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Article

Display of Linoleic Acid Isomerase on *Bacillus subtilis* Spores for Bioconversion of Linoleic Acid to t10,c12-Conjugated Linoleic Acid

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Abstract

Conjugated linoleic acid (CLA) comprises positional and geometric isomers of linoleic acid (LA) that have attracted interest due to their reported biological activities. In particular, trans-10,cis-12-CLA (t10,c12-CLA) has been associated with distinct metabolic effects, but its selective production remains challenging because conventional chemical synthesis generates mixtures of CLA isomers. In this study, the linoleic acid isomerase from *Propionibacterium acnes* (PAI) was displayed on *Bacillus subtilis* spores by fusion to spore crust proteins to develop a biocatalyst for t10,c12-CLA production. Twenty-four recombinant *B. subtilis* strains were constructed by fusing PAI to six spore crust proteins with different linker architectures. All constructs produced detectable t10,c12-CLA from 5 mg/mL LA, demonstrating that spore-displayed PAI remained catalytically active and accessible to extracellular substrate. The CgeA-L2 construct showed the highest mean CLA production, approximately 1.33 mg/mL CLA under the tested conditions. Time-course experiments revealed that product formation increased during the initial phase of the reaction before reaching a plateau, while recovered spores retained catalytic activity after one reuse cycle. Finally, spore-displayed PAI converted LA released from safflower oil by soluble lipase. Together, these results establish *B. subtilis* spores as a functional display format for PAI and provide a basis for further development of a recoverable catalyst for CLA production.

Keywords: *Bacillus subtilis*; spore surface display; linoleic acid isomerase; conjugated linoleic acid; enzyme immobilization; biocatalysis



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1. Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA; C18:2 *cis*-9,*cis*-12) characterized by conjugated double bonds. Among the numerous possible isomers, *cis*-9,*trans*-11-CLA (c9,t11-CLA) and *trans*-10,*cis*-12-CLA (t10,c12-CLA) are the most abundant and biologically studied forms. These isomers have been associated with various physiological effects including modulation of lipid metabolism, immune responses, and inflammatory pathways [1,2]. Importantly, the biological activity of CLA depends on the specific isomer present, making the ability to selectively produce defined CLA isomers essential for studying their biological functions and potential applications.

CLA occurs naturally in the meat and dairy products of ruminant animals, where microbial metabolism in the rumen converts dietary linoleic acid into conjugated isomers [3]. However, natural levels of CLA are relatively low and insufficient to meet industrial demand. As a result, most commercially available CLA is produced either by chemical isomerization of linoleic acid or by chemical dehydration of ricinoleic acid. Chemical dehydration of ricinoleic acid predominantly yields the c9,t11-CLA isomer, whereas isomerization of linoleic acid typically produces complex mixtures of CLA isomers, including both c9,t11-CLA and t10,c12-CLA along with several minor variants [4]. Such mixtures complicate the interpretation of biological effects and require additional purification steps when specific isomers are desired.

Biological production of CLA has therefore attracted increasing attention as a potential route for more selective synthesis. Several bacterial species are capable of converting linoleic acid into CLA through enzymatic pathways. In many rumen and lactic acid bacteria, CLA formation proceeds through multi-step pathways involving hydratases, dehydrogenases, and isomerases that convert LA into different CLA intermediates. In contrast, linoleic acid isomerases (LAIs) catalyze the direct one-step isomerization of linoleic acid to CLA through a regio- and stereospecific reaction. However, most characterized bacterial LAIs produce mixtures of CLA isomers, typically generating both c9,t11-CLA and t10,c12-CLA [5].

An exception is the linoleic acid isomerase from *Propionibacterium acnes* (PAI), which exhibits strict specificity for the formation of the t10,c12-CLA isomer [6,7]. This FAD-dependent enzyme catalyzes the direct conversion of linoleic acid to t10,c12-CLA without detectable formation of other CLA isomers, making it an attractive biocatalyst for selective CLA production [6,8]. Several studies have explored heterologous expression of PAI in microbial systems. For example, expression of PAI in *Escherichia coli* has enabled enzymatic conversion of LA to t10,c12-CLA in vitro [9], while metabolic engineering approaches have been used to generate CLA in yeast hosts such as *Yarrowia lipolytica* [10]. Transgenic animal models expressing PAI have also been developed to investigate the physiological effects of t10,c12-CLA in vivo [11]. Nevertheless, these approaches face several challenges. In de novo production systems, substrate transport limitations and host metabolism of fatty acids can reduce conversion efficiency [12], while CLA toxicity can restrict production in bacterial hosts [13]. Furthermore, soluble expression of PAI often results in partial misfolding or aggregation of the enzyme, limiting catalytic performance [14].

To address these challenges and simplify catalyst recovery, enzyme immobilization strategies such as cell surface display have emerged as promising alternatives. Surface display allows enzymes to be presented on the exterior of microbial cells, providing natural immobilization while maintaining catalytic activity. Previous attempts to display PAI on the surface of yeast cells have yielded mixed results. In *Pichia pastoris*, surface-displayed PAI was reported to be catalytically inactive [15], whereas *Saccharomyces cerevisiae* displayed an active enzyme but with very low conversion efficiency, producing only approximately 0.025 mg/mL t10,c12-CLA from 4 mg/mL linoleic acid [16]. These limitations highlight the need for alternative display platforms that support efficient enzyme expression and catalytic activity.

Bacillus subtilis represents a promising host for enzyme surface display due to its ability to form highly resistant endospores. These spores possess a multilayered coat structure composed of numerous proteins that can serve as anchoring points for heterologous enzymes. Spore surface display is typically achieved by genetically fusing the target protein to a native coat or crust protein, leading to immobilization of the fusion protein during sporulation. This approach offers several advantages including high robustness, resistance to environmental stresses, simplified catalyst recovery and the possibility of catalyst reuse, making *B. subtilis* spores attractive as stable whole-cell biocatalysts. Spore display has

been applied to a range of heterologous proteins and enzymes, but performance depends strongly on the selected anchor, fusion orientation, linker architecture, and properties of the displayed enzyme [17–21]. For PAI, this format may provide an alternative to soluble expression by coupling enzyme production and immobilization during sporulation and facilitating subsequent catalyst recovery. To our knowledge, PAI display on *B. subtilis* spores for t10,c12-CLA production has not previously been reported.

