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MINI-REVIEW

Advances and needs for endotoxin-free production strains

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Abstract The choice of an appropriate microbial host cell and suitable production conditions is crucial for the downstream processing of pharmaceutical- and food-grade products. Although *Escherichia coli* serves as a highly valuable leading platform for the production of value-added products, like most Gram-negative bacteria, this bacterium contains a potent immunostimulatory lipopolysaccharide (LPS), referred to as an endotoxin. In contrast, Gram-positive bacteria, notably *Bacillus*, lactic acid bacteria (LAB), *Corynebacterium*, and yeasts have been extensively used as generally recognized as safe (GRAS) endotoxin-free platforms for the production of a variety of products. This review summarizes the currently available knowledge on the utilization of these representative Gram-positive bacteria for the production of eco- and bio-friendly products, particularly natural polyesters, polyhydroxyalkanoates, bacteriocins, and membrane proteins. The successful case studies presented here serve to inspire the use of these microorganisms as a main-player or by-player depending on their individual properties for the industrial production of these desirable targets.

Keywords Food-grade Gram-positive bacteria · Lipopolysaccharide · Polyhydroxyalkanoates · Bacteriocins · Membrane proteins · Yeasts

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Introduction

To date, numerous microbial production systems have been established, benefitting a wide range of industrial applications related to food, biopharmaceuticals, welfare, energy, fine chemicals, polymeric materials, and other areas. Most eco- and bio-friendly recombinant proteins and value-added metabolites are manufactured using *Escherichia coli* as a major platform. However, the outer membrane of *E. coli* contains the potent immunostimulatory substance, lipopolysaccharide (LPS; Valappil et al. 2007; Mamat et al. 2015). LPS, also referred to as endotoxin, induces a pyrogenic response and ultimately triggers septic shock. Therefore, US Food and Drug Administration (FDA) guidelines have set a stringent limit of acceptable endotoxin levels (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM310098.pdf). The removal of bacterial endotoxin from recombinant proteins and metabolites is a challenging and expensive process that is nonetheless required to ensure the safety of the final products. Considering that FDA-approved expression hosts for recombinant therapeutic proteins, nowadays more than 150 biopharmaceuticals are largely produced by *E. coli* (30 %), yeasts (20 %), and mammalian cell lines (50 %; Bill 2014). Yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* are also important generally recognized as safe (GRAS) platforms.

In this mini-review, we focus on the advantages and requirements of endotoxin-free Gram-positive bacteria

and yeasts as generally recognized as GRAS platforms, represented by strains belonging to *Bacillus*, lactic acid bacteria (LAB) and *Corynebacterium*, and yeasts. In addition to many types of beneficial proteins, naturally occurring polyesters, polyhydroxyalkanoates (PHAs), and antibacterial bacteriocins are also good targets for manufacture by the aforementioned bacteria. PHAs are produced as energy and carbon storage materials by a large number of Gram-positive and Gram-negative bacteria (Madison and Huisman 1999). When PHAs are extracted from bacterial cells, these macromolecular substances show material properties that are similar to some common plastics, such as petrochemical polypropylene (Doi 1990; Doi et al. 1995). PHAs have, therefore, been drawing attention as a good candidate for a biodegradable and/or biocompatible plastic material that can be produced from renewable raw resources and inexpensive waste materials (Byrom 1987; Sudesh et al. 2000; Chanprateep 2010). To date, the production of PHAs has been achieved using many types of Gram-negative bacteria, such as native and recombinant strains of *Ralstonia eutropha*, *Pseudomonas* species, and recombinant *E. coli* (Steinbüchel and Füchtenbusch 1998). A trial of endotoxin removal was performed to extract PHA polymer at satisfactory levels for biomedical applications using a simple NaOH digestion method and other simple treatments (Lee et al. 1999; Furrer et al. 2007). Herein, we present several case studies on the production of PHAs and PHA-related polyesters using Gram-positive bacteria in both native and recombinant forms as endotoxin-free platforms. In addition, we address the potential of LAB as GRAS platforms, focusing on their biogenesis of nisin A, one of the antimicrobial peptides called bacteriocins, produced by *Lactococcus lactis*. Recent studies suggest there are advantages of using the *L. lactis* cell as an endotoxin-free platform for a wide variety of recombinant products, such as secretory peptides, membrane proteins, and PHA polymers (Blatteis et al. 2000).

