Engineered microbial biofuel production and recovery under supercritical carbon dioxide

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Culture contamination, end-product toxicity, and energy efficient product recovery are long-standing bioprocess challenges. To solve these problems, we propose a high-pressure fermentation strategy, coupled with in situ extraction using the abundant and renewable solvent supercritical carbon dioxide (scCO₂), which is also known for its broad microbial lethality. Towards this goal, we report the domestication and engineering of a scCO₂-tolerant strain of *Bacillus megaterium*, previously isolated from formation waters from the McElmo Dome CO₂ field, to produce branched alcohols that have potential use as biofuels. After establishing induced-expression under scCO₂, isobutanol production from 2-ketoisovalerate is observed with greater than 40% yield with co-produced isopentanol. Finally, we present a process model to compare the energy required for our process to other in situ extraction methods, such as gas stripping, finding scCO₂ extraction to be potentially competitive, if not superior.
Supercritical fluids offer many advantages over conventional solvents due to their gas-like viscosity and diffusivity with liquid-like density and solvation. Supercritical carbon dioxide (scCO₂) is especially promising as a green solvent due to its moderate critical point temperature (31.1 °C) and pressure (7.38 MPa) as well as being non-flammable, non-hazardous, abundant, and inexpensive. Numerous biocatalytic processes have taken advantage of scCO₂, including enantiomer specific lipase reactions, asymmetric alcohol reduction, and carboxylation of various substrates. The solvent properties of scCO₂ have been exploited for the separation of alcohols, aldehydes, ketones, and acids from aqueous solutions via both in situ and ex situ extraction. In situ product extraction has been shown to have a dramatic effect on bioproduction titers by alleviating end-product inhibition or toxicity. scCO₂ is especially attractive within biphasic scCO₂-aqueous systems due to its preferential extraction of alcohols with intermediate-chain length (e.g., 3 to 6 carbons), the result of decreasing water solubility with increasing number of carbons. Conversely, alcohol volatility decreases with chain length, making these molecules more attractive targets for scCO₂ extraction compared to gas stripping.

Despite the potential to couple scCO₂ for product removal with microbial synthesis of target compounds, the two have not been combined due to the broad microbial lethality of scCO₂, where cellular inactivation is thought to occur through a combination of membrane disruption, cytosolic acidification, enzyme inactivation, and cellular desiccation. Several early studies have explored processes catalyzed by whole cells under scCO₂; however, these were conducted over short time scales (< 1 day) using biomass grown in the absence of scCO₂, and without evidence of cell viability under scCO₂. Two results include carboxylation of pyrrole substrate with Bacillus megaterium cells in a biphasic mixture of buffered solution and scCO₂ after 1 h, and reduction of ketones with a high degree of enantiomer specificity using immobilized Geotrichum candidum cells in an aqueous-scCO₂ biphasic continuous flow reactor. Several additional studies have employed scCO₂-based extraction of bioproducts, but have not achieved in situ extraction due to inhibited cell growth. Using a semi-continuous process, ethanol was extracted from the spent fermentation broth of yeast and Clostridium cultures using scCO₂. Knutsen et al. attempted to produce ethanol from cellobiose in dual-phase aqueous bioreactors containing non-growing Clostridium thermocellum and pressurized scCO₂, N₂ or ethane headspaces. Significant cellobiose conversion and ethanol production were observed under pressurized nitrogen and ethane, but not under scCO₂, indicating process inhibition by CO₂.

Bioprospecting is a promising approach to recover organisms capable of active metabolism under high pressures of CO₂. Numerous environmental isolates have been characterized with tolerance to high partial pressures of CO₂, although rarely are cultures examined above atmospheric pressures (0.1 MPa). San-tillan et al. found a strain of Lactobacillus casei in a terrestrial CO₂-rich spring that can grow in CO₂ pressures up to 1 MPa. Recently, Peet et al. examined organisms capable of growth at geologic CO₂ sequestration sites, isolating several endospor-forming Bacillus species that are tolerant to scCO₂ treatment (> 10 MPa), but with low growth frequency and magnitude. Similarly, growth was reported for laboratory cultures inoculated with deep subsurface sandstone under scCO₂, although the identities of microorganisms were not determined. Motivated by the hypothesis that natural deposits of high-pressure CO₂ would harbor microorganisms adapted to actively grow in close contact with it, Freedman et al. investigated the microbial diversity at McElmo Dome, a deep subsurface CO₂ reservoir that was formed 40–72 million years ago. Laboratory enrichment cultivation of fluids collected from McElmo dome under scCO₂ enabled isolation of B. megaterium strain SR7, an endospore-forming, facultative-anaerobe. We have observed growth of SR7 cultures to a cell density of greater than 10⁷ cells ml⁻¹ after 3 weeks under scCO₂ following inoculation as endospores and supplementation with the germination inducer l-alanine. Endospores of Gram-positive bacteria are known for their resilience even when exposed directly to scCO₂, and we have hypothesized that this dormant state enables these bacteria to withstand and adapt to the scCO₂ environment prior to outgrowth as vegetative cells. Analysis of fermentation products from SR7 grown under scCO₂ indicated the production of lactate, acetate and succinate, demonstrating active metabolism under scCO₂ and the potential for microbial production to be coupled with scCO₂ extraction.

In this work, we seek to develop B. megaterium SR7 as a bioproduction host under scCO₂ for useful compounds that have already been established for in situ extraction using scCO₂ (Fig. 1), such as branched, intermediate-chain alcohols. We posit that an integrated fermentation-scCO₂ extraction process may ultimately, and simultaneously, solve three long-standing challenges in the field by (1) reducing end-product toxicity through extraction, (2) mitigating culture contamination under the highly selective conditions of scCO₂, and (3) providing an energy efficient method to recover high-purity products using scCO₂ as a sustainable solvent. Branched, intermediate-chain alcohols are not naturally made by SR7, necessitating metabolic engineering to generate them. Isobutanol is selected due to its importance as a drop-in replacement for gasoline and its favorable fuel characteristics (i.e., high energy density, suitable research octane number, low hygroscopicity). Additionally, isobutanol is an attractive molecule for in situ scCO₂ extraction as it is cytotoxic and expected to partition favorably to the scCO₂ phase. Isobutanol production requires introduction of two heterologous enzymes, an α-ketoisovalerate decarboxylase and alcohol dehydrogenase, to convert the valine-synthesis intermediate, 2-ketoisovalerate, to the final product with minimal intermediate isobutyraldehyde accumulation. We conclude our study by analyzing the energy requirements of an integrated fermentation-extraction process, comparing it to alternative in situ extraction technologies such as gas stripping, pervaporation, and adsorption, in order to address challenges associated with energy efficient product recovery.