In this study, we developed a *B. subtilis* spore-display system for the production of t10,c12-CLA using the *P. acnes* linoleic acid isomerase. Six spore crust proteins were evaluated as anchoring partners, and different linker architectures were tested to assess their effects on catalytic performance. The resulting recombinant spores were evaluated for conversion of free linoleic acid and for CLA production from safflower oil in combination with lipase-mediated hydrolysis.

2. Results

2.1. Spores Tolerate Linoleic Acid and Support Spore Coat Association of Linoleic Acid Isomerase

Vegetative *B. subtilis* cells showed sensitivity to both LA and CLA, with LA completely inhibiting growth at 4 mg/mL (Figure S1). This suggested that LA toxicity could limit the use of growing-cell biocatalysts and motivated the development of a spore-based PAI-display platform. For this, we initially used a vector-based system described before [19]. This led to construction of four recombinant strains, where the linoleic acid isomerase from *Propionibacterium acnes* (PAI) was translationally fused to various spore crust proteins (CgeA, CotV, CotW, and CotX, respectively). To assess expression and association of the PAI fusion proteins with the spore coat, coat protein extracts were prepared from purified recombinant spores and analyzed by SDS-PAGE and Western blotting. The predicted molecular weight of the fusion proteins was ~63–65 kDa. The recombinant spores showed a band at ~65 kDa which was absent in wild-type spore samples as shown in Figure 1A. The presence of the PAI fusion proteins was further verified by Western blot using anti-6xHis antibodies (Figure 1B). Specific immunoreactive bands were detected at ~65 kDa, confirming the presence of the PAI fusion proteins in the extracted spore coat fraction. No signal was detected in the wild-type KO7 control, indicating that the observed bands were specific to the recombinant constructs. These results demonstrated association of the PAI fusion proteins with the spore coat. Because analysis of extracted coat proteins does not establish the precise topology or degree of external exposure of PAI, functional accessibility of the enzyme was subsequently evaluated using intact recombinant spores in the bioconversion assays.

2.2. Construction and Functional Comparison of LAI Spore-Display Constructs

Having established association of representative PAI fusion proteins with the spore coat, we next compared the functional performance of different spore-display configurations to identify constructs that support efficient conversion of linoleic acid to t10,c12-conjugated linoleic acid. For this purpose, a library of recombinant *B. subtilis* strains was constructed in which PAI was fused to six different spore crust proteins (CgeA, CotV, CotW, CotX, CotY, and CotZ). For each anchor protein, three linker variants were evaluated in addition to constructs lacking a linker: L1 (GGGEAAAKGGG), L2 ((GGGS)₃), and L3 (EAAAK), resulting in a total of twenty-four display configurations (Supplementary Table S4). Recombinant spores were incubated with 5 mg/mL LA under standard assay conditions, and formation of t10,c12-CLA was quantified by GC-MS. All twenty-four recombinant constructs produced detectable levels of t10,c12-CLA from extracellular LA, demonstrating that PAI remained catalytically active and functionally accessible to substrate in the intact recombinant spores. Together with detection of the PAI fusion proteins in

the spore coat fraction, these results support functional display of PAI on the spores, while not resolving its precise topology or degree of external exposure. In contrast, wild-type spores lacking PAI produced no detectable CLA, indicating that abiotic or host-derived conversion under the assay conditions was negligible (Figure 2).

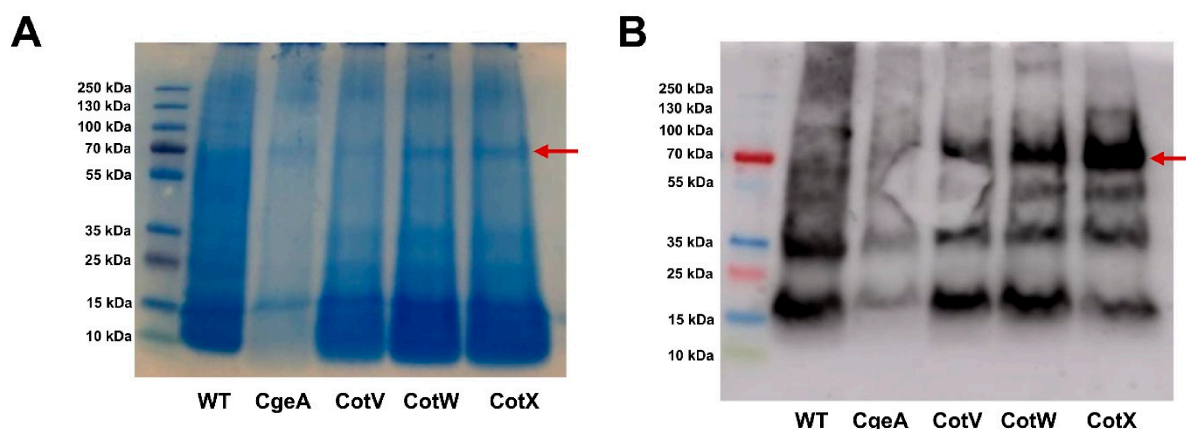


Figure 1. Qualitative detection of PAI fusion proteins in *Bacillus subtilis* spore coat extracts. (A) SDS-PAGE analysis of spore coat protein extracts. Lane 1: molecular weight marker; lane 2: wild-type spores; lanes 3–6: recombinant spores expressing PAI fused to the crust proteins (CgeA, CotV, CotW, CotX). Bands corresponding to the expected PAI fusion proteins at ~65 kDa are observed in lanes 3–6 (indicated by arrow). (B) Western blot analysis using an anti-6xHis monoclonal antibody. Immunoreactive bands at ~65 kDa (indicated by arrow) were detected in recombinant spore samples but not in the wild type control. The SDS-PAGE and Western blot analyses qualitatively verified the presence of the fusion proteins; band intensities were not used to compare display levels between constructs.

