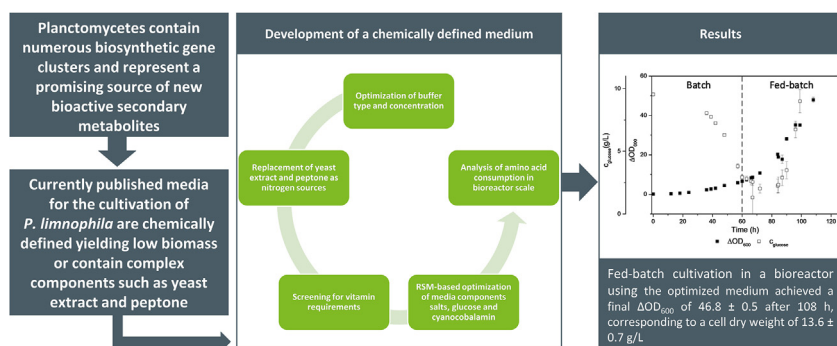




Research Article

Development of a chemically defined medium for *Planctopirus limnophila* to increase biomass productionOscar Claudius Kruppa^a, Doreen Gerlach^{a,c}, Rong Fan^{a,c,*}, Peter Czermak^{a,b,*}^a Institute of Bioprocess Engineering and Pharmaceutical Technology, University of Applied Sciences Mittelhessen, 35390 Giessen, Germany^b Faculty of Biology and Chemistry, Justus-Liebig University of Giessen, 35390 Giessen, Germany^c Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Project Group Bioresources, 35392 Giessen, Germany

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Planctomycetes is a phylum of biofilm-forming bacteria with numerous biosynthetic gene clusters, offering a promising source of new bioactive secondary metabolites. However, the current generation of chemically defined media achieves only low biomass yields, hindering research on these species. We therefore developed a chemically defined medium for the model organism *Planctopirus limnophila* to increase biomass production.

Results: We found that *P. limnophila* grows best with a 10 mM sodium phosphate buffer. The replacement of complex nitrogen sources with defined amino acid solutions did not inhibit growth. Screening for vitamin requirements revealed that only cyanocobalamin (B12) is needed for growth. We used response surface methodology to optimize the medium, resulting in concentrations of 10 g/L glucose, 34 mL/L Hutner's basal salts, 23.18 mM KNO₃, 2.318 mM NH₄Cl and 0.02 mg/L cyanocobalamin. The analysis of amino acid consumption allowed us to develop a customized amino acid solution lacking six of the amino acids present in Aminoplasmal 10%. Fed-batch cultivation in a bioreactor using the optimized medium achieved a final ΔOD₆₀₀ of 46.8 ± 0.5 after 108 h, corresponding to a cell dry weight of 13.6 ± 0.7 g/L.

Conclusions: The optimized chemically defined medium allowed us to produce larger amounts of biomass more quickly than reported in earlier studies. Further research should focus on triggering *P. limnophila* biofilm formation to activate the gene clusters responsible for secondary metabolism.

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* Corresponding authors.

E-mail addresses: rong.fan@lse.thm.de (R. Fan), peter.czermak@lse.thm.de (P. Czermak).<https://doi.org/10.1016/j.ejbt.2021.09.002>

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1. Introduction

Planctomycetes are ubiquitous bacteria that play an important role in global carbon and nitrogen cycling, including anaerobic ammonium oxidation [1,2].

Along with *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae* and some smaller groups, they belong to the Planctomycetes Verrucomicrobia Chlamydiae (PVC) superphylum [3]. Their FtsZ-free cell division mechanism which involves polar budding rather than binary fission, is unusual among bacteria [4,5]. The model organism and type species *Planctopirus limnophila* has a dimorphic life cycle, with motile daughter cells colonizing surfaces and maturing into sessile, stalked mother cells that can divide [6]. Contrary to outdated assumptions, peptidoglycan has recently been found in the cell walls of planctomycetes [7,8], and the cells can take up high-molecular-weight polysaccharides from the environment using an, as yet, unidentified mechanism [9].

