Industrial Production of Vitamin B12 withPropionibacterium freudenreichii subs shermanii

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“Once you've got a task to do, it's better to do it than live with the fear of it.”

Joe Abercrombie. The Blade Itself,

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Article-Review

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Article

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Oral presentation

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Summary

Vitamin B_{12}, also known as cobalamin (cbl) is a water-soluble vitamin essential for many organisms. Firstly, studied in 1920s as a sort of “extrinsic factor” capable of curing and preventing pernicious anemia, this highly complex molecule was isolated for the first time in 1950.

From this point, and due to its physiological importance, it has been extensively studied. Its structure (a tetrapyrrolic ring with a central Co ion similar to other prosthetic groups) and its chemical synthesis have been fully elucidated. However, due to the complexity and low yields of the synthesis route, fermentation with high-producing microorganisms is still the preferred way for the industrial production of vitamin B_{12}.

This thesis project, done in collaboration between HealtechBioactives (HTBA) and the “Grupo de Química Biológica y Biotecnológica” (GQBB) at Institut Químic de Sarrià (IQS), focuses on optimizing growth and increasing vitamin B_{12} production with a wild type of strain of the anaerobic *Propionibacterium freudenreichii subs shermanii*: NBRC 12391.

Despite the majority of the current industrial production being done with modified aerobic strains (like *Pseudomonas denitrificans*) or random mutagenesis obtained propionic-resistant *Propionibacterium* strains, this project focused on a wild type strain after considering the peculiarities of the vitamin B_{12} market, where the high health and ecological awareness of many of its consumer may lead to strategic and commercials advantages when using a wild type, GRAS and non-GMO microorganism for vitamin B_{12} production. For this reason, all the optimization and strategies described in this project were performed at a bioprocess level and no genetic engineering was involved.

Being this project the first in the research group based on the anaerobic production with *Propionibacterium* strains, many of the early efforts were centered around developing a robust and reliable culture methodology, including studying media composition, supplement addition strategies, culture conditions, pH regulation, etc.

Besides, a reliable cobalamin extraction procedure was also implemented alongside a HPLC-based analytical method for cobalamin identification and quantification.

Once the culture process was successfully implemented, several strategies were tested in order to optimize culture growth and productivity. In this sense, several points were assessed: the effect on Cbl production of the early culture oxygenation, the addition of different Cbl supplements and their addition times, the addition of nitrate, the effect of the nature of the base solution as well as the effect of propionic acid in both cell growth and production.

It was concluded that early agitation was detrimental to Cbl production most likely due to an increased oxygenation during the anaerobic phase of the culture. Besides, the addition of DMBI was deemed essential for high Cbl production. Finally, propionic acid showed a significant negative effect in both growth and production, as expected.

With this data, the process was scale-up to a laboratory-scale bioreactor in several batch and fed-batch bioprocesses at different conditions. However, production in these cultures was lower than expected due to carbon source limitations and propionic acid inhibition.

For this reason, a single-phase continuous bioprocess was developed. This was possible thanks to i) the capacity of NBRC 12391 of reaching maximum Cbl volumetric production in less than 24-h after DMBI addition and ii) the fact that NBRC 12391 can produce Cbl in anerobic conditions instead of requiring a microaerophilic phase to reach its maximum production. The continuous strategy allowed for a significant increase in Cbl volumetric productivity by 5.7 folds compared to the best batch process. This provides a new viable strategy for the development of future and sustainable strategies to produce CNCbl with wild-type GRAS *P. freudenreichii* strains without having to rely on more complicated setups like multiple-phase continuous bioprocesses or expanded bed adsorption bioreactors for propionic acid elimination.

Finally, other strategies were also assessed to further optimize Cbl production and possibly incorporate them into the single-phase continuous bioprocess like the co-culture with organic acid-consuming strains or more optimized media formulations.

Resumen

La vitamina B_{12}, también conocida como cobalamina (cbl), es una vitamina soluble en agua esencial para muchos organismos. En primer lugar, se estudió en la década de 1920 como un tipo de "factor extrínseco" capaz de curar y prevenir la anemia perniciosa. Esta molécula altamente compleja se aisló por primera vez en 1950.

A partir de este punto, y debido a su importancia fisiológica, se ha estudiado ampliamente. Su estructura (un anillo tetrapirrólico con un ión central de Co similar a otros grupos prostéticos) y su síntesis química se han elucidado completamente. Sin embargo, debido a la complejidad y los bajos rendimientos de la ruta de síntesis, la fermentación con microorganismos con una alta capacidad de producción sigue siendo el método preferido para la producción industrial de la vitamina B_{12}.

Este proyecto de tesis, realizado en colaboración entre HealtechBioactives (HTBA) y el "Grupo de Química Biológica y Biotecnológica" (GQBB) en el Instituto Químico de Sarrià (IQS), se centra en optimizar el crecimiento y aumentar la producción de vitamina B_{12} con una cepa wild type de *Propionibacterium freudenreichii subs shermanii*: NBRC 12391, un microorganismo anaeróbico.

Aunque la mayoría de la producción industrial actual se realiza con cepas aeróbicas modificadas (como *Pseudomonas denitrificans*) o cepas de *Propionibacterium* resistentes a propiónico obtenidas mediante mutagénesis aleatoria, este proyecto se centró en una cepa wild type, después de considerar las peculiaridades del mercado de la vitamina B_{12}, donde la gran conciencia sobre la salud y la ecología por parte de muchos consumidores podría comportar ventajas estratégicas y comerciales en el uso de un microorganismo wild type, GRAS (generalmente reconocido como seguro) y no modificado genéticamente para la producción de vitamina B_{12}. Por este motivo, toda la optimización y las estrategias descritas en este proyecto se llevaron a cabo a nivel de bioproceso y no se utilizó la ingeniería genética.

Resumen

Al ser este proyecto el primero en el grupo de investigación basado en la producción anaeróbica con cepas de *Propionibacterium*, muchos de los esfuerzos iniciales se centraron en desarrollar una metodología de cultivo robusta y fiable, incluido el estudio de la composición del medio, las estrategias de adición de suplementos, las condiciones de cultivo, la regulación del pH, etc. Además, se implementó un procedimiento fiable de extracción de cobalamina, así como un método analítico basado en HPLC para la identificación y cuantificación de la cobalamina.

Una vez que el proceso de cultivo se implementó con éxito, se probaron varias estrategias para optimizar el crecimiento del cultivo y la productividad de la vitamina B_{12}. En este sentido, se evaluaron varios puntos: el efecto de la oxigenación inicial del cultivo en la producción de Cbl, la adición de diferentes suplementos de Cbl y sus momentos de adición, la adición de nitrato, el efecto de la naturaleza de la solución base, así como el efecto del ácido propiónico en el crecimiento celular y la producción.

Se concluyó que la agitación inicial era perjudicial para la producción de Cbl, probablemente debido a una mayor oxigenación durante la fase anaeróbica del cultivo. Además, se consideró esencial la adición de DMBI para una alta producción de Cbl. Finalmente, se observó que el ácido propiónico tenía un efecto negativo significativo tanto en el crecimiento como en la producción, como se esperaba.

Con estos datos, el proceso se escaló a un biorreactor de escala de laboratorio en varios bioprocesos de batch y fed-batch o en diferentes condiciones. Sin embargo, la producción en estos cultivos fue más baja de lo esperado debido a las limitaciones de la fuente de carbono y la inhibición del ácido propiónico.

Por este motivo, se desarrolló un bioproceso continuo en una sola fase. Esto fue posible gracias a que i) la capacidad de NBRC 12391 de alcanzar una producción volumétrica máxima de Cbl en menos de 24 horas después de la adición de DMBI y ii) el hecho de que NBRC 12391 puede producir Cbl en condiciones anaeróbicas en lugar de requerir una fase microaerofílica para alcanzar su máxima producción. La estrategia continua permitió un aumento significativo de la productividad volumétrica de Cbl en un 5.7 veces en comparación con el mejor proceso de batch. Esto ofrece una

nueva estrategia viable para el desarrollo de estrategias futuras y sostenibles para producir CNCbl con cepas wild type de *P. freudenreichii* GRAS sin tener que depender de configuraciones más complejas como bioprocesos continuos de múltiples fases o biorreactores de adsorción en un lecho expandido para la eliminación del ácido propiónico.

Finalmente, también se evaluaron otras estrategias para optimizar aún más la producción de Cbl y posiblemente incorporarlas en el bioproceso continuo en una sola fase, como la co-cultura con cepas que consumen ácidos orgánicos o formulaciones de medios más optimizadas.

Resum

La vitamina B_{12}, coneguda també com a cobalamina (cbl), és una vitamina soluble en aigua essencial per a molts organismes. Es va començar a estudiar a la dècada de 1920 com un tipus de "factor extern" capaç de curar i prevenir l'anèmia perniciosa. Aquesta molècula altament complexa es va aïllar per primera vegada el 1950.

Des d'aquest punt, i a causa de la seva importància fisiològica, s'ha estudiat extensament. La seva estructura (un anell tetrapirròlic amb un ió central de Co similar a altres grups prostètics) i la seva síntesi química s'han elucidat completament. No obstant això, a causa de la complexitat i els baixos rendiments de la ruta de síntesi, la fermentació amb microorganismes amb una alta capacitat de producció encara és el mètode preferit per a la seva producció industrial. Aquest projecte de tesi, realitzat en col·laboració entre HealtechBioactives (HTBA) i el "Grup de Química Biològica y Biotecnològica" (GQBB) a l'Institut Químic de Sarrià (IQS), es centra en optimitzar el creixement i augmentar la producció de vitamina B_{12} amb una soca wild type de *Propionibacterium freudenreichii subs shermanii*: NBRC 12391, un microorganisme anaeròbic.

Tot i que la majoria de la producció industrial actual es fa amb soques aeròbiques modificades (com *Pseudomonas denitrificans*) o soques de *Propionibacterium* resistent a propiónic obtenides mitjançant mutagènesi aleatòria, aquest projecte es va centrar en una soca wild type, després de considerar les peculiaritats del mercat de la vitamina B_{12}, on la gran consciència sobre la salut i l'ecologia per part de molts consumidors podria comportar avantatges estratègics i comercials en l'ús d'un microorganisme wilt type, GRAS (generalment reconegut com a segur) i no modificat genèticament per a la producció de vitamina B_{12}. Per aquest motiu, tota l'optimització i les estratègies descrites en aquest projecte es van realitzar a nivell de bioproces i no es va utilitzar l'enginyeria genètica.

Sendo aquest projecte el primer en el grup de recerca basat en la producció anaeròbica amb soques de Propionibacterium, molts dels esforços inicials es van centrar en desenvolupar una metodologia de cultiu robusta i fiable, incloent l'estudi

de la composició del medi, les estratègies d'addició de suplements, les condicions de cultiu, la regulació del pH, etc. A més, es va implementar un procediment fiable d'extracció de cobalamina, així com un mètode analític basat en HPLC per a la identificació i quantificació de la cobalamina.

Un cop el procés de cultiu es va implementar amb èxit, es van provar diverses estratègies per optimitzar el creixement del cultiu i la productivitat del cultiu.. En aquest sentit, es van avaluar diversos punts: l'efecte de l'oxigenació inicial del cultiu en la producció de Cbl, l'addició de diferents suplements de Cbl i els seus moments d'addició, l'addició de nitrat, l'efecte de la natura de la solució base, així com l'efecte de l'àcid propiònic en el creixement cel·lular i la producció.

Es va concloure que l'agitat inicial era perjudicial per a la producció de Cbl, probablement a causa d'una major oxigenació durant la fase anaeròbica del cultiu. A més, es va considerar essencial l'addició de DMBI per a una alta producció de Cbl. Finalment, es va observar que l'àcid propiònic tenia un efecte negatiu significatiu tant en el creixement com en la producció, com s'esperava.

Amb aquestes dades, el procés es va escalar a un biorreactor d'escala de laboratori en diversos bioprocessos de batch i de fed-batch en diferents condicions. No obstant això, la producció en aquests cultius va ser més baixa del que s'esperava a causa de les limitacions de la font de carboni i de la inhibició de l'àcid propiònic.

Per aquest motiu, es va desenvolupar un bioproces continu en una sola fase. Això va ser possible gràcies a que i) la capacitat de NBRC 12391 d'arribar a una producció volumètrica màxima de Cbl en menys de 24 hores després de l'addició de DMBI i ii) el fet que NBRC 12391 pot produir Cbl en condicions anaeròbiques en lloc de requerir una fase microaerofílica per arribar a la seva producció màxima. L'estratègia continua va permetre un augment significatiu de la productivitat volumètrica de Cbl en un 5,7 vegades en comparació amb el millor procés de batch. Això ofereix una nova estratègia viable per al desenvolupament de futures i sostenibles estratègies per produir CNCbl amb soques wild type de *P. freudenreichii* GRAS sense haver de confiar

en configuracions més complexes com bioprocessos contínus de múltiples fases o biorreactors d'adsorció en un llit expansiu per a l'eliminació de l'àcid propiònic.

Finalment, també es van avaluar altres estratègies per optimitzar encara més la producció de Cbl i possiblement incorporar-les en el bioproces continu en una sola fase, com la co-cultura amb soques que consumeixen àcids orgànics o formulacions de mitjans més optimitzades.

List of abbreviations

AdoCbl	Adenosylcobalamin
ALA	5-Aminolaevulinic Acid
AMN	Amnionless Protein
API	Active Pharmaceutical Ingredient
ASGP-R	Asialoglycoprotein Receptor
ATP	Adenosine Triphosphate
CNCbl	Cyanocobalamin
CSL	Corns Steep Liquor
CUB	Cubam Complex
DMBI	5,6-Dimethylbenzimidazole
DOE	Design Of Experiments
EBAB	Expanded-Bed Adsorption Bioreactors
GlutCbl	Glutathionylcobalamin
GRAS	Generally Recognized As Safe
HC	Haptocorrin
IF	Intrinsic Factor
ISPR	In Situ Product Removal
MetCbl	Methylcobalamin
MMCM	Methylmalonyl-Coa Mutase
MRP1	Multidrug Resistance Protein 1
NitCbl	Nitritocobalamin
OD600	Optic Density At 600 Nm Wavelength.
OHCbl	Hydoxocobalamin
OTR	Oxygen Transfer Rate
PAB	Propionic Acid Bacteria
PBG	Porphobilinogen
QPS	Qualified Presumption Of Safety
RF	Response Factor
SAM	S-Adenosyl-L-Methione
SulCbl	Sulfitocobalamin
TCII	Transcobalamin II
Wt	Wild Type
YE	Yeast Extract

1. Vitamin B_{12}. History, synthesis, structure, and physiological significance.

1.1. Summary and historical overview of vitamin B_{12}

Vitamin B_{12}, also known as cobalamin (Cbl), is a water-soluble molecule essential in many organisms' metabolism. It has a complex structure and an elaborated biosynthesis, with over 30 biotransformation steps [1]. This biosynthetic pathway is only present in some bacteria and archaea, although the phyla capable of synthesizing vitamin B_{12} are not necessarily interrelated, so mammals, and therefore humans, are unable to synthetize it.

Vitamin B_{12} investigation began in the decade of 1920s and is directly linked to an illness first described in 1824 and known as pernicious anemia, a medical condition that causes abnormal low erythrocytes level in blood. The main symptoms include fatigue, weight loss, headaches and, in severe cases, dementia, memory loss, muscle weakness and peripheral neuropathy.

In 1926 Minot and Murphy demonstrated that patients with pernicious anemia could be successfully retained in disease remission by a special diet with high amounts of lightly cooked liver and muscle meat [1]. They theorized that the treatment was successful because of an unknown "extrinsic factor" present in animal livers. For this discovery, they were awarded the Nobel Prize in Physiology or Medicine in 1934 although it took more than two decades since the so called "extrinsic factor" was identified and isolated. In 1948, two research groups from big pharmaceutical companies (Folkers at Merck, Sharp & Dohme, and Smith at Glaxo) isolated almost at the same time a compound from animal livers that was able to cure pernicious anemia on its own [2,3]. A year later, it was also isolated from other sources such as milk, beef, and several bacterial cultures.

This red crystalline octahedral cobalt compound was called vitamin B_{12}. Years later, it was discovered that this compound was in fact one of the many isoforms of the Cbl family, cyanocobalamin (CNCbl), an artificial, physiologically inactive form of cobalamin generated in the chemical process of extraction and isolation from the liver.

Nonetheless, CNCbl was the first cobalamin isoform whose structure was solved in 1955 by Dorothy Hodgkin [4]. Years later, the same group determined the structure

of adenosylcobalamin (AdoCbl), one of the two active forms of cobalamin [4]. These discoveries granted Hodgkin the Nobel Prize in Chemistry in 1960.

Two years later, another physiologically active vitamin B_{12} isoform, methylcobalamin (MetCbl), was discovered. AdoCbl and MetCbl were found to act as cofactors in several enzyme-mediated bio-transformations and, in the years to come, many MetCbl and AdoCbl dependent enzymes were isolated and described. Some of them were also crystallized, like methionine synthase and methylmalonil CoA mutase [5,6], as well as most of the molecules responsible for vitamin B_{12} transport in mammals [7–14]

In 1973, after a long study that spanned over a decade, the complete chemical synthesis of vitamin B_{12} was described by Woodward and colleagues. The process was complex, with over 60 steps, including protection and deprotection reactions, and rendered very low yields, of less than 1% [15,16].

1.2. Cobalamin Structure and function as a cofactor

As previously stated, vitamin B_{12} is the generic name used to designate a family of compounds (cobalamins or Cbl). They share a common structure: a tetrapyrrolic corrinic ring with a central cobalt atom coordinated with four nitrogen atoms. This scaffold is similar to other prosthetic groups, such as heme in hemoglobin or cytochrome P450. This structure allows the use of the redox state of the central metallic atom, cobalt, allowing the molecule to fulfil its different functions.

As shown in Figure 1, the corrin ring is formed by four pyrrole units (C_4H_5N), joined on opposite sides by a C-CH3 methylene link, a C-H link on one side and two pyrrole units directly joined, missing the methine bridge between the A and D subunits present in other known porphyrins, such as hemoglobin.

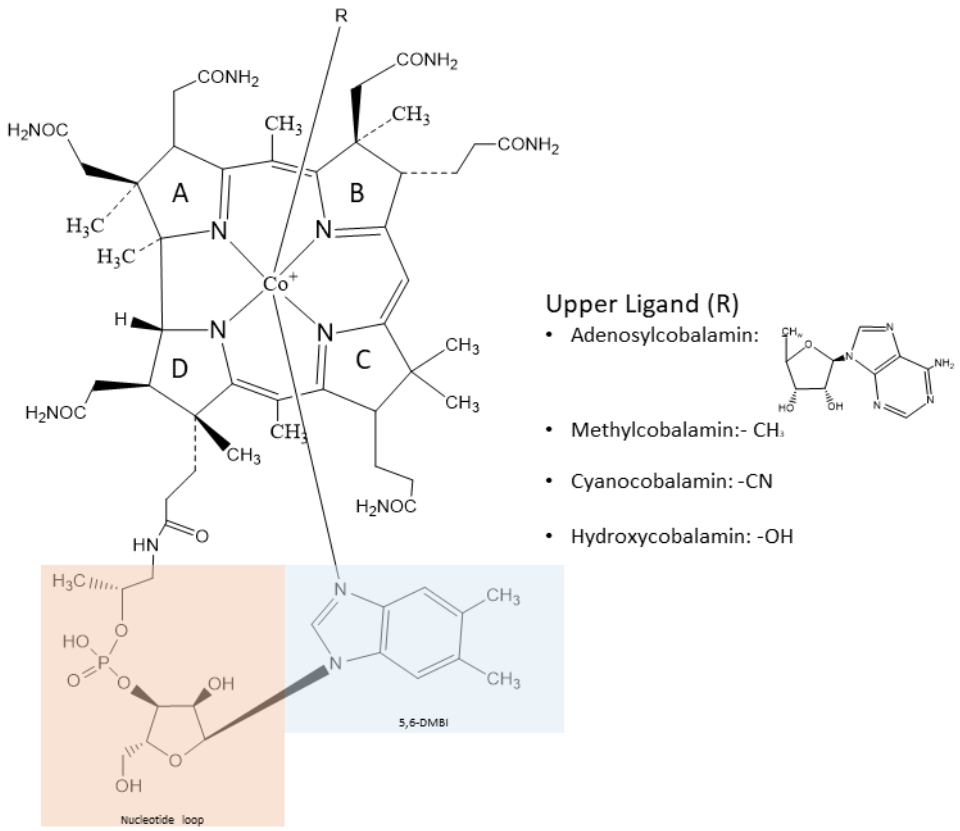

Figure 1. Schematical representation of the structure shared by all cobalamin isoforms. *The main groups that usually act as the upper ligand are also shown. 5,6-DMBI: 5,6-dimethylbenzimidazole*

Besides the four N atoms of the pyrrole units, the central Co ion is linked to two other ligands. The lower ligand is the base 5,6-dimethylbenzimidazole (DMBI) linked with the central Co ion through the N7-atom in α-axial conformation. DMBI is also linked to one of the side chains of the central corrinic structure: its phosphate group joins to an aminopropanol group that is linked to the propionic acid side chain of the pyrrole unit D of the corrinic ring.

Finally, the sixth ligand is linked to Co in the β-axial position. The nature of this chemical group is variable, presenting different physiological and catalytic functions. For example, a 5 deoxyadenosyl group in this position forms AdoCbl, while a methyl group forms the MetCbl. In fact, the C-Co found in AdoCbl was the first bond of its

type described in a biological molecule [17]. Although these two are the isoforms that present physiological activity as cofactors in humans, other forms, such as hydroxocobalamin (OHCbl) or CNCbl, exist, as well as others with less common upper ligands, such as nitritocobalamin (NitCbl), sulfitocobalamin (SulCbl) or glutathionylcobalamin (GlutCbl) [18].

As for the functions of the physiologically active isoforms, both act as a cofactor for critical metabolism reactions:

- MetCbl is a cofactor of several methyltransferases, such as methionine synthase in humans, an important cellular housekeeping enzyme that functions in two major metabolic pathways: the tetrahydrofolate-dependent one-carbon cycle and the final step in the conversion of methionine from homocysteine (See Figure 2).

  Methionine synthase is responsible for the final step in the conversion of methionine from homocysteine. This process involves a two-step reaction: First, the methyl group form MetCbl is transferred to homocysteine, forming methionine and cobalamin (Co(I)) and then, the methyl group of the CH_3-H_4 folate is transferred to the Cobalamin (Co(I)) to regenerate the methylcobalamin cofactor and form H_4folate [19]. As a side note, besides its critical function in humans and other mammals, MetCbl also is involved in the carbon dioxide fixing pathway in anaerobic acetogenic bacteria and methanogenic archaea [20].

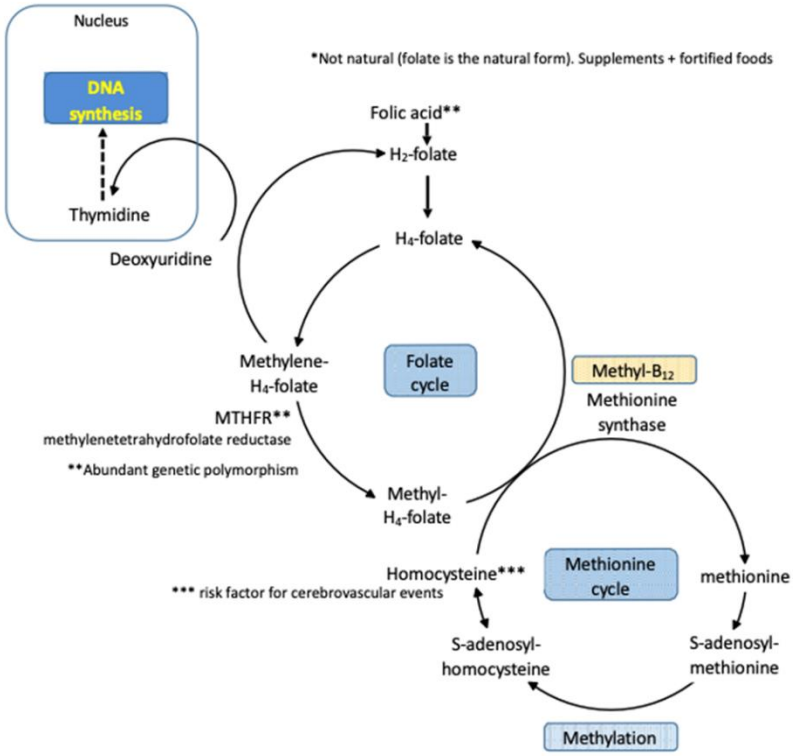

Figure 2 Methionine synthase function in the folate and methionine cycles. *Extracted from* [21]

- AdoCbl is used as a cofactor by several enzymes, mostly mutases [22], although only one AdoCbl-dependent enzyme is found in mammals: L-methylmalonyl-CoA mutase (MMCM), a critical enzyme for propionate catabolism and degradation of odd-chain fatty acids, several amino acids (valine, isoleucine, methionine, threonine) and cholesterol.

  In the mitochondria, the binding of Methylmalonil-CoA to MMCM triggers the homolysis of the Co-C bound of its cofactor, AdoCbl. This allows a radical carbon skeleton rearrangement reaction as shown in Figure 3 [23].

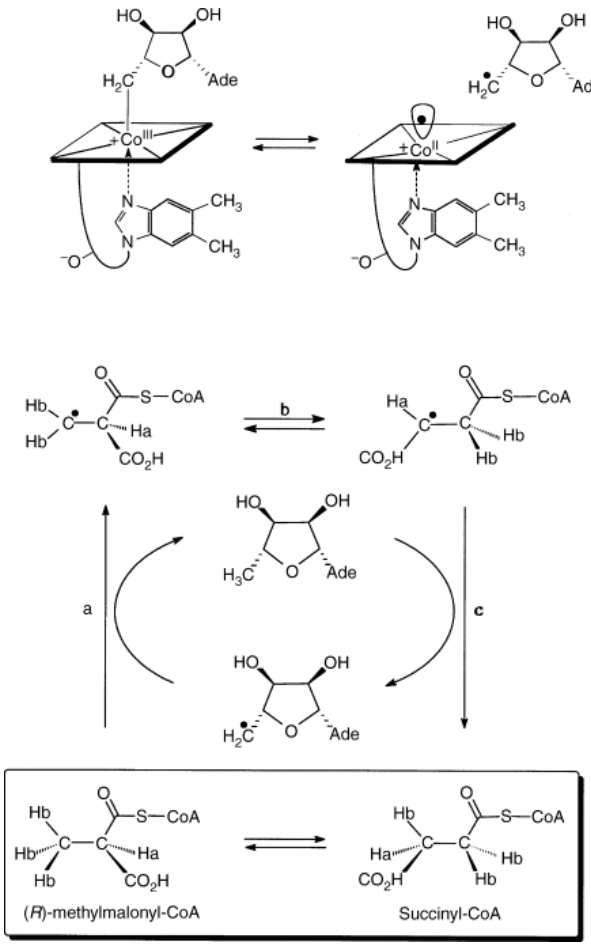

Figure 3 Schematic representation of the conversion of Methylmalonil-CoA into Succinyl CoA *mediated by MMCM and using AdoCbl as a cofactor and as a reversible source of 5'-deoxy-5'-adenosyl radical, allowing a radical rearrangement. This process is further described in* [23]

As previously stated, AdoCbl and MetCbl are the active cobalamin isoforms, but they are also known to be light-sensitive [24,25]. For this reason, the most common commercial form of vitamin B_{12} is CNCbl instead, which is more stable and readily converted in the body into an active coenzyme form [26].

1.3. Pharmacokinetics of vitamin B_{12}

B_{12} biosynthesis is limited to a small group of bacteria and archaea. For this reason, mammals and, therefore, humans, are completely dependent on diet to achieve required vitamin levels to carry out their physiologic functions. As a quick summary, the ingested Cbl is transported by a series of protein carrier until it reaches the terminal ileum, where it is absorbed and distributed to all the cells in the body through the blood.

Despite all daily Cbl requirements must be satisfied by dietary intake, symptoms of insufficient Cbl consumption can take months or even years to manifest due to a very efficient enterohepatic circulation and the Cbl reuptake at a kidney level. This allows cobalamin to stay in the body for long periods of time [27].

For this reason, besides cases of strict vegan and vegetarian diets with no Cbl supplementation, most of the vitamin B_{12} deficiencies with clinical symptoms are due to malabsorption, many times cause for a malfunction of some of the proteins involved in the absorption and transportation process [21,27]. For example, some autoimmune conditions can cause the destruction of the gastric parietal cells, leading to abnormally low levels of intrinsic factor (IF).

1.3.1. Vitamin B_{12} intake, transport, and uptake in the intestine.

Cbl absorption and distribution is done by a complex system of carriers, receptors and transporters that take the molecule from the oral cavity (where the food containing vitamin B_{12} is ingested) to each cell in the body. Cbl transportation through extracellular fluids is performed by three different carriers: Haptocorrin, (HC, transcobalamin I or R-protein), intrinsic factor (IF) and transcobalamin II (TC). These immunologically distinct proteins share a common protein core of 46 kDa [28] and a similar structure, divided into two domains. [8,29]. The Cbl molecule is bound at the interface of the two domains, where it is mostly buried and protected from the solvent (See Figure 4) [8]. This mode of binding is also found in some B_{12} dependent enzymes like methionine synthase and MMCM [30].

Briefly, haptocorrin and IF are involved in the initial uptake of vitamin B_{12}, up until its internalization at the intestine while TC is the carrier that transports the vitamin absorbed in the ileum to the different cells of the body.

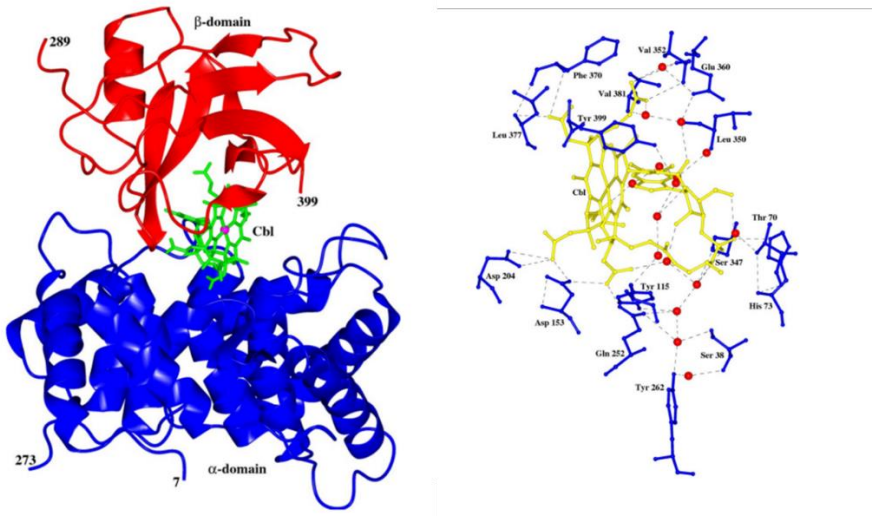

Figure 4 Ribbon diagram of the IF-Cbl complex. *Cbl is shown as ball and sticks. Right side shows Environment around the Cbl molecule at the binding site of IF. Cbl is in gold, and the water molecules are shown as red spheres. Both images extracted from* [8]**.**

Haptocorrin:

A heavily glycosylated protein present in most mammal's body fluids, mainly saliva, breastmilk and plasma [31]. It escorts Cbl from the moment the vitamin is released in the oral cavity until the duodenum (Figure 5).

HC protects the vitamin from the hydrolysis caused by the gastric environment thanks to its glycosylated pattern that is resistant to low pH [32]. Once the HC-Cbl complex arrives at the duodenum, HC is enzymatically degraded by pancreas proteases, allowing the binding between vitamin B_{12} and the IF.

In contrast to the other carrier proteins, haptocorrin has low specificity and can also bind inactive non-vitamin corrinoids that have lost their lower ligand, like cobinamide [33]. These inactive forms, also known as vitamin B_{12} analogues, often appear in fecal material and are also present in cord blood. Their physiological role remains unclear,

and no evidence of Cbl absorption in the colonic microflora has been found. However, this evidence seems to indicate that the human body, or more accurately, its microflora, is capable of synthesizing some amounts of vitamin B_{12} analogues with still unclear functions [34,35].

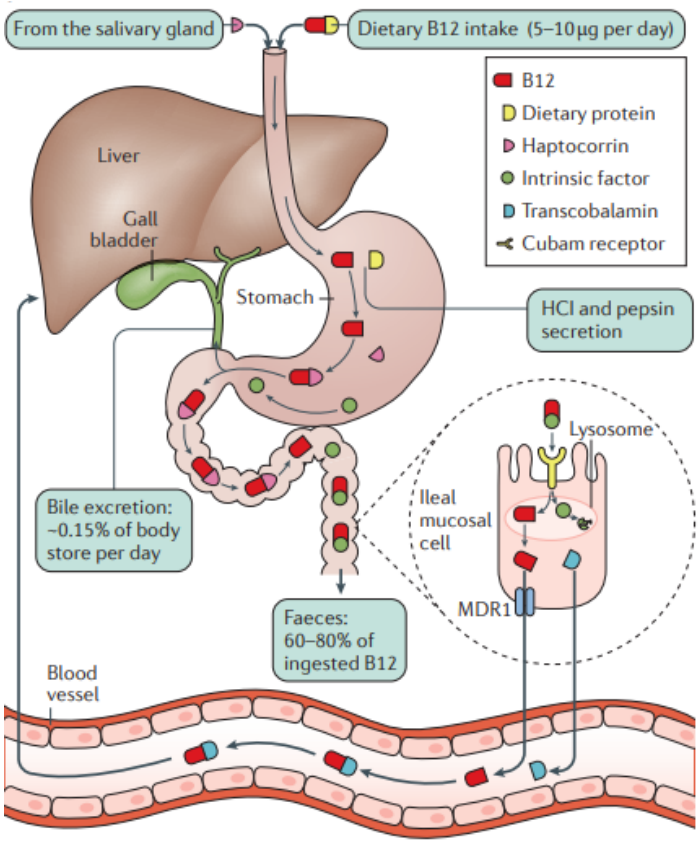

Figure 5 Schematic representation of Cbl of human vitamin B_{12} uptake until intestinal internalization. *Extracted from [27].*

The Intrinsic Factor and the internalization of vitamin B_{12} into the blood stream.

IF is also a highly glycosylated protein synthetized and secreted in the stomach by gastric parietal cells. After HC degradation, IF binds to vitamin B_{12}, forming the IF-Cbl complex. The glycosylation pattern and the amino acid sequence of the IF protect Cbl from the enzymes present in the intestine [36,37].

The IF-Cbl complex travels to the ileum, where the enterocytes synthetize and secrete the IF-Cbl receptor known as the cubam complex (CUB). The cubam complex is formed by two molecules: the amnionless protein (AMN) and cubilin. Both proteins are co-expressed in the apical membrane of the ileum by enterocytes [38–40]. Cubilin can recognize and bind the IF-Cbl complex while AMN is a transmembrane protein that mediates the IF-Cbl complex internalization. When IF-Cbl is recognized by cubilin, a receptor-mediated endocytosis takes place and the IF-Cbl complex enters the enterocyte [27] (Figure 6).

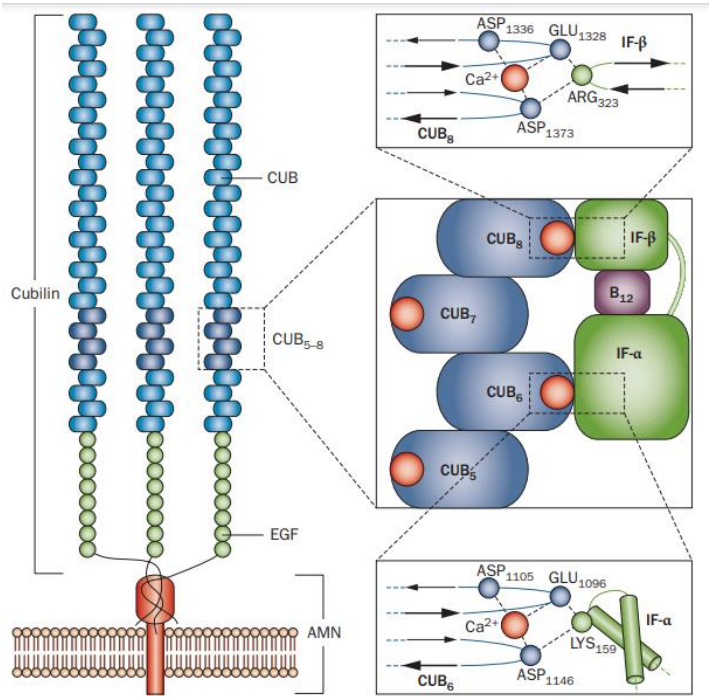

Figure 6 CUB mediated endocytosis of the IF-Cbl complex. *CUB complex is secreted by the enterocytes cells of the ileum and is formed by two proteins: AMN and cubilin. Cubilin Domains CUB5-8 are able to bind IF domains α and β, allowing a very specific endocytosis process that ends with the IF-Cbl entering the enterocyte*. [27]

The CUB complex is a key component of the vitamin B_{12} internalization pathway and is very specific towards the IF-Cbl complex, not been able to bind free forms of neither IF nor vitamin B_{12} alone [27]. It is form by two different molecules: a 460 kDa cubilin molecules that binds the IF-Cbl complex, and a 48 kDa AMN.

This high specificity is due to the structure of cubilin which is divided into three regions (Figure 6):

- Twenty-seven CUB domains with a β-barrel structure. IF-Cbl complex binds to cubilin through the CUB domains 5,6,7 and 8. CUB_6 and CUB_8 recognize IF domains α and β respectively and interact with them in a Ca^{2+} mediated binding [27].
- Eight EGF (epidermal growth factors) domains.
- An amino-terminal region that contains an α-helix and trimerizes the protein.

Once inside the enterocyte, IF is degraded by the lysosome proteases which, unlike intestinal proteases, can attack and degrade IF. After this event, free Cbl is transported through the lysosomal membrane by, presumably, a lipocalin receptor-like protein of 61 kDa known as LMBD1 [41]. Besides, an ATP-binding cassette (ABC) transporter encoded by gen ABCD4 is also theorized to have a role in the Cbl passage through the lysosomal membrane. As other ABC transports, ABCD4 couples ATP hydrolysis to the transport of molecules against its concentration gradient [42]. Several defects and malfunctions caused by mutations in this transporter support its function in the internalization of Cbl [43].

After IF degradation, free Cbl is released to the plasma from the basolateral side of the cells, mainly by a multi-specific membrane transporter of 190 kDa known as multidrug resistance protein 1 (MRP1) or ABCC1 [44]. MRP1 is a multifunctional protein and is expressed in the basolateral membranes of polarized cells, although it is also present in most non-polarized cells [42,45]. Besides MRP1 mediated export, the existence of other forms of externalization have been theorized [46]. In fact, it was found that mutations and knockouts of MRP1 only lead to a partial inhibition of Cbl secretion to plasma [47] and, although the capability of transporting vitamin B_{12} has

not been found in other similar transporters, the existence of some redundancy or even passive transportation of Cbl across the membrane seems likely [47,48].

A schematic representation of Cbl Internalization and excretion in enterocytes is shown in Figure 7 [27].

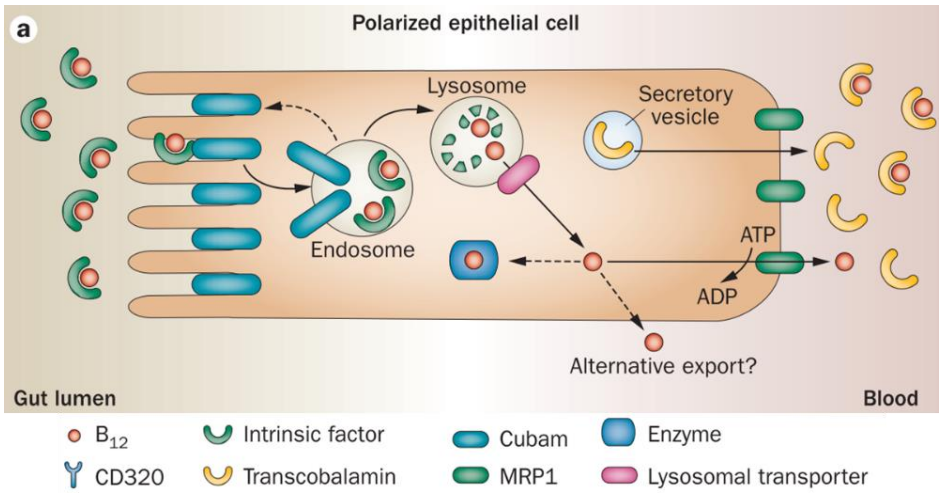

Figure 7 Cellular transport of B12 in enterocyte and other polarized cells. *Once internalized by the Cubam complex, IF-Cbl is liberated in the lysosomes where IF is degraded. Free B_{12} exit the lysosome aided by the LMBD1 transporter and then is used as a cofactor in the same cell or exported to the blood by MRP1. Alternative export processes might also exist. Extracted from* [27]

1.3.2. Vitamin B_{12} transport in plasma and cellular uptake and metabolism.

Through MDRP1 or other non-defined yet transport, Cbl is exported from the enterocytes in its free form, without forming a complex with any protein. Formerly, it was theorized that TCII bound to the free Cbl inside the cells and then the whole complex was exported to the exterior by MDRP1 [49], but more recent evidence suggests that free Cbl exists inside cells and, once in the blood stream [48], it binds to protein carriers like TCII, or, in some cases, the previously mentioned HC.

