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Biotechnological Production of Optically Pure 2,3-Butanediol by *Bacillus subtilis* Based on Dissolved Oxygen Control Strategy

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Abstract: 2,3-Butanediol (2,3-BD) is a promising platform chemical, produced from microbial cells. Oxygen availability is a crucial factor driving the formation and proportion of 2,3-BD and acetoin in 2,3-BD producing bacterial strains. In this study, the ability of *B. subtills* GD5 to produce 2,3-BD in optimized sucrose-based media was evaluated, by investigating the impact of carbon to nitrogen (C/N) ratio and the effectiveness of alternative low-cost nitrogen sources (corn steep liquor, soybean meal, and ammonium sulphate). Subsequently, different dissolved oxygen (DO) controlling regimes were assessed in batch bioreactor fermentations. The best fermentation outcomes were obtained with uncontrolled DO, achieving 5.88 g/L of optically pure (R,R)-2,3-BD (~100% purity), accompanied by a production yield of 0.43 g/g, and a productivity of 0.2 g/L/h. Additionally, the influence of the DO controlling regime on *B. subtills* key enzymes involved in the reverse activity of acetoin reductase was also monitored. A fed-batch process under the most suitable DO conditions was carried out to improve 2,3-BD production, achieving 42.31 g/L 2,3-BD with a production yield of 0.52 g/g. Thus, *B. subtilis* GD5 is a promising strain for the efficient production of pure chiral (R,R)-2,3-BD under uncontrolled DO conditions, using alternative low-cost nitrogen sources.

Keywords: 2,3-butanediol; *Bacillus subtilis*; dissolved oxygen; optical purity; fermentation; fed-batch

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1. Introduction

2,3-Butanediol (2,3-BD) is an important platform chemical that has potential applications in various industries such as in the production of antifreeze agents, printing inks, fuel additives, synthetic rubber, food flavour additives, and pharmaceutical products [1–3]. Currently, 2,3-BD is typically produced on an industrial scale from non-renewable petroleum feedstocks through chemical processes [4]. With the further development of green, sustainable technologies and continuous enhancement of environmental protection awareness, the production of bio-based chemicals inevitably has become a key trend of industrial development, even though bio-based chemicals are still generally more expensive than conventional petroleum-based ones [5]. A number of wild-type microorganisms, including those in the genera of *Klebsiella* sp., *Bacillus* sp., *Serratia* sp., *Enterobacter* sp. and *Paenibacillus* sp., have the ability to produce 2,3-BD [6–16]. Nevertheless, the pathogenic nature of *Klebsiella pneumoniae* limits their exploitation for industrial-scale production. Among those strains, *B. subtilis*, has been granted a generally regarded as safe (GRAS) status, and is considered an efficient 2,3-BD producing strain [17]. Much work has been performed using expensive nitrogen sources such as peptone, yeast extract, beef extract or tryptone to produce 2,3-BD; however, these are often associated with higher production costs and could potentially limit the translation of the bioprocess on an industrial scale [18].

The biosynthesis of 2,3-BD is part of the mixed acid fermentation pathway in bacterial cells, which produces a variety of metabolic products including acetic, lactic and succinic acid, 2,3-BD, and ethanol. The metabolic pathways of 2,3-BD have been investigated intensively and involve three key steps: firstly, two molecules of pyruvate (synthesized from the glycolytic pathway) are converted into α-acetolactate and carbon dioxide by the catalysis

of α-acetolactate synthase (ALS), then acetolactate decarboxylase (ALDC) transforms α-acetolactate to acetoin. Finally, acetoin is reduced into 2,3-BD by acetoin reductase (ACR); however, 2,3-BD could be reversibly converted back into acetoin by 2,3-BD dehydrogenase (BDH) [2,19,20]. Acetoin has one chiral center, and exists as R-acetoin or S-acetoin. 2,3-BD has two chiral centers, resulting in three stereoisomers, including the optically active form of (R,R)-2,3-BD and (S,S)-2,3-BD, and the optically inactive form of (R,S), which also designated as meso- 2,3 BD [21]. In general, a mixture of 2,3-BD stereoisomers is produced by native 2,3-BD-producing strains, which poses a challenge for the production of 2,3-BD with high chiral purity [4,22]. Specific physiochemical properties of each stereoisomer of 2,3-BD are associated with targeted industrial applications. For example, (R,R)- and (S,S)-2,3-BD could be used as antifreeze agents due to their low freezing point (−30 °C). (R,R)- and meso-2,3-BD can serve as precursors for 1,3-butadiene, methy ethyl ketone (MEK), printing ink, and softening agents. [3]. On a biochemical level, 2,3-BD plays a significant role in bacterial growth, by preventing excessive acidification of the intracellular environment, regulating the balance ratio of reduced nicotinatmide adenine dinucleotide (NADH) and nicotinatmide adenine dinucleotide (NAD^+), and serving as a carbon source for cell maintenance [23]. Since 2,3-BD, lactic acid and ethanol are NADH-dependent metabolites involved in the mixed acid fermentation pathway, NADH availability and quantity in its active form plays a critical role in directing the biosynthesis of 2,3-BD. Thus, 2,3-BD production could be enhanced by manipulating the NADH-dependent pathways [24]. Moreover, oxygen availability is a crucial factor driving the formation and proportion of 2,3-BD and acetoin. It has been suggested that relatively low dissolved oxygen (DO) saturation favour 2,3-BD production, while relatively high DO concentrations promote cell growth and acetoin production [25–27]. Thus, an effective DO control strategy is required to reduce acetoin formation whilst enhance 2,3-BD production.

The overall aim of this study was to enhance 2,3-BD production in non-pathogenic wild-type of *B. subtilis*. Initially, the effects of alternative low-cost nitrogen sources and culture C/N ratio on growth and 2,3-BD production were explored. Then, optimized media were utilised in batch bioreactor processes to assess the effect of dissolved oxygen (DO) control strategies on 2,3-BD and acetoin production, by monitoring also key-enzyme activity involved in 2,3-BD metabolic pathway. Finally, optimized culture conditions were applied in a fed-batch process targeting enhanced 2,3-BD production.

2. Materials and Methods

2.1. Microorganisms

Five non-pathogenic, wild-type, bacterial strains from the Food and Nutritional Sciences culture collection, University of Reading, were used in this study, namely *Bacillus subtilis* GD2 (FSBC 151), *Bacillus subtilis* GD5 (FSBC 322), *Bacillus licheniformis* FSBC 320, *Serratia plymuthica* FSBC 401, and *Lactococcus lactis* FSBC 64. *Bacillus* species and *S. plymuthica* were maintained on nutrient agar (NA) containing the following compounds: 2 g/L yeast extract, 1 g/L beef extract, 5 g/L peptone, 5 g/L sodium chloride, and 15 g/L bacterial agar. *L. lactis* was grown on M17 agar. All bacterial cultures were incubated at 30 °C for 18–24 h prior to inoculation.

2.2. Pre-Culture and Production Culture Media

The pre-culture medium for *Bacillus* species and *S. plymuthica* was MRS medium, consisting of 20 g/L glucose, 10 g/L peptone, 8 g/L meat extract, 4 g/L yeast extract, 2 g/L di-potassium hydrogen phosphate, 5 g/L sodium acetate, 2 g/L tri-ammonium citrate, 0.2 g/L magnesium sulphate, and 0.05 g/L manganese sulphate. The pre-culture medium for *L. lactis* was M17 broth, comprising 5 g/L lactose, 5 g/L tryptone, 5 g/L soya pep-tone, 5 g/L meat digest, 2.5 g/L yeast extract, 19 g/L disodium glycerophosphate, 0.5 g/L ascorbic acid, 0.25 g/L magnesium sulphate. A single loop from agar Petri dish was inoculated into 50 mL of sterilized (121 °C, 15 min) pre-culture medium in 100 mL Erlenmeyer flasks. The seed culture was grown with agitation (180 rpm, orbital shaker) at

30 °C for 24 h. The inoculum was centrifuged at 11,627× *g*, 4 °C for 10 min. The supernatant was removed, the cells were resuspended with sterilised 0.85% (*w*/*v*) NaCl and employed as inoculum. An initial optical density (OD_{600}) of 0.2–0.3 was used for the cultivation.

