Investigation of relationship between 2,3-butanediol toxicity and production during growth of Paenibacillus polymyxa

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\textbf{A B S T R A C T}

Understanding the capacity of \textit{Paenibacillus polymyxa} DSM 365 to tolerate increasing concentrations of 2,3-butanediol (2,3-BD) is critical to engineering a 2,3-BD-overproducing strain. Hence, we investigated the response of \textit{P. polymyxa} to high 2,3-BD concentrations. In fed-batch cultures (6-L bioreactor) 2,3-BD was accumulated to a maximum concentration of 47 g/L despite the presence of residual 13 g/L glucose in the medium. Comitantly, accumulation of acetoin, the precursor of 2,3-BD increased after maximum 2,3-BD concentration was reached, suggesting that 2,3-BD was reconverted to acetoin after the concentration tolerance threshold of 2,3-BD was exceeded. Cultures of \textit{P. polymyxa} were then challenged with levo-2,3-BD (20, 40 and 60 g/L) at 0 h in a glucose medium, and a concentration dependent growth inhibition response to levo-2,3-BD was observed. The growth of \textit{P. polymyxa} was completely inhibited by 60 g/L levo-2,3-BD. Furthermore, \textit{P. polymyxa} was challenged with incremental 2,3-BD concentrations (20, 40 and 60 g/L at 12, 24 and 36 h, respectively) to mimic 2,3-BD accumulation during fermentation. Interestingly, 2,3-BD was reconverted to acetoin when its concentration reached 60 g/L, possibly to alleviate 2,3-BD toxicity. Collectively, our findings indicate that 2,3-BD-mediated toxicity is a major metabolic impediment to 2,3-BD overproduction, thus, making it an important metabolic engineering target towards rational design of a 2,3-BD-overproducing strain.

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\textbf{I n t r o d u c t i o n}

The instability of petroleum price coupled with the finite nature of crude oil has reignited interest in bio-products as renewable replacements for their petroleum-derived counterparts. Among such products, biologically derived 2,3-butanediol (2,3-BD) is currently receiving tremendous attention because of its multifaceted industrial applications [1]. For instance, 2,3-BD is a potential crucial feedstock chemical in the production of 1,3-butadiene (1,3-BD), the monomer of synthetic rubber, currently produced by cracking of petroleum [2]. In addition, 2,3-BD is used as an anti-freeze due to its extremely low freezing point of \textdegree{}60 \textdegree{}C [3]. Further, methyl ethyl ketone an important fuel additive is a derivative of 2,3-BD [4], which also serves as a feed-stock chemical for diacetyl production, an important flavor enhancer in the food industry [5]. Different bacterial species including multiple \textit{Klebsiella} species, \textit{Paenibacillus polymyxa}, \textit{Bacillus licheniformis}, and \textit{Bacillus amyloliquefaciens}, have been shown to produce 2,3-BD from sugars [1]. \textit{P. polymyxa} was chosen for this study because of its non-pathogenicity. Most 2,3-BD producers, particularly \textit{Klebsiella} species, are pathogenic, which makes them unattractive for industrial application. In addition, \textit{P. polymyxa} produces up to 98\% levoratory 2,3-BD (levo-2,3-BD), the 2,3-BD isomer better suited to industrial applications due to its chiral nature, which makes it more amenable to a wide range of desirable chemical reactions [6–10]. For example, levo-2,3-BD is easily dehydrated to form 1,3-butadiene (1,3-BD).

Apparently, yields and titers of 2,3-BD during bacterial fermentation remain low, thereby, impeding efforts at commercializing fermentative 2,3-BD production. Co-production of multiple interfering products such as ethanol, formate, lactate, acetate, acetoin, and exopolysaccharides (EPS) account in part for the low 2,3-BD yield during fermentation because carbons are diverted away from the 2,3-BD biosynthesis pathway [7,11,12,13]. Levan is an EPS produced by \textit{P. polymyxa} during 2, 3-BD fermentation. Levan is comprised of fructans linked predominantly by $\beta$ (2-6) and $\beta$ (2-1) glycosidic bonds; and its production during fermentation turns the fermentation medium into a sticky,
colloidal solution that makes product recovery difficult [14,15]. Additionally, the co-products, formate, lactate, and ethanol drastically affect cell growth by disrupting intracellular pH and denaturing enzymes and membranes when produced in significant amounts during fermentation [16–19]. These co-products also interfere with downstream processing of 2,3-BD, thereby impacting the cost of 2,3-BD purification and ultimately, overall production cost. Consequently, efforts at improving fermentative 2,3-BD production have focused considerably on media manipulations/optimization, and strain improvement targeted at reducing the production of competing products [8,20]. Although some progress has been made with different groups reporting 2,3-BD titers between 15 and 80 g/L in batch cultures and 19.5 to 111 g/L in fed-batch cultures [8,11,12], commercialization of 2,3-BD fermentation remains to be actualized. Increasing 2,3-BD concentration during fermentation is a critical prerequisite for commercialization. To this end, we rationalized that in addition to the effects of interfering co-products, other factors may contribute to the inability of 2,3-BD-producing microorganisms to accumulate 2,3-BD titers above a certain concentration threshold.

