J Ind Microbiol Biotechnol (2011) 38:643–648
DOI 10.1007/s10295-011-0955-2

SHORT COMMUNICATION

Cinnamic acid production using *Streptomyces lividans* expressing phenylalanine ammonia lyase

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Received: 31 January 2011 / Accepted: 4 March 2011 / Published online: 22 March 2011

Abstract Cinnamic acid production was demonstrated using *Streptomyces* as a host. A gene encoding phenylalanine ammonia lyase (PAL) from *Streptomyces maritimus* was introduced into *Streptomyces lividans*, and its expression was confirmed by Western blot analysis. After 4 days cultivation using glucose as carbon source, the maximal level of cinnamic acid reached 210 mg/L. When glycerol (30 g/L) was used as carbon source, the maximal level of produced cinnamic acid reached 450 mg/L. In addition, using raw starch, xylose or xylan as carbon source, the maximal level of cinnamic acid reached 460, 300, and 130 mg/L, respectively. We demonstrated that *S. lividans* has great potential to produce cinnamic acid as well as other aromatic compounds.

Keywords Cinnamic acid · Phenylalanine ammonia lyase · *Streptomyces* · Biomass

Introduction

To construct a biomass-oriented sustainable society, it is necessary to produce chemicals and fuels from renewable resources such as biomass and industrial waste substances [3]. At present, polymer materials are made of building-block compounds usually made by chemical processes. Although various biomaterial production systems have been developed using a variety of microbes, such as ethanol production using yeasts [4, 12, 27, 28], lactic acid production using lactic acid bacteria [2, 13, 21], and amino acid production using *Corynebacterium* [9, 19, 22], there are few reports concerning production of aromatic building blocks from biomass.

Cinnamic acid is an aromatic compound widely used as a material for organic thin-film displays or as an antibacterial agent. In nature, cinnamic acid exists in plants as a precursor of various phenylpropanoids such as lignins, flavonoids, and coumarins [10]. Cinnamic acid is chemically produced via a condensation reaction of acetic anhydride and benzaldehyde in the presence of sodium acetate [10]; however, this process is energy intensive. Although cinnamic acid production systems using *P. putida* or *E. coli* have been reported previously [10, 23], cinnamic acid productivity still needs to be improved.

Here, we used *Streptomyces*, which are antibiotic producers having high tolerance for aromatic compounds, as a host for cinnamic acid production. Secondary metabolite pathways in *Streptomyces* have been widely studied, and *Streptomyces* have been used to produce various antibiotics [8, 20, 24]. *Streptomyces* have also been used as hosts for secretory production of heterologous proteins [11, 14, 17]. However, there are few reports concerning low-molecular-weight compound production using *Streptomyces*.

Phenylalanine ammonia lyase (PAL) is a ubiquitous higher-plant enzyme that catalyzes nonoxidative deamination of phenylalanine to cinnamic acid in a committed step to phenylpropanoid metabolites (Fig. 1) [16, 25]. Although plant PALs, such as from *Petroselinum crispum*,

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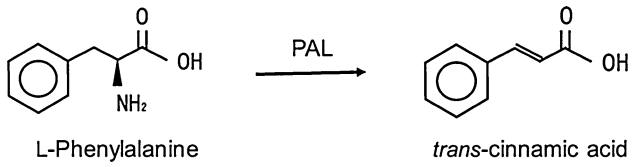

Fig. 1 PAL-catalyzed conversion of L-phenylalanine to *trans*-cinnamic acid

can convert only phenylalanine into cinnamic acid, PALs from *Rhodosporidium toruloides*, which have been thoroughly studied, can convert tyrosine to *p*-hydroxy-cinnamic acid, as well as phenylalanine into cinnamic acid [23]. Alternatively, the marine actinomycete *Streptomyces maritimus* can convert the amino acid L-phenylalanine into cinnamic acid by a novel bacterial phenylalanine ammonia lyase (PAL) in the enterocin type II polyketide synthesis process [16, 25, 26]. PAL from *S. maritimus*, having high substrate specificity for L-phenylalanine, allows for efficient cinnamic acid production [25]. Cinnamic acid production using PAL from *S. maritimus* expressing *S. coelicolor* has been previously reported [26]; however, the productivity of cinnamic acid was not high enough.

In this work, we focused on *S. lividans*, which is known as a heterologous protein producer [11, 14, 17]. We introduced PAL from *S. maritimus* into *S. lividans* and evaluated cinnamic acid productivity using not only glucose, but also glycerol, which is a potential economical feedstock for fermentation [3]. We also demonstrated direct cinnamic acid production from several kinds of biomass, such as raw starch, xylose, and xylan. One of the advantages of *S. lividans* is its biomass-assimilating ability, which is suitable for building-block compound production from biomass. This is the first report showing building-block compound production directly from various biomass resources using *Streptomyces*.

