

RESEARCH ARTICLE

Bioprocess development for endospore production by *Bacillus coagulans* using an optimized chemically defined medium

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Abstract

Bacillus coagulans is a promising probiotic, because it combines probiotic properties of *Lactobacillus* and the ability of *Bacillus* to form endospores. Due to this hybrid relationship, cultivation of this organism is challenging. As the probiotics market continues to grow, there is a new focus on the production of these microorganisms. In this work, a strain-specific bioprocess for *B. coagulans* was developed to support growth on one hand and ensure sporulation on the other hand. This circumstance is not trivial, since these two metabolic states are contrary. The developed bioprocess uses a modified chemically defined medium which was further investigated in a one-factor-at-a-time assay after adaptation. A transfer from the shake flask to the bioreactor was successfully demonstrated in the scope of this work. The investigated process parameters included temperature, agitation and pH-control. Especially the pH-control improved the sporulation in the bioreactor when compared to shake flasks. The bioprocess resulted in a sporulation efficiency of 80%–90%. This corresponds to a sevenfold increase in sporulation efficiency due to a transfer to the bioreactor with pH-control. Additionally, a design of experiment (DoE) was conducted to test the robustness of the bioprocess. This experiment validated the beforementioned sporulation efficiency for the developed bioprocess. Afterwards the bioprocess was then scaled up from a 1 L scale to a 10 L bioreactor scale. A comparable sporulation efficiency of 80% as in the small scale was achieved. The developed bioprocess facilitates the upscaling and application to an industrial scale, and can thus help meet the increasing market for probiotics.

KEYWORDS

Bacillus coagulans, bioprocess development, defined medium, optimization, spore production

Abbreviations: CP, center point; DBSM, defined *Bacillus* sporulation medium; DCW, dry cell weight; DoE, design of experiment; LAB, lactic acid bacteria; OFAT, one-factor-at-a-time; SFLAB, spore-forming lactic acid bacteria.

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1 | INTRODUCTION

1.1 | Endospores and sporulation of *Bacillus* spp.

Survival strategies are a major factor for bacterial populations to overcome long-lasting or intense environmental stress. The bacterial genera *Bacillus* are able to form endospores in a biochemically complex process [1]. Especially *Bacillus subtilis* as a model organism with the capability to sporulate was intensively examined [2].

Endospores are metabolically dormant cell types formed cell-density dependent after nutrient depletion or other environmental stress such as UV-radiation, acid or heat [1, 3–5]. In a sporulating cell an asymmetric division is performed, separating it into mother cell and forespore. After an eight step process the autolysis of the mother cell releases the matured endospore [1, 2, 6–9]. This cell type is largely dehydrated [10] and includes a typical biomarker called dipicolinic acid [6, 11–13]. Especially the nutrient depletion acts as the main trigger for sporulation and, thus prevents active growth and sporulation at the same time [1, 3].

1.2 | Characteristics and biotechnological application of *B. coagulans*

1.2.1 | Overview of the organism *B. coagulans*

Lactic acid bacteria (LAB) such as *Lactobacillus* are often used in the dairy industry and as probiotics in food and feed supplements [3]. A subcategory to the LAB are spore-forming lactic acid bacteria (SFLAB) such as *B. coagulans* which show as endospores a higher acid tolerance and overall resistance to environmental stress [3, 14]. Belonging to the SFLAB, *B. coagulans* was first isolated in spoiled tinned milk [3, 14, 15] and is a GRAS (generally regarded as safe) organism [16, 17]. The homofermentative lactate producer is facultative anaerobe and facultative thermophile [3]. Its acid tolerance and nutrient needs are phylogenetically linked to the relationship with the genera *Lactobacillus* [3, 18].

1.2.2 | Application of *B. coagulans* as a probiotic

The applications of LAB and SFLAB often overlap due to their similar metabolites and characteristics. However, the endospores formed by SFLAB show a great advantage for industrial application [19–23]. Probiotics contribute to improved host health by actively preventing colonization of the gut against pathogens. They also interact with the

PRACTICAL APPLICATION

Endospores gain importance in various fields of application due to their unique characteristics and endurance of harsh environmental stress. Of particular note is the organism *Bacillus coagulans*, which combines probiotic properties of *Lactobacillus* with the resistance of *Bacillus* endospores. Due to an increasing demand for this organism as a probiotic, bioprocess development for *B. coagulans* is particularly interesting but currently still economically challenging. In this work, a strain-specific bioprocess for *B. coagulans* was developed and upscaled. For this purpose, a strain selection was followed by a media screening. Afterwards, a defined medium was tested and further modified, which ensures high sporulation efficiency. This bioprocess, which was validated by robustness testing in a design of experiment (DoE), enables the sometimes laborious and strain-dependent endospore-production of *B. coagulans* with economic productivity

immune system and produce metabolites and antimicrobial substances that are beneficial to the host [24–26]. *B. coagulans* possesses important properties that are useful for the application as a probiotic [12, 19, 27–29]. The survival of endospores in gastric acid leads to germination of endospores and multiplication in the intestine of the host [19–22, 30]. The production of lactate and other metabolites allows the bacterium to proliferate as a commensal in the intestine [19, 30]. The heat tolerance of *B. coagulans* leads to a higher vitality of endospores during product processing and also prolongs the shelf life [16, 31–33].