We rationalized that a likely reason for this may be 2,3-BD-mediated toxicity to the fermenting microorganisms. Hence, in this study, we investigated the tolerance of P. polymyxa DSM 365 to increasing 2,3-BD concentrations added at 0 h and pulse-fed at 12, 24 and 36 h of fermentation. Our results suggest that 2,3-BD-mediated toxicity likely poses a roadblock to the accumulation of higher 2,3-BD concentrations during fermentation. Therefore, we infer that understanding the mechanism of action of this roadblock might aid efforts targeted at engineering 2,3-BD-overproducing strains.

**Fed-batch fermentations**

Fed-batch fermentations were conducted in a 6-L Bioflo 3000 Bioreactor (New Brunswick Scientific, Edison, NJ). The fermenter was equipped with sensors for measuring pH, agitation speed, and temperature. Mixing was achieved by means of 2 Rushton impellers (3-plate). Sterile air was sparged into the medium through a 0.2 μm PTFE Acro® 50 sterile filter ( Pall Corporation, Ann Arbor, MI) using a Masterflex L/S® Pump (Cole-Parmer Instrument Company, Vernon Hills, IL) connected to the top of the bioreactor at a flow rate of 150 mL/min. The Production medium contained (g/L): 100 glucose, 5.0 YE, 5.0 tryptone, 3.0 (NH₄)₂SO₄, 3.5 KH₂PO₄, 2.75 K₂HPO₄, 0.2 MgSO₄, 1.5 NH₄ acetate, 0.05 CoCl₂, 10.0 3-(N-morpholino) propanesulfonic acid (MOPS), and 6 mL of the trace element solution. All medium components were separately and filter-sterilized using a 500 mL polystyrene non-pyrogenic sterile filter bottle (0.2 μm; Corning Incorporated, NY), with the exception of glucose, YE and tryptone that were separately autoclaved at 121 °C for 15 min. After cooling, the medium components were constituted under aseptic condition. The fed-batch fermentations were initiated with a starting working volume of 2 L (inoculated with 10% v/v seed culture) at an initial pH of 6.5 ± 0.1 that was externally controlled with 12.5% NH₄OH or 6.5% H₃PO₄ when pH dropped below 6.0 ± 0.1 or increased above 6.5 ± 0.1. The fermentation medium was stirred at 300 rpm and the bioreactor was fed when broth glucose concentration dropped below 20 g/L. Each feeding was accompanied by addition of half strength of all the original medium components.

**Materials and methods**

**Microorganisms and culture conditions**

*Paeonibacillus polymyxa* DSM 365 used in this study was procured from the German Collection of Microorganisms and Cell Culture, Braunschweig, Germany (DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen). The lyophilized stock was reactivated by inoculating into Luria Bertani (LB) broth, grown overnight (12 h), and then stored as glycerol stock (50% sterile glycerol) at −80 °C. Inoculums for fermentation were prepared by inoculating 1 mL of *P. polymyxa* stocks into 30 mL of pre-culture medium containing (g/L): 20.0 glucose, 5.0 yeast extract (YE; Sigma-Aldrich, St Louis, MO), 5.0 tryptone (Sigma-Aldrich, St Louis, MO), and 3.0 (NH₄)₂SO₄. The pre-culture was supplemented with 0.09 mL of phosphate buffer (pH 6.5) and 0.09 mL trace element solution. The phosphate buffer (pH 6.5) contained (g/L): 3.5 KH₂PO₄, 2.75 K₂HPO₄, 0.2 MgSO₄, while the trace element solution was prepared by dissolving 0.4 g/L FeSO₄ into 3 mL 25% HCl, followed by addition of 500 mL double-distilled H₂O and then addition of (g/L): 0.8H₃BO₃, 0.04 CuSO₄·5H₂O, 0.04 NaMoO₄·2H₂O, 5.0 MnCl₂·4H₂O, 0.1 ZnSO₄·7H₂O, 0.08 Co(NO₃)₂·6H₂O, 1.0 CaCl₂, 2H₂O, 0.01 biotin. When the optical density (OD₆₀₀nm) of the pre-culture reached 1.0–1.2, 10 mL of actively growing cells were transferred into 90 mL of sterile pre-culture medium and incubated aerobically for another 2–3 h until OD₆₀₀nm reached 1.0–1.2, after which it was transferred to the production medium. Glucose, yeast extract and tryptone were prepared and sterilized separately at 121 °C for 30 min. Phosphate buffer and trace element solution were prepared separately and filter-sterilized using 0.22 μm PES filter (Corning Incorporated, Corning, NY). All media components were constituted after sterilization.

