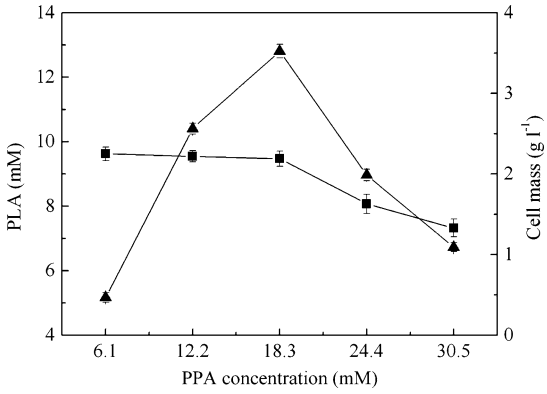

Fig. 3 Effect of PPA concentration on PLA production by *Lactobacillus* sp. SK007 growing cells. PLA (▲) and cell mass (■) were monitored at various PPA concentrations for 24 h at 30°C. Data were expressed as mean ± SD from three independent experiments

Bioconversion of PPA to PLA using resting cells of *Lactobacillus* sp. SK007

PLA production from PPA using *Lactobacillus* sp. SK007 resting cells was investigated. PLA concentration reached a maximum after 4 h and could not be improved with prolonged incubation time (Fig. 4). Compared with growing cells, resting cells lead to a lower yield in PLA production. However, biotransformation using resting cells has some advantages: the product separation is more convenient, the reaction time can be shortened, and the process can be run under non-sterile conditions.

A cosubstrate is necessary to drive the internal cofactor regeneration cycles in the cell for the NAD(P)H-dependent dehydrogenase (Haberland et al. 2002). Therefore, various cosubstrates were tested for their effects on PLA production using *Lactobacillus* sp. SK007 resting cells. As shown in Fig. 5, all the cosubstrates significantly increased the amount of PLA, and glucose achieved the highest yield. The optimal conditions for PLA conversion were: 22.5 g resting cells l^{-1} 35°C, and pH 7.5, respectively (data not shown). The effect of PPA concentration on PLA yield using resting cells was also studied (Fig. 6). When PPA was added up to 30.5 mM, PLA production increased while the conversion yield decreased gradually. These results suggested that further studies are needed to develop a PLA production process using immobilized cells.

Conclusion

Phe transamination was a limiting factor in PLA production by *Lactobacillus* sp. SK007 and this

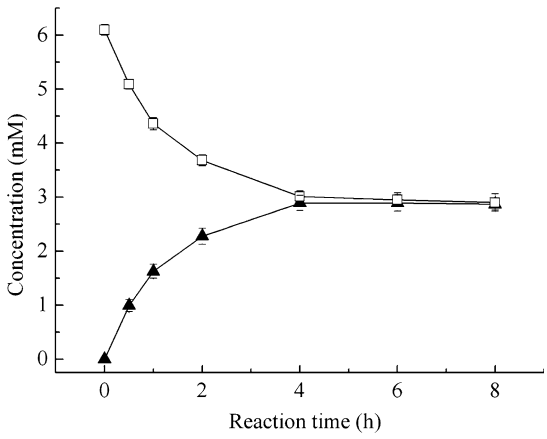

Fig. 4 PLA production from PPA by *Lactobacillus* sp. SK007 resting cells. PLA (▲), PPA (□); reactions were carried out at 30°C for 8 h with shaking at 150 rpm in tubes containing 5 ml of 50 mM Tris/HCl buffer (pH 7.1) with 6.1 mM PPA and 11.25 g dry cell wt l^{-1} of resting cells. Data were expressed as mean ± SD from three independent experiments

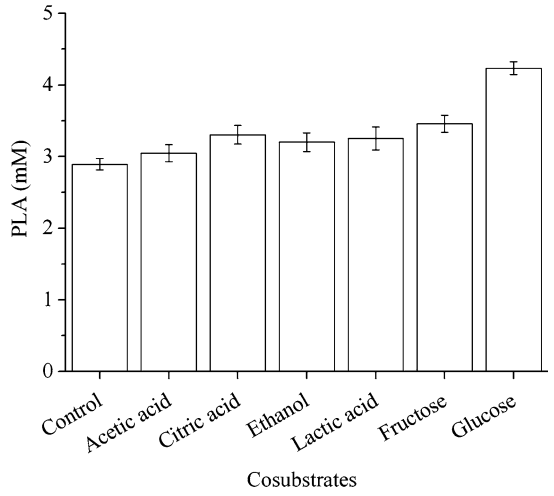

Fig. 5 Effect of cosubstrate on PLA production by *Lactobacillus* sp. SK007 resting cells. Reactions were carried out at 30°C for 4 h with shaking at 150 rpm in tubes containing 5 ml of 50 mM Tris/HCl buffer (pH 7.1) with 6.1 mM PPA, 11.25 g dry cell wt l^{-1} of resting cells and 55 mM of each cosubstrate. Data were expressed as mean ± SD from three independent experiments

