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RESEARCH

Defined and refined: development of a minimal medium for *Clostridium pasteurianum*

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Abstract

Even though microorganisms can often grow in defined media, they are frequently cultured in rich media containing complex components like yeast extract. A drawback of using rich media is the effect of secondary substrates from complex components on the metabolism, which can change the anabolism and the formation of products, complicating the interpretation of experimental results. Rich media containing yeast extract is generally used to grow *Clostridium pasteurianum*. In this work, we describe the development of a minimal medium for *C. pasteurianum* combining rational media design, transfers in batch bottles and continuous bioreactors experiments. The media were designed based on literature, the elements needed in the metabolism, and a general chemical formula for the composition of biomass. The media were tested by cultivations in chemostat and batch bottles. Microbial growth was not sustained in an inorganic medium with glucose over batch bottles transfers. In contrast, a medium with glucose supplemented with para-aminobenzoic acid (PABA), biotin and cysteine, supported growth in chemostat (12 retention times) and in batch bottles transfers. Growth was also maintained in the same medium without cysteine during the 12 retention times of chemostat operation, but at a lower dilution rate, showing that cysteine enhanced the growth rate of *C. pasteurianum* despite not being essential. Microbial growth was sustained through batch bottle transfers in media with PABA only and with biotin only, apart from glucose and cysteine. Therefore, it was concluded that PABA and biotin are essential for the growth of *C. pasteurianum* without yeast extract, only one—any—amongst both being needed.

Key points

- *Clostridium pasteurianum is auxotrophic on B vitamins.*
- *Both biotin and PABA suffice to support growth in an otherwise inorganic medium.*
- *Cysteine was not essential but increased its growth rate.*

Keywords *Clostridium pasteurianum* · Medium development · Essential nutrients · Biotin · Para-aminobenzoic acid · Cysteine

Abbreviations

CDM	Chemically defined medium or media
Cum. RT	Cumulative retention times
D	Dilution rate
DMbc	Defined medium with biotin and cysteine
DMbp	Defined medium with biotin and PABA
DMbpc1	Defined medium with biotin, PABA and cysteine 1 (used in batch bottles – high pH buffer concentration)
DMbpc2	Defined medium with biotin, PABA and cysteine 2 (used in bioreactors – low pH buffer concentration)
DMc	Defined medium with cysteine
DMpc	Defined medium with PABA and cysteine
GS	Glycerol stock
GSMM	Genome-scale metabolic model(s)
MM	Minimal medium or media
OD600	Optical density at 600 nm
PABA	Para-aminobenzoic acid
RT	Retention times
T1	Transfer 1
T2	Transfer 2
T3	Transfer 3
TES	Trace elements solution

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UM1	Undefined medium 1 (used in batch bottles—high pH buffer concentration)
UM2	Undefined medium 2 (used in bioreactors—low pH buffer concentration)
YE	Yeast extract

Introduction

The media composition affects whether a microorganism can grow, as well as its growth rate, product spectrum and biomass yield. When cultivating any specific microorganism or community, the medium should be carefully designed for the experiment to give the intended information. At the same time, a medium for growth should provide all essential inorganic and organic nutrients in excess concentrations yet below toxicity, except for one known nutrient that limits growth (typically the carbon source in heterotrophic growth). Only in this way can one directly discern the dependency of the stoichiometry and kinetics of the process on the concentration of the growth-limiting substrate. Inorganic nutrients should be supplied in an oxidation state that can be used by the microbe to make biomolecules (N, P, S, Fe) and as coenzymes, i.e., Fe and Mg (Frieden 1985; Silva and Williams 2001; Merchant and Helmann 2012). Organic compounds such as amino acids or vitamins are needed by the microbes that have auxotrophies or metabolic insufficiencies on these compounds.

Complex ingredients such as yeast extract (YE) are commonly added to media, because they are cheap and supply many nutrients simultaneously, both inorganic and organic. They are chemically undefined and their composition is highly sensitive to the production process (Tao et al. 2023). This results in composition variations between YE brands and even batches, that can substantially impact microbial behaviour (Sparviero et al. 2023). YE variability can compromise the comparability between experiments and hinders the supply chain of an industrial process. The obscure nutrient composition complicates the interpretation of experimental results, and can limit the full analysis of the metabolic activity of the culture or a comprehensive study of microbial growth. Additionally, a high YE dose might be needed to provide enough essential nutrients, which can lead to relatively high costs and a more difficult downstream processing (Grosvenor 2012; Elliott et al. 2013). For these reasons, avoiding the introduction of complex ingredients such as YE in the system is usually preferred. A chemically defined medium (CDM) reduces uncertainties, simplifies the interpretation of experimental results, and ensures full traceability of the metabolic activity and growth of a culture. A minimal media (MM) is a specific case of CDM that exclusively contains the essential nutrients to sustain growth.