Planctomycetes are found in soils worldwide [10], including mangrove wetlands [11], saline coastal soils in China [12], the rhizosphere in the Amazon rainforest [13], and forested tundra soil in Siberia [14]. Furthermore, planctomycetes are part of the microbiome of white shrimp (*Litopenaeus vannamei*) [15], soil-feeding termites (*Cubitermes* spp.) [16] and bighead carp (*Aristichthys nobilis*) [17]. Nevertheless, most species in the phylum Planctomycetes live in aquatic habitats [1], where they often colonize biotic surfaces such as microalgae and macroalgae to form biofilms [18,19,20,21,22,23,24,25]. Planctomycetal abundance often correlates with algal blooming events, suggesting that planctomycetes are involved in the degradation of algal biomass [26,27,28,29]. Planctomycetes can metabolize a variety of complex carbon sources, such as those provided by algae [24,30,31], and are resistant to many antibiotics [32], which may confer an evolutionary advantage over other heterotrophs. Given the slow growth of planctomycetes (some species have doubling times of 1 month [33]), the abundance of these bacteria in such highly-competitive niches is unexpected, especially because other heterotrophs (e.g., *Roseobacter* spp. [34]) divide much more rapidly and should out-compete them [35]. This has prompted research into planctomycetal defense mechanisms against competitors. The genomes of various planctomycetal strains contain many silent biosynthetic gene clusters and giant genes [1,30,36,37,38,39] encoding enzymes potentially for the synthesis of bioactive secondary metabolites [40,41]. Planctomycetes therefore offer a source of novel bioactive substances, and the first such metabolites were recently documented. For example, planctomycetal extracts were shown to display antimicrobial activity against Gram-positive and Gram-negative bacteria [35], and also contain phytotoxins [31] and stielericinins [42] that are thought to be involved in the modulation of biofilms.

Most microbial biosynthetic gene clusters remain silent under laboratory conditions [43] and are only activated under certain environmental conditions. We found that cultivation processes are the bottleneck in Planctomycetes research because the current generation of chemically defined media promotes slow growth and generates small amounts of biomass to work with. Controlled process conditions and the use of chemically defined media can facilitate more reproducible and traceable results. Here we describe the development of a chemically defined medium for the fresh-water

strain *P. limnophila* to promote biomass production, and a bioreactor-scale cultivation process. Different buffer systems and amino acid solutions were compared to determine their effect on cell growth, and a screening approach was used to identify vitamin requirements. We used response surface methodology (RSM) to identify inhibitory and growth-limiting concentrations of media components, and adjusted them to meet the needs of *P. limnophila*. Based on amino acid consumption during cultivation, we also developed a customized amino acid solution. Finally, we established a fed-batch cultivation process using the optimized chemically defined medium to confirm the improvement of *P. limnophila* biomass production.

2. Materials and methods

2.1. Strain

Planctopirus limnophila strain DSM 3776 was obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ), and was used as a model strain for medium development.

2.2. Media

Medium development began with the comparison of different buffers in complex PYGV medium. A modified version of medium 621 was used for this purpose, as recommended by DSMZ, and mineral salts solution (Hutner's salts) and vitamins were prepared according to DSMZ medium 621. The metal salts solution "Metals 44" for the mineral salts was prepared according to DSMZ medium 590. The PYGV medium was supplemented with 1 g/L peptone, 1 g/L yeast extract, 20 mL/L Hutner's salts, 5 mL/L vitamin solution and 5 g/L filter-sterilized glucose. Because medium 621 does not contain a buffer, we added HEPES, Tris-HCl or sodium phosphate (pH 7.5) at concentrations of 10 mM and 50 mM. 200 mM sodium phosphate buffer (pH 7.5) was prepared in deionized water. For further comparisons with chemically defined media, PYGV was supplemented with 10 mM sodium phosphate buffer.

The chemically defined medium D1 was developed by replacing yeast extract and peptone (complex nitrogen sources) with 13.6 mM KNO₃ and 1.36 mM NH₄Cl. The medium was supplemented with 20 mL/L Hutner's salts solution, 5 mL/L vitamin solution, 5 g/L filter-sterilized glucose and 10 mM sodium phosphate buffer (pH 7.5) as above.

We tested two commercial amino acid solutions: 10 mL/L of Aminoplasmal 10% (B. Braun, Melsungen, Germany) and 38.5 mL/L MEM 50x amino acids solution (Sigma-Aldrich, St. Louis, MO, USA), achieving a final amino acid concentration of 1 g/L in each case. Aminoplasmal 10% contains isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, glycine, aspartic acid, glutamic acid, proline, serine and tyrosine. MEM amino acids solution contains arginine, cysteine, proline, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine.

The D1AP base medium (D1 plus 10 mL/L Aminoplasmal 10%, 20 mL/L Hutner's salts solution, 13.6 mM KNO₃, 1.36 mM NH₄Cl, 5 g/L filter-sterilized glucose and 10 mM sodium phosphate buffer, pH 7.5) was used to screen for vitamin requirements and the

components were also varied for RSM optimization, yielding the modified D1AP medium for bioreactor cultivations (10 mM sodium phosphate buffer pH 7.5, 10 mL/L Aminoplasmal 10%, 34 mL/L Hutner's salts solution, 23.18 mM KNO₃, 2.32 mM NH₄Cl, 10 g/L glucose, 0.02 mg/L cyanocobalamin). For fed-batch cultivation, the Aminoplasmal 10% was replaced with 50 mL/L of a customized amino acid solution (ASO, Table S1) to produce D1ASO medium. All chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany).