Results

Heterologous protein expression in aerobically grown SR7. As strain B. megaterium SR7 is an environmental isolate, its genetic modification required optimization and customization of protocols previously developed for laboratory strains and model organisms. A transformation protocol for SR7 was developed by modifying a method based on protoplast-osmotic shock (Supplementary Note 1). The xylose-inducible promoter system (pXyl) was selected for use in SR7 due to its simplicity, high expression level, and widespread use in the B. megaterium literature. Additionally, the IPTG-inducible hyperspank promoter system (pHysp) and the p43-growth based promoter (p43) were evaluated, both of which have shown effective protein production in B. subtilis (Fig. 2a).

All three promoters were functional in SR7 (Fig. 2b and Supplementary Fig. 1), with pXyl and pHysp promoters resulting in strong GFP expression of 25- and 20-fold greater than uninduced cultures, respectively. The xylose promoter was activated most rapidly, achieving maximal fluorescence 3 h post induction, similar to other B. megaterium strains using this promoter system. For induced pXyl GFP and pHysp gfp cultures, a large proportion...
**Fig. 1** Schematic of integrated fermentation and extraction under supercritical CO$_2$. A scCO$_2$-tolerant microbe can be engineered to produce compounds, such as medium-chain alcohols (C4-C5), that may serve as biofuels and may be preferentially extracted from aqueous media into the scCO$_2$ phase. Collection of scCO$_2$ followed by partial de-pressurization will facilitate high-purity biofuel extraction. The presented biphasic scCO$_2$ separation strategy is expected to simultaneously provide a contaminant-free environment for the engineered organism due to the broad microbial lethality of scCO$_2$, and continuously strip microbially produced biofuels to eliminate end-product toxicity. scCO$_2$ is only weakly soluble in the aqueous media phase (<1%) and vice versa, resulting in a biphasic system.

**Fig. 2** Heterologous protein expression and promoter evaluation in SR7. **a** Schematic of the promoters tested in SR7. pXyl is a xylose-inducible promoter and contains the xylR gene and xylA promoter from *B. megaterium* strain DSM 319. pHysp contains the IPTG-inducible hyperspank promoter and *lacI*, both from plasmid pDR111. The p43 promoter is taken from *B. subtilis* strain KS438 and is growth-associated. Expected inducers are indicated in parentheses. **b** Bulk fluorescence measurements of SR7 populations containing gfp-encoding plasmids, with (circles, solid lines) or without (diamonds, dashed lines) inducers. Error bars represent the standard deviation of biological triplicate samples. **c** Fluorescence populations of SR7 cells expressing gfp at 4 h post induction measured by FACS (Alexa Fluor 488). A GFP positive gate was established using the autofluorescence of SR7 cells containing an empty plasmid control. **d** Phase-contrast and fluorescence microscopy of SR7 pXyl gfp cells with and without xylose induction. Images were taken 4 h post induction. Source data are provided as a Source Data file.
(> 95%) of the population was found to express GFP (Fig. 2c, d and Supplementary Table 1), which is greater than that observed for other B. megaterium strains using pXyl. Uninduced pXyl gfp containing cells have many members (40%) that fall into the GFP positive gate, providing evidence that this promoter is leaky when grown aerobically in rich Luria-Bertani (LB) medium (Supplementary Table 1 and Supplementary Fig. 2), in contrast to previous work showing tight regulation of pXyl within B. megaterium strain WH3203. As expected, the growth-associated p43 promoter was activated as the cells entered exponential growth phase (Supplementary Fig. 1e, f), but showed weaker GFP expression, only sixfold greater than the empty plasmid-containing control strain. Inducible production of GFP for the pXyl and pHySp promoters was also observed for cells cultured in semi-defined medium supplemented with xylose and/or glucose, with maximal fluorescence observed for cells containing the pXyl promoter grown in xylose-amended medium (Supplementary Fig. 3). Expression of gfp from the xylose promoter was shown to be lowered when both glucose and xylose sugars are present, likely due to catabolite repression, as observed for other strains of B. megaterium. Elements for individual colonies (Supplementary Table 2 and Supplementary Fig. 5). The anaerobic functionality of the xylose promoter was next established for SR7 grown under 0.1 MPa CO2 (i.e., atmospheric pressure) as well as a 10 MPa headspace of scCO2. Several challenges were anticipated when moving from aerobic to scCO2-based culturing, including slower and less predictable growth due to stresses associated with scCO2, and reduced maintenance of non-native plasmids through sporulation, germination, and outgrowth. Since the formation of the GFP chromophore is oxygen dependent, the β-galactosidase protein (LacZ) from E. coli was selected as a reporter for anaerobic cultures due to its previous use in B. megaterium, its oxygen independence, and its easy and sensitive detection via enzymatic assay. After confirming heterologous lacZ expression (Fig. 3) and plasmid maintenance under 0.1 MPa CO2 (Supplementary Note 2), the functionality of the pXyl promoter was assessed for SR7 pXyl lacZ cells grown under 10 MPa scCO2 at 37 °C in semi-defined medium containing glucose as a carbon source and L-alanine as a germination inducer. Xylose was added as an inducer at the time of inoculation rather than during early exponential growth since high-pressure cultivation of SR7 in stainless steel columns precludes non-destructive amending of samples during cultivation. After 21 days of incubation under scCO2, LacZ activity was measured in crude lysates derived from columns that displayed growth. Cells grown under 0.1 MPa and 10 MPa CO2 with xylose displayed similar LacZ specific activities of 0.56 and 0.40 U mg⁻¹, respectively (Fig. 3). The 15-fold increase in LacZ expression under 10 MPa CO2 relative to uninduced cultures (p = 0.0059) is similar in magnitude to the increase in induced-expression observed under 0.1 MPa (Fig. 3).

