Short Communication

Enhancement of lactate fraction in poly(lactate-co-3-hydroxybutyrate) synthesized by *Escherichia coli* harboring the D-lactate dehydrogenase gene from *Lactobacillus acetotolerans* HT

(Received August 24, 2018; Accepted September 11, 2018; J-STAGE Advance publication date: January 31, 2019)

Saki Goto,¹ Naoyuki Suzuki,² Ken'ichiro Matsumoto,³ Seiichi Taguchi,⁴
Kenji Tanaka,² and Hiromi Matsusaki^{1,*}

¹ Department of Food and Health Sciences, Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100, Tsukide, Higashi-ku, Kumamoto 862-8502, Japan

² Department of Biological and Environmental Chemistry, Faculty of Humanity-Oriented Science and Engineering, Kindai University, 11-6 Kayanomori, Izuka City, Fukuoka 820-8555, Japan

³ Division of Applied Chemistry, Graduate School of Engineering, Hokkaido University, Kita 13, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0808, Japan

⁴ Department of Chemistry for Life Sciences and Agriculture, Faculty of Life Sciences, Tokyo University of Agriculture, 1-1-1 Sakuraoka, Setagaya-ku, Tokyo 156-8502, Japan

For enhancing the lactate (LA) fraction of poly(lactate-co-3-hydroxybutyrate)s [P(LA-co-3HB)s], an exogenous D-lactate dehydrogenase gene (*ldhD*) was introduced into *Escherichia coli*. Recombinant strains of *E. coli* DH5 α , LS5218, and XL1-Blue harboring the *ldhD* gene from *Lactobacillus acetotolerans* HT, together with polyhydroxyalkanoate (PHA)-biosynthetic genes containing a lactate-polymerizing enzyme (modified PHA synthase) gene, accumulated the P(LA-co-3HB) copolymer from glucose under microaerobic conditions (100 strokes/min). The LA fraction of copolymers synthesized in the strains of DH5 α , LS5218, and XL1-Blue were 19.8, 15.7, and 28.5 mol%, respectively, which were higher than those of the strains without the *ldhD* gene (<6.7 mol% of LA units). Introduction of the exogenous *ldhD* gene into *E. coli* strains resulted in an enhanced LA fraction in P(LA-co-3HB)s.

Key Words: biodegradable plastics; lactate dehydrogenase; polyhydroxyalkanoate; poly(lactic acid) (PLA)

Poly(lactic acid)s (PLAs), a biodegradable plastic, have recently attracted considerable attention because they are

producible from renewable resources and have excellent properties, such as transparency (Jambunathan and Zhang, 2016). PLAs are normally synthesized by chemical processes using heavy metal catalysts (Garlotta, 2001). Whereas, polyhydroxyalkanoates (PHAs), a biodegradable plastic, are accumulated in various bacteria as intracellular carbon and energy storage materials (Sudesh et al., 2000). Recently, a new microbial platform for producing PLA-like polyester has been established based on a PHA biosynthetic pathway by applying an LA-polymerizing enzyme (LPE), which is an engineered PHA synthase, to recognize D-lactyl-CoA (D-LA-CoA) as a substrate (Taguchi et al., 2008). This bio-process is an ecofriendly method to produce PLA-like polyesters without heavy metal catalysts. In addition, poly(lactate-co-3-hydroxybutyrate)s [P(LA-co-3HB)s], PLA-like polyesters consisting of LA and 3HB units, exhibit transparency, an attractive feature, depending on their LA fraction (Yamada et al., 2011). Attempts have been made to enhance the LA fraction by modifying cultivation, using an LA-overproducing *pflA* knockout mutant of *E. coli*, which shuts off the pathway of formate production from pyruvate, and applying a further-engineered LPE with enhanced LA-polymerizing capacity (Matsumoto and Taguchi, 2013).