The probiotic efficacy of *B. coagulans* has been investigated and proven in a large number of different studies. Here, secreted enzymes cause a positive effect on host health [16, 17, 34, 35]. In addition, an improved food availability can be proven, which is based on the release of amino acids from the food by *B. coagulans* [19]. Further, probiotic administration of *B. coagulans* can reduce inflammation in the digestive tract and thus facilitate nutrient absorption [19, 36].

1.2.3 | Bioprocess development for endospore production of *Bacillus* spp.

The biotechnological production of endospores is a non-trivial task, as the process should support both growth

and sporulation in equal parts. In addition to strain selection [3, 33], media composition is a particularly important issue here [37–39]. Essential for sporulation are the mineral salts such as manganese and calcium [6, 38, 40–46]. Likewise, a too high glucose concentration or a too rich medium can trigger catabolite repression [47, 48]. Further, the cultivation parameters should be carefully investigated, which can then be upscaled from shake flask in laboratory scale to a controlled bioreactor in industrial scale [6, 49–55]. Special attention should be paid to pH-control [5, 48, 56–63] of the process as well as aeration [5, 6, 58, 64–66] and temperature [3, 19, 48, 49, 51, 55, 57, 58, 62, 67–71]. Based on the preliminary work, a screening and adjustment of the parameters should therefore be carried out. Eventually, a strain-specific bioprocess can be developed resulting in high sporulation efficiencies [3].

In this presented work, a bioprocess was developed which supports growth and sporulation of *B. coagulans* in a chemically defined medium. First, strain selection and initial cultivation parameter screenings in shake flasks were conducted. Further, the bioprocess was transferred to a bioreactor and was eventually upscaled.

2 | MATERIALS AND METHODS

2.1 | Chemicals

For the conducted experiments, bulk chemicals for media preparation were obtained by Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma-Aldrich (St. Louis, Missouri, USA) and Merck (Darmstadt, Germany). All media were prepared with deionized water from a Sartorius Arium device (Sartorius Stedim Biotech, Göttingen, Germany).

2.2 | Media composition

All media compounds were dissolved in purified water and the pH was adjusted. Sterilization of the media was ensured by autoclaving at 121°C for 30 min. Glucose, mineral salt and vitamin solutions were sterilized using a 0.2 µm filter (Filtropur S, Sarstedt) and added under sterile conditions before inoculation of the media.

2.3 | Storage medium

For the preparation of storage medium (after DSMZ (German Collection of Microorganisms, *Deutsche Sammlung von Mikroorganismen und Zellkulturen*)) the following

chemicals were used [g L⁻¹]: 5.0 peptone (meat) and 3.0 meat extract. The was adjusted to a pH of 7.0 by using 1 mol L⁻¹ potassium hydroxide.

2.4 | Standard medium

The standard medium after DSMZ was prepared with the following chemicals [g L⁻¹]: 7.8 peptone (meat), 7.8 tryptone/peptone (casein), 5.6 sodium chloride, 2.8 yeast extract, 1.0 D(+) glucose. The pH was adjusted to pH 7.5 by using 3 mol L⁻¹ potassium hydroxide.

2.5 | PNY medium

For the preparation of PNY medium following media components were dissolved in purified water [g L⁻¹]: 5.0 peptone (casein), 5.0 yeast extract, 5.0 D(+) glucose, 0.5 KH₂PO₄, 0.5 K₂HPO₄ and 0.3 MgSO₄. After sterilization a mineral salt solution (1% v/v) was added containing [g 50 mL⁻¹]: 0.5 NaCl, 0.8 MnSO₄·5 H₂O, 0.9 FeSO₄·7 H₂O, 0.08 CuSO₄·5 H₂O, 0.08 ZnSO₄·7 H₂O, 0.08 CoSO₄·7 H₂O. The pH of the completed medium was adjusted to pH 5.8 by using 1 mol L⁻¹ hydrochloric acid.

2.6 | Super broth medium

The super broth medium was prepared with the following chemicals [g L⁻¹]: 24.0 yeast extract, 20.0 tryptone (casein), 0.5 sodium hydroxide, 0.19 potassium chloride, 0.24 MgSO₄. The pH was adjusted to pH 7.3 using 3 mol L⁻¹ potassium hydroxide.

2.7 | Defined *Bacillus* sporulation medium (DBSM)

For the media preparation the following L-amino acids with the concentration of 1 g L⁻¹ were used: alanine, arginine, cysteine, glutamic acid, histidine, isoleucine, leucine, methionine, threonine, tyrosine, valine. Additionally, DBSM contained the following chemicals [g L⁻¹]: 0.5 MgSO₄·7 H₂O, 0.05 MnSO₄·H₂O, 0.02 FeSO₄·7 H₂O, 0.3 KH₂PO₄, 0.3 K₂HPO₄·7 H₂O, 2.0 sodium acetate, 1.0 ammonium citrate (dibasic). Furthermore, 5 mL (v/v) vitamin mix containing 5 mg L⁻¹ of the following vitamins respectively was added after sterilization: folic acid, thiamine-HCl, biotin, pyridoxine-HCl, riboflavin, nicotinic acid. The medium was also supplemented with 10 g L⁻¹ D(+) glucose after sterilization. The pH was adjusted using 2 mol L⁻¹ hydrochloric acid to pH 6.0.