Materials and methods

Plasmid construction

The basal expression vector for the PAL protein in *S. lividans* as a host was constructed by polymerase chain reaction (PCR) using PrimeSTAR HS (Takara, Shiga, Japan). The gene fragment encoding the promoter region of the PLD open reading frame from *Streptoverticillium cinnamoneum* [11, 14] was amplified by PCR using the *Stv. cinnamoneum* genome (NBRC 12852) as a template with the following two primers: 5′-aaaagcttacgtcatggcgggtctctctcgtc-3′ (*Hin*dIII-promoter/Fw) and 5′-ttaagcttcccccgtcatgcgccccggaacgtcc gggcgctcagtcgtcagatcttgtgcatccttaaacgaagtaacgattccg-3′ (*Hin*dIII-terminator-*Bgl*II-promoter/Rv). The amplified fragment was subcloned into the *Hin*dIII site of pUC18. The vector was named pUC18-prom-*Bgl*II-term.

The gene fragment encoding *enc*P from *S. maritimus* was amplified by PCR using the *S. maritimus* genome (DSMZ 41777) as a template with the following two primers: 5′-aaagaattcagatctccgtcgagtccaccgattctccggccgcaa agg-3′ (*Eco*RI-*Bgl*II-*enc*P/Fw) and 5′-tttaagcttagatctgc ggccgcctcgagaggccttcagtgcgccgccacggcagc-3′ (*Hin*dIII-*Bgl*II-*Not*I-*Xho*I-*Stu*I-*enc*P/Rv). The amplified fragment was subcloned into the *Hin*dIII and *Eco*RI sites of pUC18. The vector was named pUC18-*enc*P. pUC18-*enc*P was digested with *Bgl*II, and the digested fragment encoding *enc*P was subcloned into the *Bgl*II site of pUC18-prom-*Bgl*II-term. The vector was named pUC18-prom-*enc*P-term. Finally, pUC18-prom-*enc*P-term was digested with *Hin*dIII, and the digested fragment encoding prom-*enc*P-term was subcloned into the *Hin*dIII site of pUC702. The vector was named pUC702-p-*enc*P. The vector for expression of His-tagged *enc*P was constructed as follows. The gene fragment encoding *enc*P-$(His)_6$ was amplified by PCR using pUC18-*enc*P as a template with the flowing primers: 5′-aaaagatctatgaccttcgtcatagagctcgaca-3′ (*Bgl*II-*enc*P-$(His)_6$/Fw) and 5′-tttagatcttcagtggtggtggtggtggtggtg cgccgccacggcagcgtcgacg-3′ (*Bgl*II-*enc*P-$(His)_6$/Rv). The resultant plasmid was named pUC702-p-*enc*P-$(His)_6$.

Bacterial strain, transformation, and cultivation

Protoplasts of *S. lividans* 1326 were prepared according to the method of Hopwood et al. [5]. Briefly, mycelium of *S. lividans* 1326 was treated with 1 mg/mL lysozyme solution (Wako, Osaka, Japan), and the suspended mycelium was used as protoplasts. The plasmids pUC702-p-*enc*P and pUC702-p-*enc*P-$(His)_6$ were introduced by the polyethylene glycol (PEG) method. Selection of transformants was carried out by overlaying soft agar containing 50 μg/mL thiostrepton as antibiotic. After cultivation for 5 days, selected transformants were named *S. lividans*/p-*enc*P and *S. lividans*/p-*enc*P-$(His)_6$, respectively.

One spore of *S. lividans*/p-*enc*P was inoculated in a test-tube containing 5 ml TSB medium [17 g/L pancreatic digest of casein, 3 g/L papaic digest of soybean meal, 2.5 g/L glucose, 5.0 g/L sodium chloride, 2.5 g/L dipotassium phosphate (BD Diagnostic Systems, Sparks, MD, USA)] supplemented with 5 μg/mL thiostrepton (Sigma, St. Louis, MO), followed by cultivation at 28°C for 3 days. Then, 5 ml of the preculture medium was seeded into a shake flask with a baffle containing 100 ml TSB medium with 5 μg/mL thiostrepton, 15 g/L glucose, 15–30 g/L glycerol, 15 g/L raw starch, 15 g/L xylose, 15 g/L xylan from birch wood (Sigma) as carbon source, and 15–50 g/L tryptone as nitrogen source, and incubated at 28°C for 4–8 days.