2.3. Shake-flask cultivations

Bacteria were cultivated at 28°C in 500-mL baffled shake flasks with an operating volume of 50 mL shaking at 100 rpm in a Multitron Standard orbital shaker (Infors, Bottmingen, Switzerland). If not stated otherwise, cultures were inoculated with cryopreserved cells to an initial ΔOD_{600} of 0.1 and then incubated until cells reached stationary phase. The initial pH of the medium was 7.5 but no pH control was applied during cultivation.

2.4. Bioreactor cultivations

Bacteria were cultivated at 28°C in a 0.5-L MiniBio 500 stirred-tank bioreactor (Applikon, Delft, Netherlands) with a 0.3-L working volume. The culture was agitated at 200 rpm using two Rushton impellers and was aerated with a microsparger at 0.1 vvm. The bioreactor was equipped with pH, temperature, and dissolved oxygen probes, and the pH was maintained at 7.5 by adding 1 M NaOH as required. The initial ΔOD_{600} was 0.1.

Batch experiments were carried out using modified D1AP medium as described above. Hutner's salts, KNO₃ and NH₄Cl and deionized water were added to the bioreactor before autoclaving, and the remaining components (Aminoplasmal 10%, cyanocobalamin, sodium phosphate buffer and glucose) were then added via a syringe fitted with a sterile 0.2- μm filter.

Fed-batch experiments were carried out using D1ASO medium as described above. After batch cultivation for 60 h, the fed-batch phase was initiated with an initial feed rate calculated using Equation 1:

$$F(t_0) = m_x \times \left(\mu_{\text{set}} / Y'_{X/S} \right) / c_S \quad \text{Equation 1}$$

where m_x [g] is the current biomass in the vessel, μ_{set} [h⁻¹] is the growth rate, $Y'_{X/S}$ [g/g] is the observed biomass yield from substrate (0.35 g/g), and c_S is the glucose concentration in the feed reservoir (250 g/L). The subsequent exponential feed rate was calculated using Equation 2:

$$F(t) = F(t_0) \times e^{\mu_{\text{set}} \times t} \quad \text{Equation 2}$$

2.5. Screening for vitamin requirements

The vitamin requirements of *P. limnophila* were investigated using a two-level minimum run resolution IV screening design in Design Expert v11 (Stat-Ease, Minneapolis, MN, USA). The ΔOD_{600} was used as the response factor. We tested upper levels of 0.002 mg/L biotin, 0.002 mg/L folic acid, 0.1 mg/L pyridoxine hydrochloride, 0.05 mg/L riboflavin, 0.05 mg/L thiamine hydrochloride, 0.05 mg/L nicotinamide, 0.05 mg/L calcium pantothenate, 0.001 mg/L cyanocobalamin (B12) and 0.05 mg/L *p*-aminobenzoic acid according to the screening design (Table S2) and the lower levels were set to zero. The design consisted of 23 runs including five center points. Pre-cultures were grown in 500-mL baffled shake flasks containing D1AP medium until the ΔOD_{600} reached 1. Cultures for screening experiments were inocu-

lated to $\Delta\text{OD}_{600} = 0.01$ with pre-culture and were incubated as described in Section 3.3.

2.6. RSM-based media optimization

Media optimization was based on an I-optimal design with 25 runs (five center points included) in DesignExpert v11. The growth rate μ [1/h] was used as the response factor given the weakly-buffered medium. Hutner's salts solution, KNO₃ and NH₄Cl were combined as a single factor (*salts*). The factors cyanocobalamin (0.0005–0.02 mg/L), salts (0.5–2-fold the volume used in D1AP medium) and glucose (1–30 g/L) were mixed according to the design layout (Table S3). Pre-cultures were grown in 500-mL baffled shake flasks containing D1AP medium with 0.001 mg/L cyanocobalamin until the ΔOD_{600} reached 1. Cultures were inoculated to $\Delta\text{OD}_{600} = 0.05$ with pre-culture and were incubated as described in Section 3.3.

2.7. Analytical methods

Optical density was measured using a BioSpectrometer basic (Eppendorf, Hamburg, Germany) at a wavelength of 600 nm. For absorbance readings > 0.3, samples were diluted in PYGV-medium (for fermentations in complex medium) or 0.9% NaCl (for fermentations in chemically defined medium).

Cell dry weight [g/L] (CDW) was calculated using an experimentally determined correlation between ΔOD_{600} and CDW, as shown in Equation 3:

$$\text{CDW} = 0.2905 \times \Delta\text{OD}_{600} + 0.0294 \quad \text{Equation 3}$$

This was derived by plotting ΔOD_{600} against CDW for a dilution series, followed by linear fitting at $R^2 = 0.9999$. Dilutions were prepared in triplicates.