To develop a CDM or MM, the nutrient needs of the microorganism have to be defined. A common approach is to perform genome-scale metabolic models (GSMM) and single omission and addition experiments. GSMM are used to predict essential and stimulatory compounds. However, while some studies reported that these predictions matched in vivo experiments (Song et al. 2008; Devendran et al. 2019), others found falsified predictions due to network deficiencies and incomplete biosynthetic pathways (Fan 2014; Chen 2016). The predictions of essential compounds by GSMM are limited by the accuracy of the model, related to the completeness of genome annotation and information on the enzymatic expression. Single omission and addition experiments do not consider interrelations between compounds, and can lead to media unable to sustain growth. For example, while single omissions of glutamine and glutamic acid did not affect growth, the absence of both reduced it (Chen 2016).

Clostridium pasteurianum is a soil anaerobic bacterium that has been studied for over a century for its nitrogen-fixing capability and solvent production (butanol, 1,3-propanediol). Its energy conservation metabolism has sparked interest due to its electron bifurcation complex BcdA-EtfBC, which oxidizes NADH and bifurcates the released electrons into acceptors of high and low potential, granting an increased ATP yield (Buckel and Thauer 2013). Many different media have been used to grow *C. pasteurianum*, mostly being chemically undefined, with little to no information on the essential, enhancing and growth-limiting compounds. The different media composition hampers the comparability between studies, their reproducibility and the full understanding of microbial activity as the essential nutrients are still undefined.

The present study describes the design, test and validation of a carbon-limited minimal medium for *C. pasteurianum*. Carbon-limited media with different complexities were designed based on literature, and tested through experiments in batch bottles and bioreactors. Essential and enhancing medium compounds were identified, and the experiments were evaluated for media validation.

Materials and methods

Medium design

A literature review of the media used to grow *Clostridium pasteurianum* is presented in Table S1 (Carnahan and Castle 1958; Carnahan et al. 1960; Lovenberg et al. 1963; Mackey and Morris 1972; Robson et al. 1974; Minton and Morris 1983; Dabrock et al. 1992; Choi et al. 2014; Kolek et al. 2015; Sarchami et al. 2016; Sabra et al. 2016; Gallardo et al. 2017; Schmitz et al. 2019; Johnson and

Rehmann 2020; Arbter 2022; Berthomieu et al. 2022; Joseph and Sandoval 2023; Behera et al. 2023; Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures; Sargeant et al. 1968). The composition of selected media with glucose as main carbon source and up to 1 g/L of YE, and of medium 54b (recommended by the supplier DSMZ of C. pasteurianum) was analysed (Tables S2 and S3) including YE, whose composition was estimated from literature (Table S4; Ogoshi et al. 2014; Thompson et al. 2017; Tomé 2021; Tao et al. 2023; Sigma-Aldrich (Merck) 2025). Vitamin B9 intermediate para-aminobenzoic acid (PABA) and biotin (vitamin B7) were almost always present in the media without YE, and often supplemented to media containing YE. Therefore, PABA and biotin were selected as potentially essential compounds when removing YE from the medium. The reducing amino acid L-cysteine hydrochloride was often used as reducing agent. It was selected as a potentially influential compound due to the importance of the redox potential of the medium on the growth of anaerobic bacteria. Based on these outcomes of the literature review, six types of media were designed: (1) an undefined medium with glucose and YE as organic components (UM), and defined media (2) with glucose, biotin, PABA and cysteine as organic compounds (DMbpc), (3) with glucose, biotin, and PABA; DMbp), (4) with glucose, biotin, and cysteine (DMbc), (5) with glucose, PABA, and cysteine (DMpc), and (6) with glucose and cysteine (DMc; Table 1). The goal of these types of defined media was to find the simplest composition able to sustain the growth of *C. pasteurianum* without yeast extract.

The elemental composition of the media was devised to be limiting in carbon, based on the general elemental composition of biomass (Roels 1980) $CH_{1.8}O_{0.50}N_{0.20}Ca_{3.1\cdot10^{-5}}Cu_{3.0\cdot10^{-6}}Fe_{1.6\cdot10^{-5}}K_{8.0\cdot10^{-3}}Mg_{1.0\cdot10^{-2}}Mn_{2.3\cdot10^{-5}}Na_{6.5\cdot10^{-3}}S_{2.0\cdot10^{-2}}P_{3.0\cdot10^{-2}}Zn_{3.9\cdot10^{-4}}$ (Table S5). The amounts of Ni, Co, Se and Mo required per C-mol of biomass are unknown. Their concentrations were established based on an average between selected glucose-based media from literature with up to 1 g/L of YE (Table S2), closest to the aimed glucose-based minimal medium. The other elements that can be found in yeast extract are Al, Sr, Pb, Cr, W, Sr and V (Tao et al. 2023). Except for the latter, they were not found to be needed for *C. pasteurianum* (Scherer and Thauer 1978; Pors Nielsen 2004; Santos 2008; Sobolev and Begonia 2008; Exley and Mold 2015; Castelvecchi 2016; Gutiérrez-Corona et al. 2016; Alshammari et al. 2021; Ikeda 2021; Go et al. 2023), so were not included. V acts as coenzyme for N_2 fixation (Berthomieu et al. 2022). Since no N_2 is fixed when there is enough ammonium in the medium, V was excluded from the media. The media used for the batch bottle transfers (UM1, DMbpc1, DMbc, DMpc, DMc) needed a pH buffer to limit the pH decrease over growth, while the media used the chemostat in bioreactors (UM2, DMbpc2, DMbp) relied on automatic pH adjustment. That is why the media