Western blotting

S. lividans/p-*enc*P was cultured at 28°C for 72 h in 100 ml TSB medium. After cultivation, cells were centrifuged at 21,880×*g* for 10 min at 4°C. Cells were washed and resuspended in 700 µl phosphate-buffered saline (pH 7.2) containing 1% (vol/vol) Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). Cells were then disrupted using a Multi Beads Shocker (Yasuikikai, Osaka, Japan) according to a previously described protocol [22]. The insoluble fraction and glass beads were removed by centrifugation at 21,880×*g* for 10 min at 4°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer was added to the supernatant, followed by boiling at 100°C for 5 min. Proteins were analyzed by SDS–PAGE using an SDS–polyacrylamide gel (15%: w/v), after which proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore Co., Boston, MA, USA) and were allowed to react with primary rabbit anti-$(His)_6$ and secondary goat anti-rabbit immunoglobulin G alkaline-phosphatase-conjugated antibodies (Promega Co., Madison, WI, USA). The membrane was then stained with nitroblue tetrazolium (Promega) and 5-bromo-4-chloro-3-indolylphosphate (Promega) according to the manufacturer's protocol.

Analytical methods

Cinnamic acid concentration was simultaneously determined by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) using a Cholester column (Nacalai Tesque, Kyoto, Japan). The operating conditions were 30°C, acetonitrile:phosphate buffer (50 mM, pH 2.5) (30:70) mobile phase, and flow rate 1.2 ml/min; cinnamic acid concentration was determined using a ultraviolet absorbance detector (Shimadzu SPD-20AV). Culture supernatant was separated from the culture broth by centrifugation at 21,880×*g* for 20 min and then analyzed by HPLC.

Results and discussion

Expression of *enc*P using *S. lividans*

To confirm expression of *enc*P, $(His)_6$-tagged *enc*P was expressed using *S. lividans*. The intracellular fraction of *S. lividans*/p-*enc*P-$(His)_6$ cells after 5 days of cultivation was analyzed by Western blotting. A band corresponding to PAL (calculated molecular mass, 67 kDa) was clearly observed (Fig. 2, lane 2), whereas no band corresponding to PAL was observed in the case of *S. lividans*/pUC702 (Fig. 2, lane 1). These results show successful intracellular expression of *enc*P using *S. lividans*.

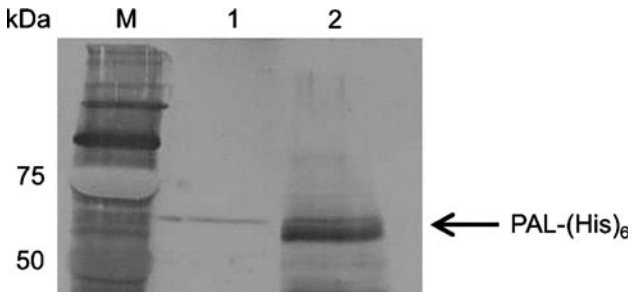

Fig. 2 Western blot analysis of PAL-$(His)_6$: *lane 1*: *S. lividans*/pUC702; *lane 2*: *S. lividans*/p-*enc*P-$(His)_6$

Production of cinnamic acid from glucose using *S. lividans*/p-*enc*P, and optimization of the supplied nitrogen source

We evaluated cinnamic acid production using modified TSB medium (TSB medium with additional 15 g/L glucose and 15 g/L tryptone) using *S. lividans*/p-*enc*P. *S. lividans*/pUC702 cells were also used as a control. Figure 3a shows time-courses of dry cell weight during cultivation. The maximum dry cell weight of *S. lividans*/p-*enc*P was 5.0 g/L after 2 days cultivation, which is almost the same as that of *S. lividans*/pUC702 (6.0 g/L after 3 days cultivation). The cell growth rate of *S. lividans*/p-*enc*P was slightly higher than that of *S. lividans*/pUC702. These data suggest that introduction of the *enc*P gene did not inhibit cell growth.

Figure 3b shows the amount of cinnamic acid produced. *S. lividans*/p-*enc*P successfully produced about 80 mg/L cinnamic acid from 15 g/L glucose, whereas *S. lividans*/pUC702 did not produce cinnamic acid at all. Increasing the initial glucose concentration led to low cell growth and lower cinnamic acid productivity (data not shown), and hence 15 g/L glucose was suitable for efficient cinnamic acid production using *S. lividans*/p-*enc*P.