Glucose concentrations were determined using a Biosen C_line analyzer (EKF Diagnostics, Cardiff, UK) according to manufacturer's instructions.

Amino acid concentrations were determined using the EZfaast Amino Acid Analysis Kit KGO-7165 (Phenomenex, Torrance, CA, USA) according to the manufacturer's instructions and were analyzed on an Agilent 7890B gas chromatography system (Agilent Technologies, Santa Clara, CA, USA).

3. Results and discussion

3.1. Optimization of buffer type and concentration

To maintain the optimal pH of *P. limnophila* cultures, we tested HEPES, Tris and sodium phosphate buffers at concentrations of 10 mM and 50 mM in modified PYGV medium. After cultivation for 72 h, the comparison of ΔOD_{600} values revealed that optimal growth occurred with 10 mM sodium phosphate buffer, with much slower growth at the higher concentration of 50 mM, a trend also observed for HEPES and Tris (Fig. 1). This may reflect the previously documented low salt tolerance of this freshwater strain [44]. However, a contrasting report claims that *P. limnophila* growth is unaffected by exposure to 100 mM sodium phosphate buffer [35]. Further medium optimization was therefore carried out with 10 mM sodium phosphate buffer.

3.2. Replacement of yeast extract and peptone with defined amino acids

Yeast extract and peptone are often used as sources of vitamins, amino acids and nitrogen in media such as PYGV, whereas KNO₃ and NH₄Cl are the only nitrogen sources present in the chemically

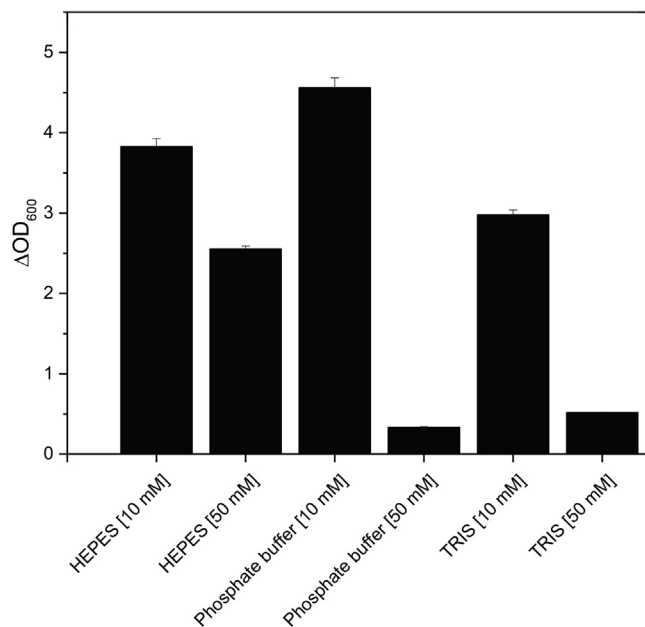


Fig. 1. Influence of HEPES, Tris and sodium phosphate buffer on the growth of *P. limnophila* at concentrations of 10 and 50 mM in PYGV medium. The ΔOD_{600} of *P. limnophila* cultures is shown after cultivation for 72 h in baffled shake flasks. Data are means \pm standard deviations ($n = 3$ biological replicates).

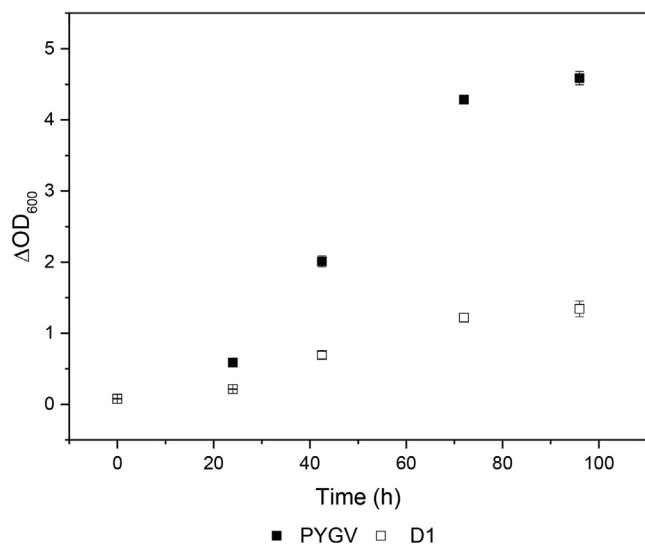


Fig. 2. Elimination of complex nitrogen sources (yeast extract and peptone) and their replacement with KNO_3 and NH_4Cl . The ΔOD_{600} of *P. limnophila* cultures is shown after cultivation for 96 h in baffled shake flasks containing PYGV and D1 media. Data are means \pm standard deviations ($n = 3$ biological replicates).

defined medium D1. We compared the growth of *P. limnophila* in these media, and found that the ΔOD_{600} after 96 h was 1.3 ± 0.1 in D1 medium compared to 4.6 ± 0.1 in PYGV medium (Fig. 2). A chemically defined medium developed for *P. limnophila* achieved ΔOD_{600} values comparable to our results without a complex nitrogen source [35], suggesting that *P. limnophila* can synthesize all amino acids needed for biomass production. Even so, the slow growth we observed in D1 compared to PYGV suggests that inorganic nitrogen sources are insufficient to promote optimal growth and that amino acid supplements are required.