The case for *Bacillus* species

The high-level expression of recombinant proteins in bacterial cells is an indispensable technique for protein engineering in academic and industrial research. *E. coli* is still widely used and is often the first choice of organisms; however, this species presents several obstacles, including the generation of endotoxins, that limit its final application as a cell factory for the production of recombinant proteins, especially the ones that are of pharmaceutical interest (Liu et al. 1997; Schmidt 2004; Ferrer-Miralles et al. 2009). Thus, considering the limitations imposed by the use of *E. coli* for pharmaceutical applications, *Bacillus subtilis* is among the best-characterized endotoxin-free Gram-positive bacteria in terms of biochemical, genetic, and molecular biological studies (Zweers et al. 2008). *B. subtilis* is attractive for industrial use for a variety of reasons, including its excellent ability to secrete proteins and safe antibacterial compounds (Westers et al. 2004). Many studies on the industrial secretory production of enzymes, such as alkaline α-amylase and alkaline cellulose (Manabe et al. 2011), and medicinal drugs, such as myo-inositol and scyllo-inositol (Tanaka et al. 2013), have been reported using this bacterium. This excellent secretion ability is related to the accessibility of the expressed protein to the host membrane and suggests another advantage of this bacterium. Proteins overexpressed in *E. coli* often tend to form inclusion bodies (Singh et al. 2015), and resolving this problem of inclusion bodies has been gaining increasing significance. Many genome sequences are available for the functional analysis of unknown genes. Approximately 25 % of genes identified in all genomes are thought to encode membrane proteins (Wallin and von Heijne 1998), which could perform a wide variety of functions, such as signal sensing and transduction, and could provide important information for drug targets (Overington et al. 2006). However, the function of the vast majority of membrane proteins has not been assigned. This lack is mainly due to the difficulty of recombinant expression of membrane proteins in *E. coli* cells (Frelet-Barrand et al. 2010).

As representative polymers, glycogen (polysaccharide), polyphosphate (polyanhydride), cyanophycin and polyglutamic acid (polyamide), or PHA are naturally occurred and recombinantly synthesized inside the bacterial cells. Many microorganisms can produce biodegradable and biocompatible PHA polymers, which serve as carbon and energy storage material under nutrient-limiting conditions with excess carbon (Rehm 2010). In the 1920s, French microbiologist Dr. Maurice Lemoigne discovered the first natural polyester, poly(3-hydroxybutyrate) [P(3HB)] in Gram-positive bacterium *Bacillus megaterium* (Lemoigne 1926). After that, P(3HB) has been extensively studied as the most common type of PHA. As illustrated in Fig. 1, PHA polymer has a variation in the length and composition of the side chains, capable of making over 150 possible monomeric constituents including 3HB, and consequently, producing a wide range of technical and medical applications, being potential alternatives to petroleum-derived thermoplastics (Anderson and Dawes 1990; Steinbüchel and Valentin 1995; Doi et al. 1995; Madison and Huisman 1999). For the synthesis of P(3HB), two molecules of acetyl-coenzyme (CoA) are firstly condensed to acetoacetyl-CoA by β-ketothiolase (PhaA). Acetoacetyl-CoA then is reduced to (*R*)-3-hydroxybutyryl (3HB)-CoA by the NADPH-dependent acetoacetyl-CoA reductase (PhaB), and 3HB-CoA is polymerized into P(3HB) by PHA synthase (PhaC) (Steinbüchel et al. 1992). This is the metabolic core pathway, which is commonly shared among Gram-positive and

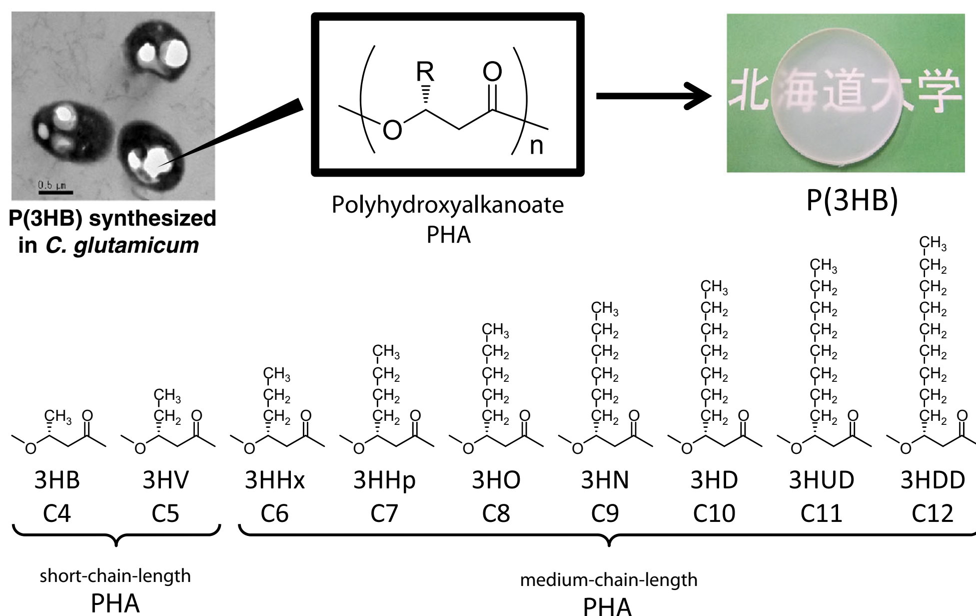
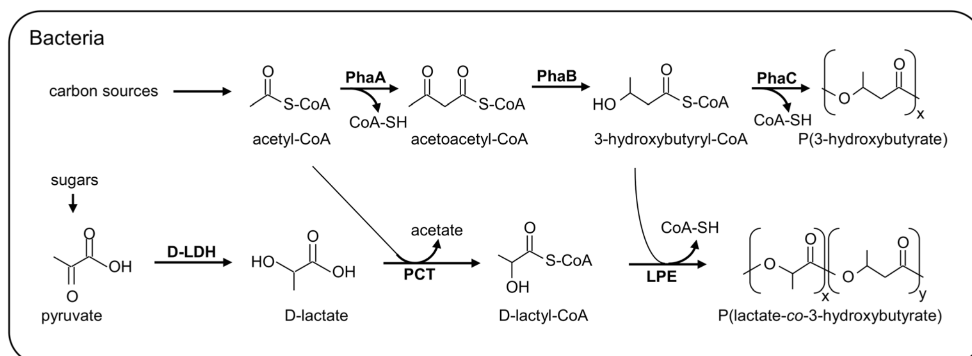