Modified MRS (MMRS) medium was used as production medium, containing 20 g/L sucrose instead of glucose Initial screening of bacterial strains was done in aerobic and anaerobic conditions. Aerobic conditions were investigated in 250 mL Erlenmeyer flasks with a working volume of 100 mL of MMRS medium, incubated in a shaking incubator with agitation speed of 180 rpm, at 30 °C for 48 h. The pH remained uncontrolled during the fermentation period.

In the case of anaerobic conditions, 250 mL Duran bottles with a working volume of 100 mL culture media were used. Duran bottles were placed in an orbital shaker with agitation speed of 80 rpm, at 30 °C for 48 h. The pH remained uncontrolled during the fermentation period.

2.3. Effect of Different Nitrogen Sources on 2,3-BD Production

Low-cost nitrogen sources, including corn steep liquor (CSL) (Sigma-Aldrich, Gillingham, UK) and soybean meal (SBM) (Target feed Ltd., Whitchurch, UK), were employed individually at 20 g/L to replace the original nitrogen sources in the MMRS medium. Ammonium sulphate (AS) (10 g/L), representing an inorganic source of nitrogen, with and without vitamin supplementation, was also used for comparison reasons. The vitamin supplementation solution was prepared based on the vitamin composition of yeast extract [23], and was based on the amount of yeast extract in MRS medium, which contained 0.14 mg/L thiamine, 4.0 mg/L inositol, 0.07 mg/L panthothenic acid, 0.02 mg/L vitamin B6, 0.09 mg/L riboflavin, and 0.41 mg/L niacin. Fermentations were performed in 250 mL Erlenmeyer flasks, containing 100 mL of sterilised medium (121 °C/ 15 min), at 30 °C, 180 rpm.

2.4. Effect of Carbon to Nitrogen (C/N) Molar Ratio on 2,3-BD Production

Various C/N ratios were evaluated to establish a suitable ratio for bacterial growth and 2,3-BD production. In all experiments, sucrose concentration was constant at 20 g/L, while the concentration of CSL varied (from 50, 20, 10 to 5 g/L) corresponding to C/N ratios of 6, 11, 17, and 22, respectively. The contribution of other carbon- or nitrogen ingredients in MMRS was also taken into account when calculating the C/N ratio. The fermentations were conducted in 250 mL Erlenmeyer flasks, containing 100 mL of sterilised medium (121 °C/ 15 min), at 30 °C, 180 rpm.

2.5. Effect of Dissolved Oxygen Control Strategies in Growth and 2,3-BD in 2-L Bioreactor

Different DO controlling strategies were assessed during batch fermentations in a 2-L benchtop bioreactor (Biostat® B, Sartorius, Germany), in which sucrose (20 g/L) and CSL (20 g/L) were employed as carbon and nitrogen sources, respectively, at 30 °C, pH 6, and aeration of 1.0 vvm. The DO controlling regimes included: (i) DO at 5% achieved through cascade agitation, (ii) DO at 10%, achieved through cascade agitation, (iii) uncontrolled DO at fixed stirring rate of 180 rpm, and (iv) a two-stage DO control, starting with DO at 5% with cascade agitation from 0–15 h, followed by anaerobic conditions (no oxygen input into the bioreactor). DO and pH were measured using polarographic and potentiometric sensors, respectively (Hamilton, Bonaduz, Switzerland). 10% (*w*/*v*) Antifoam B (Sigma-Aldrich, Gillingham, UK) was fed into the bioreactor as needed. Samples were collected at various time intervals for measuring cell growth, sugar, and metabolites.

2.6. Fed Batch Cultivation for 2,3-BD Production in 2-L Bioreactor

The most effective DO control strategy from the previous experiment was selected for fed-batch fermentation in a 2 L benchtop bioreactor (Biostat® B, Sartorius, Germany), with an initial working volume of 1.2 L, in MMRS medium using sucrose (20 g/L) and CSL (20 g/L) as carbon and nitrogen sources, respectively. Fed-batch was conducted at 30 °C,

pH 6.0 and aeration of 1.0 vvm. Concentrated sucrose solution (500 g/L) was fed when residual sugar concentration dropped to 10 g/L. Samples were collected at various time intervals for the determination of cell growth, sugar, and metabolites.

2.7. Determination of Acetoin Reductase Butanediol Dehydrogenase (ACR/BDH) and NADH Oxidase (NOX) Activities

2.7.1. Cell Preparation

Bacterial cells were harvested from the fermentation broth by centrifugation at 9418× *g* for 10 min at 4 °C and washed twice with 50 mM potassium phosphate buffer (pH 6.5). Then, the cell pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.5) containing a protease inhibitor cocktail (Sigma-Aldrich, Gillingham, UK). Then, the cell suspension was introduced to a Vibra cell™ ultra-sonicator (Sonics, Newtown, CT, USA) with 4 cycles of pulse for 30 s, intermittent for 5 s, on ice. Cell debris was removed by centrifugation at 9418× *g*, 4 °C for 10 min and a crude protein solution was obtained for enzyme assays.

2.7.2. Acetoin Reductase and Butanediol Dehydrogenase (ACR/BDH) Activities

The ACR/ BDH activity was determined spectrophotometrically by monitoring the change of absorbance at 340 nm and 30 °C corresponding to the oxidation of NADH and reduction of NAD^+ [28–30]. The enzyme activity determination was performed in accordance with previously reported methods, with some modifications [17,29,31]. Briefly, the ACR activity was determined in a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 25 mM acetoin, and 0.25 mM NADH. The BDH activity was determined using 50 mM glycine-NaOH buffer (pH 8.5) containing 25 mM 2,3-butanediol, and 0.25 mM NAD^+. In all assays, the reaction was initiated by the addition of crude enzyme after adding the substrate. One unit of activity (U) was defined as the amount of crude enzyme that reduced (for ACR) or generated (for BDH) 1 µmol of NADH per minute. A correction was made for the spontaneous degradation of NADH. The protein concentration was measured using the Bradford protocol with bovine serum albumin as a standard [32]. The specific activity of ACR/BDH was expressed as enzyme unit (U) per enzyme protein (mg).

2.7.3. NADH Oxidase (NOX) Activity

The NOX activity was determined using the assay kit (Fluorometric) ab273345 from Abcam (Cambridge, UK) according to the manufacturer's instructions. NOX activity couples the oxidation of NADH by NOX and the reduction of a colorless probe to a brightly colored fluorescent product. The generated fluorescence corresponded to the NOX activity in the samples. The fluorescence was detected using an Infinite® 200 PRO microplate reader (Tecan, Männedorf, Switzerland). The reaction mechanism relies on the oxidation of lactate to generate NADH, which is in turn used by the NADH oxidase, catalyzing the reduction of the probe into a fluorescent molecule, which an emission can be read at 587 nm. One mole of oxidized lactate generates one mole of NADH from β-NAD^+. The NADH is subsequently used in the reduction of the substrate by NOX. One unit of NOX activity (U) can be defined as the amount of enzyme that catalyses the reduction of 1 µmol of substrate per minute under the assay conditions and the specific enzyme activity was expressed as µU per mg of protein.