TCII is secreted by the vascular endothelial cells and binds active forms of vitamin B_{12} with high specificity to form holotranscobalamin II, the bioavailable form of vitamin B_{12} [50]. Holotranscobalamin II is, then, carried through the blood stream to their final cell destination, where an endocytic receptor mediates its internalization. Once inside

the cell, TCII is degraded in the lysosome in a similar manner than the IF degradation in the enterocytes. Afterwards, free Cbl is either stored in the cell, used as a cofactor in the various B_{12} mediated reactions or exported again in a similar way to that described for enterocytes (Figure 8).

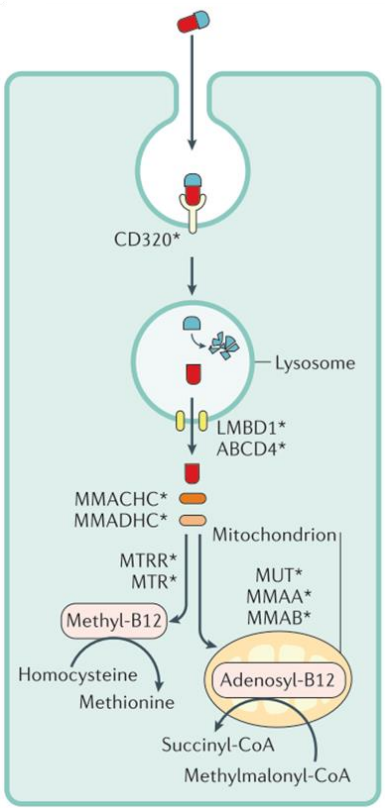

Figure 8 Cellular uptake of Vitamin B12 and metabolism. *The different proteins involved in Vitamin B_{12} cellular trafficking and processing are shown. Adapted from [27]*

Cellular uptake of vitamin B_{12} involves an endocytic receptor present in the cell surface of all tissues [51] known as CD320, although B_{12} internalization by other not yet defined receptors is also possible [52]. CD320 is a 58 kDa that belongs to the LDL receptor family [53]. It seems that CD320 expression is regulated according to the proliferative and differentiative status of the cell, presenting increased expression levels during cell proliferation [54].

Few Cbl exits the lysosome helped by two transporters: LMBDR1 and ABCD4 and enters the cytoplasm. Once there, it follows a complex cellular trafficking pathway that involves several chaperones that deliver the final active form of vitamin B_{12} (MetCbl or AdoCbl) to the final molecular targets, cytosol or mitochondria respectively [55].

1.3.3. Vitamin B_{12} recycling and reabsorption at the kidney.

Vitamin B_{12} present in body fluids is the fraction bound to TCII, the so called holotranscobalamin. However, up to an 80% of the total Cbl in the bloodstream is linked to HC [56]. Besides being the first carrier protein that binds Cbl in the oral cavity, HC is also present in high quantities in the blood steam. Because of its low specificity and its ability to bind a wide arrange of different Cbl molecules and inactive Cbl forms is theorized that HC is involved in the removal of damaged vitamin and the storage and recycling of Cbl in the liver. This theory is reinforced by the existence of HC receptors exclusively in hepatocyte cell membranes known as the asialoglycoprotein receptor or ASGP-R [31]. Thus, Cbl and Cbl analogues reabsorption and storage is performed by hepatocytes. Besides HC specific receptor ASGP-R, hepatocytes also have the CD320 receptors present in other cell types for the internalization of holotranscobalamin. Therefore, they are capable of internalizing both TCII-Cbl and HC-Cbl.

Inside the hepatocyte, the carrier protein (HC or TCII) is degraded by the lysosome and the free Cbl is either stored in the liver [56] or secreted to the bile to be reabsorbed at the intestine. In the bile, HC synthetized by the liver binds the Cbl and escorts it until the ileum, where it is once again degraded by intestinal proteases. After that, free Cbl binds to IF and can be reabsorbed by the enterocytes or excreted in the feces. A schematic representation of the recycling process of Vitamin B_{12} can be seen in Figure 9 [56].

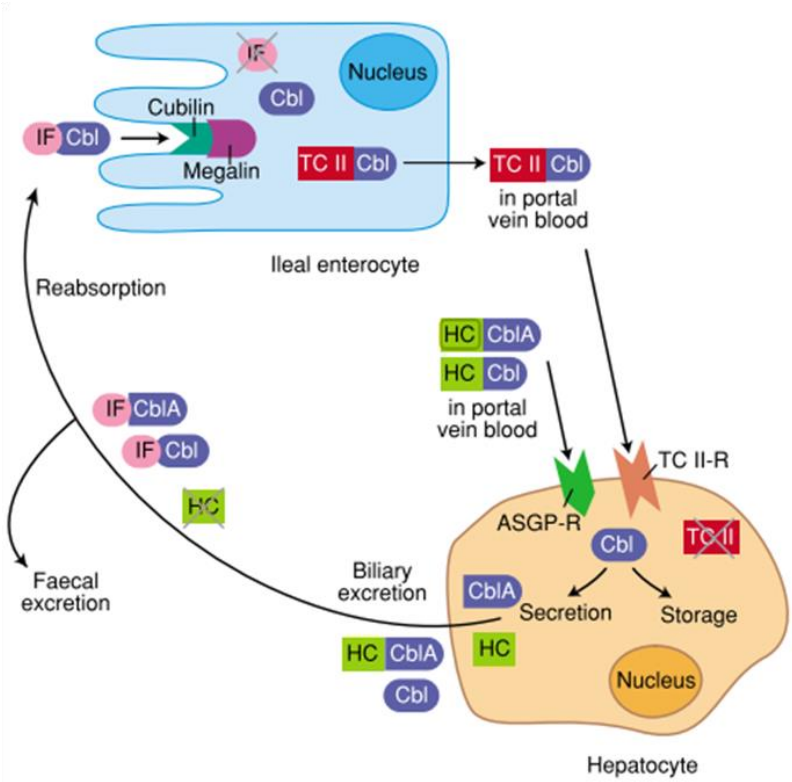

Figure 9 Schematic representation of the recycling pathway of Vitamin B_{12} at the liver. *Adapted from* [56].

1.4. Vitamin B_{12} Functions. Deficiency symptoms and causes.

1.4.1. Functions and daily recommended intakes

Vitamin B_{12} was first discover as the Extrinsic Factor capable of curing pernicious anemia. This disease gained the adjective pernicious for the severe and ultimately fatal consequences that happened when the illness was not treated correctly. Taking this into consideration, it is obvious that Vitamin B_{12} has a capital role in human health and physiology and that it must be consumed and absorbed in a sufficient amount to maintain a healthy status.

Due to its involvement in several critical enzymatic conversion steps, maintaining a healthy Cbl levels is critical for the correct development of some critical physiological functions, such as:

- Red blood cell formation [57].
- Immunomodulator, protection against viral infections [58] and necessary for the development and correct functioning of the immune system as a whole [57].
- Cbl and some of its non-physiological functional analogues may have importance in maintaining a healthy gut microbiota [59].
- As inferred for its function as a coenzyme, it is also critical in protein synthesis, nucleic acid synthesis [57] and cellular respiration.

To correctly maintain its functions, a daily intake of Cbl of around 2.4 µg for men and non-pregnant women and 2.6 µg for pregnant women is usually recommended [60]. Although the typical diet in western developed countries should be enough to maintain appropriate intake, vegetarian and vegan diets may be at risk of insufficient intake without Cbl supplementation.

Cbl is absent in vegetables [61], and the main dietary sources are foods derived from animal products. Although B_{12} is present in dairy products and eggs (products suitable for vegetarian diets), their quantities are quite low compared with other options (approximately 0.4 µg/100 g in milk and 1.3 µg/100 g in eggs vs. 9.4 µg/100 g in some meats, 8.9 µg/100 g in fish and 52.4 µg/100 in shellfish [62]). This fact, together with the assumed bioavailability of only 50% of all the Cbl obtained from food sources [63] and the losses that can occur during food processing (cooking, exposure to light, pasteurization, etc.) [61], make reaching the recommended daily dietary intake of 2.4 µg a difficult task for those on pure vegetarian diets.

Vitamin B_{12} deficiency is also prevalent in low- or middle-income countries with mainly plant-based diets and low meat consumption [64]. In high-income countries, there are also several population groups at high risk of B_{12} deficiency. This specially affects the elderly, with around 20% of people over 60 suffering from it in the USA and in the UK according to the NIH [65]. In the case of the elderly, deficiency is mostly due to lower intake and a high prevalence of food-bound malabsorption, caused by age-related gastric atrophy and lower IF levels [63,66].

Other high-risk populations are pregnant and lactating women, children, and patients with autoimmune diseases that cause gastric complications, such as atrophic gastritis or decreased stomach acid secretion [62].

1.4.2 Vitamin B_{12} shortage causes, symptoms, clinical manifestations, and biological consequences.

There are several causes that can lead to vitamin B_{12} deficiency, from an inadequate intake to malabsorption or the disruption of some key component in B_{12} trafficking and transportation. Some of the main causes are summarized in Table 1.

The first of this possible cause is insufficient dietary intake. Cbl dairy requirement of 2-3 µg is easily satisfied in diets with large amounts of animal products. However, as B_{12} is exclusively found in food obtained from animal sources (meat, eggs, dairies…), vegan diets only obtain around 0-0,25 µg of vitamin B_{12} daily. For people with these kind of diet vitamin B_{12} supplements are mandatory. The dietary recommended intakes can vary depending on the age or different physiological status or conditions (e.g. pregnancy, elite athletes). Besides, vitamin B_{12} bioavailability can vary widely between different persons according to the condition of the gastrointestinal track, overall amount of vitamin ingested, etc. [67]. Food source is also a relevant factor for bioaccessibility because the release of vitamin B_{12} from its food matrix depends on the protein that it is bound to. For example, bioaccessibility of vitamin B_{12} in milk is far better than from other animal sources [68].

Exposure to nitrous oxide can cause chemical inactivation of vitamin B_{12} through an irreversible oxidation of MetCbl. Patients chronically exposed to this gas can present symptoms of vitamin B_{12} deficiency [69].

Table 1. Main causes of Vitamin B_{12} deficiency. *Information collated from various sources* [21,27,70]

Vitamin B_{12} deficiency main causes	
Cause	**Comments**
Inadequate dietary intake	Prevalent in low-income countries and strict vegan and vegetarian diets. Alcoholism.
Malabsorption due to a deficit of IF or impaired IF absorption	Many causes: pernicious anemia, gastric diseases, bacterial overgrowth, parasitic infections, stomach bypass surgery, medications, cholestyramine, etc.
Inability to release Cbl from the food matrix in the stomach	Low pepsin activity in the stomach. Probably due to atrophic chronic gastritis and long-term treatments with antiacids or pump proton inhibitors.
Inability to degrade HC	Low pancreatic enzymatic activity due to pancreatic diseases or pancreatectomy
Malabsorption due to villous atrophy	Caused by inflammation bowel diseases like Crohn's disease or Coeliac disease.
Decreased expression, binding activity or affinity of receptors and proteins involved in Cbl trafficking and processing	Caused by several genetic disorders such as alterations on TCN2, MMACHC, MMADHC, etc.
Other causes	Nitrous oxide chemical inactivation, HIV…

However, the main cause for Cbl deficiency is an alteration of B_{12} absorption. Because of the complex pathway that Cbl follows from the moment it is ingested until the active forms act as cofactors in the cells, malabsorption can be due to several causes. Main examples include:

- **Pernicious anemia**. This severe disease is caused for an almost complete absence of gastric IF. This causes deficient absorption of both dietary Cbl and recycled bile Cbl and leads to a progressive exhaustion of the body Cbl reserves. Pernicious anemia is often caused by an autoimmune gastritis, caused by the apoptosis of parietal cells. This apoptotic process leads to the release of gastric H^+/K^+ ATPase, activating naïve $CD4^+$T cells. These activated cells then migrate to the gastric mucosa where they bind to MHC Class II molecules and activate FAS-dependent mechanisms, leading to tissue damage and the destruction of more parietal cells. The loss of these IF producing parietal cells, coupled with the presence of anti-IF autoantibodies lead to a sharp decrease in IF levels, causing pernicious anemia [71].
- **Disrupted transport and cell trafficking alterations**: Several genetic disorders can alter one or more steps in Cbl assimilation, transport and/or intracellular processing. For example, mutations in TCN2 (the gene codifying TCII expression) are often associated to severe Cbl deficiency diseases and symptoms [72].

  Other mutations can affect the Cbl release from the lysosome or the function of chaperones involved in Cbl trafficking and processing like MMACHC or MMADHC leading to an impairment in the synthesis of AdoCbl, MetCbl or both depending on the specific mutation [73,74].
- **Impaired protein degradation:** As explained before, HC is a key carrier protein for Cbl transport to the duodenum, where it is degraded, allowing the free Cbl to bind to IF. However, a low pH in the intestine caused by several alterations like the Zollinger-Ellison syndrome or an exocrine pancreatic insufficiency can prevent HC degradation by the pancreatic released proteases [75].

  Besides, several causes, such a reduced acid secretion caused by a chronic gastritis or a long-term treatment with proton pump inhibitors, may limit the release of dietary B_{12} from its food carrier proteins [76,77].
- **Impaired intestinal internalization**: Several genetic disorder can cause mutations in key proteins for Cbl internalization. For example, the Imerslund-Gräsbeck syndrome is caused by mutations in CUBN and AMN (that codified

for cubilin and the amnionnless protein respectively) and therefore causes and impairment in Cbl internalization due to changes in the CUBAM receptor complex (decreased binding affinity, decreased activity, lower expression and receptor instability) [78].

- **Bowel Inflammation**: Inflammatory bowel diseases such as Coeliac or Chron's disease can affect the ileum integrity and, therefore, cause malabsorption [71].
- **Infections**: In human infection caused by *Giardia lamblia*, *Helicobacter pylori* or *Diphyllobothrium latum*. Those parasites can trap the ingested vitamin B_{12} and lead to its shortage [79].
- **Bacterial overgrowth**: most of the bacteria present in human microflora are B_{12} consumers. Extreme conditions of bacterial overgrowth can lead to a Cbl deficit due to the increased heterotrophic exhaustion of the consumed vitamin [80].
- **Medications**: Some medications, like the previously mentioned proton pump inhibitors, can also lead to B_{12} malabsorption. Other examples of medications that interfere with a correct B_{12} absorption are cholestyramine, a drug use to treat hypercholesterolemia that can chelate IF, and drugs like colchicine or some antibiotics that can act as inhibitors of the internalization of the IF-Cbl complex [69].

All these alterations could eventually lead to several physiological consequences and clinical manifestations. It also must be considered that, because the very low daily vitamin B_{12} requirement and the efficiency of the kidney storage and reabsorption system, these symptoms could take months or even years to appear.

Pernicious anemia is probably the most common clinical manifestation of vitamin B_{12} shortage. Its symptoms included fatigue, weight loss, headaches and, in severe cases, dementia, memory loss, muscle weakness and peripheral neuropathy, which can become lethal without treatment.

Besides pernicious anemia, a vitamin B_{12} deficiency could also lead to other symptoms, as summarized in Table 2.

Table 2. Main clinical manifestations of vitamin B_{12} deficiency

Main clinical manifestations and symptoms		
Hematological disorders	• Megaloblastic anemia • Leukopenia • Pancytopenia • Thrombocytopenia • Thrombocytosis	• Muscle weakness • Nauseas • Diarrhea • Numbness in extremities
Neurological disorders	• Areflexia • Cognitive impairment • Gait abnormalities • Olfactory impairment • Irritability • Depression	• Loss of proprioception and vibratory sense • Dementia • Psychosis • Impaired sensory and peripherical nerve function • Subacute combined degeneration of the spinal cord.
Other symptoms	• Vitiligo • Glossitis • Hyperpigmentation • Hearing loss • Jaundice • Macular degeneration	

1.5 Vitamin B_{12} Biosynthesis

The discovery of the structure and biological functions of the different vitamin B_{12} compounds in the 1970s focused the attention of many researchers on the biosynthetic pathways of Cbl-producing organisms. The structural complexity, as it was later established, was due to a large and convoluted biosynthesis that involves more than thirty genes and many enzymatic steps for the “de novo” synthesis of the molecule. This pathway is thought to be exclusive for some bacteria and archaea, as there is no genetic evidence of any eukaryotic organism being capable of producing any isoform of Cbl [81,82].

Although some intermediates were found and isolated earlier [83], it was not until the 1990s that the complete biosynthetic pathway was described in *Pseudomonas denitrificans* [84]. The genes involved in Cbl synthesis were given the prefix cob and a letter that refers to each gene position in the operon. In the following years, the cob enzymes and cobalamin intermediates of *Pseudomonas denitrificans* were

characterized and isolated by the French company Rhône-Poulenc Santé, nowadays Sanofi [85].

Later, genes involved in Cbl biosynthesis were characterized in other organisms, such as *Bacillus megaterium*, *Salmonella enterica* and *Propionibacterium freudenreichii*. From the beginning, it was clear that the pathway found in the later organisms was similar to the one found in *P. denitrificans* but genetically different. Key differences included the lack of a monooxygenase and a different cobaltochelatase. Taking this into account, two different pathways for Cbl biosynthesis were established: (i) an aerobic or late cobalt insertion pathway, performed by *Pseudomonas denitrificans* [86] and, as it was later discovered, by other microorganisms such as *Ensifer casida* and *Sinorhizobium meliloti*; and (ii) an anaerobic or early cobalt insertion pathway, performed mainly by *Propionibacterium freudenreichii*, *B. megaterium* and *S. enterica* [87].

Independently of the biosynthetic pathway, tetrapyrrole synthesis begins with the synthesis of 5-aminolaevulinic acid (ALA). Thereafter, ALA conversion to a tetrapyrrolic macrocycle structure is performed by three different enzymatic reactions. First, an ALA dehydratase (EC 4.2.1.24), a Zn^{2+} and Mg^{2+}-dependent enzyme catalyzes the condensation reaction between two ALA molecules to form porphobilinogen (PBG) [88]. Then, a PBG deamynase (EC 4.3.1.8) polymerizes four molecules of PBG into a linear tetrapyrrole. Finally, a uroporphyrinogen III synthase (EC 4.2.1.75) can invert the final pyrrole unit and link it to the first pyrrole unit of the linear tetrapyrrole, forming uroporphyrinogen III, an unsymmetrical hexahydro porphyrin isomer [89]. This molecule is the last intermediate shared with other prosthetic groups, such as heme and chlorophyll groups [90].

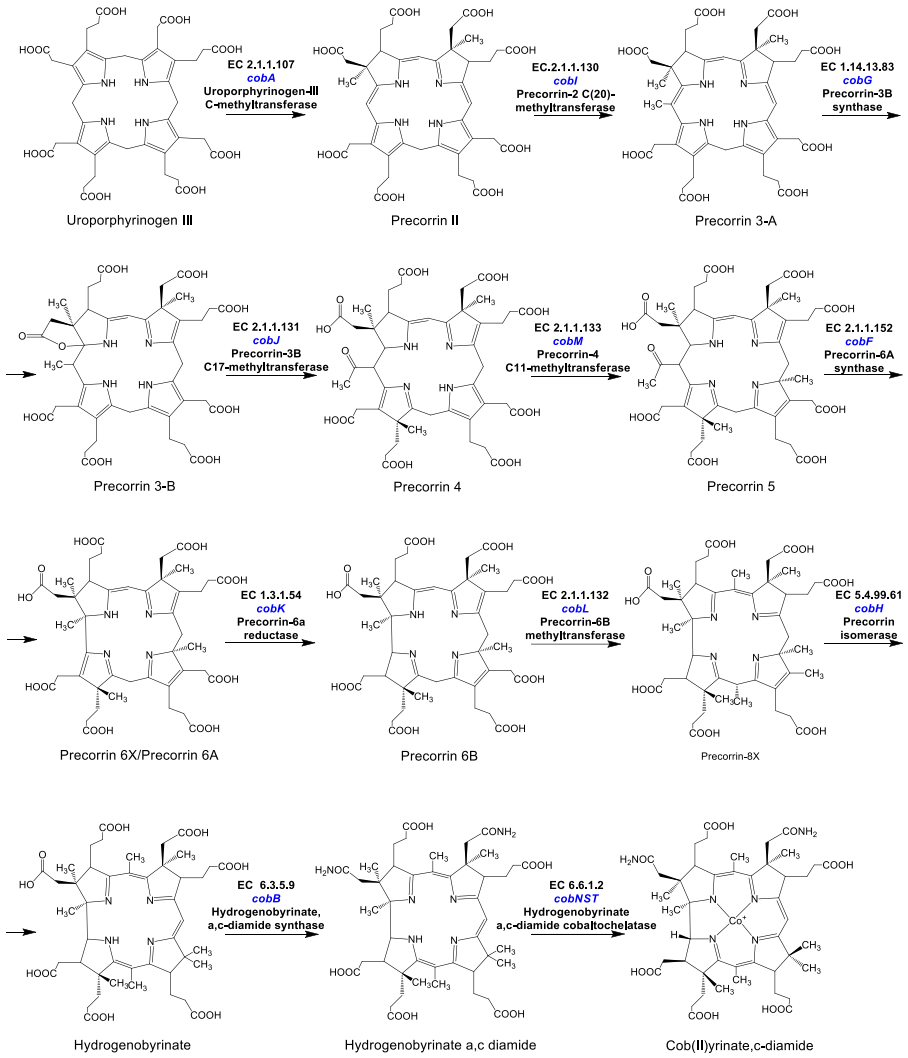

Figure 10 Aerobic pathway for cobalamin biosynthesis. *The EC number, the gene and the reaction name are provided for each step.*

The transformation of uroporphyrinogen III to precorrin-2, the first molecule in Figure 10, is catalyzed by an uroporhyrinogen III methyltransferase (EC 2.1.1.107), which requires S-adenosyl-L-methione (SAM) as the methyl donor. More specifically, the enzyme methylates at C-2 of the uroporphyrinogen III forming precorrin I and, after a phototrophic tautomerization, the same enzyme is able to methylate at C-7, obtaining precorrin-2, which is the last common intermediate for coenzyme siroheme, P450 and vitamin B_{12} [90,91].

The main differences between the aerobic and anaerobic pathways are in the ring contraction and cobalt chelation steps (see Figure 10 and 11). On one hand, the ring contraction in the aerobic pathway requires a molecule of oxygen plus a monooxygenase (CobG) to form precorrin-3B, a hydroxylated γ-lactone intermediate that undergoes a masked pinacol rearrangement during the ring contraction, extruding the methylated C20 position. The ring is then totally contracted, and an acetic acid molecule is liberated in the process [84]. On the other hand, the ring contraction takes place at a later stage in the anaerobic pathway when the cobalt has already been inserted in the molecule. This step is catalyzed by the enzyme codified by the *cbiH* gene, with no molecular oxygen needed. Thereafter, a SAM-dependent at C17, promoting the extrusion of the already methylated C20 position and methylation takes place forming a δ-lactone ring [28].

Cobalt chelation is also very different in both pathways. In the aerobic pathway, there is a "late" insertion of the cobalt atom once the ring has been fully contracted. This insertion is catalyzed by an ATP-dependent multienzyme complex (cobNST) in the presence of magnesium [28,33]. In the anaerobic pathway, this step takes place at an earlier point on the route, when a cobalt chelatase, encoded by either *cbiX* or *cbiK* genes, with the ring still in a non-contracted state [28].

The final steps of both biosynthetic pathways are, again, fairly similar between both pathways as shown in Figure 12.

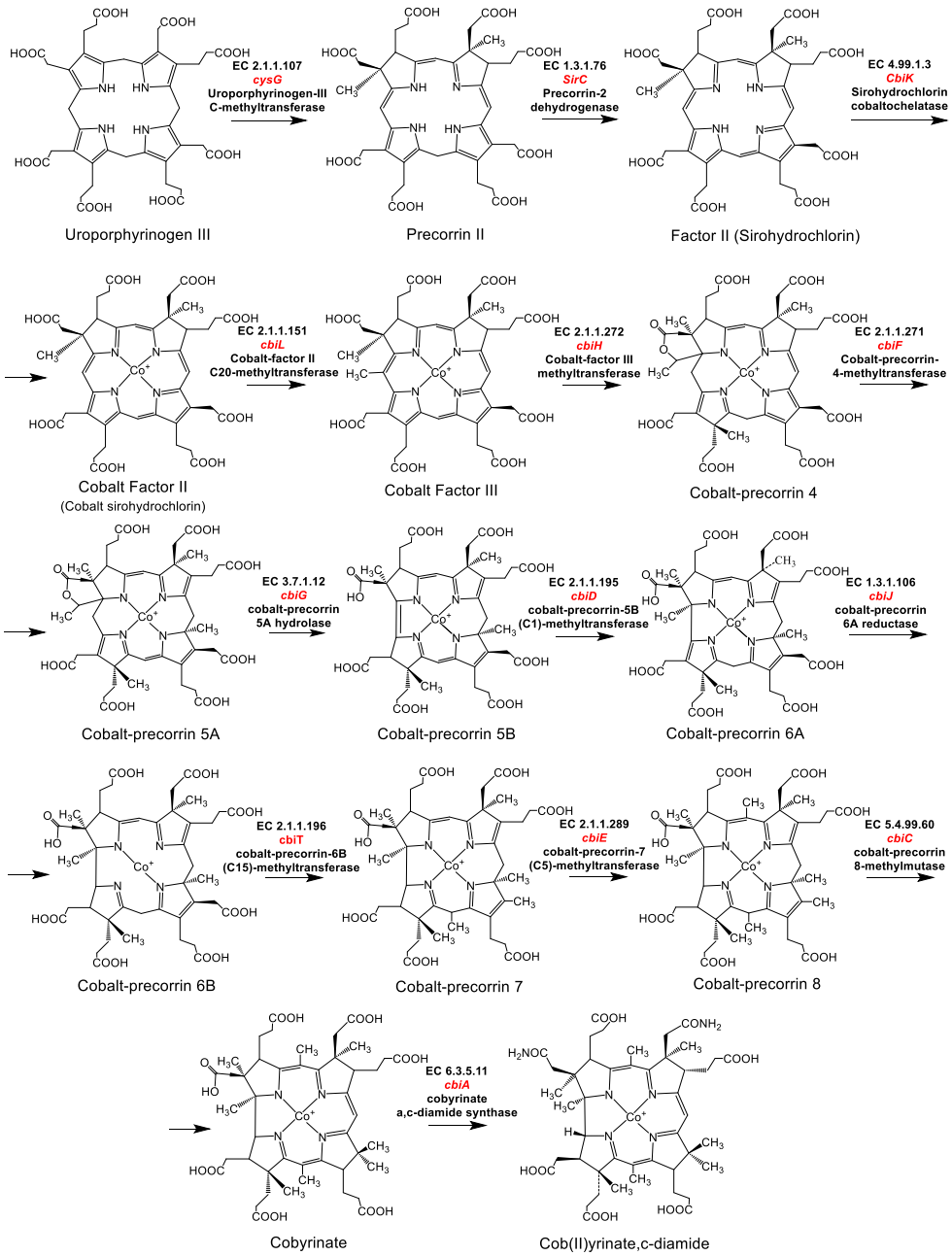

Figure 11 Anaerobic pathway for cobalamin biosynthesis. *The EC number, the gene and the reaction name are provided for each step.*

In addition, independently of the biosynthetic pathway of the corrin ring, DMBI is produced separately to be later attached in α-axial conformation. The lower ligand synthesis has been described recently and also presents two clearly differentiated routes (the aerobic and the anaerobic pathway), depending on the needs of oxygen.

On one hand, the aerobic biosynthesis of DMBI is catalyzed by the 5,6-dimethylbenzimidazole synthase BluB (EC 1.13.11.79), which performs the fragmentation and contraction of the bound flavin mononucleotide cofactor and the cleavage of the ribityl tail to form DMBI and D-erythrose 4-phosphate in the presence of molecular oxygen. Later, the phosphoribosyltransferase CobU/T (EC 2.4.2.21) introduces the DMBI via a nucleophilic substitution reaction [34]. This pathway was firstly described for *S. meliloti* [35] and later found in the majority of Cbl-producing bacteria [36], including the two most important industrial producers, *Propionibacterium freudenreichii* [37] and *Pseudomonas denitrificans* [38]. This fact highlighted the incapacity of *Propionibacterium freudenreichii* to completely produce Cbl anaerobically without any external addition of DMBI.

On the other hand, the anaerobic biosynthesis of DMBI is catalyzed by the gene products of the *bzaA-bzaB-cobT-bzaC-bzaD-bzaE* operon, which promote the formation of DMBI with 5-hydroxybenzimidazole, 5-methoxybenzimidazole and 5-methoxy-6-methylbenzimidazole as intermediates. This route was described in the obligate anaerobic bacteria *Eubacterium limosum* [39] and *Acetobacterium woodii* [34].

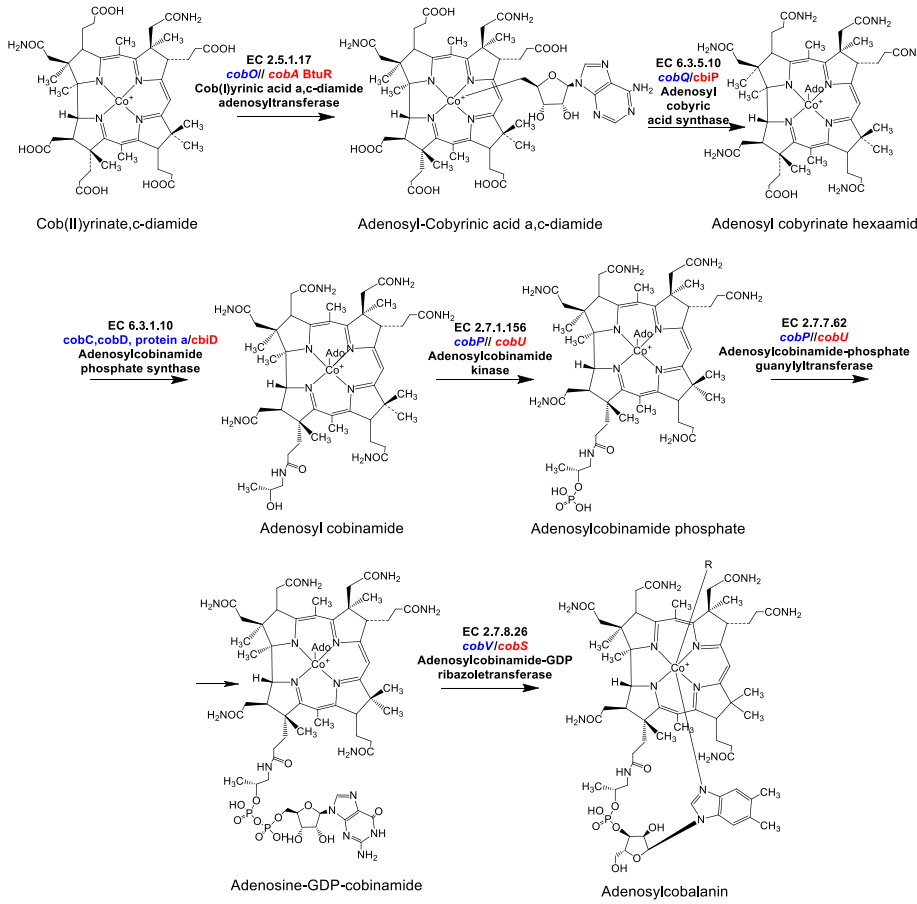

Figure 12 Final steps for cobalamin biosynthesis. *Genes from aerobic and anaerobic pathways are shown in blue and red respectively.*

1.6 Industrial production of vitamin B_{12}

The demand for cobalamin by the food, beverage, dietary and nutraceutical industries has increased sharply in recent years due to the increased health awareness of the general population as well as the growing popularity of alternative diets, such as vegan and vegetarian diets. For this reason, many efforts have been made in strain and process optimization for cyanocobalamin production over the years [81,92].

Historically, the microorganisms used for cobalamin production at industrial scale were strains with high natural productivity, mainly different strains

of *Propionibacterium freudenreichii* and *Pseudomonas denitrificans*, as well as related strains, such as *Pseudomonas nitroreducens* and *Ensifer casida* [81,92].

For many years, a common strategy to improve these strains has been the usage of random mutagenesis techniques to increase vitamin B_{12} productivity or resistance to toxic intermediates present in the media [93]. Nevertheless, overexpression of genes involved in cobalamin biosynthesis [94], heterologous expression of foreign genes [95] and downregulation [96] of several genes have also generated better producer strains. Furthermore, it is worth noting the appearance of new productive strains with promising results, such as *Bacillus megaterium* [97] and *Acetobacter pasteurianus* [98], and the heterologous expression of the biosynthetic pathway in other well-known cell platforms, such as *E. coli*, the later extensively reviewed in Fang and coworkers' study [92]. Recently, Balabanova and co-workers have extensively reviewed the genetic and biosynthetic regulation as well as the genetic tools that have been used with the aim of improving cobalamin production in different cell factories [99].

In contrast, there are also many examples of advances in vitamin B_{12} microbial production by bioprocess optimization. Table 3 summarizes the most relevant innovations performed at a bioprocess level for increased Cbl production and new strategies for Cbl production in new platforms or media. Studies focused on increased production via genetic engineering of known strains are not presented because the objective of this section is to provide an update on the main bioprocess innovations for biotechnological cobalamin production. A summary of culture conditions, media specifications and volumetric productions and productivities is included.

Overall, sharp differences in cobalamin production can be found amongst different producing microorganisms. In this sense, volumetric productions and productivities obtained with *Pseudomonas denitrificans* are clearly superior to the ones obtained with other producers, while *Propionibacterium freudenreichii* productions vary widely between strains and culture conditions. In the latter case, strategies based on decreasing the propionic acid inhibitory effect seem to be the most effective.

1.6.1 Microbial Production in *Pseudomonas denitrificans*

Pseudomonas denitrificans is a Gram-negative bacterium that uses the aerobic biosynthetic pathway to produce vitamin B_{12}. Despite not having a Generally Recognized as Safe (GRAS) status, *Pseudomonas denitrificans* is currently the main vitamin B_{12} producer used by industrial manufactures, such as Sanofi in Europe [100] or the Huarong Pharmacy Corporation in China.

On one hand, the Sanofi strain was originally generated by a combination of random mutagenesis and molecular biology techniques and, although no official information about its volumetric production is available, taking into consideration other aerobic strains, it is tempting to speculate that it may produce around 200–300 mg/L [101]. The optimized strain was originated from a natural, high-producing strain known as MB-580, first described and patented in 1962 (US3018225A)[102]. Over several years, the French company Rhône-Poulenc amplified several of the cob genes involved in vitamin B_{12} biosynthesis until certain high-producing strains were created—SBL27 and, eventually, SC510 [85]. Sanofi, former Rhône-Poulenc, is now the main European vitamin B_{12} manufacturer.

The dominant worldwide producers of vitamin B_{12} on the market are, however, based in China and include the North China Pharmaceutical Company, the Henan Luyuan Pharmaceutical Company, the Hebei Yuxing Bio-Engineering Company and the Chinese CSPC Huarong Pharmaceutical Company, with a combined reported vitamin B_{12} production in 2020 of approximately 31.41 tons and an estimated value of USD 339.8 million. The origin of the strains used in their industrial productions is not precisely known but assumed to be an aerobic strain due to different publications from research groups related to the Huarong Pharmaceutical Company [103–105].

Besides genetic modifications, vitamin B_{12} productivity improvement in *Pseudomonas denitrificans* has also been achieved with culture media optimization and changes in bioprocessing conditions. For example, the effects of trace elements in media, pH, dissolved oxygen control and the addition of several supplements have been tested. In this sense, the addition of Zn^{2+} has been reported to have a significant positive effect on the synthesis of ALA and PBG, two of the main precursors of cobalamin, while

supplementation with Co^{2+} and DMBI, the base that is incorporated into the nucleotide loop, positively affects production [106]. Optimization of the initial amounts of these three compounds by design of experiments led to a 13% increase in cobalamin production [106].

Media composition affected the pH stability of cultures and showed a significant effect on vitamin production. In order to better control pH, a feeding strategy with glucose as carbon source and betaine as methyl donor was developed and was found to be beneficial for vitamin production when applied to 120 m^3 bioreactor cultures [103–105]. Moreover, although it is well known that betaine acts as a methyl donor for vitamin B_{12} biosynthesis [107] and enhances the formation of several key intermediates, such as ALA, glutamate, glycine and methionine [108], high concentrations of betaine can also inhibit cell growth [105,108]. Therefore, a proper betaine feeding strategy was further developed to balance the negative effect on cell growth and the positive effect on cobalamin production which was later successfully implemented at industrial scale [105].

Oxygen transfer rate (OTR) has also been a major subject of bioprocess optimization in *Pseudomnas denitrificans*. Higher OTRs during initial culture stages enhance cell growth, while lower OTRs in later stages were found to be critical for higher productivity [109]. Later studies revealed that the increased production observed in lower oxygenation conditions can be related to alterations in cell morphology, stimulating change from the cell growth phase to an elongation state that presents higher vitamin B_{12} production [110]. Taking this into consideration, several multi-steps dissolved oxygen control strategies were developed, in which aeration and agitation were gradually reduced until dissolved oxygen values fell below 2%, obtaining an improvement in production around 17% [104,110]. Furthermore, the addition of respiratory chain inhibitors, such as rotenone, could also enhance vitamin production despite a detrimental impact on cell growth [67].

Finally, different carbon and nitrogen sources, such as glucose, maltose syrup, beet molasses and corn steep liquor, have been tested as cheaper alternatives to more expensive refined sucrose and glucose. Some of these compounds could negatively affect pH stability and therefore the final vitamin production [111]. Nevertheless, a

combination of maltose syrup, corn steep liquor and betaine has been reported as a successful and cheaper alternative to the traditional media compositions [112].

1.6.2 Microbial Production in *Propionibacterium freudenreichii*

Propionibacterium freudenreichii strains comprise Gram-positive rod-shaped bacteria named after their capacity to synthesize large quantities of propionic acid by the Wood–Werkman pathway. In contrast to aerobic vitamin B_{12} producers, *Propionibacterium freudenreichii* has the advantage of GRAS status having been granted by the FDA and Qualified Presumption of Safety (QPS) status granted by the EFSA.

Some genetic engineering approaches were tested in *Propionibacterium freudenreichii* to obtain higher amounts of vitamin B_{12}. For example, the overexpression of some of the main genes involved in cobalamin synthesis [94] and a genome shuffling approach [113] were reported to improve cobalamin production. However, the main industrial strains were usually obtained by random mutagenesis using different mutagenic agents, such as UV light or chemical compounds, to obtain better cobalamin producers. In *Propionibacterium freudenreichii*, these high-yield strains usually present higher tolerance and resistance to propionic acid [93].

Propionibacterium freudenreichii are facultative anaerobic strains that follow the anaerobic biosynthetic route for cobalamin production. Despite their only producing high cobalamin yields at very low-oxygen conditions, oxygen is needed for DMBI synthesis and its attachment to the corrinic ring [114]. For this reason, the culture is usually divided into two stages: a first stage in which the cells are cultured in complete anaerobic conditions and a second stage, usually after 72–96 hours of cultivation [114–116], in which gentle aeration is provided by agitation to generate the microaeration needed for DMBI synthesis and cobalamin production [100].

The GRAS status of these vitamin B_{12} producers allowed the expansion of their market scope by allowing their direct use in the production of food products. In this sense, in situ food fortification with *Propionibacterium freudenreichii* has been successfully tested using food-like media, such as in cheese-like propionic medium or whey-based liquid medium [116,117], cereal matrices [118] and in situ fortification of tempeh

[119]. Although final cell densities and reported production levels are low compared to other traditional media, in the context of food fortification, it allows an increase in cobalamin content using non-traditional sources and the achievement of the recommended daily vitamin B_{12} consumption levels with only small amounts of fermented products [117].

As mentioned before, these bacteria have the ability to produce large quantities of propionic acid, which eventually becomes toxic and limits cell growth [120]. Therefore, several bioprocess optimization strategies for decreasing propionic accumulation have been tested. In particular, in situ product removal (ISPR) techniques have shown promising results for the simultaneous production of propionic acid and vitamin B_{12}. Among ISPR techniques, the use of expanded-bed adsorption bioreactors (EBABs) with high biocompatibility resins, such as ZGA330, has been reported to support vitamin B_{12} volumetric production levels between 40 mg/L and 60 mg/L [120]. In EBABs, adsorption occurs when the column is expanded, allowing the culture to pass through the chromatographic column without clogging, while propionic acid is retained in the resin [121]. Different culture conditions [121], carbon and nitrogen sources [122] and the addition of media supplements, such as DMBI [109], have been tested for the simultaneous improvement of propionic acid and vitamin B_{12} production. In an EBAB system, the combination of glucose and glycerol [121] and corn stalk hydrolysates [122] have proven to be efficient carbon sources, with reported volumetric CNCbl production levels of 43.2 mg/L and 47.6 mg/L, respectively.