Table 1 Name and composition of the used media

Medium name		**UM1**	**DMbpc1**	**DMbc**	**DMpc**	**DMc**	**UM2**	**DMbpc2**	**DMbp**
Used in…		Bottles	Bottles	Bottles	Bottles	Bottles	Bioreactors	Bioreactors	Bioreactors
Glucose	g/kg	5.05	5.89	5.92	6.12	6.02	5.03	5.73	6.00
YE[ii]	g/kg	1.01	-	-	-	-	1.01	-	
NaCl	g/kg	0.109	0.124	0.125	0.129	0.126	0.110	0.126	
$(NH_4)_2SO_4$	g/kg	2.63	3.00	3.02	3.12	3.07	2.62	2.86	3.07
$MgSO_4.7H_2O$	g/kg	0.404	0.427	0.429	0.443	0.441	0.403	0.436	
$CaCl_2.2H_2O$	mg/kg	2.99	2.58	2.59	2.68	2.61	2.62	2.60	
KH_2PO_4	g/kg	1.21	1.22	1.21	1.22	1.20	0.231	0.232	
K_2HPO_4	g/kg	3.03	3.00	3.03	3.03	3.00	0.580	0.578	
TES[iii]	mL/kg	4.88	5.38	5.38	5.38	5.38	4.88	5.38	
Biotin	mg/kg	-	7.95	7.78	-	-	-	0.645	
PABA	mg/kg	-	2.46	-	2.45	-	-	0.904	
Cysteine	g/kg	-	0.500	0.500	0.500	0.500	-	0.500	-

[i]UM stands for undefined medium (with yeast extract), DM for defined medium (without yeast extract), b for biotin, p for PABA and c for cysteine. The numbers distinguish the different versions of each type of medium

[ii]Gibco™ Bacto™ Yeast Extract

[iii]TES = trace elements solution. It contains 0.549 g/kg $FeSO_4{\cdot}7H_2O$, 4.11 g/kg $ZnSO_4{\cdot}7H_2O$, 0.564 g/kg $MnCl_2{\cdot}4H_2O$, 0.303 g/kg H_3BO_3, 0.041 g/kg $CoCl_2{\cdot}6H_2O$, 0.068 g/kg $CuCl_2{\cdot}2H_2O$, 0.717 g/kg $NiSO_4{\cdot}6H_2O$, 0.161 g/kg $Na_2MoO_4{\cdot}2H_2O$, 0.023 g/kg $HNaSeO_3$, 0.292 g/kg HCl. The trace elements were added to the media by a trace elements solution (TES) just before inoculation, to limit the adsorption of heavy metals on glass or polypropylene (2020a, 2020b; Fan et al. 2021; Han et al. 2021; Liu et al. 2022). The TES was filter-sterilized and stored in a polycarbonate container

for batch bottle transfers have higher concentrations of potassium dihydrogen phosphate and dipotassium hydrogen phosphate than the media for chemostat in bioreactors. The biotin and PABA concentrations were chosen based on the literature average amount of moles of PABA or biotin per C-mol from the main carbon source. The pH of all media was 7 ± 0.1.

Medium validation

Medium validation by transfers in batch bottles

Five media (UM1, DMbpc1, DMbc, DMpc and DMc) were tested in batch bottle transfers, based on the ability to sustain microbial growth over the transfers. The cell pellet used as inoculum was washed with DMc during the transfers, to minimize nutrient carryover from previous media. UM1 served as a positive control since it contained 1 g/L YE.

A glycerol stock of *C. pasteurianum* was revived by letting it thaw in the fridge for 1 h. An 8-s centrifugation pulse was done to settle the $CaCO_3$ precipitate and remove it by transferring the supernatant into a new sterile Eppendorf tube. The supernatant was centrifugated at 13,000 rpm for 10 min. The cell pellet was then resuspended in 0.5 mL DMc and inoculated in 10 mL DMbpc1. After 12 h since inoculation, 1 mL of the culture was introduced into four Eppendorf tubes and centrifugated at 10,000 rpm for 10 min. The supernatants were discarded and the pellets were resuspended in 2 mL DMc. The centrifugation, supernatants removal and cell pellets resuspension in DMc were repeated three times as washings. In the last washing, the cell pellets were resuspended in 1 mL DMc, and 0.5 mL were used as inocula for duplicate bottles containing 10 mL of DMbpc1, DMbc, DMpc and DMc. After 11 h since inoculation, the pellets from 0.5 mL of each culture were washed three times as described previously, and the washed pellets resuspended in 0.5 mL DMc were used to inoculate bottles containing 10 mL fresh media. After 14 h since inoculation, the cultures were transferred once more into fresh media, with three pellet washings and in the same way. Samples were taken to measure pH, the optical density at 600 nm (OD600) and glucose concentration. The UM1 medium was tested similarly over two transfers from another glycerol stock.