**Fig. 1.** Fed-batch 2,3-BD fermentation by *P. polymyxa*. Concentrations of fermentation products (total 2,3-BD, acetoin, ethanol, exopolysaccharide: EPS; A) and glucose utilization profile (B) during fed-batch fermentation by *P. polymyxa*. Error bars show standard deviations of means (n = 3).
The tolerance of _P. polymyxa_ to levo-2,3-BD was tested in batch cultures in sterile 125 mL Pyrex bottles containing 30 mL of the production medium. Since levo-2,3-BD is by far the predominant isomer produced by _P. polymyxa_, 2,3-BD toxicity bioassay was first conducted with this isomer. The production medium contained 80 g/L glucose, while the other medium constituents were same as in the medium described above for the fed-batch process. Two separate sets of experiments were conducted to test the tolerance of _P. polymyxa_ to levo-2,3-BD. First, levo-2,3-BD was added to the medium at 0 h. In this set of experiments, levo-2,3-BD was added to triplicate cultures to final concentrations of 0, 20, 40 and 60 g/L levo-2,3-BD, respectively. The medium with 0 g/L levo-2,3-BD served as control. Second, levo-2,3-BD was pulse-fed into the fermentation medium in a step-wise manner aimed at mimicking the pattern of 2,3-BD accumulation by _P. polymyxa_ during fed-batch fermentation. Levo-2,3-BD pulse-feeding was commenced at 12 h, when growth had reached sufficient cell density to withstand possible levo-2,3-BD-mediated toxicity. Levo-2,3-BD was pulse-fed at 12, 24 and 36 h to make up 2,3-BD concentrations in the cultures to 20, 40 and 60 g/L, respectively. Twenty seven milliliters of the production medium was inoculated with 3 mL of pre-culture (10% inoculum; OD₆₀₀nm of 1.0–1.2). All experiments were started at pH 6.5 and incubated in an Innova™ 4000 rotary shaker (New Brunswick Scientific, Edison, NJ) agitated at 200 rpm, and temperature was maintained at 37 °C. To test whether both levo- and meso-2,3-BD are equally toxic to _P. polymyxa_, cultures of _P. polymyxa_ were supplemented in triplicate with 20, 40 and 60 g/L of either levo- or meso-2,3-BD at 0 h and cell growth was measured by monitoring cell density as described below (analytical methods).

**Batch fermentation, and levo- and meso-2,3-BD toxicity bioassay**

**Analytical methods**

Cell growth was determined by measuring optical density (OD₆₀₀nm) in a DU™ Spectrophotometer (Beckman Coulter Inc., Brea, CA). Changes in pH were measured using an Acumen® Basic pH meter (Fisher Scientific, Pittsburgh, PA). The concentrations of 2,3-BD (Levo- and meso-), acetoin, ethanol, and acetic acid were determined using a 7890A Agilent gas chromatograph (Agilent Technologies Inc., Wilmington, DE, USA) equipped with a flame ionization detector (FID) and a J × W 19091 N-213 capillary column [30 m (length) × 320 μm (internal diameter) × 0.5 μm (HP-Innowax film thickness)]. The carrier gas was nitrogen, and the inlet and detector temperatures were maintained at 250 and 300 °C, respectively. The oven temperature was programmed to span from 60 to 200 °C with 20 °C min⁻¹ increments, and a 5-min hold at 200 °C. Samples (1 μL) were injected with a split ratio of 10:1.

Glucose concentrations were determined by HPLC using a Waters 2796 Bioseparations Module equipped with an Evaporative Light Scattering Detector (ELSD; Waters, Milford, MA) and a 9 μm Aminex HPX-87P column; 300 mm (length) × 7.8 mm (internal diameter) connected in series to a 4.6 mm (internal diameter) × 3 cm (length) Aminex deashing guard column (Bio-Rad, Hercules, CA). The column temperature was maintained at 65 °C. The mobile phase was HPLC-grade water maintained at a flow rate of 0.6 mL/ min. The ES was produced during fermentation was quantified as previously described [21]. Culture broth was centrifuged at 8000 × g for 10 min to pellet the cells while ES remained in the supernatant. EPS was precipitated with 95% cold ethanol (4 °C), 3 × the volume of the supernatant. The supernatant-ethanol mixture was kept overnight at 4 °C, followed by centrifugation at 8000 × g for 10 min. The EPS pellet was dried in the oven at 60 °C and

![Fig. 2. Tolerance of _P. polymyxa_ to levo-2,3-BD challenge at 0 h: A: Optical density; B: Total 2,3-BD concentrations with different treatments; C: Acetoin profile; D: Glucose profile. Cultures were challenged with 0, 20, 40, and 60 g/L levo-2,3-BD at 0 h. Error bars show standard deviations of means (n = 3).](image-url)
weighed afterwards on a Mettler AE 166 weighing balance (Mettler, Toledo, OH).