Growth of SR7 from endospores under scCO2 has been demonstrated to be at least a tenfold increase in cell number and presence of vegetative cells, as determined by direct cell counts using fluorescence microscopy. SR7 pXyl lacZ cultures amended with xylose grew in 8 of 55 columns (15%) while growth was observed in 5 of 34 unamended columns (15%) (Supplementary Table 3), which together represent a 4.4-fold decrease in growth frequency relative to wild-type SR7 (64%) under the same conditions (i.e., 5 ml culture volume with 3 × 10⁵ spores ml⁻¹ inoculum and 5 ml scCO2 headspace). Previous studies have shown bacteria transformed with exogenous plasmids are more susceptible to cell death than wild-type strains, especially under harsh culture conditions, consistent with our results. Improving growth of genetically modified SR7 remains an important target for further optimization.
cultures shown in S. cerevisiae scCO₂, cultures with at least a tenfold increase in cell number, as enumerated by microscopy, were analyzed for alcohol production, sugar consumption and in SR7 samples shown in respectively. Cultures were grown aerobically in LB medium in the presence and absence of 5 g l⁻¹ xylose as an inducer and 5 mM αKIV as substrate for the pathway. For all aerobic experiments, error bars represent the standard deviation of biological triplicate cultures. Co-production of isopentanol measured in SR7 samples shown in b. Accumulation of the isobutyraldehyde intermediate at short culture times, 4 h post induction. Alcohol dehydrogenases ADH6 from S. cerevisiae, AdhA from L. lactis, AdhP from E. coli, YqhD from E. coli, and AdhA from B. megaterium SR7 were evaluated to decrease the buildup of isobutyraldehyde intermediate for aerobic cultures fed 5 mM αKIV and induced with xylose. Production of isobutanol at 24 h post induction for the cultures shown in d. Production of isobutanol for SR7 cultures grown under 0.1 MPa CO₂ and 10 MPa scCO₂ at 37 °C. Under scCO₂, cultures with at least a tenfold increase in cell number, as enumerated by microscopy, were analyzed for alcohol production, sugar consumption and fermentation product generation. Average isobutanol titers from the aqueous phase of cultures showing at least 1 mM glucose consumption (classified as high activity; Supplementary Fig. 11) are provided. Source data are provided as a Source Data file (Fig. 4b). In addition to the target isobutanol molecule, isopentanol (Fig. 4c and Supplementary Figs. 6 and 7) and phenylethyl alcohol (PEA) (Supplementary Fig. 8a) production were also observed, with the isopentanol titer being greater than that of isobutanol. Accumulation of these products is not surprising given the known promiscuity of KivD towards other amino acid synthesis pathway intermediates (Supplementary Fig. 8b).

Biosynthesis of alcohols entails conversion of aldehyde intermediates (Fig. 4a) that tend to be highly soluble in scCO₂, partitioning approximately tenfold more readily into scCO₂ than their cognate alcohols. Thus, any accumulation of aldehyde is hypothesized to result in premature extraction of this compound under scCO₂, resulting in lower alcohol production. Under aerobic conditions at short induction times of 4 h, isobutyraldehyde buildup was observed, even for cells that contained the alcohol dehydrogenase ADH6Sc, (Fig. 4d). Four additional alcohol dehydrogenases were screened for reduced aldehyde accumulation: three previously shown to be functional within the context of the isobutanol pathway, and a predicted alcohol dehydrogenase native to SR7 (AdhA₈₉) with high homology to AdhA, from L. lactis. The YqhD enzyme from E. coli, known to be highly effective for producing isobutanol, was identified as the superior variant, with the lowest amount of aldehyde accumulation at short culture times (4 h) (Fig. 4d), over 80% yield of isobutanol from αKIV (Fig. 4e), and maintained capacity for isopentanol production (Fig. 4f).

The optimized two-enzyme isobutanol production pathway was evaluated aerobically in semi-defined medium containing 4 g l⁻¹ glucose and 5 g l⁻¹ xylose (conditions previously shown to support heterologous enzyme production in SR7 under anaerobic conditions). The final isobutanol titer was approximately half of that observed in LB medium (Supplementary Fig. 9a), possibly due to lower heterologous enzyme expression and more αKIV substrate being directed towards biomass in the lower nutrient semi-defined medium. Increasing the glucose concentration to 10 g l⁻¹ in the semi-defined medium increased the titers of all three alcohols, suggesting that cellular resources may be directed towards these products when carbon is provided in greater excess.
Neither consumed sugar substrate nor generated meaningful consumption were classified as having less than 10-fold growth, but less than 1 mM glucose lactate and acetate (Supplementary Fig. 11b). Other samples that showed at least 10-fold increase in titers upon addition of KIV substrate (Supplementary Fig. 9c). When combined with xylose, the addition of 100 mM alanine (used under anaerobic CO₂ conditions for endospore germination) increased cell growth (OD₆₀₀ > 15) and more than doubled isobutanol production (Supplementary Fig. 9d), likely due to the deamination of alanine to pyruvate, a precursor for isobutanol via the valine-synthesis pathway and part of central carbon metabolism.

**Isobutanol production in SR7 under scCO₂.** After optimizing aldehyde conversion and alcohol production for SR7 under aerobic conditions, functionality of the two-step isobutanol pathway was assessed in semi-defined medium under anaerobic conditions (Fig. 4f). Seed cultures of SR7 pXyl *kivD yhgd* cells passed under 0.1 MPa CO₂ into fresh medium amended with xylose and 5 mM KIV substrate generated 4.43 mM isobutanol after 48 h of incubation. The final isobutanol titer under anaerobic 0.1 MPa CO₂ headspace is greater than the 2 mM isobutanol generated by aerobic cells grown in the same medium conditions. Isopentanol and PEA titers were 1.8 and 0.21 mM, respectively (Supplementary Fig. 10). As expected, no isobutanol or isopentanol was observed for SR7 cultures that contained an empty plasmid (Fig. 4f and Supplementary Fig. 10).

Biofuel production was next evaluated for SR7 cultures grown under supercritical CO₂ at 10 MPa and 37 °C. Cultures were grown for 21 days in semi-defined medium from SR7 endospores containing the desired biofuel pathway or empty plasmid, with 4 g/l−1 glucose, 5 g/l−1 xylose, 5 mM KIV, and 100 mM alanine added at the time of inoculation. As was found for SR7 pXyl *lacZ*, the addition of a plasmid resulted in lower frequency growth (27%) as compared to SR7 wild-type (64%) (Supplementary Table 3). For cultures that displayed growth, glucose consumption was not correlated with increases in cell number (Supplementary Fig. 11a), possibly due to consumption of yeast extract and l-alanine. Glucose consumption was found to correlate strongly with the production of fermentation products lactate and acetate (R² = 0.97) (Supplementary Fig. 11b), which have previously been shown to accumulate in SR7 cultures grown under scCO₂. This finding led to classifying samples that consumed more than 1 mM glucose as having high activity, as all of these samples also produced greater than 1 mM combined lactate and acetate (Supplementary Fig. 11b). Other samples that showed at least tenfold growth, but less than 1 mM glucose consumption were classified as having low activity since they neither consumed sugar substrate nor generated meaningful amounts of fermentation products (Supplementary Fig. 11b).