Fig. 1 Chemical structure and transmission electron microscopy image of the intracellular accumulation and solvent cast film of PHA. PHAs are primarily linear, head-to-tail polyesters consisting of 3-hydroxy fatty acid monomers, where the pendant group (*R*) varies from methyl to dodecyl. *3HB* 3-hydroxybutyrate, *3HV* 3-hydroxyvalerate, *3HHx* 3-hydroxyhexanoate, *3HHp* 3-hydroxyheptanoate, *3HO* 3-hydroxyoctanoate, *3HN* 3-hydroxynonanoate, *3HD* 3-hydroxydecanoate, *3HUD* 3-hydroxyundecanoate, *3HDD* 3-hydroxydodecanoate

Gram-negative bacteria (Fig. 2), via acetyl-CoA supplied from glycolysis pathway and fatty acid degradation pathway (Taguchi et al. 2001).

Bacillus spp. serve as a good platform for the production of 3HB homopolymer [P(3HB)] and its copolymers in both native and recombinant forms, as summarized in



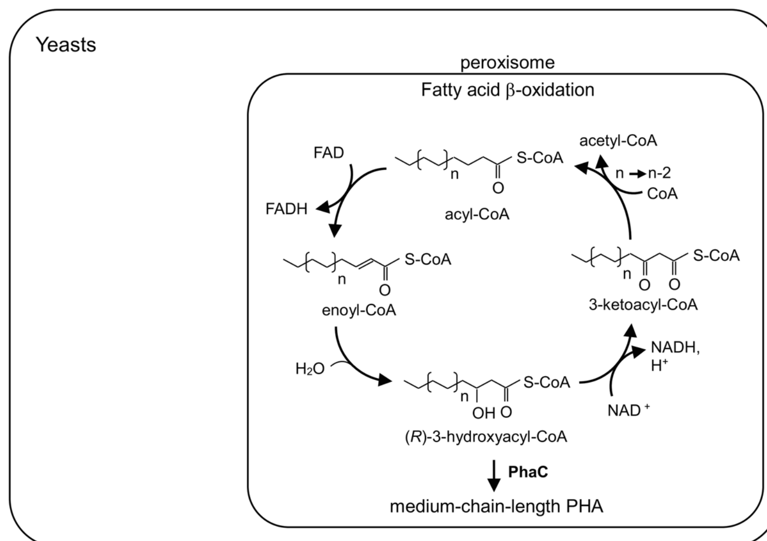


Fig. 2 Metabolic pathway for the production of PHAs and P(LA-*co*-3HB) in bacteria and yeast. *PhaA* β-ketothiolase, *PhaB* acetoacetyl CoA reductase, *PhaC* PHA synthase, *D-LDH* D-lactate dehydrogenase, *PCT* propionyl CoA transferase, *LPE* lactate polymerizing enzyme derived from PhaC1 from *Pseudomonas* sp. 61-3

several reviews (Valappil et al. 2007; Singh et al. 2009; Kumar et al. 2013). It should be noted that a wide range of nutrient substrates are available for the production of PHAs by more than fifteen species of *Bacillus* spp. Naturally, the yield and monomeric composition of the PHA polymers is highly varied depending on the type strain, feed material, culture conditions, and a combination of these factors. As shown in Fig. 1, the constituent monomers of PHA have been traditionally classified as short-chain-length (C4 and C5) and medium-chain-length (C6≦) hydroxyalkanoates (HAs; Steinbüchel and Valentin 1995). A variety of monomers are generated in the bacterial cells depending on the substrate specificity of the monomer-supplying enzymes and PHA synthases. In copolymerization with 3HB units, HA unit types 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HH), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 4-hydroxybutyrate (4HB), and 6-hydroxyhexanoate (6HH) have been frequently observed in the polymeric backbones formed by *Bacillus* spp. Because of its capacity for accepting a variety of monomers, *Bacillus* spp. can be considered a target-adjustable platform. The copolymerization of 3HB with other HA monomers can solve the problem of high-crystallinity, which causes brittleness and stiffness of the P(3HB) homopolymer (Nakamura et al. 1992; Doi et al. 1995).