Statistical analysis and calculations

Analysis of variance (ANOVA) using Tukey’s method for pairwise comparisons between treatments was conducted using Minitab 16 [22]. Maximum product concentrations, yields and productivities were analyzed at 95% confidence interval.

Results

Production of 2,3-BD by P. polymyxa in fed-batch cultures

To determine the 2,3-BD production capacity of P. polymyxa, fed-batch fermentations (three separate experiments) were conducted. Fresh glucose medium was fed into the bioreactor when concentrations fell below 20 g/L (Fig. 1B). The sum of levorotary and mesorotary 2,3-BD is presented as total 2,3-BD. Approximately 47 g/L total 2,3-BD was produced by P. polymyxa in fed-batch fermentations (Fig. 1A). The concentration of 2,3-BD increased with time until 72 h when concentration began to decrease, with concomitant increase in acetoin concentration (Fig. [1A]). Prior to 72 h, acetoin concentrations remained less than 6 g/L and increased to 10.7 g/L afterwards (Fig. 1A). Similar to 2,3-BD, ethanol concentration increased steadily until 72 h when concentration plateaued. Residual glucose concentration in the range of 10–16 g/L remained in the fermentation broth at the end of fermentation (Fig. 1B). Extra glucose medium was fed into the bioreactor at 24, 42, and 60 h, thereby increasing glucose concentrations to 53, 45, and 35 g/L, respectively (Fig. 1B). The rate of 2,3-BD production reduced markedly after 60 h before a drop in concentration was observed at 72 h (Fig. 1A). A total of 190 g/L of glucose was consumed by P. polymyxa during fed-batch fermentation. EPS concentration increased with time and plateaued after 72 h fermentation (Fig. 1A). However, it appears that a fraction of the produced EPS was utilized during the course of the fermentation given the oscillatory trend of the EPS concentration in the bioreactor (Fig. 1A).

Tolerance of P. polymyxa to levo-2,3-BD during 2,3-BD fermentation

To evaluate the tolerance of P. polymyxa to levo-2, 3-BD (the major 2,3-BD isomer produced by this microorganism) during fermentation, different concentrations of levo-2,3-BD (0, 20, 40, and 60 g/L) were either added to the fermentation medium at 0 h or pulse-fed into the culture at 12, 24 and 36 h. The concentrations of choice and time points of levo-2,3-BD addition were informed by the patterns of 2,3-BD accumulation observed in the fed-batch experiment (Fig. 1A). Levo-2,3-BD affected the growth of P. polymyxa in a concentration dependent manner. Whereas 20 g/L 2,3-BD added at 0 h had no effect on the final cell density in cultures of P. polymyxa relative to the control (0 g/L 2,3-BD), a 46% reduction in exponential growth rate (at 12 h) was observed with an extended lag phase in cultures supplemented with 20 g/L 2,3-BD (Fig. 2A). With 40 and 60 g/L levo-2,3-BD, cell growth reduced 32% (at 36 h) and 100%, respectively, relative to the control (Fig. 2A). The rate of 2,3-BD production was approximately 1.2- and 1.9-fold faster in the control than in cultures supplemented with 20 and 40 g/L levo-2,3-BD, respectively, where extended concentration-dependent lags in 2,3-BD production were observed (Fig. 2B). Whereas maximum total 2,3-BD concentrations (this refers to 2,3-BD accumulated by the growing cells, excluding supplemented 2,3-BD) were produced in the cultures treated with 0 and 20 g/L levo-2,3-BD were approximately 23 and 19 g/L, the cultures supplemented with 40 g/L levo-2,3-BD produced only
58% of the total 2,3-BD (13.34 g/L) produced by the control with no levo-2,3-BD supplementation (Fig. 2B). The cultures challenged with 60 g/L levo-2,3-BD at 0 h did not produce 2,3-BD. With the exception of cultures challenged with 60 g/L levo-2,3-BD, which exhibited no growth, hence no metabolic activity, acetoin concentrations showed an irregular pattern, increasing and decreasing at different time points (Fig. 2C). Interestingly, initial rise and drop in acetoin concentrations (12 h) occurred only in cultures challenged with 20 g/L levo-2,3-BD (6 g/L), whereas acetoin increased continuously in 40 g/L levo-2,3-BD-challenged cultures (Fig. 2C). Acetoin concentrations increased sharply towards the end of the fermentation, beginning at 48 h for the controls and the cultures challenged with 20 g/L levo-2,3-BD, and at 12 h for the 40 g/L levo-2,3-BD-challenged cultures (Fig. 2C). Although residual glucose concentrations in the 0 g/L- and 20 g/L levo-2,3-BD-challenged cultures were the same, the initial rate of glucose utilization was 3.9-fold faster in the control fermentations [12 h of fermentation; (Fig. 2D)]. Residual glucose concentration in the cultures supplemented with 40 g/L levo-2,3-BD at 0 h was 9.5- and 7.6-fold higher than those in cultures supplemented with 0 g/L and 20 g/L, respectively. Despite low glucose consumption, 2,3-BD yield was considerably low. This is likely as a result of diversion of carbon to the EPS and ethanol biosynthetic pathways (Table 1).