2.8 | Bacterial strains and culture conditions

Four strains of *B. coagulans* were tested in a strain selection before developing a bioprocess for the cultivation of endospores and were provided by a biotechnological company in Germany. In the following experiments the selected strain was used exclusively. The bacteria cultures were stored in 20% (v/v) glycerol at -80°C . All shake flasks contained baffles for better aeration.

2.9 | Preculture

Precultures were conducted in 125 or 500 mL shake flasks with 1.6% (v/v) glycerol culture in standard medium (2.2.2), respectively. The flasks were incubated for 17 h at 150 rpm and thermophilic temperatures between $45\text{--}50^{\circ}\text{C}$.

2.10 | Main culture in shake flask cultivations

From the preculture 125 or 500 mL shake flasks were inoculated with a start OD_{600} of 0.3 rel.AU in 12.5 or 50 mL medium, respectively. The media composition varied depending on the experimental setup. The main cultures were incubated at thermophilic temperatures and 150 rpm if not otherwise stated.

2.11 | Batch-mode bioreactor cultivations

The bioreactor cultivations were conducted in a 2 L Biostat A plus (Sartorius AG) using 1 L working volume of DBSM. Inoculation was set to an OD_{600} of 0.3 rel.AU from 17 h old precultures cultivated in standard medium. A constant control of pH, temperature and aeration was maintained. For this, the pH was set to 6.0, temperature to thermophilic ranges and the dissolved oxygen (DO) was maintained above 30% with air by varying the agitation between 200–500 rpm. An antifoaming agent was added as needed. The pH was adjusted by using 1 mol L^{-1} hydrochloric acid or 3 mol L^{-1} sodium hydroxide.

2.12 | DoE

The DoE was conducted in a 2 L Biostat A plus (Sartorius AG) using 1 L working volume in DBSM. First an Ishikawa diagram was made and a FMEA (failure mode and effect analysis) was conducted (both in [Supporting information](#)). In this approach the classification of proba-

bility and severity (level 1–5) was used. The factor numbers were multiplied, resulting in the risk priority number (RPN). The three parameters with the highest RPN (temperature, incubation time of preculture and pH, data concluded in [Supporting information](#)) were chosen and tested in a quadratic model. The output-value was selected as sporulation efficiency. To limit the needed experiments, a 3D partial factorial design was chosen.

2.13 | Analysis of offline bioprocess data

The cultivations were assessed using offline methods as seen in the following subsections or online data acquisition during bioreactor cultivations.

2.14 | Optical density

The optical density at 600 nm (OD_{600}) was measured with a Libra S50 (Biochrom GmbH) spectrophotometer using 0.9% (w/v) NaCl solution as a blank. Higher data values than 0.5 rel.AU were diluted using 0.9% (w/v) NaCl solution.

2.15 | Dry cell weight

The dry cell weight (DCW) was determined in 2 mL reaction tubes which were dried at 70°C for 24 h minimum. The reaction tubes were subsequently cooled down to room temperature in a desiccator and then weighed with a precision balance (Sartorius AG) for tare weight determination. After sampling, 1 mL was transferred to the reaction tubes and centrifuged at 13,300 rpm and 4°C for 5 min. The supernatant was further used for the determination of glucose and lactate concentration (2.4.3). The pellet was subsequently dried at 70°C for 3 days and cooled to room temperature in a desiccator for several hours. In the end, the samples were weighed with a precision balance and the DCW was calculated by subtracting the tare weight.

2.16 | Glucose and lactate concentration

Glucose and lactate concentration from bioreactor cultivations using chemically defined medium were simultaneously determined using an YSI 2500 (Kreienbaum Neoscience). This device measures glucose and lactate enzymatically. 200 μL of the supernatant resulting from DCW determination were transferred to a 96 well plate and measured cell-free at room temperature.

2.17 | Calculation of growth rate and doubling time

For the comparison of different media, the growth rate μ_{\max} during exponential growth was calculated following Equation (1) ($\ln(\text{OD}) = \ln$ of the OD_{600} from the end or start of exponential phase; $t = \text{time [h]}$ of the end or start of exponential phase).

$$\mu_{\max} \text{ [h}^{-1}\text{]} = \frac{\ln(\text{OD})_{\text{end}} - \ln(\text{OD})_{\text{start}}}{t_{\text{end}} - t_{\text{start}}} \quad (1)$$

The doubling time t_d was calculated as stated in Equation (2).

$$t_d \text{ [h]} = \frac{\ln(2)}{\mu_{\max}} \quad (2)$$

2.18 | Microscopic cell and spore count

Cell and spore count were determined using a dark-lined Petroff chamber (depth 0.02 mm). Microscopic determination was performed using a phase-contrast microscope with a magnification of 40x. If necessary, the samples were diluted with 0.9% (w/v) NaCl solution and applied to the counting chamber. For statistical analysis, four large squares with 16 small squares each were counted separately for vegetative cells, endospores and their precursors. The cell or spore number was calculated using Equation (3).