The glycerol stocks of *C. pasteurianum* were produced from an active culture purchased at the DSMZ collection (strain DSM 525; Leibniz Institute DSMZ) and grown in 54b medium (see Supplementary Materials and Methods for more information). All experiments were conducted under sterile conditions to ensure the purity of the culture. Serum bottles with maximal working volume of 10 mL were used, closed with butyl rubber stoppers and capped with aluminium rings. The microbial cultures in bottles were incubated in a rotary shaker at 37 °C and 150 rpm for growth. Bottles with media were prepared identically to the culture bottles but without being inoculated, as negative controls to detect eventual contaminations. The statistical analysis, consisting of Anova and Tukey HSD tests, was conducted on R.

Media validation in chemostat bioreactors

The media UM2, DMbpc2 and DMbp were tested in duplicate bioreactors operated in chemostat, based on the ability to maintain growth over a high amount of retention times (RT).

A glycerol stock was washed in DMc: it was centrifugated at 10,000 rpm for 10 min, the supernatant was discarded and the pellet resuspended in 2 mL DMc. The glycerol stock was then revived in 10 mL UM1 as done previously. After 12 h, 8 mL of culture were used to inoculate a bottle with 106 mL UM1. After 12 h, 50 mL were used as inoculum for each of the two duplicate bioreactors containing UM2 sparged with N_2 overnight. Once the CO_2 and H_2 contents in the off-gas decreased forming a clear peak, the chemostat phase was started by activating the feed and effluent pumps. The chemostats were operated with UM2 for 12 retention times, then the feeds were switched to DMbpc2. The chemostats went on with DMbpc2 for 12 RT: the first 11.2 RT took place at a dilution rate of 0.18 h^{-1}, and the following 0.61 RT at 0.08 h^{-1}. Then, the DMbpc2 feeds were switched to DMbp. The chemostats were operated with DMbp for 12 RT at dilution rates of 0.18 h^{-1} (2.55 RT), 0.08 h^{-1} (2.02 RT) and 0.06 h^{-1} (7.26 RT). The bioreactors were maintained at 37 °C and their pH adjusted to 7 with automatic addition of 4 M KOH. More information on the bioreactors setup can be found in the Supplementary information – Materials & methods.

Results

Medium validation by transfers in batch bottles

Media were evaluated based on the ability of *C. pasteurianum* to sustain growth over transfers in batch bottles. Before inoculation, the pellet was washed to minimize nutrient carryover. For UM1, DMbpc1, DMbc and DMpc, the final OD600 did not significantly change over the transfers (Figs. 1A and 2). The final OD600 were also not significantly different between these media the end of each transfer (Fig. 3 and Figs. S1 and S2). However, the final OD600 in DMc was significantly lower compared to the other media, in all three transfers (Figs. 1 and 3). The final OD600 in DMc was also significantly different between the first and the third transfers (Fig. 2), and became increasingly different and lower compared to the final OD600 in the other media

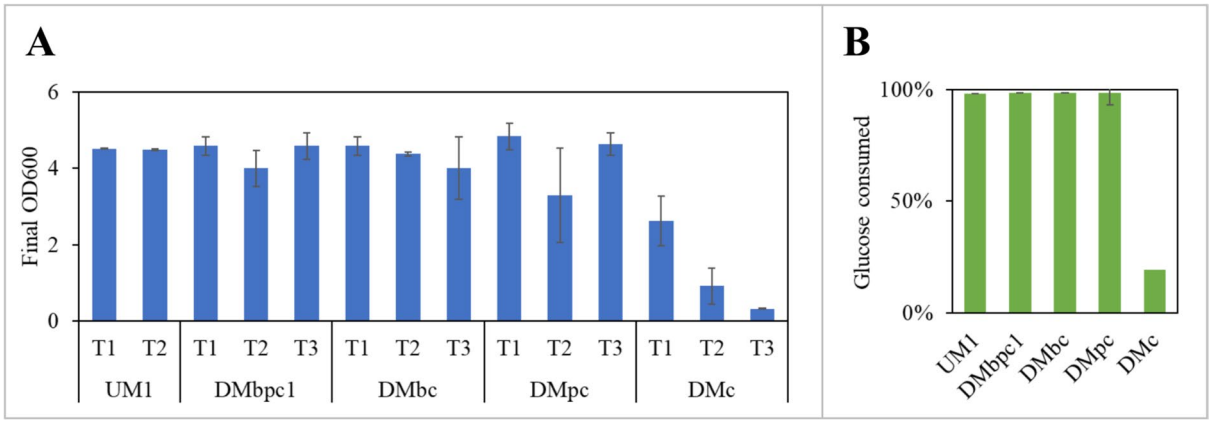

Fig. 1 **A** Average final optical density at 600 nm of the *C. pasteurianum* culture duplicates, over the transfers (T1, T2, T3) in five different media (UM1, DMbpc1, DMbc, DMpc, DMc). **B** Average glucose consumed in the culture duplicates in each medium after the last transfer. The error bars correspond to the standard deviation between the duplicates

(Fig. 3 and Figs. S1 and S2). The final pH values after the third transfer were substantially higher for DMc than the other media (Fig. S3). At the end of the last transfer, more than 98% of glucose is consumed in UM1, DMbpc1, DMbc and DMpc, while only the 19% is in DMc (Fig. 1B).