In an attempt to mimic the pattern of 2,3-BD production by P. polymyxa, we pulse-fed levo-2,3-BD into the fermentation broth at different time points. Pulse-feeding commenced at 12 h (late exponential phase) when the cultures had grown significantly (Fig. 3A). Pulse-feeding of levo-2,3-BD exerted lesser effect on cell growth and 2,3-BD production by P. polymyxa relative to cultures challenged with levo-2,3-BD at 0 h. Following the initial pulse-feeding (12 h), cell density of P. polymyxa cultures decreased (Fig. 3A). Whereas 2,3-BD concentration decreased in P. polymyxa cultures challenged with levo-2,3-BD (pulse-feeding from 12 to 36 h and 0 h supplementation), this effect was more pronounced in cultures challenged with levo-2,3-BD at 0 h. For instance, cultures of P. polymyxa pulse-fed between 12 and 36 h to bring the total concentration of levo-2,3BD to 20 g/L (12 h), 40 g/L (24 h), and 60 g/L (36 h) exhibited a 19% decrease in total 2,3-BD concentration (relative to the controls, Fig. 3B), while cultures challenged with levo-2,3-BD (20 and 40 g/L) at 0 h showed decrease (16 and 42%, respectively) in total 2,3-BD concentration (Fig. 2B). Further, cultures challenged with 60 g/L levo-2,3-BD at 0 h were completely inhibited while the same concentration at 36 h under pulse-feeding condition only decreased cell growth, glucose utilization, and 2,3-BD production by 16.5 and 19%, respectively, relative to the controls. Concomitantly, acetoin concentration increased approximately 2-fold in the challenged cultures relative to the controls after maximum total 2,3-BD concentration was attained in the challenged culture through 2,3-BD supplementation and production (Fig. 3C). The glucose profiles in both the challenged and unchallenged cultures were similar pre-challenge, however, upon initial levo-2,3-BD pulse-feeding, the rate of glucose utilization decreased 1.1-fold in the pulse-fed cultures when compared to the controls (Fig. 3C). Similarly, residual glucose concentration in the challenged cultures was 1.6-fold higher than those in the control fermentations.

**Levo-2,3-BD supplementation alters the ratio of levo- to meso-2,3-BD produced by P. polymyxa**

Addition of levo-2,3-BD to the cultures of P. polymyxa, regardless of whether the addition is at 0 h or by pulse-feeding alters the ratio of levo- to meso-2,3-BD produced by this microorganism during fermentation, and this effect appears to be concentration-
dependent. While <20 g/L levo-2,3-BD added at 0 h did not alter production of the levo-2,3-BD isomer by *P. polymyxa*, resulting in a ratio of levo-2,3-BD to meso-2,3-BD of 23:0 (levo-2,3-BD, 23.00 g/L; meso-2,3-BD, 0 g/L), the ratios of produced 2,3-BD in the 20 g/L levo-2,3-BD-supplemented culture at 0 h decreased from approximately 25:1 (levo-2,3-BD, 14.47 g/L; meso-2,3-BD, 0.58 g/L) to 11:1 (levo-2,3-BD, 9.72 g/L; meso-2,3-BD, 0.91 g/L) from 60 to 72 h of fermentation. Further, the ratio of levo- to meso-2,3-BD decreased from approximately 12:1 (levo-2,3-BD, 11.72 g/L; meso-2,3-BD, 0.94 g/L) to 4:1 (levo-2,3-BD, 8.28 g/L; meso-2,3-BD, 2.30 g/L) when the concentration of supplemented levo-2,3-BD at 0 h increased to 40 g/L (Fig. 4A). For the pulse-feeding experiments, the control cultures (0 g/L levo-2,3-BD supplementation) produced 24.27 g/L levo-2,3-BD and 0 g/L meso-2,3-BD, resulting in a levo-2,3-BD to meso-2,3-BD ratio of 24:0 (Fig. 4B). However, when cultures were pulse-fed with levo-2,3-BD leading to a final 2,3-BD concentration of 60 g/L, the concentrations of 2,3-BD isomers produced at the end of the fermentation were 15.63 g/L levo-2,3-BD and 2.59 g/L meso-2,3-BD, resulting in levo-2,3-BD to meso-2,3-BD ratio of approximately 6:1 (Fig. 4B).