E. coli converts pyruvate to D-LA by the endogenous D-lactate dehydrogenase (D-LDH), encoded as *ldhA*, which is highly expressed under anaerobic conditions and at a low pH (Bunch et al., 1997; Mat-Jan et al., 1989). In con-

*Corresponding author: Hiromi Matsusaki, Department of Food and Health Sciences, Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100, Tsukide, Higashi-ku, Kumamoto 862-8502, Japan.
Tel: +81-96-321-6697 Fax: +81-96-384-6765 E-mail: matusaki@pu-kumamoto.ac.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Table 1. Strains and plasmids used in this study.

Bacterial strains or plasmids	Characteristics	Reference or source
Bacterial strains		
<i>Lactobacillus acetotolerans</i> HT	Wild strain	Tanaka et al. (2015)
<i>Escherichia coli</i> DH5 α	<i>deoR</i> , <i>endA1</i> , <i>gyrA96</i> , <i>hsdR17</i> ($r_K^-m_K^+$), <i>recA1</i> , <i>relA1</i> , <i>supE44</i> , <i>thi-1</i> , $\Delta(lacZYA-argFV169)$, $\Phi80\Delta lacZ\Delta M15$, F ⁻ <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> , <i>supE44</i> , <i>relA1</i> , <i>lac</i> [F ⁺ <i>proAB</i> , <i>lac^{ts}</i> , <i>lacZ\Delta M15::Tn10</i> (Tet ^r)]	Clontech
<i>E. coli</i> XL1-Blue	<i>fudR601</i> , <i>atoC2</i> (Con)	Stratagene
<i>E. coli</i> LS5218		Spratt et al. (1981)
Plasmids		
T-vector pMD20	<i>E. coli</i> cloning vector, Ap ^r , <i>lacPOZ</i> , SP promoter	TaKaRa
pBluescript II KS ⁺	<i>E. coli</i> cloning vector, Ap ^r , <i>lacPOZ</i> , T7 and T3 promoter	Stratagene
pBBR1MCS-2	Km ^r , broad host range, <i>lacPOZ</i>	Kovach et al. (1995)
pTV118NpctC1(STQK)AB	pTV118N derivative; P _{lac} , <i>pct</i> , P _{Re} , <i>phaC1</i> (STQK), <i>phaA</i> , <i>phaB</i> , T _{Re}	Taguchi et al. (2008)
pBS- <i>ldhD</i>	pBluescript II KS ⁺ derivative; <i>ldhD</i> gene of <i>Lb. acetotolerans</i> HT	Goto et al. (2018)
pRKmAX- <i>ldhD</i>	pBBR1MCS-2 derivative; <i>ldhD</i> gene of <i>Lb. acetotolerans</i> HT	This study
pTV118NpctC1(STQK)ABdP _{Re}	pTV118N derivative; P _{lac} , <i>pct</i> , <i>phaC1</i> (STQK), <i>phaA</i> , <i>phaB</i> , T _{Re}	This study
pTV118NpctC1(STQK)ldhDABdP _{Re}	pTV118N derivative; P _{lac} , <i>pct</i> , <i>phaC1</i> (STQK), <i>ldhD</i> , <i>phaA</i> , <i>phaB</i> , T _{Re}	This study

Ap^r, ampicillin resistance gene; Km^r, kanamycin resistance gene.

trast, under aerobic conditions, the production of acetyl-CoA is increased, which is able to serve as a CoA donor for the generation of D-LA-CoA (Matsumoto and Taguchi, 2013). In addition, LPE can recognize D-LA-CoA as a substrate and incorporate the LA unit into the polymer chain only when (R)-3HB-CoA is present. Therefore, we introduced an exogenous D-LDH gene (*ldhD*) into *E. coli* in order to make D-LA constitutively produced even under aerobic conditions as well as anaerobic conditions.

In our previous study, we cloned and identified the *ldhD* gene of *Lactobacillus acetotolerans* HT (Goto et al., 2018). The recombinant *E. coli* DH5 α with the introduced *ldhD* gene from *Lb. acetotolerans* HT produced a higher amount of D-LA than the control under a shaking culture. Hence, the aim of the present study was to enhance the LA fraction in P(LA-co-3HB) synthesized via the *ldhD*-introducing recombinant *E. coli*.