Media validation in chemostat bioreactors

Two bioreactor duplicates operated in chemostat were fed on UM2, then the feed was switched to DMbpc2 for 12 RT, and afterwards to DMbp for another 12 RT. The CO_2 and H_2 contents with DMbpc2 became stable over the 11.2 RT at a dilution rate of 0.18 h^{-1} (Fig. 4). When the DMbpc2 feed was replaced by DMbp at 0.18 h^{-1} dilution rate, the CO_2 and H_2 contents steadily decreased over 2.55 RT, after which the dilution rate was lowered to 0.08 h^{-1} then to 0.06 h^{-1} to prevent the washout of the biomass. Over the following 7.26 RT at 0.06 h^{-1}, the CO_2 and H_2 contents remained stable.

Discussion

Microbial growth in DMc declined over the batch bottle transfers due to the lack of essential nutrients, which carried over from previous media and became increasingly diluted.

Media with YE (UM1), with both biotin and PABA (DMbpc1), with only biotin (DMbc), with only PABA (DMpc), and with no biotin nor PABA (DMc) were tested in batch bottles to find the simplest one able to sustain the growth of *C. pasteurianum*. All media had the same elemental composition, with the only difference being the presence or absence of YE, biotin and PABA. Washings were done on the inocula to decrease the carryover of nutrients through the transfers. UM1, containing 1 g/L YE, served as positive control medium, as it was seen to sustain growth over numerous retention times in a chemostat.

Microbial growth in batch bottles with DMc stopped at decreasing OD600 values over the three transfers, leaving glucose unconsumed. The decreasing final OD600 values across transfers suggest that an essential nutrient is becoming increasingly limiting. This is explained by the lack of essential compounds in DMc, which did not have biotin, PABA nor YE.

Growth was still possible in DMc thanks to the carryover of the missing essential compounds from previous media. The media previously used were 54b, containing 10 g/L YE and employed to produce the glycerol stocks, and DMbpc1, used to revive the glycerol stock. Nutrient carryover was visible despite the intensive washings conducted on the pellet when transferring the cultures, which aimed to minimize the carryover of compounds from previous media. This stresses the importance of monitoring growth over enough transfers, washing the inoculum and performing a statistical analysis in order to accurately test media. The number of transfers and washings needed depends on the microbial requirement of the eventual missing compound and its concentration in the previous media.

Biotin and PABA sustain the growth of *C. pasteurianum* in the absence of YE

The final OD600 values reached in the batch bottles with UM1, DMbpc1, DMbc and DMpc were not significantly different between the transfers nor between the media. The washings and transfers decreased the nutrient carryover sufficiently to visibly reduce growth when essential nutrients were missing in the tested media, as observed in DMc. Therefore, growth was supported in DMbpc1, DMbc and DMpc due to all essential nutrients being present in