2.8. Analytical Methods

Cell growth was determined by optical density at 600 nm (OD_{600}) using a spectrophotometer (Life Technologies Ltd., Paisley, UK). Dry cell weight (DCW) was estimated using a standard calibration curve established between optical density and dry cell weight data. Collected samples were centrifuged at 11,627× *g* for 10 min, and the cell-free supernatant was hydrolysed with 10% (*v*/*v*) sulphuric acid at 100 °C for 30 min and filtered (0.2 µm, Whatman, SLS, Nottingham, UK) before HPLC analysis [33]. The concentration of glucose,

fructose, 2,3-BD, acetoin, succinic acid, lactic acid, acetic acid, and ethanol were determined by an Agilent Infinity 1200 high-performance liquid chromatography (HPLC) system (Agilent, Didcot, UK) with an aminex HPX-87H (300 mm × 7.8 mm, Biorad, Watford, UK) column coupled to refractive index (RI) and DAD detectors. The mobile phase consisted of 5 mM H_2SO_4 with a flow rate of 0.6 mL/min. The column temperature was controlled at 65 °C. The concentrations of sugar and metabolites were quantified using external calibration curves while the dilution of samples mixed with sulphuric acid was taken into consideration.

2.9. Identification of 2,3-BD and Acetoin Stereoisomeric Forms by GC-FID

Supernatants were extracted with ethyl acetate using Fast prep 24™ homogenizer (MP Biomedicals, Eschwege, Germany), and the solvent was evaporated by nitrogen gas. The concentration of acetoin stereoisomers (R- and S- forms) and (R,R-), (S,S-), meso-2,3-BD were analysed in a GC system Agilent 7890B (Agilent, Denver, CO, USA). The system consisted of an FID detector and a chiral column (CP-Chirasil-Dex DB 25 m length, and 0.25 mm inner diameter, Agilent Technologies, Denver, CO, USA). The operating conditions were developed based on the method of Caligiani et al. [34], and were as follows: helium was used as a carrier gas at a flow rate of 1.2 mL/min; the injection port temperature and the detector temperature were set at 250 °C; and the column temperature was maintained at 60 °C for 1 min, then raised to 120 °C at a rate of 10 °C/min followed by a ramp of 40 °C/min to 200 °C. The concentrations of acetoin and 2,3-BD in the supernatant were determined using standard curves of known solutions of commercial samples (Sigma-Aldrich, Gillingham, UK).

3. Results

3.1. Screening of Bacterial Strains for 2,3-BD Production in Sucrose-Based Media

The five bacterial strains were grown under aerobic and anaerobic conditions, and the results are presented in Table 1. In terms of growth, aerobic conditions seemed favourable for most strains. *B. subtilis* GD5 presented the highest growth, achieving an OD_{600} of 5.34 (3.11 g/L DCW) at 24 h. Bacillus species are known as facultative anaerobic bacteria, and they can grow in either anaerobic or aerobic conditions. However, under the presence of oxygen, these species grow better compared to conditions where oxygen is limited or absent.

Under anaerobic conditions, *S. plymuthica* exhibited the highest growth, reaching an OD_{600} of 2.61 (1.95 g/L DCW) at 24 h. Serratia spp., like other members of Enterobacteriaceae, can grow well in synthetic media under both anaerobic and aerobic conditions. As observed in Table 1, most bacterial strains produced higher concentrations of 2,3-BD under aerobic compared to anaerobic conditions. Interestingly, *B. subtilis* GD5 produced the highest 2,3-BD concentration (7.28 g/L) under aerobic conditions, whereas *S. plymuthica* produced relatively high 2,3-BD (>5 g/L) under both conditions. In the case of *B. subtilis* GD5, *B. licheniformis* and *S. plymuthica* strains all of which grew very well under aerobic conditions, maximum concentrations of 2,3-BD were obtained during the growth phase (24 h), indicating that BD is a growth-associated metabolite. Subsequently, 2,3-BD declined, indicating the reversible conversion of 2,3-BD to acetoin under conditions of carbon depletion. 2,3-BD production is favoured under slightly acidic conditions (pH 5.0–6.5) and is a mechanism for bacteria to limit the decrease of external pH that is caused by the formation of organic acids such as acetic, lactic and succinic acid, from pyruvate. The reverse conversion of 2,3-BD to acetoin occurred in the stationary phase (24–48 h), when the pH of the medium increased to higher than 7. *L. lactis* produced lactic acid as a major metabolite under both conditions. Moreover, acetoin was not produced by any strain under anaerobic conditions.

With regards to 2,3-BD stereoisomers, *B. subtilis* GD5 produced pure (R,R)-2,3-BD after 24 h of fermentation. *B. licheniformis* also exhibited notable production of pure (R,R) 2,3-BD (5.70 g/L), whereas *S. plymuthica* produced 95% meso-2,3-BD and 5% (S,S)-2,3-BD.

Moreover, it was observed that *B. subtilis* GD5 and *S. plymuthica* produced R and S-acetoin, *L. lactis* generated only R-acetoin, whereas no detection of acetoin in *B. licheniformis* at 24 h was noted. Considering the above results with regard to total 2,3 BD and stereoisomers from all screened bacterial strains, *B. subtilis* GD5 was selected as the most suitable strain to be used in further experiments.

Table 1. Cell growth, pH and 2,3-BD production in sucrose-based media by various bacterial strains under aerobic (shake-flask) and anaerobic (Duran bottles) conditions.

Growth Conditions	Bacterial Strain	Time (h)	Substrate Consumed (g/L)	$OD_{600\ nm}$ (Abs)	pH	2,3-BD (g/L)	Acetoin (g/L)	Lactic Acid (g/L)
Aerobic	*B. subtilis* GD2	24	1.45 ± 0.03	1.27 ± 0.19	6.31 ± 0.05	0.87 ± 0.13	ND	ND
		48	10.23 ± 0.03	2.41 ± 0.07	5.94 ± 0.03	3.78 ± 0.00	ND	ND
	B. subtilis GD5	24	16.10 ± 0.01	5.34 ± 0.15	5.82 ± 0.07	7.28 ± 0.25	0.17 ± 0.05	0.96 ± 0.00
		48	18.18 ± 0.01	5.28 ± 0.26	7.54 ± 0.05	3.46 ± 0.01	3.21 ± 0.01	ND
	B. licheniformis	24	17.55 ± 0.00	5.18 ± 0.30	6.40 ± 0.06	6.27 ± 0.00	ND	0.86 ± 0.00
		48	18.19 ± 0.00	3.53 ± 0.37	8.04 ± 0.11	1.77 ± 0.01	5.32 ± 0.01	ND
	S. plymuthica	24	18.50 ± 0.00	4.04 ± 0.03	5.87 ± 0.01	5.88 ± 0.03	1.74 ± 0.04	0.15 ± 0.00
		48	18.73 ± 0.00	5.78 ± 0.10	7.63 ± 0.03	2.63 ± 0.01	4.17 ± 0.02	ND
	L. lactis	24	12.92 ± 0.14	4.16 ± 0.08	4.90 ± 0.06	0.46 ± 0.00	0.48 ± 0.00	3.47 ± 0.00
		48	14.68 ± 0.14	4.50 ± 0.38	4.46 ± 0.03	ND	1.20 ± 0.01	4.46 ± 0.01
Anaerobic	*B. subtilis* GD2	24	2.63 ± 0.01	0.68 ± 0.04	6.11 ± 0.01	0.21 ± 0.03	ND	ND
		48	3.17 ± 0.28	0.84 ± 0.08	6.33 ± 0.03	0.19 ± 0.00	ND	ND
	B. subtilis GD5	24	3.44 ± 0.09	1.16 ± 0.04	5.94 ± 0.02	1.73 ± 0.01	ND	0.28 ± 0.00
		48	7.15 ± 0.27	1.16 ± 0.07	5.86 ± 0.04	3.02 ± 0.02	ND	1.30 ± 0.02
	B. licheniformis	24	3.30 ± 0.25	0.91 ± 0.13	5.73 ± 0.03	1.32 ± 0.01	ND	0.66 ± 0.03
		48	5.68 ± 0.88	0.71 ± 0.30	5.64 ± 0.09	2.15 ± 0.04	ND	1.66 ± 0.07
	S. plymuthica	24	17.56 ± 0.18	2.61 ± 0.08	6.00 ± 0.04	7.08 ± 0.12	ND	1.47 ± 0.04
		48	17.61 ± 0.23	2.08 ± 0.27	6.18 ± 0.19	6.96 ± 0.02	ND	1.29 ± 0.01
	L. lactis	24	7.97 ± 0.51	0.96 ± 0.01	6.21 ± 0.07	0.16 ± 0.01	ND	1.82 ± 0.11
		48	13.61 ± 0.04	2.11 ± 0.27	5.17 ± 0.17	1.56 ± 0.02	ND	6.57 ± 0.10

Culture conditions: Temperature 30 °C, agitation rate 180 rpm, initial pH 6.8–7.0, initial sucrose concentration 20 g/L. ND: Not detected.