Regarding the molecular weight of polymers, the research group of Dr. Tsuge recently reported an interesting phenomenon: there is a time-dependent decrease in the molecular weight of P(3HB) synthesized in recombinant *E. coli* harboring the gene encoding PHA synthase from *Bacillus cereus* YB-4 (Mizuno et al. 2010; Tomizawa et al. 2011). Through detailed experiments, this molecular weight reduction was determined to result from the alcoholytic cleavage of PHA chains by the *B. cereus* YB-4 PHA synthase induced by endogenous ethanol (Hyakutake et al. 2014). The PHA synthase from *Bacillus megaterium* also exerted weak alcoholysis activity for PHA chains. These results suggest that this type of PHA synthase may share alcoholysis activity as a common inherent feature. Site-specific mutagenesis at the putative catalytic sites in the *B. cereus* YB-4 PHA synthase revealed that this enzyme has the dual activities of polymerization and alcoholysis (Hyakutake et al. 2015).

Regarding the downstream process for polymer extraction from bacterial cells, a cell-autolysis procedure should provide cost-effective recovery of intracellular accumulation products such as PHAs. For this purpose, a cellular self-disruption system was installed into *B. megaterium* to spontaneously occur in the presence of a xylose inducer upon completion of glucose consumption-dependent PHA biosynthesis using holin and endolysin of the *Bacillus amyloliquefaciens* phage (Morita et al. 2001; Hori et al. 2002). In the further studies of this regulatory tool, mutations in the cell wall-associated protein YoeB were shown to enhance cell autolysis in response to antibiotic stress. Such cell disruption can provide an alternative to conventional treatment for PHA recovery using chemical reagents or enzymes, thereby reducing the cost of polymer production on an industrial scale.

The case for lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive, low-GC bacteria defined by their common ability to produce lactic acid as the major metabolic product of carbohydrate fermentation. LAB include species of various genera, such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Many of these LAB are associated with the traditional and industrial production of fermented foods, beverages, and animal feed. Interesting examples include the manufacture of cheese, yogurt, and sausage from animal origin as well as sauerkraut, soy sauce and silage from vegetable origin. Moreover, some LAB are exploited as producers of flavoring enzymes, peptides with antibacterial activity, or metabolites that contribute to the flavor, conservation, or texture of foods. Because LAB have not been associated with pathogenic effects, most LAB are now GRAS according to the US FDA. Several LAB are also currently marketed as probiotic strains with health-promoting capacity. Therefore, LAB have attracted increased attention as endotoxin-free hosts for pharmaceutical products as an alternative to *E. coli*, which contains endotoxins in the membrane that are pyrogenic in humans (Blatteis et al. 2000).

In the past two decades, genetic studies in LAB have resulted in the development of genetic techniques, transformation protocols, and sophisticated vectors (De Vos 1999a). A variety of constitutive or inducible gene expression systems in LAB have also been developed (Van de Guchte et al. 1992; Kok 1996; Kuipers et al. 1998; De Vos 1999a; Mierau and Kleerebezem 2005; Karlskås et al. 2014). Among them, bacteriocin-inducible expression systems in *Lactococcus lactis* (De Ruyter et al. 1996) and *Lactobacillus* spp. (Mathiesen et al. 2004) have been reported to be complementary and alternative systems for difficult cases in *E. coli* systems. However, successful cases of recombinant protein expression in LAB systems are not well known. Here, we describe the nisin-controlled gene expression (NICE) system with *L. lactis* as the host cell (De Ruyter et al. 1996; De Vos 1999b; Mierau et al. 2005; Mierau and Kleerebezem 2005) and discuss the advantages of LAB host cells for the functional expression of recombinant protein, especially membrane proteins, which are important for drug targets, as described above.

Some strains of *L. lactis* produce an antimicrobial peptide (bacteriocin) known as nisin (Klaenhammer 1993; Nes et al. 1996; Cheigh and Ryun 2005). Nisin is a small (3.4 kDa), cationic, hydrophobic, and 34-amino acid peptide which contains one lanthionine and four β-methyl-lanthionine, and unusual residues as dehydroalanine and dehydrobutyrine (De Vos et al. 1995; Kuipers et al. 1995; Siegers and Entian 1995; Kleerebezem et al. 1999). Only nisin A is approved as a food preservative among the bacteriocins from LAB although some nisin variants have been reported (Zendo 2013; Perez et al. 2014). Nisin containing its variants inactivates a wide variety of bacterial cells by permeabilizing their cytoplasmic membrane (Breukink et al. 1999). Nisin A is expressed on the pathway encoded by the *nisABTCIPRKEFG* operon (Kuipers et al. 1993) in which the expression is precisely controlled by an auto-regulatory system. The nisin expression system has been developed by using this auto-regulatory system consisting of *nisA* promoter and *nisRK* genes encoding a two-component regulatory system. The receptor histidine kinase (NisK) autophosphorylates by binding of extracellular nisin A and activates the transcriptional response regulator (NisR) that activates the *nisA* promoter (Zhou et al. 2006). When nisin A is added to the culture of *L. lactis*, a target gene downstream of the *nisA* promoter on a plasmid is overexpressed. The applications of the NICE system using *L. lactis* as the host are listed in Table 1.