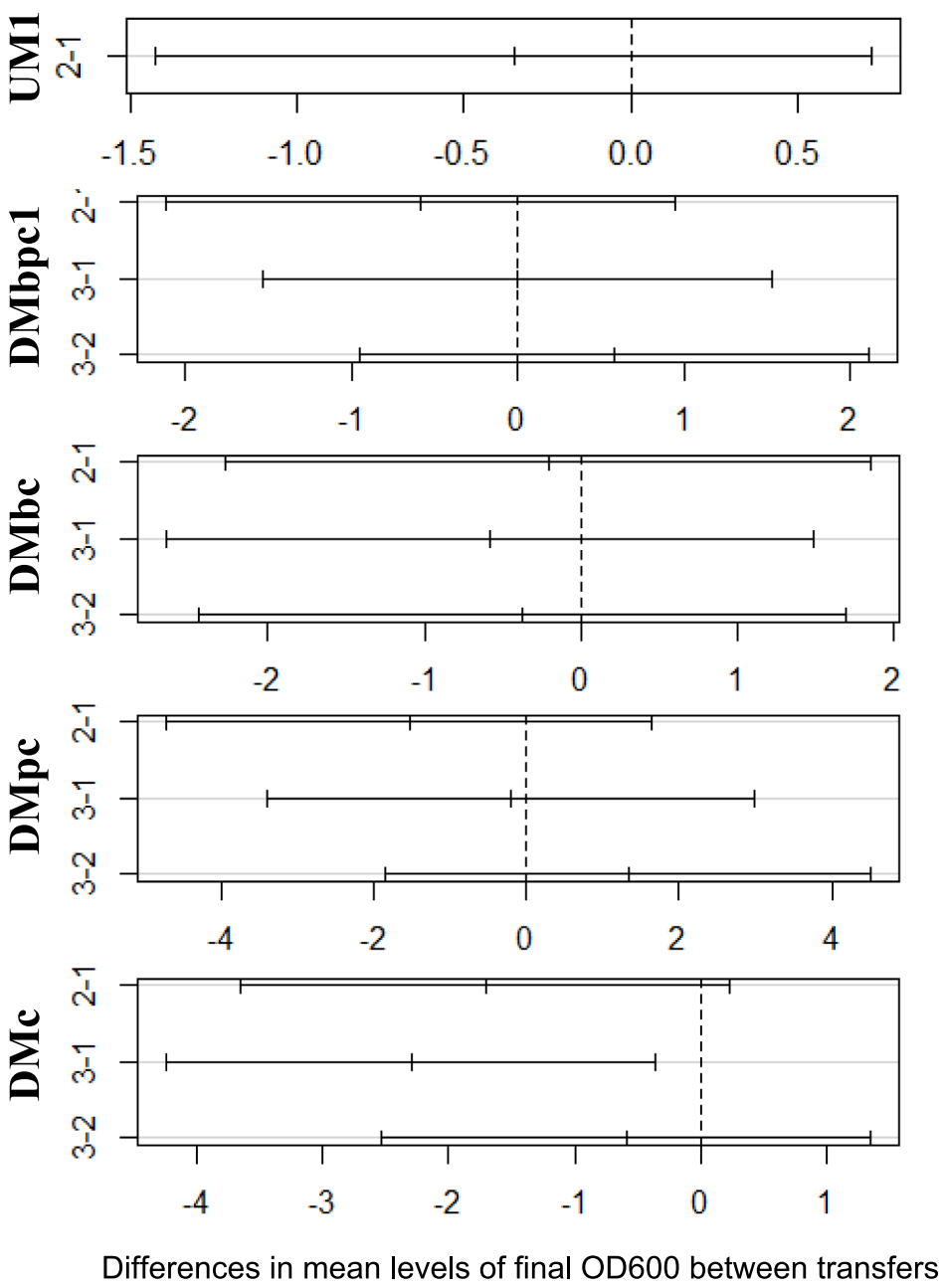

Fig. 2 Tukey HSD plot comparing the final OD600 between the transfers in UM1, DMbpc1, DMbc, DMpc and DMc media, for a 95% family-wise confidence level. For each pairwise comparison, the middle point of the confidence interval corresponds to the difference in OD600 means between each pair of transfers. If the confidence interval includes zero, the difference in final OD600 between the pair of transfers is not significant

the media, and not due to nutrient carryover from previous media. Growth stopped due to the depletion of glucose, the limiting compound in all these media. The final OD600 values in DMbpc1, DMbc and DMpc were not significantly different than the ones in UM1, which suggests that biotin and/or PABA replaced YE in the medium without diminishing the biomass yield.

Relationship between biotin and PABA

Biotin and PABA seem to be individually able to maintain growth, thus interchangeable. This was not expected, because even if they are both involved in vitamin pathways, they play different metabolic roles. Biotin is a cofactor for biotin-dependent enzymes, such as the anaepleoretic pyruvate carboxylase, which catalyses the production of oxaloacetate from pyruvate in *C. pasteurianum* (Sabra et al. 2016). Oxaloacetate serves as a direct precursor for aspartate, asparagine, lysine, threonine and β-alanine (pantothenate), and as an indirect precursor for arginine, histidine, purine, nicotinamide and nicotinate (Sabra et al. 2016). The biosynthetic route for biotin appears to be incomplete in *C. pasteurianum* (Kanehisa et al. 2015a). All media without YE reported in literature contain biotin (Table S1).

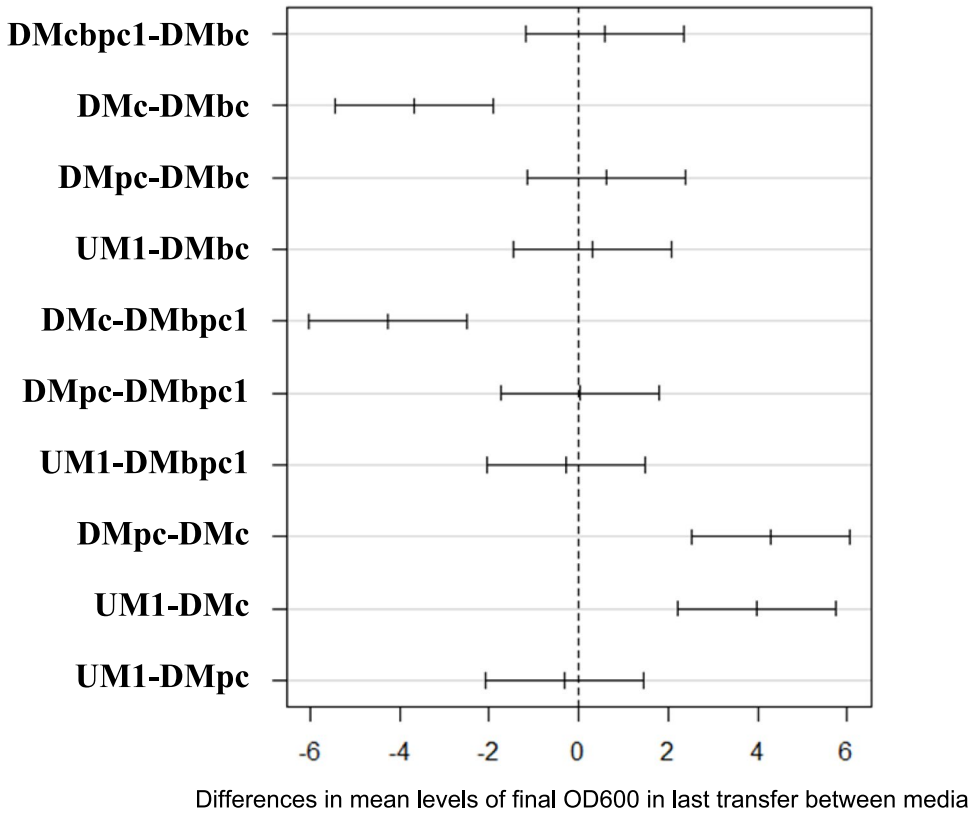

Fig. 3 Tukey HSD plot comparing the final OD600 between the different media in the end of the last transfer, for a 95% family-wise confidence level. For each pairwise comparison, the middle point of the confidence interval corresponds to the difference in OD600 means between the pair of media. If the confidence interval includes zero, the difference in final OD600 between the pair of media is not significant