3.2. Effect of Alternative Low-Cost Nitrogen Sources on Cell Growth and 2,3-BD Production

Low-cost nitrogen sources, including corn steep liquor (CSL), soybean meal (SBM), and ammonium sulphate (AMS) with and without vitamin addition, were employed individually to replace more costly nitrogen sources, such as peptone, yeast extract and meat extract which are found in MRS medium. As shown in Table 2, relatively high bacterial growth was observed when organic sources of nitrogen were employed compared to inorganic nitrogen sources (i.e., ammonium sulphate). CSL was the most suitable nitrogen source, as it supported adequate cell growth and 2,3-BD production from *B. subtilis* GD5, reaching 3.52 g/L at 24 h. It was noted that less acetoin was produced when CSL was used as nitrogen source compared to the control (unmodified MRS medium). The highest 2,3-BD production yield of 0.39 g/g was achieved after 24 h of fermentation when CSL was employed as nitrogen source. CSL is a major by-product of the wet-milling process of corn starch production [35]. It is an inexpensive nutrient-rich source and has been employed as a supplement in various microbial fermentations [36,37]. Apart from amino acids, vitamins, and minerals, 2.5% (*w*/*w*) invert sugars are also present in CSL composition. [38]. Yang et al. [39] also reported that CSL exhibited a positive effect on 2,3-BD production over other N-sources, such as soy peptone and beef extract. They also demonstrated that organic nitrogen sources such as CSL and yeast extract not only stimulate the conversion of acetoin to 2,3-BD, but also promote cell growth in *B. subtilis*. Soybean meal has been reported as a suitable nitrogen source to replace yeast extract for 2,3-BD and acetoin production [9]. However, it was not comparable to the results of this study; CSL seemed to be more easily assimilated than SBM by *B. subtilis* GD5. In the case of inorganic nitrogen, the results showed that even with vitamin supplementation, 2,3 BD production

was not improved. Thus, corn steep liquor was selected as a promising alternative nitrogen source for further studies.

Table 2. Effect of different nitrogen sources on biomass and 2,3-BD production by *B. subtilis* at 24 h cultivated under aerobic conditions in MMRS medium.

N-Source [a]	DCW (g/L)	pH	2,3-BD (g/L)	Acetoin (g/L)	Lactic Acid (g/L)	2,3-BD Production Yield (g/g)
Control	3.14 ± 0.09	6.14 ± 0.02	3.58 ± 0.93	2.24 ± 0.69	0.23 ± 0.03	0.27 ± 0.08
CSL	3.52± 0.04	6.97 ± 0.05	5.42 ± 0.16	1.32 ± 0.25	0.51 ± 0.03	0.39 ± 0.01
SBM	2.70 ± 0.34	6.78 ± 0.02	4.38 ± 0.10	0.59 ± 0.00	0.37 ± 0.03	0.34 ± 0.02
AMS 20 g/L	1.35 ± 0.03	7.08 ± 0.01	0.15 ± 0.01	1.02 ± 0.08	ND	0.03 ± 0.01
AMS 10 g/L	1.50 ± 0.07	7.13 ± 0.07	0.20 ± 0.01	0.84 ± 0.04	ND	0.04 ± 0.01
AMS 10 g/L + Vitamins	1.56 ± 0.04	7.11 ± 0.00	0.14 ± 0.02	0.85 ± 0.02	ND	0.03 ± 0.01

Culture conditions: T = 30 °C; initial pH = 7.0; agitation rate = 180 rpm. [a] Control: unmodified MRS, CSL: Corn steep liquor, SBM: Soybean meal, AMS: Ammonium sulphate. ND: Not detected. Data shown are means ± SD of duplicate independent fermentations.

3.3. Effect of Different Carbon-to-Nitrogen (C/N) Molar Ratios on Cell Growth and 2,3-BD Production

Microbial metabolism requires carbon for the provision of energy and carbon and nitrogen for cell synthesis. In order to investigate the impact of the C/N ratio on 2,3-BD production, media with varying concentrations of CSL were used, whilst other ingredients, as well as sucrose concentration in the culture medium, were fixed.

As seen in Table 3, the lowest dry cell mass (2.2 g/L) and lowest specific growth rate (0.111 h^{-1}) were obtained in cultures with a C/N ratio of 6. Moreover, trials with C/N ratios of 6 and 22 provided the lowest 2,3-BD production yield and productivity. This might reflect an imbalanced C/N ratio in the media for *B. subtilis* GD5, either an overabundant (C/N 6) or inadequate (C/N 22) provision of nitrogen. Maximum biomass (3.5 g/L), specific growth rate (0.125 h^{-1}), 2,3-BD production yield (0.35 g/g) and productivity (0.22 g/L/h) were obtained at 24 h in media with a C/N ratio of 11. Considering those parameters, a C/N ratio of 11, provided an excellent balance between carbon and nitrogen provision during batch fermentation, and thus was therefore selected for further study. It has been reported that the C/N ratio has a great impact on cell growth and metabolite production in various microorganisms [40,41]. Thus, it should be finely adjusted in fermentation media to enhance microbial growth and promote the biosynthesis of desired products. Yang et al. [39] reported that the initial concentration of CSL affected 2,3-BD and acetoin production and also the ratio of 2,3-BD to acetoin. They claimed that with an increase in CSL concentration, cell growth of *B. subtilis* was increased, acetoin reductase (ACR) was stimulated, 2,3-BD concentrations improved by 78.6%, acetoin concentration decreased by 61.9%, and the ratio of 2,3-BD to acetoin enhanced by 3.69-fold.

Table 3. Effect of different carbon to nitrogen (C/N) molar ratios on bacterial growth and 2,3-BD production by *B. subtilis* at 24 h cultivated under aerobic conditions in MMRS medium.

C/N Ratio	DCW (g/L)	Specific Growth Rate (h^{-1})	2,3-BD (g/L)	2,3-BD Production Yield (g/g)	2,3-BD Productivity (g/L/h)
6	2.24 ± 0.15	0.111± 0.01	3.09 ± 0.04	0.27 ± 0.02	0.13 ± 0.00
11	3.53 ± 0.04	0.125 ± 0.02	5.39 ± 0.01	0.35 ± 0.02	0.22 ± 0.02
17	3.39 ± 0.02	0.120 ± 0.01	4.65 ± 0.01	0.33 ± 0.01	0.19 ± 0.01
22	2.98 ± 0.01	0.115 ± 0.01	3.25 ± 0.15	0.27 ± 0.03	0.14 ± 0.01

Data shown are means ± SD of duplicate independent experiments.