$$\begin{aligned} &\text{cell number [cells mL}^{-1}\text{]} \\ &= \frac{\emptyset \text{ cell count (small squares)} \times \text{dilution factor}}{\text{volume small square (} 5 \times 10^{-8} \text{ mL)}} \quad (3) \end{aligned}$$

The total cell count is the sum of the cell and spore counts. Sporulation efficiency was calculated as in Equation (4).

$$\begin{aligned} &\text{sporulation efficiency [\%]} \\ &= \frac{\text{spore number [spores mL}^{-1}\text{]}}{\left(\text{cell number [cells mL}^{-1}\text{]} + \text{spore number [spores mL}^{-1}\text{]}\right)} \quad (4) \end{aligned}$$

3 | RESULTS

3.1 | Strain selection

The aim was to characterize the growth behavior of four different *B. coagulans* strains and further to perform strain

TABLE 1 Comparison of four different strains concerning the maximal growth rate μ_{\max} [h^{-1}] and doubling time t_d [h].

Strain	μ_{\max} [h^{-1}]	t_d [h]
1	0.11	6.41
2	0.24	2.91
3	0.22	2.78
4	0.27	2.57

selection. The strain was selected based on the achieved cell density and growth rate during exponential growth (μ_{\max}). All four genetically unmodified strains were cultivated in shake flasks with baffles at different temperatures. A cultivation temperature of 37°C did not support the cell growth and was not used in further cultivations. Thermophilic temperatures between 45–55°C showed higher cell densities and an improved μ_{\max} . The nutrient-poor storage medium supports the growth of all four strains at thermophilic temperatures (Table 1). A maximal OD_{600} of 2.18 rel.AU (± 0.07) and a $\mu_{\max} = 0.27 \text{ h}^{-1}$ was achieved with the selected strain (no. 4).

3.2 | Culture condition screening in shake flasks

Further, different cultivation conditions were investigated with the selected model strain. For this purpose, a focus was placed on the agitation speed, working volume in the flasks and the inoculation volume in the shake flask cultivation. All cultivations were conducted in standard medium in triplicates. Based on the publications of Posada-Urbe et al. [5], an agitation of 150 rpm was determined and experimentally confirmed. The working volume was chosen between 10% and 20% flask volume. Due to the relationship to *Lactobacillus*, a reduced oxygen consumption was accepted. The cultivations with 20% working volume were carried out and reproduced without negative influences due to an oxygen limitation. Furthermore, the cultivation temperature was set in a thermophilic range between 45–55°C. All cultivations were conducted in triplicates, except when otherwise stated.

3.3 | Media development and modification

The storage medium has a limited availability of nutrients, which is why three different nutrient-richer complex media were tested. With the previously described cultivation conditions, the growth behavior in standard medium, super broth medium and PNY medium was investigated

TABLE 2 Comparison of the three used complex media concerning the maximal growth rate μ_{\max} [h^{-1}] and doubling time t_d [h].

medium	μ_{\max} [h^{-1}]	t_d [h]
Standard	0.36	1.90
Super broth	0.40	1.72
PNY	0.39	1.77

in a shake flask. The μ_{\max} and doubling time t_d were compared and can be found in Table 2.

Complex media often have a nutrient-rich composition and therefore support the growth of many *Bacillus* spp. [1, 3, 4]. In contrast, sporulation relies on nutrient depletion and thus, a more nutrient-poorer medium could optimize this biochemical process. Here, all three media supported the growth of the organism and shortened the doubling time compared to the storage medium. The highest cell density was observed with the standard medium. Furthermore, the doubling time of the super broth medium was shorter. This medium is significantly richer than the standard medium and thus supports growth in a shorter time period.

The studied complex media on the other hand turned out to be too nutrient-rich for a successful sporulation of this *B. coagulans* strain. Therefore, and for the reason of comparability of media composition, a chemically defined medium was adapted to the organism. Initially, the defined medium MCDM3++ of Chen et al. [72] was used (showed in supplements). But this medium was designed for lactate formation by *B. coagulans*. First, calcium carbonate was replaced with calcium chloride due to solubility issues and glucose was reduced to 10 g L^{-1} . In addition, high glucose concentrations can cause catabolic repression, which inhibits growth [47, 48]. Furthermore, a glucose concentration higher than 10 g L^{-1} can inhibit the spore formation [57, 64].

A reduction of the media components by half led to a decrease in growth, which is why the medium published by Chen et al. with an amino acid concentration of 0.5 g L^{-1} [72] was prepared with an approach of twice the amino acid and vitamin concentrations (1 g L^{-1}). This resulted in a 34% increase in cell growth, which was increased up to 78% with further addition of the vitamins biotin, pyridoxine, riboflavin and niacin compared with the medium according to Chen et al. [72] and is depicted in Figure 1A). Folic acid, biotin, and niacin were identified as essential media components for *B. coagulans* growth [73–76].