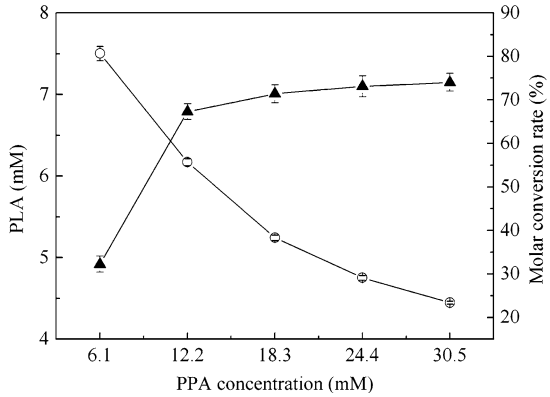

Fig. 6 Effect of PPA concentration on PLA production by *Lactobacillus* sp. SK007 resting cells. PLA (▲), molar conversion rate (○); reactions were carried out at 35°C for 4 h with shaking at 150 rpm in tubes containing 5 ml of 50 mM Tris/HCl buffer (pH 7.5) with 55 mM glucose, 22.5 g dry cell wt l^{-1} of resting cells and various PPA concentrations. Data were expressed as mean ± SD from three independent experiments

bottleneck was overcome using PPA instead of Phe as substrate. With growing cells, PPA addition significantly stimulated PLA formation and shortened the fermentation time to 24 h. PLA production using resting cells was effective with glucose addition as cosubstrate. These properties provide the possibility of utilizing PPA as substrate in PLA production using either growing or resting cells of *Lactobacillus* sp. SK007.

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References

Dieuleveux V, Van Der Pyl D, Chataud J, Gueguen M (1998a) Purification and characterization of anti-*Listeria* compounds produced by *Geotrichum candidum*. Appl Environ Microbiol 64:800–803

Dieuleveux V, Lemarinier S, Gueguen M (1998b) Antimicrobial spectrum and target site of D-3-phenyllactic acid. Int J Food Microbiol 40:177–183

Dieuleveux V, Gueguen M (1998) Antimicrobial effects of D-3-phenyllactic acid on *Listeria monocytogenes* in TSB-YE medium, milk, and cheese. J Food Prot 61:1281–1285

Gupta RK, Prasad DN (1989) Antibiotic activity of nisin in food preservation: a review. Microbiol Aliment Nutrit 7:199–208

Haberland J, Kriegesmann A, Wolfram E, Hummel W, Liese A (2002) Diastereoselective synthesis of optically active (2*R*,5*R*)-hexanediol. Appl Microbiol Biotechnol 58:595–599

Lavermicocca P, Valerio F, Evidente A, Lazzaroni S, Corsetti A, Gobbetti M (2000) Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus* strain 21B. Appl Environ Microbiol 66:4084–4090

Lavermicocca P, Valerio F, Visconti A (2003) Antifungal activity of phenyllactic acid against molds isolated from bakery products. Appl Environ Microbiol 69:634–640

Matsunaga T, Higashijima M, Sulaswatty A, Nishimura S, Kitamura T, Tsuji M, Kawaguchi T (1987) Repeated batch production of L-phenylalanine from phenylpyruvate and NH_4Cl by immobilized cells of *Nocardia opaca* under hydrogen high pressure. Biotechnol Bioeng 31:834–840

Schnurer J, Magnusson J (2005) Antifungal lactic acid bacteria as biopreservatives. Trend Food Sci Technol 16:70–78

Strom K, Sjogren J, Broberg A, Schnurer J (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-*trans*-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 68:4322–4327

Then J, Doherty A, Neatherway H, Marquardt R, Deger H-M, Voelskow H, Wohner G, Prave P (1987) Production of L-phenylalanine from phenylpyruvate using resting cells of *Escherichia coli*. Biotechnol Lett 9:680–684

Thierry A, Maillard MB (2002) Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii*. Lait 82:17–32

Valerio F, Lavermicocca P, Pascale M, Visconti A (2004) Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. FEMS Microbiol Lett 233:289–295

Vermeulen N, Ganzle MG, Vogel RF (2006) Influence of peptide supply and cosubstrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451^T and *Lactobacillus plantarum* TMW1.468. J Agric Food Chem 54:3832–3839

Yu JM, Xue F, Dai HJ (1991) Synthesis of ''Danshensu'' derivatives. Acta Pharmaceutica Sinica 26:552–556 (in Chinese)