3.4. Effect of Dissolved Oxygen Control Strategy on Cell Growth and 2,3-BD Production in 2-L Batch Bioreactor

The impact of four DO control strategies on *B. subtilis* GD5 cell growth and 2,3-BD production was investigated in a 2-L bioreactor operated under batch mode (Figure 1). Unless stated otherwise, the fermentations were carried out at 30 °C, pH 6.0 and an aeration rate of 1.0 vvm throughout the cultivation. Higher growth levels were obtained when DO concentrations were maintained at 5 and 10% with cascade agitation throughout the fermentation, compared to fermentations with uncontrolled DO. This indicated that higher stirrer speed generated higher oxygen saturation and provided better mixing of bacteria and medium in the bioreactor, stimulatin bacterial growth. It is noted that *B. subtilis* entered the exponential growth phase after 6 h and maximum DCW (5–6 g/L) was reached within 24 h in both DO control levels. In uncontrolled DO fermentations, cell growth increased gradually up until 30 h. In the case of the two-stage DO control strategy, higher growth (maximum DCW 2.1 g/L) was observed during the first stage with cascade agitation at 5% until 15 h. However, during anaerobic conditions (after 15 h), bacterial growth decreased slightly, possibly due to the absence of dissolved oxygen in the medium. In terms of maximum specific growth rate (μ_{max}), the highest μ_{max} of 0.37 h^{-1} was achieved in the runs with controlling DO at 10% followed by a μ_{max} of 0.23 h^{-1} when controlling DO at 5%, whereas μ_{max} values of 0.14 and 0.16 h^{-1} were obtained in cultures with uncontrolled %DO and the two-stage DO control regimes, respectively.

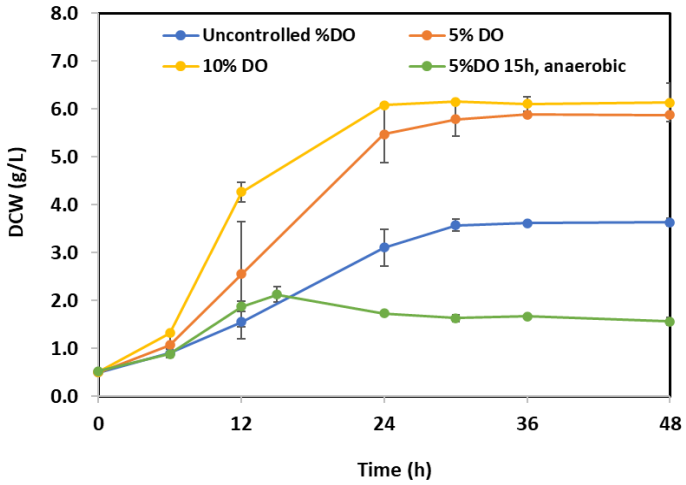


Figure 1. Cell growth of *B. subtilis* at 30 °C, pH 6 in a 2 L benchtop bioreactor under different DO control strategies: controlling DO with cascade agitation at 5%, controlling DO with cascade agitation at 10%, uncontrolled DO at fixed stirrer speed at 180 rpm throughout fermentation, controlling DO with cascade agitation at 5% during the first 15 h, followed by anaerobic conditions.

As shown in Figure 2, the cells consumed sucrose faster under high agitation and high DO conditions (Figure 2a,b) compared to the fermentations with uncontrol DO (Figure 2c) and the two-stage DO control strategy (Figure 2d).

Additionally, the effect of different DO control strategies on 2,3-BD and acetoin production was evaluated. Controlling the DO at 5 or 10% contributed to the high oxygen concentration in the bioreactor, and resulted in the accumulation of acetoin (approximately 5 g/L), whereas very low levels of 2,3-BD (≤1 g/L) were obtained. This finding is in agreement with the study of Qureshi et al. who reported that excessive aeration-agitation led to the production of acetoin at the expense of 2,3-BD [42]. Moreover, after sucrose was depleted, acetoin concentration decreased to nearly zero (Figure 2a,b). This phenomenon was in agreement with Xiao [43] who claimed that acetoin could be reutilised during the stationary phase after the depletion of other carbon sources, providing further evidence that microorganisms produce 2,3-BD and acetoin as carbon and energy storage [43,44].

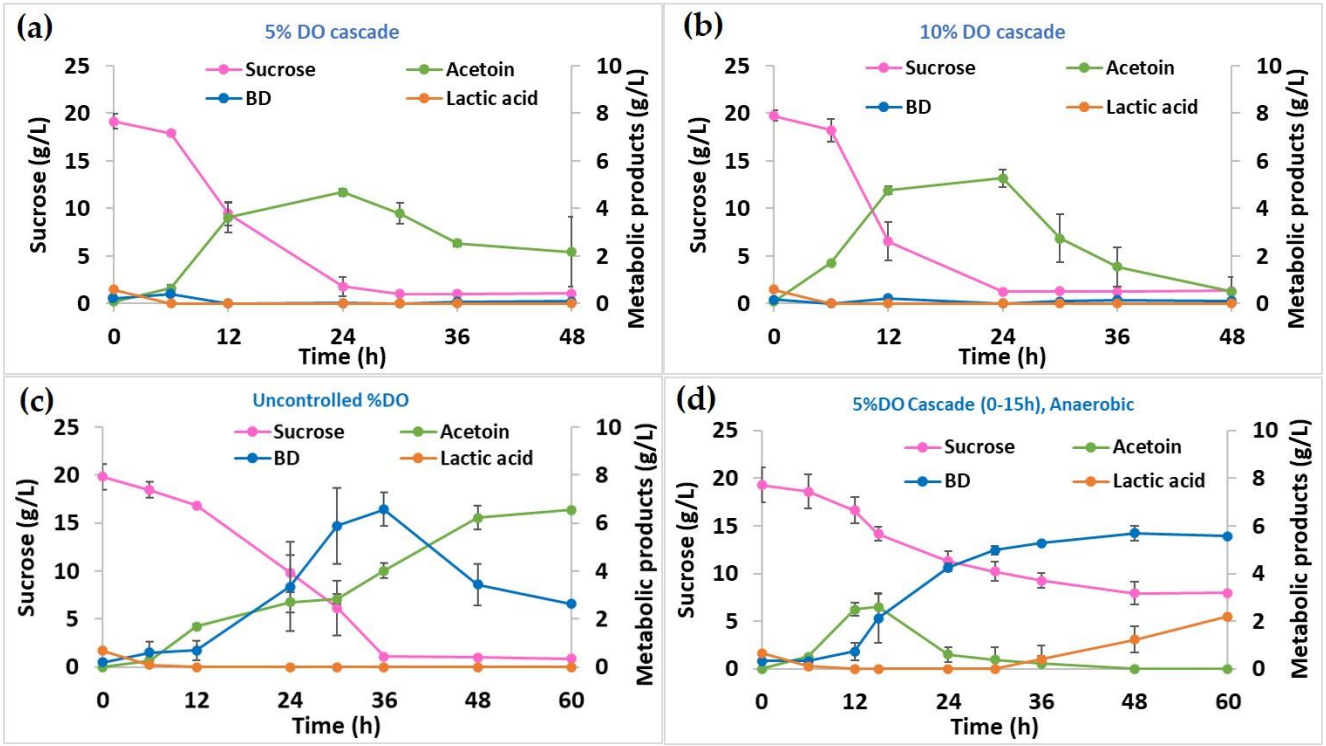


Figure 2. Time courses of substrate utilisation and production of metabolic products by *B. subtilis* from sucrose-based medium in 2 L bioreactor at 30 °C pH 6, aeration rate 1.0 vvm under (**a**) controlling DO with cascade agitation at 5%, (**b**) controlling DO with cascade agitation at 10%, (**c**) uncontrolled DO at fixed stirrer speed at 180 rpm throughout fermentation, (**d**) controlling DO with cascade agitation at 5% during the first 15 h, followed by anaerobic conditions.