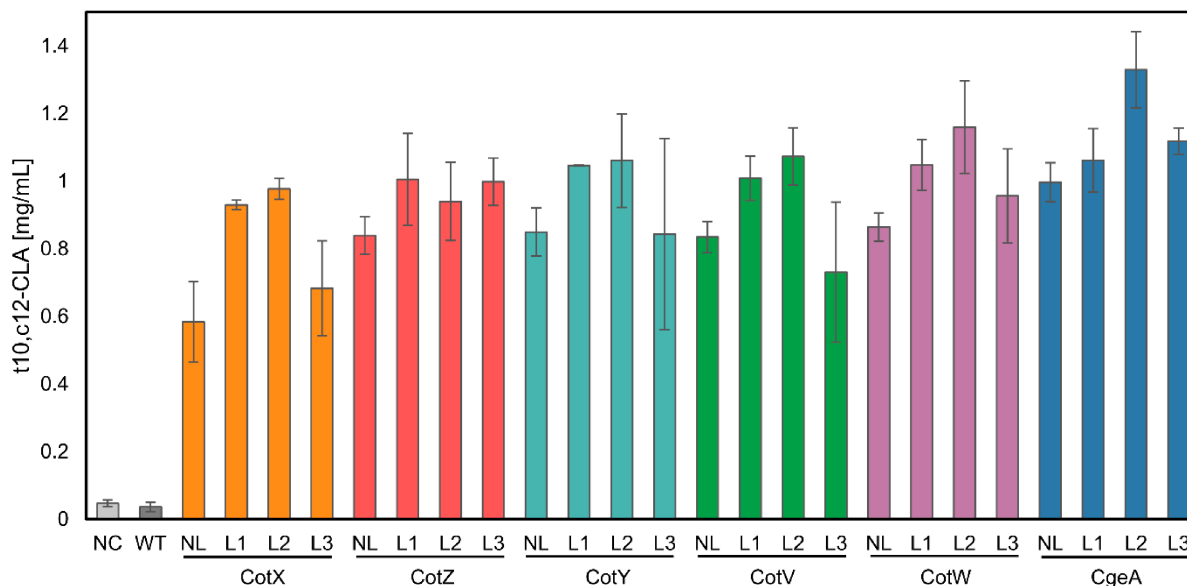


Figure 2. Screening of PAI-displaying *Bacillus subtilis* spore constructs. Recombinant *B. subtilis* spores displaying PAI fused to different crust proteins (CgeA, CotV, CotW, CotX, CotY, CotZ) and linker variants were evaluated for their ability to convert linoleic acid (5 mg/mL) to t10,c12-conjugated linoleic acid (CLA). Reactions were performed at 35 °C for 6 h, and CLA production was quantified by GC–MS. Constructs are grouped according to anchoring crust protein, with linker variants shown within each group, and are colored according to the anchoring crust protein. Wild-type (WT) and no-spore control (NC) are included as controls. All engineered strains produced substantially more CLA than the wild type and no-spore control. CgeA-L2 showed the highest mean CLA production under the tested conditions.

Comparison of CLA production across the construct library revealed relatively modest differences in catalytic performance. Overall, constructs lacking a linker tended to produce slightly lower amounts of t10,c12-CLA, whereas constructs containing flexible linkers performed somewhat better. Among the linker variants, the flexible (GGGS)₃ linker (L2) showed the highest mean CLA production. Similarly, constructs using the CgeA anchor generally produced higher CLA levels than the other crust proteins, although the differences between anchors were limited. Across the full construct library, the CgeA-L2 configuration showed the highest mean t10,c12-CLA production, corresponding to approximately 1.33 mg/mL CLA, equivalent to ~26.6% of the nominal initial linoleic acid concentration. In contrast, the lowest-performing construct, CotX with no linker, produced approximately 0.58 mg/mL CLA, equivalent to ~11.7% of the nominal linoleic acid loading.

2.3. Catalytic Properties of the Selected Spore Biocatalyst

As CgeA-L2 showed the highest mean CLA production under the tested conditions, this construct was selected for further characterization. Bioconversion assays were performed using 5 mg/mL linoleic acid (LA) and 5×10^9 spores/mL under standardized reaction conditions, and product formation was quantified by GC-MS. The 5 mg/mL LA concentration refers to the nominal initial substrate loading. The combined analytically recovered LA and CLA concentrations were lower than this nominal value, including at the operational 0 h time point. Because the experiment was not designed as a complete mass-balance analysis, the origin of the unrecovered fraction could not be determined. A time-course experiment revealed that formation of t10,c12-conjugated linoleic acid (CLA) increased steadily during the initial phase of the reaction, reaching approximately 1.2 mg/mL after about two hours (Figure 3A). Based on the net increase in CLA concentration between the operational 0 h and 2 h time points, the average volumetric productivity during this initial reaction period was $0.52 \text{ g L}^{-1} \text{ h}^{-1}$. After this point, CLA levels plateaued despite continued incubation. The observed plateau at later time points suggests that the reaction becomes limited at later time points, potentially due to enzyme inactivation or physicochemical constraints of the reaction system.

To examine whether increasing enzyme loading could overcome this limitation, bioconversion assays were performed using different spore concentrations ranging from 2.5×10^9 to 2×10^{10} spores/mL while maintaining the substrate concentration at 5 mg/mL LA. Product formation was quantified after 6 h incubation (Figure 3B). Increasing the spore concentration from 2.5×10^9 to 2×10^{10} spores/mL did not result in a corresponding increase in mean endpoint CLA production under the tested conditions.

The activity of the spore-displayed enzyme after one reuse cycle was evaluated by recovering the spores and using them in a second reaction. After completion of an initial bioconversion reaction, spores were recovered, washed, and resuspended in fresh LA substrate for a second reaction cycle under identical conditions. Recovered spores produced approximately 1.1 mg/mL CLA during the second reaction cycle (Figure 3C), demonstrating that the spore-displayed PAI retained catalytic activity after one reuse cycle.

Together, these observations suggest that the plateau observed after approximately two hours is unlikely to result primarily from rapid irreversible enzyme inactivation and that physicochemical limitations of the reaction system may contribute.