Isobutanol production was measured from the aqueous phase of scCO₂ cultures grown in the presence and absence of xylose (Supplementary Fig. 12). Xylose-containing high activity cultures produced an average of 1.23 mM isobutanol, whereas those without xylose generated approximately half that amount (Fig. 4f and Supplementary Table 4). Isobutanol titers normalized to cell density were comparable under 0.1 MPa CO₂ and 10 MPa scCO₂, at 2.2 × 10⁻⁸ μmol cell⁻¹ and 3.1 × 10⁻⁸ μmol cell⁻¹, respectively (Supplementary Table 5). While the high activity, empty vector control cultures consumed up to 1.9 mM KIV under 10 MPa scCO₂, isobutanol was not detected in any of these samples (Supplementary Table 4), an identical outcome to aerobic and 0.1 MPa CO₂ cultures. The isobutanol yield from KIV ranged from 37 to 45% in the aqueous phase for xylose-induced high activity biofuel cultures, 2.8-fold higher than for those that lacked xylose (Supplementary Table 4). Isopentanol generation was also observed for samples grown under 10 MPa scCO₂, reaching an average titer of 0.31 mM (Supplementary Table 4), while only trace amounts of PEA were detected under scCO₂. The amount of isobutanol produced by low activity cultures was on average 35-fold less than for the high activity cultures (Supplementary Table 4).

**Extraction of isobutanol using scCO₂.** Inspired by our demonstration of microbially generated alcohols under scCO₂, we attempted to recover product from SR7 cultures using scCO₂. Partitioning of biologically produced isobutanol into the scCO₂ headspace was observed for the aforementioned SR7 cultures (Supplementary Fig. 13), albeit at marginal concentrations. Isopentanol was not detected in the headspace samples. Our proof of concept demonstration for isobutanol extraction motivates adoption of two engineering solutions to increase recovery: (1) use of flowing scCO₂ to increase mass transfer and (2) controlled de-pressurization to capture scCO₂-solubilized alcohol. To evaluate these solutions, we assessed the recovery of isobutanol in the absence of microbial cells using a stirred two-phase reactor that is designed for in situ extraction using scCO₂ stripping (Supplementary Fig. 14), as previously done for n-butanol and n-pentanol. Similar to straight-chained alcohols, the rate of isobutanol extraction from aqueous solutions increases with low scCO₂ flow rates, reaching a maximum at a flow rate of 3.2 ml min⁻¹, most likely due to a tradeoff between convective and diffusive mass transfer. Isobutanol extraction rate and efficiency using scCO₂ was found to be approximately 25% greater than that for n-butanol (Supplementary Fig. 14). Taken together, these data indicate that scCO₂ is effective for rapid extraction of isobutanol from dilute aqueous solutions and identifies scale up of cultures to a similarly designed system as a priority for further development.

**Process analysis for extraction using scCO₂.** The experimental component of this study established growth and heterologous bioproduction in the presence of scCO₂ and extraction of isobutanol into the scCO₂ phase in a stirred reactor. Next, we constructed a fermentation-extraction process model to assess technological potential using detailed mass and energy balances. The objective of the analysis was calculation of isobutanol recovery energy requirements for comparison with previously reported analyses of butanol recovery using other methods. In practice, Oudshoorn suggested a benchmark of 5 MJ kg⁻¹ as the maximum energy requirement for butanol recovery. Unfortunately, previous studies of butanol recovery from dilute fermentation broths (<20 g l⁻¹) using a wide range of conventional technologies, such as gas stripping, adsorption, and liquid–liquid extraction, report energy requirements in the range of 10–20 MJ kg⁻¹. By comparison, Tompsett et al. estimated the energy demand for the CO₂ compression required for scCO₂ extraction of dilute n-butanol to be 4.0 MJ kg⁻¹. Since CO₂ compression is expected to represent the majority of the energy required by the scCO₂ extraction process, these results suggest that the energy requirement of scCO₂ extraction might compare favorably with alternative technologies—especially since efforts to minimize CO₂ compression requirements were not considered. Moreover, this previous study did not analyze the performance of an integrated fermentation-extraction process since bioproduction in the presence of scCO₂ has never been reported prior to this work. Accordingly, the current study evaluated approaches to reduce CO₂ compression requirements, the advantages associated with
coupled fermentation-extraction (Supplementary Note 3), and the intrinsic performance of the scCO2 extraction process.

With emphasis on evaluating energy demand for recovering a high-purity product, we developed an Aspen Plus simulation model (Fig. 5a and Supplementary Fig. 15a) to analyze a process consisting of integrated fermentation and extraction using scCO2. Isobutanol extraction performance was simulated using data reported in the current work, literature data on n-butanol extraction12,43,44, and established thermodynamic properties45,46 (Supplementary Note 3). Sensitivity analysis revealed that the most influential parameter in determining isobutanol energy requirement was the CO2:isobutanol feed ratio since it directly relates to the amount of CO2 that must be compressed for extraction (Supplementary Fig. 15b and Supplementary Note 3). Combining our isobutanol extraction data with values reported on n-butanol44, we calculate that 99.73% isobutanol recovery could be achieved at a CO2:isobutanol feed ratio of 3:1 as a base case. To reduce CO2 compression energy demand, product recovery via partial de-pressurization of the CO2 stream was explored, finding >94% pure isobutanol product could be recovered by partially de-pressurizing the stream exiting the fermenter to between 3.5 and 6.5 MPa45. In this pressure range, liquid–liquid phase splitting occurs, resulting in isobutanol-rich and water-rich liquids45. Partial de-pressurization allows recyling of a high-pressure CO2 stream, further reducing CO2 compression demand and thereby energy consumption (Supplementary Fig. 15b). Lastly, the sensitivity of the process to the ratio of recycled to purged CO2 was investigated using an optimized intermediate pressure of 6.5 MPa (Supplementary Fig. 15b).