The bacterial strains and plasmids used in this study are listed in Table 1. To construct pRKmAX-*ldhD*, pBS-*ldhD* was digested with *ApaI* and *XhoI*, and the DNA fragment containing the SD sequence was ligated with the *ApaI* and *XhoI*-digested pBBR1MCS-2. The plasmid pRKmAX-*ldhD* was introduced into *E. coli* DH5 α together with pTV118NpctC1(STQK)AB, which harbors the genes of propionyl-CoA transferase (Pct) from *Megasphaera elsdenii*, the Ser325Thr/Gln481Lys mutated PHA synthase from *Pseudomonas* sp. 61-3 [PhaC1(STQK)] as a LPE, and the 3HB monomer supplying enzymes [β -ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB)] from *Ralstonia eutropha*.

To remove the promoter (P_{Re}) of *R. eutropha* from pTV118NpctC1(STQK)AB, inverse PCR was performed using primers phaC1(STQK)(UP)-f1 (5'-GAATCTAGAAATAATTTTGTCTTAACCTTTAA-3') and pct(DS)-r1 (5'-CCCGGGATCCGGTTATTTTTT-CAGTCCCAT-3'). The amplified DNA fragment was phosphorylated using the T4 Polynucleotide Kinase (Toyobo), followed by self-ligation to yield pTV118NpctC1(STQK)ABdP_{Re}, in which all inserted genes were expressed under control of the *lac* promoter (P_{lac}). The *ldhD* gene was amplified by PCR from the genomic DNA of *Lb. acetotolerans* HT with primers *ldhD*-UP(*PstI*)-f (5'-AACTGCAGAAAGGGTCATATCATTA-TTTTTTTTGGGAGG-3') and *ldhD*-*PstI*(TAA)-r (5'-AACTGCAGGAATAGAAAATTATGCATCTAA-3') (underlined sequences indicate *PstI* site). The PCR product was cloned into the pMD20-T vector. The 1.0-kb *PstI* fragment containing the *ldhD* gene from the pMD20-T derivative was introduced into pTV118NpctC1(STQK)ABdP_{Re} at the *PstI* site to give pTV118NpctC1(STQK)ldhDABdP_{Re}. The plasmid pTV118NpctC1(STQK)ABdP_{Re} or pTV118NpctC1(STQK)ldhDABdP_{Re} was introduced into *E. coli* DH5 α , XL1-Blue and LS5218, respectively.

For P(LA-co-3HB) production, the recombinant strains of *E. coli* were grown in 1.5 mL lysogeny broth (LB) medium (Sambrook et al., 1989) at 30°C for 15 h. When needed, ampicillin (100 mg/L) and kanamycin (50 mg/L) were added to the medium. Following this, 1 mL of culture broth was transferred to 300-mL conical flasks with 100 mL LB medium. Cells were cultivated at 30°C in a

Table 2. P(LA-co-3HB) production by the recombinant strains of *E. coli* DH5 α .

Plasmid	Shaking speed (strokes/min)	Dry cell weight (g/l)	Polymer content (wt%)	Polymer composition (mol%)		pH
				LA (C3)	3HB (C4)	
pTV118NpctC1(STQK)AB	0	0.35 $\pm$ 0.02	22.5 $\pm$ 5.0	29.6 $\pm$ 0.9	70.4 $\pm$ 0.9	4.77 $\pm$ 0.09
	60	0.55 $\pm$ 0.17	31.7 $\pm$ 4.8	30.0 $\pm$ 0.9	70.0 $\pm$ 0.9	4.80 $\pm$ 0.08
	100	1.30 $\pm$ 0.05	46.9 $\pm$ 3.3	9.8 $\pm$ 0.9	90.2 $\pm$ 0.9	4.94 $\pm$ 0.06
pTV118NpctC1(STQK)AB and pRKmAX-ldhD	0	0.39 $\pm$ 0.03	33.5 $\pm$ 4.1	36.6 $\pm$ 1.3	63.4 $\pm$ 1.3	4.70 $\pm$ 0.08
	60	0.77 $\pm$ 0.06	40.5 $\pm$ 5.3	36.4 $\pm$ 0.4	63.6 $\pm$ 0.4	4.74 $\pm$ 0.08
	100	1.80 $\pm$ 0.11	45.3 $\pm$ 0.6	19.8 $\pm$ 0.8	80.2 $\pm$ 0.8	4.87 $\pm$ 0.05