Many prokaryotic membrane proteins, such as ABC transporters, major facilitator superfamily transporters, mechanosensitive channels, lipoproteins, and peptide transporters of the PTR family, have been successfully expressed in the NICE system (Kunji et al. 2003). Furthermore, several successful cases of the functional expression of eukaryotic membrane proteins, such as plant chloroplast envelope protein, mitochondrial carrier, and human KDEL receptor, have been reported (Frelet-Barrand et al. 2010; Kunji et al. 2003). To date, certain features of the *L. lactis* cell could account for the successful expression of these membrane proteins. One of the major advantages of *L. lactis* is that it does not form inclusion bodies (Monné et al. 2005), mainly due to the simple structure of the cell, with a single cytoplasmic membrane and a relatively small genome (2.4 Mbp; Bolotin et al. 2001). It has been reported that inclusion bodies could contain host cell components such as proteins and RNA (Singh et al. 2015), and thus, *L. lactis* could be an excellent host cell due to its limited genomic redundancy. In addition, the *L. lactis* membrane composition appears to be suitable for the functional expression of chloroplast membrane proteins because it contains glycolipids (Oliveira et al. 2005) and is much more similar to the chloroplast inner membrane than the *E. coli* membrane, which does not contain any glycolipids (Frelet-Barrand et al. 2010). The NICE system has also been studied for enhanced bacteriocin expression in food products as a biopreservative (Abee et al. 1995; Chikindas et al. 1995; Horn et al. 2004) as well as recombinant hyaluronic acid production (Chien and Lee 2007). Microbial hyaluronic acid is commonly produced by pathogenic *Streptococcus* (Leonard

Table 1 Overview of applications of the NICE system using *Lactococcus lactis* as a host

Category	Function	Product	Source organism	Yield[a]	Reference
Membrane proteins	Transporter	LmrA, LmrO, OpuA, $GlnP_2Q_2$, DtpT, MleP, CitP, and OppBCDF	*Lactococcus lactis*	1–30 %	Kunji et al. (2003)
	Mitochondrial carrier	CTP1 and AAC3	*Saccharomyces cerevisiae*	~5 %	Kunji et al. (2003)
	Hydrogenosomal carrier	AAC_{hyd}	*Neocallimastix patriciarum* (rumen fungus)	<1 %	Kunji et al. (2003)
	Chloroplast envelope quinone oxidoreductase	ceQORH	*Arabidopsis* (plant)	20–30 %	Frelet-Barrand et al. (2010)
	KDEL-receptor	Erd2	*Homo sapiens*	<0.1 %	Kunji et al. (2003)
Secretory protein	Antibacterial protein	Lysostaphin	*Staphylococcus simulans*	100 mg/L	Mierau et al. (2005)
Bacteriocins	Antibacterial protein	Pediocin PA-1	*Pediococcus acidilactici*	540 μg/L	Horn et al. (2004)
	Antibacterial protein	Colicin V	*Escherichia coli*	834 BU/mL[b]	Horn et al. (2004)
Polysaccharide	Hyaluronic acid synthase and uridine diphosphate-glucose dehydrogenase	Hyaluronic acid	*Streptococus equi* subsp. *zooepidemicus*	0.65 g/L	Chien and Lee (2007)
Biopolymer	Biopolyester	Poly(3-hydroxybutyrate)[c]	*Cupriavidus necator* (*Ralstonia eutropha*)	6 wt%	Mifune et al. (2009)

[a] Percentage of total membrane protein for membrane proteins, weight per culture medium for antibacterial protein, or weight percentage per dry cell weight for poly(3-hydroxybutyrate)

[b] One BU [bacteriocin unit] is defined as the reciprocal of the highest dilution causing 50 % growth inhibition of the indicator organism

[c] Poly(3-hydroxybutyrate) was final product from a recombinant synthesis pathway with *phaCAB* operon derived from *Cupriavidus necator.*

et al. 1998). Recently, its production was achieved in *L. lactis* coexpressing hyaluronic acid synthase and uridine diphosphate-glucose dehydrogenase derived from *Streptococcus equi* subsp. *zooepidemicus* (Chien and Lee 2007).

The research group of Dr. Rehm extensively engineered the bacterium *L. lactis* as a platform to display various bio-active proteins linked with PHA synthase molecules covalently attached to the surface of intracellularly generated P(3HB) granules (Mifune et al 2009). These functionalized polyester beads have previously been constructed only by recombinant Gram-negative bacteria such as *E. coli* or *Pseudomonas aeruginosa*. There has been considerable effort to develop methods for removing the endotoxin contaminants from P(3HB) beads manufactured in these bacteria, although these methods are considered too harsh. In this sense, *L. lactis* is a good candidate in that this bacterium has been traditionally used for the production of a variety of fermented milk products and heterologous proteins for medical applications. In this study, the functionalization of P(3HB) beads was successfully achieved using the immunoglobulin G binding ZZ domain of *Staphylococcus aureus* protein A based on the NICE system. Similar studies on PHA beads have been conducted in other types of bacteria (Draper and Rehm 2012; Dinjaski and Prieto 2015).