The resulting medium containing 1 g L^{-1} amino acids and the vitamins folic acid, thiamine, pyridoxine, riboflavin and niacin (2.2.5) will be further referred to as Defined *Bacillus* Sporulation Medium (DBSM). For the better understanding of the amino acid requirements of

the strain, the one-factor-at-a-time (OFAT) method of the medium was performed with the standard concentration of 1 g L^{-1} . For this purpose, the amino acids were decreased to 0.1 or increased to 5.0 g L^{-1} (Figure 1B). Since glutamic acid, leucine and tyrosine are only partially soluble in water, only an increase of concentration to 2.5 g L^{-1} was performed. The growth of *B. coagulans* was then subsequently assessed by microscopic cell count and OD_{600} . This showed that individual increases in amino acid concentrations did not produce a significant increase in growth (Figure 1D). However, an increased cysteine concentration reduced growth by 23% compared to the standard of 1 g L^{-1} . Free exogenous cysteine, even in low concentrations, is reactive and interferes with different amino acid pathways in microorganisms [77–80]. In *E. coli* free cysteine blocks the isoleucine synthesis and is thus growth inhibitory and cytotoxic [78, 81, 82]. A decreased cell number due to increased cysteine concentration also had a negative effect on sporulation. This circumstance can be explained by quorum sensing, in which sporulation is induced via signal induction in a cell number-dependent manner [3].

Decreasing individual amino acids also showed no significant growth inhibition (Figure 1C). However, valine at lowered concentrations was found to inhibit growth by up to 77%. The reduced growth due to the reduction of valine can be explained by the fact that this amino acid can stimulate the growth of the thermophilic *B. coagulans* [83]. The reduction of alanine leads to an increase in spore yield, since alanine, like other purines, acts as an inducer of germination. Valine can also have this influence on sporulation [6]. The difference in measured cell density between OD_{600} and microscopic cell count determination with glutamic acid reduction is striking. However, glutamic acid has a stimulating effect on the growth of *B. coagulans* [72].

Further, single-omission experiments could be performed. Here, one amino acid at a time would not be added to the medium. This would provide a better understanding of the amino acid requirement of the *B. coagulans* strain.

3.4 | Batch-mode bioreactor cultivation

Upscaling to a bioreactor was performed with the DBSM. For this purpose, a parameter screening was carried out in a 1 L scale. Initially, the parameters used were based on the reactor cultivations in the 1 L scale with the super broth medium, since growth was successfully supported here in pre-experiments. The aeration was controlled by agitation and air to maintain the DO above 30%. Boniolo et al. noted in their work that the DO should be set above 30% [65]. Monteiro et al. and Sarrafzadeh et al. also used this setting for reactor cultivations to support growth and sporulation [57, 58].