Cells were cultivated at 30°C for 48 h in a 300-ml conical flask containing 100 ml of LB medium. 2% (w/v) glucose was added to the medium after 8 h of cultivation.

Fig. 1. P(LA-co-3HB) production by the recombinant strains of *E. coli* DH5 α .

Cells were cultivated at 30°C for 48 h at a shaking speed of 100 strokes/min in a 300-mL conical flask containing 100 mL of LB medium. 2% (w/v) glucose was added to the medium after 8 h of cultivation. 1, pTV118NpctC1(STQK)AB; 2, pTV118NpctC1(STQK)AB and pRKmAX-ldhD; Diamond, D-LA concentration; white bar, 3HB unit in the polymer; gray bar, D-LA unit in the polymer.

Fig. 2. Relationship between the D-LA concentration in the culture supernatant and LA molar fraction of P(LA-co-3HB)s accumulated in the cells.

White diamond, DH5 α /pTV118NpctC1(STQK)AB; black diamond, DH5 α /pTV118NpctC1(STQK)AB and pRKmAX-ldhD.

static culture (anaerobically) or a shaking culture (60 or 100 strokes/min, microaerobically). A filter-sterilized glucose solution was added to the medium at a concentration of 2% (w/v), as the sole carbon source, after 8 h of cul-

Fig. 3. P(LA-co-3HB) production by the recombinant strains of *E. coli*.

Cells were cultivated at 30°C for 48 h at a shaking speed of 100 strokes/min in a 300-mL conical flask containing 100 mL of LB medium. 2% (w/v) glucose was added to the medium after 8 h of cultivation. *ldhD* (-), the recombinant strains of *E. coli* harboring pTV118NpctC1(STQK)ABdP_{RE}; *ldhD* (+), the recombinant strains of *E. coli* harboring pTV118NpctC1(STQK)ldhDABdP_{RE}; White bar, 3HB unit in the polymer; gray bar, D-LA unit in the polymer.

vation, and with continued cultivation for a total of 48 h. A determination of the cellular polymer composition and content by gas chromatography was performed as reported previously (Kato et al., 1996). The concentration of D-lactic acid in the medium was determined using a D-Lactic acid/L-Lactic acid F-kit (Roche) according to the manufacturer's instructions. The cultivation runs were performed at least in triplicate.

With a shaking of 100 strokes/min, 0.13 g/L D-LA was secreted from *E. coli* DH5 α harboring pTV118NpctC1(STQK)AB and pRKmAX-ldhD into the medium, whereas the D-LA concentration produced by *E. coli* DH5 α harboring pTV118NpctC1(STQK)AB was very low (<0.01 g/L) (Fig. 1). Moreover, the LA fraction (19.8

mol%) in the P(LA-co-3HB) obtained by *E. coli* DH5 α harboring pTV118NpctC1(STQK)AB and pRkmAX-ldhD was higher than the strain harboring pTV118NpctC1(STQK)AB only (9.8 mol%), indicating that the high D-LA concentration in the culture supernatant led to an increase in the LA fraction of P(LA-co-3HB) accumulated in the cells. In other words, the production of D-LA would be increased by the expression of the exogenous *ldhD* gene, and, therefore, it would increase the LA fraction in P(LA-co-3HB).