To enhance cinnamic acid production using *S. lividans*, we tried to optimize the initial nitrogen source. *S. lividans*/p-*enc*P was grown in TSB medium with additional 15, 30 or 50 g/L tryptone as nitrogen source. As shown in Fig. 3b, increasing the initial tryptone concentration enhanced cinnamic acid productivity, and the cinnamic acid concentration reached up to 210 mg/L after 5 days cultivation of *S. lividans*/p-*enc*P with 15 g/L glucose and 50 g/L tryptone. Cinnamic acid productivity was not enhanced when larger amounts of tryptone were used (data not shown). Although initial cell growth and cinnamic acid production rates become slower with increasing initial tryptone concentrations, the maximum dry cell weight and produced cinnamic acid were improved. These results demonstrated that the nitrogen source is a key factor to enhance the production rate and maximal concentration of cinnamic acid.

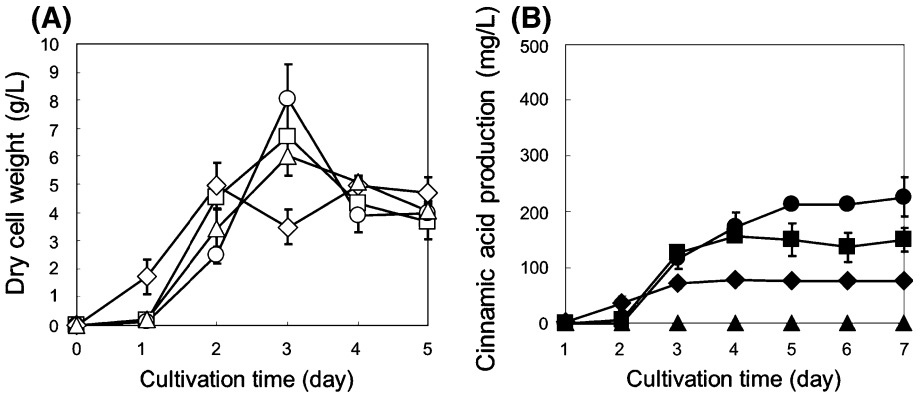

Fig. 3 **a** Time-courses of dry cell weight using glucose as sole carbon source: *S. lividans*/pUC702 in TSB medium with 15 g/L glucose and 15 g/L tryptone (*open triangles*); *S. lividans*/p-*enc*P in TSB medium with 15 g/L glucose, and 15 g/L tryptone (*diamonds*), 30 g/L tryptone (*open squares*), and 50 g/L tryptone (*open circles*). **b** Time-courses of produced cinnamic acid in culture: *S. lividans*/pUC702 in TSB medium with 15 g/L glucose and tryptone (*closed triangles*); *S. lividans*/p-*enc*P in TSB medium with 15 g/L glucose, and 15 g/L tryptone (*closed diamonds*), 30 g/L tryptone (*closed squares*), and 50 g/L tryptone (*closed circles*). Each data point shows the average of three independent experiments, and *error bars* represent standard deviation

Production of cinnamic acid from biomass in *S. lividans*/p-*enc*P, and optimization of the nutrient source concentration

According to our finding that increasing the initial glucose concentration leads to low cell growth and low cinnamic acid productivity, we tried to use glycerol, a potentially economical feedstock for fermentation, as carbon source [3]. Some *Streptomyces* can utilize glycerol more effectively than glucose [18]; however, the glycerol-assimilating ability of *S. lividans* had not previously been tested.

Figure 4a shows time-courses of dry cell weight using modified TSB medium with additional 15 g/L glycerol. In the case of wild-type *S. lividans*, the initial cell growth rate and maximum dry cell weight were significantly improved using glycerol as sole carbon source, implying that *S. lividans* possesses a glycerol utilization operon as well as *S. coelicolor* A3 (2). Similarly, the maximum dry cell weight of *S. lividans*/p-*enc*P with glycerol was 6.0 g/L after 2 days cultivation, showing notable improvement in cell growth. As shown in Fig. 4b, cinnamic acid production in TSB medium with 15 g/L glycerol was at maximal levels after 3 days, whereas 4 days were needed in the case of glucose (Fig. 3b). The amount of produced cinnamic acid from 15 g/L glycerol was 120 mg/L, which was 1.5-fold higher than that using glucose. This result shows that glycerol is a more suitable carbon source for *S. lividans* than glucose. Along with increasing glycerol and