On the other hand, uncontrolled DO at a fixed agitation speed of 180 rpm (Figure 2c), led to DO reduction at 0% within 5 h, and favoured 2,3-BD production, resulting in a maximum 5.88 g/L of 2,3-BD, with a yield of 0.35 g/g sucrose, and productivity of 0.20 g/L/h after 30 h of cultivation. The enzymes involved in the 2,3-BD production pathway (α-acetolactate synthase, α-acetolactate decarboxylase, acetoin reductase) are generally activated under micro-aerobic conditions, and can be inactivated by high oxygen supply under fully aerobic conditions. To this end, it has been suggested that 2,3-BD production yield could be maximised by regulating a suitable oxygen supply in order to limit microbial respiration [45]. The levels of produced 2,3-BD and acetoin seem to be biochemically driven by oxygen requirement. As shown in Figure 2c, notable concentrations of 2,3-BD were obtained during growth phase (0–30 h), indicating that 2,3-BD is a growth associated metabolite for this particular strain. Thereafter, 2,3-BD steadily declined and was reversibly converted to acetoin, providing further evidence on the reverse activity of acetoin reductase in the *B. subtilis* GD5 strain. Considering the metabolic pathways of acetoin and 2,3-BD production in bacteria cells, acetoin and 2,3-BD could be transformed into each other directly [8,26,46,47]. Initially, the produced acetoin is converted into 2,3-BD by acetoin reductase (ACR), then 2,3-BD is reversibly transformed to acetoin by butanediol dehydrogenase (BDH) [48]. In this reaction, one molecule of NADH is regenerated through the BDH activity to maintain bacterial growth [26]. Zhang [49] reported that the reverse activity of ACR/BDH takes place when *B. subtilis* enters the late stationary phase. They showed that a pH around 6 was optimum for the reduction of acetoin into 2,3-BD. The reverse activity of acetoin reductase can be also explained by cell metabolism. When the carbon source is depleted, the NADH required for microbial metabolism is generated through the conversion of 2,3-BD to acetoin for maintaining a constant redox state [26,50,51]. This phenomenon has also been reported by Tian [26] who produced acetoin from sugar-

based substrates using *B. subtilis*, but also for other 2,3-BD-producing strains such as *B. licheniformis* and *Serratia marcescens* [26,51,52].

In order to further verify the effect of dissolved oxygen on 2,3-BD and acetoin production, a two-stage DO control strategy was investigated (Figure 2d). The DO was controlled with a cascade agitation at 5% for 15 h, followed by anaerobic conditions. Under these conditions, the strain consumed less sucrose and at a slower rate, particularly after anaerobic conditions were applied (no oxygen input to the bioreactor), compared to the other DO control strategies. Moreover, during the first 15 h, acetoin production was superior to 2,3-BD as more DO was present in the culture. In this phase, the NADH generated from the glycolytic pathway was mostly oxidised through the electron transport chain to regenerate NAD^+, thus the pathway of acetoin to 2,3-BD conversion was not active [26,53]. On the other hand, when the cultivation mode switched to anaerobic conditions, the pathway for transformation from acetoin to 2,3-BD was actived. As a result, 2,3-BD concentration reached a peak at 30 h (4.98 g/L), and then remained steady until the end of fermentation. Moreover, 2 g/L of lactic acid was produced, whereas acetoin decreased to zero at the end of cultivation. Besides acetoin and lactic acid, traces of succinic acid (less than 0.2 g/L) were also detected in the culture medium.

3.5. Determination of 2,3-BD and Acetoin Stereoisomeric Forms

To further understand the mechanism of ACR/BDH enzymes involved in the metabolic pathway of 2,3-BD production, the isomers of the produced acetoin and 2,3-BD were identified. R-acetoin is produced under anaerobic conditions from acetolactate, whilst S-acetoin is the product of aerobic fermentations from diacetyl. The existence of various ACR/BDHs in native 2,3-BD producers leads to different 2,3-BD isomers [54]. Interestingly, *B. subtilis* produced high optical purity of R- acetoin (100%) and (R,R)-2,3-BD (100%) throughout the cultivation under uncontrolled DO regime (Figure 3). This could imply that the limited presence of oxygen promoted the (R,R)-ACR enzyme that catalyses the conversion of R-acetoin to (R,R)-2,3-BD during the exponential growth phase. On the contrary, during the late stationary phase and after sugar depletion, (R,R)-BDH seemed to be active and converted (R,R)-2,3-BD to R-acetoin. This is the first study that reports on optically pure (~100% purity) R-acetoin and (R,R)-2,3-BD isomers produced by the non-pathogenic wild-type strain *B. subtilis* DG5. This finding was consistent with the work of Fu et al. [55] who revealed that the wild-type *B. subtilis* 168 generated only (R,R)-2,3-butanediol (purity > 99%) under low oxygen conditions [55]. Most studies in the literature have reported that wild-type strains of *B. subtilis* produce a mixture of (R,R) and meso-2,3-BD with a ratio of 3:2 [1,2,56–58]. Optically active forms of 2,3-BD are industrially important as chiral groups for the production of high-value chemicals and pharmaceutical products [59].

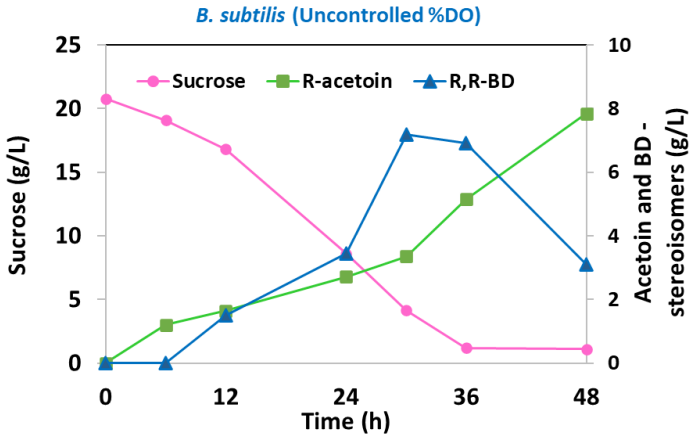


Figure 3. Time courses of acetoin and 2,3-BD isomers produced by *B. subtilis* from sucrose-based medium in 2 L bioreactor with uncontrolled DO at 30 °C, pH 6, aeration rate 1.0 vvm.

3.6. Investigation of Metabolic Pathways in B. subtilis FSBC 322 by Enzyme Activity Assay

As one pair of cofactors, NADH and NAD^+ play a vital role in more than 300 biochemical reactions including oxidation and reduction [60,61], which help maintain a constant redox state for microbial cell metabolism [62]. The 2,3-BD pathway participates in the regulation of NAD^+/NADH ratio in bacterial cells. As shown in Figure 4, acetoin can be converted into 2,3-BD by acetoin reductase (ACR) with the concomitant oxidation of NADH to NAD^+. Thus, the biosynthesis of 2,3-BD is likely driven by the proportion of a rate-limiting factor, particularly ACR activity, and/or NADH levels [24]. During the catalytic reaction of butanediol dehydrogenase (BDH), 2,3-BD is reversibly transformed to acetoin concomitantly with the reduction of NAD^+ to NADH. Consequently, NAD^+ is depleted, whilst NADH and acetoin are produced [53]. Thus, a driving force carried out by NADH oxidase (NOX) is required to regenerate NAD^+ from NADH to maintain a constant redox state in the bacterial cell. NOX reduces oxygen to produce either water or hydrogen peroxide [63].