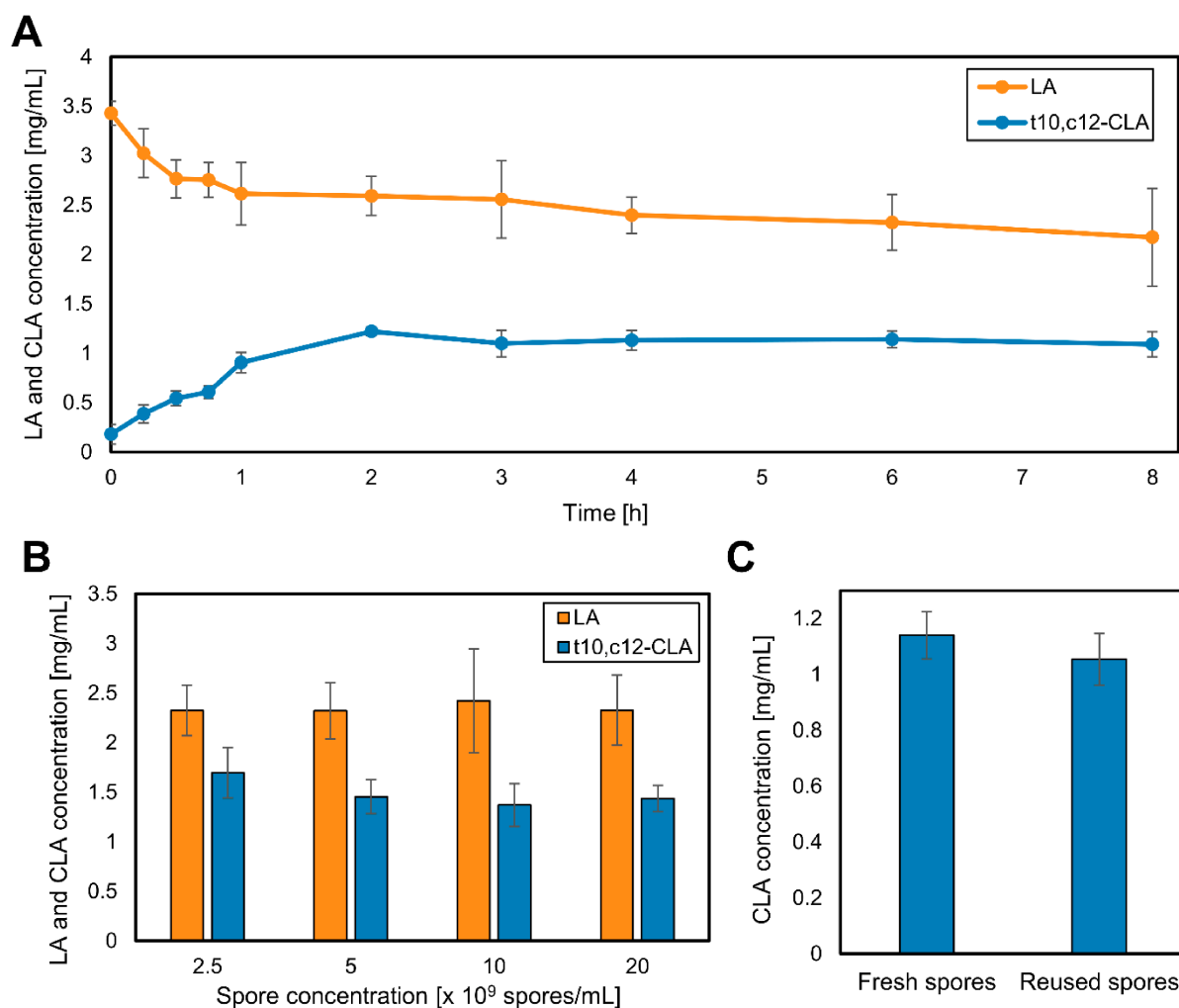


Figure 3. Catalytic behavior and reuse of the spore biocatalyst. **(A)** Time course of linoleic acid (LA) conversion to t10,c12-conjugated linoleic acid (CLA) by CgeA-L2 spores. **(B)** Effect of spore concentration on CLA production. Bioconversion reactions were performed using increasing concentrations of CgeA-L2 spores. **(C)** Activity of the spore-displayed catalyst after one reuse cycle. CLA production was compared between fresh and reused spores after 6 h of reaction.

2.4. Application of the Spore-Displayed Catalyst for CLA Production from Safflower Oil

While free LA is commonly used as a model substrate for CLA bioconversion, it represents a simplified and relatively costly system compared to natural lipid matrices. Several edible plant oils are naturally rich in LA and therefore represent attractive substrates for generating CLA-containing oils for food applications. Among these, safflower oil is particularly rich in LA, with reported contents of approximately 70% [22], making it a relevant substrate for evaluating the applicability of the spore-displayed PAI system beyond free fatty acids.

In plant oils, LA is predominantly present as triglycerides and must first be released as a free fatty acid prior to isomerization. Because PAI is active only toward free LA and cannot utilize esterified fatty acids [23], an exogenous lipase was included in the reaction system to hydrolyze triglycerides and release free LA. This established a two-enzyme process in which lipase-mediated hydrolysis generates free LA that could subsequently be converted to t10,c12-CLA by spore-displayed PAI. A safflower oil emulsion corresponding to 7 mg/mL oil was used, approximating the total LA content applied in the free LA experiments (5 mg/mL) (Figure 4). In control reactions containing lipase but no spores, the measurable free LA concentration reached only 1.88 mg/mL, indicating incomplete

triglyceride hydrolysis under the tested conditions. The CgeA-L2 strain was used to evaluate CLA production from safflower oil. Under free LA conditions, CgeA-L2 produced approximately 1.14 mg/mL t10,c12-CLA, whereas reactions using safflower oil yielded 0.46 mg/mL CLA. Because triglyceride hydrolysis and the total amount of free LA released during the coupled reaction were not determined directly, conversion of the available LA could not be determined directly. Nevertheless, these results demonstrate that co-application of lipase and spore-displayed PAI enables conversion of a natural oil substrate, providing proof of concept for CLA production from edible plant oils.

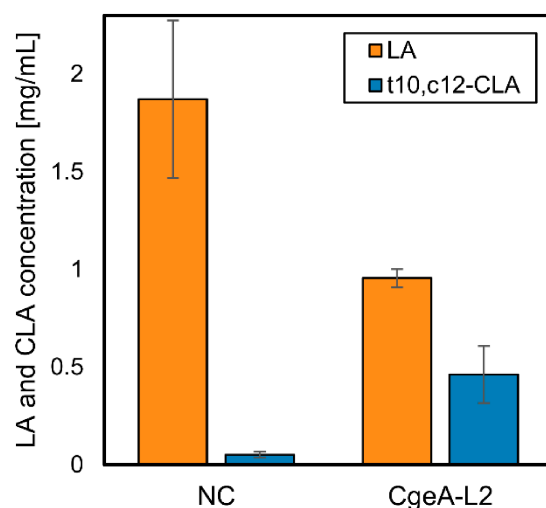


Figure 4. CLA production from safflower oil. Safflower oil was hydrolysed with lipase and used as a natural source of linoleic acid (LA) for bioconversion by CgeA-L2 spores. Residual LA and produced t10,c12-conjugated linoleic acid (CLA) were quantified after 6 h and compared with a no-spore control (NC).