The case for *Corynebacterium glutamicum*

In the 1950s, *Corynebacterium glutamicum* was first isolated from Japanese soil in response to the need to isolate natural l-glutamate producers (Kinoshita et al. 1957). This bacterium has also been used as a GRAS endotoxin-free organism that is advantageous for the production of food-grade amino acids, such as l-glutamate and l-lysine, for more than 50 years (Leuchtenberger et al. 2005). There is considerable accumulated experience regarding high-performance fermentation conditions that can be used to optimize the manufacturing process of amino acids (Krämer 1994). Additionally, the *C. glutamicum* genome has been sequenced and made publicly available (Kalinowski et al. 2003). With its transition from an amino acid producer to a wider-spectrum platform, *C. glutamicum* has recently become a leading player in white biotechnology. Together with these milestones, current developments, such as rational strain design, genome bleeding and metabolic pathway engineering, have greatly facilitated the process of product diversification to include fuels, fine chemicals and polymeric materials such as PHAs in addition to enzymes for food processing.

Among the industrial enzymes produced in *C. glutamicum*, the microbial protein cross-linking enzyme transglutaminase (MTG; protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) is a typical successful target. MTG is commercially available and widely used in the food industry, biopolymers and cosmetics, clinical applications, and wool textiles (Yokoyama et al. 2004; Strop 2014). After much effort, production of greater than 1.2 g/l is currently achieved by the secretion system of *C. glutamicum* based on effective approaches such as the overproduction of Tat pathway-related TatABC (Yokoyama et al. 2000; Taguchi et al. 2002a; Kikuchi et al. 2009) and ^{13}C metabolic flux analysis (Umakoshi et al. 2011). The leading Japanese company, Ajinomoto Co. Ltd., produces food-grade MTG for food processing on an industrial scale. The excellent secretion capacity of *C. glutamicum* has been used to provide many types of proteinaceous substances (Burkovski 2015).

The genome sequence of *C. glutamicum* revealed the absence of genes related to PHA synthesis in this organism. Thus, a set of P(3HB) synthetic genes should be heterologously introduced into *C. glutamicum* host cells to produce P(3HB). There is a metabolic linkage between the glutamate and the P(3HB) synthetic pathways in *C. glutamicum*, both of which competitively utilize acetyl-CoA. Biotin limitation is a known key to promoting glutamate formation. In fact, a recombinant *C. glutamicum* strain harboring the P(3HB) synthetic gene operon derived from *R. eutropha* produced 22.5 wt% P(3HB) from glucose under biotin limitation conditions (Jo et al. 2006). P(3HB) production was improved in *C. glutamicum* by codon optimization of the N-terminal-encoding region in the PHA synthase gene (Jo et al. 2007), and the replacement of wild-type *phaC* with a beneficial mutation was shown to enhance the expression and production of PhaC and P(3HB) in *E. coli* (Normi et al. 2005). The production of P(3HB) was further synergistically increased up to 52.5 wt% by the gene dosage effect of monomer-supplying enzymes (Jo et al. 2009). In a related study, a newly designed synthetic pathway involving the generation of acetoacetyl-CoA catalyzed by acetoacetyl-CoA synthase (AACS; Okamura et al. 2010) was developed to produce P(3HB) in *C. glutamicum* (Matsumoto and Taguchi 2009; Matsumoto et al. 2011).

To diversify the production system of *C. glutamicum* and maximize carbon source utilization, the co-production of intracellular P(3HB) along with extracellular glutamate has been attempted (Jo et al. 2009). Based on two-step cultivation, recombinant *C. glutamicum* cells expressing the *phaCAB* operon accumulated 36 wt% P(3HB) and secreted glutamate up to 18 g/l. In a separate study, the P(3HB) synthesis genes exerted a positive effect on glutamate production in *C. glutamicum* (Liu et al. 2007). These results suggest that *C. glutamicum* has potential as a host for the simultaneous production of a number of bio-products, thereby decreasing the cost of P(3HB) production. The closeness of the melting temperature (T_m) and the thermal degradation temperature along with the brittleness of P(3HB) limits its processability and range of applications (Kemnitzer et al. 1993; VanderHart et al. 1995;

Valentin et al. 1999). However, the T_m of P(3HB-*co*-3HV) varies in the range of 65–170 °C depending on the 3HV fraction (Inoue and Yoshie 1992; Inoue 1998; Ivanova et al. 2009). The incorporation of 3HV into P(3HB) was achieved in the *C. glutamicum* platform by the addition of propionate, which is commonly used as a structurally related secondary precursor (Matsumoto et al. 2010).