Accordingly, we supplemented D1 medium with Aminoplasmal 10% (D1AP) or MEM 50 x amino acids solution (D1MEM), in both

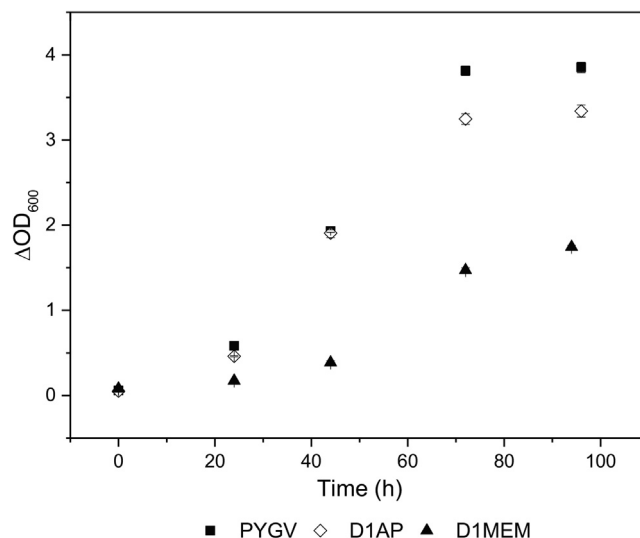


Fig. 3. The influence of different amino acid sources on the growth of *P. limnophila* in shake flasks. The ΔOD_{600} of *P. limnophila* cultures is shown after cultivation for 96 h in baffled shake flasks containing PYGV medium or D1 medium supplemented with Aminoplasmal 10% (D1AP) or MEM 50x amino acids solution. Data are means \pm standard deviations ($n = 3$ biological replicates).

cases with a total amino acid concentration of 1 g/L. The growth rate of *P. limnophila* was comparable in D1AP and PYGV media, although final ΔOD_{600} was lower in D1AP (Fig. 3). In contrast, the growth rate in D1MEM was much lower. D1AP and D1MEM differed in their amino acid composition, with MEM lacking alanine, glycine, aspartic acid, glutamic acid, proline and serine. The unmet demand for at least one of the missing amino acids is therefore likely to explain the slow growth in D1MEM. Further medium optimization was therefore carried out with Aminoplasmal 10% as the source of amino acids.

3.3. Screening for vitamin requirements

We screened nine different vitamins over a range of concentrations, but the only one that was essential for the growth of *P. limnophila* was cyanocobalamin (Fig. 4A). This showed a significant effect on growth ($p < 0.0001$), and cultures without this vitamin showed no growth at all after 96 h. The importance of cyanocobalamin has also been reported for other planctomycetal strains [45,46,47]. Analysis of variance (ANOVA) verified the significance of the model with a nonsignificant lack of fit, a significant model p -value (< 0.0001) and an adjusted R^2 value of 0.9879. We confirmed these results by cultivating *P. limnophila* in D1AP supplemented with the entire vitamin solution from PYGV or cyanocobalamin alone, revealing no significant difference in growth rate (Fig. 4B).

3.4. RSM-based optimization of media components

The composition of D1AP was optimized within the boundaries of the experimental design using the RSM approach to maximize the growth rate of *P. limnophila*. We also determined the inhibitory concentrations of the investigated factors. We used an exponential feeding strategy in the bioreactor, so it was desirable to provide a high initial glucose concentration in order to accumulate enough biomass at the feed start and to minimize the change in volume.

The glucose concentration and the volume of salt solution were identified as significant factors ($p < 0.0001$ in both cases). Even at the highest investigated concentration of 0.02 mg/L, cyanocobalamin showed no significant influence on the growth rate although