Another interesting approach to decrease propionic acid concentration is the co-fermentation of *Propionibacterium freudenreichii* with other microorganisms capable of metabolizing propionic acid. For example, the co-culture of *Propionibacterium. freudenreichii* and *Ralstonia eutropha* showed an improved cobalamin production from 6.73 mg/L to almost 19 mg/L [123]. Moreover, co-fermentation has also been successfully applied not only to reduce propionic acid but also to either produce more than one product simultaneously or to fortify other cell cultures. Simultaneous production of both folate and vitamin B_{12} was achieved with the co-cultivation of *Propionibacterium freudenreichii* and *Lactobacillus plantarum* (currently

named *Lactiplantibacillus plantarum* [124]) [125], and a co-fermentation of a *Basidiomycota* strain plus *Propionibacterium freudenreichii* has been recently patented to simultaneously produce vitamin D and B_{12} [126]. A food fortification example would be the in-situ vitamin B_{12} production in bread dough performed in whey-based media with the co-cultivation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* (currently named *Levilactobacillus brevis* [124]) to ensure microbial safety and stability [127].

Supplementation with cobalamin precursors is another common strategy for increasing productivity. The addition of common precursors and needed compounds, such as ALA and Co2+, has often been described as beneficial for vitamin production [81]. Although all *Propionibacterium freudenreichii* strains are capable of synthetizing DMBI on their own, the biosynthesis of this base is low. Moreover, DMBI formation is not possible in strictly anaerobic conditions, as oxygen is needed for its synthesis [128]. If the availability of DMBI is restricted, the active form of vitamin B_{12} is not formed, and the cells begin to accumulate incomplete forms, such as cobinamide or pseudovitamin B_{12}. Thus, the addition of DMBI or even DMBI precursors, such as Riboflavin or Nicotinamide, has been consistently reported as a positive factor in cobalamin production [81]. In addition, other groups have found that the addition of vitamin B_{12} analogues can decrease feedback inhibition and increase cobalamin production [129].

Finally, *Propionibacterium freudenreichii* cultures are also interesting in industrial settings for their ability to grow in a wide range of complex carbon and nitrogen sources and even waste and spent media, such as molasses [130], crude glycerol [131], waste frying sunflower oil [132], tomato pomace [133], liquid acid protein residue of soybean [134] and vegetable juice spent media[135].

Table 3 Summary of reported productions with the industrial cobalamin producing strains.

Microorganism Strain	Main media components	Scale	Summary/Innovation	Volumetric production	Volumetric productivity (mg/L/h)	Reference
Bacillus megaterium DSM 319	Terrific Broth Media	250mL Shake Flask	Precursor supplementation and pO_2 control	0.21 mg/L[3]	0.006 mg/L/h	[97]
Lactobacillus reuteri ZJ03	Soymilk	250 mL Shake Flask	Different carbon sources supplementation	0.204 mg/L	0.003 mg/L/h	[136]
P. freudenreichii subsp. shermanii NRRL-B-4327, 3523 and NRRL-B-3524	Sodium Lactate broth	250 mL Shake Flask	Vitamin B_{12} analogues addition	31 mg/L	0.51 mg/L/h	[129]
Propionibacterium freudenreichii CICC 10019	Glucose, CSL[1]	7L stirred tank bioreactor	Expanded-bed-bioreactor (EBAB) with crop stark hydrolysates	47.6 mg/L	0.18 mg/L/h	[122]
Propionibacterium freudenreichii CICC 10019	Glucose, CSL	7L stirred tank bioreactor	EBAB bioreactor	43.4 mg/L	0.27 mg/L/h	[120]
Propionibacterium freudenreichii CICC 10019	Glucose, CSL	1,5 L stirred tank bioreactor	EBAB bioreactor and DMBI addition	58.8 mg/L	0.59 mg/L/h	[109]
Propionibacterium freudenreichii CICC 10019	Glucose/glycerol, CSL	5-L stirred tank bioreactor	EBAB bioreactor, glycerol as Carbon source and Crop stalk hydrolysate as nitrogen source	43 mg/L	0.36 mg/L/h	[121]
Propionibacterium freudenreichii DF13	Supplemented Whey Permeate	1L stirred tank bioreactor	Co-culture with Lactobacillus plantarum SM39 for simultaneous folate and Cbl production	0.75 mg/L	0.004 mg/L/h	[125]

Propionibacterium freudenreichii DSM 20271 // *Lactobacillus brevis* ATCC 14869	Wheat bran dough	n.d.[2]	Co-fermentation in wheat bran dough for in situ production of Vitamin B_{12}	332 ng/g[3]	n.d.[2]	[127]
Propionibacterium freudenreichii IFO 12424 //*Ralstonia eutropha* H16 (ATCC17699)	Polypeptone, Casein, Yeast extract	5L stirred tank bioreactor	Cell recycling system and co-culture with Ralstonia eutropha for decreasing propionic acid inhibition	8 mg/L[3]	0.14 mg/L/h	[123]
Propionibacterium freudenreichii PTCC 1674.	Tryptone, Yeast extract, different carbon sources	100 cm3	Waste frying sun oil as a carbon source for vitamin B_{12} production	2.74 mg/L	0.02 mg/L/h	[132]
Propionibacterium freudenreichii subs shermanii ATCC 13673	Glucose, Yeast extract	2L stirred tank bioreactor	Inoculum volume, pH control and substrate concentration optimization	0.087 mg/L	0.002 mg/L/h	[115]
Propionibacterium freudenreichii subs shermanii CICC 10019	Glucose, CSL	100 L fermenter	Addition of DMBI precisely with Ado-Cbl control strategy	39.15 mg/L	0.32 mg/L/h	[137]
Propionibacterium freudenreichii subs shermanii	Glycerol, Tryptone, Casein, DMBI	200 mL Shake-Flask	Media optimization by Design of Experiments with crude glycerol as the main carbon source	4.01 mg/L	0.024 mg/L/h	[131]
Propionibacterium freudenreichii subs shermanii	Whey Based Media	20 mL Tubes	DMBI, Nicotinamide and Riboflavin supplementation	5.3 mg/L	0.03 mg/L/h	[116]
Propionibacterium freudenreichii subs shermanii	Food-like media (cereal matrices)	n.d.	Precursor supplementation in different cereal like matrices	1.5 mg/Kg	0.009 mg/Kg/h	[138]

Propionibacterium freudenreichii subs shermanii 2067	Cheese-based propionic media/Whey based liquid media	50 mL Erlenmeyer Shake-Flask	Production in food like conditions without DMBI addition	0.124 mg/L[3]	0.0013 mg/L/h	[117]
Pseudomonas denitrificans	Maltose, peptone, betaine	250 mL Shake Flask	Addition of rotenone as a respiration inhibitor for enhanced production	54.7 mg/L	0.57 mg/L/h	[139]
Pseudomonas denitrificans	Beet molasses, sucrose, betaine	120 m3 fermenter	Glucose-betaine feeding. pH control strategy	214.13 mg/L[3]	1.27 mg/L/h	[111]
Pseudomonas denitrificans	Glucose, CSL, betaine	120 m3 fermenter	Stepwise oxygen uptake rate control strategy	188 mg/L	1.12 mg/L/h	[103]
Pseudomonas denitrificans	Glucose, CSL, betaine	50 L fermenter	Effects of specific oxygen consumption rate in cell morphology and production	213.1 mg/L	1.88 mg/L/h	[110]
Pseudomonas denitrificans	Maltose, peptone, betaine	250 mL Shake Flask	Betaine supplementation	58.61 mg/L	0.48 mg/L/h	[[108]
Pseudomonas denitrificans	Maltose syrup, CSL, betaine	120 m3 fermenter	Maltose syrup and CSL as the main substrates.	198.27 mg/L	1.10 mg/L/h	[112]
Pseudomonas denitrificans	Glucose, CSL, betaine	120 m3 fermenter	pO_2 stepwise control	198.80 mg/L	1.18 mg/L/h	[104]

The main microorganism, strain, scale, and media compounds are shown as well as a brief summary of the main innovation and the volumetric productions. Volumetric productions are presented in mg/L. Volumetric productivities were calculated using data from the original publication. [1]CSL: Corn Steep Liquor; [2] n.d.: not determined; [3]Values were converted to mg/L using the data available from the original publication.

1.7. Vitamin B_{12} Market Applications and the State of the Market

The most important market for B_{12} products is the feed and food industry, where its efficiency and security has been extensively verified [140], although its usage is also extensive in the supplement and pharmaceutical industry.

In the feed and food industry, CNCbl is commonly added to poultry, pig and calf feeds at dosage levels between 10 to 30 mg/t in almost all Europe and the USA [100]. It is also used as an additive in several food products, for example, in cereal, where its organoleptic properties and chemical properties, such as odorlessness, tastelessness and solubility in water, are an advantage for the fortification of several products. Nevertheless, its bright red color can present a challenge to its addition in other foods, such as white bread [141].

Regarding its usage in the supplement industry, vitamin B_{12} has been gaining relevance in later years, especially with the rise in the popularity of vegetarian and vegan diets [142]. CNCbl is the most used form mainly because of its stability, price, proven safety [143] and its similar efficiency compared to other forms [142,143].

Besides direct supplementation, B_{12} is also widely used for the fortification of different food products. In this case, CNCbl is again the preferred form due to its higher stability when processed and cooked [144]. B_{12}-fortified products are common in the United States and other countries where, for example, B_{12}-fortified cereals and milk provide a significant amount of the total daily Cbl requirement [144]. Other alternatives, such as flour fortification, have also been considered.

Vitamin B_{12} is also widely used in the pharmacological sector, where, besides CNCbl, other forms, such as OHCbl, AdoCbl and MetCbl, are also produced and distributed due to their higher uptake and more sustained serum levels [100]. Pharmacological B_{12} is presented in different forms, such as nasal sprays, oral and sublingual products, and even direct injections to treat pernicious anemia, B_{12} deficiency, cyanide poisoning and lower homocysteine levels. There are also several claims that have been made regarding its positive effect in patients with Alzheimer's disease and as a stimulant of the immune system, though more evidence is needed [100,145].

Considering all these different usages and markets, it should not be a surprise that vitamin B_{12} total worldwide production and market volume have been steadily increasing, although the exact worldwide market values are difficult to obtain due to the scarcity of reliable information. However, it is safe to assume a great increase in overall production in the last decades. In 1989, the overall production was around 3 tons per year [100] and, by 2005, it had already increased to 10 tons and had a market value of approximately EUR 77 million [141]. As mentioned before, production in China in 2020 reached 31.41 tons with a market value of USD 339.48 million, while some projections have been made that the vitamin B_{12} market will reach a total value of USD 410 million by 2027. The progressive increase in the elderly populations, the rise of alternative vegan and vegetarian diets and the scarcity of animal food products are factors that explain this sharp market increase and are also the reasons why the B_{12} market is expected to continue to grow in the future.

1.8. Patents. State of the art of Industrial Cbl production.

Research in cobalamin production has been extensively patented since its very beginning, with thousands of patents being published, although most of them are no longer active. Due to the large number of publications and the fact that nowadays most of the production and industrial advances are being made in China, providing a comprehensive list of all the currently active and used patents is difficult and beyond the scope of this review. Instead, Table 2 aims to provide a historical overview of some of the most important and relevant patents for the current industrial strains. We include the state of each patent—as expired, abandoned, or active—in addition to the main innovations claimed and, if available, volumetric production figures.

In 1962, one of the first relevant patents related to the subject after the discovery of the extrinsic factor was [102], where the discovery of a natural, high-producing strain (*Pseudomonas denitrificans* MB580) was described. This strain was extensively researched and many high-producing strains, such as SC510, were obtained through genetic engineering approaches, as described in US20060019352A1 [146] In fact, researchers associated with Rhône-Poulenc used MB580 and its derived strains to study the genes behind aerobic Cbl biosynthesis and presented the complete aerobic biosynthetic pathway in 1990 [85]. Nowadays, the precise aerobic strains used for

industrial cobalamin production are not known but are thought to be closely related to SC510. More recent aerobic strain-related patents cover all stages of bioprocess development: (i) screening and identification of new producer strains (CN111254173 A [147]), (ii) media and bioprocess optimization (CN108949866 A [148], CN110205350 A [149], CN109837320 A [150]) as well as (iii) downstream processing (CN111808158 A [151]).

On the other hand, most of the earliest anaerobic- and *Propionibacterium freudenreichii*-related patents were focused on strain optimization for CNCbl production. In this sense, one of the most significant early patents is US4544633A [93], where the generation of a propionic-resistant producing strain by random mutagenesis is described. Besides strain enhancement, later patents often focused on bioprocess optimization and the use of EBABs for the simultaneous production of CNCbl and other compounds of interest, such as propionic acid (US6492141B1 [152]).

In addition, the possibility of using Cbl-producing strains of *Propionibacterium freudenreichii* as probiotics has also been patented (US7427397B2 [153]). Interestingly, the latest patents related to anaerobic strains are focused on either co-cultivation strategies (US9938554 [154], US20200149084A1 [126]) or co-production (CN206828509U [155], IN201827044769 A [156]). The latter patent, IN201827044769 A [156], claims a volumetric production of 76.13 mg/L, which is the maximum production reported for a *Propionibacterium freudenreichii* strain.

Finally, there are a number of patents with alternative producing strains, such as *B. megaterium* (US2576932A [112]), several *Lactobacillu*s strains (WO2011154820A2 [157]), *S. meliloti* (CN104342390 A[158], CN110804598 A [158]) and even *E. coli* (WO2019109975A1 [159]. The production levels of most of these microorganisms are quite low compared to the traditional producers, and patents are often focused on strain identification or strain enhancement by genetic engineering or heterologous expression of the main genes involved in Cbl biosynthesis. However, the exception is the *S. meliloti* strain (CGMCC 9638), which has a vitamin B_{12} production level in the range of 50–115 mg/L [158].

Table 4. Main patents for vitamin B_{12} industrial production.

	Patent Application number (Reference)	Name	Microorganism strain	Innovation	Volumetric production	Year
Propionibacterium genus	US4544633A [93] (Expired)	Process for producing vitamin B_{12} by the fermentation technique, and vitamin B_{12}-producing microorganism	*Propionibacterium freudenreichii* (IFO 12424, IFO 12391, IFO 12426)	Creation of propionic resistant strains (*Propionibacterium freudenreichii* FERM-86 and FERM-87) for enhanced CNCbl production.	15 mg/L	1983
	US6492141B1 [160] (Expired)	Process for the production of vitamin B_{12}	*Propionibacterium freudenreichii* CBS 929.97	O_2 effect in production during the anaerobic phase and a "Fill and Draw" strategy for enhanced production.	19 mg/L	1999
	US6187761B1 [161] (Expired)	Production and use of compositions comprising high concentrations of vitamin B_{12} activity	*Propionibacterium freudenreichii subs shermanii and Pseudomonas denitrificans*	Method for producing Vitamin B_{12} and making highly concentrated compositions	10 mg/L	1999
	US7427397B2 [153] (Expired)	Probiotic *Propionibacterium*	*Propionibacterium jensenii* 702	*Propionibacterium jensenii* as a probiotic	0.0012 mg/L	2004
	EP2376644B1 [162] (Active)	Process for the preparation of a fermentation broth	*Lactobacillus plantarum* DSM 22118 and *Propionibacterium freudenreichii* DSM 22120	Fermentation media optimization and co-culture for folate and Vitamin B_{12} production	1.07 mg/L	2009
	CN206828509U [155] (Active)	A device for producing propionic acid and co-producing vitamin B_{12} by semi-continuous fermentation	*Propionibacterium freudenreichii*	Simultaneous production of propionic acid and Vitamin B_{12} in a semicontinuous fermentation with propionic acid separation.	20.12 mg/L	2017
	US9938554 [154] (Active)	Co-cultivation of *Propionibacterium* and yeast.	*Propionibacterium freudenreichii* (ATCC 6207) and yeast cells (DSM 28271)	Co culture of *Propionibacterium* and propionic resistant yeast to decrease the chemical oxygen load (COD) of spent media	16 mg/L	2018
	US20200149084A1[126] (Active)	Sequential co-culturing method for producing a vitamin- and protein-rich food product	*Basidiomycota* and *Propionibacterium freudenreichii*	Co-culture of *Basidiomycota* genus strains and vitamin B_{12} producing strains for in-situ food fortification	0.0014 mg/L[1]	2020
	IN201827044769 A [156] (Active)	Continuous process for co-production of vitamin B_{12} and organic acids	*Propionibacterium freudenreichii* (ATCC 13673)	Co-production of vitamin B_{12} and organic acids in a continuous	76.13 mg/L	2020

				fermentation with a single bioreactor		
	WO21041759 A1[163] (Active)	Modified *Propionibacterium* and methods of use	*Propionibacterium freudenreichii* (P. UF 1)	Generation of a vitamin B_{12} overproducing strain by introducing a mutation that decreases the activity of the cbiMcbl riboswitch	n.d.[2]	2021
Pseudomonas denitrificans	US3018225A [164] (Expired)	Production of vitamin B_{12}	*Pseudomonas denitrificans* MB-580	A process por Vitamin B_{12} production with a high yield strain (*Pseudomonas denitrificans* MB-580)	2.4 mg/L[1]	1962
	US20060019352A1 [146] (Abandoned)	Methods of increasing the production of cobalamins using cob gene expression	*Pseudomonas denitrificans*	Overexpression of several genes involved in Cob biosynthesis. Generation of several over-producing strains like SC-510	65 mg/L	1990
	US6156545A [165] (Expired)	Biosynthesis method enabling the preparation of cobalamins	*Pseudomonas denitrificans* G2650	Enhanced Cob production by the heterologous overexpression o precursors like DMBI and O-phospo-L-threonine	7.9 mg/L	1996
	CN101538599A [149] (Active)	Method for improving yield of denitrified pseudomonas vitamin B_{12}	*Pseudomonas denitrificans J741*	Enhance cob production by betaine addition optimization	177.49 mg/L	2008
	CN102399845A[166] (Active)	Vitamin B_{12} fermentation production control process based on CO_2 concentration in tail gas	*Pseudomonas denitrificans* MB-580	Vitamin B_{12} enhanced production through a carbon dioxide control strategy during fermentation	164.6 mg/L	2010
	CN101748177 A [167] (Active)	Optimized method for producing vitamin B_{12} through *Pseudomonas denitrificans* fermentation and synthetic medium	*Pseudomonas denitrificans*	Development and optimization of media and bioprocess conditions for improved vitamin B_{12} production	77 mg/L	2010
	CN102021214 A [168] (Active)	Oxygen consumption rate-based vitamin B_{12} fermentation production control process	*Pseudomonas denitrificans*	Vitamin B_{12} production optimization through an oxygen control strategy	171,4 mg/L	2011
	CN102453740 A [169] (Active)	Culture medium for producing vitamin B_{12} by fermenting *Pseudomonas denitrificans* and fermentation method thereof	*Pseudomonas denitrificans*	Use of artificial molasses and bioprocess optimization for a more stable fermentation yield.	198 mg/L	2012
	CN108949866 A [148] (Active)	Multi-stage rotating speed regulating policy for improving *Pseudomonas denitrificans* fermentation for production of vitamin B_{12}	*Pseudomonas denitrificans*	Vitamin B_{12} production improved by optimization of the culture media and	246 mg/L[1]	2018

				the stirring speed of the bioprocess		
	CN108913739 A [170] (Active)	Method for producing vitamin B_{12} by using *Pseudomonas denitrificans* based on pH value control	*Pseudomonas denitrificans*	Improved vitamin B_{12} production by optimization of the bioprocess trough pH value control	248 mg/L	2018
	CN110205350 A [171] (Active)	Method for improving yield of vitamin B_{12} based of regulation of ammonia nitrogen index	*Pseudomonas denitrificans*	A method for improved Cbl production by supplementation with yeast extract controlled by the ammonia nitrogen index	167 mg/L[1]	2019
	CN109837320 A [150] (Active)	Method for promoting *Pseudomonas denitrificans* to generate vitamin B_{12}	*Pseudomonas denitrificans*	Optimization of media and culture conditions for improved vitamin B_{12} production	198 mg/L	2019
	CN111808158 A [151] (Active)	Preparation method of vitamin B_{12} crude product	*Pseudomonas denitrificans*	Downstream process improvement for AdoCbl extraction	n.d.[2]	2020
	CN111254173 A [172] (Active)	Screening method and screening culture medium for bacterial strain for high yield of vitamin B_{12} produced through fermentation production with *Pseudomonas denitrificans*	Several high yield strains of *Pseudomonas denitrificans*	Screening for high vitamin B_{12} producing *Pseudomonas denitrificans* strains and culture medium screening for high vitamin B_{12} production	281 mg/L[1]	2020
Other producers	US2650896A [173] (Expired)	Cyanide ion in production of vitamin B_{12}	*Streptomyces griseus*	Effects of cyanide ion in B_{12} production	Biological assay	1953
	US2576932A [174] (expired)	Fermentation process for production of vitamin B_{12}	*Bacillus megaterium* B-938	Vitamin B_{12} production with *Bacillus megaterium* in a nutrient media with sucrose	0.45 mg/L	1983
	US20050227332A1 [175] (Expired)	Method for producing vitamin B_{12} from hydrogen-metabolizing methane bacterium	A mesophilic methane bacterium obtained from digested sludge	The culture is acclimatized in a H2/CO media and grown in an immobilized bed bioreactor	25.2 mg/L	2005
	US20060105432A1 [176] (Abandoned)	Method for the production of vitamin B_{12}	*Bacillus megaterium* DSMZ509	Genetically modified *Bacillus megaterium* strain	0.008 mg/L[1]	2006
	WO2011154820A2 [157] (Application granted)	Vitamin B_{12} producing probiotic bacterial strains	*Lactobacillus reuteri* (DSM 17938, DSM 16143, ATCC 55730)	In situ food fortification for increased vitamin B_{12} production with *Lactobacillus reuteri* strains	0.018 mg/L[1]	2011
	CN104342390 A [159] (Active)	*Sinorhizobium meliloti* strain and composition and application of Sinorhizobium meliloti strain	*Sinorhizobium meliloti* (CGMCC 9638)	A *Sinorhizobium melitolli* strain capable of producing vitamin B_{12} and optimization of the bioprocess for vitamin B_{12} production	At least 50 mg/L	2015

	WO2019109975A1 [159] (Active)	Recombinant strain of *Escherichia coli* for de novo synthesis of vitamin B_{12}, construction method therefor and application thereof	*Escherichia coli*	Recombinant *E. coli* for the de novo synthesis of Vitamin B_{12}	89 µg/g DCW	2018
	CN110804598 A [158] (Active)	Procorrin-2C(20)-methyltransferase mutant and mutant gene and application thereof in preparing vitamin B_{12}	*Sinorhizobium* (CGMCC 9638)	Generation of a vitamin B_{12} overproducer strain by overexpressing the precorrin-2C(20)-methyltransferase gene	115 mg/L	2020

[1] Values were converted to mg/L using the data available from the original publication; [2] n.d.: not determined

2. Objectives

This thesis project was made possible by the AGAUR Grant and the Doctorats Industrials program, which aims to enhance the competitiveness and internationalization of the Catalan industry by attracting talented individuals to develop R&D&I projects in companies. According to the Doctorats Industrials own webpage: "*The objective of the Industrial Doctorates Plan is to contribute to the competitiveness and internationalization of the Catalan industry, strengthen the tools to attract the talent generated by the country and place future doctors in a position to develop R&D&I projects in a company*."

In collaboration with both the Institut Quimic de Sarria (IQS) and Healtech Bioactives (HTBA), the project sought to generate valuable scientific knowledge for both academic and industrial purposes.

HTBA, formerly a part of Interquim, was established in 2019 and has since been growing, expanding, and developing new technologies to ensure the quality and sustainability of its products and solutions. The company specializes in the production of Active Pharmaceutical Ingredients (APIs), taste modulation molecules, and natural products for functional health and animal nutrition, particularly made from flavonoids and other plant-based substances.

Besides, HTBA is also specialized in the production of vitamin B_{12} derivates that aim to treat vitamin B_{12} deficiency and contribute to other health-related functions like red cell formation, cognitive and immune systems functions, anemia prevention, vegan and vegetarian supplementation, etc.

In this sense, several vitamin B_{12} related products are produced and commercialized by HTBA like mecobalamin, hydroxocobalamin acetate, hydroxocobalamin sulphate, adenosylcobalamin and hydroxocobalamin hydrochloride.

The Doctorats Industrials thesis projects have a primary objective of bridging the gap between the industry and the academic community. To achieve this goal, all the research work was carried out within the Biological Chemistry and Biotechnology Group (Grupo de Química Biológica y Biotecnológica - GQBB) affiliated with the Institut Químic de Sarrià (IQS).

GQBB is a multidisciplinary group consisting of professors and researchers from IQS's Bioengineering department, led by Dr. Antoni Planas. With a strong emphasis on integrating basic and applied research, GQBB conducts research in various fields of study, with a special focus on Carbohydrate Active Enzymes (CAZymes).

The research conducted by GQBB encompasses several main lines of investigation, including enzyme engineering and biocatalysis, bioinformatics and modeling, metabolic engineering of different microorganisms, synthetic biology, and bioprocess development and optimization.

GQBB's multidisciplinary approach and commitment to collaborating with the industry to apply research advancements to industrial applications (evidenced by its inclusion in the IQS Tech Transfer program) have led to fruitful long-term relationships between GQBB and commercial companies seeking to enhance their research and development capabilities. In this regard, GQBB has enjoyed a successful collaboration with HTBA (formerly Ferrer S.A) for over 11 years, resulting in several Doctorats Industrials projects. This thesis project is the latest example of such collaborations.

As described in Chapter 1 of this thesis project, despite being the physiological functioning forms, AdoCbl and MetCbl present several difficulties in their production and, most importantly, its storage. For this reason, the usual way to produce the different vitamin B_{12} derivates is by several transformation processes, property of HTBA, that begin with the much more shelf stable CNCbl. Thereby, the main raw material for their products is CNCbl, usually produced by recombinant high-producing aerobic strains like *Pseudomonas denitrificans* SC510 or its derived strains.

Besides some production still happening in Europe by Sanofi, most of the CNCbl output in the world is done in China and the exact nature of the strains used is not exactly known. For this reason and taking into consideration the peculiarities of the market share where many final customers prefer a non-GMO-based production, the production of its own raw material became a project of both strategic and economic interest for HTBA.

Besides vitamin B_{12} deficiencies caused by absorption or transportation malfunctions as the cases thoroughly described in Chapter 1, many vitamin B_{12} derived products are

destined for the vegan and vegetarian populations, a section of the market that has wide ecological and health awareness. Therefore, developing a CNCbl production process based on a GRAS strain became the main objective of this thesis project.

Possible alternatives to the usually employed aerobic strains commonly used in the current industrial setting are anaerobic strains like *Propionibacterium freudenreichii*. Although their slower growth and lower productivity compared to the aerobic strains, several propionic-resistant *Propionibacterium* strains have been employed in Cbl production over the years. These propionic resistant strains were not obtained by genetic engineering but rather by random mutagenesis process like the ones described in Kojima et al. 1985, where different parental strains were submitted to UV radiation and other mutating inducing stressors to be afterwards selected based on their increased resistance to propionic acid. Unfortunately, these resistant industrially capable strains are still company owned and not accessible in public banks.

For all the reasons described above, it was decided to develop and optimize a CNCbl production methodology with a wild-type strain that could be accessed and purchased in public cell banks like NBRC, ATTC or DMSZ.

In this scenario, even in the propionic resistant strains, high propionic acid concentration still inhibits growth and hindered production in the late stages of the culture. Moreover, even industrially capable anaerobic strains are still far from achieving the values obtained by aerobic strains. Therefore, before considering the alternative to the current use of aerobic strains, there is still ample opportunity for improvement in the production of anaerobic strains such as implementing innovative bioprocess improvements, even in wild-type strains, that could potentially lead to the development of a propionic resistant strain that the company could acquire in the future.

All points discussed above were considered in the design of this thesis and summarized in the following objectives:

- Find a wild-type Propionibacterium strain easily accessible in public cell banks and suitable for cell growth characterization and CNCbl production improvement.

- Develop the methodology for anaerobic cell cultures, specifically for *Propionibacterium freudenreichii* strains.
    - Develop and optimize a suitable fermentation media for *Propionibacterium freudenreichii* strains growth and CNCbl production. Select a suitable and industrially affordable carbon and nitrogen source.
    - Adapt the laboratory conditions to facultative anaerobic microorganisms and study the effect of the different experimental conditions (Erlenmeyer Shake Flask with or without agitation, different shake flasks culture volume, Falcon tube cultures).
    - Assess the efficiency of different published methodologies for Cbl extraction from whole biomass.
    - Develop proper analytics for Cbl identification and quantification based on HPLC.
    - Adapt the usually described two-stage culture process for Cbl production in *Propionibacterium* to the selected strains.
- Developed strategies for increased Cbl production in a non-propionic resistant *Propionibacterium* strain.
    - Study the effect of propionic acid on growth and Cbl production.
    - Optimize supplement composition and timing for increased Cbl production.
    - Study the oxygen needs during the microaerobic phase in the selected strains.
- Scale-up of the bioprocess to a laboratory-scale bioreactor.
    - Test several bioreactor settings and strategies to increase production.
    - Apply the obtained knowledge during the optimization process in Erlenmeyer shake flask in the scale-up process.
- Try to minimize the effect of aeration and propionic acid by different scale-up strategies. Improve production by better characterizing the metabolic needs of the chosen *Propionibacterium* strain.

- Analyze the metabolic needs of the chosen strain and try to develop a defined or semi-defined media that substitutes the traditionally used nitrogen complex source-based medias.

- Overall comparison between publicly available aerobic-producing strains and the best condition obtained by the selected anaerobic-producing strain.

3. *Propionibacterium freudenreichii subs shermanii* as a vitamin B_{12} producer. Growth and production characterization, media composition optimization and analytical methods suitability

3.1. Introduction

As stated in Chapter 2, the main objective of this thesis project is to characterize and optimize the production of vitamin B_{12} via bioprocess improvements with a wild type (wt) strain.

Historically, the main microorganisms used for cobalamin production at industrial scale were different strains of *Propionibacterium freudenreichii* and *Pseudomonas denitrificans*, as well as related strains, such as *Pseudomonas nitroreducens* and *Ensifer casida* [81,92].

A comparison of the different production strains is provided in Table 1 and 2 of Chapter 1. It should be noted that, despite the sharp differences in behavior and overall faster growth and production of aerobic strains like *Pseudomonas denitrificans*, the main efforts of this project in understanding and increasing the productivity of a wild type strains with improvements at a bioprocess level.

Propionibacterium freudenreichii was selected as the producer strain for this study due to several reasons. It is a traditional and extensively researched vitamin B_{12} producer, with a wealth of information available regarding its growth conditions and production characteristics. This existing knowledge provides a solid foundation for the optimization of bioprocesses in this context.

However, it is important to note that most of the research conducted on *Propionibacterium freudenreichii* for vitamin B_{12} production has focused on developing high propionic acid-resistant strains, rather than improving and optimizing bioprocesses to achieve higher yields using non-modified strains. This is significant because *Propionibacterium freudenreichii* is a generally recognized as safe (GRAS) microorganism capable of naturally producing vitamin B_{12}. In a market sector with high health concerns, having the ability to develop a process that yields high productions with a non-modified strain offers a clear commercial and economic advantage compared to the current main industrial process, which involves Class I genetically modified aerobic strains or propionic acid-resistant strains obtained through random mutagenesis and other genetic engineering approaches.

Furthermore, the predominant focus on strain improvement has created a gap in the study of *Propionibacterium freudenreichii* bioprocesses. This thesis project aims to fill that gap by developing and optimizing bioprocess conditions, with the goal of narrowing the productivity gap between non-modified strains and propionic acid-resistant ones.

It is important to emphasize that any improvements achieved at the bioprocess level could potentially be applied to propionic acid-resistant strains in the future, facilitating the industrial implementation.

As explained in Chapter 1, despite there have been several studies aimed to increase production by genetic engineering, mainly by the overexpression of genes involved in the biosynthetic pathways, the main industrial producers were obtained by increasing the microorganism's resistance to propionic acid with random mutagenesis approaches. However, and due to the GRAS status of *Propionibacterium freudenreichii*, other strategies that aim to maintain the wild type strains and optimize production have gain some attraction over the years.

Some of these strategies involve propionic acid concentration reduction by EBABs reactors [120–122] or even the co-culture with propionic consuming strains like *Ralstonia eutropha* [123].

Before implementing these and novel strategies, it was necessary to develop a growth platform for *Propionibacterium freudenreichii* as this was the first time that this microorganism was implemented in our laboratory. In fact, this project was also the first time that anaerobic strains were studied in the group from a bioprocess viewpoint so developing a proper experimental set-up was the first milestone of the project.

As described in Chapter 1, *Propionibacterium* cultures intended for Cbl production are often divided into two different culture stages: an anaerobic stage intended for biomass production and a microaerophilic or production stage where DMBI is synthesized and couple to the cobinamide Cbl precursor. For optimal growth and production, it is important to maintain a constant temperature of 30 ºC and a close to neutral pH (7). Taking into consideration that the *Propionibacterium* generates significant amounts of propionic acid (and acetic acid in lower quantities), pH must be

controlled, usually with the addition of base solutions to the culture media. Propionic acid can also be removed from the culture broth, for example a Expanded-Bed bioreactor as described in [120,122]. Finally, optimal Cbl production often implies supplementation with important nutrients or precursors. For example, DMBI supplementation is very often described as necessary at the beginning of the production phase while other compounds like glycine, cobalt ions, threonine, etc have also been described as beneficial in many producing strains [182].

Furthermore, choosing a suitable wild type producing strain from the publicly available collections was also deemed important, and the initial efforts were focused on the characterization of two different producing strains.

The selected strains were DSM Z4902, acquired from the German Collection of Microorganisms and Cell Cultures GmbH and NBRC 12391 acquired from the National Institute of Technology and Evaluation (NITE, Japan). Both strains were selected because the existence of studies supporting their natural capabilities as vitamin B_{12} producers. Besides, NBRC 12391 is one of the parental strains described in Kojima et al 1985 [93], where several propionic resistance *Propionibacterium* strains were obtained from wild type producing microorganism through random mutagenesis.

3.2. *Propionibacterium* genus. Characteristics, uses and metabolism.

3.2.1. Characteristics and types.

Genus *Propionibacterium* has been known since the beginning of the twentieth century, when it was isolated and described for the first time in the early twenty century.

Since then, it has been traditionally divided in two main groups: cutaneous -or acne- *Propionibacterium* and classic -or diary- *Propionibacterium*. Cutaneous *Propionibacterium* strains, such as P*ropionibacterium acnes, Propionibacterium avidum* or *Propionibacterium propionicum*, are usually considered pathogens to humans [177] and are, therefore, out of the scope of this study.

On the other hand, classic *Propionibacterium* strains are non-pathogenic and some of them are used for the production of several products due to its interesting metabolism like Swiss cheese production. Classic Propionibacterium includes *Propionibacterium jensenii*, *Propionibacterium thoenni* -both of them now included in the genus *Acidipropionibacterium* [178]- and several strains of *Propionibacterium freudenreichii*.

All *Propionibacterium* subspecies, from now Propionic Acid Bacteria or PAB, are Gram positive bacilli. They are nonmotile, polymorphic (usually club or coccoid shaped and presenting rounded ends) and have a mean length of 1-5 µm and a usual diameter of 0.8 µm [179]. They are either anaerobic or aerotolerant microorganism with slow culture growth, often achieving the highest growth rate 48 hours after inoculation [180]. Moreover, they are generally catalase positive and present high C+G content [181].

The following characteristics are shared by all PABs:

- The optimal pH value varies between 4.5-8.0 (usually around 7) [182].
- Most of them are mesophiles, with optimum growth temperatures close to 30ºC, although they tolerate wider temperature ranges (from 20ºC to 70ºC) for a short period of time [182].
- Their growth can be inhibited by several environmental factors such as high or low acidity, high or low temperature and high salt concentration in the media [182].
- They are heterologous fermenters, being capable of metabolizing various simple and complex carbon sources. Although glucose or saccharose are often the preferred choice, they are capable of metabolizing alcohols [181] and some organic acids, like lactic acid [183]. However, apart from the carbon source, they present rather strict nutritional needs, and a proper microelement supplementation is mandatory in order to achieve proper growth.

Even if all optimal conditions are achieved, PAB growth is often very slow with culture times between one or two weeks. In solid media, growth is even slower, and it is only achieved in a strict anaerobic environment.

Most importantly, several microorganisms from the *Propionibacterium* genus are GRAS and QPS which has boosted numerous valuable industrial applications. For example, they are capable of synthetizing interesting compounds such as trehalose, intracellular peptidases, bacteriocins and preservatives such as propionic and acetic acid, as well as Vitamin B_{12} [184] in high amounts. In addition, they can use several cost-effective carbon and nitrogen sources and even waste and spent media, such as molasses [130], crude glycerol [131], waste frying sunflower oil [132], tomato pomace [133], liquid acid protein residue of soybean [134] and vegetable juice spent media [135]. Because of all these advantages dairy PABs are widely used for production in the health, food and cosmetic industries and they are also used as additives in feeds for livestock.

3.2.2. Dairy PABs. Main strains, characteristics and uses.

The main natural habitats on which classic *Propionibacterium* can be found are raw milk and cheese, but they also can be obtained from vegetables suitable for human consumption and from ruminal contents of different animals (cows, pigs, calves, hens) [185]. The current isolated and described species are *P. acidipropionici, P. cyclohexanicum, P. freudenreichii, P. jensenii, P. microaerophilum, P. thoenii*. Of these, *P. jensenii, P. thoenii* and *P. acidipropionici* have been recently classified in their own unique genus: *Acidipropionibacterium* [186].

Industrial use of several strains of diary *Propionibacterium* is common. The oldest application is possibly the usage of this microorganism as a starter and/or supplement for the production of Swiss type cheeses (e.g. Compté, Emmental, Gruyère), where it is responsible for the characteristic flavor of those kind of cheeses [187]. The nutty and fruity flavor is partially due to PAB production and accumulation of propionic acid and other metabolites. PAB also contribute to cheese ripening and maturation by hydrolyzing fats and forming free fatty acids [188]. They also have a role in the formation of the typical “eyes” or “holes” in the cheeses.

Of all classic *Propionibacterium* strains, *P. freudenreichii* is possibly the most interesting from an industrial point of view.

Traditionally the distinction between the two subspecies of *Propionibacterium freudenreichii* (*Propionibacterium freudenreichii freudenreichii* and *Propionibacterium freudenreichii shermanii*) was based in two phenotypical criteria: [189]

- *Propionibacterium freudenreichii freudenreichii* (lac+/nit−) were those strains incapable of lactose fermentation but that have nitrate reductase activity.
- *Propionibacterium. freudenreichii sherm*anii (lac-/nit+) strains could metabolize lactose but do not have nitrate reductase activity.

However, more recent discoveries and the reported existence of other two possible phenotypes (lac+/nit+ and lac-/nit-) [190,191] have made this classification no longer suitable, and nowadays it is no longer considered correct to classify them into different subspecies based on these criteria [192].

3.2.3. Dairy PABs as industrial metabolite producers

Propionic acid and other microbiocides

Propionic acid ($C_3H_6O_2$) is a colorless fluid with a characteristic pungent odor. Propionic acid and many of its salts (propionates) are FDA-approved, GRAS compounds with numerous applications in the chemistry, food, cosmetic and pharmaceutical industries [193], mainly as a preservative for its strong antimicrobial properties, allowing a significant increase in the products shelf life.

Many propionates are used as components in cleaning products because they are effective mold growth suppressors and can strongly inhibit the growth of pathogenic bacteria such as *Listeria monocytogenes* [194]. Propionic acid is also used to produce textiles, air filters or membranes [195]. Moreover, it is also used in the cosmetic industry to prolong the shelf-life of many perfumes and, in the pharmaceutical industry, for the treatment of infected wounds in animals [196]. Lastly, it can also be used as a herbicide [197].

Currently, most of the worldwide propionic acid is produced from crude oil through a petrochemical route, but a bio-based alternative would allow the product to be labeled as “natural preservative”. For this reason, numerous studies are being carried

out in order to enhance microbial propionic production, mainly focusing on strain and process optimization [180]. In this sense, PAB capability of synthetizing propionic acid via the Wood-Werkman cycle [198] makes them a very interesting candidate for bio production of propionic acid at an industrial setting. In fact, nowadays, some commercial propionic acid products obtained by fermentation are already in the market as food preservers, for example MicrogardTM [199].

Besides propionic acid, PAB are also capable of synthetizing different kinds of antimicrobial agents, such as bacteriocins, reinforcing the potential value of PAB supplementation as a safe bio preservative [200].

Vitamin B_{12}

All PAB are also producers of Vitamin B_{12}, as this molecule is needed as a cofactor for the Wood-Werkman cycle, the main fermentation pathway followed by *Propionibacterium* for energy obtention.