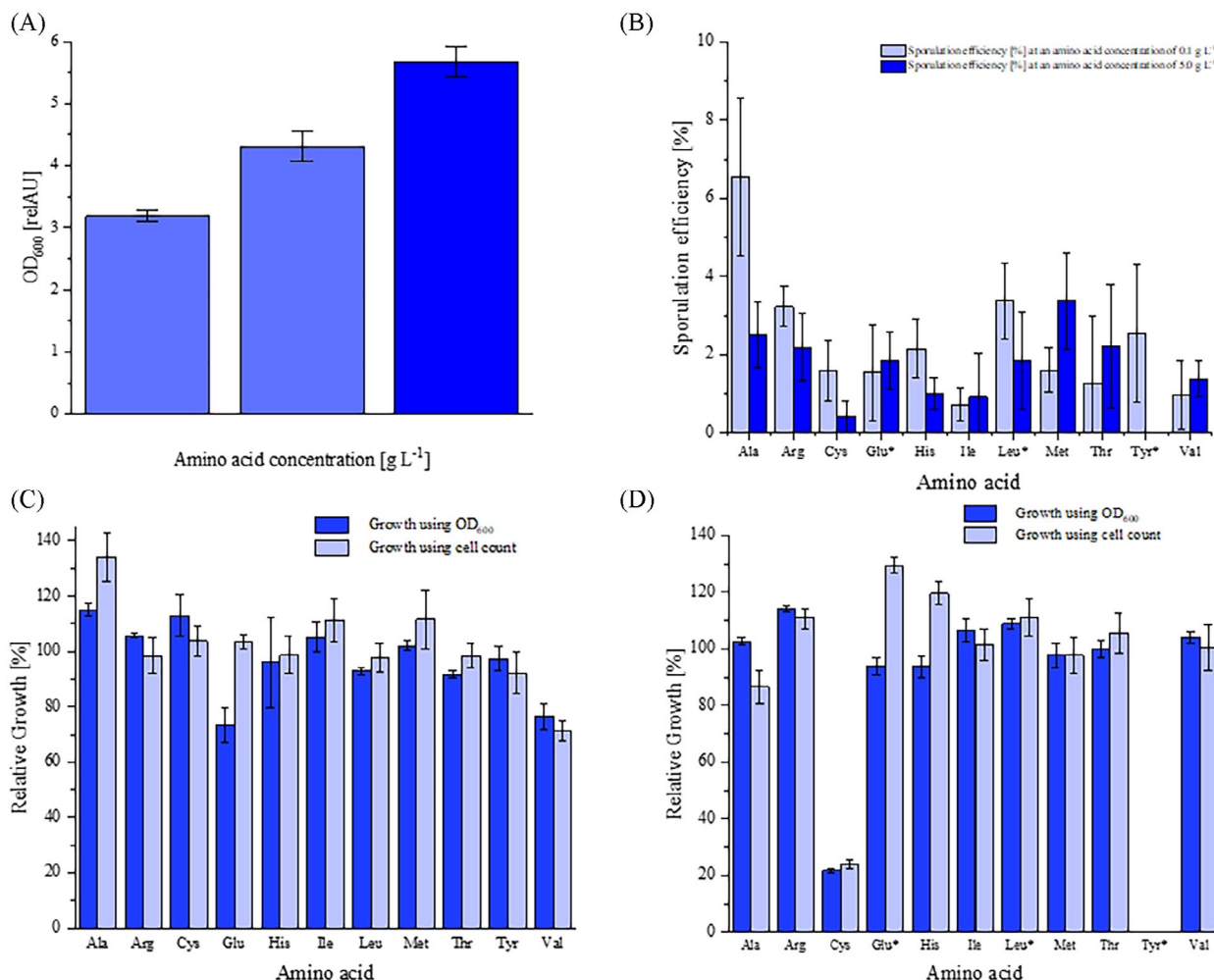


FIGURE 1 (A) Cultivation of *B. coagulans* in the defined medium after Chen et al. (A) with the amino acid concentration of Chen et al. of 0.5 g L⁻¹, (B) with an amino acid concentration of 1.0 g L⁻¹, and (C) with an amino acid concentration of 1.0 g L⁻¹ and an additional vitamin solution containing 5 mg L⁻¹, respectively. (B) Depiction of the sporulation efficiency [%] in a medium with decreased amino acid concentrations to 0.1 g L⁻¹ (light blue) or an increase in amino acid concentration to 5.0 g L⁻¹ (dark blue). The efficiency results as the percentage of spores in the total cell count. *) due to limited solubility the amino acid concentration was limited to an increase of 2.5 g L⁻¹ instead of 5.0 g L⁻¹. (C) Illustration of the relative growth [%] when the concentration of individual amino acids was decreased to 0.1 g L⁻¹. The relative values of OD₆₀₀ and microscopically determined cell number compared to DBSM with the standard concentration of 1.0 g L⁻¹ of the respective amino acid are indicated. (D) Illustration of the relative growth [%] when the concentration of individual amino acids was increased to 5.0 g L⁻¹. The relative values of OD₆₀₀ and microscopically determined cell number compared to DBSM with the standard concentration of 1.0 g L⁻¹ of the respective amino acid are indicated.

The pH-control proved to be a particularly important parameter for successful spore generation and higher cell density. The control of the pH value was especially important to counteract cell lysis due to strong acidification of the medium after lactate formation. The pH-control can improve growth [56] and also increase sporulation [48, 57–60]. A minimum pH value of 5.0 is necessary for sporulation. In addition, pH-control could also contribute to a more synchronized sporulation process [57]. A lack of pH-control caused a greatly reduced sporulation efficiency of 1.4% instead of 80%–90% in DBSM.

With the selected parameters in the reactor cultivation, a sporulation efficiency of 80%–90% was generated

(Figure 2). A $\mu_{\max} = 0.184 \text{ h}^{-1}$ and $t_d = 3.77 \text{ h}$ were calculated for the developed bioprocess and shows a higher sporulation efficiency than the complex media. In the complex medium a sporulation efficiency of 10% was achieved. In these reactor cultivations, no decisive lag phase could be detected after the preculture was transferred to the reactor. An increased sporulation efficiency at the beginning of the cultivation can be explained by a possible transfer of endospores from the preculture into the main culture.

Diauxia was also detected in the defined medium after 15 h of process time. In this case, a metabolite is consumed after glucose utilization, which leads to the typical diauxic growth curve. Diauxia typically occurs in *B. coagulans*.