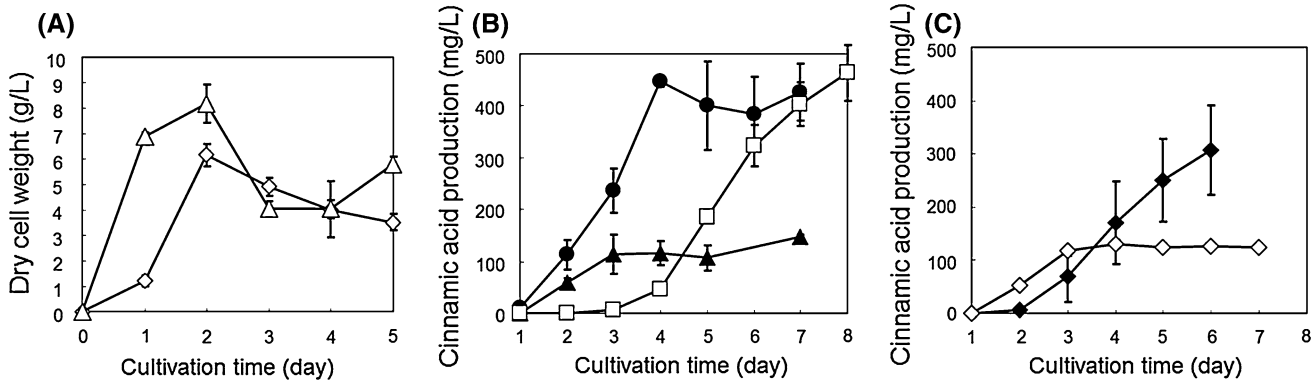

Fig. 4 **a** Time-courses of dry cell weight using glycerol as sole carbon source: *S. lividans* in TSB medium with 15 g/L glycerol and 15 g/L tryptone (*open triangles*); *S. lividans*/p-*enc*P in TSB medium with 15 g/L glycerol and 15 g/L tryptone (*open diamonds*). **b** Time-courses of produced cinnamic acid in culture: *S. lividans*/p-*enc*P in TSB medium with 15 g/L glycerol and 15 g/L tryptone (*closed diamonds*), 30 g/L glycerol and 50 g/L tryptone (*closed circles*), and 15 g/L raw starch and 50 g/L tryptone (*open squares*) **c** 15 g/L xylose and 50 g/L tryptone (*closed diamonds*), and 15 g/L xylan and 50 g/L tryptone (*open diamonds*). Each data point shows the average of three independent experiments, and *error bars* represent standard deviation

tryptone (30 g/L glycerol and 50 g/L tryptone), the maximal levels of produced cinnamic acid also increased and reached up to 450 mg/L after 4 days cultivation, which is higher than those of *E. coli* (120 mg/L) or batch-cultured *P. putida* S12 (93 mg/L) [10, 23]. Furthermore, we also confirmed cinnamic acid production from raw starch using *S. lividans*/p-*enc*P. From Fig. 4b, the production rate of cinnamic acid at the initial stage of cultivation is lower than that of glycerol, which may be caused by the lag time of raw starch degradation. The amount of cinnamic acid produced from 15 g/L raw starch and 50 g/L tryptone was 460 mg/L after 8 days cultivation.

Encouraged by these findings, we tried to carry out cinnamic acid production from xylose or xylan, which represents the most abundant hemicellulosic polysaccharide, using *S. lividans*/p-*enc*P. Although the initial rate of cinnamic acid production was lower than that with 15 g/L glucose, the maximal amount of produced cinnamic acid from 15 g/L xylose was 300 mg/L, which was 1.5-fold higher than that using glucose. When arabinose was used as sole carbon source, the amount of cinnamic acid produced was lower compared with other carbon sources (data not shown). This result implies that *S. lividans* also has an only xylose-assimilating pathway, like other *Streptomyces* species [1, 7, 15]. In addition, the amount of cinnamic acid produced from 15 g/L xylan reached 130 mg/L after 4 days cultivation, due to active-form xylanases produced by *S. lividans* [6]. This is the first report concerning production of biocompounds from various types of biomass using *Streptomyces*, and we also demonstrated that *S. lividans* has a great advantage for as a host for production of biocompounds from various carbon sources due to its glycerol-, xylose-, xylan-, and starch-assimilating abilities. In addition, *S. lividans* can grow in the presence of more than 1 g/L cinnamic acid, whereas *E. coli* cannot grow in only 0.1 g/L cinnamic acid (data not shown). This result implies that *S. lividans* has tolerance to cinnamic acid and is a promising host for cinnamic acid overproduction.

In conclusion, we successfully demonstrated cinnamic acid production using engineered *S. lividans* along with optimization of the carbon and nitrogen source. To improve cinnamic acid productivity, L-phenylalanine availability in the cell should be enhanced, because addition of small amounts of L-phenylalanine improved cinnamic acid productivity (data not shown). *S. lividans* can potentially be used as a host to produce various aromatic building-block compounds.

Acknowledgments This work was supported by the Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovation Bioproduction Kobe), MEXT, Japan.

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