3. Discussion

The present study demonstrates that *B. subtilis* spores can serve as a functional display platform for linoleic acid isomerase (PAI), enabling direct bioconversion of linoleic acid to the t10,c12-conjugated linoleic acid (CLA) isomer. All spore-displayed constructs produced detectable levels of t10,c12-CLA, indicating that PAI retains catalytic activity when fused to spore coat proteins. This contrasts with previous attempts to display PAI in eukaryotic hosts, where enzymes were reported to be either catalytically inactive [15] or to exhibit very low bioconversion efficiencies [16]. Evidence for functional spore display is based on two complementary observations: PAI fusion proteins were detected in the extracted spore coat fraction, and intact recombinant spores converted extracellular LA to t10,c12-CLA. Although these findings demonstrate spore coat association and functional accessibility of PAI, they do not resolve the precise topology or degree of exposure of the enzyme on the outer spore surface. The results therefore support *B. subtilis* spores as suitable for display of catalytically active PAI. In the *S. cerevisiae* surface-display system, approximately 0.025 mg/mL t10,c12-CLA was produced from 4 mg/mL LA [16], corresponding to approximately 0.6% relative to the supplied LA, whereas the present spore-display system produced up to 1.33 mg/mL from a nominal initial LA concentration of 5 mg/mL, equivalent to approximately 26.6% of the nominal LA loading. Because the systems differ in catalyst format and experimental conditions, these values reflect overall catalyst performance rather than intrinsic enzyme activity. Spore surface display may offer several advantages over soluble enzyme systems, including concurrent enzyme production and immobilization, straightforward catalyst recovery, and elimination of separate enzyme purification and external immobilization supports [24]. These properties make

spore-displayed enzymes attractive for biocatalytic applications in which catalyst recovery and potential reuse are desirable.

An additional advantage of PAI is its product specificity. Chemical CLA production methods often generate mixtures of isomers that require downstream separation [25], whereas PAI catalyzes the selective conversion of linoleic acid to t10,c12-CLA. This enables production of a defined CLA isomer.

The variation in mean activity among crust protein fusions suggests that anchor choice may influence catalytic performance. The slightly improved performance of CgeA-based constructs may reflect its positioning in the outermost crust layer, which could enhance enzyme accessibility to the linoleic acid present at the oil–water interface. In contrast, CotX forms filamentous substructures within the crust that may restrict enzyme exposure or limit substrate diffusion to the active site [26,27]. However, the relative performance of crust proteins appears to be highly context dependent. CotX has previously been used successfully for surface display of several heterologous enzymes, including lactulose-converting enzymes [28]. Similarly, in one study, CotY and CotZ were reported as highly effective anchors for heterologous protein display, whereas CgeA exhibited lower activity [19]. These observations suggest that no single crust protein can be universally considered superior. Instead, optimal anchor selection likely depends on the physicochemical properties of the fused enzyme, including its size, folding characteristics, and fusion orientation (N- vs. C-terminus) [19,20,29].

Linker architecture also influenced the catalytic performance of the spore-displayed enzyme. Constructs incorporating the flexible (GGGGS)₃ linker (L2) generally exhibited higher catalytic activity than the corresponding constructs containing shorter or more rigid linkers. Flexible glycine–serine linkers are widely used in fusion proteins because they reduce steric hindrance between domains and allow greater conformational freedom, which can improve folding and substrate accessibility [18]. In contrast, rigid α -helical linkers such as (EAAAK) may restrict domain mobility and limit the effective interaction between enzyme active sites and substrates [30]. These observations underscore the importance of linker design when optimizing surface-displayed enzymes. The display constructs were evaluated through fusion-protein detection and catalytic performance, while possible effects of PAI display on spore coat organization and resistance properties were not assessed.

The catalytic performance of the spore-displayed system should be interpreted in relation to the markedly different catalyst formats used in previous PAI studies. Recombinant *E. coli* and *Lactococcus lactis* converted approximately 40% and more than 50%, respectively, of externally supplied LA, but at substrate concentrations of only 0.1–0.5 mg/mL and after 72 h [31]. By comparison, the present catalyst produced approximately 1.33 g/L t10,c12-CLA from 5 g/L LA within 6 h, although the fractional conversion was lower. Much higher process performance has been achieved with extensively developed enzyme systems. A coupled system based on separately produced and immobilized PAI and lipase yielded 109 g/L t10,c12-CLA and 90.5% conversion of LA from 200 g/L sunflower oil after 36 h. This corresponds to an average volumetric productivity of approximately 3.0 g L^{−1} h^{−1} over the reported 36 h reaction, compared with 0.52 g L^{−1} h^{−1} over the initial 0–2 h interval in the present system. That study also reported substantial formation of PAI inclusion bodies, followed by purification and carrier-based immobilization of the soluble enzyme [9]. In another approach, recombinant PAI solubility was increased from 29% to 97% through GroESL co-expression, and a cell-autolysis-based recovery system was developed. Following optimization of enzyme loading and reaction conditions, the system achieved a maximum yield of 75.4% and a maximum titer of 9.4 g/L CLA from 15 and 25 g/L sunflower oil, respectively [32]. In contrast, the present system couples PAI production and immobilization during sporulation and therefore avoids separate

purification and carrier-based immobilization of PAI. The current spore-displayed system does not match the catalytic performance of these optimized PAI systems and should be regarded as proof of concept rather than a process-optimized catalyst. Its main contribution is the demonstration that catalytically active PAI can be produced and immobilized in a recoverable spore-display format, providing a basis for further development.

Characterization of the catalytic properties of the selected construct revealed that CLA formation increased approximately linearly during the initial phase of the reaction but plateaued after approximately two hours. Increasing the spore load over the tested range did not result in a corresponding increase in mean endpoint CLA production. Because the number of displayed PAI molecules per spore was not quantified, catalytic performance was evaluated relative to spore loading rather than enzyme-specific activity. PAI is an FAD-dependent flavoprotein, but the extent of FAD loading of the spore-displayed enzyme was not assessed in the present study. Cofactor availability during PAI synthesis and sporulation could therefore influence the fraction of catalytically competent enzyme and may represent an additional factor affecting catalyst performance. Recovered spores remained catalytically active when used in a second reaction cycle. Together, these observations suggest that insufficient catalyst loading or rapid enzyme inactivation was not the sole cause of the reaction plateau. Instead, the plateau may reflect physicochemical constraints of the reaction system, including limited substrate accessibility within the oil–water emulsion. Although measurable amounts of unreacted linoleic acid remained in the reaction mixture, not all substrate may have been accessible to the enzyme at the interface between the aqueous phase and oil droplets.