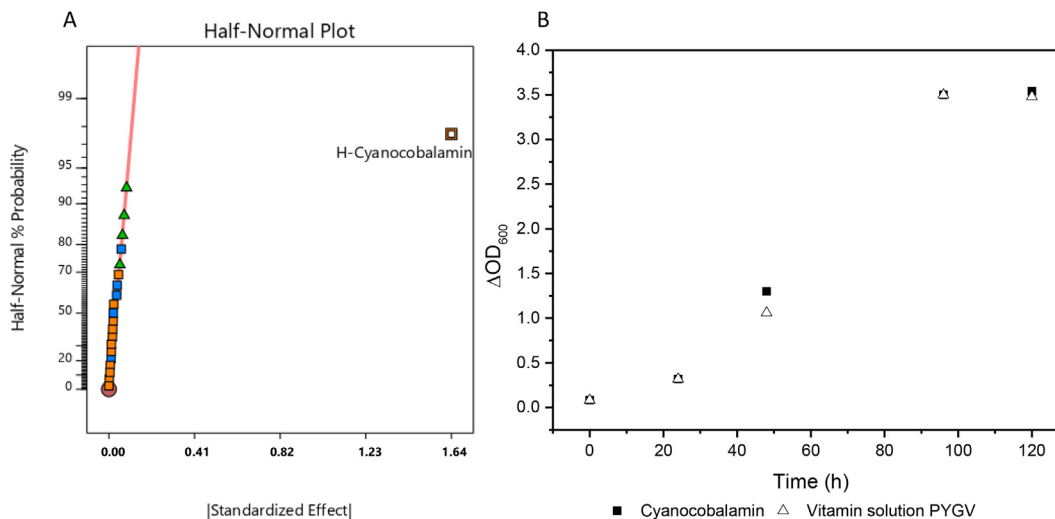


Fig. 4. Screening vitamin requirements for the cultivation of *P. limnophila*. (A) Half-normal plot with biotin, folic acid, pyridoxine-HCl, riboflavin, thiamine-HCl, nicotinamide, D-Ca-pantothenate, cyanocobalamin (B12) and *p*-aminobenzoic acid. (B) Growth kinetics of *P. limnophila* in D1AP medium supplemented with the PYGV vitamin solution or cyanocobalamin alone in baffled shake flasks.

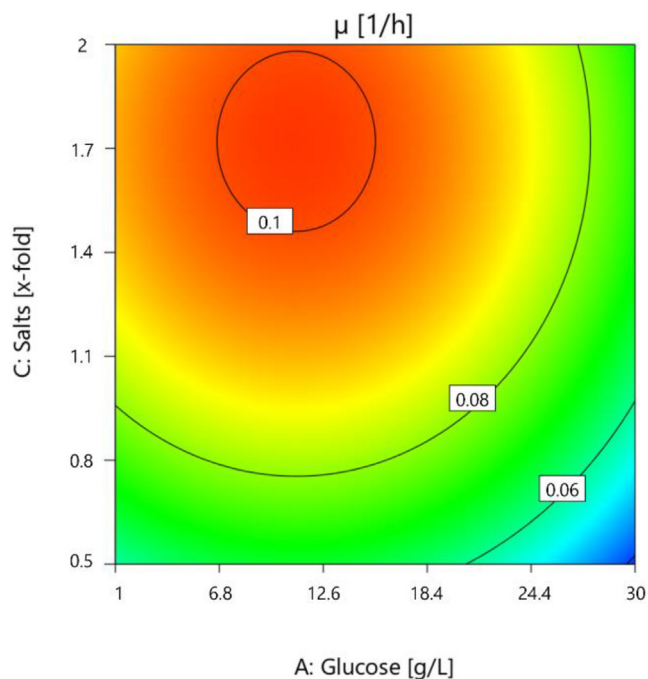


Fig. 5. Optimization of *P. limnophila* medium composition by varying three factors (salts, glucose and cyanocobalamin) to improve growth rate μ . The contour plot of the growth rate μ shows responses to different combinations of salts and glucose at a fixed cyanocobalamin concentration of 0.02 mg/L.

it was essential for growth. The lower level of cyanocobalamin was 0.0005 mg/L. It is possible that saturation is already reached at this low concentration, hence an increase would not affect the growth rate. ANOVA confirmed the significance of the model, with a non-significant lack of fit ($p = 0.14$), a significant model p -value (<0.0001) and an adjusted R^2 value of 0.90. Numerical optimization indicated an optimum within the selected limits, recommending an ideal composition of the factors at 1.7-fold salt volume, 10 g/L glucose and 0.02 mg/L cyanocobalamin (Fig. 5). The mean of three confirmation runs, under the predicted optimal conditions, was $\mu = 0.108 \pm 0.006$ 1/h and fitted thereby within the 95% confidence interval of the model.

Given the previously reported low salt tolerance of *P. limnophila* [2], the enhanced growth rate achieved by increasing the salt concentration 1.7-fold was unexpected. Planctomycetes cell division is coupled to a lifestyle switch from motile daughter cells to sessile, stalked mother cells. Only the mother cells can divide by polar budding [3,7,8]. It is possible that increasing the abundance of ions in the chemically defined medium limits the repulsive forces between individual cells and allows them to make contact to form rosettes.