Subsequently, cells were relatively anaerobically cultivated in a 60 strokes/min shaking culture and in a static culture. The relationship between the D-LA concentration in the culture supernatant and the LA fraction of P(LA-co-3HB)s accumulated in the cells under all culture conditions is shown in Fig. 2. In both recombinant strains, the D-LA concentrations in the static culture (1.93 and 2.07 g/L) were higher than those in the 60 strokes/min shaking culture (1.35 and 1.49 g/L); whereas the LA fractions (30 and 36 mol%) in P(LA-co-3HB)s synthesized were almost the same in the static culture and the 60 strokes/min shaking culture. The results indicated that the LA fraction in P(LA-co-3HB) could not be increased any more, even though the cells were cultivated in the static culture. In a previous study, under anaerobic conditions by nitrogen aeration, the *pflA*-deficient *E. coli* strain JW0885 harboring pTV118NpctC1(STQK)AB produced 5.7 g/L LA and accumulated P(47% LA-co-3HB), whereas the amount of LA in the polymer was decreased compared with the microaerobic culture (Yamada et al., 2011). This phenomenon has been interpreted as a decreased LA-CoA supply due to reduced acetyl-CoA, as CoA is the LA donor, under anaerobic conditions.

Table 2 summarizes the results of P(LA-co-3HB)s production in all culture conditions. DH5 α /pTV118NpctC1(STQK)AB and pRkmAX-ldhD showed the highest LA fraction (36.6 mol%) in a static culture; however, the dry cell weight (0.39 g/L) was lower than those in a microaerobic culture (100 strokes/min). This result indicated that the LA produced inhibited cell growth. Although LA production increased in *E. coli* as the host under anaerobic conditions, the cell growth decreased as compared with the microaerobic condition (de Graef et al., 1999; Matsuoka and Kurata, 2017; Yamada et al., 2011). PhaC1(STQK) is capable of polymerizing the unusual substrate D-LA-CoA into a polyester; however, no polymer containing LA units has been synthesized without a supply of the natural substrate (R)-3HB-CoA to the system (Matsumoto and Taguchi, 2013; Taguchi et al., 2008; Yamada et al., 2011). For these reasons, the exogenous *ldhD*-expressing recombinant strains would be suitable for synthesizing P(LA-co-3HB) with a relatively high LA fraction under microaerobic conditions since sufficient cell growth and the presence of (R)-3HB-CoA were observed.

To further investigate the effects of introducing *ldhD* gene in other *E. coli* strains, P(LA-co-3HB) copolymers were produced using *E. coli* XL1-Blue and LS5218 as hosts. pTV118NpctC1(STQK)ABdP_{Re} or pTV118NpctC1(STQK)ldhDABdP_{Re}, in which all genes were expressed under control of the *lac* promoter (P_{lac}),

was introduced into *E. coli* DH5 α , XL1-Blue, and LS5218. All recombinant strains synthesized P(LA-co-3HB) copolymers under microaerobic conditions (100 strokes/min) (Fig. 3). Introduction of the *ldhD* gene into *E. coli* strains resulted in the synthesis of P(LA-co-3HB)s with relatively high LA fractions. That is, P(LA-co-3HB)s with an LA fraction of 14.3–28.5 mol% were synthesized by the recombinant strains harboring pTV118NpctC1(STQK)ldhDABdP_{Re}. In particular, *E. coli* XL1-Blue/pTV118NpctC1(STQK)ldhDABdP_{Re} accumulated 15.7 wt% P(28.5% LA-co-3HB), resulting in a higher polymer content and LA fraction than those of other recombinant strains harboring pTV118NpctC1(STQK)ABdP_{Re}. This indicates that polymers with relatively high LA fractions would be synthesized by introducing the *ldhD* gene, even into *E. coli* strains. Further, *E. coli* DH5 α harboring both pTV118NpctC1(STQK)AB and pRkmAX-ldhD accumulated 45.3 wt% LA-based polymer (LA fraction: 19.8 mol%) (Table 2), resulting in a much higher polymer content than that of *E. coli* DH5 α harboring pTV118NpctC1(STQK)ldhDABdP_{Re} (7.6 wt%, LA fraction: 14.3 mol%) (Fig. 3). pTV118NpctC1(STQK)ldhDABdP_{Re} was constructed by eliminating P_{Re}, the promoter of the *phaCAB* operon in *R. eutropha*, between the *pct* and *phaC1(STQK)* genes, from pTV118NpctC1(STQK)AB and inserting the *ldhD* gene. Thus, the difference in the plasmids was presumed to affect the expression level of these genes, including the *phaC1(STQK)* gene.