Based on these sensitivity analyses, four scenarios were assessed using the process model, with specific emphasis on evaluating the extent to which partial de-pressurization and CO2 recycling could be used to decrease energy requirements of isobutanol extraction (Fig. 5b and Supplementary Table 6). Atmospheric-pressure fermentation followed by scCO2 extraction (scenario A) required

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**Fig. 5** Isobutanol energy recovery requirements for integrated fermentation-extraction under scCO2. a Simplified process schematic of the envisioned scCO2 bioprocess used to develop an Aspen Plus model, including compression of CO2 to scCO2, fermentation/extraction, de-pressurization/biofuel recovery, and CO2 recycle. Further detail is provided in Supplementary Fig. 15a. b Energy requirement for isobutanol recovery as MJ kg−1 for several scenarios. Separate fermentation and scCO2 recovery processes represent the base case (scenario A). Scenario B is coupled fermentation and extraction without use of partial extraction stream de-pressurization or scCO2 recycle. Scenario C de-pressurizes the extraction stream to 6.5 MPa to induce formation of an isobutanol-rich stream, which permits recycling of compressed CO2, set at 50% (by mass). Scenario D again de-pressurizes to 6.5 MPa and recycles the maximum possible amount of CO2 (87%) that results in no accumulation of CO2 in the system. Values for b can be found in Supplementary Table 6. c Comparison of the energy requirements for isobutanol production found for the scCO2 process relative to published literature values for alternative in situ recovery methods28. The scCO2 extraction energy requirement in c is for coupled fermentation and extraction with partial de-pressurization and full recycle (i.e., Scenario D in b). Source data are provided as a Source Data file.
an estimated 6.80 MJ kg\(^{-1}\), with compression accounting for approximately 66% of the energy demand. An integrated process, with complete de-pressurization to 0.1 MPa and no CO\(_2\) recycling (scenario B), reduced the energy burden to 4.4 MJ kg\(^{-1}\), with most of the improvement stemming from reduced compression requirements. Intermediate de-pressurization to 6.5 MPa combined with 50% CO\(_2\) recycle (scenario C)\(^{43}\), further reduces the energy requirement to 2.4 MJ kg\(^{-1}\), again largely due to diminished compression requirements. Finally, placing the CO\(_2\) recycle ratio at its maximum value (87%) (scenario D) and de-pressurizing the extraction stream to 6.5 MPa reduces the energy requirement to 1.4 MJ kg\(^{-1}\). As expected from previous arguments, the newly established energy requirement is lower than that previously reported for stand-alone fermentation and scCO\(_2\) extraction, and much less than any figure reported for conventional technologies such as gas stripping, liquid–liquid extraction, or adsorption (Fig. 5c). Although the energy requirement estimated here for integrated extraction-fermentation has not yet been demonstrated in practice, the current findings motivate further evaluation, with our model serving as a basis for follow-on techno-economic, emissions, and life cycle assessment studies.

**Discussion**

In this work, we report the production of alcohol biofuels under supercritical carbon dioxide. Using an environmentally isolated strain (*Bacillus megaterium* SR7) native to a deep subsurface CO\(_2\) reservoir, a transformation protocol was developed and heterologous protein expressed from three promoters. Furthermore, inducible β-galactosidase production was observed for cells grown under scCO\(_2\). SR7 was engineered to produce isobutanol from αKIV through a two-step metabolic pathway that simultaneously produced isopentanol. Both isobutanol and isopentanol have potential as drop-in biofuels or fuel additives, with comparable energy densities (29 MJ l\(^{-1}\) and 28 MJ l\(^{-1}\) respectively) and research octane numbers (98 and 105)\(^{47}\). Similar to isobutanol, isopentanol is predicted to be highly miscible in scCO\(_2\)\(^{12}\). For cultures grown under scCO\(_2\), that showed high activity, isobutanol titers were greater than 1 mM, with 42% average yield on consumed αKIV substrate. Importantly, while total titers were reduced at 10 MPa, specific titers under scCO\(_2\) were similar to those observed under 0.1 MPa conditions, at 2.2 × 10\(^{-8}\) µmol cell\(^{-1}\) and 3.1 × 10\(^{-8}\) µmol cell\(^{-1}\), respectively. Recovery of microbially generated isobutanol using scCO\(_2\) was demonstrated, but limited, due to both the configuration of the high-pressure culturing vessels and the low titers achieved. Separation of dilute isobutanol from aqueous media using scCO\(_2\) is possible using a two-phase extractor-fermenter, as previously observed for straight-chain alcohols\(^{12}\). Lastly, process analysis shows that using scCO\(_2\) to extract biofuels is energetically feasible and comparable, if not better, than other in situ extraction techniques if sufficiently high product titers can be achieved. As such, this work serves as a starting point for bioproduction under scCO\(_2\) with numerous avenues for continued optimization.

Further development of the genetic toolbox for SR7 will enable future metabolic engineering of SR7 for enhanced biochemical production. Isobutanol pathway optimization was briefly explored by screening five alcohol dehydrogenases and selecting the variant that resulted in highest substrate conversion (> 80%) and minimal aldehyde accumulation at short incubation times. Additionally, isopentanol and phenylethyl alcohol production were achieved through the simple addition of a decarboxylase, possibly due to upregulation of the leucine and phenylalanine synthesis pathways in SR7. Other strains of *B. megaterium* are known to accumulate the amino acids lysine\(^{38}\) and possibly valine\(^{49}\); however, evidence for leucine and phenylalanine accumulation was not previously observed\(^{49}\). The leucine biosynthesis pathway may become a starting point for future engineering efforts to increase isopentanol titers or make additional bioproducts due to the already presumably high flux of the pathway\(^{30}\).

To ensure that coupled fermentation-extraction technology is utilized to its full potential, initial screening of subsequent compounds to be made by SR7 will be determined based on their preferential partitioning into scCO\(_2\) and toxicity to bioproduction hosts.