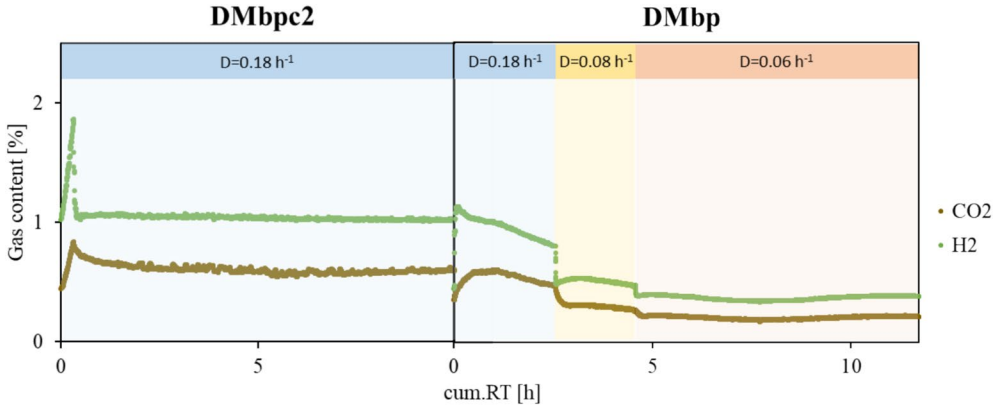

Fig. 4 CO_2 and H_2 content in the off-gas during the chemostat in bioreactor duplicate 1, with DMbpc2 as feed, which after 12 RT was switched to DMbp. Cum.RT stands for cumulative retention time. The dilution rates are shown in the top (abbreviated D). The bioreactor duplicate 2 showed a similar gas profile, available in Fig. S4

PABA is produced from chorismate and is a precursor of folate. Folate activates and transfers 1 C units for biosynthetic processes such as purine and thymidine synthesis (Ducker and Rabinowitz 2017). *Clostridium pasteurianum* appears to lack an enzyme in the synthesis of chorismate (converting 3-dehydroshikimate into shikimate (Kanehisa et al. 2015b)). Chorismate serves as precursor for aromatic amino acids (Wegkamp et al. 2007; Kanehisa et al. 2015b). All media without YE reported in literature contain PABA (Table S1), except for Arbter et al. (2022), though PABA carryover from the previous media was highly probable.

However, unexpected vitamin relationships are not new. Annan et al. (2019) studied the auxotrophies of *Clostridium authoethanogenum* and *Clostridium ljungdahlii* for panthothenate, biotin and thiamine. They found biotin was produced in presence of pimelic acid, despite some required

enzymes missing. Similar observations applied for thiamine. This suggests that either alternative genes encoding these enzymes were present or completely different mechanisms existed. Previous omission experiments also suggest unknown relationships: *Bacterium linens* was unable to grow without vitamins, but could grow when either PABA or pantothenate were present (Purko et al. 1951). If a relationship between PABA and pantothenate is also present in the metabolism of *C. pasteurianum*, it could explain a potential connection between biotin and PABA since biotin is an indirect precursor for pantothenate (Sabra et al. 2016).

Cysteine was not essential but increased the observed growth rate in chemostat operation

The chemostat operation reached stable CO_2 and H_2 productions with both media DMbpc2 and DMbp. Such stable gas productions evidence stable growth, which were maintained throughout the RTs as leftover YE and cysteine concentrations from previous media decreased to the µg/kg or ppb range. The stable gas production when the leftovers of YE and cysteine from previous media were practically depleted shows that DMbpc2 (with PABA, biotin and cysteine) and DMbp (with PABA and biotin) are both able to support the growth of *C. pasteurianum*. The growth rate in DMbp seemed lower, as the gas production decreased after the feed was switched from DMbpc2 to DMbp over the 2.55 RT at the same dilution rate. Since the only difference between DMbpc2 and DMbp was the presence of cysteine, it seems that this component increased the growth rate by acting as a nutrient or as a reducing agent.

The use of cysteine as energy and carbon source is unlikely: even if some Clostridia grow on amino acids by Stickland fermentations (Lu and Imlay 2021), this has not been reported in the presence of carbohydrates nor for *C. pasteurianum* (Pavao et al. 2022). It has been proposed that cysteine enhances the growth rate by substituting the amino acid synthesis and saving the required energy (Chen 2016). However, the energy cost of importing amino acids through the membrane was estimated to exceed the energy cost of building the amino acid by more than three-fold—assuming one mole of ATP is needed to import one amino acid (Stouthamer 1973). The transport cost and the fact that cysteine represents a small part of all monomers in a cell, make it unlikely that cysteine enhanced the growth rate by acting as a nutrient.