3.5. Analysis of amino acid consumption

Batch fermentations in a 0.5-L bioreactor were used to investigate the amino acid consumption of *P. limnophila* when cultivated in the modified D1AP medium under well-controlled conditions, including a constant pH. The EZfaast Amino Acid Analysis Kit was unable to quantify arginine. Tyrosine, histidine and tryptophan were not detected in the first sample, but were present in subsequent samples (Fig. S1). After cultivation for 51 h, aspartic acid ($\Delta c = 0.032$ g/L) and glutamic acid ($\Delta c = 0.024$ g/L) were identified as the most consumed amino acids (Fig. 6A). Tyrosine, threonine, tryptophan, histidine, proline and lysine were excluded from further experiments because less than 5% of the available pool of each was consumed after 51 h. Based on the quantity of amino acids consumed after 51 h, we developed a customized amino acid solution (ASO) for *P. limnophila*. An earlier screening also showed that a higher final ΔOD_{600} was achieved when arginine was added to ASO at same concentration as aspartate (data not shown). Further cultivations were therefore carried out using modified D1 medium supplemented with ASO (Table S1). A confirmation experiment in shake flasks using modified D1 medium supplemented with either Aminoplasmal 10% or ASO (Fig. 6B) demonstrated the comparable growth of *P. limnophila* even though ASO lacked six of the amino acids present in Aminoplasmal 10%.

3.6. Bioreactor fed-batch cultivation

The analysis of planctomycetal genomes has revealed the presence of many silent biosynthetic gene clusters and giant genes [1,30,36,37,38] suggesting these bacteria could be exploited as sources of novel bioactive secondary metabolites [40,41]. However, research is hindered by slow growth and low biomass yields, hence

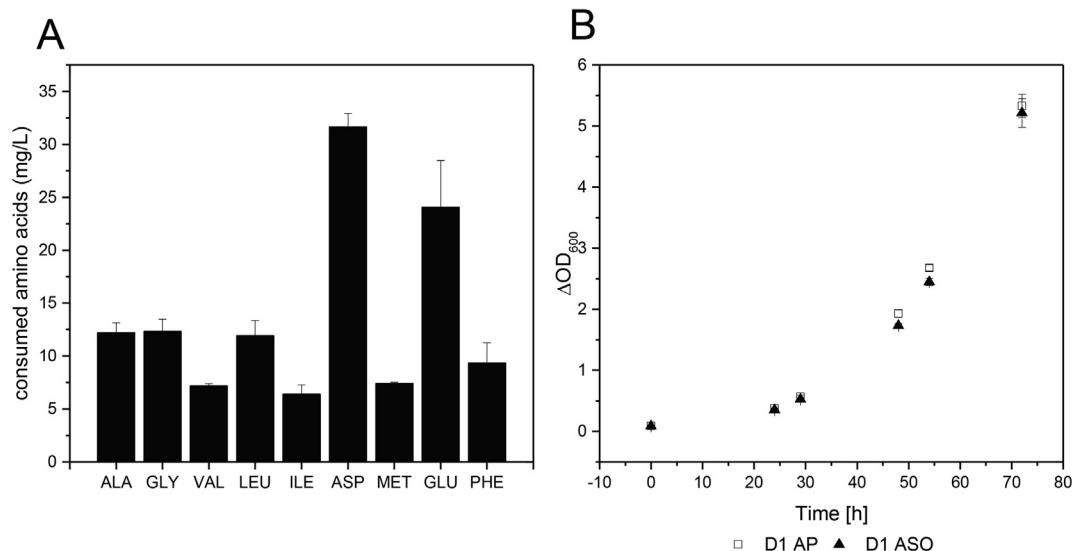


Fig. 6. Development of a customized amino acid solution for *P. limnophila*. (A) Consumption of amino acids after cultivation for 51 h. (B) Growth kinetics of *P. limnophila* cultivated in baffled shake flasks containing modified D1 medium supplemented with Aminoplasmal 10% or the customized amino acid solution ASO. Data are means \pm standard deviations ($n = 3$ biological replicates).