The properties of the emulsion system may play an important role in determining overall conversion efficiency. In this study, Tween 20 was used as the emulsifying agent. Tween 20 is a nonionic surfactant with a high hydrophilic–lipophilic balance (HLB $\approx$ 16.7) that efficiently stabilizes oil-in-water emulsions and facilitates substrate dispersion [33]. However, at high concentrations, surfactants can form dense layers around oil droplets that reduce enzyme access to the lipid substrate [34]. Systematic optimization of the emulsification conditions, including surfactant concentration and alternative surfactants, may therefore improve substrate availability and overall reaction efficiency. Further studies should systematically evaluate substrate loading, temperature, pH, product effects, and catalyst performance over additional reuse cycles to identify the factors limiting conversion and determine the operational stability of the catalyst.

Finally, the safflower oil experiment showed that the spore-displayed CgeA-L2-PAI could convert LA released from a natural triglyceride substrate when combined with lipase-mediated hydrolysis. Safflower oil contains approximately 70% linoleic acid [22], corresponding to about 4.9 mg/mL potentially available LA with the oil concentration used here. In the lipase-only control, 1.88 mg/mL free LA was detected, indicating that triglyceride hydrolysis was incomplete. Formation of 0.46 mg/mL t10,c12-CLA corresponded to an apparent conversion of approximately 24% when calculated relative to the 1.88 mg/mL free LA measured in the separate lipase-only control. Because the total amount of free LA released during the coupled lipase/PAI reaction could not be determined directly, this value should be regarded as an estimate rather than a complete mass-balance-based conversion. Nevertheless, the apparent conversion was within the same range as that observed when free LA was supplied directly. These results suggest that incomplete triglyceride hydrolysis likely contributed to the lower overall CLA yield from safflower oil. Because the lipase was used in soluble form, the present setup does not represent a fully reusable two-enzyme catalyst, but it demonstrates that spore-displayed PAI remains functional with oil-derived LA. Thus, the present experiment should be viewed as a preliminary demonstration that spore-displayed PAI can convert oil-derived LA, rather than as an optimized, fully reusable

two-enzyme process. Further development should evaluate lipase type, enzyme loading, and hydrolysis time and, where complete catalyst recovery is required, the use of an immobilized lipase or a bifunctional spore catalyst co-displaying lipase and PAI.

4. Conclusions

Overall, this study demonstrates that *B. subtilis* spores can serve as a functional platform for the display of linoleic acid isomerase and the production of t10,c12-CLA. The spore-displayed enzyme was catalytically active, remained active after one reuse cycle, and converted both free LA and oil-derived LA released by lipase-mediated hydrolysis. Quantitative PAI abundance on the spores was not determined, catalyst reuse was evaluated over only one additional reaction cycle, and substrate availability in the safflower oil experiment was limited by incomplete triglyceride hydrolysis. Nevertheless, by coupling PAI production and immobilization during sporulation, the system provides a recoverable catalyst format that may simplify catalyst preparation compared with approaches requiring separate enzyme purification and immobilization. Although the present conversion efficiency remains below that of extensively optimized PAI systems, the high performance achieved in such systems indicates substantial scope for improving the spore-based catalyst through optimization of substrate accessibility, reaction conditions, and catalyst reuse.

5. Materials and Methods

5.1. Bacterial Strains and Culture Conditions

Escherichia coli Mach1 was used for plasmid construction. All genetic modifications were carried out in *B. subtilis* KO7 (1A1133) which is deficient in seven extracellular proteases [35,36]. All bacterial strains were grown in low-salt Luria–Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl). For solid media, 1.5% (*w/v*) agar was added. Cultures were incubated at 37 °C with shaking at 200 rpm. When required, antibiotics were added at final concentrations of 50 µg/mL kanamycin or 100 µg/mL ampicillin for *E. coli* and 5 µg/mL chloramphenicol for *B. subtilis*.

5.2. Transformation

Transformation of *E. coli* was performed by heat shock using chemically competent cells [37]. *B. subtilis* was transformed by natural competence triggered by nutrient downshift, as described previously, with the modification that the recovery time was increased to two hours [38].

5.3. Chemicals

Linoleic acid ($\geq 99\%$ purity, CAS No. 60-33-3), *trans*-10,*cis*-12 conjugated linoleic acid (t10,c12-CLA, 96% purity, CAS No. 2420-56-6), *cis*-9,*trans*-11 conjugated linoleic acid (c9,t11-CLA, 98% purity, CAS No. 544-71-8), and oleic acid (98% purity, CAS No. 112-80-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Safflower oil (CAS No. 8001-23-8) was purchased from Thermo Fisher scientific (Waltham, MA, USA). Commercial fatty acid methyl esters, including linoleic acid methyl ester (CAS No. 112-63-0) and conjugated linoleic acid methyl ester, were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received without further purification. Methyl tricosanoate (CAS No. 2433-97-8) was used as an internal standard for fatty acid analysis. Organic solvents used for fatty acid derivatization, including chloroform, methanol, and *n*-hexane, were LC–MS grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipase from *Candida rugosa* (L1754) used for safflower oil hydrolysis was purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.4. DNA Manipulation and Plasmid Construction

All primers used in this study were purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA) and are listed in Supplementary Table S1. The promoters, genes, and linker sequences used are provided in Supplementary Table S2, and all plasmids constructed or used in this work are listed in Supplementary Table S3. Strains are listed in Supplementary Table S4. All supplementary tables are provided in a single Excel file (Additional File S1). The native coding sequence of linoleic acid isomerase from *Propionibacterium acnes* (PAI; GenBank accession no. AX062088) was codon optimized for *B. subtilis* and synthesized by General Biosystems (Durham, NC, USA). The p1CSV-based plasmids used in this study for translationally fusing PAI with various spore coat proteins were constructed by Bartels and co-workers [19]. These vectors contain the P_{cotYZ} promoter, specific crust protein fusion partners, and terminator elements and confer chloramphenicol resistance for *B. subtilis* selection and ampicillin resistance for *E. coli*. The plasmids used included p1CSV-CgeA-N, p1CSV-CotV-N, p1CSV-CotW-N, p1CSV-CotX-N, p1CSV-CotY-N, and p1CSV-CotZ-N.