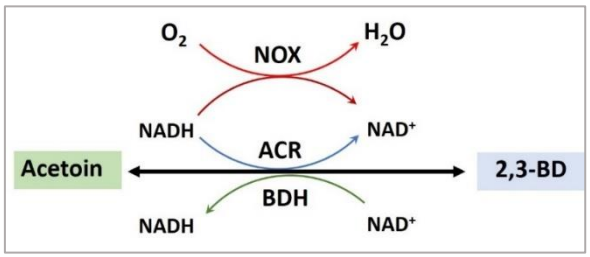


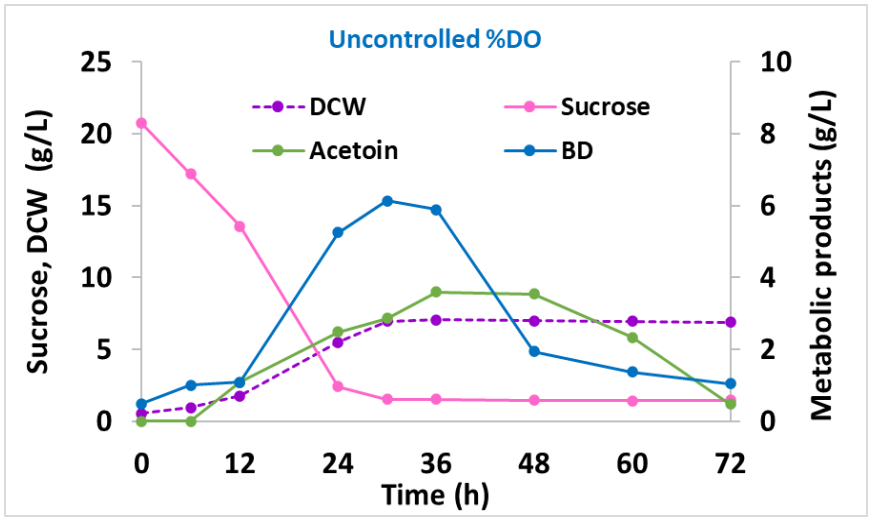
Figure 4. Enzymatic NAD regeneration system in microbial cell for the production of 2,3-BD and acetoin [5]. ACR: acetoin reductase; BDH: 2,3-butanediol dehydrogenase; NOX: NADH oxidase.

Considering the above, in order to evaluate the enzyme activities involved in *B. subtilis* acetoin reduction and 2,3-BD oxidation, the reductive reaction utilising acetoin and NADH as well as the oxidative reaction using 2,3-BD and NAD^+ were determined in crude extracts of *B. subtilis* cultures obtained from various DO control patterns. As shown in Table 4, particularly, at 36 h, the highest specific activity of ACR (13.79 U/mg) was obtained with uncontrolled DO, followed by the two-stage DO control (10.17 U/mg), whereas only 0.97 U/mg of ACR was detected with controlling DO at 5% with cascade agitation throughout the cultivation. These results were correlated to the concentrations of the produced 2,3-BD in each DO control strategy at 36 h (Figure 5), where higher concentrations of 2,3-BD (~6 g/L) were produced under uncontrolled DO and during the anaerobic phases of the two-stage DO control techniques. On the other hand, very low concentrations of 2,3-BD (<0.5 g/L) were detected when controlling DO at 5% throughout cultivation.

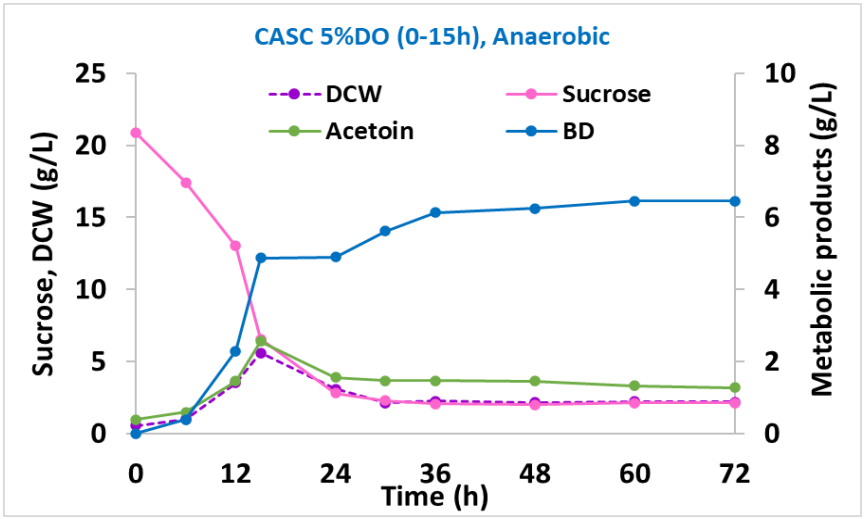
Table 4. Specific activities of ACR, BDH, and NOX obtained in *B. subtilis* during batch fermentations with different DO control strategies.

Cultivation Conditions	Fermentation Time (h)	Specific Enzyme Activity		
		ACR (U/mg)	BDH (U/mg)	NOX (mU/mg)
Uncontrolled DO	12	2.51 ± 0.40^{ab}	0.00 ± 0.00^{a}	2.69 ± 0.39^{a}
	36	13.79 ± 0.51^{A}	5.92 ± 0.81^{A}	4.01 ± 0.46^{A}
Cascade 5%DO (15 h), Anaerobic	12	4.19 ± 0.06^{a}	0.28 ± 0.12^{a}	2.00 ± 0.04^{a}
	36	10.17 ± 0.59^{B}	0.00 ± 0.00^{B}	0.92 ± 0.00^{B}
Cascade 5%DO throughout	12	1.29 ± 0.61^{b}	0.00 ± 0.00^{a}	14.69 ± 0.22^{b}
	36	0.97 ± 0.13^{C}	0.33 ± 0.47^{B}	12.79 ± 0.14^{C}

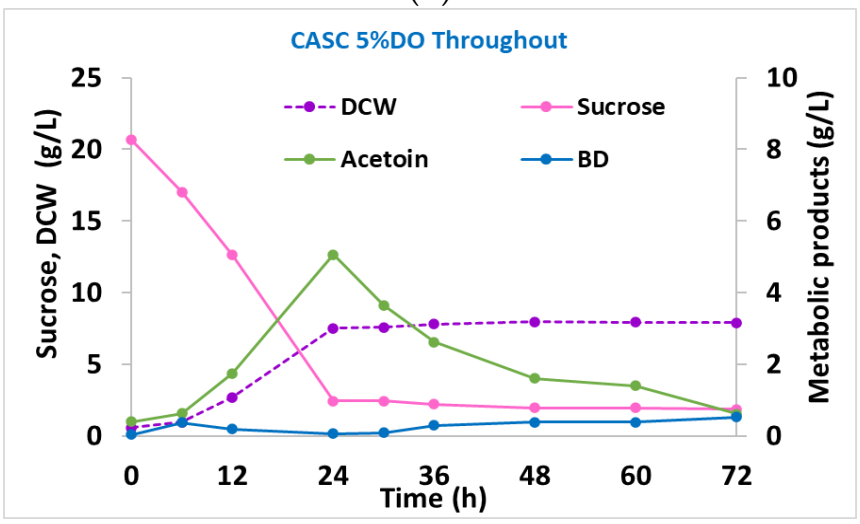
Indicated values are reported as means ± standard deviation. Values with the different superscript letters (within same column) are significantly different ($p < 0.05$). Data are average values and standard deviations of duplicate measurements. All culture conditions were conducted in 5-L bioreactors at 30 °C, pH 6.0 using MMRS with sucrose 20 g/L and corn steep liquor 20 g/L.



(**a**)



(**b**)



(**c**)

Figure 5. Metabolite production during cultivation with (**a**) uncontrolled DO, (**b**) two-stage DO regime, and (**c**) ontrolling DO at 5% throughout using *B. subtilis* in MMRS medium in 5 L benchtop bioreactor at 30 °C, pH 6.0, agitation speed 180 rpm, aeration 1.0 vvm.