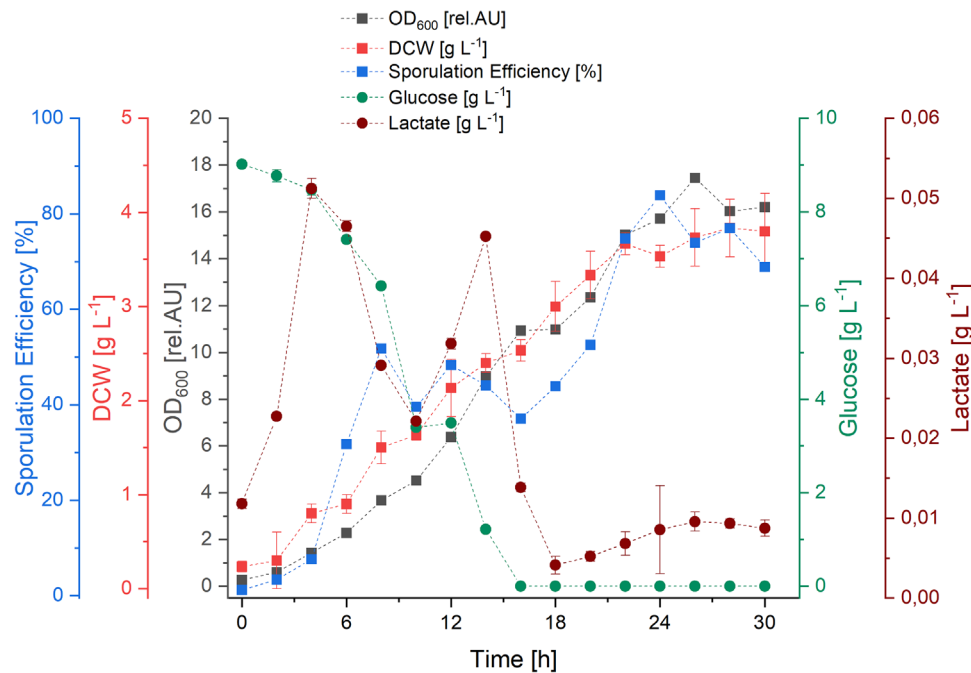


FIGURE 2 Process parameters of the developed bioprocess for *B. coagulans*. Depicted are the sporulation efficiency [%], dry cell weight (DCW) [g L⁻¹], OD₆₀₀ [rel.AU], glucose and lactate concentration [g L⁻¹] against the process time [h].

Here, lactate is formed by the bacteria in the exponential growth phase, while glucose is consumed for cell mass build-up. At the same time, a drop in pH is detected in a pH-uncontrolled bioprocess. The diauxic shift then indicates the consumption of lactate into the stationary phase and leads to a brief additional growth of the bacteria [59].

3.5 | Design of experiment robustness testing

For further upscaling of the bioprocess, the robustness of it was tested by using design of experiment (DoE). Based on the understanding of the process, an Ishikawa diagram of the process-influencing parameters was created and a risk assessment with FMEA was performed (Supporting information). Thereupon a DoE concept was created, which uses a 3D partial factorial design matrix. The standard process conditions were used as center points (CP) and carried out in triplicates. The selected parameters for robustness testing are temperature, incubation time of the preculture and pH. The sporulation efficiency after 28 h of incubation was used as an output value. A summary of the conducted DoE is depicted in Figure 3.

The developed bioprocess generated a reproducible sporulation efficiency of 80%–90% as shown in the response contour plot in Figure 3. The cultivation conditions of the CP are located in the red area in the response contour plot. Furthermore, the harvest time of

the endospores after 24 h was validated due to the highest concentration of endospores at this sample time point.

3.6 | Upscaling

The robustness-tested bioprocess was upscaled to a 10 L working volume scale in a 30 L Biostat C plus bioreactor. Further upscaling to a larger scale is important for process application and transfer to industrial production. The upscaling could be carried out successfully, as more DCW per liter and a higher cell density were achieved in the same process time compared to the 1 L scale (Figure 4). In addition, sporulation efficiency is highest at 24–26 h of about 80% process time, showing comparable results to the small scale. Also, more lactate was formed in the larger scale per liter when compared to the small scale. Taking this further, an increased working volume or scale-up can help to transfer the bioprocess more easily into an industrial application.

4 | CONCLUDING REMARKS

In this work, a bioprocess was developed for the organism *B. coagulans* which is used as a probiotic. For this purpose, a strain selection was successfully carried out in a nutrient-poor medium. Subsequently, the growth of this strain was tested in three different complex media. The growth rate,