As a renewable carbon source available in large quantities, starch is an inexpensive substrate candidate. Prior to fermentation, starch usually needs to be hydrolyzed to glucose by a two-step process, i.e., liquefaction and saccharification. The direct utilization of starch instead of enzyme-treated starch (Rusendi and Sheppard 1995; Quillaguaman et al. 2005) would be desirable for reducing costs. Cell surface display technology is a powerful platform for meeting this goal, as it allows proteins fused with anchoring motifs to be localized on the external surface of the bacterial cell membrane. Additionally, the displayed enzymes have been reported to maintain their activity during long-term cultivation (Yamakawa et al. 2012), which could provide an advantage over enzyme-secreting systems. For example, an α-amylase displaying *C. glutamicum* strain was demonstrated to produce L-lysine from starch (Tateno et al. 2007) and has been applied to the direct production of P(3HB) from starch. The productivity of P(3HB) from starch was even slightly higher than that from glucose (Song et al. 2013).

The well-known polyester poly(lactic acid) (PLA) has good mechanical properties, a high transparency, and a low environmental impact and thus is commonly used in packaging containers and stationery and as a component of nano-alloys in creating engineering plastics (Kricheldorf 2001; Auras et al. 2004; Tokiwa and Calabia 2006; Ozkoc and Kemaloglu 2009; Nampoothiri et al. 2010). However, its wide application in medicine and food production is limited by the potential remnants of toxic heavy metal catalysts used for the chemical ring-opening polymerization of the lactide to form PLA (Auras et al. 2004). For the first time, the one-pot biosynthesis of PLA and its derivatives has been achieved in *E. coli* using an engineered PHA synthase (lactate-polymerizing enzyme: LPE; Taguchi et al. 2008; Yamada et al. 2009; Matsumoto and Taguchi 2013a, b) discovered through the in vitro polymerization system (Tajima et al. 2009). LPE has gained two beneficial mutations in PHA synthase from *Pseudomonas* sp. 61-3 (Taguchi et al. 2008). LPE is one of the many artificial mutant enzymes developed during the last decade (Taguchi et al. 2002b; Taguchi and Doi 2004; Takase et al. 2004; Tsuge et al. 2004; Nomura and Taguchi 2007). Similar studies have followed using the same LPE or the homologous PHA synthases with the same mutations (Park et al. 2011).

To upgrade this prototype system to the practical scale, especially for food-grade and biomedical demands, *C. glutamicum* has been considered as a suitable GRAS candidate for the production of LA-based polyester (Takors et al. 2007). A key factor in the construction of the metabolic pathway for producing LA-based polyester in *C. glutamicum* was the stereochemistry of LA, as it has been demonstrated that LPE has strict stereospecificity towards D-LA-CoA (Taguchi et al. 2008; Tajima et al. 2009; Yamada et al. 2009). However, the *C. glutamicum* strain is known to produce mainly L-LA, as reported in several studies (Inui et al. 2004; Okino et al. 2008; Toyoda et al. 2009), whereas *E. coli* can produce D-LA (Bunch et al. 1997; Chang et al. 1999). Therefore, *C. glutamicum* was remodeled as a D-LA producer by the introduction of D-LDH from *E. coli*. The recombinant *C. glutamicum* expressing D-LDH together with the related biosynthetic genes synthesized P(LA-*co*-3HB) with an extremely high LA fraction of 99.3 mol%, nearly a PDLA homopolymer (Song et al. 2012).

The case for yeast

To date, more than 150 recombinant biopharmaceuticals including monoclonal antibodies, hormones, and growth factors have been approved by the US FDA since human insulin was expressed in *E. coli* as the first licensed drug in 1982. Yeast produces approximately 20 % of these pharmaceuticals, whereas *E. coli* and mammalian cell lines produce 30 and 50 %, respectively (Bill 2014). Yeast cell is widely used because of its GRAS status and providing a good middle ground between *E. coli* and mammalian cell lines, which combines advantages of ease of manipulation with eukaryotic protein processing (Byrne 2015). For example, recombinant human insulin is nowadays produced in *E. coli* and *S. cerevisiae*. In *E. coli* system, the insulin precursors are produced as inclusion bodies and require solubilization procedure to be functional, whereas *S. cerevisiae* system secretes the soluble precursors in the culture supernatant (Baeshen et al. 2014). *S. cerevisiae* is the prominent recombinant host in yeasts and produces hormones (insulin, non-glycosylated human growth hormone somatotropin, and glucagon), vaccines (hepatitis B virus surface antigen), urate oxidase from *Aspergillus flavus*, granulocyte-macrophage colony stimulating factor, albumin, hirudin of *Hirudo medicinalis*, and human platelets derived growth factor (Gerngross 2004). Recently, *P. pastoris*, approved as a host for biopharmaceutical production by FDA in 2009, has been known as an alternate yeast strain that has ability to achieve high cell density, in some cases more than 150 g dry cell weight per liter (Vogl et al. 2013), and produce soluble and correctly folded proteins by its strong methanol-inducible AOX1 promoter (Gonçalves et al. 2013). It is well known that *P. pastoris* has been used with great success to produce recombinant human G protein-coupled receptors and ion channels (Hedfalk 2013), which led to an increasing trend in its usage in academic reports, and this strain