Increased production of meso-2,3-BD by *P. polymyxa* following levo-2,3-BD supplementation led us to ask whether the change in the levo-2,3-BD to meso-2,3-BD ratio produced by this microorganism is as a result of reduced toxicity of the meso-2,3-BD isomer relative to levo-2,3-BD. Reduced toxicity of the meso isomer relative to the levo isomer would explain increased accumulation of the meso isomer following addition of levo-2,3-BD to fermentation cultures, as a means of alleviating stresses stemming from levo-2,3-mediated feedback inhibition. Thus, we evaluated the effects of different concentrations (0, 20, 40, and 60 g/L) of the two isomers (meso- and levo-2,3-BD) on the growth of *P. polymyxa* using cultures with no 2,3-BD supplementation (0 g/L 2,3-BD) as controls. When the fermentation medium was supplemented with 20 g/L meso- and levo-2,3-BD, the meso-2,3-BD treated cultures exhibited the same maximum optical density as the control cultures, while the maximum growth attained by the levo-2,3-BD-treated cultures showed a slight reduction in optical density (2.3%) relative to the control and the meso-2,3-BD treated cultures (Fig. 5A). At 40 g/L, the maximum growth achieved by the levo-2,3-BD treated cultures was 24% lesser than that reached by the meso-2,3-BD treated cultures (Fig. 5A). More strikingly, while 60 g/L levo-2,3-BD completely inhibited the growth of *P. polymyxa*, cultures supplemented with 60 g/L meso-2,3-BD grew after an extended lag phase (Fig. 5C).

**Discussion**

Final product concentrations achieved during fermentation significantly influence the economics of large-scale

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**Fig. 4.** The concentrations of levo- and meso-2,3-BD in cultures of *P. polymyxa* following levo-2,3-BD challenge (0 h-addition and pulse-feeding): A: 0 h addition of levo-2,3-BD; B: Pulse-feeding of levo-2,3-BD. Broken lines represent meso-2,3-BD with different scales (secondary axis). Since meso-2,3-BD was absent in most cultures, the values (0 g/L) are merged with the x-axis, hence, not visible. Error bars show standard deviations of means (n = 3).

**Fig. 5.** Relative inhibitory effects of levo- and meso-2,3-BD on the growth of *P. polymyxa*. A: The growth profiles of *P. polymyxa* challenged with 20 g/L levo- and meso-2,3-BD, relative to the control (0 g/L 2,3-BD); B: The growth profiles of *P. polymyxa* challenged with 40 g/L levo- and meso-2,3-BD, relative to the control; C: The growth profiles of *P. polymyxa* challenged with 60 g/L levo- and meso-2,3-BD, relative to the control. Error bars show standard deviations of means (n = 3).
biotechnological operations. The base chemical, 2,3-BD is a versatile petroleum-derived raw material, and as global fossil fuel reserve drops, biological routes for 2,3-BD production are attracting increasing attention as renewable alternatives to fossil-based feedstock. Increasing 2,3-BD concentration in the fermentation broth will exert a significant impact on the commercialization of biological 2,3-BD production. In this study, we investigated the effect of 2,3-BD-mediated feedback inhibition on product concentrations obtained in cultures of P. polymyxa. As expected, a higher concentration of total 2,3-BD was achieved in fed-batch fermentations in the bioreactor than in batch fermentations in shaken flasks. Additional glucose supply, mixing, and pH control in the reactor are some of the factors responsible for increased product accumulation in the bioreactor (fed-batch) than in the flasks (batch). Clearly, increase in 2,3-BD concentration during fermentation by P. polymyxa significantly limits product accumulation below a toxic threshold of 50 g/L. This is evidenced by (i) termination of fermentation when total 2,3-BD concentration reached ~47 g/L in fed-batch cultures, despite glucose repletion in the broth (Fig. 1A), (ii) increased accumulation of acetoin in fed-batch cultures when total 2,3-BD concentration reached ~47 g/L (Fig. 1A) and in the early stages of batch cultures supplemented with 20 and 40 g/L levo-2,3-BD at 0 h (Fig. 2C), and in the later stages of batch cultures pulse-fed with levo-2,3-BD (Fig. 3B, C); (iii) dose-dependent decrease in glucose utilization with levo-2,3-BD supplementation (0, 20, 40, 60 g/L; Fig. 2D, D), and (iv) altered ratio of levo- to meso-2,3-BD produced by P. polymyxa in levo-2,3-BD-challenged cultures (Fig. 4).