Initially, the PAI-encoding gene was inserted into the p1CSV vector backbone using standard restriction-based cloning, generating constructs in which PAI was fused directly to the respective crust proteins without a linker. These vectors contain homology regions enabling site-specific chromosomal integration into the *amyE* locus of *B. subtilis*.

To construct crust protein–PAI fusion variants containing peptide linkers, the USER (Uracil-Specific Excision Reagent) cloning system was used to enable seamless assembly [39]. PCR fragments of the codon-optimized PAI gene and the p1CSV backbone were amplified using uracil-containing primers and Phusion® U Hot Start DNA Polymerase (Thermo Fisher Scientific (Waltham, MA, USA)). Following DpnI digestion, purified PCR products were combined with USER® enzyme mix (New England Biolabs (Ipswich, MA, USA)) and incubated sequentially at 37 °C and 25 °C to generate recombinant plasmids. Three linker variants were incorporated: L1 (GGGEAAAKGGG), a flexible α -helix-forming linker [17]; L2 ((GGGGS)₃), a flexible long linker; and L3 (EAAAK), a rigid linker [40]. This approach generated 18 linker-containing fusion plasmids combining six crust proteins (CgeA, CotV, CotW, CotX, CotY, and CotZ) with the three linker variants. Together with the six corresponding constructs lacking a linker, this resulted in a total of 24 PAI display configurations.

All recombinant plasmids were verified by Sanger sequencing (Eurofins Genomics, (Ebersberg, Germany)). Plasmid DNA was purified using the NucleoSpin® Plasmid Easy-Pure Kit (Macherey-Nagel, (Düren, Germany)), and genomic DNA was isolated using the PureLink™ Genomic DNA Mini Kit (Invitrogen (Carlsbad, CA, USA)) according to the manufacturers' instructions.

5.5. Bacillus Spore Preparation

Spores were prepared using Schaeffer's sporulation medium (SSM; 8 g/L Difco nutrient broth, 1 g/L KCl, 1 mM MgSO₄·7H₂O, 10 μ M MnCl₂, 0.5 mM CaCl₂, and 1 μ M FeSO₄ (pH 7)) [41]. Overnight *B. subtilis* cultures were inoculated into SSM and incubated at 37 °C with shaking at 200 rpm for 48 h or until >90% sporulation was observed by phase-contrast microscopy. After sporulation, cultures were centrifuged at 7000 $\times$ g for 10 min, and the pellet was treated with 0.5% lysozyme at 37 °C for 1 h to lyse residual vegetative cells. The spores were successively washed with 1 M NaCl, 1 M KCl, and sterile water, then resuspended in 200 mM Tris-HCl buffer (pH 7.0) and stored at 4 °C until further use. Purified spores were counted using a disposable hemocytometer (MilliporeSigma (Burlington, MA, USA)) according to the manufacturer's instructions.

5.6. Spore De-Coating and Extraction of Spore Crust Proteins

Spore coat proteins were extracted by chemical de-coating [42]. Briefly, 100 mg (wet weight) spores were incubated in 1 mL of de-coating buffer (0.1 M NaCl, 0.1 M NaOH, 1% sodium dodecyl sulfate (SDS), and 0.1 M dithiothreitol) for 30 min at 70 °C, followed by sonication for 5 min with 30 s on/off intervals. The samples were then centrifuged at $12,000\times g$ for 15 min at 4 °C, and the resulting supernatant was mixed with ice-cold acetone at a ratio of 1:4. After gentle mixing, samples were incubated overnight at −20 °C to precipitate proteins. The precipitated proteins were collected by centrifugation at $12,000\times g$ for 15 min at 4 °C, washed with cold acetone and air-dried for 10 min. The pellet was resuspended in $4\times$ Laemmli sample buffer and used for SDS–PAGE analysis.

5.7. SDS–PAGE and Western Blotting

Protein samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 20% Mini-PROTEAN[®] TGX Stain-Free[™] Protein Gels (Bio-Rad (Hercules, CA, USA)). Protein concentrations were determined using the Bradford assay (Bio-Rad (Hercules, CA, USA)) with bovine serum albumin (BSA) as the standard. For Western blot analysis, proteins were transferred from SDS–polyacrylamide gels onto polyvinylidene difluoride (PVDF) membranes (MilliporeSigma (Burlington, MA, USA)) using an iBlot[™] 2 Gel Transfer Device (Thermo Fisher Scientific (Waltham, MA, USA)) according to the manufacturer's instructions. Membranes were blocked with 5% (*w/v*) skim milk in TBST buffer for 1 h at room temperature. His-tagged PAI protein was detected by incubating the membrane with a mouse anti-6xHis monoclonal antibody (4A12E4; 1:1000; Invitrogen (Carlsbad, CA, USA)) at 4 °C overnight, followed by incubation with an HRP-conjugated goat anti-mouse IgG secondary antibody (1:10,000; Sigma-Aldrich (St. Louis, MO, USA)) for 1 h at room temperature. Membranes were washed three times with TBST for 5 min after each antibody incubation step. Bands were visualized using an enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific (Waltham, MA, USA)) and imaged with an iBright[™] Imaging System (Thermo Fisher Scientific (Waltham, MA, USA)).

5.8. Bioconversion of LA to *t*10,*c*12-CLA

Linoleic acid (LA) and safflower oil emulsion stocks were prepared at concentrations of 10 mg/mL (*w/v*) and 14 mg/mL (*w/v*), respectively, in 200 mM Tris–HCl buffer (pH 7.0). Emulsions were stabilized with 1% (*v/v*) Tween 20 (Thermo Fisher Scientific (Waltham, MA, USA)) and sonicated using a probe sonicator (Sonics Vibra-Cell) (Sonics & Materials, Inc. (Newtown, CT, USA)) for 3 min with 30 s on/off intervals to ensure uniform dispersion. For safflower oil hydrolysis, lipase from *Candida rugosa* (Type VII, ≥ 700 U/mg) was added to the 14 mg/mL safflower oil emulsion at a final concentration of approximately 700 U/mL and incubated for 1 h to release free linoleic acid from triglycerides. The prepared emulsions were then mixed with the spore suspension in 200 mM Tris–HCl buffer to obtain a nominal initial substrate concentration of 5 mg/mL LA or 7 mg/mL safflower oil. The LA concentration was selected during preliminary assay development to provide readily quantifiable CLA formation. Unless otherwise stated, the bioconversion was done using 5×10^9 spores/mL for 6 h. For the initial construct-screening experiments, wild-type KO7 spores and reactions without spores were included as controls under otherwise identical conditions. To minimize oxidative degradation of fatty acids, the headspace of each glass vial was flushed with nitrogen gas and the reactions were protected from light throughout incubation. Enzymatic bioconversion was carried out at 35 °C with shaking at 200 rpm, a temperature previously reported to support efficient PAI activity [9]. Reactions were terminated by centrifugation at $5000\times g$ for 5 min to separate spores from the reaction