Taking advantage of scCO\(_2\) for properties beyond its well-established extractive\(^{12}\) and sterilizing capabilities\(^{13}\) would confer additional utility to this technology. For example, scCO\(_2\) has been used for the depolymerization of lignocellulosic biomass to release fermentable sugars\(^{31}\). Combining the two technologies could provide an inexpensive substrate for SR7 growth and bioproduction. Carboxylation reactions may also be possible in SR7, directly using CO\(_2\) or HCO\(_3^-\) as found in solution for bioproduction\(^{22,23}\). Even with the low solubility of scCO\(_2\) in water\(^{24}\), the approximate HCO\(_3^-\) concentration of 1.1–1.3 mM (based on Henry’s law and the reaction of CO\(_2\)(aq) with water) is advantageous relative to other studies that use air as a CO\(_2\) source for carbon fixation through heterologous overexpression of a carboxylase\(^{55,56}\). SR7 contains several predicted carboxylases, including pyruvate carboxylase, two PEP carboxylases and a biotin-dependent acetyl-CoA carboxylase, each of which could serve as pathways to route HCO\(_3^-\) into central carbon metabolism\(^{25}\). Additionally, the SR7 genome encodes two predicted carbonic anhydrases for converting CO\(_2\)(aq) to HCO\(_3^-\). Lastly, *Bacillus megaterium* strains are known for their ability to secrete proteins\(^{57}\), conferring further biotechnological utility to this host for ex vivo reactions.

One of the limitations to using scCO\(_2\) for bioprocessing is the slow and infrequent growth observed for SR7, especially when bearing an exogenous plasmid. Significant improvements were previously made for scCO\(_2\) culturing using SR7 through media optimization and recognition of endospore germination as a key step for acclimation of this strain to conditions associated with scCO\(_2\) exposure\(^{25}\). Future work will be conducted to better understand the factors limiting growth of SR7 under scCO\(_2\) and to select for strains of SR7 that demonstrate more robust and rapid outgrowth into vegetative cells. Enhanced understanding and substantial improvement of SR7 growth will similarly inform economic, environmental, and energy analysis of the integrated process, due to the inter-relationships between growth, productivity, titer, scCO\(_2\) volumes and extraction efficiency, energy requirements, and ultimately cost. Numerous studies have shown the ability to improve the tolerance of microbes to harsh environments, including through the use of adaptive laboratory evolution\(^{38}\). Many of these studies, however, found that increased tolerance of strains does not necessarily correlate with enhanced specific titers when used for bioproduction\(^{39–41}\). For SR7, the fact that specific titer is comparable under atmospheric and scCO\(_2\) conditions suggests that scCO\(_2\) does not impair specific productivity and that improvements in titer observed under 1 atm CO\(_2\) will positively translate into higher titers under scCO\(_2\). Using the plasmid bearing strains in this work, future selection of SR7 mutants that have a higher growth capacity despite the burden associated with plasmid maintenance and expression may also be possible\(^{62}\). Additionally, genomic integration could alleviate plasmid-maintenance burden for SR7 strains, and recently developed Cas9-based genomic modification strategies for *B. subtilis* strains\(^{63,64}\) provide a starting point for integrating and removing desired and competing pathways, respectively, in SR7.

The isolation, characterization and genetic engineering of an scCO\(_2\)-tolerant strain of *B. megaterium* establishes exciting...
biotechnological opportunities for in vivo high-pressure CO₂ bioprocessing coupled with in situ product extraction. While low-frequency growth from Bacillus spp. spores and enzymatic catalysis have previously been shown under scCO₂, this work demonstrates combined heterologous enzyme expression and bioprocess synthesis under these harsh conditions. Proven scCO₂ stripping of biofuels and favorable energy requirements of scCO₂-based bioprocessing lend credence to the adoption of this process for commercial applications, motivating continued work to improve titers, yields, and productivities. Achieving such improvements would enable demonstration of the integrated extraction-fermentation process at a larger scale, facilitating collection of data needed to form the basis for full technoeconomic and life cycle analyses, and more detailed comparisons to other extraction strategies and renewable energy sources.

Methods

Plasmid construction. For plasmid construction (Supplementary Table 7), restriction enzymes, T4 DNA ligase, and Q5 High-Fidelity PCR MasterMix were purchased from NEB and used according to manufacturer recommendations. The pXyl gfp plasmid is based on the pRBBm34 shuttle vector (purchased from Addgene), which contains a xylose-inducible promoter, the xylose repressor (Ampr) for selection in E. coli, tetracycline resistance marker (Tet) for selection in B. megaterium, ampicillin resistance marker (lacI) under control of its native B. megaterium promoter, and a mChar expression cassette (YqhD). The hyperspank promoter and gfp is amplified from pRBBm34 and ligated into E. coli DH5α, which was subsequently plated on LB agar containing 100 μg ml⁻¹ carbenicillin (Sigma). Plasmids were purified (Zippy plasmid miniprep kit, Zymo research) prior to verification by DNA sequencing (Genewiz). All primers were designed using the software Primer3 (Supplementary Table 8).

The xylose repressor and promoter of pRBBm34 were replaced with a hypospank promoter and lacI using circular polymerase extension cloning (CPEC). The pRBBm34 plasmid was PCR linearized with two sets of primers to remove xyrl and the xylose promoter: pRBBm34 for/for rev and bla for/pRBBm34 rev. The hypospank promoter and lacI were PCR amplified from pDR111 (gift from Alan Grossman, MIT Department of Biology) using primers pDR334pHspf for and lacI-pRBBm34. A standard CPEC protocol was used to assemble the three PCR products into the pHsp vector. Plasmid gfp was PCR amplified from pXyl gfp using primers Sull RBS gfp for and gfp NheI rev. The gfp PCR products were digested with NheI and SalI, then purified using MBI Express and ligated into the oligonucleotide sets RBS-start for/for rev and bla for/pRBBm34 rev. The promoter PCR amplified from Bacillus subtilis strain K5438 using primers pDR334pHspf for and BH44 SaI rev. Two RBS-start for/for rev for pRBBm34 plasmids were created by assembling the three PCR fragments using CPEC.