As explained in Chapter 1, vitamin B_{12} is only produced by some bacteria and archaea and have to be incorporated into the diet by cobalamin rich food products.

Different *Propionibacterium freudenreichii* strains have been used over the years for vitamin B_{12} production. Despite their already mentioned disadvantages compared with competitors' aerobic strains like *Pseudomonas denitrificans* (lower growth rates, propionic inhibition), its GRAS status has allowed the expansion of their market scope by allowing their direct use in the production of food products. In this sense, in situ food fortification with *Propionibacterium freudenreichii* has been successfully tested using food-like media, such as in cheese-like propionic medium or whey-based liquid medium [116,117] cereal matrices [118] and in situ fortification of tempeh [119]. Although final cell densities and reported production levels are low compared to other, traditional media, in the context of food fortification, it allows an increase in cobalamin content using non-traditional sources and the achievement of the recommended daily vitamin B_{12} consumption levels with only small amounts of fermented products [117].

Despite being facultative anerobic microorganisms that follow the anaerobic biosynthetic route for cobalamin production, *Propionibacterium freudenreichii* still

needs small quantities of oxygen for the synthesis of the lower ligand of the molecule and its attachment to the rest of the corrinic ring [114]. For this reason, the culture is usually divided into two stages: a first stage in which the cells are cultured in complete anaerobic conditions and a second stage, usually after 72–96 h of cultivation [114–116] in which gentle aeration is provided by agitation to generate the microaeration needed for DMBI synthesis and cobalamin production [100].

Other biotechnological applications of PABs

PABs can also synthetize a wide arrange of interesting compounds with potential market applications such as conjugated linoleic acid (or CLA) [201], exopolysaccharides (or EPS) [202], or other vitamins (B_7, B_9, B_2, porphyrin, etc.) [203]. Some PAB strains can produce trehalose, a widely used food sweetener. It is reported that trehalose production can be stimulated in PABs by media stressors, such as an acidic pH, high salt concentration or the presence of oxygen radicals [204].

PABs as probiotics

Lastly, the numerous health-promoting metabolites that some GRAS strains of *Propionibacterium* can produce are increasing the interest for possible probiotic applications. Some of the potential beneficial effects of PAB as probiotics are microbiota modulation, immunomodulation, and inflammation control [205].

3.2.4. Propionic acid biosynthesis in *Propionibacterium* genus: the Wood-Werkman pathway

Many organisms can produce small amounts of propionic acid when fermentations are performed in complex media: mainly as a product of different catabolic and anabolic pathways of several amino acids like valine, threonine, isoleucine, methionine [206]. However, there are few organisms that produce propionic acid as their primary fermentation product.

There are three main pathways for propionic production in these microorganisms: the acrylate pathway, the 1,2-propanediol (POD) associated pathways and the succinate pathways [206].

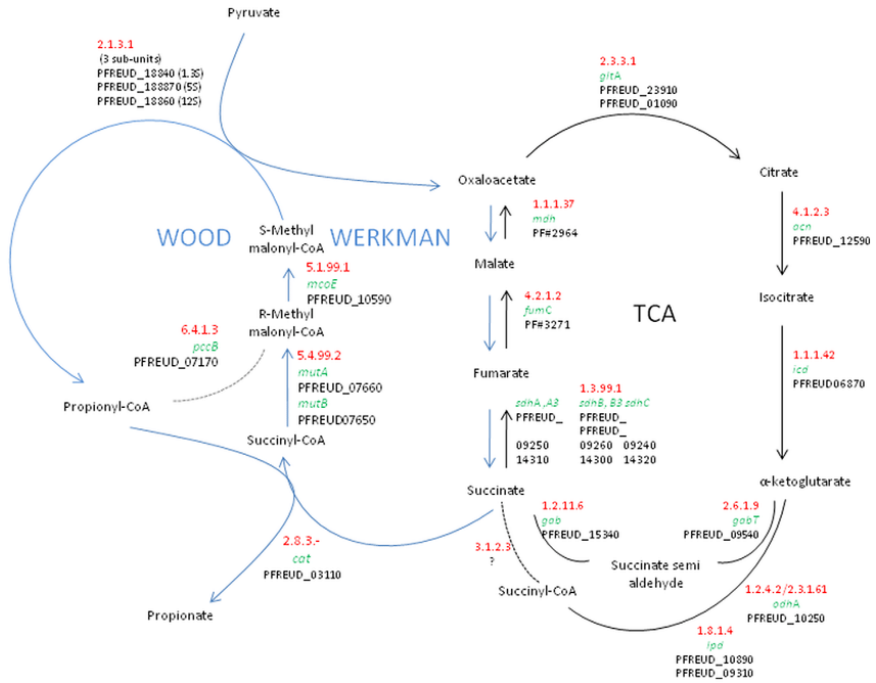

Figure 13. Schematic representation of the Wood-Erkman Cycle. *Extracted from* [207]

The Wood-Werkman pathway is a succinate pathway and is clearly considered the most important of all the different propionic acid biosynthetic mechanisms for a couple of important reasons: a) the yields and productivities of the Wood-Werkman cycle are the highest of all the propionic producing pathways [208] and b) some of the microorganisms that use this pathway as their primary source of energy during fermentation have a notorious industrial importance (like, for example, *Propionibacterium freudenreichii)*

In the Wood-Werkmann cycle, the pyruvate obtained during glycolysis is converted into oxaloacetate with the action of a methylmalonyl CoA carboxytransferase. Oxalacetate is later reduced to malate, fumarate and, finally, succinate.

Succinate is transformed by a succinyl CoA mutase into succinyl CoA, that is later transformed by a methylmalonyl CoA mutase (a key enzyme that uses vitamin B_{12} as a co-factor) into methylmalonyl CoA, leading to the formation of propionyl-CoA. Finally, a CoA transferase releases the CoA group and produces propionate.

The key point of the cycle (as shown in Figure 13) is the transcarboxylation reaction catalyzed by methylmalonyl-CoA carboxyltransferase. In this step, the carboxylic group from methylmalonyl CoA is transferred to pyruvate, generating oxaloacetic acid and propionyl-CoA. The latter is then converted into propionic acid. Methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1) is a multiunit enzyme that needs biotin as a cofactor [209].

3.3. Results and discussion.

As mentioned in the introduction, the main goal of this initial chapter is select a suitable strain for the project and develop a culture methodology for *Propionibacterium freudenreichii* growth and production characterization.

Taking into consideration that this was the first project that involved production in a facultative anaerobic strain in the research group, all different culture systems had to be adapted to anaerobic cultures. For this reason, it was decided to reproduce the experimental set up described in the Patent US4544633A by Kojima et al, 1985 [93]. As mentioned before, in this patent several parental wild type Cbl-producing strains are subjected to different random mutagenesis processes in order to obtain a propionic resistant over-producing strain. Despite that this thesis project aims to improve and optimize Cbl producing at a bioprocess level with a wt non-improved strains, the experimental set-up described in Patent US4544633A (culture conditions, bioreactor configuration, Cbl extraction and quantification and media composition) were still found suitable as a starting point for future improvements.

More specifically, the main targets were:

- Select the most suitable strain for growth characterization and Cbl production optimization between the two main candidates: DSM Z4902 and NBCR 12391.
- Evaluate the growth parameters of the selected strain in a working fermentation media. Determine the composition of the fermentation media for optimal growth and production: Initial carbon source concentration, nitrogen source composition and concentration (YE or CSL).

- Establish optimal culture conditions for cell growth and production, taking into consideration strain requirements like the addition of DMBI supplements at different times to stimulate Cbl production.
- Develop all the analytics to quantify the metabolites consumed and produced externally by *P. freudenreichii* in our culture conditions.
- Develop the methodology for Cbl culture extraction and implement and optimize the quantification of vitamin B_{12} in our laboratory.

With the objective of having a good system for culture condition optimization, conditions described in Patent US4544633A were not only replicated in a bioreactor setting, but also in smaller scales, such as Falcon tubes and Erlenmeyer Shake Flask.

In fact, during this first step of the project, the main effort was to establish a proper experimental set up at a lab-scale laboratory level, mainly in Shake Flask. This was done in a middle-throughput system that allowed for many different growth conditions.

Bioreactor cultures were also used during this first step of the process with the intention of confirming the results obtained at Erlenmeyer Shake Flash cultures in a more controlled environment.

3.3.1. Initial strains growth characterization. Strain selection.

The main candidate was NBRC 12391 because this is one of the parental strains described in Kojima et al 1985. However, before committing with one strain, an initial growth characterization in Erlenmeyer Shake flask fill with Media 1 (See Materials and Method Section for more detailed information) was performed for both strains (Figure 14). This media is a complex media with glucose, YE and tryptone recommended by the NBRC bank for NBRC 12391 storage and grow in agar plates.

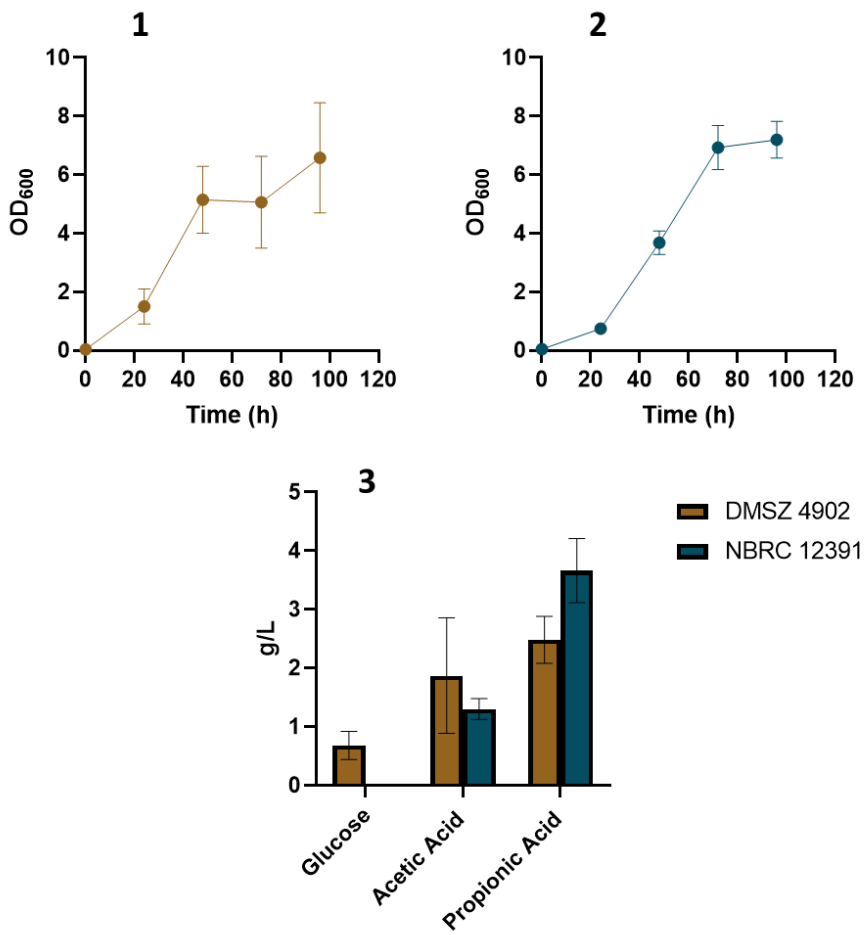

Figure 14 Erlenmeyer Shake flasks cultures of DMSZ Z4902 (A) and NBRC 12391 (B). *Media 1 was used in both cultures. Glucose, Acetic Acid and Propionic acid quantified at 96 culture h are also shown for both strains (C). Error bars represent the standard deviation of three replicates.*

Figure 14 shows obvious differences between both strains. Although final OD_{600} values were quite similar, cultures with NBRC 12391 were much more reproducible, with very little variation between cultures. This variance can also be appreciated in the metabolite production, with a great difference in acetic acid between replicates for DMSZ 4902 cultures. Propionic acid production seemed to be very similar between both strains. DMSZ 4902 propionic acid production seemed to be slightly smaller than NBRC 1291, but not by a significant amount.

It should be noted that, for DMSZ 4902 cultures, not all the glucose was depleted at 96 culture hours and small amounts were remaining in all 4 replicates. This led us to question the capability of the strain to growth and consume carbon source efficiently in a high carbon source concentration media like the ones described in Kojima et al 1985 [93](with up to 50 g/L initial glucose concentration). For this reason, new cultures in Falcon tubes filled with 45 mL of Media 2, rich media from Kojima et al, 1985[93] that contained 50 g/L of glucose, were performed. The results for this experiment can be seen in Figure 15.

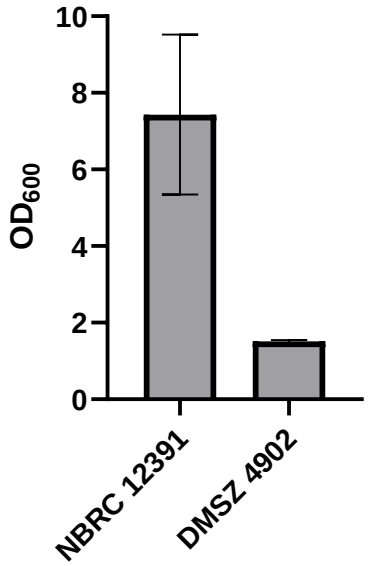

Figure 15 OD_{600} values at 72 culture hours for Falcon tubes cultures with Media 2 of NBRC 12391 and DMSZ 4902. *Error bars represent the standard deviation of three replicates.*

As shown in Figure 15, while NBRC 12391 showed a significant growth during the first 72 culture hours, the growth of DMSZ 4902 was much lower, and stagnate at around 1.5 OD_{600} values. Taking into consideration these results, the variation and low glucose consumption and the fact that NBRC 12391 had already been described as a successful Cbl overproducer, we decided to continue using this later strain for further characterization and optimization.

3.3.2. Falcon tube and initial Erlenmeyer Shake Flask cultures characterization.

We intended to develop a culture system that allowed for multiple conditions testing at a fast pace. The original idea was to set up several cultures in Falcon tubes filled with fermentation media and harvest and measured samples at the final cultures points in order to have a middle-throughput screening system. However, the significant variation in OD_{600} values between replicates in initial experiments made us doubt of falcon tube configuration.

In order to achieve the most optimal Falcon tube configuration, several cultures were carried out at different media volumes to check if it had an impact in cell growth and culture stability. Taking into consideration that NBRC 12391 is a facultative anaerobic microorganism, we intended to fill the tubes as much as possible to ensure the minimal presence of oxygen in the media but ensuring proper culture homogenization. These cultures were carried out in Media 1 using three different media volumes: 25 mL, 35 mL, and 45 mL. In all cases, the cultures were carried out in 50 mL Falcon tubes with 150 rpm agitation at 30 ºC (Figure 16).

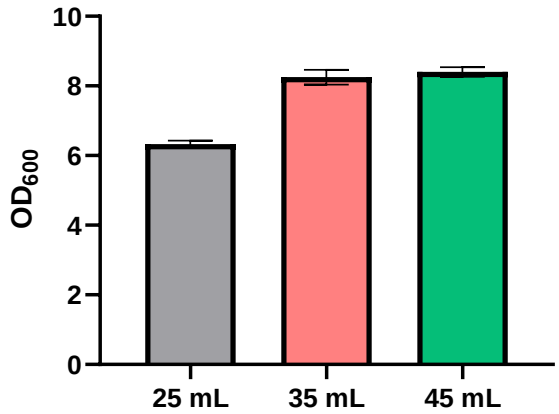

Figure 16 50 mL Falcon tube cultures of *Propionibacterium freudenreichii subs shermanii* NBRC 12391. *Three different media volumes were tested: 25 mL, 35 mL and 45 mL. OD600 was measured at 96 culture hours. Error bars represent the standard deviation of three replicates.*

As shown in Figure 16, Falcon tubes filled with 35 mL and 45 mL showed a fairly similar behavior, reaching OD_{600} values or around 8 while the Falcon filled with only 25 mL showed a significant decrease in max OD_{600} values. However, both values were quite low in reference to those found in the bibliography. Furthermore, there were also very similar to the values obtained in the cultures performed in Erlenmeyer Shake flask to characterize the candidate strains (Figures 14)

For this reason, before continuing to study Falcon tubes as a platform for culture screening, the growth of NBRC 12391 in carbon rich media and in Erlenmeyer shake flask was studied. Besides OD_{600}, acetic acid, propionic acid and glucose were also measured at several points during the culture as described in materials and methods. For these cultures, Media 2, with up to 50 g/L of glucose and 40 g/L of Yeast extract (YE) was used.

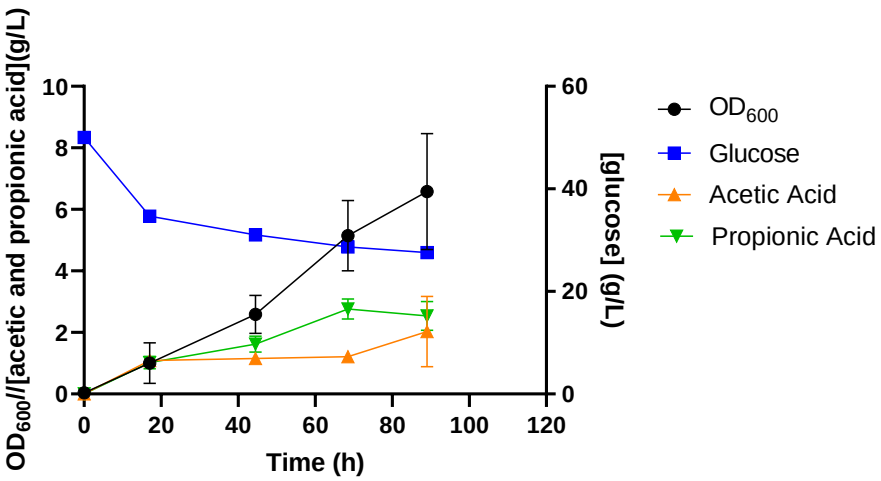

Figure 17 Erlenmeyer shake flask cultures of NBRC 12391 with Media 2. *OD_{600} glucose (g/L), acetic acid (g/L) and propionic acid (g/L) are represented. Error bars represent the standard deviation of three replicates.*

As shown in Figure 17, the OD_{600} values in Erlenmeyer, even with higher initial glucose concentration (5 g/L vs 50 g/L) are very similar to those obtained with Media 1 in Erlenmeyer Shake Flask and Falcon tubes. Obtaining similar OD_{600} values than in much

more carbon source media was concerning. Moreover, at 96 hour the pH of the culture presented values of around 5.

This decrease in pH was most probably due to the production of propionic and acetic acids, reaching values of 2.5 ± 0.5 g/L and 2.0 ± 1.1 g/L, respectively.

These pH values are significantly lower than the recommended 6.8 to 7. If cell growth was inhibited by the lower values, pH regulation became mandatory for proper culture assessment and optimization. This would render Falcon tube cultures ineffective for culture testing because, due to the low volume, pH adjustment by adding a base solution is very impractical and could potentially increase the variance in the cultures.

For this reason, the growth between cultures with and without pH adjustment was compared in Erlenmeyer shake Flask. The cultures were carried out in Media 2 and pH was adjusted daily when required with a 2M NaOH solution. The result of this experiment can be seen in Figure 18.

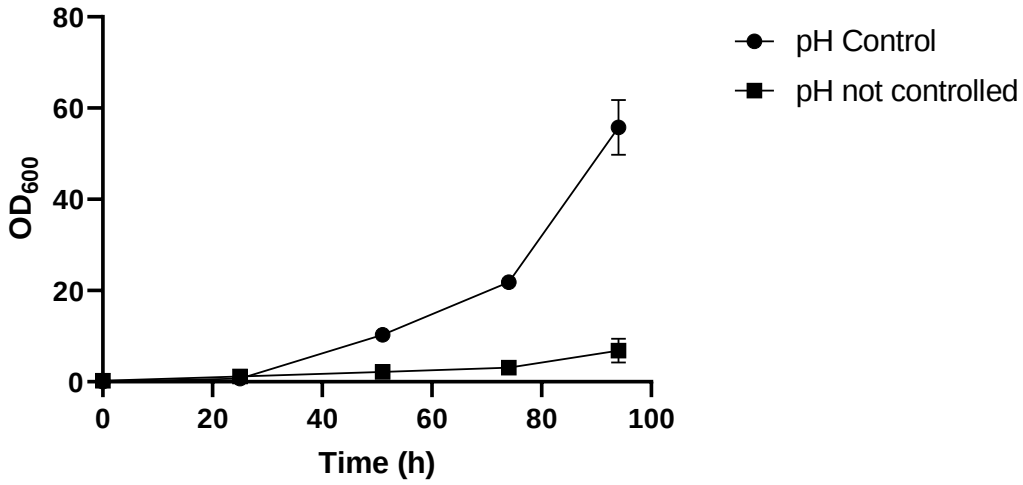

Figure 18 Erlenmeyer shake flask cultures of NBRC 12391 with and without pH adjustment. *The pH was adjusted daily by the addition of a 2M NaOH. OD_{600} and pH values were measured and recorded before every daily adjustment. Error bars represent the standard deviation of two replicates.*

Results of figure 18 strongly indicate that pH must be analyzed and corrected during the culture for correct growth and that pH values of around 5 are inhibiting NBRC 12391 growth. This, in turn, makes using Falcon tubes as a screening method for different culture conditions very troublesome, so it was decided that further characterization was directly perform in Erlenmeyer Shake flaks.

3.3.2 Growth and Cbl production characterization and optimization for NBRC 12391.

Once Erlenmeyer shake flasks were chosen as the set up for growth characterization and optimization, further efforts to improve the composition and concentration of the initial culture media were carried out.

Initial carbon source concentration

Media 2 is an adaptation from the media used by Kojima and colleagues for *Propionibacterium freudenreichii shermanii* growth and production and has glucose as the sole carbon source at 50 g/L.

Taking into consideration that during the cultures carried out in Figure 18, glucose was found to be almost completely depleted after the first 96 culture hours, it was decided to study the fortification of the initial media with higher glucose quantities. With this objective, 6 different conditions were tested out: 25g/L, 50 g/L, 70 g/L, 90 g/L, 100 g/L and 125 g/L. pH were adjusted daily and OD_{600} was also measured and recorded daily. The results from these cultures, alongside the μ_{net} for each condition can be found in Figure 19.

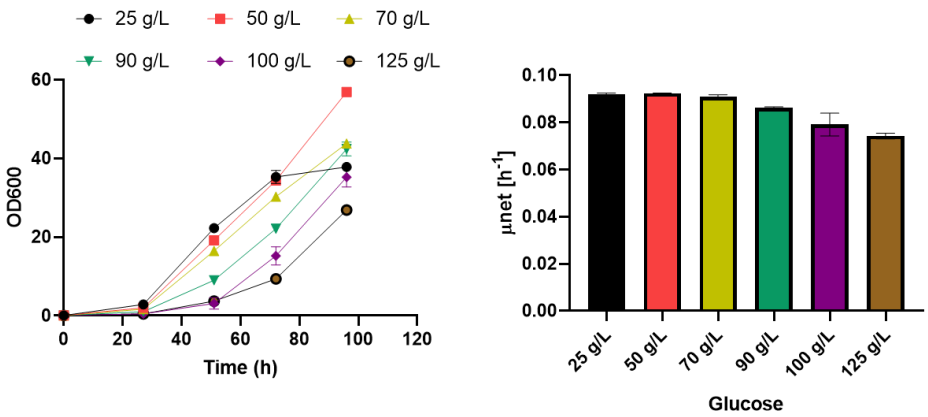

Figure 19 NBRC 12391 Erlenmeyer shake flask cultures at 6 different glucose concentrations: 25 g/L, 50 g/L, 70 g/L, 90 g/L, 100 g/L and 125 g/L. *OD_{600} values (left) and μnet (right) are shown. Error bars represent the standard deviation of three replicates.*

Several conclusions can be reached from the previous experiment:

- At 25 g/L μ_{net} is very similar to the one obtained in the base conditions, but glucose is depleted sooner, and final OD_{600} values are significantly lower.
- From 90 g/L to 125 g/L there seems to be a gradual decrease in μ_{net}. Besides, every value above 50 g/L showed a delay in growth and lower OD_{600} values at 96 hours despite there was some glucose remaining in the media.

Taking into consideration these results, it was decided to continue using 50 g/L as the initial glucose source and not try top fortified the media in this regard but rather use fed-batch strategies to try to maintain the glucose at a sufficient level for cell growth but without causing inhibition.

Before trying to characterize the cell growth and production in a bioreactor with glucose feeding, a methodology for detecting and quantifying Cbl had to be developed.

3.3.4 Cobalamin detection. System suitability and quantification of all possible Cbl types present in the culture broth.

As described in chapter one, what is usually called vitamin B_{12} is in fact a family or similar molecules that share the same central pyrrolic ring structure.

From what is described in the field about *Propionibacterium freudenreichii* vitamin B_{12} production [81,210,211] and its metabolic needs, we expected to find mainly AdoCbl. Moreover, AdoCbl is the molecule used as a coenzyme by the enzyme methylmalonil-CoA mutase to transform succinyl-CoA into methylmalonil CoA [212] during the Wood-Werkman cycle.

However, AdoCbl, as well as MetCbl (the other form with physiological functions in humans) are notoriously light sensible and can be rapidly degraded into OHCbl in the presence of light [24,25]. For this reason, they are usually converted into CNCbl by a cyanidation process.

Besides AdoCbl photosensitivity, there was also the possibility of other Cbl and cobalamin compounds and precursors in the culture broth. This could difficult the identification and quantification of the Cbl produced and hamper the HPLC analysis.

Nonetheless, it was decided to evaluate our HPLC system in front of several Cbl that could be present in the culture samples. In this sense, calibration curves with several Cbl natural occurring standard were performed following the methodology described in the materials and methods chapter. Response factor were also calculated for each standard to better assess system suitability. CNCbl standards were also measured although is not naturally present in case cyanidation was finally necessary for proper Cbl quantification.

Figure 20 shows the 5-point calibration curve of OHCbl, AdoCbl, MetCbl and CNCbl, alongside their respective response factor in each concentration and a dotted line indicating the average response factor (RF) in all points.

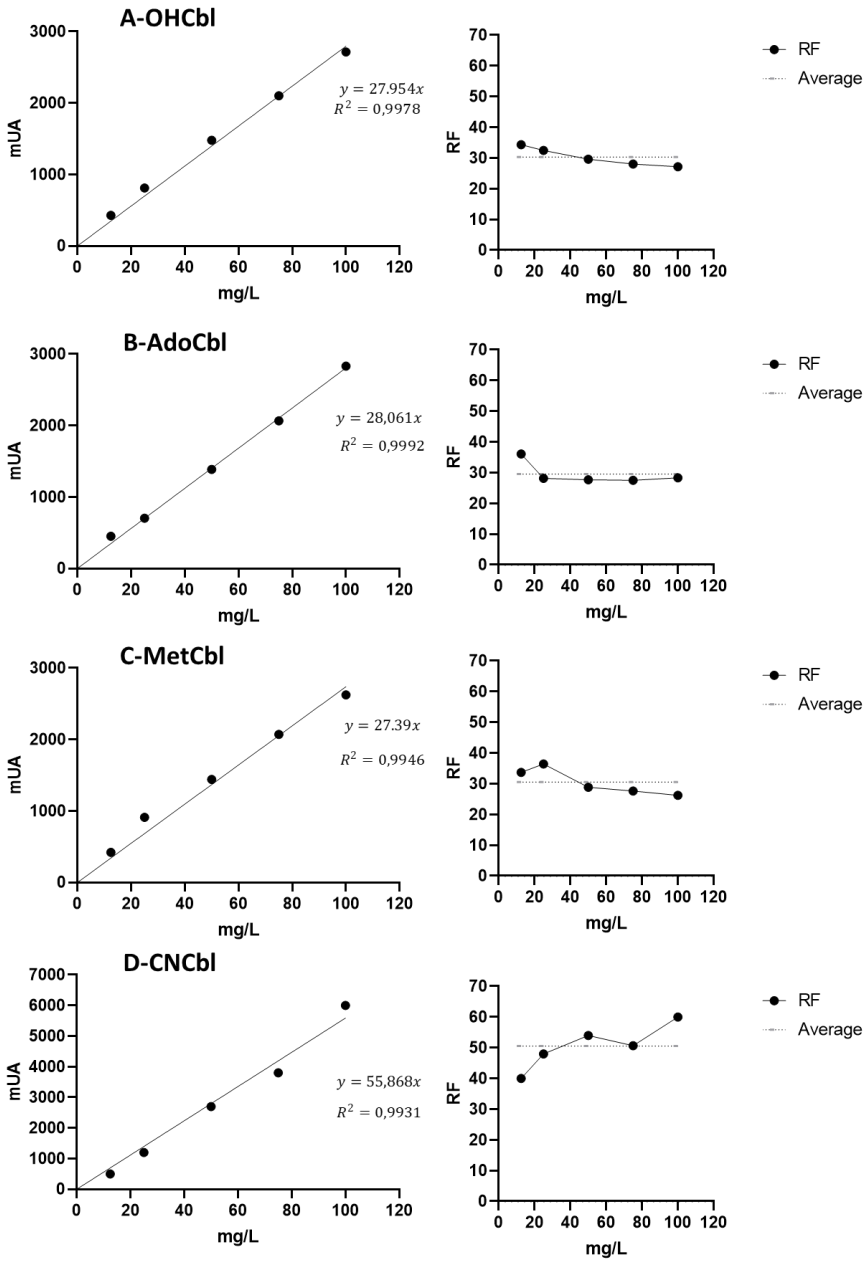

Figure 20 5-point calibration curves obtained by HPLC measurements of different known standard concentrations. *RF is shown in the right. A) OHCbl, B) AdoCBL, C) MetCbl and D) CNCbl. In each case, the 5 points of the curve were: 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L and 12,5 mg/L.*

For the initial Cbl quantifications of the cultures it was decided that the target molecules for identification by HPLC would be AdoCbl and other possible Cbl natural occurring subtypes to avoid any extra steps in the downstream processing of the samples like a treatment with cyanide.

3.3.5 Fed-Batch cultures with Media 2.

Up until this point, all pH regulated cultures were performed with Media 2, an adaptation from the media described by Kojima and colleagues [93] where CSL was substituted by YE. This change was mainly motivated by several reasons.

Corn steep liquor or CSL is a byproduct of the corn milling industry that is commonly uses in industrial fermentation process due to its availability and the fact that it is considerable cheaper than other complex nitrogen sources. However, one of the main problems of its use is the great variability between different commercial brands and even between batches due to its nature as a byproduct. Besides its unknown composition, CSL usually presents a very high solid fraction. In a laboratory setting, this means an extra treatment of the media in order to remove all these solids that can precipitate during autoclave sterilization or pH adjustment and impaired OD_{600} measurements and samples harvesting. Finally, although cheaper at an industrial setting, CSL usually has to be bought in higher bulks than other alternatives and becomes significantly more expensive that other more common alternatives like YE.

For all these reasons, the initial characterizations of growth and Cbl productions were all performed with a YE based media. At this stage, a fed-batch process was developed and carried out. The main objective of this fed-batch was two-fold:

- Perform NBRC 12391 in a more controlled environment, where the pH could be measured and adjusted constantly instead on relying in a once every 24 hours regulation. Considering the great impact shown by low pH values during the Erlenmeyer shake flask cultures, being capable of more tightly controlling the pH during the culture seemed necessary for proper growth and production characterization.

- Maintaining a glucose level that allowed for cell growth and Cbl production without being too highly concentrated and inhibiting growth during the early stages of the culture. As the results from Figure 19 showed, initial concentrations higher than 50 g/L were detrimental for cell growth but in all cultures carried out with 50 g/L the glucose was almost depleted by the 96-hour culture time. So, glucose was fed to enlarge the growth.

pH was controlled by a pH probe and the addition of 2M NaOH solution when necessary. Glucose was constantly added from a 200 g/L glucose stock solution from 48 hours until the end of the experiment at 144 hours at a 6.6 mL/h fed rate. Samples were harvested every 24 hours and target metabolites (acetic and propionic acid) and Cbl were measured. To stimulate Cbl production, a DMBI supplement (100 μM) was added at 96 culture hours.

For Cbl detection, as previously stated, the target molecule was AdoCbl. However, we could not discard some degree of degradation during the extraction and samples preparation and other known subtypes were also quantify.

The results from these cultures are shown in Figure 21.

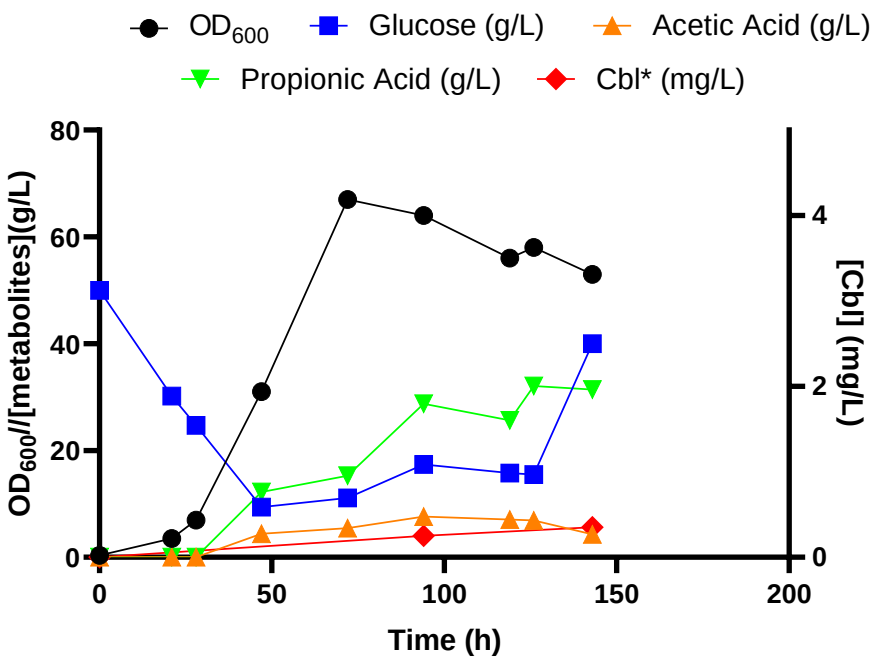

Figure 21 Bioreactor culture fed-batch of NBRC 12391 with constant pH adjustment by 2M NaOH and constant 200 g/L stock glucose solution addition from 48 culture hours. *OD_{600}, glucose, acetic acid, propionic acid and Cbl concentrations over time are shown. The reported cobalamin concentration is the sum of all Cbl types detected in the samples. Error bars represent the standard deviation of two replicates.*

Besides the low Cbl concentration, its detection and quantification were also troublesome for a couple of reasons. First, despite a fresh preparation in a low light environment, MetCbl and AdoCbl standards presented some degree of degradation into OHCbl. Besides, several peaks were found in the samples themselves, with similar retention times to different Cbl standards. Finally, OHCbl and AdoCBl were identified and quantify separately, but the total amount of Cbl could have been underestimated. As we observed some degree of transformation between the different Cbl, we preferred to report the sum of all of them (See Figure 21).

Several conclusions can be extracted from the cultures of figure 21:

- OD_{600} max values were higher than those obtain in Erlenmeyer shake flask cultures. However, it should be considered that a significant amount of extracellular precipitation was found in the samples making a correct assessment of the exact OD_{600} troublesome. These precipitates could be exopolysaccharides (reported to be secreted by Propionibacterium at high cellular densities) or other unknown compounds.
- Propionic acid concentration was also higher than in previous cultures, reaching maximum values over 30 g/L.
- Glucose concentration was kept stable between 10 and 20 g/L during the first 124 hours. However, by the end of the culture the levels began to rise until values of more than 40 g/L were achieved by 144 hours.
- Besides, the maximum OD_{600} of almost 70 was found at 72 hours and, from then on, it steadily declines until 53. This fact, alongside the previous point seem to indicate some limitation in culture growth unrelated to the carbon source.
- Finally, although high cell densities were achieved, total Cbl production was very low, 0.35 mg/L at the end of the process, compared with other examples found in the bibliography.

Taking into consideration these results, it was decided that before continuing with the process optimization, exploring Cbl production with other alternative nitrogen sources more similar to the ones described in bibliography was necessary. Besides, developing a more trustworthy system for Cbl detection and quantification was also deemed a necessity.

3.3.6 Media optimization. Selecting a more suitable complex N source for Cbl production.

As previously explained, working with CSL in a lab environment was rather troublesome, mainly because the large solid fraction. During initial preparations, a very significant precipitation occurred after autoclaving the CSL stock solution. Besides, even after discarding the solid fraction by centrifugation in sterile conditions after autoclaving, more precipitates were formed when the complete media pH was adjusted with the addition of a 2M NaOH solution.

Finally, a clear CSL solution that did not caused precipitation in the complete media when the pH was adjusted was achieved by adjusting the pH of the rather acidic CSL solution (around 3.5) up to a pH value of 8. After that, the solution was sterilized by autoclaving and later centrifuged to discard the solid fraction.

Once this methodology was established, several CSL concentrations were tested in Erlenmeyer Shake flask to try to establish an initial concentration that did not limit growth at 50 g/L of glucose. 40 g/L, as described in Kojima et al was tested alongside two lower CSL concentrations (10g/L and 5g/L) in order to see if 40 g/L of nitrogen source was in excess for our culture conditions.

The results from these cultures, alongside their respective μ_{net} are shown in Figure 22.

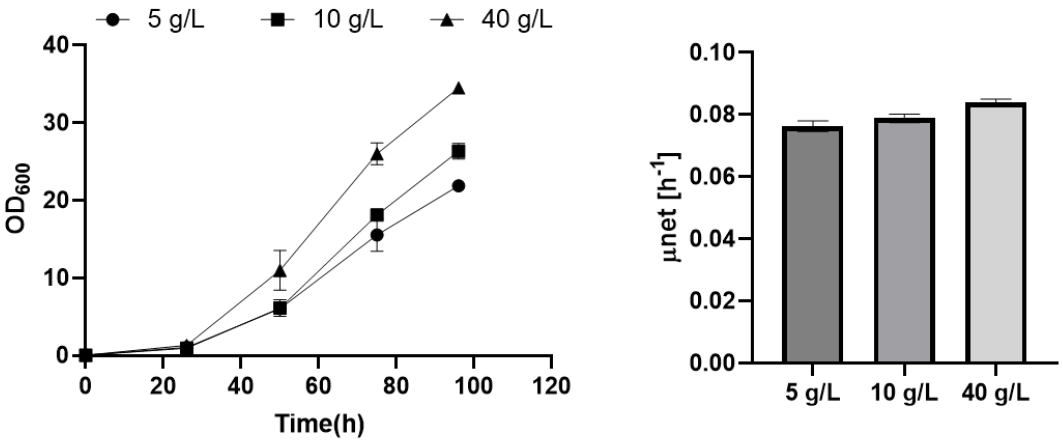

Figure 22 NBRC 12391 cultures with different amounts of CSL: 5 g/L, 10 g/L and 40 g/L. *OD_{600} values (left) and μnets are shown for each condition. Error bars represent the standard deviation of three replicates.*

As shown in Figure 22, 40 g/L of CSL allowed for higher OD_{600} values at 96 culture hours while not inhibiting cell growth so it was decided to establish 40 g/L as the initial nitrogen source concentration for further optimization.

Before committing to a fed-batch bioreactor, the behavior of the strain regarding glucose consumption and propionic and acetic acid consumption with media with 40 g/L CSL (Media 3) was studied in an Erlenmeyer shake flask culture.

As shown in Figure 23, around 96 hours, $OD_{600\ reached}$ values of around 27.5 while propionic acid concentration was around 12.5 g/L. Glucose was not completely depleted from the media, showing a mean value of 11 g/L.

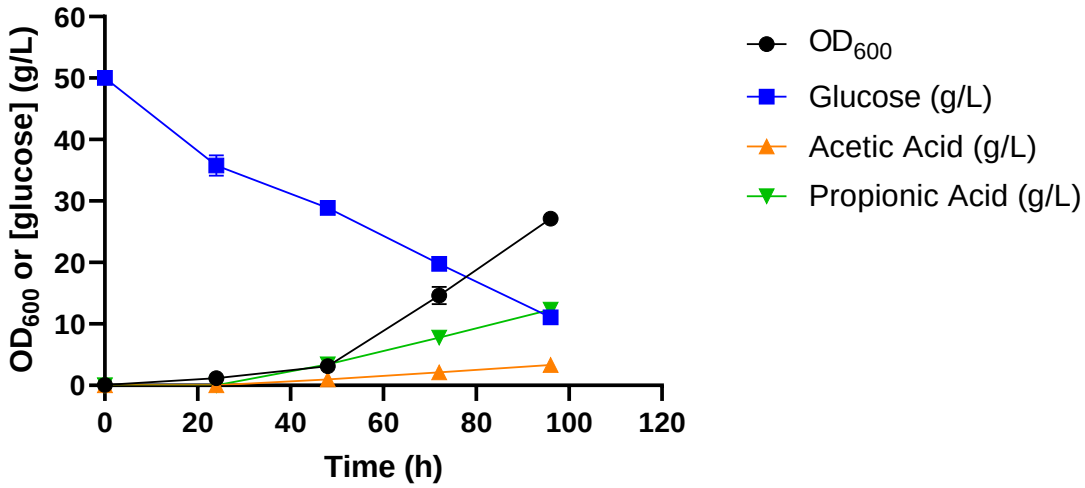

Figure 23 NBRC 12391 Erlenmeyer shake flask cultures with Media 3 (40 g/L CSL). *OD_{600} and glucose (g/L), acetic acid (g/L) and propionic acid (g/L) are shown. Error bars represent the standard deviation of three replicates.*

The objective of the experiment shown in Figure 23 was to study glucose consumption and metabolite production, so cobalamin was not measured during the culture. A similar fed-batch set up to the one described for Figure 21 was used to study the cell growth and Cbl production in a controlled bioreactor with constant glucose addition (Figure 24).