accounted for 11 % of the articles in the field of recombinant expression published in 2013 (Bill 2014). *P. pastoris* also produces more than 70 commercialized products that are on the market or in late stage development including kallikrein inhibitor, human insulin, human serum albumin, hepatitis B vaccine, interferon-alpha 2b, microplasmin, anti-IL6 receptor single domain antibody fragment, anti-RSV single domain antibody fragment, heparin-binding EGF-like growth factor, and collagen (Kim et al. 2015). Another advantage for using yeast expression systems is potential of posttranslational modification on therapeutic proteins. It is known that yeasts are capable of performing glycosylations such as *N*-glycosylation that is involved in protein folding, in vivo half-life, and protein function. Mammalian cells and yeast share the initial biosynthetic pathway for *N*-glycans synthesis; however, the further pathways in the Golgi diverge significantly as the protein proceeds through the secretory pathways. In mammalian cells, mannose residues are trimmed by several mannosidases. In contrast, yeasts do not trim but rather extend mannose sugars to generate hyper-mannosylated glycans, could result in poor half-life or immunogenic, in the case of therapeutic antibodies, lack of the functions (Hamilton and Gerngross 2007; Gonçalves et al. 2013). In the hyper-mannosylation issue, *P. pastoris* is attractive because of less glycosylation activity that generates much shorter mannose residues than those in *S. cerevisiae* (Baeshen et al. 2014) and has been genetically explored to develop more humanized host strain suitable for recombinant production of therapeutic proteins, although glycosylation is complex and needs to be evaluated on a case-by-case basis (Krainer et al. 2013).

Polyhydroxyalkanoates (PHAs) have also been produced by engineered yeast strains. As far as we know, yeasts do not produce PHAs, and therefore, many studies have attempted to heterologously express bacterial PHA biosynthetic genes in yeasts. For this purpose, PHA biosynthetic pathway has to be designed based on the background pathways of each organelle. As examples, the research group of Dr. Poirier reported the PHA production in the peroxisome of *S. cerevisiae* (Poirier et al. 2001; De Oliveira et al. 2004) and *P. pastoris* (Poirier et al. 2002). Peroxisome, which contributes to the fatty acid metabolism, operates β-oxidation cycle of fatty acids and produces (*R*)-3-hydroxyacyl-CoAs (3HA-CoAs) as metabolic intermediates (Fig. 2). The cytosolic synthesis of PHA by engineered *S. cerevisiae* cells was also reported by the research group of Dr. Srienc (Zhang et al. 2005, 2006). Thus, the expression of PHA synthase gene in yeast cells alone conferred the capacity of synthesizing PHA as shown in Fig. 2. Engineered *S. cerevisiae* and *P. pastoris* accumulated approximately 0.5–1 wt% of short-chain-length and medium-chain-length PHAs. The polymer content was lower than that of PHA-producing bacteria. This could be due to the limited number and volume of peroxisome compared to the cell size. However, it should be noted that PHA biosynthetic pathway also acted as a detector of intracellular metabolites in yeasts, because the monomer composition of the synthesized PHAs are affected by the metabolite levels of monomer substrates, the 3HA-CoAs. In fact, Haddouche et al. proposed the occurrence of partial mitochondrial β-oxidation in *Yarrowia lipolytica* based on the PHA synthesis by this strain (Haddouche et al. 2010, 2011). In conclusion, PHA productivity in yeasts is not high compared to prokaryote systems yet. However, the PHA biosynthetic pathway has potential as a tool for analyzing metabolic pathways in eukaryotic hosts.

Outlook

For the microbial production of value-added compounds, *E. coli*, *Pseudomonas* spp., *R. eutropha*, *B. subtilis*, LAB, *C. glutamicum*, *Streptomyces*, and other organisms are representative super-bugs that can satisfy multiple requirements, such as versatile host–vector systems, high-cell density cultivation, and robustness against various environments. In addition, endotoxin-free bacteria with GRAS status, such as *B. subtilis*, LAB, and *C. glutamicum*, are also desirable for eco-friendly and biomedical applications, as described in this review. Currently, as an alternative to endotoxin removal treatment, the engineering of endotoxin-free *E. coli* has been accomplished by incorporating a total of seven non-reverting genetic deletions that disrupt Kdo biosynthesis (Mamat et al. 2015). The constructed strains successfully produce recombinant proteins with negligible endotoxin contamination. This strategy would be applicable for other types of Gram-negative bacteria that have good potential properties as platforms but retain endotoxins. LAB have also been explored as live vectors for mucosal vaccination (Enouf et al. 2001; Le Loir et al. 2005; Wyszynska et al. 2015). The health-promoting properties and high degree of safety of LAB would make them an attractive alternative to other vectors used for the construction of vaccines, including attenuated strains of various species of pathogenic microorganisms, liposomes and microparticles. Thus, GRAS microorganisms have a great deal of potential in a variety of industries. Such whole-cell utilization holds promise for extensive future applications in health care-related areas.

These current advances in the development of endotoxin-free production strains will encourage us to achieve the practical manufacture of eco- and bio-friendly compounds in response to bioindustrial needs.

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