In conclusion, we succeeded in enhancing LA fractions of P(LA-co-3HB)s synthesized by various *E. coli* strains through the introduction of the *ldhD* gene from *Lb. acetotolerans* HT. This is the first report on LA-based polymer synthesis by exogenous D-LDH gene introduction in *E. coli*. The introduction of an exogenous D-LDH gene (*ldhD*) into *E. coli* would be preferable for effective LA-based polymer production and cell growth under microaerobic conditions. This method enables us to enhance the LA fraction of the LA-based polymer without using a host such as a *pflA*-knockout strain.

Acknowledgments

This work was partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society. We would like to thank Editage (www.editage.jp) for English language editing.

References

- Bunch, P. K., Mat-Jan, F., Lee, N., and Clark, D. P. (1997) The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. *Microbiology*, **143**, 187–195.
- de Graef, M. R., Alexeeva, S., Snoep, J. L., and de Mattos, M. J. T. (1999) The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. *J. Bacteriol.*, **181**, 2351–2357.
- Garlotta, D. (2001) A literature review of poly(lactic acid). *J. Polym. Environ.*, **9**, 63–84.
- Goto, S., Motomura, A., Kawahara, A., Shiratsuchi, H., Tanaka, K. et al. (2018) Cloning and heterologous expression of lactate dehydrogenase genes from acid-tolerant *Lactobacillus acetotolerans* HT. *Food Sci. Technol. Res.*, **24**, 861–868.
- Jambunathan, P. and Zhang, K. (2016) Engineered biosynthesis of biodegradable polymers. *J. Ind. Microbiol. Biotechnol.*, **43**, 1037–1058.
- Kato, M., Bao, H. J., Kang, C. K., Fukui, T., and Doi, Y. (1996) Produc-

- tion of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. *Appl. Microbiol. Biotechnol.*, **45**, 363–370.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A. et al. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*, **166**, 175–176.
- Mat-Jan, F., Alam, K. Y., and Clark, D. P. (1989) Mutants of *Escherichia coli* deficient in the fermentative lactate dehydrogenase. *J. Bacteriol.*, **171**, 342–348.
- Matsumoto, K. and Taguchi, S. (2013) Enzyme and metabolic engineering for the production of novel biopolymers: crossover of biological and chemical processes. *Curr. Opin. Biotechnol.*, **24**, 1054–1060.
- Matsuoka, Y. and Kurata, H. (2017) Modeling and simulation of the redox regulation of the metabolism in *Escherichia coli* at different oxygen concentrations. *Biotechnol. Biofuels*, **10**, 183.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Spratt, S. K., Ginsburgh, C. L., and Nunn, W. D. (1981) Isolation and genetic characterization of *Escherichia coli* mutants defective in propionate metabolism. *J. Bacteriol.*, **146**, 1166–1169.
- Sudesh, K., Abe, H., and Doi, Y. (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.*, **25**, 1503–1555.
- Taguchi, S., Yamada, M., Matsumoto, K., Tajima, K., Satoh, Y. et al. (2008) A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. *Proc. Natl. Acad. Sci. USA*, **105**, 17323–17327.
- Tanaka, K., Tajiri, S., Sawada, R., Kawamoto, Y., Matsubara, T. et al. (2015) Acid-tolerant lactic acid bacterium isolated from rice vinegar. *Int. J. Res. Appl. Nat. Soc. Sci.*, **3**, 29–36.
- Yamada, M., Matsumoto, K., Uramoto, S., Motohashi, R., Abe, H. et al. (2011) Lactate fraction dependent mechanical properties of semitransparent poly(lactate-co-3-hydroxybutyrate)s produced by control of lactyl-CoA monomer fluxes in recombinant *Escherichia coli*. *J. Biotechnol.*, **154**, 255–260.