All the remaining plasmids were made using standard restriction enzyme digestion and ligation construction; the pairs of restriction enzymes used are indicated in the oligonucleotide primer names. The gene encoding β-galactosidase from E. coli was PCR amplified from the plasmid pKS45 lacZae (lab stock) using primers Spel-lacZae for and lacZae-SphI rev. The ketosolverase decarboxylase gene from L. lactis was amplified from pCOLA (Fjoh-2967 (kindly) with primers Spel-lkvdD for and kvdD-BamHI SplI rev for the construction of pXyl kvdD). The ADH6 gene from Saccharomyces cerevisiae was amplified from pACYC (car SphI) (ADH6Sph, kindly) with primers Sull RBS ADH6 for and ADH6-BamHI ADH6 rev. For all the alcohol dehydrogenases studied, the RBS 5'–aggggaaa-3' was used. The adhA4 gene was amplified from the L. lactis subsp. lactis (ATCC 19435D) genome (primers: BamHI-adhA4 for and adhA4-SphI rev), adhDc, and YphDc from the E. coli MG1655 genome (primers: BamHI-adhDc for and adhDc-SphI rev, BamHI-yphDc for and yphDc-SphI rev, respectively), and L. lactis adhAae, and adhAae-SphI rev from the B. megaterium SR7 genome (primers: BamHI-adhAae for and adhAae-SphI rev). All alcohol dehydrogenases were ligated between the BamHI and SphI sites in pXyl kvdD.

Transformation of SR7 and verification. Protoplast transformation of SR7 was adapted from an established method by Biedendiek et al.28. All buffers described in this protocol were identical to those in Biedendiek et al.28 and were made with chemicals from Millipore-Sigma unless otherwise described. An overnight culture of SR7 was grown from the inoculation of 50 ml of LB medium (BD Difco) with a single SR7 colony, and was incubated at 37°C and 250 rpm. SR7 was subcultured at a dilution of 1:100 in fresh LB medium and grown at 37°C and 250 rpm until an OD₆₀₀ reading of 1.0 was reached (~3 h) (Implen NanoPhotometer). Cells were collected by centrifugation at 4000 × g for 10 min and the cell pellet was retained. SR7 was resuspended in 1 ml 1x SMMP and 1 ml 1x SMMP buffer (Alfa Aesar), 1.6 g l⁻¹ NaOH, 4.06 g l⁻¹ MgCl₂–6H₂O, 171.16 g l⁻¹ sucrose, 17.5 g l⁻¹ antibiotic medium number 3 (AB3) (BD Difco). Lysozyme (Sigma) was added to a concentration of 30 μg ml⁻¹, which was tenfold lower than used in previous methods29. Protoplast formation was performed at 37°C for 10 min at 70 r.p.m. and visualized by microscopy.

Protoplasts were collected by centrifugation at 1300 r.p.m. for 10 min, followed by a wash with 5 ml 1x SMMP. Protoplasts were resuspended gently in 5 ml 1x SMMP and were aliquoted into 0.5 ml fractions. Each sample was transformed with M13K07 (NEB)-labeled plasmid DNA and subjected to a CPEC reaction. The PCR primers were designed for a 1:1 ratio of GC-grade ethyl acetate (Sigma), vortexing vigorously for 5 min, centrifuging at 21000 × g for 5 min, and recovering the ethyl acetate fraction.
Plasmid maintenance. For plasmid maintenance studies, a 25 ml culture of SR7 pXyl gfp was grown in LB medium with 5 μg ml⁻¹ tetracycline in a 250 ml baffled flask. Cultures were grown with 250 ml of prewarmed culture and were subsequently incubated at 37 °C and 250 rpm. Protein induction occurred at 3 h post subculture using 5 g l⁻¹ xylose. For each time point, an OD₅₀₀ reading was recorded, and cells were diluted to a uniform OD₅₀₀ in a black-lined 96-well plate (Costar) using LB medium prior to reading GFP fluorescence on a plate reader (Tecan infinite 200 pro, ex: 485/20nm, em: 535/25 nm). To confirm plasmid maintenance at 74 h post induction, dilutions of cells were made in 1x M9 salts and were plated on LB agar with and without 5 μg ml⁻¹ tetracycline to determine colony forming units. Colony PCR was performed using primers RepU seq for and RepU rev to amplify the RepU fragment, and primers pXyl seq for and pXyl rev to amplify gfp (Supplementary Table 8). A negative control PCR reaction did not contain DNA template and a positive control used 1 ng μl⁻¹ purified pXyl gfp.

FACS and microscopy. Samples for fluorescence activated cell sorting (FACS) were collected 4 h post induction for cultures (or 7 h total culturing time for p43-β-galactosidase production experiments) for cell pellets and for microscopy (for cultures grown aerobically). Cells were visualized at 1000x by diluting 5 μl of culture into 200 μl 1x M9 salts in a 96-well plate. FACS measurements were performed on a FACS Canto (BD) equipped with a 505 nm long pass mirror and an X-Cite Series 200 fluorescence filter set. For SR7 and SR7 with pXyl grown aerobically in LB medium were used to establish forward and side scatter gate to select events corresponding to the morphology of SR7. Fluorescence data was collected for 10,000 cells for each sample. FACS data was recorded using FlowJo software and a GFP positive gate was established for fluorescence values above all the negative control samples where SR7 contained an empty plasmid.

Samples for microscopy of SR7 expressing gfp were also collected 4 h post induction for cultures grown aerobically in LB medium. Each sample was prepared as a wet-mount on a slide below a coverslip that had been prearranged with poly-L-lysine (Sigma) to enable cells to adhere. Cells were visualized at 1000x magnification using an epifluorescence microscope (Zeiss Axioskop 2). Cell morphology was observed using phase-contrast microscopy and GFP fluorescence was visualized using a 480/30 nm excitation and 535/40 emission FITC filter set with an S30x long pass mirror and an X-Cite Series 200 fluorescence source. Images were captured on a Nikon D100 camera using the NKRremote live-imaging software.

SR7 endoreplication preparation. Overnight cultures of SR7 grown in LB medium aerobically were diluted 1:50 in modified G medium (2 g l⁻¹ yeast extract, 2 g l⁻¹ (NH₄)₂SO₄, 0.025 g l⁻¹ CaCl₂2H₂O, 0.5 g l⁻¹ KH₂PO₄, 0.2 g l⁻¹ MgSO₄7H₂O, 0.05 g l⁻¹ MnSO₄·H₂O, 0.005 g l⁻¹ ZnSO₄·7H₂O, 0.0005 g l⁻¹ FeSO₄·7H₂O, pH 7.1). For SR7 cells that contained a plasmid, both the vegetative cell growth medium and sporulation medium were supplemented with 5 μg ml⁻¹ tetracycline. Sporulation was induced by incubating cultures in modified G medium for 72 h at 37 °C. Endospores were collected by centrifugation at 4000 x g and 4 °C for 15 min, and stored at −80 °C. For SR7 cultures with Δp43, Sporulation was done manually by differential centrifugation of 200 μl of 4 mg ml⁻¹ freshly prepared, β-galactosidase substrate o-nitrophenyl-β-D-galactoside (ONPG) (Sigma). Absorbance at 420 nm was collected every 15 s for 10 min using a Beckman DU600 spectrophotometer. The volume of lysed culture was adjusted to ensure the concentration of ONPG was not limiting during the assay. The initial rate of absorbance change was calculated for each sample and corrected to 1 mg ml⁻¹ using 2130 ± 1 cm⁻¹ as the extinction coefficient for ONPG. Rates were normalized by total protein added to each reaction to generate a specific activity per unit total protein for each sample.