mixture. For the nominal 0 h sample in the time-course experiment, substrate and spores were mixed and the sample was immediately processed for reaction termination by centrifugation. Thus, this sample represents an operational 0 h time point rather than instantaneous quenching of enzyme activity. The supernatant was collected using glass Pasteur pipettes for subsequent lipid extraction and analysis.

5.9. Preparation of Fatty Acid Methyl Esters (FAMES)

Fatty acids were extracted from linoleic acid emulsions using a modified Folch method [43]. Samples containing LA and CLA emulsions were mixed with chloroform-methanol (2:1, *v/v*; 5 mL per 1 mL of emulsion) in pre-washed 10 mL glass tubes. The mixtures were sonicated for 5 min, flushed with nitrogen gas, and sealed for 30 min at room temperature to facilitate lipid extraction. Following extraction, 0.88% KCl solution (0.25 mL/mL of fatty acid–chloroform–methanol mixture) and methyl tricosanoate (internal standard; 1 mg/mL solution, 200 μ L/mL of initial fatty acid emulsion) were added. Samples were vortexed and centrifuged at 1500 rpm for 2 min to induce phase separation. The lower chloroform phase containing fatty acids was carefully collected using a glass Pasteur pipette (Fisher Scientific (Pittsburgh, PA, USA)). The remaining aqueous phase and biomass were re-extracted twice with fresh chloroform–methanol (2:1, *v/v*) under identical conditions, and all organic phases were pooled. The combined lipid extracts were evaporated under a stream of nitrogen. For derivatization, the dried fatty acid extracts were reacted with 1.5 mL sulfuric acid–chloroform–methanol solution (0.3:2.0:1.7, *v/v/v*) at 60 °C for 2 h to convert fatty acids into their corresponding fatty acid methyl esters (FAMES). Sulfuric acid-based methylation has previously been shown to result in minimal alteration of CLA isomer composition under comparable derivatization conditions [44]. After cooling to room temperature, LC-MS-grade water was added and the lower organic phase containing FAMES was collected. Residual aqueous fractions were re-extracted twice with HPLC-grade chloroform, and the organic layers were combined and dried over anhydrous sodium sulfate. The final derivatized extracts were evaporated under nitrogen at room temperature and stored at –80 °C until GC–MS analysis. Prior to analysis, samples were re-dissolved in hexane.

5.10. GC-MS Analysis

Fatty acid methyl esters (FAMES) were analyzed by gas chromatography–mass spectrometry (GC-MS) using a GCMS QP2020 NX system (Shimadzu, Japan) equipped with a ZB-FAME column (100 m $\times$ 0.25 mm I.D., 0.20 μ m film thickness; Phenomenex, UK). Samples (1 μ L) were injected in split mode (split ratio 1:1). The injector temperature was maintained at 250 °C. Helium was used as the carrier gas and operated in constant pressure mode at 262.6 kPa. The oven temperature program was as follows: initial temperature 120 °C held for 10 min; ramped to 180 °C at 3 °C/min; ramped to 200 °C at 1.5 °C/min and held for 3 min; and finally ramped to 220 °C at 2 °C/min and held for 24 min. The transfer line temperature was maintained at 250 °C and the ion source temperature at 230 °C. Electron impact ionization (70 eV) was used. FAMES were detected by full-scan acquisition over a mass range of *m/z* 50–600, and by single-ion monitoring (SIM) of characteristic ions at *m/z* 81, 95, 263, and 294. Chromatographic data were processed and quantified using GCMSsolution software (version 4.2, Shimadzu Corporation (Kyoto, Japan)) with the QuantBrowser module. LA and t10,c12-CLA were quantified using calibration standards prepared from authentic LA and t10,c12-CLA over the range of 6–700 μ g/mL, with separate calibration curves generated for each analyte. Methyl tricosanoate was included at the same concentration in standards and samples as an internal standard, and LA and t10,c12-CLA peak areas were normalized to the internal-standard peak area before calibration and

quantification. The retention positions of c9,t11-CLA and t10,c12-CLA were established using CLA FAME standards containing both isomers. Compound identity was further supported by comparison of retention times with authentic standards and mass spectra with the NIST11 spectral library. For free-LA bioconversion experiments in which both residual LA and t10,c12-CLA were quantified, apparent recovery was calculated as the sum of measured LA and CLA relative to the nominal initial LA concentration of 5 mg/mL.

5.11. Data Analysis and Presentation

All graphs and calculations were performed using GraphPad Prism (version 10.0.0; GraphPad Software, Boston, MA, USA). Bioconversion experiments were performed as duplicate reaction measurements using aliquots from the same spore preparation, with each reaction processed and analyzed separately. Data are presented descriptively as mean $\pm$ standard deviation (SD). Because the duplicate reactions did not represent independent biological replicates, inferential statistical tests were not applied. The individual measured values underlying the bioconversion experiments are provided in Supplementary Table S5 (Additional File S1).

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/catal16090826/s1>, **Figure S1.** Sensitivity of vegetative *Bacillus subtilis* cells to fatty acids. (A–B) Growth of *B. subtilis* in the presence of increasing concentrations of linoleic acid (A) and conjugated linoleic acid (CLA) (B). Growth was monitored at concentrations ranging from 0 to 10 mg/mL. **Table S1.** Primers used in this study. **Table S2.** Promoters and gene sequences used in this study. **Table S3.** List of plasmids. **Table S4.** List of strains. **Table S5.** Source data for figures. Tables S1–S5 are found in Additional File S1.

Author Contributions: C.J. and A.B. conceived the study. S.N. and C.J. performed the experiments. All authors contributed to data analysis and interpretation. S.N. and C.J. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Author Adriana Botes was employed by the company Azotic Technologies. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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