As with Figure 21, Cbl quantification was troublesome, and the total cobalamin had to be estimated from the identified AdoCbl and OHCbl peaks. However, as shown in Figure 24, Cbl production with Media 3 was significantly higher than the values obtained from Media 2: 0.85 mg/L vs 0.35 mg/L. Besides this higher Cbl production, a significant lower OD_{600} (26.35 vs 70) and much lower glucose consumption were

obtained. In fact, despite that the glucose addition was performed in the exact same way as Figure 21, with a constant addition of a 200 g/L glucose solution from the 48 hours culture hours until the end of the culture, glucose levels in the broth were much higher than those obtained previously, with values around the 50 g/L from 48 hours on.

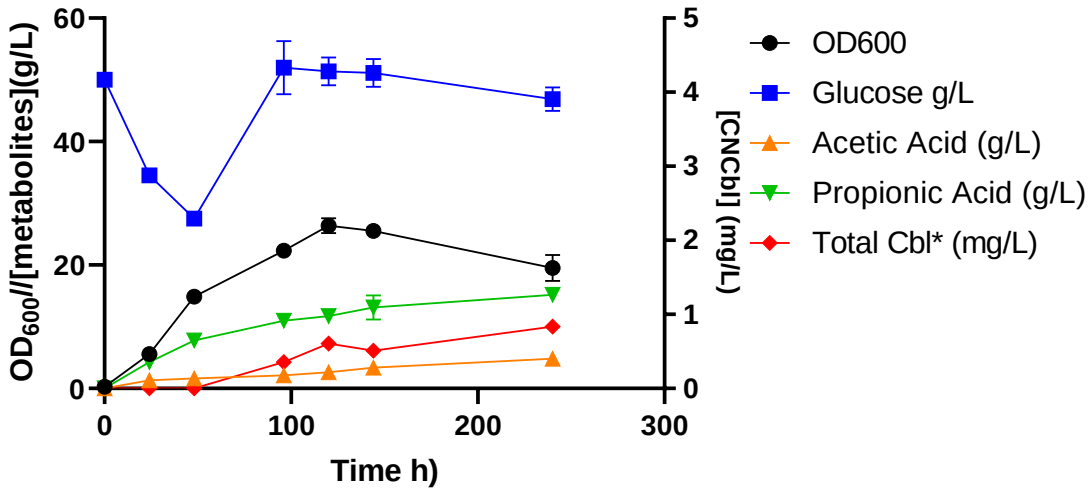

Figure 24 NBRC 12391 bioreactor culture with Media 3. *OD_{600} and glucose (g/L), acetic acid (g/L), propionic acid (g/L) and total Cbl (mg/L) are shown. Error bars represent the standard deviation of two replicates.*

Considering the improved production and much better glucose/Cbl yields, Media 3 seemed a better choice for production optimization than Media 2, despite the lower OD_{600} values. However, Cbl identification and quantification was still an issue that hindered proper assessment of the cells production capabilities.

3.3.7. Developing a methodology for Cbl extraction, cyanidation, and quantification.

Cobalamin cyanidation.

As mentioned previously, CNCbl is the most usual commercial form of vitamin B_{12} due its higher shelf life and lower light sensitivities compared to other natural occurring forms like MetCbl or AdoCbl.

CNCbl is usually obtained during the downstream process, where all species of corrinoids are extracted by heating at 80–120 °C and a pH of 6.5–8.5 for 10–30 min [100]. Usually, potassium cyanide or thiocyanate are added in the presence of heat [114,141]. After that, the already formed CNCbl is subsequently clarified by one or more filtration (microfiltration and/or nanofiltration) and adsorption processes (XAD resin) and even more adsorption processes with different resins (IRA, Alumina, etc) can be performed if the product is destined to pharmaceutical uses.

Nonetheless, most of the conversion processes described are performed during Cbl extraction from the cells, usually performed by boiling or even autoclaving the culture samples. However, given the large amount of samples and in order to minimize as much as possible the risks of using cyanide solutions during the experiments, it was decided to developed a safer cyanidation method that did not involve boiling samples with cyanide. In this sense, the proposed protocol as described in more detail in the materials and methods chapter involved adding a NaCN solution to the already extracted samples.

To correctly determine the amount of Cyanide needed to a total conversion of all Cbl present and avoid as much as possible any leftover free cyanide in the samples, three different NaCN solutions were prepared in ultrapure H_2O: 1%, 0.1% and 0.01%.

Standards of different Cbl types (OHCbl, MetCbl and AdoCbl) were prepared and treated with the three different NaCN solutions. It should be noted that, despite being prepared fresh from the crystalline stocks kept at 4ºC and protected from light, AdoCbl and MetCbl stock solutions were mostly converted to OHCbl by the time the samples were analyzed by HPLC. Nevertheless, according to the bibliography [100], NaCN should be able of transforming all Cbl types into CNCbl.

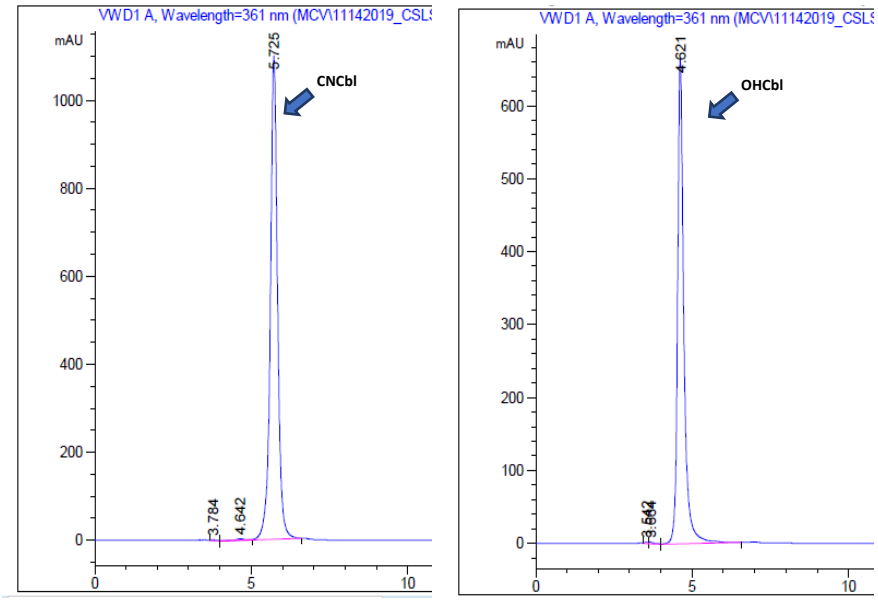

Figure 25 CNCbl standard chromatogram showing a peak with a retention time of 5.725. *Right: OHCbl standard chromatogram showing a peak with a retention time of 4.621.*

Figure 25 shows the CNCbl standard and the OHCbl standard solutions chromatograms obtained by HPLC. Both have clearly different retention times so identification of the converted Cbl should be feasible.

On the other hand, Figure 26 shows that treating the standard OHCbl solution with either a NaCN 1% or a NaCN 0.1% resulted in a total conversion of all the cobalamin present in the sample to CNCbl. However, when a NaCN 0.01% solution was used, some OHCbl could still be detected in the sample.

All samples were prepared at a 100 mg/L concentration. Considering that, the total conversion using NaCN 1%, and 0.1% solutions makes sense as, taking into account the molecular weight of each standard, these solutions should be capable of transforming up to 2g/L and 200 mg/L respectively.

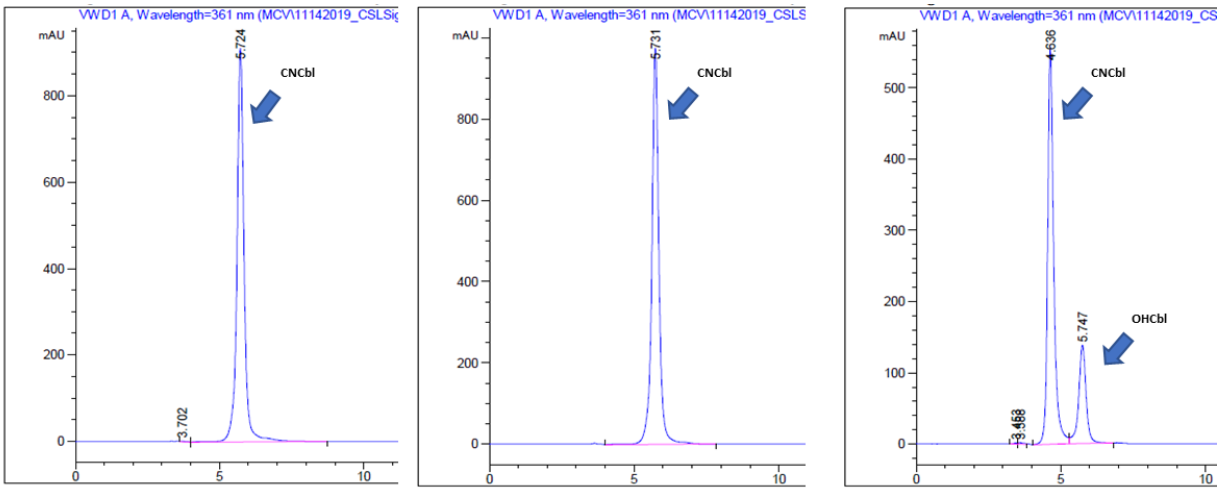

Figure 26 Chromatograms of cyanided OHCbl standard samples. ***From left to right the following NaCN solutions were used: NaCN 1%, NaCN 0,1% and NaCN 0,01%.***

With the actual Cbl productions by our cell platform in mind and the fact that even propionic resistant strains like the ones described by Kojima and colleagues [93] did not reach 200 mg/L, it was considered that using the NaCN 1% solution would translate into a significant amount of free cyanide in the treated samples. For this reason, NaCN 0.1% solution was chosen as the cyanide agent. Transforming all free Cbl into CNCbl allowed us to quantify Cbl production more easily by using CNCbl standard 5-point calibration curve like the one shown in Figure 20-D.

Biomass samples from the same culture and time point were extracted with and without NaCN 0.1% addition after boiling in order to assess the conversion capacity of the cyanidation treatment in real samples. The resulting chromatograms can be seen in Figure 27.

Both the identified OHCbl present in the sample and several peaks most likely corresponding to other Cbl subtypes like AdoCbl where completely transformed into a clear CNCbl. This conversion greatly facilitates Cbl identification and quantification in real samples, so it was decided to implement cyanidation with a 0.1% NaCN solution in every Cbl extraction.

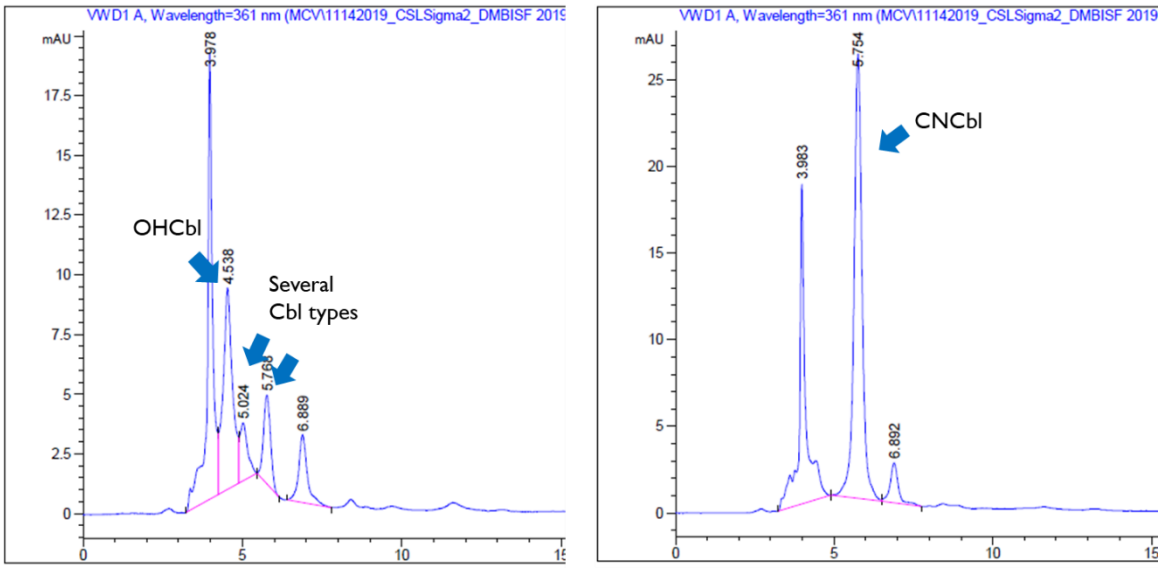

Figure 27 **Extracted samples from an Erlenmeyer Shake Flask Culture of NBRC 12391.** *Left: non-cyanidated sample. Right: Sample cyanidated with a NaCN 0,1% solution. Identify peaks of OHCbl, CNCbl and other unidentified Cbls are indicated by arrows.*

Optimizing Cobalamin extraction.

Until this moment, cultures were extracted by a protocol adapted from the bibliography, mainly [120] and [100]. However, many different extraction conditions are described regarding extraction temperature (from 80ºC to 120ªC), pH of the samples (between 6.5 and 8.5) and even extraction time (from 10 to 20 minutes). Extraction buffer also varies, with some publications using ultrapure water while others employ acetate buffers. Nitrate addition is also often added during the heat extraction in aerobic strains [114].

With all this in mind, it was possible that the low Cbl quantities found in previous experiments were not totally due to a low intracellular production but rather to an insufficient Cbl extraction.

Considering the large number of different variables that could be affecting extraction, (extraction time, temperature, buffer used in the extraction, etc.) it was decided to

check as many of them using the statistical tools provided by Design of Experiments (DoE).

DoE is a branch on applied statics most commonly used to evaluate the impact that several factors and their interactions could have in one or other parameters. It allows to study several inputs effect with the same experiments, avoiding repeating experiments like traditional "one-by-one" factor approaches while also study all the possible interactions that could be happening between different factors.

In order to study the influence of several factors in the extraction and quantification of CNCbl from culture samples a DoE approach was designed and executed. More specifically, four different factors (Extraction buffer, time, temperature, and sample volume) at two levels were considered as described in Table 5. A complete 2^4 design was proposed because the importance of factor interaction was unknown and the number of experiences needed for the complete design was manageable, with 16 extraction experiments to be performed at the same time.

All samples analyzed were obtained from the culture showed in Figure 24 at 240 hours. At this time point the OD_{600} value was 19 representing a biomass conversion of around 7.6 g/L as explained in Materials and Methods.

After all extractions were performed and the CNCbl quantified by HPLC, the results were statistically analyzed by an ANOVA study and represented graphically.

. Contributions of each factor and interaction were studied and a Half Normal Probability Plot representation of each term was also used to help study the importance of each individual factor.

Table 5 Experiments, Factors, and Levels proposed for a complete 2^4 DOE to assess the best conditions for CNCbl extraction. CNCbl quantification of each condition is also provided.

Experiment	A=Extraction Buffer	B=t (min)	C=T (ºC)	D= Culture volume (mL)	[CNCbl] (mg/L)
1	UPW	10	90	1	0.91
2	Acetate Buffer	10	90	1	0.90
3	UPW	20	90	1	0.93
4	Acetate Buffer	20	90	1	1.08
5	UPW	10	100	1	0.94
6	Acetate Buffer	10	100	1	0.95
7	UPW	20	100	1	0.97
8	Acetate Buffer	20	100	1	0.93
9	UPW	10	90	2	1.01
10	Acetate Buffer	10	90	2	0.94
11	UPW	20	90	2	0.98
12	UPW	20	90	2	0.92
13	Acetate Buffer	10	100	2	0.99
14	UPW	10	100	2	0.93
15	Acetate Buffer	20	100	2	0.92
16	UPW	20	100	2	0.95

A graphical representation of this study can be found in Figure 28. The two the factors that had the biggest impact in response (CNCbl concentration obtained) were factor C (Temperature) and factor D (sample volume). Both factors had a negative effect in the response: in their highest level, response was negatively affected suggesting potential degradation of cobalamins at higher Temperature and inefficient extraction at higher biomass concentrations.

The next term in order of importance was the triple interaction ABC (Extraction Buffer, Time, and Temperature). This kind of interactions is rarely important so the difference in respond in this term can probably by explain by experimental variation. Although at first glance experimental error can seem large (10-15 % of the variation), all experience responses are close to each other (Minimum quantification: 0.90 mg/L; Maximum quantification: 1.08 mg/L). This may indicate that all conditions tested were suitable for CNCbl sample extraction although high Temperature and higher Biomass quantities seem to have a minimal negative effect in the final CNCbl amount obtained.

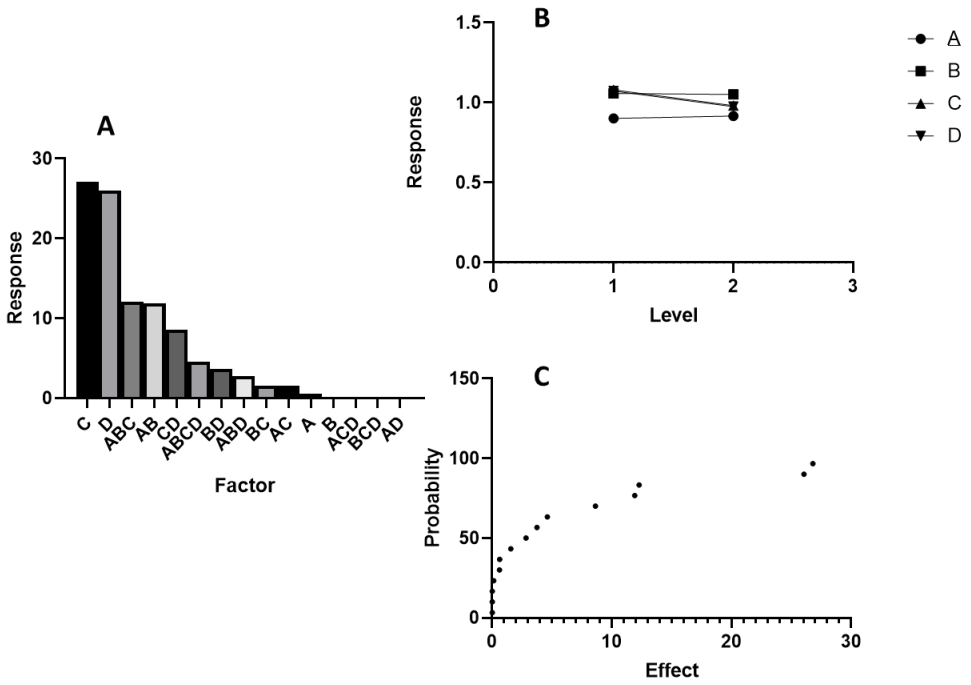

Figure 28 DOE study for CNCbl extraction *A.- Contributions of the different terms (Factors and interactions) of the 2^4 design proposed for studying CNCbl extraction optimization; B- Graphical representation of the effect of each factor; C.- Half Normal Probability Plot*

Taking into consideration these results and the need for a simple and robust extraction method, the following conditions were established for further experiments:

- Time: 10 minutes per sample
- Bath Temperature: 90ºC
- Extraction Buffer: ultrapure H_2O
- Samples volumes of 1 mL of culture broth.

4. Optimizing the production of CNCbl in wild type NBRC 12391.

Aeration effect during the early stages on CNCbl production. Developing a single-stage continuous process strategy for vitamin B_{12} production with *Propionibacterium freudenreichii*

4.1. Introduction

In Chapter 3 a suitable strain for Vitamin B_{12} production was selected and growth and production were thoughtfully characterized with several media and conditions. NBRC 12391 was chosen as the wild-type strain for further improvements in this chapter.

As previously mentioned, the main objective of this thesis project was to optimize the production by bioprocesses strategies instead of genetic modifications. The reason behind this mainly stems from the particularities of the target market of many potential vitamin B_{12}-based products, supplements, or fortified foods.

Many final vitamin B_{12} consumers, besides patients affected by pernicious anemia or other diseases, are vegans or vegetarians and people with high health and ecological awareness, so providing a solution based on a wild type of GRAS strain is a valuable asset that increases its market appeal, especially when compared with the current main industrial producers.

As said in previous chapters, nowadays most of Cbl industrial production is done in China by companies that use *Pseudomonas denitrificans-related* strains that have been submitted to genetic engineering to enhance their productivity. Taking into consideration that even the propionic resistant and overproducing *Propionibacterium* strains have been displaced as traditional industrial producers by this more efficient aerobic microorganism, focusing on other aspects and strengths of *Propionibacterium* besides raw productivity seems like a necessity to find a useful market niche for *Propionibacterium* produced Cbl products.

In this sense, marketing Cbl supplements as non-GMO can be valuables. Besides, and considering its traditional use in Swiss cheese production, the in-situ fortification of food products like breads or flours with direct cultures of Propionibacterium can also become a future economically viable application. This possibility is further reinforced by the promising probiotic properties described for some *Propionibacterium freudenreichii* strains: microbiota modulation, immunomodulation and the production of several nutraceutical compounds, such as trehalose, naphthoic acid and short-chain fatty acids.

4.2. Results

During Chapter 3, a strain was selected among overproducing candidates and its growth was characterized. Several glucose concentrations were tested and CSL was choosen as the complex nitrogen source, despite the lower OD_{600} values obtained, due to its industrial appeal (cheaper price) and the fact that more Cbl was found in fed-batch CSL cultures than in YE fed batch cultures even with lower OD_{600} and glucose consumption.

This optimization of the culture by media composition improvement was necessarily accompanied by the development of suitable extraction and quantification methodologies. Cyanidation with a 0.1% NaCN solution after a 10-minute extraction of the culture pellets at 90ºC was found suitable to extract the maximum amount of Cbl from the samples, although other extraction conditions provided similar results. And HPLC 1100 with a C18 column and a UV detector, as described in Materials and Methods was used to quantify all the Cbl production of the cells converted to the more stable CNCbl.

Considering that, at this point of the project, the growth was already characterized and optimize from a OD_{600}/Biomass point of view and that an extraction and quantification method had already been established, it was decided to focus the attention on trying to optimize CNCbl production by several mechanisms.

4.2.1. DMBI supplement addition time effect and first Fed-batch with CNCbl quantification.

Before repeating a bioreactor culture with the new extraction and cyanidation methodology, the effect of the DMBI addition on cell growth was tested in Erlenmeyer shake Flask cultures to establish the most optimal time for the supplement addition.

DMBI importance in Cbl production has been widely described in the bibliography. DMBI is the nucleotide that acts as a lower ligand for the central Co ion of the cobalamin molecule, bonding to the corrinic ring through the N7-atom in an axial conformation.

Despite the fact that *Propionibacterium* is a facultative anaerobic microorganism, it still follows the aerobic biosynthesis pathway for DMBI production: BluB (EC 1.13.11.79), performs the fragmentation and contraction of the bound flavin mononucleotide cofactor and the cleavage of the ribityl tail to form DMBI and D-erythrose 4-phosphate in the presence of molecular oxygen [213].

For this reason, Cbl production with *Propionibacterium freudenreichii* needs to find a balanced between the oxygen needs of the biomass producing phase and the Cbl production phase: very low oxygen conditions are needed for high cobalamin yields but oxygen is needed for DMBI synthesis and formation of the complete molecule by DMBI linkage to the corrinic ring [114].

This is the main reason behind the traditional double stage fermentation process, where after an initial complete anaerobic phase of 72 to 96 hours [114–116], cultures are usually micro-aerated by agitation [100]. However, despite having the necessary genes for DMBI synthesis on their own, *Propionibacterium* intrinsic production of this key nucleotide is very low [128] and, without any supplementation, incomplete Cbl forms tend to accumulate. For this reason, DMBI supplementation is widely extended and has been described to significantly increased total Cbl yields when coupled with proper culture conditions [81].

Many groups described a 96-hour addition, just at the beginning of the agitated production phase when the culture have had time to develop in completely anaerobic conditions [100]. In the case study, the effect of the addition time of this supplement was considered important for several reasons:

- Being a non-propionic resistant strain unlike other more traditional industrial strains, the concentration of propionic acid at 96-hours could potentially inhibit Cbl production.
- In many previous cultures, the glucose was almost depleted at 96 hours and the lack of a proper C source could also hinder production.
- We wanted to assess NBRC 12391 capability of producing CNCbl in lower time frames than traditional approaches.

For all these reasons, several cultures were carried out in Erlenmeyer shake flaks where DMBI was added at different times. Three different conditions were tested with Media 3.

- DMBI addition at 96 hours culture time.
- DMBI addition at 72 hours culture time.
- DMBI addition at 48 hours culture time.

In each case, 100 µM of DMBI was added at the described times as described in the materials and methods section. As in other previous Erlenmeyer Shake flask cultures, OD_{600} was followed by spectrophotometry and the pH was daily regulated with the addition of a 2M NaOH solution. Figure 29 shows the OD_{600} values of the three tested conditions next to cobalamin volumetric production at the end of the culture (168 h).

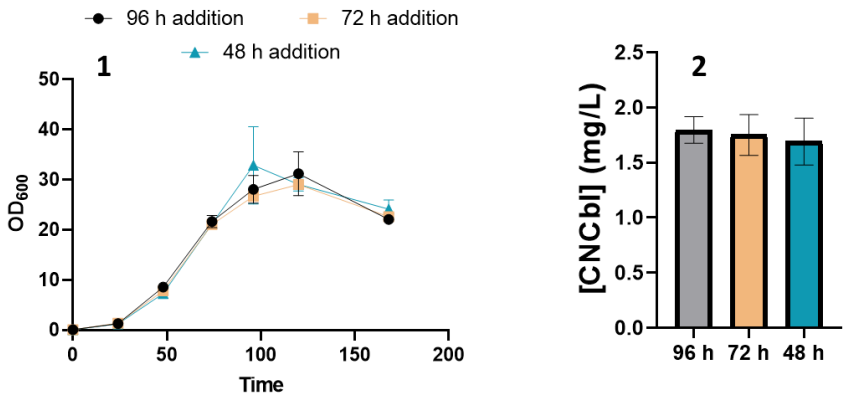

Figure 29 NBRC 12391 Erlenmeyer Shake Flask cultures at different 100 µM DMBI supplement addition times: 48 hours, 72 hours and 96 hours.*1: OD_{600} values, 2: volumetric CNCbl production at 168 culture h. Error bars represent the standard deviation of two replicates.*

As shown in Figure 29, different addition times of DMBI did not have any significant effect on Cell growth (with very similar max OD_{600} values between 27 and 32 approximately) nor on CNCbl production being the volumetric production very similar and around 1.7 mg/L. Considering these results, a new fed-batch approach with DMBI addition at 96 hours was developed to correctly assess CNCbl production in a pH and

C source controlled environment with conditions as close as possible to the ones described elsewhere (Figure 30).

As in previous experiments, the culture was carried out in a 2L bioreactor with a working volume of 1.5L and an initial volume of 80% of the final volume. Fed began at 48 h to avoid limitation due to lack of C source and the pH was constantly maintained at 7 by the addition of a 2M NaOH solution. 100 µM of DMBI were added at 96 h.

Glucose was constantly added from a 200 g/L glucose stock solution from 48 hours of culture until the end of the experiment at 144 hours at a 6.6 mL/h fed rate.

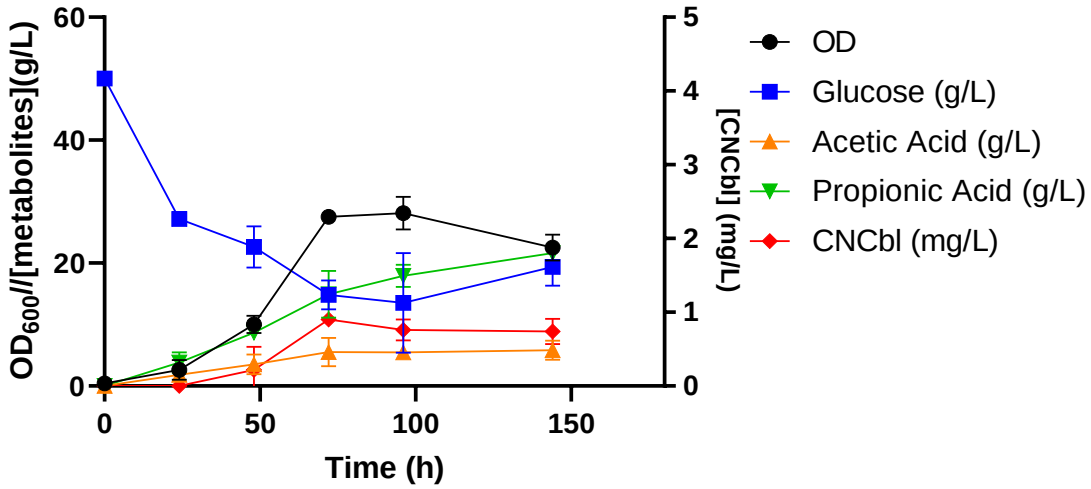

Figure 30 NBRC 12391 fed-batch bioreactor. Glucose was added from 72 h and measured with an Y15 analyzer (Biosystems). *OD_{600}, glucose (g/L), acetic acid (g/L), propionic acid (g/L) are depicted using the left y axis while CNCbl (mg/L) is shown in the right Y axis. Error bars represent the standard deviation of two replicates.*

First of all, it should be noted that Cbl quantification was more accurate and reliable in this later bioreactor than in previous experiments thanks to the cyanidation process. All Cbl was quantified using a single clearly distinct peak and the quantifications problems derived from the degradation of AdoCbl (both in the samples and in the standards) into OHCbl were avoided.

However, despite that the growth of the cells was clearly similar than those obtained in previous cultures with media 3 (although slightly lower, with max values of 27.5 ± 0.7), CNCbl was still lower than expected, 0.91 mg/L. In fact, two important facts are appreciated in Figure 30.

- CNCbl is lower in the fed batch approach than in the Erlenmeyer shake flask cultures shown in Figure 29 (0.91±0.01 vs 1.8±0.12)
- Maximum CNCbl production in Figure 30 was obtained before DMBI addition.
- Propionic acid was already at 18 ± 2 g/L at 96 hours when DMBI was added.

This result seems to suggest that something other than DMBI was inhibiting CNCbl production.

At this point, having established that neither the lack of DMBI nor an insufficient amount of C source were the caused for the low CNCbl productions, several possibilities were hypothesized:

- At 72 h propionic acid concentration were already significantly high (14.81 ±2.36 g/L) and by 96 h it already reached values very close to 20 g/L (18 ± 2 g/L). This high amount of propionic acid in the culture broth could be inhibiting the production of the cells, especially considering that we are working with a non-propionic resistant strain.
- Despite previous experiments pH regulation were performed as described elsewhere , we wanted to discard a possible negative effect of the constant addition of NaOH 2M solution and explore other alternatives for base addition.
- Finally, despite being a facultative anaerobic microorganism, we expect some amount of oxygen to be present in the bioreactor culture in the microaerated phase, probably in higher amounts than in Erlenmeyer shake flask. This slight oxygenation during the second microaerobic phase could hinder the production.

4.2.2. Propionic acid effect on cell growth and CNCbl production

Taking into consideration the results shown in Figure 30, understanding the effect of propionic acid on NBRC 12391 growth and production was deemed necessary.

Knowing the inhibitory concentration range of propionic acid and the effect that it had in growth, production or both would allow us to design strategies to avoid this limitation. A series of cultures were carried out to assess the effect on propionic acid accumulation and whether NBRC 12391 could develop and produce further without this limitation.

Besides the control condition, carried out as described previously, two different settings were tested in Erlenmeyer Shake Flask cultures.

- At 72 hours a concentrated solution of propionic acid was added to the media to increase the total concentration of this metabolite in the broth by 8.5 g/L.
- At 72 hours, the broth was centrifuged, the supernatant was discarded, and the cells were resuspended in fresh media.

With this experiment, we aimed to investigate the effect of propionic acid with opposing conditions: one where the limiting concentrations were achieved sooner and other where the cells were allowed to growth in a less propionic concentrated media for a longer time.

The results of these experiments are shown in Figure 31. OD_{600} are display next to the propionic acid concentration and the CNCbl volumetric production at 168 h.

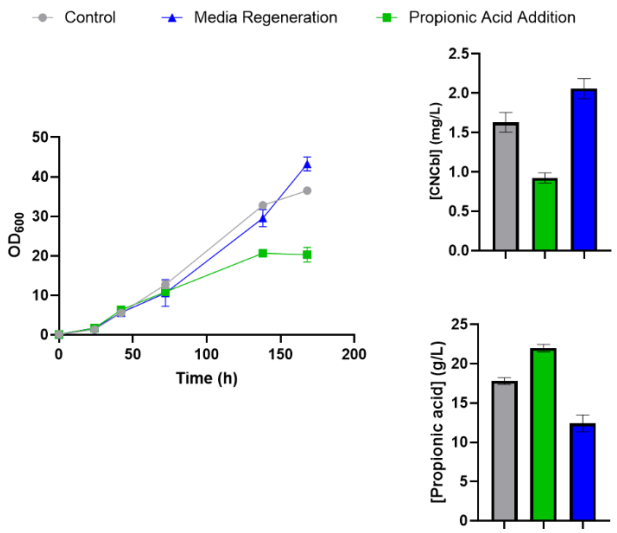

Figure 31 NBRC 12391 Erlenmeyer shake flask cultures. Three different conditions are shown: control, 8.5 g/l propionic acid increase at 72 hours and media regeneration at 72 hours. *1: OD_{600} values, 2: propionic acid concentration (g/L) at 168 h and 3: CNCbl (mg/L) concentration at 168 h). Error bars represent the standard deviation of three replicates.*

The results from Figure 31 clearly indicate that the addition of propionic acid was detrimental to both cell growth and production. The OD_{600} values of the cultures where propionic acid was added at 72 hours were significantly lower than those of the control and media regeneration conditions and only reached max values of around 20.8 ± 0.7 at 138 h. Besides, CNCbl production was also significantly lower than in the control condition: 0.93 ± 0.05 mg/L vs 1.62 ± 0.09 mg/L (P=0.0003). Propionic acid concentration was algo higher: 22.14 ± 0.42 g/L vs 18.03 ± 0.5 g/L (P=0.0004) although the control condition at that point was almost reaching the 20 g/L. These results seem to indicate that propionic acid concentrations near 20 g/L are inhibitory for both cell growth and CNCbl production.

Besides, cultures where the media was regenerated at 72 h also provide some valuable information regarding the effect of propionic in the growth. OD_{600} was significantly higher than in the control (36.1 ± 0.5 vs 43.2 ± 1.2 respectively), (P=0.001) and CNCbl production in the later condition was also significantly higher than in the

control (2.05 ± 0.10 mg/L vs 1.62 ± 0.09 mg/L) (P=0.005). In addition, even though the higher values, propionic acid concentration was lower than in the control (12.6 ± 0.8 g/L) and very possibly under the inhibitory range of propionic acid. This could mean that if the culture had been extended more time, even higher OD_{600} and CNCbl volumetric productions could have been obtained.

With the objective to study the maximum values that could possibly be obtained with this strategy and if further media regenerations could achieve even higher production values, a new experiment where the media was regenerated at two points during the culture (72 hours and 168 h) was performed and compared to a control culture. The results from this experiment are shown in Figure 32.

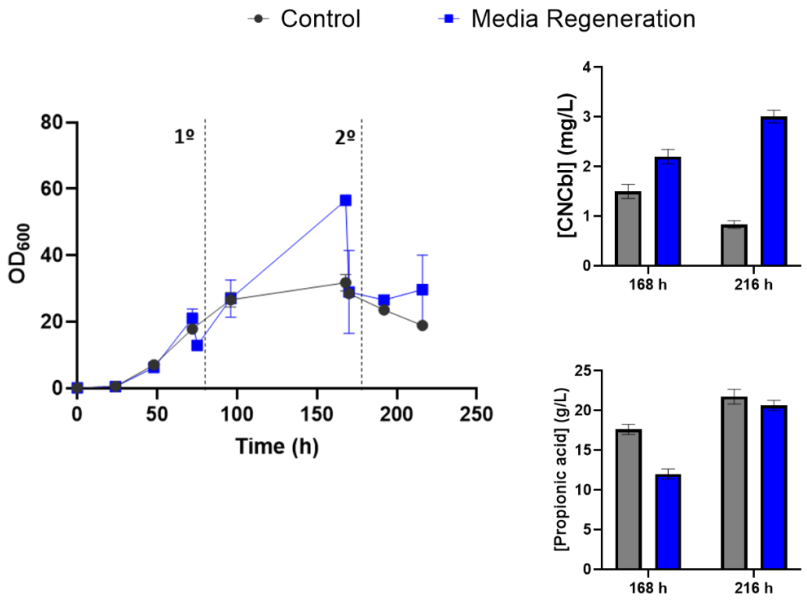

Figure 32 NBRC 12391 Erlenmeyer shake flask cultures. Two different conditions are shown: control and media regeneration at 72 h and 168 h. *The media regeneration times are visually indicated with a dotted line. 1: OD_{600} values, 2: CNCbl (mg/L) concentration at 168 h) and 3: propionic acid concentration (g/L) at 168 h and 216 h. The cultures were performed by duplicate.*

As shown in Figure 32, the differences in OD_{600} values were higher in this second media regeneration experiment than in Figure 31, partly because the lower max value of the

control culture and partly because the culture that had been resuspended in fresh media reached higher values.

However, when the cells were centrifuged and resuspended again at 216 h, the OD_{600} sharply fell, and the culture did not restore its previous max value. The final OD_{600} values for the cultures where the cells were resuspended in fresh media twice were 29 ± 10 and its maximum value at 168 h was 56 ± 1.

At 168 h, CNCbl production was higher in the resuspended cells than in the control culture (2.20 ± 0.14 mg/L vs 1.5 ± 0.14 mg/L) while propionic acid concentration was significantly lower (12.0 ± 0.6 g/L vs 17.7 ± 0.6 g/L).

Even after the already mentioned sharp decreased in OD_{600} after the second culture centrifugation, propionic acid concentration levels had raised to similar levels of those of the control culture: 20.6 ± 0.6 g/L in the regenerated cultures vs 21.7 ± 0.9 g/L in the control ones.

More importantly, CNCbl had also reached higher values despite the lower OD_{600} with a value of 3.01 ± 0.13 mg/L.

In addition, it is worth notice that CNCbl concentrations decreased in the control culture from 168 to 216 h (from 1.5 ± 0.14 mg/L to 0.84 ± 0.10 mg/L), thereby, suggesting that once the culture is inhibited by propionic acid not only it did not produce further CNCbl, but the latter got degraded.

These results seem to indicate that even though greater growth and production values can be obtained through refreshing the media as a way to eliminate propionic acid, this strategy is not sustainable for more than one regeneration, at least in a Erlenmeyer shake flask setting. Taking into consideration the state of the cells at 168 h, where most probably some amount of cellular lysis had already taken place, may explain the sharp descent in OD_{600}. Besides, centrifugation and resuspending the culture at this high OD_{600} was rather troublesome due to the fragility of the cultures. Therefore, some of these results may be explained also by an inappropriate experimental set up.

Nevertheless, the results of Figures 31 and 32 clearly suggests a strong inhibitory effect of propionic acid over cell growth and CNCbl production and point out a possible strategy to diminish this effect.

To first asses the viability of reducing the already established inhibition effect on the culture growth and production, and initial media-based strategy was first developed and try out in Erlenmeyer Shake Flasks. We thought that an interesting approach could be trying to reduce propionic acid production in the early stages of the cell culture by favoring alternative energy pathways instead of the main Wood-Werkman fermentation pathway. To do so, a nitrate supplementation was proposed as a method to stimulate anaerobic respiration instead of fermentation during the early stages of the culture. Theoretically, this could lead to a situation were similar OD_{600} values than those obtained in previous cultures were achieved but with lower propionic acid concentration.

For this strategy to work, NBRC 12391 needs to present nitrate reductase activity. As explained in Chapter 3, the traditional classification between the different subspecies of *Propionibacterium freudenreichii* (*Propionibacterium freudenreichii freudenreichii* and *Propionibacterium freudenreichii shermanii)* was based in their ability to metabolize lactose and their nitrogen reductase ability. Following these criteria, *Propionibacterium freudenreichii subs shermanii* NBRC 12391 phenotype would be lac-/nit+. However, as established in Chapter 3, recent discoveries and the reported existence of other two possible phenotypes (lac+/nit+ and lac-/nit-) [190,191] have made this classification no longer suitable, and nowadays it is no longer considered correct to classify them into different subspecies based on these criteria [192]. Therefore, establishing if NBRC 12391 was capable of nitrate reductase activity and if this activity influenced the fermentation and anaerobic respiration ratios needed to be tested.