Compound quantification. Glucose, xylose, aKIV, and fermentation products (acetate, lactate, formate) were detected using an HPLC (Agilent 1200) with an Aminex HPX-87H anion exchange column (Bio-Rad). Cell-free supernatants were loaded onto the column with an inlet and outlet temperature of 30 °C, a flow rate of 0.6 ml min⁻¹ under isocratic conditions with 5 mM sulfuric acid. Compound concentrations were determined by integrating the refractive index detection chromatogram and comparison to a standard curve generated with known concentrations of each commercially available standard (Sigma). Purified standards were used to determine retention times: glucose—9 min, lactate—10.2 min, and acetate—15.6 min. Alcohols and isobutyraldehyde were detected using a gas chromatography system (Agilent 7890B GC) equipped with a VF-wax column (Agilent 30 m × 0.25 mm × 0.50 μm), flame-ionization detector (FID) and mass spectrometer (MS). All compounds were extracted into ethyl acetate from cell-free supernatants prior to analysis. The temperature program for the GC oven was 80 °C hold for 3 min, 5 °C min⁻¹ ramp to 210 °C, and 210 °C hold for 3 min. Compound concentrations were determined by integrating the FID chromatogram and comparison to a standard curve generated with known concentrations of each commercially available standard (Sigma). Purified standards were used to determine retention times: glucose—9 min, lactate—10.2 min, and acetate—15.6 min. Alcohol and isobutyraldehyde were detected using a gas chromatography system (Agilent 7890B GC) equipped with a VF-wax column (Agilent 30 m × 0.25 mm × 0.50 μm), flame-ionization detector (FID) and mass spectrometer (MS). Alcohols and isobutyraldehyde were detected using a gas chromatography system (Agilent 7890B GC) equipped with a VF-wax column (Agilent 30 m × 0.25 mm × 0.50 μm), flame-ionization detector (FID) and mass spectrometer (MS). All compounds were extracted into ethyl acetate from cell-free supernatants prior to analysis. The temperature program for the GC oven was 80 °C hold for 3 min, 5 °C min⁻¹ ramp to 210 °C, and 210 °C hold for 3 min. Compound concentrations were determined by integrating the FID chromatogram and comparison to a standard curve generated with known concentrations of each commercially available standard (Sigma). Purified standards were used to determine retention times: glucose—9 min, lactate—10.2 min, and acetate—15.6 min. Alcohols and isobutyraldehyde were detected using a gas chromatography system (Agilent 7890B GC) equipped with a VF-wax column (Agilent 30 m × 0.25 mm × 0.50 μm), flame-ionization detector (FID) and mass spectrometer (MS). All compounds were extracted into ethyl acetate from cell-free supernatants prior to analysis.
Isobutanol was also extracted from a custom built extractor-fermenter using scCO2. A 1% v/v isobutanol (Sigma) solution was prepared in deionized water and extraction was carried out at scCO2 flow rates of 1.3, 3.2, 5.4, and 9.0 ml min⁻¹. The scCO2 was de-pressurized through chilled methanol to collect the isobutanol, which was quantified by GC-FID. Recovery rate was determined by fitting the natural log of the fraction recovered as a function of time.

**scCO2 fermentation extraction process model.** Aspen Plus (AspenTech) was used to simulate the isobutanol extraction process (full process: Supplementary Fig. 15a, simplified process schematic: Fig. 5a). Preliminary calculations and comparison with published data suggested use of the Soave-Redlich-Kwong equation of state to model phase equilibrium in all cases except for scCO2 extraction of isobutanol. For which our own measurements on isobutanol were used in concert with literature data on n-butanol separation performance. Thermodynamic properties were modeled using the Lee-Kessler-Plocker equation of state, as recommended by Aspen for supercritical fluids. The process feeds were CO2 at 0.1 MPa and a stream of aqueous glucose (media and substrate). The water feed (P1) was pumped to 10 MPa in a single stage. Consistent with typical engineering practice, CO2 pressurization to 10 MPa was completed in four stages (Cl-4), with each stage accomplishing a threefold pressure increase. In all cases, the thermodynamic efficiency of the pumps/compressors was 85% and their mechanical efficiency was 35%. Heat exchangers (HE1-3) were used to maintain CO2 temperature at 40 °C after compression and for feeding the fermenter. The fermenter was simulated as consisting of a continuously fed stirred tank reactor and a separator (V2) configured in series. The reactor was modeled as achieving a steady state isobutanol concentration of 20 g l⁻¹, consistent with reported titers of isobutanol. The entire contents of the reactor were fed to the separator (V2), a countercurrent extractor column, which separated the feed into water-rich and CO2-rich streams. The isobutanol composition of the exit streams were set to that reported by Laitinen and Kaunisto and modified using our performance data for isobutanol (i.e., 99.73% butanol recovery) and the exiting water and CO2 streams. The isobutanol-rich, water-rich, and CO2-rich streams, as suggested by de Filippi and Moses. Pressures tested in the three-phase separator ranged from 0.1 to 6.5 MPa, the pressure at which an isobutanol-rich phase first appears. The CO2-rich stream exiting the three-phase separator was either recycled or purged, with the ratio between these streams set by the splitter (V5). The CO2 recycle ratio was defined by the relative mass flow rates of the CO2 streams exiting the splitter: e.g., 50% CO2 recycle implies that 50% of the CO2 entering the splitter is recycled and 50% is off gassed. The isobutanol-rich phase was further de-pressurized to 0.1 MPa (V4), if necessary, to recover high-purity isobutanol liquid. Finally, the water exiting the three-phase separator was purged, though it could be recycled in an industrial process.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this Article.

**Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request. A Reporting Summary for this Article is available as a Supplementary Information file. The source data underlying Figs 2, 3, 4, and 5 and Supplementary Tables S1–S15, Supplementary Tables 1–6 are provided as a Source Data file.

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