To better understand the oscillatory trend of the EPS concentration in the bioreactor during fermentation (Fig. 1A), we searched the genome of P. polymyxa for the presence of the gene that codes for levananse, the enzyme that catalyzes the hydrolysis of levan to fructose residues [23]. Our bioinformatic analysis showed that P. polymyxa possesses a single copy of levananse gene (1593 bp) that encodes a polypeptide with 530 amino acid residues. A fraction of EPS produced by P. polymyxa via the activity of levansucrase may be hydrolyzed to fructose residues by levananse, and thus, may account for the decrease in the EPS concentration in the bioreactor as the fermentation progressed.

In the fed-batch fermentations, 2,3-BD production terminated at 72 h with concomitant increase in acetoin production and accumulation. Notably, P. polymyxa cells were still viable (60–100 h) given the upward trend of acetoin accumulation and glucose consumption after 72 h fermentation. Indeed, after glucose (25 g/L) supplementation at 60 h, only minimal increase in 2,3-BD concentration was observed (8%), while acetoin concentration increased 3-fold during the same period (Fig. 1). Biosynthesis of 2,3-BD proceeds via acetoin reduction by 2,3-BD dehydrogenase, a dual-functional enzyme that also converts 2,3-BD back to acetoin [24–27]. While the 2,3-BD dehydrogenase of P. polymyxa can catalyze both forward (acetoin to 2,3-BD direction) and reverse (2,3-BD to acetoin direction) reactions, the ratio of 2,3-BD to acetoin produced during fermentation seem to suggest that the forward reaction is favored over the reverse reaction. However, it appears that the reverse reaction becomes more favorable when the immediate environment is saturated with 2,3-BD (Fig. 6). This may explain why 2,3-BD-challenged P. polymyxa cultures produced predominantly acetoin after maximum tolerable limit for 2,3-BD concentration was attained (Fig. 3C).

Conceivably, conversion of 2,3-BD to acetoin is a likely stress-mitigating mechanism evolved by P. polymyxa to alleviate 2,3-BD toxicity since the bioassay of acetoin toxicity against P. polymyxa showed that acetoin is less inhibitory than 2,3-BD (data not shown). Additionally, accumulation of acetoin in the cultures supplemented with levo-2,3-BD at 0 h coincided with a lag in cell growth (Fig. 2A), especially in the cultures challenged with 40 g/L levo-2,3-BD, indicating probable reduction of 2,3-BD toxicity (by conversion to acetoin) to facilitate cell growth. This assumption is in line with the acetoin production profile of P. polymyxa cultures pulse-fed with levo-2,3-BD (Fig. 3C) or control cultures after

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![Fig. 6. Schematic representation of 2,3-BD-mediated feedback inhibition during fermentation by P. polymyxa.](image-url)

(A) The prominent operating pathways during 2,3-BD biosynthesis from sugars as carbon sources; (B) activity of butanediol dehydrogenase catalyzing the reversible acetoin–2,3-BD interconversions. At low 2,3-BD levels in cultures, the rate of acetoin production and conversion to 2,3-BD is relatively constant (rate of acetoin production equals rate of its conversion to 2,3-BD Bi), whereas above toxic 2,3-BD threshold, 2,3-BD is re-converted to acetoin by the culture (Bi); (C) Proposed mechanism for the switch from levo- to meso-2,3-BD formation in P. polymyxa (modified, based on [29]). Figures represent the following enzymes: α-acetolactate synthase, 1; α-acetolactate decarboxylase, 2; butanediol dehydrogenase, 3; diacetyl reductase, 5. The arrows in green and blue represent levo- and meso-2,3-BD formation pathways. The red arrow shows irreversible reduction of diacetyl to R-acetoin by butanediol dehydrogenase.
relatively high concentrations of 2,3-BD had accumulated early in cultures (Fig. 2C). Whereas acetoin accumulation occurred early during fermentation in cultures supplemented with 2,3-BD at 0 h, considerable accumulation of acetoin was not observed until the latter stages of fermentation in cultures pulse-fed with levo-2,3-BD at 12, 24 and 36 h (Fig. 2C and C). With incremental increase in levo-2,3-BD in the pulse-fed fermentations, 2,3-BD-mediated toxicity increased remarkably when total 2,3-BD concentration in the fermentation broth exceeded the toxic threshold (48 g/L), thereby necessitating backward conversion to acetoin, possibly to alleviate 2,3-BD toxicity (Fig. 3C). In cases where reduction in 2,3-BD concentration occurred after the toxic threshold for 2,3-BD had been exceeded, they were accompanied by increase in acetoin concentration. Clearly, backward conversion of 2,3-BD contributes to this trend (increase in acetoin concentration). This is logical given that acetoin is less toxic than 2,3-BD, thus, acetoin biosynthesis is less likely to stall due to 2,3-BD toxicity. However, it is worthy of note that acetoin accumulation after 2,3-BD concentrations exceeded toxic levels may stem in part from acetoin biosynthesis from pyruvate as reductions in 2,3-BD concentration do not completely account for increases in acetoin concentrations.