In terms of the specific BDH activities, the highest BDH activity of 5.92 U/mg was detected in uncontrolled DO cultures and was 2-fold lower than the specific ACR activity (13.79 U/mg) at 36 h of cultivation, indicating that the reversible reaction of BDH was also active during this period of time; however, the reductive reaction was more favourable than the oxidative reaction. Thus, the strain produced reasonable concentrations (6 g/L) of 2,3-BD, with lower concentrations of acetoin (3.9 g/L). These results were consistent with Cho et al. [64], in which the ACR activity of *Klebsiella oxytoca* M1 was higher than BDH activity, leading to higher 2,3-BD concentrations compared to acetoin. This demonstrates that ACR is a key enzyme for acetoin conversion to 2,3-BD [17,65]. Moreover, the reversible reaction of BDHs was not active in the other two DO control strategies as no BDH activity was detected. The rationale could be that under oxic conditions in the case of controlling the DO at 5% throughout cultivation, relatively high acetoin was accumulated and was not reduced to 2,3-BD, thus NAD^+ was not generated. Consequently, the BDH enzyme was not active due to the limited presence of substrate (2,3-BD) and coenzyme (NAD^+). During anaerobic conditions (36 h), the ACR enzyme was much more active (10.17 U/mg), than BDH (0.0 mU/mg). This indicated that *B.subtilis* could not effectively convert 2,3-BD to acetoin with low or no BDH activity and the limited presence of NAD^+ [5].

Considering NADH oxidase (NOX), it should be mentioned that both ACR and NOX are NADH-dependent enzymes; ACR is activated under low oxygen levels, whilst NOX is activated under high oxygen availability. At low DO levels, considerably lower levels of NOX activity were noted (4.01 mU/mg for uncontrolled DO and 0.92 mU/mg during the anaerobic phases of the two-stage DO control strategies), resulting in less competition for NADH in the cell and redirection of the carbon flux towards 2,3-BD production. A possible reason for this was that NOX was not substantially active under low oxygen conditions [66]. High levels of NOX activity (12.79 mU/mg) were noted at cultures where DO was controlled at 5%, at 36 h. This could imply that intracellular NADH was oxidised and that NAD^+ was continuously regenerated by NOX activity. Thus, the intracellular NAD^+ pools in *B.subtilis* increased. In the meantime, NADH was regenerated by BDH enzyme for maintaining a constant redox state, thus kept persisting on acetoin production to 3 g/L. This result is in consistent with Bao [5] who demonstrated that NAD^+ was regenerated by over-expression of NOX.

3.7. Enhancing 2,3-BD Production by Fed Batch Fermentation

As observed in batch fermentation, sugar depletion appeared to have a critical role in influencing the conversion of 2,3-BD back into acetoin. To further improve the production of 2,3-BD and reduce the formation of acetoin, a fed-batch process was performed. Sucrose was fed into the bioreactor when sucrose dropped to around 10 g/L until no further increase in 2,3-BD production or sucrose consumption was observed. As shown in Figure 6, maximum cell biomass of 8.64 g/L was obtained at 108 h. The maximum concentration of 2,3-BD obtained from fed-batch fermentations was 42.31 g/L with a yield of 0.52 g/g and a productivity of 0.33 g/L/h at 130 h of fermentation. Interestingly, even though acetoin was still observed and seemed to increase towards the end of fermentation, the ratio of acetoin to 2,3-BD (0.20) in the fed batch fermentation was considerably reduced (~60%) compared to its ratio (0.48) in the batch fermentations. It is likely that high sugar provision was enough to cover NADH demand for microbial cell metabolism, therefore the reverse transformation of 2,3-BD to acetoin was no longer necessary for NADH regeneration [43]. Other by-products, i.e., ethanol, lactic acid, succinic acid were only produced in traces. The increase of acetoin towards the end of cultivation, concurring with a decline in 2,3-BD, could be the reason that the sucrose consumption rate seemed to reduce after 96 h and hence, the BDH that converts 2,3-BD to acetoin was activated to regenerate NADH for cell maintenance.

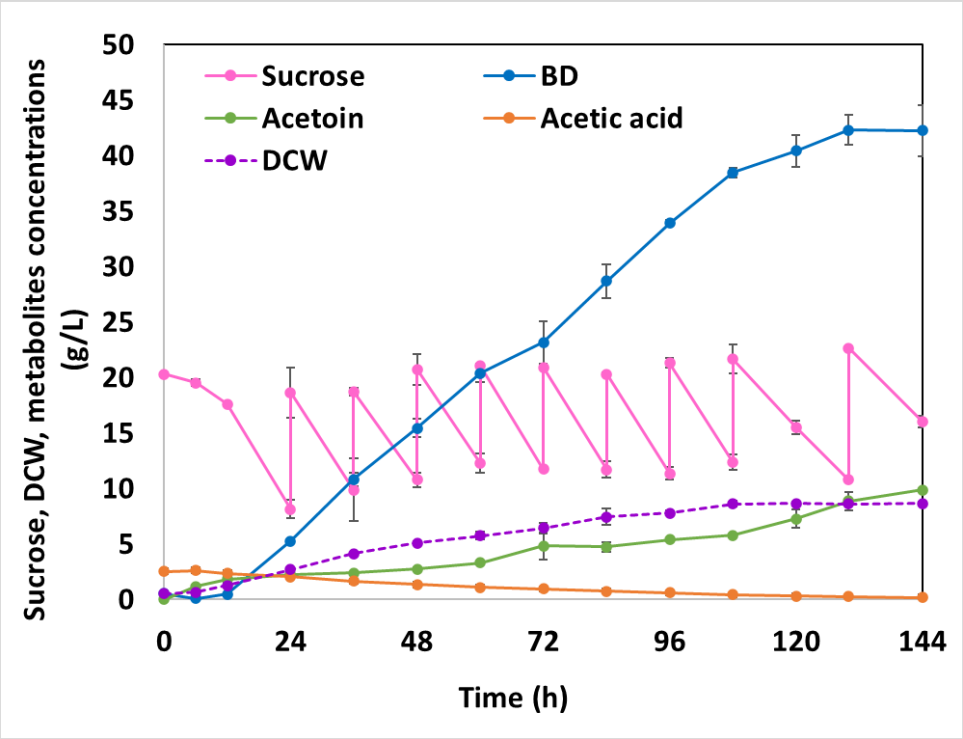


Figure 6. Fed-batch fermentation by *B. subtilis* in MMRS medium in 2 L benchtop bioreactor with uncontrolled %DO and a constant residual sugar feeding method, at 30 °C, pH 6.0, agitation speed 180 rpm, aeration 1.0 vvm.

4. Conclusions

Increasing concerns for sustainability and global climate change have driven the development of sustainable production bio-based chemicals. 2,3-BD is a promising compound with a wide range of industrial applications and can be produced by various microorganisms. Microbial 2,3-BD production still experiences production challenges through fermentation due to several factors. Oxygen supply conditions are one of the most critical parameters affecting 2,3-BD production. In the present study, *B. subtilis*, a non-pathogenic wild-type strain was used for evaluating 2,3-BD production in sucrose-based media. The result showed that corn steep liquor could be employed as an alternative low-cost nitrogen source to replace more expensive nitrogen (yeast extract, meat extract, and peptone) in Modified MRS medium. This could contribute to the reduction of production cost on large scale 2,3-BD production. Moreover, the evaluation of dissolved oxygen concentrations showed that the limited presence of oxygen produced a higher concentration of 2,3-BD, whilst the presence of oxygen or microaerophilic conditions promoted bacterial growth and acetoin (by-product) formation. Thus, batch cultures with uncontrolled DO have the potential for developing an efficient and economically viable process for the production of high optically pure (R,R)-2,3-BD (purity ~ 100%). Fed batch processes can further enhance 2,3-BD production, by overcoming metabolic changes related to substrate depletion and reverse activity of acetoin reductase.

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