Instead, we might observe a higher growth rate in the presence of cysteine due to its activity as reducing agent. Even if the culture and feed were sparged with 99.999% nitrogen (v/v), O_2 traces might still impact the anaerobium. Reduced cysteine may react abiotically with O_2 making H_2O_2 (Lu and Imlay 2021), which also reacts with reduced cysteine converting its thiol group in sulfenic, sulfinic or sulfonic acid (Alcock et al. 2018). In this way, cysteine lowers the O_2 and reactive oxygen species concentrations, decreasing damage on the enzymes and DNA (Lu and Imlay 2021). With less enzyme and DNA damage by O_2, the cell has more resources available for growth, increasing the observed growth rate. Previous research has often added reduced cysteine to the medium to grow *C. pasteurianum* and usually in the same concentration of 0.5 g/L (Table S1). This concentration of cysteine is precisely what is required to reduce all dissolved oxygen at 1 atm and 37 °C, supporting its use as a reducing agent.

Chemostats support a more controlled media validation at the cost of large number of retention times needed, in opposition to media validation in batch bottle transfers

Media were tested in batch bottle transfers and by chemostat operation in bioreactors. A key requirement of both approaches for a correct media validation is that the carryover of eventual missing nutrients from previous media should be low enough to show a decrease in microbial growth. The nutrient carryover can be sufficiently reduced both in chemostat and in batch bottle transfers, if the amount of RT, and the amount of transfers and washings are high enough (Fig. 5).

Batch bottle transfers and growth in chemostat bioreactors present opposite advantages and drawbacks for media validation. Validating media in bioreactors saves sampling and transferring labour, and provides a constant pH and minimal exposure to O_2 due to the absence of washing steps. The drawbacks are the large feed and effluent volumes required, the eventual need to replace feed and effluent bottles, and the limited number of media that can be tested (based on bioreactor availability and experimental time constrains). An additional difficulty of batch bottle cultivation is that it requires a pH buffer ensuring maintenance of a pH that allows full growth (until depletion of of the limiting nutrient).

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Author contributions NNA, RGC and RK conceived and designed the research. NNA and DCV conducted the experiments and analysed data. NNA wrote the first manuscript. All authors contributed and approved the final manuscript.

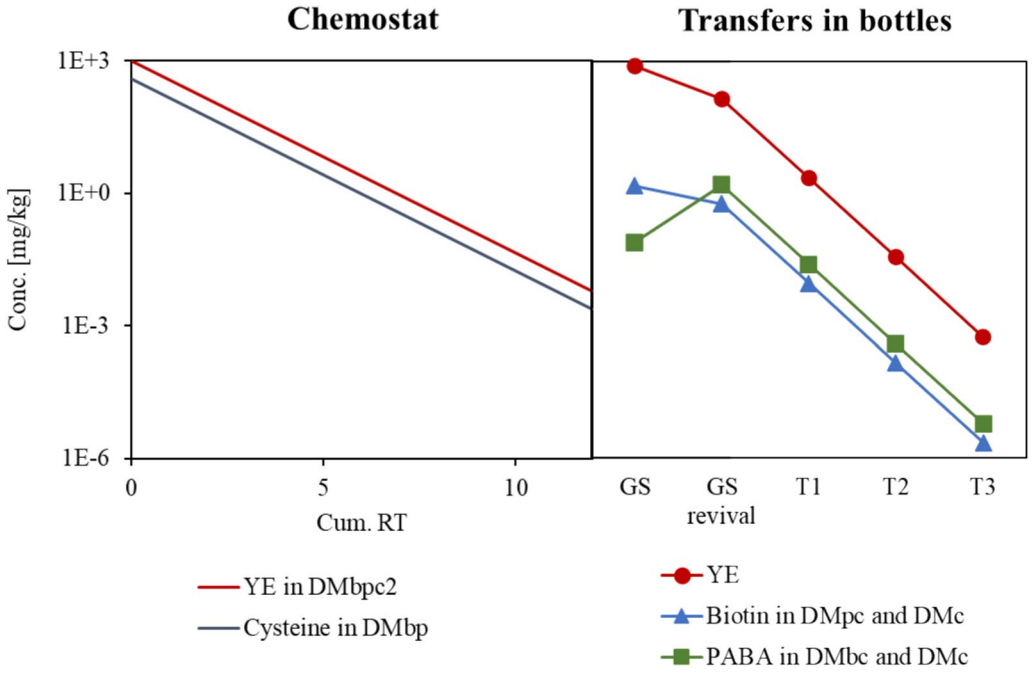

Fig. 5 Estimated concentrations of YE, cysteine, biotin and PABA over transfers to media without YE, cysteine, biotin and PABA respectively, over retention times (RT) in the chemostat and over the glycerol stock (GS) revival and the three transfers (T1, T2 and T3). In the glycerol stock revival, 0.2 mL were assumed to be carried over from the glycerol stock to the 10 mL DMbpc1. Over the three transfers, 0.1 mL of culture was assumed to be carried over to 10 mL fresh medium. The calculations assumed no consumption, yielding maximal concentrations (see Supplementary Set of Eqs. 1)

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

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