Further, the patterns of glucose consumption in the levo-2,3-BD-challenged cultures support the observation that the greater the concentration of 2,3-BD in the fermentation broth the lower the growth of P. polymyxa and 2,3-BD production (Fig. 2 and 3). The presence of 2,3-BD (>20 g/L) in the broth in the early stages of fermentation led to a decreased rate of glucose utilization and consequently reduction in total glucose consumed at the end of fermentation (Fig. 2D). Interestingly, while the test and control fermentations (levo-2,3-BD-challenged and unchallenged cultures) exhibited similar glucose utilization profile prior to pulse-feeding (0–12 h, Fig. 3D), the rate of glucose utilization decreased in the treatment cultures following the first round of levo-2,3-BD pulse-feeding (20 g/L at 12 h), resulting in 3.4% decrease in total glucose consumed at the end of fermentation relative to the control fermentations.

Interestingly, levo- and meso-2,3-BD isomers were determined to exert varying degrees of growth inhibitions on P. polymyxa. Our results show that the meso-2,3-BD isomer is less inhibitory to P. polymyxa than the levo-2,3-BD isomer (Fig. 5). Although P. polymyxa produces the levo-2,3-BD isomer predominantly during fermentation, the concentration of the meso-2,3-BD isomer in the fermentation medium increased towards the end of the fermentation when concentrations of levo-2,3-BD had reached toxic levels (Figs. 2 C and 3 C). A similar pattern (delayed production of meso-2,3-BD) has been reported previously for P. polymyxa ZJ-9 [9]. This may be additional stress-mitigating mechanism to reduce levo-2,3-BD mediated toxicity in line with the results obtained in the current study.

The mechanism by which P. polymyxa switches from levo- to meso-2,3-BD biosynthesis upon accumulation of toxic concentrations of levo-2,3-BD is not clear, hence, this requires further study. However, a likely mechanism for this switch may lie in the dual-pronged nature of the 2,3-BD pathway (Fig. 6C). For instance, butanediol dehydrogenase, the enzyme that catalyzes acetoin reduction to 2,3-BD has been shown to generate the 2 isomers of 2,3-BD [28] by utilizing different acetoine stereoisomers as substrates [29]. When acetolactate decarboxylase acts on α-acetolactate, R-acetoin is generated, which is then reduced to levo-2,3-BD by butanediol dehydrogenase. Conversely, S-acetoin generated from diacetyl is reduced to meso-2,3-BD, also by butanediol dehydrogenase (Fig. 6C). Since levo-2,3-BD concentrations reduced during fermentation with concomitant increases in acetoin and meso-2,3-BD concentrations (Figs. 2 and 3), it is likely that at high concentrations of levo-2,3-BD, butanediol dehydrogenase oxidizes levo-2,3-BD back to R-acetoin. Therefore, accumulation of R-acetoin by backward conversion (of levo-2,3-BD) and biosynthesis (of acetoin) via pyruvate may limit the activity of acetolactate decarboxylase, which would lead to spontaneous (non-catalytic) conversion of α-acetolactate to diacetyl (Fig. 6C). Spontaneous conversion of α-acetolactate to diacetyl is well documented in the literature [30]. Ultimately, diacetyl is converted to S-acetoin and subsequently to meso-2,3-BD by butanediol dehydrogenase (Fig. 6C).

Conclusion

Our results underscore the role of 2,3-BD-mediated feedback inhibition in limiting the accumulation of higher concentrations of 2,3-BD in fermentation cultures of P. polymyxa. If biological production of 2,3-BD is to reach large-scale commercialization, it is therefore, imperative to tackle this bottleneck by process development and rational design of a 2,3-BD over-producing strain (metabolic engineering). In light of the results presented here, overcoming 2,3-BD toxicity and abolishing catalysis of 2,3-BD dehydrogenase in the reverse direction (2,3-BD conversion to acetoin) are key metabolic engineering targets worth pursuing. Further, since the levo-2,3-BD is the industrially desirable isomer of 2,3-BD, production of small amounts of meso-2,3-BD may represent considerable losses in large-scale operations. Therefore, eliminating the meso-2,3-BD biosynthesis route may further improve the economics of 2,3-BD fermentation.

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References

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