4.2.3. Nitrate as alternative electron acceptor

Media 3 already presents some amount of nitrate in its composition in the form of NH_4NO_3, so it was decided to study the effect of larger concentrations by increasing the concentration of this compound by 2 g/L (25 mM) and 5 g/L (62 mM). To discard any possible negative effect of the NH_4 added to the media, 37mM NH_4Cl were also

added in other cultures. The results from these cultures compared to a control culture with regular Media 3 are shown in Figure 33.

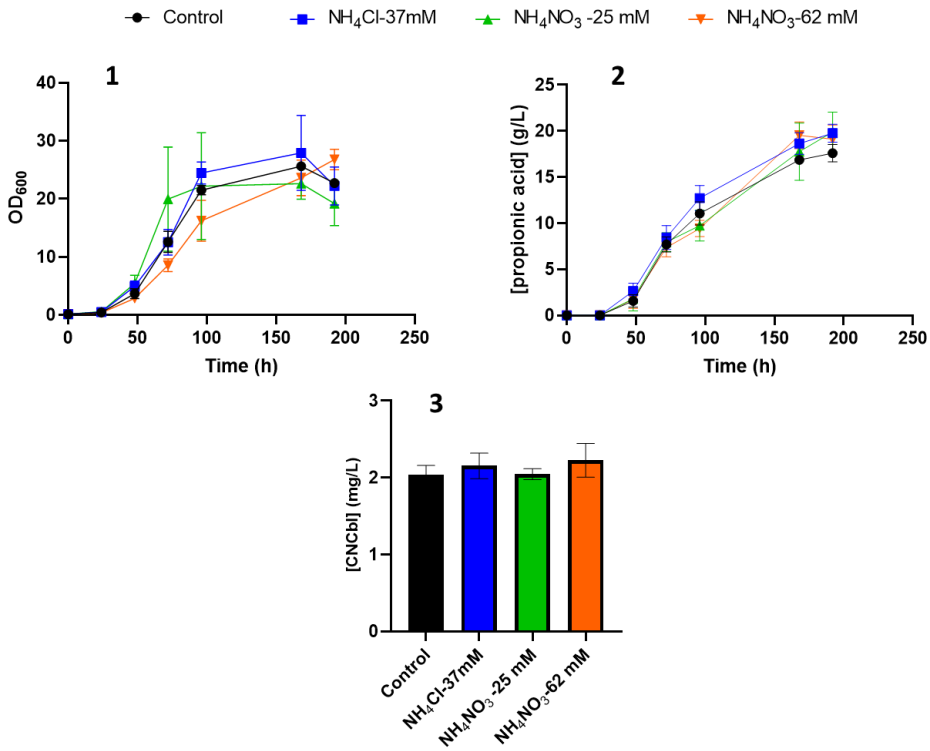

Figure 33 NBRC 12391 Erlenmeyer shake flask cultures with different nitrate additions. NH_4Cl was also tested as a negative control. 1. *OD_{600} values. 2. Propionic concentration (g/L) over time of the different conditions. 3. CNCbl volumetric production (mg/L) at 192 culture h Error bars represent the standard deviation of three replicates.*

As shown in Figure 33, nitrate addition does not seem to have a significant effect in neither cell growth nor propionic acid production. Besides, CNCbl volumetric production values of all the conditions were also very similar.

This seems to indicate that either NBRC 12391 does not have nitrate reductase activity or that the addition of nitrate at the tested concentrations did not have a significant effect on displacing the balance between fermentation and anaerobic respiration.

4.2.4. Effect of the base solution used for daily pH adjustment.

To discard any possible negative effect on the base solution used for pH adjustment of the cultures (NaOH 2M), Erlenmeyer shake flask cultures where different bases were added for the daily pH adjustment were performed. As shown in Figure 34, they were not significant changes in growth between the different base solutions. CNCbl production at 168 was also very similar between all three conditions (with values of around 1.5 mg/L) so the nature of the base solution does not seem to have an effect on the culture.

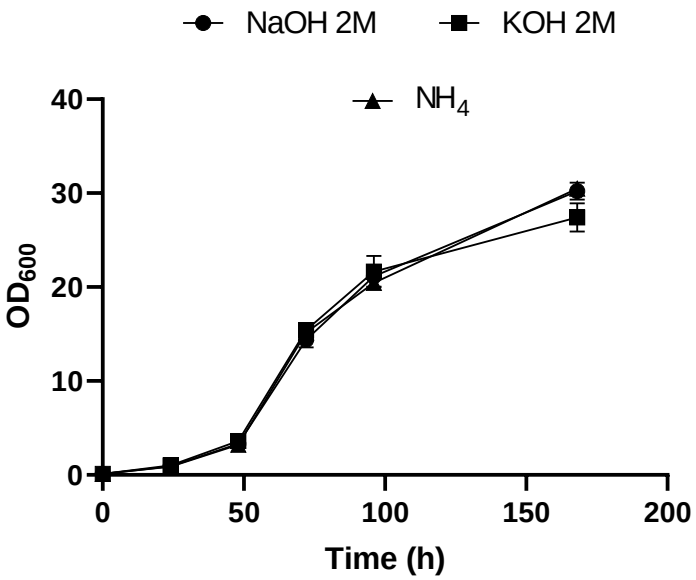

Figure 34 NBRC 12391 Erlenmeyer shake flask cultures with different bases added for the daily pH adjustment. *NaOH 2M, KOH2M and NH4 solutions were added to the media every 24 hour until a pH of around 7 was achieved. Error bars represent the standard deviation of three replicates.*

Taking that into account, future experiments were performed with NaOH 2M as previously, as this is the base more commonly used in the bibliography.

4.2.5. Early agitation effect on cell growth and CNCbl production.

There is no denying in that agitation is mandatory in any culture system to get a homogeneous system and not promote nutrient gradients or cell sedimentations. This

is especially true when the process are scale up to a bioreactor setting, where agitation by mechanical or order means becomes a very important topic to control and optimize.

However, in the case of our cultures with NBRC 12391, one of the possible explanations to the rather low CNCbl productions, besides the propionic acid clear inhibition effect, is the uncomplete anaerobiosis during the early phases of the culture. In fact, although *Propionibacterium freudenreichii* is described as a facultative anaerobic microorganism, it can grow and produce in ambient with small amounts of oxygen, at least in liquid cultures. As previously mentioned, solid agar-plate cultures are much more sensible [100] to the presence of oxygen and the use of anaerobic jars of anaerobic incubator becomes a necessity in these cases.

Even in liquid media, it has been reported that an oxygenation or agitation during the early stages of the culture could hinder Cbl production during the production stage [160]. Considering the experimental set up where all Erlenmeyer cultures were carried out, we decided to study the effect of the agitation during the early stages in NBRC 12391 cultures in terms of growth and production.

As explained in Materials and Methods, until this point all Erlenmeyer shake flask cultures were carry out in the following manner: 250 mL Erlenmeyer Shake Flask will fill with 200 mL of media and tightly close with screw caps. The ratio between total volume and culture volume was set at 80% to reduce the oxygenation capacity of the system to obtain conditions as close as anaerobic as possible without the aid of an anerobic incubator. However, the culture was constantly agitated at a gentle 150 rpm and, taking into consideration that even very low amounts of oxygen may have an effect on production as described in Hunik. 2001 [160], this agitation could be increasing the amount of oxygen available in the media and hindering the potential production of the cells. Besides, we must consider that cultures were open daily under sterile conditions to adjust their pH so further oxygenation of the broth could be taking place. Assessing the effect of agitation during the culture became, then, a necessity to try to optimize CNCbl production further and to develop suitable scale up strategies.

Control experiments with constant gentle agitation at 150 rpm were compared with cultures that were kept static during the first 96 culture hours, the anaerobic biomass production stage of the culture. After 96 hours, both conditions were supplemented with 100 μM DMBI and agitated at 150 rpm to create the microaerobic conditions that are described to be necessary for proper CNCbl production. The results from this experiment can be seen in Figure 35.

From the OD_{600} profile of Figure 35, a slightly delayed growth was observed on agitated cultures from the beginning, but both conditions reached similar maximum values at 96 h, OD_{600} of 29.1±0.3 and 26.1±1.0 for the static and agitated cultures respectively. These results seem to suggest that agitation may have a slightly negative effect on cell growth, especially during the early stages of the culture.

By the 96 hours and before DMBI addition to the media, our strain was already producing a significant amount of CNCbl even without DMBI, but only in the condition that was kept under agitation from the beginning of the culture. This volumetric production of 0.71±0.03 mg/L contrast with the static condition where CNCbl, if any, was under the quantification limit of our quantification method (0.3 mg/L).

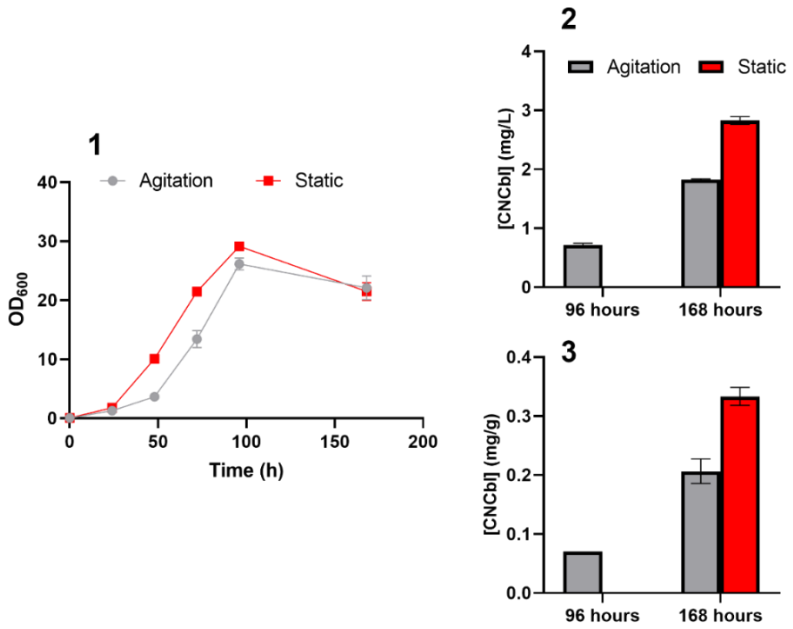

Figure 35 1 OD_{600} values, 2 volumetric productions (mg CNCbl/L) and 3 specific productions (mg CNCbl/g Biomass) at 96 and 168 h of both conditions, agitated or static. *Agitated cultures were kept at 150 rpm during the whole culture while static cultures were kept without any kind of agitation during the first 96 h and gentle agitated at 150 rpm afterwards. In both cases, 100 µM DMBI was added at 96 h to promote Cbl production. Error bars represent the standard deviation of three replicates.*

However, at the end of the culture at 168 h and after several days after DMBI addition, the static condition presented significantly higher volumetric and specific production than in the control: 2.83±0.06 mg/L vs 1.82 ± 0.02 mg/L ($P<0.0001$) and 0.33±0.02 mg CNCbl/g Biomass vs 0.21 ±0.02 mg CNCbl/g ($P=0.001$) .This seems to indicate that the final production depends on the culture condition before the DMBI addition.

This volumetric production was more similar (although slightly lower, but this could be due to the different strains used) to other values described in the bibliography for wild-type non propionic resistant strains. For example, Liu et al. reported productions with their non-optimized media of around 3.81 mg/L and Chamgalain and coworkers reported a production of 5.3 mg/L in whey-based media [116].

We are aware of the necessity of agitation in an eventual scale up of the process. However, considering the significant difference in volumetric production when the cultures were not agitated, we decided to continue optimizing the growth and production in Erlenmeyer shake flask using the static conditions. If, as theorized, this inhibitory effect of agitation during the early stages is due to a minor oxygenation of the culture that compromises the anaerobic environment, when scaling up the process in a bioreactor set-up this oxygenation can be avoided and the anaerobic condition better ensured by the constant addition of N_2 to the vessel.

4.2.6. Supplementation of CNCbl precursors

DMBI supplementation and supplementation time effect were already assessed in Erlenmeyer Flask as described earlier in this chapter. However, this study was performed in conditions that, after the conclusions reached by experiments performed in Figure 7, might not be ideal for CNCbl production. For this reason, we wanted to correctly assess the effect of DMBI supplementation in our strain. Furthermore, besides DMBI, other supplements such as riboflavin (RF) or nicotinamide (NAM) have been reported to have an effect on the production of other wild type Cbl producing strains [116].

To define the best supplementation approach for strain NBRC 12391, 100 µM of RF, 100 µM of NAM or 100 µM of DMBI were added at two different culture stages, at 0 and 96 h. Moreover, 50 µM of RF plus 50 µM of NAM were combined to test potential synergistic effects of both precursors. The results of this experiments are shown in Figure 36.

From all conditions tested, only supplementation with DMBI at time 0 seemed to have a slightly negative effect on cell growth during the early stages of the culture. Nevertheless, despite this inhibitory effect, the final absorbance achieved was not significantly different from the rest of the tested conditions, with OD_{600} values of around 30 at 168 h.

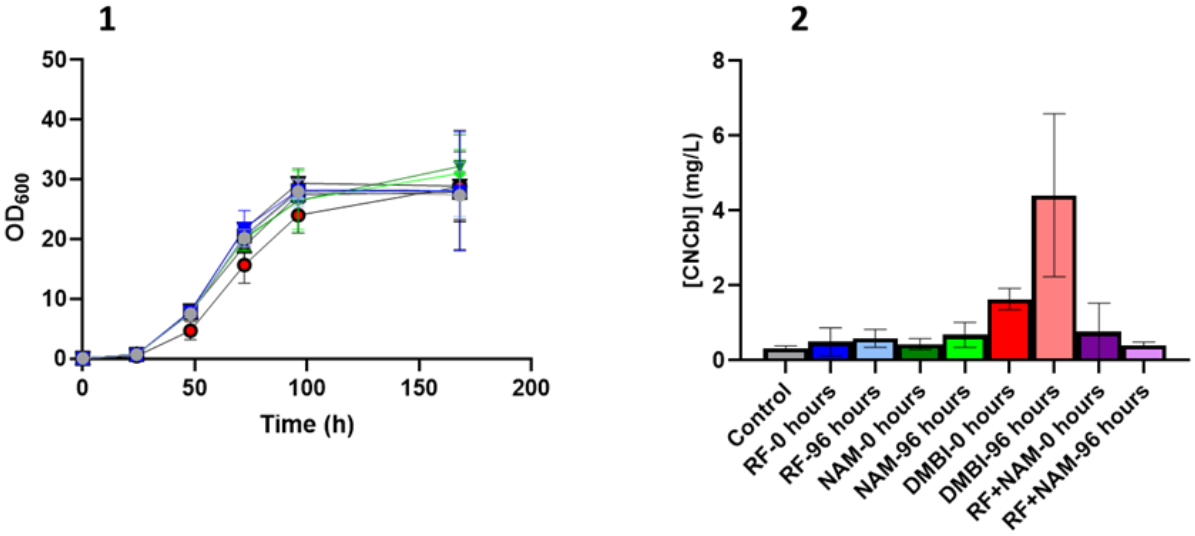

Figure 36 Study of the effect of the addition of different CNCbl precursors at different times. *1: OD_{600} values and 2: volumetric productions (mg CNCbl/L) at 168 h of NBRC 12.391 cultures where different Cbl precursors were added to the culture at 0 or 96 h. Control (without precursors), Riboflavin (RF), Nicotinamide (NAM), 5,6-dimethylbenzemidazole (DMBI) and a combination of both riboflavin and Nicotinamide (RF+NAM). Error bars represent the standard deviation of three replicates*.

On the other hand, CNCbl production was assessed only at 168 h, the final point of the culture after 72 h of gently agitation. At this time, all conditions tested including the control condition without any addition showed some amount of CNCbl production. However, except for DMBI, the addition of precursors, independently of when they were added, did not significantly different from the control condition and low volumetric production were obtained.

Nevertheless, the addition of DMBI had a positive effect on CNCbl production, both when added at time 0 and at 96 culture h. Besides, as usually described the addition of DMBI at 96 h seemed to be better for CNCbl volumetric production, as evidenced in the difference on production between the two conditions at 168 h: 1.64 ± 0,29 mg/L when DMBI was added at time 0 vs 4.41± 2.17 mg/L when it was added at 96 h (P=0.09). However, the variance in the CNCbl obtained when DMBI was added at 96

hours made assessing the difference in production between this condition and when DMBI was added at 0 h not statistically different.

It was decided to further assess the effect of different addition times with the new static experimental set up. With that objective, four different addition times were tested, 0, 48, 72 and 96 h. The results from this experiment are shown in Figure 37.

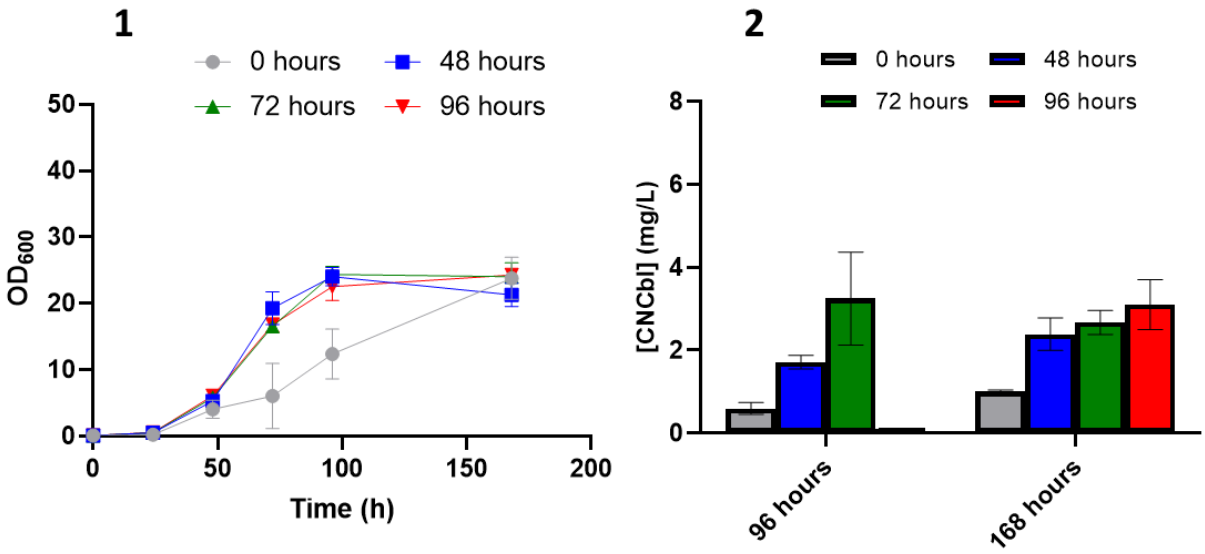

Figure 37 Study of the effect of adding DMBI at different times. *1. OD_{600} values and 2. volumetric productions (mg CNCbl/L) at 168 h of NBRC 12391 cultures where DMBI was added at four different times: 0, 48, 72 and 96 h. Error bars represent the standard deviation of two replicates.*

As shown in Figure 37, production was evaluated at 96 and 168 h for all conditions. As expected, at 96 h, all conditions where DMBI was added showed some amount of Cbl, being the induced culture at 72 h the best presenting already 3.3 ± 1.2 mg/L of CNCbl just 24 h after the induction. This is significant because, at 168 h, the production of the culture induced at 96 h was very similar to this value (3.11±0.6 mg/L), suggesting that after certain OD_{600} value of around 20 is achieved, the maximum CNCbl volumetric production in NBRC 12391 cultures can be obtained just 24 h after DMBI addition.

This conclusion could mean that, for our non-propionic resistant strain, culture could be greatly shortened to just 24 hours before DMBI addition and that DMBI could also be added 24 h before the usually described addition times. This could also open the possibility for several scale up strategies that differ from the two-stage culture described in the media where DMBI is added at 96 h and the culture enters a microaerobic production stage until 168 h.

4.2.7. Scale up of NBRC 12391 cultures for CNCbl production. Batch and fetch batch processes.

As previously explained, a non-agitated bioreactor culture is not feasible due to the improper homogenization and the cell sedimentation that it would imply. For this reason, all the scale-up strategies described in this section were performed in a gentle agitated bioreactor (150 rpm). To maintain a proper anaerobic environment and avoid the inhibition in production detected by an early oxygenation of the cultures, the bioreactor vessel was pumped with a constant N_2 flow of 0.15 vvm.

The main objective of the scale up was to develop a strategy that allowed for higher production and productivity than those obtained in Erlenmeyer shake flasks in a more tightly controlled environment. A bioreactor setting, besides constant pH regulation provided by a pH probe control, also allows to solve common bioprocesses limitation like the lack of C source for an eventual industrial application of the project. All the experiments in bioreactor described in this section were performed in a lab-scale bioreactor with a 1L working volume.

Batch with DMBI induction at 96 h

The first characterization in a completely anaerobic growth phase was performed according to the most common set up described in the field. That meant that the initial growth phase was extended for 96 h. After that time, 100 µM DMBI was added to the culture and N_2 addition was stopped to promote micro aeration. The results of this first bioreactor are shown in Figure 37.

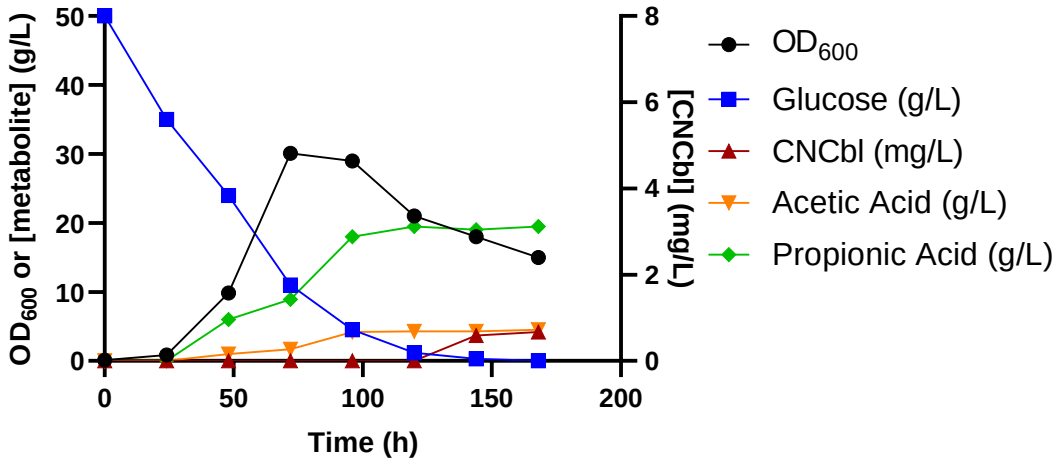

Figure 38 NBRC 12391 batch with DMBI addition at 96 hours. *OD_{600} values, glucose (g/L), acetic acid (g/L), propionic acid (g/L) and CNCbl (mg/L) are provided. Due to the results and the early glucose depletion from the media, this experiment was only carried out once instead of by duplicate.*

Unlikely following experiments, results from Figure 37 were only performed once instead of by duplicate or triplicate due to the obvious performance issues of NBRC 12391 and the low and unexpected CNCbl production during the culture. As shown in Figure 10, CNCbl production in a batch process with DMBI addition at 96 h was much lower than the one obtained in Erlenmeyer shake flask with maximum values of only 0.63 mg/L.

An explanation for this low production can be that the DMBI addition was inadequate, despite that similar additions times in Erlenmeyer shake flasks rendered better production results. Looking at the growth, the obtained data shows that our strain presented faster growth rates in the bioreactor, even though it was agitated, compared to shake flasks studies as the maximum OD_{600} was obtained sooner, at approximately 72 h. This can be easily explained by the more controlled pH, that was constantly adjusted to 7 instead of every 24 h. However, this increased growth also meant that, by the time DMBI was added at 96 h, glucose was almost depleted from the media, presenting values of around 4 g/L. Moreover, under the microaeration stage, glucose was completely depleted from the media and OD_{600} values began to

decrease, most probably due to some degree of cell lysis. The lack of C source could explain the diminish production and seems to indicate that, for our strain a DMBI addition at 96 h without any kind of extra C source was not a suitable scale-up strategy.

Besides glucose and CNCbl, the acetic and propionic acid accumulations were also followed through the process. As expected, propionic acid was the principal fermentation by-product mainly produced during the anaerobic phase reaching 18.4 g/L at 96 h. This value remained stable in the microaerobic phase (reaching a maximum value of 19.5 g/L at 168 h) most probably because glucose was already almost depleted.

Batch with DMBI induction at 72 h

Taking into consideration the experiments showed in Figure 37 where it was proved that maximum volumetric production values could be obtained with earlier DMBI addition if certain OD_{600} values were achieved and the negative effect of an insufficient amount of glucose during the production stage, a new batch process where DMBI was added at 72 hours was performed. The rest of the conditions were the same as the ones described for the bioreactor culture in Figure 10. The results from this batch are shown in Figure 39.

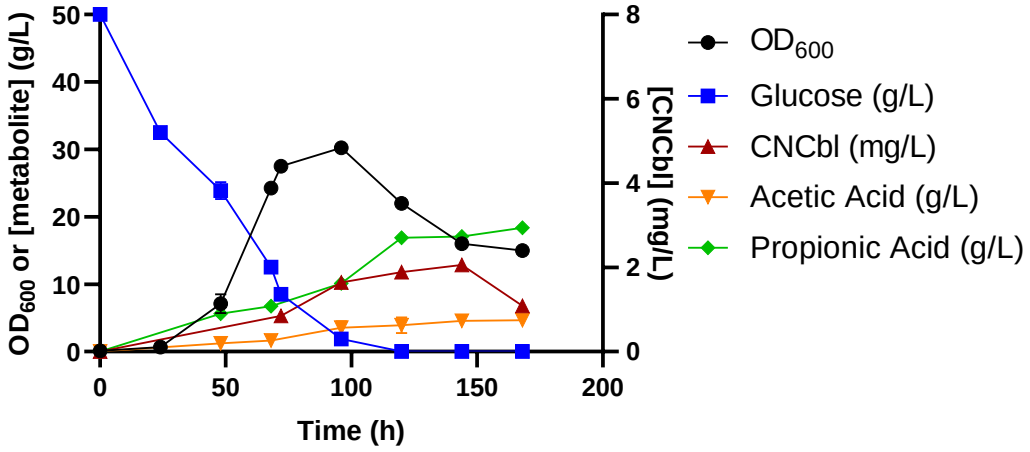

Figure 39 NBRC 12391 batch with DMBI addition at 72 h. *OD_{600} values, glucose (g/L), acetic acid (g/L), propionic acid (g/L) and CNCbl (mg/L) are provided. Error bars represent the standard deviation of two replicates.*

As shown in Figure 38, at 72 h when DMBI was added, OD_{600} values were already quite high at 27.5 but there was still a significant amount of glucose remaining in the medium (8.5±0.7 g/L). After DMBI addition, CNCbl began and interestingly, at 75 h, just 3 h after the DMBI addition we could already quantify 0.85 mg/L ± 0.02 of CNCbl suggesting a fast accumulation of the vitamin. Besides, the maximum CNCbl volumetric production was significantly higher than the one obtained in Figure 10, and 2.05 mg/L ± 0.3 were obtained in a 48-h period.

Like always, propionic acid and acetic acid were followed through the culture and the maximum and final values are very similar to the ones obtained in the batch induced at 96 h, suggesting that the increased CNCbl production is due to the increased C source concentration in the media during the microaeration stage rather than a lower propionic acid inhibition.

Nevertheless, although better than in previous attempts, the CNCbl volumetric production obtained in Figure 39 were still lower than the maximum values of around

4 mg/L obtained in Erlenmeyer shake flasks. Further improvements were still needed until a proper scale-up strategy was established.

Fed-batch with DMBI induction at 72 h

Due to the impact of carbon source limitation on both OD_{600} and CNCbl volumetric production, a fed-batch bioprocess with constant glucose addition after 72 h was assessed. The glucose was added from a 250 g/L at a constant rate of 6.66 mL/h. A total of 200 mL of the solution were added to the bioreactor vessel.

At the time the glucose feeding began, DMBI supplementation (100 µM) was also added to the vessel and the microaeration phase began. The results from this experiment are shown in Figure 40.

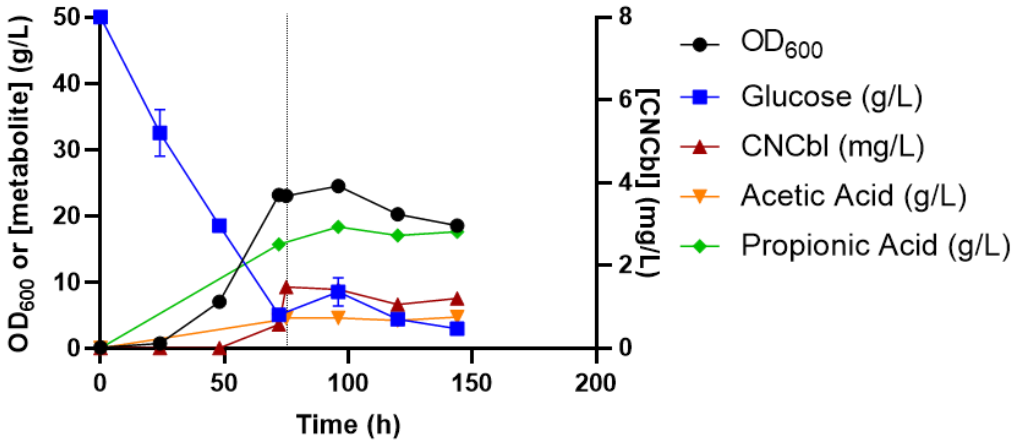

Figure 40 NBRC 12391 fed-batch with DMBI addition at 72 h. *OD_{600} values, glucose (g/L), acetic acid (g/L), propionic acid (g/L) and CNCbl (mg/L) are provided. The vertical dashed line represents when feeding was started. Error bars represent the standard deviation of two replicates.*

Several conclusions can be extracted from Figure 40. First, despite the increased amount of C source in comparison with previous bioreactor cultures, OD_{600} did not reach higher values and, in fact, OD_{600} max (24.5 ± 0.7) was lower than in previous experiments. Besides, maximum volumetric production was also lower (1.5 ± 0.5

mg/L). Propionic acid, in the other hand presented slightly lower maximum values than in previous conditions, but was still quite high and very near to 20 g/L.

These results seem to suggest that C source limitation is not the only factor that could hinder the production in the bioreactor. Other factors, like a possible propionic acid inhibition could also be affecting the production.

This inhibition could be explaining the low OD_{600} and CNCbl values: by the time the fed began, propionic acid was already at high enough concentrations to limit cell growth and production and even cause some amount of cell lysis. This way, when a concentrated glucose stock solution was added to the vessel, the cells were incapable of continuing replicating like before and the broth got diluted, explaining the lower and counterintuitive OD_{600} values when compared with batch processes.

Considering this inhibition, a fed-batch strategy without a mechanism for propionic acid elimination/removal (for example, an expanded bed reactor like the one used in [120]) did not seem to be a viable strategy for cobalamin production.

As the main objective of the thesis project is to optimize production with wild type non-propionic resistant strains, engineering NBRC 12391 to further withstand propionic acid is out of the scope of this work. Besides, implementing complicating solutions to eliminate propionic acid directly from the broth that the one described in [120] and in other similar papers was also not contemplated due to the increased cost in an eventual industrial scale-up of the process. For this reason, alternative ways of limiting the propionic acid inhibition effect of the culture were studied.

4.2.8. Development of a single-stage continuous bioprocess in lab-scaled bioreactor for increased CNCbl volumetric productivity.

In Chapter 3 of this thesis project, an increased growth and production by centrifugating the cells and resuspending them in fresh media was described. This was performed with the previous experimental and constantly agitated Erlenmeyer Shake Flask set up but could open the possibility to an alternative scale up strategy.

Taking into consideration that decreasing the propionic acid amount in the broth during later CNCbl-producing stages seemed to be a necessity for obtaining higher

productions, a continuous bioreactor process, where the spent media was constantly extracted from the vessel and renew with fresh media, seemed like and interesting approach to the problem.

This possibility was initially discarded due to the usually described two stage process for CNCbl production with *Propionibacterium freudenreichii*. On one hand, the two-stage production process with an anaerobic growth phase and a microaerobic production phase severely hinders the viability and increases the complexity of a continuous bioprocess. Besides, production stages up to 72 h were also incompatible with a constantly refreshed media, they would imply low dilution rates in chemostat cultures (D).

On the other hand, the experiments performed in Figure 38 showed that the maximum CNCbl production could be achieved only 24 h after the DMBI addition so, theoretically, a continuous bioprocess with regeneration times of under a day could be used to increase the productivity of the culture.

Media regeneration cultures in Erlenmeyer Shake flask.

To test the capacity of the cell culture to continue growing in fresh media, a similar experiment to the one described in Figures 31 and 32 was performed in Erlenmeyer shake flasks. In contrast with these previous experiments, here the Erlenmeyer shake flasks were not agitated until the production was induced with DMBI at the first media exchange at 72 h. This way, after the first 72 h of growth, the whole culture was centrifuged, the supernatant discarded, and the cell pellet resuspended in fresh media and supplemented with 100 µM DMBI. The results from this experiment are shown in Figure 41.

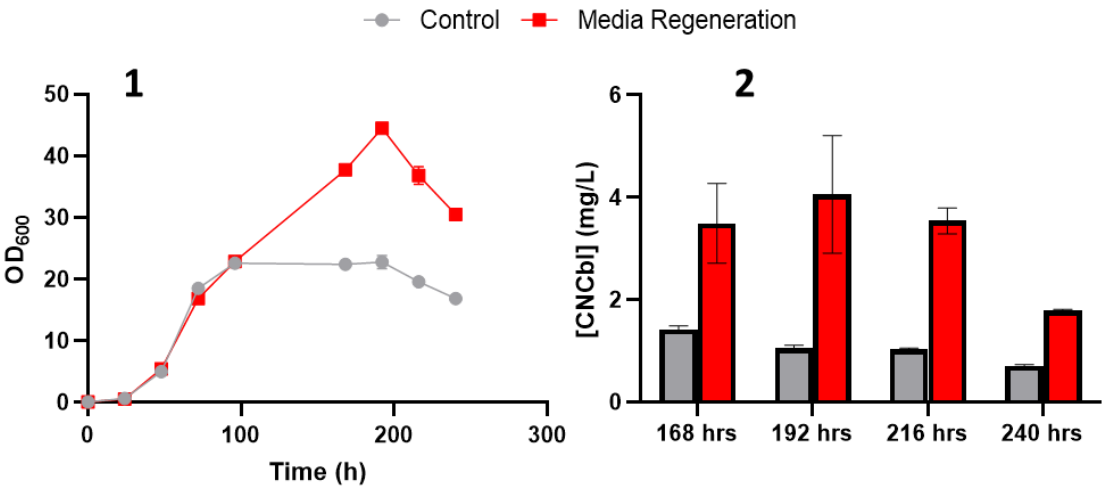

Figure 41 1. OD_{600} values and 2. volumetric productions at 168, 192, 216 and 40 h of NBRC 12391 control cultures and cultures where the cells were centrifuged and resuspended in fresh media at 96 h. ***In both cases, 100 µM DMBI was added at 96 h to promote Cbl production. Error bars represent the standard deviation of three replicates*.**

As shown above, resuspending the cells in fresh media allowed to reach not only higher OD_{600} values, 44.5±0.7 vs 22.75 ± 1.1 in the control culture ($P < 0.0001$) but also higher CNCbl volumetric production, 4.1 ± 1.1 mg CNCbl/L vs 1.42 ± 0.07 mg CNCbl/L ($P = 0.01$). By eliminating the propionic acid from the media, the cells were allowed to growth and producer further than in control circumstances, hinting the viability of a continuous bioprocess.

CNCbl anaerobic production in Erlenmeyer shake flasks.

As previously explained, a two-stage production bioprocess, with an anaerobic growth phase and a producing microaerobic stage severely hinders the viability of a continuous bioreactor strategy. The need to change conditions multiple time for each retention time makes developing a proper strategy much complex. A single-phase process, with constant anaerobic conditions to ensure cell growth, would be preferable. Nevertheless, for this strategy to be feasible, our strain should be capable of producing CNCbl in anaerobic conditions without a microaerobic production stage.

Taking into consideration that oxygen is needed mainly for DMBI biosynthesis and its linkage to the rest of the Cbl molecule, and that DMBI is added in excess during all cultures, the possibility of a single-phase anaerobic production became an interesting possibility.

The capacity of NBRC 12391 to produce CNCbl in a single anaerobic phase was assessed first in Erlenmeyer shake flask. For this, several cultures were kept static during the whole 168 h and their production was compared with control cultures that were agitated after the first 96 h. In each case, 100 µM DMBI were added to the broth at 96 h to stimulate CNCbl production. The results for these cultures are shown in Figure 42.

As a result of the experiments shown below, it was concluded that OD_{600} values and CNCbl volumetric and specific productions were not significantly different (max OD_{600} P= 0.57; CNCbl productions P= 0.15) between single phase and double phase cultures, thereby allowing a single anaerobic process.

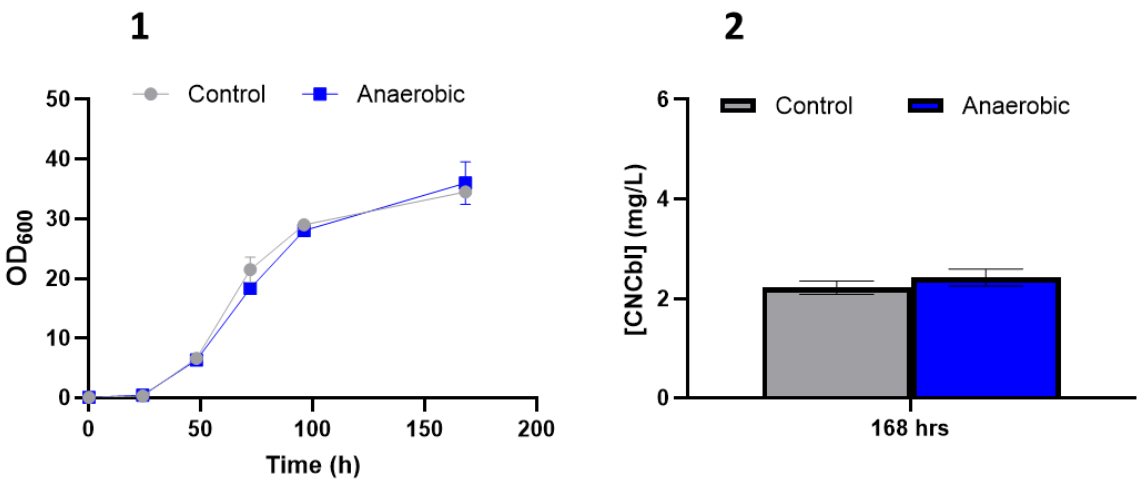

Figure 42 1. OD_{600} values and 2. volumetric productions at 168 h of NBRC 12391 control cultures and cultures that were kept anaerobic through the whole process. *In both cases, 100 µM DMBI was added at 96 h to promote Cbl production. Error bars represent the standard deviation of three replicates.*

Continuous bioprocess in bioreactor

From the previously described experiments, two main conclusions were reached:

- The cells could continue growing in fresh media even after being exposed to high propionic acid concentrations as shown in Figure 40.
- NBRC 12391 can produce similar amounts of CNCbl in complete anerobic conditions (with added DMBI) that the ones obtained in a two-phase culture with an microaerobic production phase in our experimental Erlenmeyer shake flask, as shown in Figure 41.

With this, a single-phase continuous process was developed with the objective of reducing the propionic acid inhibition in the vessel at steady state. The exact experimental set-up is described in detail in the materials and methods section of this chapter. Briefly, the initial media and the fed used for this culture had lower amount of glucose and CSL than previous experiments. Specifically, they contained 25 g/L glucose and 20 g/L CSL instead of 50 g/L and 40 g/L. This was done to limit the growth and propionic acid production of the culture. Thereby, we were expecting to limit OD_{600} values to around 15 and propionic acid to 9 g/L at steady state assuming equivalent yields as in previous Batch processes.

After an initial batch phase of 48 hours and before the initial glucose was complete depleted from the medium, the continuous bioprocess began with the constant addition of fresh media at a constant rate of 50 mL/h. Besides, 100 µM DMBI were also added to the vessel. It should be noted that, from this point on, all the fresh media fed to the bioreactor vessel already incorporated 100 µM DMBI in its composition to avoid CNCbl production limitations due to insufficient amounts of precursor. This is especially important considering that all the culture was carried out in a single anaerobic phase and therefore greatly limiting the cells capacity to produce DMBI on its own. The result for these cultures is shown In Figure 43.