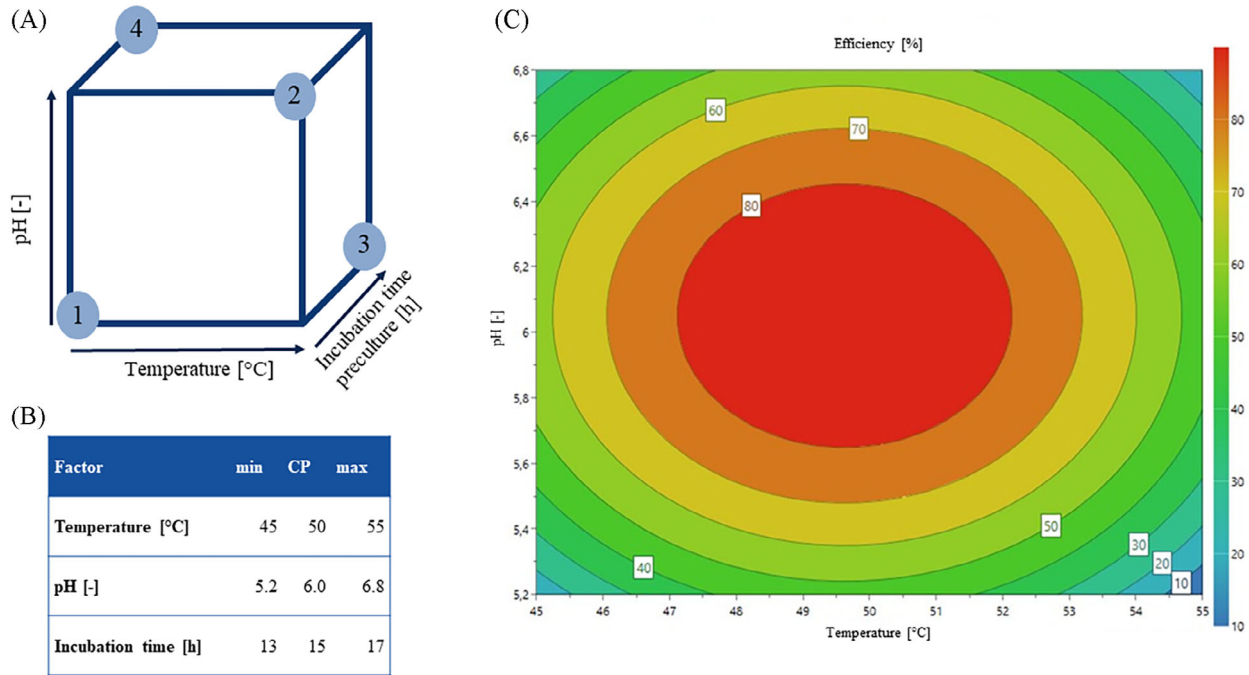


FIGURE 3 (A) Overview of the used fractional factorial 3D DoE in a quadratic model for the factors pH, temperature and incubation time of the preculture. (B) Table with the factor settings based on the described design in (A). (C) Response contour plot of the designed fractional factorial design at 28 h process time. The depicted factors are temperature and pH against the sporulation efficiency and were statistically evaluated using MODDE.

doubling time and sporulation were compared. Growth was positively influenced, but insufficient sporulation was observed in the more nutrient-rich media. Therefore, the chemically defined media according to Chen et al. [72] was tested and modified for strain-specific growth and sporulation.

The modified medium supports the growth and sporulation of *B. coagulans*. To gain a better understanding of amino acid requirements, OFAT screening was performed. Especially alanine showed an important influence on sporulation, leading to improved sporulation when decreased. Cysteine showed a negative influence

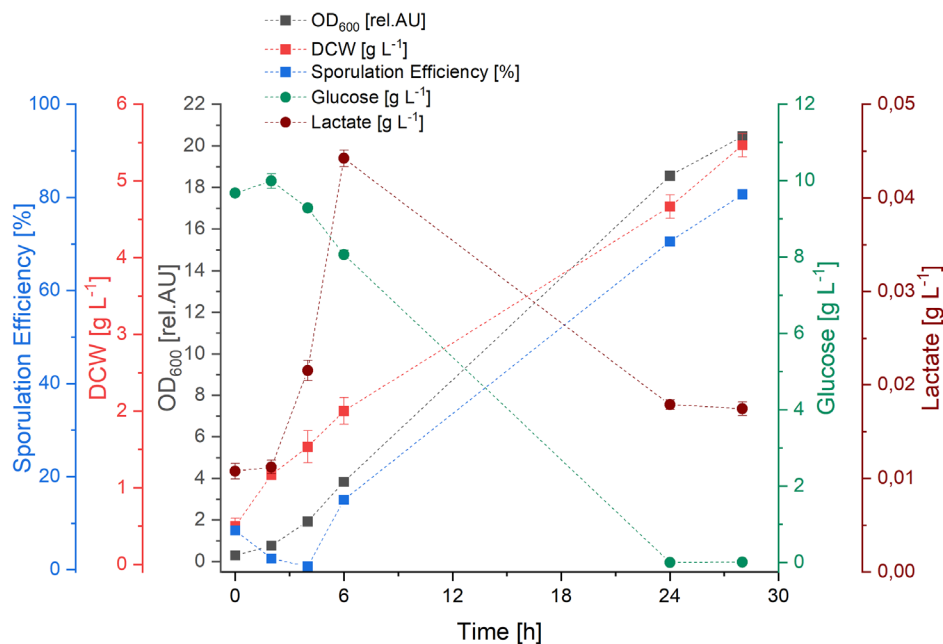


FIGURE 4 Upscaled bioprocess in 10 L working volume. Depicted are the sporulation efficiency [%], dry cell weight (DCW) [g L⁻¹], OD₆₀₀ [rel.AU], glucose and lactate concentration [g L⁻¹] against the process time [h].

in increased concentrations on the growth of this organism. Through these experiments, important influences and possible turning screws for further medium optimization could be found. In addition, the strain-specific amino acid requirement was determined.

Starting from the shake flask, the cultivation with the medium was transferred to a bioreactor and the cultivation parameters for this were screened. The control of the pH showed a great influence on the sporulation and the prevention of the lysis of the bacteria by over-acidification. The medium and the selected process parameters achieved a sporulation efficiency of 80%–90% in a 1 L scale. Neither a feed solution nor a change in the cultivation conditions was necessary, which facilitates the process in handling and application.

In process development, the deep understanding of it and the possible finding of weaknesses is especially important. Therefore, a DoE-based robustness testing of the process was performed. This confirmed the reproducibility of the CP and verified a reliable sporulation efficiency of 80%–90%.

The robustness-tested bioprocess was then transferred to a 10 L scale. In this upscaling, improved DCW and lactate concentrations were observed. Furthermore, comparable sporulation efficiencies were achieved compared to the smaller laboratory scale.

The easy and successful upscaling of the developed bioprocess may facilitate the way to transfer it to an industrial scale. Simplification of the medium by cost-effective complex media components could be a vital step in the transfer to an economic industrial scale production.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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