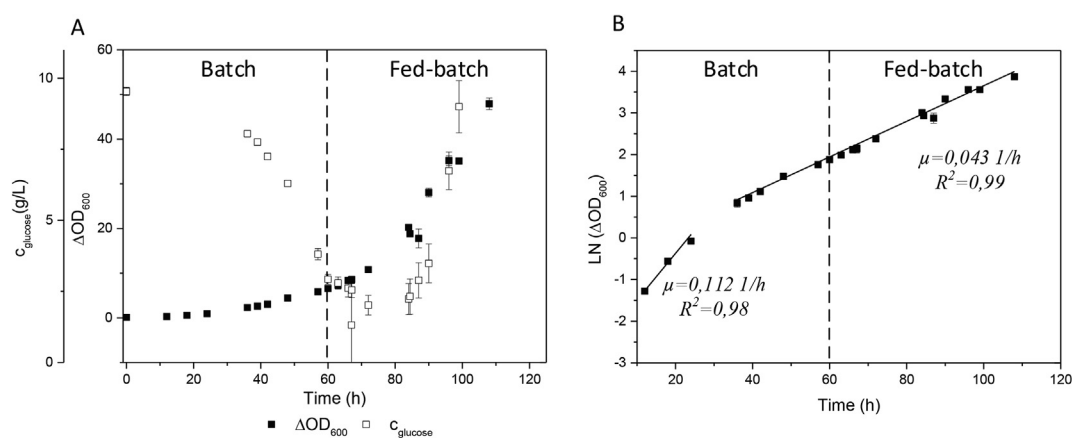


Fig. 7. Fed-batch cultivation of *P. limnophila* in customized D1ASO medium. (A) Growth kinetics and glucose consumption. The fed-batch phase started after 60 h with exponential glucose feeding. (B) Logarithmic plot of ΔOD_{600} (batch phase exponential growth at $\mu_{\text{batch}} = 0.112 \text{ 1/h}$, and fed-batch phase exponential growth at $\mu_{\text{set}} = 0.04 \text{ 1/h}$). Data are means \pm standard deviations ($n = 2$ biological replicates).

our objective to develop and optimize the chemically defined D1ASO medium to support biomass production of *P. limnophila* in a 0.5-L stirred tank bioreactor.

We therefore evaluated the potential of D1ASO medium in fed-batch cultivations using an exponential glucose feeding strategy adapted to the empirical growth rate. The customized D1ASO medium achieved a final ΔOD_{600} of 46.8 ± 0.5 after 108 h (Fig. 7A), corresponding to a calculated cell dry weight of $13.6 \pm 0.7 \text{ g/L}$. We observed two phases during batch cultivation, defined by a declining growth rate (0.112–0.043 1/h) after 24 h (Fig. 7B), which may reflect the dimorphic life cycle of *P. limnophila*. The glucose feed was started at $t = 60 \text{ h}$ and 15 mL of ASO was added to the bioreactor at $t = 67, 84$ and 90 h , synchronized with the doubling times to avoid limitation. After 84 h, glucose started to accumulate in the culture broth. The quantity of amino acids we added corresponded to the initial concentration, and the cyanocobalamin concentration in the bioreactor was 20-fold higher than we used in the shake flasks for vitamin screening (Fig. 4A). Nutrient limitation therefore seems unlikely to explain the declining growth rate, and the formation of growth-inhibiting metabolites such as acetate may be

responsible instead. However, further experiments are required to determine the cause of glucose accumulation.

Few bioreactor-based processes for the axenic cultivation of planctomycetes have been described thus far. The cultivation of *P. limnophila* in 5-L and 10-L bioreactors containing a chemically defined minimal medium without an organic nitrogen source achieved cell dry weights of 1.8 g/L ($\Delta OD_{600} = 0.99$) after 6 d cultivation at the 10-L scale and 2.9 g/L after 17 d at the 5-L scale (ΔOD_{600} data not available) [35].

Another fermentation process is described by Panter et al. [31] in complex medium for the cultivation of the recently isolated strain 10988 belonging to the order of Planctomycetales but no biomass data were published for the reported 1-L and 5-L scale processes [31]. The planctomycete *Rhodopirellula baltica* has been cultivated in a 1-L chemostat [48].

4. Conclusions

The production of secondary metabolites by microbes is usually associated with the stationary phase, when the corresponding

enzymes are expressed [49]. However, rapid growth during the exponential phase is required to generate sufficient biomass for the recovery of bioactive products. Accordingly, bioprocesses for the production of secondary metabolites often consist of at least two phases, the first for biomass accumulation and the second for the induction of product synthesis [50,51].

As a step towards bioreactor-based processes for the production of planctomycetal secondary metabolites, we therefore optimized a chemically defined medium to improve *P. limnophila* biomass production in a fed-batch bioreactor. A design of experiments screening approach revealed that cyanocobalamin is absolutely required for growth. We also found that *P. limnophila* can grow slowly when complex nitrogen sources (yeast extract and peptone) are replaced with KNO_3 and NH_4Cl , but that rapid growth comparable with complex media require the addition of selected amino acids. Fed-batch fermentations in a bioreactor resulted in a cell dry weight of 13.6 ± 0.7 g/L, which is 4.7–7.6 times higher than previously described processes and was achieved over a much shorter cultivation time [35]. Research in the future should focus on triggering biofilm formation to activate the silent gene clusters responsible for the synthesis of bioactive secondary products.

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Conflict of interest

All authors declare that there are no conflicts of interest.

Supplementary material

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