Before the beginning of the continuous phase at 58 h, an OD_{600} of 13.05 ± 0.07 were reached. During the continuous phase, the total volume addition rate was 55 mL/h due to the constant addition of 2 M NaOH (5 mL/h) to control pH. These values imply a dilution rate of 0.055 h^{-1} and a mean residence time of 18.2 h. CNCbl production began just after DMBI addition, reaching a volumetric production of 1.33±0.26 mg/L

during the first residence time. After 5 retention times, a stable OD_{600} value of around 24 was achieved, while glucose concentration remained stable at approximately 1.2–1.5 g/L. Propionic acid also presented a stable value of around 7.5 g/L, as expected much lower than the 20 g/L obtained in previous cultures and not in the inhibitory concentration range. Moreover, OD_{600} and propionic acid values were better than the expected ones of 15 and 9 g/L respectively, so the continuous state not only allowed to control propionic acid at lower levels but also improved the carbon source usage towards biomass formation. Moreover, CNCbl volumetric production was stable from the first residence time at 1.33±0.26 mg/L.

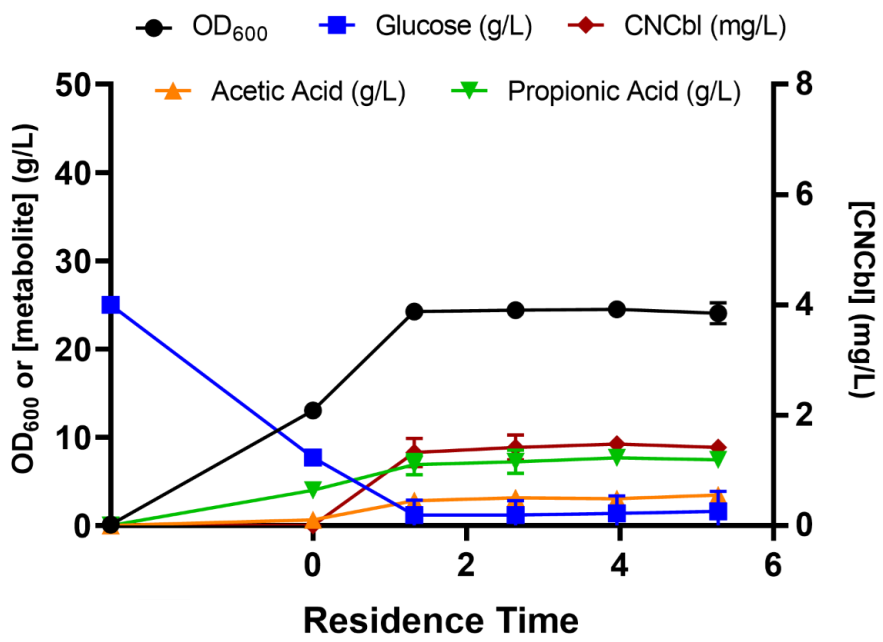

Figure 43 Continuous bioprocess of NBRC 12391. *The number of residence times is shown on the X-axis. According to the calculated addition of both fresh media and NaOH solution, each residence time corresponds to 18.2 h. Fresh media containing 100 µM DMBI addition began at residence time 0. OD_{600} values, glucose (g/L), acetic acid (g/L), propionic acid (g/L) and CNCbl (mg/L) are provided. Error bars represent the standard deviation of two replicates.*

The maximum obtained volumetric productions are not much higher than the ones obtained in other scale up strategies and were, in fact, lower than the ones obtained in the batch induced at 96 h. This means that they were also lower than the maximum

productions obtained in the Erlenmeyer Shake flask cultures with the best culture and DMBI induction conditions. However, the fact that NBRC 12391 could produce maximum amounts of CNCbl in just one bioreactor retention time and in complete anaerobic conditions allowed the continuous culture to reach much higher volumetric productivities than any other tested alternative.

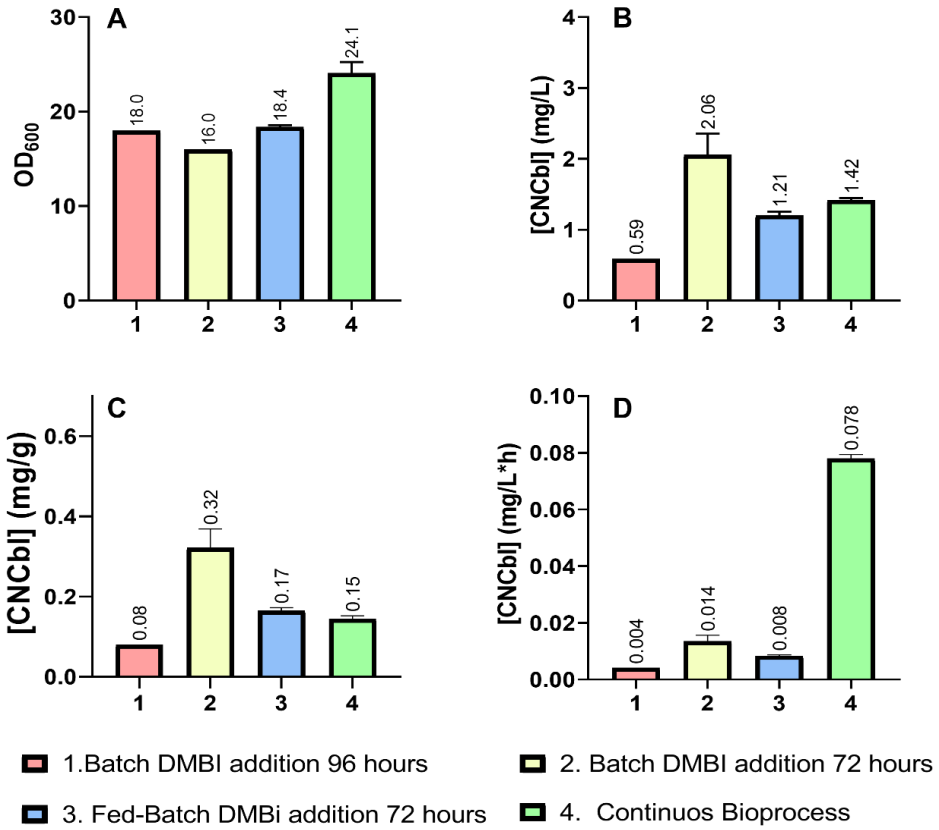

Figure 44 Comparison of several bioprocess parameters between the 4 scale-up strategies tested: batch with DMBI addition at 96 h, batch with DMBI addition at 72 h, fed-batch with DMBI addition at 72 h, and continuous bioprocess. *A OD_{600}; B Volumetric production; C Specific production; D Volumetric productivity.*

In fact, as shown in Figure 44 comparing the two batch bioprocesses (with DMBI added at 96 and 72 h respectively), the fed-batch process and the newly developed continuous bioprocess at 144 h, volumetric and specific production values showed values in the same range independently of the culture strategy. However, the

volumetric productivities in the continuous process were 5.7-fold higher than the best batch process, 0.078±0.001 vs 0.014± 0.002 mg CNCbl/(L·h).

When comparing the results achieved through the developed continuous process with data from recent publications, as summarized in Table 3 of Chapter 1 and in a recent publication by our research group [214], it becomes evident that volumetric yields remain either similar to or lower than previously reported figures for wild-type Propionibacterium strains that do not involve complex propionic limiting mechanisms, such as EBAB bioreactors. For instance, our CNCbl value of 1.33 mg/L compares to values of 5.3 mg/L [116], 2.74 mg/L [132], or 0.75 mg/L [125] from previously published studies. Nevertheless, it's worth noting that volumetric productivity data significantly exceeds these previous cases, with a value of 0.078 mg/L/h as opposed to 0.03 mg/L/h [116], 0.02 mg/L/h, or 0.004 mg/L/h, respectively.

Both the volumetric production and productivity values are nonetheless significantly inferior to those obtained in process involving EBAB or other systems for propionic acid elimination from the culture broth like cell recycling systems. For example, the obtained volumetric production value of 1.33 mg/L compares to values of 43.4 mg/L [120], 47.6 mg/L [122], 58.8 mg/L [109] or 8 mg/L [123]. Similarly, the respective volumetric productivity values also fall short when compared to our obtained 0.078 mg/L/h, as opposed to values like 0.27 mg/L/h [120], 0.18 mg/L/h [122], 0.59 mg/L/h [109], or 0.14 mg/L/h [123]. However, it should be noted that all these cases involved dedicated broth recycling systems unlike the developed single-phase continuous reactor bioprocess.

Finally, and as expected due to the nature of the producing strains, the results obtained are significantly inferior to those obtained in industrially used aerobic strains like Pseudomonas denitrificans, where volumetric production values of 213.1 mg/L with volumetric productivities of 1.88 mg/L/h [110] are reportedly achieved.

Overall, despite the inherent limitations of a slow growing and low producing strain like NBRC 12391 and the difficulties attached to a continuous bioprocess, a single

phase, easy to replicate and to scale up, continuous bioprocess with much higher volumetric productivities than other alternatives has been developed.

Although there is still work to be done regarding the optimization of the production, evidence by the fact that the highest production obtained in this thesis project was found in Erlenmeyers Shake flasks and no in a bioreactor setting, this provides the initial layout of a new viable strategy for the development of future and sustainable strategies to produce CNCbl with wild-type GRAS *P. freudenreichii* strains without having to rely on more complicated set-ups like multiple-phase continuous bioprocesses or expanded bed adsorption bioreactors for propionic acid elimination.

To further optimize this process, studying the starting conditions of the bioreactor culture may be a viable approach. In these cultures, the maximum propionic acid concentrations were 7.5 g/L, much lower than the estimated inhibitory range of around 20 g/L. Trying different starting glucose and CSL concentrations and/or extending the initial batch phase and starting the continuous bioprocess at higher OD_{600} values may render higher volumetric productions and even higher volumetric productivities. Any other improvement in the supplementation strategy, the media composition or the culture conditions can be also easily implemented in this new continuous methodology.

5. Comparison with other vitamin B_{12}-producing strains and other strategies for improved production through bioprocess optimization

5.1. Other vitamin B_{12} producing strains. Growth and production characterization of two Cbl producing aerobic strains: DMSZ 1650 and NBRC 102608.

During the development of a culture methodology for improved vitamin B_{12} production described in previous chapters, it was decided to obtain more information about some of the aerobic producing strains in order to compare.

Nowadays, most of the vitamin B_{12} production worldwide is done in China using genetically modified aerobic strains that can reach volumetric productions as high as 200 mg/L [215]. Taking into consideration that the main objective of this thesis project was improving the productivity of wild-type strains, we wanted to assess the Cbl production capabilities of non-modify aerobic strains to compare their growth, culture settings, metabolic needs and total Cbl production to the *Propionibacterium freudenreichii* NBRC 12391 strain.

There is a significant amount of confusion in the bibliography regarding the specific aerobic strains that are used today at industrial settings. In order to choose a suitable and publicly accessible wild-type strain, the bibliography about the subject was thoughtfully reviewed.

The fact is that it still remains unclear whether the current vitamin B_{12} producers are *Pseudomonas denitrificans* or other aerobic strains. *Pseudomonas denitrificans* has been traditionally used as a taxonomy name to describe vitamin B_{12}-producing aerobic strains. However, as most of the industrial production occurs in China with strains that are not publicly available, there is very little information available about the actual process and strain. As such, determining the exact parental wild-type strains and any possible modifications is an unrealistic task. Nevertheless, early discoveries and advances in vitamin B_{12} aerobic production can provide useful guidance in the search for a suitable wild type producer.

Based on this early bibliography about the subject, it seems that the first described natural over-producing wild type strain is known as *Pseudomonas denitrificans* MB-580, as shown in the 1962 patent US3018225A [216]. This strain, initially owned by the French company Rhône-Poulenc (now Sanofi, the only company that continues to

produce some amount of vitamin B_{12} in Europe), was further studied and modified by research groups linked to Rhone-Poulenc.

MB-5800 genes involved in Cbl synthesis were cloned, analyzed [218] and, eventually, overexpressed to create genetically engineered Cbl overproducing strains like SBL27 and SC510 [165].

Despite many of these early articles alluding to strain MB-580 or its derived SC-510, at a certain point, the exact name of the strains used in the aerobic production of vitamin B_{12} are no longer specified and described only as "industrial producing". This ambiguity is intentional and a well-known fact in the bibliography studying vitamin B_{12} production trough fermentation. For example, in Industrial Biotechnology of Vitamins, Biopigments, and Antioxidants by Vandamme and Revuelta [100] the following extract alluding to the difficulty in knowing the exact nature of the aerobic producing strains can be read:

"*The species ambiguity is known, as explained in the following statement: 'although the taxonomic validity of the species P. denitrificans is questionable, we retain this taxonomic definition for strains derived from MB580' (Cameron et al.,1989)* [217]. *Recent papers on B_{12} production by P. denitrificans use 'industrial production 'strain without any further references. The exact species of the production strains is not clear, nor their relation to each other and to other strains assigned to the former species P. denitrificans*."[100]

There are several articles published in the last ten years by Chinese research groups focused on the large-scale (120 000 L tanks) production of vitamin B_{12} and the optimization of several parameters (addition of a betaine feeding, control of dissolved oxygen levels, concentration of Zn and Co salts in the medium...) that describe culture media, bioprocess feeding, DO control, and the effect of these variants on bioprocess yield [104–106,112]. The final Cbl yields described in these publications are around 200 mg/L. However, these articles do not specify the strain they use but refer to it as "an industrial use strain". They may be derived strains of SC-510 or SC-510 itself but without more information confirming the exact strain used. Nonetheless, considering the very high productions described it seems safe to assume that all the strains described in these papers and currently used at industrial setting (both by Chinese

producers like the Huarong Company or European like Sanofi) had been genetically engineered by some of the methodologies described in the bibliography[84].

Nevertheless, even the MB-580 is still privately owned and not available in publics cell banks, in some of the bibliography describing the early genetic improvements of MB-580 there is information that may be useful to identify other potential producing strains. For example, in [85] a 5.4 kba DNa fragment of *Pseudomonas denitrificans* SC-510 containing five cob genes (coba, cobb, cobc, cobd and cobe) is identified and described.

The strain known as *Ensifer adhaerens* CSBa is described in Williams et al 2017 [218] as a gram-positive bacterial firstly isolated in 1980 and, at first sight, it does not seem to have many similarities with the usually described *Pseudomonas denitrificans*. However, in the same publication the whole genome of the strain is described and a very similar DNA fragment to the 5.4 kbs sequence described in [85] can be found. *Ensifer adhaerens* CSBa also known as *Sinorhizobium morelense* have been confirmed as a Cbl producer and up to 22 cob genes very similar to the ones described for SC-510 have been found in its genome [219]. The strain is publicly available in the NBRC cell bank with the reference NBRC 108628: *Ensifer adhaerens* Casida 1982 so it was acquired to study its growth and Cbl production.

Furthermore, in order to not only rely on a sole producer, other candidate strains were also studied. In this sense, *Pseudomonas nitroreducens* ATCC 13687 (also available in other cell banks like the DSMZ under the reference DSM 1650) was also considered, although it has been primarily studied for its ability to reduce nitrite, rather than for cobalamin production. But, its genome has been fully described [220] and contains all the genes involved in adenosylcobalamin biosynthesis. Additionally, there is a wealth of information available about the strain and its phylogenetic relationship to other closely related strains [221]. The ability of ATCC 13687 has been described in several papers [220,221] and patents [221]. For this reason, it was decided to also acquire this strain (from now on DSM 1650).

5.1.1 Initial growth characterization of NBRC 108628 and DSM 1650 in Erlenmeyer shake-flask.

Once the candidate strains were selected, initial characterization cultures were carried out. The methodology of this experiments was based on the experiments described by research groups that have been conducting bioprocess improvements in aerobic producing strains at an industrial level (120,000 L fermenters) for several years such as [215,223–226].

The media used during the experiments was based on the fermentation media described by some of this articles, specifically the one appearing in [225] with the following composition: sucrose, 80 (g/L); CSL (g/L), 30; betaine, 15 (g/L); $(NH_4)_2SO_4$, 2 (g/L); $MgSO_4$, 1.5 (g/L); KH_2PO_4,0.75 (g/L); $ZnSO_4 \cdot 7H_2O$, 0.08 (g/L); $CoCl_2 \cdot 6H_2O$, 0.14 (g/L); DMBI, 0.075 (g/L) and a pH value of around 7.20–7.40.

This media incorporates some amount of betaine as it has been described that it has a positive effect on vitamin B_{12} production, acting as a methyl group donor for the formation of methionine that is later involved in Cbl and other porphyrinic compounds synthesis [224]. However, betaine has also been described as an inhibitor for the cell growth, so its concentration has to be carefully regulated. In fact, in [224] is described that it was best to incorporate the betaine in the bioreactor feeding in order to ensure that it was not present at inhibitory concentration. In our experimental setting, we decided to incorporate 15 g/L of betaine directly into the initial culture media as this is described in the bibliography and our intention was to assess the initial growth and production of DSM 1650 and NBRC 108628. In the eventual scale-up of the process, the strategy of betaine addition could be reassessed and adapted.

The initial cultures using the media directly adapted from [225] are shown in Figure 45. Several issues raised during these experiments. First and foremost, there was a rather large lag phases in cultures with both candidate strains, and even though DSM 1650 seemed to reach a final OD_{600} value of around 6 after 24 culture h, NBRC 108628 did not show almost any growth in the same time frame. Furthermore, this OD_{600} values could not be considered accurate as a significant amount of precipitation was

formed in the media broth over time when some of the salt solutions described in the media composition were added.

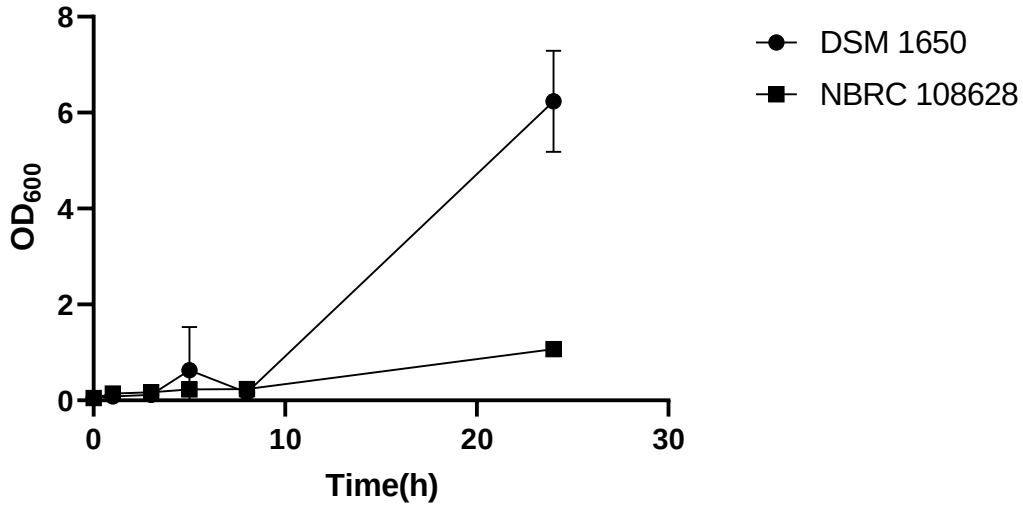

Figure 45 Initial cultures of DSM 1650 and NBRC 108628 in Erlenmeyer shake flask with media adapted from [225]**.** *15 g/L of betaine and 0.08 g/L $ZnSO_4$ and 0.14 g/L $CoCl_2$. The error bars represent the standard deviation of three replicates*.

$ZnSO_4 \cdot 7H_2O$ and $CoCl_2 \cdot 6H_2O$ influence in Cbl production have been studied in [226] and deemed as very important for optimization of vitamin B_{12} production. This is most likely the reason of why they appearance in such high relative quantities in later publications. Other possible explanation is that this media is intended to be used in high producing modified strains that reach over 200 mg/L of production and in which the high amount of Co may be needed for Cbl synthesis. However, when the stock solution containing these two compounds was added to the media as described in the materials and methods chapter, a significant amount of white precipitation began to form over time. Besides, as shown in Figure 45, there is a very significant lag phase. For this reason, it was decided to further adapt the media described in [225], considerably reducing the total amount of both salts.

This way, new cultures with a reduced amount of $ZnSO_4 \cdot 7H_2O$ and $CoCl_2 \cdot 6H_2O$ were carried out in Erlenmeyer shake flask. The final concentration of both compounds

was 0.02 g/L and no precipitation could be appreciated in the preparation of the culture media, even after letting the media rest for 4 hours at room temperature. The results of these cultures are shown in Figure 46.

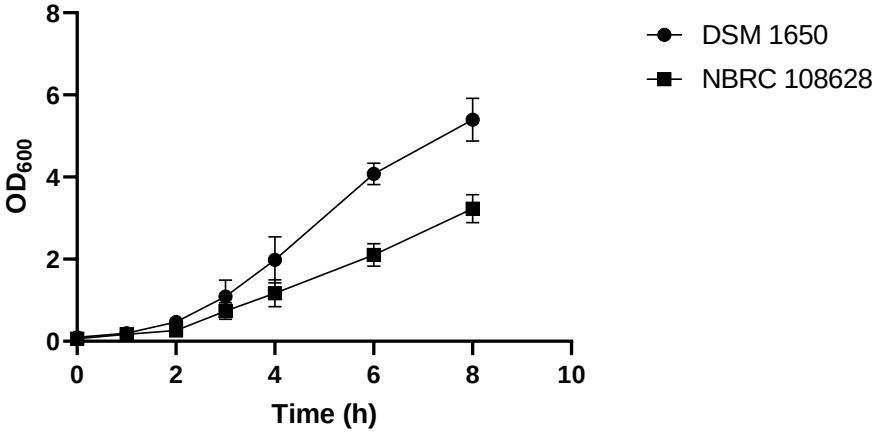

Figure 46 Cultures of DSM 1650 and NBRC 108628 in Erlenmeyer shake flask with media adapted from [225]**.** *15 g/L of betaine and 0.02 g/L $ZnSO_4$ and 0.02 g/L $CoCl_2$. The error bars represent the standard deviation of three replicates.*

In the reduced $ZnSO_4$ and $CoCl_2$ media, the cells showed a significant improvement in growth in the initial hours of the culture with almost no lag phase and μ_{nets} of 0.52 h^{-1} (DMS 1650) and 0.48 h^{-1} (NBRC 108628). NBRC 108628, however, seemed to have a slower growth during the initial 8 h, and it also reached lower final OD_{600} values at 24 h: 5.40 ± 0.52 (DSM 1650) vs 3.23 ± 0.34 (P = 0.0038)

Nevertheless, despite the presence of DMBI in the initial culture media, no CNCbl was detected in either of the strains after 24 hours.

Being aerobic organisms, the aeration limitations of an Erlenmeyer shake-flask experimental set up are surely affecting the cells capability of growing and reaching higher biomass levels. This may explain why no Cbl could be found during these cultures; it may be possible that there was not a sufficient amount of biomass present in the cultures to produce CNCbl in concentrations higher than the Limit of quantification (LoQ) of our analytical system (0.3 mg/L).

For this reason, it was decided to scale the process for both strains to a bioreactor setting, where aeration could be more easily followed and control as described in the materials and methods section of this chapter. The results of these experiments can be seen in Figure 47.

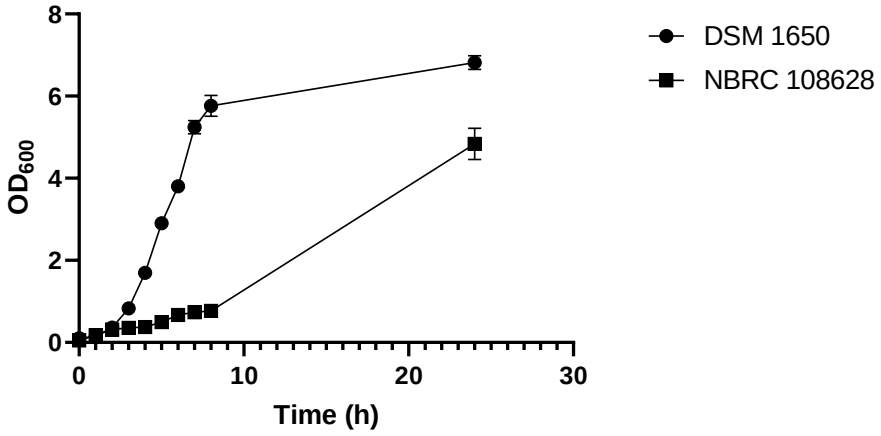

Figure 47 2L Bioreactor cultures of DSM 1650 and NBRC 108628 with media adapted from [225]**.** *15 g/L of betaine and 0.02 g/L $ZnSO_4$ and 0.02 g/L $CoCl_2$. The error bars represent the standard deviation of three replicates.*

First, in this new bioreactor setting, NBRC 108628 showed a significant lag phase during the first 8 h of the culture and, although some growth was obtained after the first 24 h (OD_{600} values of 4.84 ± 0.38), much lower than anticipated and very similar to the ones obtained in Erlenmeyer shake flask. On the other hand, although DSM 1650 showed no lag phase and rather positive initial growth rate during the initial h (μ_{net}= 0.55 h^{-1}), the cells stopped growing after the first 8 h and the OD_{600} value at 24 h were only slightly higher to those obtained in Erlenmeyer (6.82 ± 0.17). Unfortunately, like previous experiment, no CNCbl was found in either of the strains at 8 or 24 culture h.

These results seemed to indicate that there is a lack of some nutrient in the media that is hindering cell production. Considering both C and N source are added in excess and surely no depleted by the modest cell growth at 24 h, it is possible that the reduced amount of $ZnSO_4$ and $CoCl_2$ in the media cost the non Cbl production.

However, increasing the concentration of these two compounds to the values described in the bibliography was also not feasible due to the precipitation.

We suspected that the precipitation when adding $ZnSO_4$ and $CoCl_2$ was due to an interaction with the CSL. CSL has a rather large solid fraction and needs to be processed before and after autoclaving to avoid precipitation, as described in the materials and methods chapter. Due to the known variability between batches and manufacturing companies, it is possible that the composition of the CSL used by [225] was significantly different and did not cause the issues we were facing at high concentrations of $ZnSO_4$ and $CoCl_2$.

Considering the low growth showed by both strains on Figure 47 it was decided to use another complex nutrient-rich N source that allowed the addition of 0.08 g/L $ZnSO_4 \cdot 7H_2O$ and 0.14 g/L of $CoCl_2 \cdot 6H_2O$. As the main objective of these experiments was to assess the growth and production of aerobic wild type strains to compare them with NBRC 12391, and not to optimize growth or make the cultures economically viable at an industrial scale, it was decided to use a complex media as nutrient rich as possible. With that into account, the following media composition was used in the bioreactor cultures showed in Figure 48: glucose, 30 (g/L); Yeast Extract, 15 (g/L); tryptone, 15 (g/L); $MgSO_4$, 1.5 (g/L); $ZnSO_4 \cdot 7H_2O$, 0.08 (g/L); $CoCl_2 \cdot 6H_2O$, 0.14 (g/L); DMBI, 0.02 (g/L); betaine, 15 (g/L).

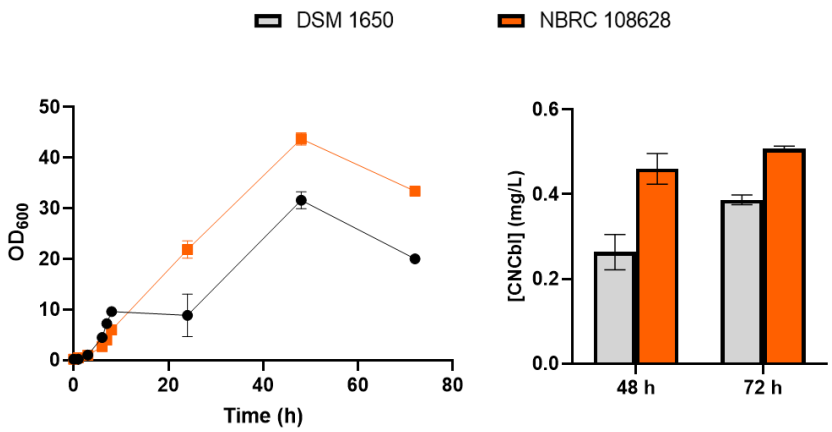

Figure 48 2-L bioreactor cultures of DSM 1650 and NBRC 108628 with a complex media with YE and Tryptone as N sources, 15 g/L betaine and 0.02 g/L $ZnSO_4$ and 0.02 g/L $CoCl_2$. *Left: OD600 values; Right: CNCbl volumetric production (mg/L) at 48 h and 72 h). The error bars represent the standard deviation of three replicates.*

In this later bioreactor cultures, the growth was significantly faster and much higher max OD_{600} values were reached. Furthermore, in both strains the cells continued to growth after the first 24h and the maximum growth was found at 48 hours.

As in Figure 48, DSM 1650 showed a faster growth rate during the initial culture hours and reached a higher OD_{600} value than in previous cultures. However, after the first 24 h, NBRC 108268 showed a much steadier growth and reached higher max values than DSM 1650: 43.73 ± 1.19 vs 31.6 ± 1.67 (P=0.0005). After 48 h, both strains showed a decrease in OD_{600} most likely caused by the depletion of some nutrient in the culture broth. Furthermore, unlikely previous cultures with aerobic strains, CNCbl was identified in the extracted pellets and quantify at both 48 h and 72 h (Figure 48, right).

Both strains presented similar amounts of CNCbl at 72 h, being the volumetric production of NBRC 108628 slightly higher than DSM 1650: 0.51 ± 0.01 vs 0.39 ± 0.01 mg/L (P<0.0001. Both volumetric productions are, however, lower than those obtained with NBRC 12391 at similar OD_{600} values in a bioreactor (between 1.5 and 2 mg/L).

It is very likely that this growth and production values could be further improved by a more suited aeration and feeding strategy. However, taking into consideration that both DSM 1650 and NBRC 108268 are wild type non overproducing strains and the volumetric production obtained at OD_{600} values of 30 to 40, it seems very unlikely that they would be able to reach the volumetric productions of around 200 mg/L described for the genetically modified aerobic strains.

It was deemed that the data obtained during this initial characterization of wild-type aerobic strains producing vitamin B_{12} was sufficient to allow for comparison with NBRC 12391. Based on the findings in this section, the production of NBRC 12391 is comparable to that of unmodified aerobic strains, while considering that further optimization of the process would still be necessary to achieve higher yields with the aerobic strains.

5.2. Developing a chemically defined media for NBRC 12391

Until this moment all cultures have been performed with a complex nitrogen source, mainly CSL, a by-product of the corn milling industry. Using a complex media presents several advantages, one of the main ones being the fact that complex nitrogen sources also have a large amount of other needed nutrients for cell growth, like vitamins and nucleic acids. Besides, at an industrial setting, employing complex and cheaper sources like CSL translates into a more economical bioprocess that can also synergizes with other producing industries and reduce the overall waste of resources.

However, a chemical defined media (CDM), in which all the compounds and its concentrations are precisely known, allows to a better understanding of the culture metabolic needs as well as providing a wider toolbox for improving the bioprocess by culture media optimization [212,227]. Besides, to the best of our knowledge, another reason that make the development of a chemical defined media for NBRC 12391 interesting is the fact that no such media has been defined for vitamin B_{12} production with *Propionibacterium freudenreichii*.

Considering than *Propionibacterium freudenreichii* seems to share a similar ecological niche with related strains like some lactic acid bacteria (LAB), it was decided to base

the initial composition of the proposed CDM in some of the medias used for LAB cultures.

Besides, developing a CDM aims to substitute CSL with a known composition of amino acids, nucleic acids, vitamins, and other micronutrients (salts, trace elements, etc.), so the nature of the compounds present in CSL should also be considered when developing a new CDM. The exact composition of the CSL used during the cultures performed in this thesis is unknown, but a qualitative approximation can be made based on publications that analyze the exact composition of different CSL [228]. Furthermore, the composition of SOLULYS® 095 (Roquette, France), a brand name for a CSL specifically intended to be used in bioprocesses and with known composition and concentrations was also assessed during this process.

Table 6 provides the exact composition of three CDM developed for PAB [229–231] alongside a qualitative composition of the most likely composition of CSL based on the bibliography findings.

Table 6 Composition of three CDM for LAB described in Zhang et al 2009, Wegkamp et al 2010 and Terrade et al 2009. *A qualitative composition of CSL based on bibliography and the composition of brand CSL Solulys 095 is also provided. The different nutrients are classified in the following groups. Sugar (Or carbon source in the case there is no sugar in the composition like in CSL), Amino acids, phosphate-based buffer salts, important vitamins, mineral salts, other minor vitamins, fatty acids, nucleic acids and trace minerals.*

		Concentration (g/L)			
	Compound	**Zhang et al.**	**Wegkamp et al.**	**Terrade et al.**	**CSL**
Sugar	Glucose	15	11	(Ribose) 100	Lactic acid
Amino acids	L-Histidine	0.17	0.15	0.5	Yes
	L-Isoleucine	0.24	0.21	0.2	Yes
	L-Leucine	1	0.475	0.2	Yes
	L-Methionine	0.06	0.125	0.15	Yes
	L-Valine	0.7	0.325	0.2	Yes
	L-Arginine	0.72	0.125	0.75	Yes
	L-Glutamic Acid	0.72	0.5	0.5	Yes
	L-Phenylalanine	0.48	0.275	0.2	Yes
	L-Proline	0.84	0.675	0.5	Yes
	L-Aspartic acid	0.06	0.42	0.35	Yes
	L-Serine	0.6	0.34	0.4	Yes
	L-Threonine	0.5	0.225	0.35	Yes
	L-Cysteine	0.24	0.13	0.5	Yes
	L-Alanine	0.48	0.24	0.2	Yes
	L-Glycine	0.36	0.175	0.5	Yes
	L-Lysine	0.6	0.44	0.25	Yes
	L-Tyrosine	0.36	0.25	0.2	Yes
Phosphate buffers	KH_2PO4/K_2HPO_4	3.6/7.3	No	1	YEs
Important vitamin group	Biotin	0.006	0.0025	0.002	Yes
	Calcium pantothenate	0.0012	0.001	0.002	No (Pantothenic ac.)
	Pyridoxal HCL	0.0048	0.005	(Pyridoxine) 0.002	(Pyridoxine)
	Choline	No	No	0.002	Yes
	Riboflavin	0.0009	0.001	0.002	Yes
Important mineral group	$MgSO_4{\cdot}7H_2O$	1	(MgCl2) 0.2	0.002	Yes
	$FeSO_4{\cdot}7H_2O$	0.004	(FeCl2) 0.005	0.002	Yes
	$ZnSO_4{\cdot}7H_2O$	0.005	0.005	0.1	Yes
Other vitamins group	p-Aminobenzoic acid	0.000056	0.001	0.0001	Yes
	Thiamine HCl	0.00056	0.001	0.001	Yes
Fatty acid group	Nicotinic acid	No	0.001	0.002	Yes
	Potassium acetate	0.9	(Sodium acetate) 1	No	Yes
Nucleic acid base group	Adenine	0.011	0.01	0.01	Yes
	Guanine	0.0056	0.01	0.01	Yes
	Uracil	0.023	0.01	0.01	Yes
	Xanthine	0.0038	0.01	0.005	Yes
Trace mineral group	$(NH_4)Mo_7O_{24}{\cdot}H_20$	0.00019	0.0025	No	Yes
	$MnSO_4{\cdot}4H_2O$	0.00038	(MnCl2) 0.016	0.1	(MnCl2)
	$CaCl{\cdot}2H_2O$	0.04	0.05	0.44	Yes
	$CoCl_2{\cdot}6H_2O$	0.00019	(CoSO4) 0.0025	No	(CoSO4)
	$CuSO_4{\cdot}5H_2O$	0.00019	0.0025	1.50E-05	Yes

Some of the bibliography research for the design of the CDM shown in table 7 (from now on, CDM-1) was performed by the master student Carlos Jiménez during his master's thesis project. Besides, the development of the methodology used for the media formulation described in the materials and methods chapter was also performed by Carlos Jiménez.

Table 7 Composition and concentrations of proposed rich Chemical Defined Medium (CDM).

	Compound	CDM Proposal [g/L]
Sugar	Glucose	50
Amino acids	L- Histidine	0.27
	L-Isoleucine	0.22
	L-Leucine	0.56
	L-Methionine	0.11
	L-Valine	0.41
	L-Arginine	0.53
	L-Glutamic acid	0.57
	L-Phenylalanine	0.32
	L-Proline	0.67
	L-Aspartic acid	0.28
	L-Serine	0.45
	L-Threonine	0.36
	L-Cysteine	0.29
	L-Alanine	0.31
	L-Glycine	0.35
	L-Lysine	0.43
	L-Tyrosine	0.27
Phosphate buffers	KH_2PO_4 K_2HPO_4	3.6
Important vitamin group	Biotin	0.004
	Calcium pantothenate	0.005
	Pyridoxal HCl	0.004
	Choline	0.002
	Riboflavin	0.001
Important mineral group	$MgSO_4 \cdot 7H_2O$	0.5
	$FeSO_4 \cdot 7H_2O$	0.01
	$ZnSO_4 \cdot 7H_2O$	0.01
Other vitamins	p-Aminobenzoic acid	0.001
	Thiamine HCI	0.0009
Fatty acid group	Nicotinic acid	0.002
	Potassium acetate	1
Nucleic acid base group	Adenine	0.010
	Guanine	0.009
	Uracil	0.014
	Xanthine	0.006
Trace mineral group	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.001
	$MnSO_4 \cdot 4H_2O$	0.005

After some initial characterization of cell growth in Falcon tubes, it was shown that growth in CDM-1 was very slow, with lag phases that could take several days. Besides, media preparation was rather troublesome, especially the addition of the different individual amino acids that had to be kept in individual stock solutions and could easily precipitate when added to the media.

For these reasons, the first step in optimizing the proposed CDM was trying to reduce the final number of amino acids used in the composition. Initial characterization showed that a media containing only seven of the initial amino acids, mainly Asp, Glu, Phe, Tyr, His, Val and Leu, rendered very similar results in terms of cell growth compared to the initial CDM.

These finds were further assessed in Erlenmeyer shake flask cultures as shown in Figure 49. Here, the growth profiles in the CDM described in Table 7 were compared to those obtained in a CDM with only Asp, Glu, Phe, Tyr, His, Val and Leu. Besides, with the objective of simplifying further the CDM and increase its viability in a potential scale up, two other conditions were also tested. In this sense, the four conditions studied in Figure 49 are as follows:

- Defined Media 1: CDM as described in Table 7.
- Defined Media 2. CDM with only Asp, Glu, Phe, Tyr, His, Val and Leu as amino acids.
- Defined Media 3: CDM with only Glu, Phe, Tyr, His, Val and Leu as amino acids. Asp was removed compared to Defined Media 2.
- Defined Media 4: CDM with only Glu, Phe, Tyr, Val and Leu as amino acids. Asp and His were removed compared to Definded Media 2.

In all conditions 100 µM DMBI was added to the media at 200 culture hours to induce Cbl production. The original experimental design was to add DMBI at 96 h like in previous experiments but at that point no significant growth was detected in any of the conditions tested due to a very long initial lag phase.

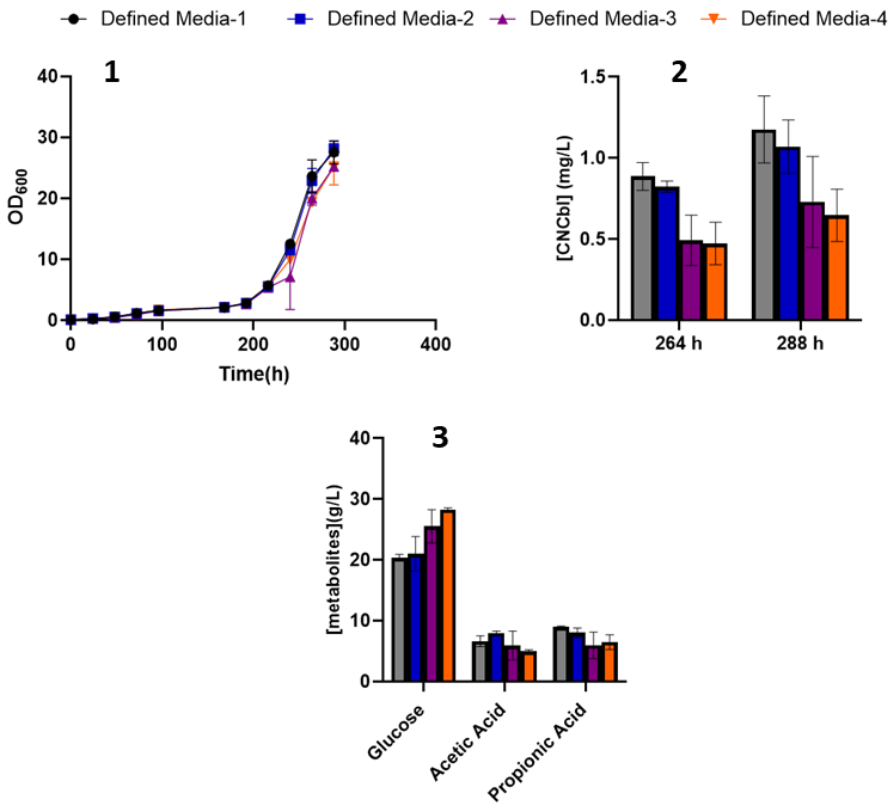

Figure 49 NBRC 12391 Erlenmeyer Shake Flask Cultures with 4 different CDM compositions (1,2,3 and 4). *1: OD_{600} values; 2: CNCbl volumetric production (mg/L) at 264 h and 288 h; 3: Glucose (g/L), acetic acid (g/L) and propionic acid(g/L) concentrations at 288 h. The error bars represent the standard deviation of three replicates.*

As in the initial experiments carried out by Carlos Jiménez in Erlenmeyer Shake Flasks, the growth of all the different conditions was very slow, with an initial lag phase of over 8 days or more than 200 h. After the initial 200 h, all cultures began to show a steady increased in growth rate until maximum OD_{600} values of around 25 to 30.

NBRC 12391 seems to need a very long adaptation phase to the CDM media even with the inoculum culture also cultured in the same media, as described in the materials and methods chapter. This translates to a significant increase in cultures times that make the cultures with the proposed CDM not viable at an industrial setting.

However, it should also be noted that some amount of CNCbl was quantified at 266 hours and 288 hours in the four conditions tests as shown in Figure 49. Results seems to indicate a significant decreased in CNCbl volumetric production when not His or neither His nor Asp were added to the media, although we obtained a rather large variability between replicate cultures. Nonetheless, max CNCbl production of 1.0 ± 0.2 mg/L was obtained at 288 h in Defined Media 1 cultures, significantly lower than in the CSL based media used in previous studies. Interestingly, in all conditions there were very low propionic acid productions considering the final OD values obtained.

One possible explanation of this slow growth could be that the media lacks some of micro element or does not have enough concentration of one or more essential nutrients. This could be hindering cell growth and increasing the time it takes NBRC 12391 to adapt to the CDM.

With the intention of reducing this long lag phase, another experiment was carried out in Erlenmeyer shake flask. In these cultures, three different small concentrations of CSL were added to the media to try to dilucidated if some nutrient present in the CSL was missing in our chemical defined media composition and it was needed in trace quantities for a proper growth. More specifically, three different concentrations of CSL were used, all of them much smaller than the 40 g/L used in the production fermentation media used in the formulation of the complex media used un previous chapters:

- 1 g/L CSL
- 0.5 g/L CSL
- 0.2 g/L CSL

Besides cultures with different CSL concentrations, another condition where DMBI was added at a concentration of 100 µM from time 0 was also tested. The objective behind this last condition was to test whether or not the media had the proper composition to allow the production of trace quantities of vitamin B_{12} during the early stages of the culture as Cbl is needed as a coenzyme for the Wood-Werkman cycle [15,232]. Taking into consideration that most industrial cultures have the need to add this base as a supplement for high volumetric productions, we wanted to ensure that

our CDM allowed to produce active forms of vitamin B_{12} in enough quantities to guarantee the fulfillment of the microorganism primary fermentation pathway. In the case growth was improved by early DMBI addition, this could mean that some key component for DMBI synthesis (and therefore, active Cbl form synthesis) is lacking from our CDM.

In order to induce Cbl production and to replicate as accurately as possible the conditions of the cultures performed in complex media, 100 µM DMBI was added in all conditions at 96 culture h. The results for these experiments are shown in Figure 50.

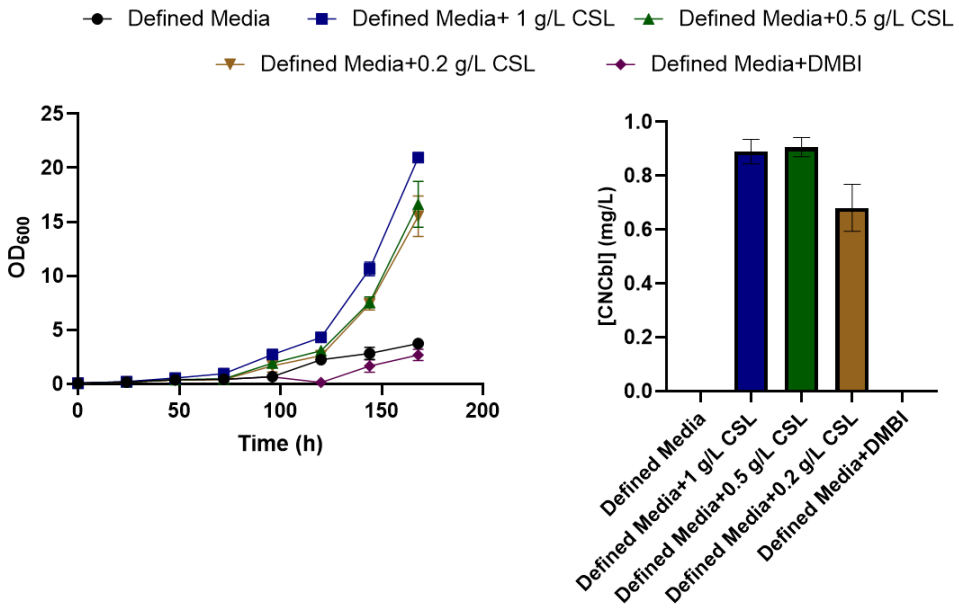

Figure 50 NBRC 12391 shake flask cultures with CDM and addition of different small concentrations of CSL or DMBI. *Left OD_{600} values of the culture over time. Right: CNCbl volumetric production at 168 h of all the different conditions. The error bars represent the standard deviation of three replicates*.

As the main objective of the experiment was to limit the lag phase and, therefore, the total culture time, it was decided to stop the culture at the final point employed in cultures made with complex media: 168 h.

As seen in Figure 50, all conditions tested still showed a significant lag phase. However, from the 100-h culture point, all conditions were CSL was added to the media began

to grow faster and entered to an exponential growth phase that allowed maximum OD_{600} values of 20.95 ± 0.05 (CDM+1 g/L CSL), 16.63 ± 2.13 (CDM+0.5 g/L CSL) and 15.5 ± 1.5 (CDM+0.2 g/L CSL). These values are still lower than those obtained from complex media cultures at the same culture times (from 30 to 40) but significantly greater than the CDM without CSL addition and the CDM with DMBI addition (3.77 ± 0.25 and 2.71 ± 0.43 respectively). Regarding the last condition, DMBI addition at time 0 seemed to have a slight negative effect on cell growth when compared to the control culture.

Besides greater and faster growths, the three conditions where CSL was added were the only ones to show detectable amounts of CNCbl at 168 h: 0.89 ± 0.05 mg/L (CDM + 1g/L CSL), 0.91 ± 0.04 mg/L (CDM+0.5 g/L CSL) and 0.68 ± 0.09 mg/L (0.2 g/L CSL). CNCbl volumetric productions of these three conditions are fairly similar although it seems to be a slight decrease in production when the amount of CSL in the media is lower than 0.5 g/L.

These results seem to suggest several conclusions:

- The proposed CDM is missing one of more critical nutrients that are present in CSL and needed in trace amounts for cell growth and CNCbl production.
- Even when CSL is added to the media, NBRC 12391 still needs a rather large adaptation time to the less complex media, which translates in very significant lag phases of over 100 h.
- Besides, even after some growth and production is achieved at 168 h when CSL is added in small concentrations to the media, their values are still significantly lower when compared to those obtained in media with 40 g/L CSL.

Considering that the main components described in the bibliography for both proposed CDM for LAB cultures and typical CSL compositions, finding the missing micronutrients was considered a significant resource commitment and out of the scope of this project.

Besides, although some amount of growth and production where finally achieved with a mostly defined media, there are still some unknown components in the form of trace

amounts of CSL. Finally, the initial lag phases made the adaption of these culture media to the continuous bioreactor culture described in Chapter 4 not feasible.

For this reason, it was decided to not continue to pursue the optimization of a CDM for *Propionibacterium freudenreichii* NBRC 12391 and focus the effort in developing other process improvement strategies.

5.3. Co-culture with propionic consuming strains.

At this point of the project, the main strategies for increasing CNCbl production have been the reduction of the inhibitory effect of propionic acid trough modifications at a bioprocess level. Of those, the strategy that showed the better results in terms of volumetric productivity was a continuous bioreactor process where the cells were kept at OD_{600} where propionic acid concentration were not inhibiting growth and production.

With the intention of continuing the improvement of this process, other strategies to further limit the hindering effect of propionic acid concentrations were also studied. Of those, one strategy that has been described in the bibliography but not yet tested in this project is the co-culture with bacterial strains capable of assimilating propionic acid as a carbon source.

Miyano et al, 2000 [123] used a microorganism called *Ralstonia eutropha* capable of consuming propionic acid and increased CNCbl volumetric production from 6.73 µg/mL to 19 µg/mL when co-cultured with *Propionibacterium freudenreichii* IFO 12424. The same strain of *Ralstonia eutropha*, now known as *Cupriavidus necator* (ATCC 17699/DSM 468) was acquired and tested in co-culture with NBRC 12391.

As described in Materials and methods DSM 468 pre-inoculum was first cultured in the media recommended by the DSMZ bank. After, the inoculum was used to seed a CSL media for 24 h until an OD_{600} of 5-6 was obtained. 20 mL of this culture were added to a 200 mL flask filled with 180 mL of CSL media in which NBRC 12391 was being cultured for 72h. As usual, 100 µM DMBI was added at 96 culture h to induce CNCbl production.

The results of this co-culture experiment are shown in Figure 51.

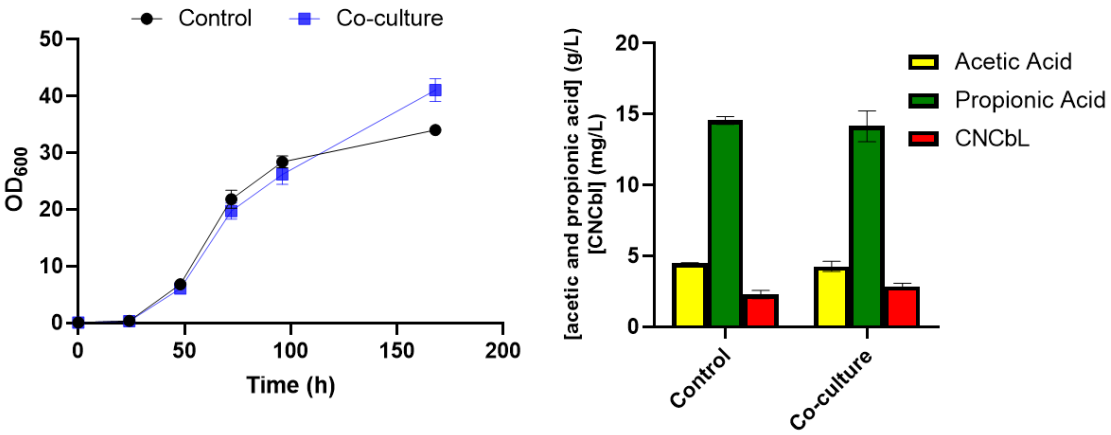

Figure 51 NBRC 12391 cultures in Erlenmeyers Shake Flasks. In the co-culture condition, 20 mL of a culture of DSM 468 with OD600 values of around 5 to 6 were added to the broth at 72 culture h. *Left: OD600 values; Right: Acetic acid (g/L), propionic acid (g/L) and CNCbl (mg/L) values at 168 h of both conditions. The error bars represent the standard deviation of three replicates*.

As seen in Figure 51, it seems to be a significant increase in OD_{600} at 168 h when comparing the control condition with the co-culture with DSM 468: 34.0 ± 0.9 vs 41 ± 2 respectively (P = 0.0204). However, even with the increased OD_{600} of the co-culture, the final concentration of propionic acid at 168 h was not significantly different in both conditions: 14.5 ± 0.3 g/L in the control vs 14.1 ± 1.1 g/L in the co-culture (P = 0.65). So, the apparent positive effect on growth did not correlate with less propionic acid accumulation. Nevertheless, a slightly increase in terms of CNCbl was observed in co-culture, 2.3 ± 0.3 mg/L (Control) vs 2.8 ± 0.2 mg/L (Co-culture) (P = 0.28), but it was not conclusive enough.

These results were not sufficient to assess the viability of the co-culture for propionic acid reduction, especially considering that the difference in values could be caused by culture-to-culture variability and no by a real effect of the co-culture. In order to better assess the effect of the co-culture with DSM 468, a new set of Erlenmeyer shake flask cultures were carried out. In this case, the behavior of the cultures was more tightly studied during the 96 h to 168 h time range in order to better estimate the effect of the co-culture in both propionic acid and CNCbl production.

Besides *Cupriavidus necator* DSM 468, it was decided to test other potential propionic consuming strains to see if they were more suitable for co-cultures with NBRC 12391. *Azotobacter vinelandii* DSM 366 was chosen because it was easily available in the lab and has been described as capable of metabolizing acetic acid and other organic acids as primary carbon sources [233,234].

The co-cultures were prepared following the same methodology previously described and, at 72 culture h, 20 mL of an already grown culture of either DSM 468 or DSM 366 were added to 180 mL of NBRC 12391 culture.

The results from these co-culture experiments are shown in Figure 52. As shown below, in these set of experiments the co-culture with DSM 468 did not show an increased growth or better CNCbl production. At 72 h and before the addition of the 20 mL of grown DSM 468, OD_{600} were already slightly inferior to the results of the control culture so this difference can be explained by culture-to-culture variability. However, the maximum volumetric production of CNCbl found at 144 h were 3.8 mg/L ± 0.1 (control), 1.6 ± 0.5 mg/L (co-culture with DSM 468) and 2.3 ± 0.3 mg/L (co-culture with DSM 366). At 168 h, CNCbl values did not change significantly compared to 144 h. Therefore, any of the co-cultures increased Cbl production.

Furthermore, propionic acid concentrations between all different conditions remained similar, with final values at 168 h of 18.2 ± 0.9 g/L (control), 17.7 ± 0.1 g/L (Co-culture with DSM 468) and 17.2 ± 0.8 g/L (Co-culture with DSM 366).

Taking into account these results, it was decided to not continue pursuing the co-culture of NBRC 12391.

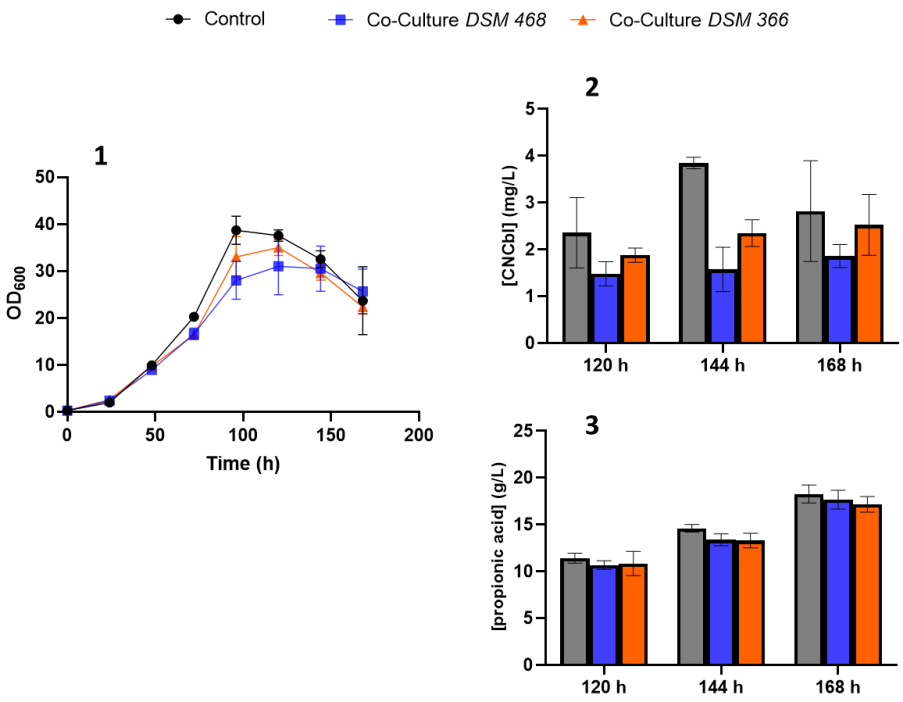

Figure 52 NBRC 12391 cultures in Erlenmeyers Shake Flasks. 20 mL of cultures of either DSM 468 or DSM 266 with OD600 values of around 5 to 6 were added to the broth at 48 culture h. *1: OD_{600} values; 2: CNCbl volumetric production (mg/L) values at 120h, 144h and 168 h for all conditions; 3: Propionic acid concentration (g/L) values at 120 h, 144 h and 168 h for all conditions. The error bars represent the standard deviation of three replicates*

5.4. YE and CSL combination media as a starting point for further bioprocess improvement

During Chapter 3 of this thesis project, both YE and CSL were tested as complex nitrogen sources for NBRC 12391. Despite an increased growth with YE as nitrogen source, CNCbl production was significantly higher in the cultures performed in CSL. For this reason, and despite the increased complexity of working with CSL instead of YE at a lab setting, it was decided to continue using CSL as the only complex nitrogen source.

Besides the increased production, other consideration when choosing CSL was that this is the nitrogen source more widely described in the bibliography for vitamin B_{12} production with *Propionibacterium freudenreichii*, including Kojima et al 1985 [93] where NBRC 12391 is used as one of the parental strains for the development of propionic resistant over-producing strains.

Nonetheless, during the final steps of this thesis project, an optimized media combining both YE and CSL was described by Liu et al [235]. Adding glycine to the media also showed a significant effect on CNCbl production. With this changes, Liu and colleagues achieved an increase of 120% with *Propionibacterium freudenreichii* CICC10019.

In order to test if the optimize production of Liu and colleagues could be replicate in NBRC 12391, a new set of Erlenmeyer shake flask cultures were carried out to compare growth and production between cells culture in the standard CSL based media vs cells cultures in the media described by Liu et al.

The composition of this media is described in the materials and methods chapter, and the results for these experiments are shown in Figure 53. As usual, DMBI was added at 96 h to induced CNCbl production.

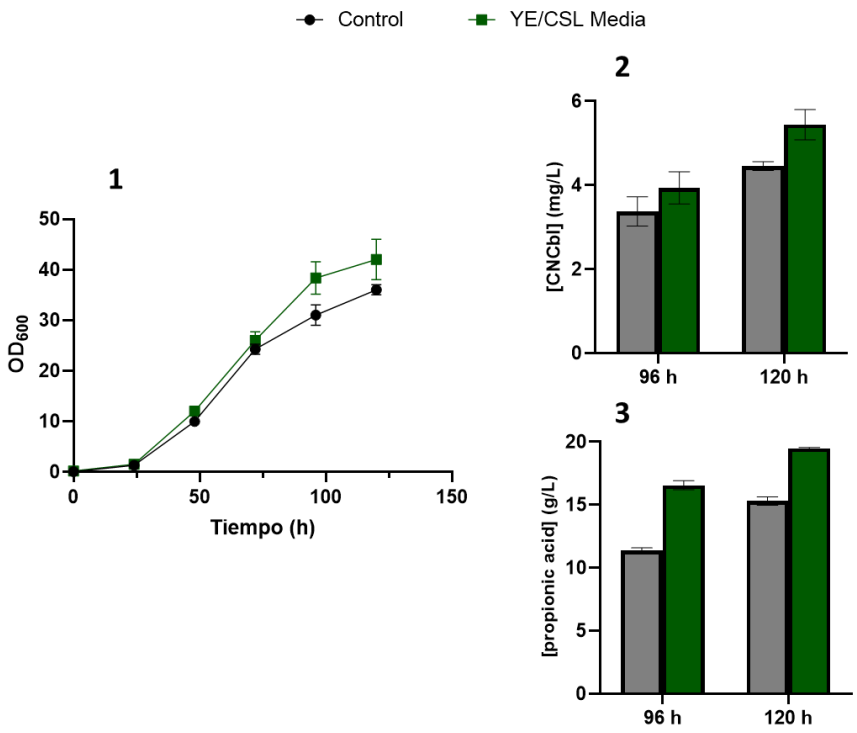

Figure 53 NBRC 12391 Erlenmeyer shake flask cultures with CSL media, and the media described in Liu et al 2021[235]. *1: OD_{600} values; 2: CNCbl volumetric production (mg/L) values at 96h and 120 h for all conditions; 3: Propionic acid concentration (g/L) values at 96h and 120 h for all conditions. The error bars represent the standard deviation of three replicates.*

As shown in Figure 53, there seems to be a slight increase in both growth and production at 96 h and 120 h when the YE/CSL media is used: 4.5 ± 0.1 mg/L (CSL) vs 5.4 ± 0.4 mg/L (YE/CSL) (P = 0.0106). Furthermore, propionic acid concentration at this point were also significatively higher: 15.2 ± 0.3 g/L (YE/CSL) vs 10.5 ± 0.1 g/L (CSL) (P = 0.0019.

These results seem to indicate that combining YE/CSL and/or adding glycine to the media composition have a positive effect on the growth and Cbl production of NBRC 12391.

This strategy could not be investigated further due to the time constrain of the PhD project.

6. Future Steps and Conclusions.

6.1. Conclusions.

- After an extensive bibliography research and the study of two potential wild type candidates for Cbl production optimization, NBRC 12391 was selected for further characterization and optimization as it exhibited better performance in terms of culture stability and glucose consumption.
- As a result of the initial characterization of NBRC 12391 growth and production, a culture methodology and media composition were established. 50 g/L glucose and 40 g/L CSL were chosen as the initial C and N source respectively and a pH control strategy was also implemented.
- A HPLC-based analytical system capable of identifying and quantifying the main Cbl subtypes was developed and implemented following the optimization of CNCbl extraction conditions with DOE.
- Several possibilities were studied to try to determine the low CNCbl production values obtained in early characterization. Consequently, the inhibitory effect of propionic acid of both growth and CNCbl production was confirmed. Additionally, we observed that agitation during the early stages of the culture had an adverse effect on CNCbl production. Cultures agitated from the outset yielded volumetric and specific production values of 2.83 ± 0.06 mg/L and 0.33 ± 0.02 mg CNCbl/g, respectively, compared to 1.82 ± 0.02 mg/L and 0.21 ± 0.02 mg CNCbl/g in non-agitated conditions.

  These results seemed to suggest that the early agitation was causing a slight oxygenation of the culture and hindering production.

  With this taken into account, a new experimental set-up more suitable for CNCbl production where Erlenmeyer cultures were kept static during the first 96-h. For bioreactor cultures, limiting the oxygenation during the early stages was addressed by constantly pumping N_2 trough a 0.22 µm filter to displace the oxygen.
- DMBI had a significant positive effect in CNCbl production. Other precursors like RF or NAM, described to affect production in some strains, did not show any effect on NBRC 12391 cultures. Volumetric productions of 4.41 ± 2.17

mg/L were obtained when DMBI was added at 96 h. In the other hand, only 0.33 ± 0.07 mg/L were obtained in the control culture without DMBI addition. The time at which DMBI was added was also important, with a slightly negative effect on cell growth when added a time 0 and maximum CNCbl volumetric productions just 24 h after DMBI addition, at 72 or 96 h depending on the culture strategy.

- In a bioreactor environment, supplementing DMBI at the 72-h mark yielded superior results in CNCbl production compared to adding it at 96 h. This was primarily because, towards the end of the anaerobic phase, there was a depletion of the carbon source. Attempting a fed-batch process to overcome carbon source limitations led to inferior outcomes when compared to the batch method with DMBI incorporated at 72 hours. It appears that this strategy is not viable without a mechanism in place for the elimination or removal of propionic acid.
- By implementing a single-phase continuous bioprocess, we achieved a 5.7-fold increase in volumetric productivity compared to other scale-up strategies tested. Furthermore, this volumetric productivity of 0.078 mg/L/h surpasses previously reported values for wild-type strains without EBAB systems. The successful development of this single-phase continuous bioprocessing approach was made feasible thanks to the ability of NBRC 12391 to achieve maximum CNCbl volumetric production in less than 24 hours following the addition of DMBI in a fully anaerobic environment.
- Co-cultures of NBRC 12391 with propionic and other organic acids assimilating strains (*Cupriavidus necator* DSM 468 and *Azotobacter vinelandii* DSM 366) did not showed any positive effect in neither cell growth nor CNCbl production.
- A chemical defined media for NBRC 12391 was developed but deemed unsuited for Cbl production due to causing a long lag phase of over 100-h and very small CNCbl productions that required trace amounts of CSL added to the culture media.

6.2. Future steps for further improving Cbl production by bioprocess optimization.

In this thesis project, a methodology for culturing and producing vitamin B_{12} with the anaerobic *Propionibacterium freudenreichii* subs *shermanii* NBRC 12391 was successfully implemented in the laboratory. This included not only developing a suitable experimental set-up for anaerobic cultures but also a robust a reliable analytical method for Cbl extraction, identification and quantification.

Furthermore, after characterizing the growth and production and identifying one plausible cause for the initial low Cbl production (excessive aeration during the early stages of the culture) the process was scale-up with several strategies until a single-use continuous bioprocess was developed, allowing an increased in volumetric productivity of 5.7-fold when compared with other strategies.

However, due to time and resources constraints in the thesis project, further improvements over the proposed continuous strategy were not carried out. Overall, these results suppose a new viable strategy for the development of future and sustainable bioprocess to produce CNCbl with wild-type GRAS *Propionibacterium freudenreichii* strains without having to rely on more complicated setups like multiple-phase continuous bioprocesses or expanded bed adsorption bioreactors for propionic acid elimination.

In this sense, future projects should continue to research the single-use continuous bioprocess conditions to achieve further optimization in this regard.

In the current work, 25 g/L glucose and 20 g/L CSL were chosen as the starting points of C source concentration and N source concentration respectively for the continuous bioprocess. This was done to avoid reaching inhibiting propionic acid concentrations in the broth. However as shown in Figure 43, propionic acid values were lower than expected and faraway of the inhibiting 20 g/L threshold encounter in previous experiments. For this reason, a continuous bioprocess with higher concentrations of both glucose and CSL could reach higher stable OD_{600} and, presumably, render higher

CNCbl volumetric productions and therefore further increasing the volumetric productivity of the process.

Furthermore, despite the efforts described in this work to optimize the composition of the culture media by studying the C and N source nature and composition, the effect of adding several Cbl precursors at different times and other possible significant conditions, it is possible that the media used in most of this project could be further improved.

As shown in Figure 53, a combination of YE and CSL and the addition of glycine have a slight positive effect both in cell growth and Cbl production. Applying this optimized media composition and other future improvements to our single-use strategies could also allow higher Cbl production than the ones described in this work.

The continued development of a defined medium (CDM) for *Propionibacterium freudenreichii* has the potential to advance our knowledge of the microorganism's metabolic requirements. This, in turn, would facilitate the exploration and creation of novel bioprocess strategies.

Despite reaching a media formulation that allowed cell growth, the very long lag and adaptation phase and the fact that small amounts of CSL were still needed for Cbl production seem to indicate that there are still some key micronutrients missing in the proposed CDM. A more exhaustive analysis of CSL composition could allow us to identify the missing compounds and truly develop a viable CDM. This, in turn, could be apply to the single-use phase continuous strategy and render a more controlled bioprocess.

Finally, despite an initial growth characterization of two aerobic Cbl producing wt strains, further optimizing their growth and production was deemed out of the scope of this thesis project. However, a better understanding of the production ceiling of its wt aerobic counterparts would allow us to better propose an end-goal production objective for *Propionibacterium freudenreichii*.

At the start of this thesis project, we chose to optimize production of a wt *Propionibacterium* strains due to several qualities that were considered a market

advantage. We also wanted to focus on developing bioprocess level strategies for production optimization rather than cell modification.

However, the improvements described in this work and, specifically, the single-phase continuous bioprocess could also be applied to propionic resistant strains obtained through genetic engineering of random mutagenesis processes.

Despite their increased propionic acid resistant, this compound is still inhibiting their growth and production at sufficiently high levels so a simple and cheap strategy to reduce its concentration could render higher yields.

7. Materials and Methods.

7.1. Microorganisms and media

7.1.1. Anaerobic strains

Anaerobic cultures described in the different chapters were mainly perform with *Propionibacterium freudenreichii* NBRC 12391 acquired from Japan's National Biological Resource Center (NBRC) (the parental strain used by Kojima and co-workers in their Patent from 1985 [93] also named IFO 12391).

DSM Z4902 was acquired from the German Collection of Microorganisms and Cell Cultures GmbH was also used for the initial strains selection and characterization.

For the co-cultures with NBRC 12391 described in Chapter 5, two different strains were acquired from the DMSZ cell bank: *Cupriavidus necator* (ATCC 17699/DSM 468) and *Azotobacter vinelandii* DSM 366.

Cells were stored in pre-culture media supplemented with 25% glycerol at -80°C and in agar-solid media.

7.1.2. Aerobic Strains

Two different strains were used: *Pseudomonas nitroreducens* ATCC 13687/DSM 1650 and *Ensifer adhaerens* Casida 1982, NBRC 108628.

Both strains were stored at -80ºC and in agar plates in Media 1. For the storage at -80ºC, a final concentration of 25% glycerol was also added.

7.1.3. Media composition

All products used in the different preparations described were purchased from Sigma-Aldrich unless stated otherwise.

Media 1. Used as for storing the cells at -80ºC, agar plates, initial growth characterizations and pre-culture when more complex medias were used for the final culture. Its composition follows NBRC and DMSZ guidelines and it was: 5 g/L glucose, 5 g/L yeast extract, 5 g/L tryptone and 1 g/L $MgSO_4$.

For cultures in solid media, 15 g/L agar were added to Media 1. To provide an adequate anaerobic environment for cell growth, solid-media cultures were performed in a 2.5 L Oxoid Anaerojar (Thermo Fisher Scientific, Alcobendas, Spain).

Media 2: Adaption of the media described in Kojima et al 1985 [93] with YE as the sole complex nitrogen source. Its composition was: 50 g/L glucose, 40 g/L YE, 3 g/L NH_4NO_3, 1.5 g Na_2HPO_4, 0.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 mg/L $MnSO_4 \cdot 4H_2O$, 10 mg/L $FeSO_4 \cdot 7H_2O$, 10 mg/L $ZnSO_4 \cdot 7H_2O$, 0.05 mg/L $CuSO_4 \cdot 5H_2O$, 0.01 mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 15 mg/L $Co(NO_3) \cdot 6H_2O$ and 5 mg/L Calcium pantothenate.

Glucose and YE concentrated stocks (250 g/L and 200 g/L stock solutions respectively) were autoclaved separately from the rest of the media components. All salts except NH_4NO_3, Na_2HPO_4, and KH_2PO_4 were prepared in a separated solution and sterilized by filtration with a 0.22 μm filter (Teknokroma, Sant Cugat del Valles, Spain).

Media 3: Adaption of the media described in Kojima et al 1985 [93] with CSL as the sole complex nitrogen source. Its composition was: 50 g/L glucose, 40 g/L CSL, 3 g/L NH_4NO_3, 1.5 g Na_2HPO_4, 0.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 mg/L $MnSO_4 \cdot 4H_2O$, 10 mg/L $FeSO_4 \cdot 7H_2O$, 10 mg/L $ZnSO_4 \cdot 7H_2O$, 0.05 mg/L $CuSO_4 \cdot 5H_2O$, 0.01 mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 15 mg/L $Co(NO_3) \cdot 6H_2O$ and 5 mg/L Calcium pantothenate.

As before, glucose and YE concentrated stocks (250 g/L and 200 g/L stock solutions respectively) were autoclaved separately and all salts except NH_4NO_3, Na_2HPO_4, and KH_2PO_4 were prepared in a separated solution and sterilized by filtrated. To prevent precipitation of the CSL solid fraction, the pH of the CSL solution was adjusted to 8 before autoclaving and, after sterilization, centrifuged at 18.000 g's for 30 minutes to recover just the supernatant.

Media 4: described by [235] was also used for the experiments described in section 5.4 of chapter 5. Its composition is as follows: 54.3 g/L glucose, 30 g/L CSL, 17.6 g/L YE, 2.7 g/L KH_2PO_4, 3.5 g/L glycine, 0.005 g/L $CoCl_2 \cdot 6H_2O$, 2 g/L $(NH_4)_2SO$.

For the cultures carried out with aerobic strains described in Chapter 5, three different media were employed. Media 1 was used for agar plate solid cultures and strain storage at -80ºC.

Aerobic Media 1: described by [19]: sucrose, 80; corn steep liquor, 30; betaine, 15; (NH4)2SO4, 2; MgSO4, 1.5; KH2PO4, 0.75; ZnSO4 · 7H2O, 0.08; CoCl2 · 6H2O, 0.14; DMBI, 0.075 and a pH value of around 7.20–7.40.

Aerobic Media 2: sucrose, 80; corn steep liquor, 30; betaine, 15; (NH4)2SO4, 2; MgSO4, 1.5; KH2PO4, 0.75; ZnSO4 · 7H2O, 0.02; CoCl2 · 6H2O, 0.02; DMBI, 0.075 and a pH value of around 7.20–7.40.

Aerobic Media 3: Glucose 30 g/L, Yeast Extract 15 g/L, Tryptone 15 g/L. MgSO4 1.5 g/L, ZnSO4·7H2O 0.08 g/L, CoCl2·6H2O 0.14 g/L, DMBI 0.02 g/L, Betaine 15g/L.

Glucose, CSL, YE and Tryptone concentrated stock solutions (250 g/L, 200 g/L, 200 g/L and 100 g/L) were autoclaved separately from the rest of the media components. All salts except NH4NO3, Na2HPO4, and KH2PO4 were prepared in a separated solution and sterilized by filtration with a 0.22 µm filter (Teknokroma, Sant Cugat del Valles, Spain).

Chemical Defined Media preparation:

Several Chemical Defined Media (CDM) were also used in the section 5.2 of Chapter 5.

CDM-1: Composition described in Table 5.

CDM 2: as CDM-1 with only Asp, Glu, Phe, Tyr, His, Val and Leu as amino acids.

CDM-3: as CDM-1 CDM with only His, Glu, Phe, Tyr, Val and Leu as amino acids.

CDM-4: as CDM-1 CDM with only Glu, Phe, Tyr, Val and Leu as amino acids.

The CDM was prepared by dividing its components in several groups, that were sterilized by autoclaving or filtration and finally combined.

Glucose was prepared in a 200 g/L stock solution.

Amino acids were prepared individually in KOH 2.5 M as explained solution with the exception of Cysteine, that is known to form disulphide bonds at basic pH. For this reason, Cysteine was prepared at a pH value of 3.5. Stock solution and final culture concentrations are shown in Table 7.

Table 8 Stock and culture concentrations of amino acids for CDM

Amino acid	Stock Solution (200x) [g/L]	Final culture medium [g/L]
L-Histidine	55	0.275
L-Isoleucine	43	0.215
L-Leucine	112	0.56
L-Methionine	22	0.11
L-Valine	82	0.41
L-Arginine	106	0.53
L-Glutamic acid	115	0.575
L-Phenylalanine	64	0.32
L-Proline	134	0.67
L-Aspartic acid	55	0.275
L-Serine	89	0.445
L-Threonine	72	0.36
L-Cysteine	58	0.29
L-Alanine	61	0.305
L-Glycine	69	0.345
L-Lysine	86	0.43
L-Tyrosine	54	0.27

Vitamins were prepared in a 100x stock solution and sterilized by filtration with a 0.22µm filter. The stock and final concentrations are shown in Table 8. Biotin was solubilized in deionized water under heat (without boiling it) and stirred until totally dissolved. Then, the solution was cooled to room temperature and the rest of the vitamins were added.

Table 9 Stock and culture concentrations of vitamins for CDM

Vitamin	Stock concentration (g/L) [100x]	Final culture concentration (g/L)
d-Biotin	0.4	0.004
Thiamine*HCl	0.1	0.001
Calcium Pantothenate	0.5	0.005
Choline Chloride	0.2	0.002
Riboflavin	0.13	0.0013
Pyridoxine	0.4	0.004
p-Aminobenzoic acid	0.12	0.0012
Nicotinic acid	0.15	0.0015

Nitrogenous bases stock solutions were prepared individually in different solvents as described in Table 9 and sterilized by filtration with a 0.22µm filter.

Table 10 Stock and culture concentrations of nitrogenous bases for CDM

Base	Stock concentration (100x) [g/L]	Culture concentration (g/L)	Solvent
Adenine	1	0.01	HCl 1M
Guanine	1	0.01	HCl 5M
Uracil	1	0.01	NaOH 1M
Xhantine	1	0.01	NaOH 2M

All the different salts were separated in three different solutions as shown in Tables 10, 11 and 12 and sterilized by filtration with a 0.22µm filter.

Table 11 Salt solution 1 for CDM.

Component	Stock solution [g/L]	Culture medium [g/L]
NH4NO3	30	3
Na2HPO4·H2O	15	1.5
KH2PO4	4	0.4

Table 12 Salt solution 2 for CDM.

Component	Stock solution [g/L]	Culture medium [g/L]
MgSO4	50	0.5

Table 13 Salt solution 3 for CDM.

Component	Stock solution [mg/L]	Culture medium [g/L]
MnSO4 · 4H2O	500	5
FeSO4 · 7H2O	1000	10
ZnSO4 · 7H2O	1000	10
CuSO4 · 5H2O	5	0.05
(NH4)6Mo7O 24 · H2O	1	0.01
Co(NO3)2 · 6H2O	1500	15
Calcium pantothenate	500	5

Finally, 1 g/L of potassium acetate was dissolved in deionized water and autoclaved.

7.2. Falcon tube cultures

For the initial assessment of both strains, Falcon tube cultures were performed. The tubes were filled with three different volumes of Media 2: 25 mL, 35 mL and 45 mL and incubated for 96 hours at 30ºC with 150 rpm agitation in an INFORS HT incubator (Biogen, Madrid, Spain).

Samples were harvested at the final culture point. OD_{600} was measured and recorded, and the samples were centrifuged at 10.000 g's for 10 minutes and supernatants were separated, filtrated through 0.45 µm PVDF filters (Teknokroma, Sant Cugat del Valles, Spain) and stored at -20°C.

7.3. Erlenmeyer flask cultures.

The initial cultures described in Chapter 3 were performed in 250 mL Erlenmeyer Shake flask filled with 200 mL of the corresponding media. The Erlenmeyer were closed with a lid with two ports, capped by two short length silicon tubing and two 0.22 µm filters (Figure 16). The objective of this construction was to displace the oxygen present in the media to ensure anaerobic conditions. In this sense, previous

culture inoculation and after sampling, the media was pumped through the 0.22 µm filters with a 0.150 L/min constant current of N_2 for 1 min. After N_2 pumping, the silicon tubes were tightly closed with Hoffmann clamps. This configuration, however, seemed to affect greatly the growth of the cells so they were substitute for regular screwcaps.

Figure 54 Initial Erlenmeyer Shafe Flask configuration for *Propionibacterium freudenreichii* culture.

All Erlenmeyer Shake Flask cultures described in Chapter 3 were performed in 250 mL shake flasks filled with 200 mL of media at 30°C and 150 rpm in an INFORS HT incubator (Biogen, Madrid, Spain) unless stated otherwise. The ratio between the volume of culture and the maximum volume of the shake flask was fixed at 80% to reduce the oxygenation capacity of the system as anaerobic and microaerobic conditions were desired.

An aliquot of the cryopreserved cells was grown in 10 mL of pre-culture media for 24 hours. These cultures were used to inoculate another 10 mL of culture media for 48 hours. Finally, these cultures were used to inoculate the main cultures used in the studies.

All cultures were performed, at least, in triplicate unless stated otherwise.

7.3.1. pH control

After the initial cultures, pH was measured and adjusted daily with the addition of a 2M NaOH solution. During this adjustment, the cultures were not mixed with the cap open to avoid any extra oxygenation of the media.

From this point, all cultures pH were measured and adjusted daily with the addition of a 2 M NaOH solution.

7.3.2. Initial glucose concentration determination.

Six different glucose concentrations were tested: 25 g/L, 50 g/L, 70 g/L, 90 g/L, 100 g/L and 125 g/L. The rest of the media composition was the same as described in Media 2. The glucose was prepared in a stock solution and autoclaved separately. Samples were harvested every 24 hours and OD_{600} were measured and recorded.

7.3.4. Initial CSL concentration determination

Three different CSL concentrations were tested: 5 g/L, 10 g/L and 40 g/L. The rest of the media composition was the same as described in Media 3. The glucose and CSL were prepared in a stock solution and autoclaved separately. Samples were harvested every 24 hours and OD_{600} were measured and recorded.

7.3.5. DMBI supplement addition time effect

100 µM of DMBI was added at 48 h, 72 and 96 h. DMBI was added from a 100X tock solution of pure ethanol due to the insolubility of the compound in water.

7.3.6. Propionic acid effect on cell growth and CNCbl production

A 100X concentrated solution of propionic acid was prepared and added in enough volume to the media to increase its propionic concentra5tion by 8.5 g/L at 72 hours. The solution was sterilized by 0.22 µM filters.

To further asses the propionic inhibition and to study the possibility of obtaining better growth by removing this limiting factor, in other cultures the media was

replaced at 72 h by centrifugation at 3.900 g for 30 min and resuspending the cell pellets in 200 mL of fresh media.

Furthermore, in later cultures this media regeneration strategy was performed at 72 h and 168 h.

7.3.6. Nitrate as alternative electron acceptor

To assess the viability of using different compounds to displace fermentation for anaerobic respiration, NH_4NO_3 was prepared in 100X stock solutions, filtrated by 0.22 µM filters and added to the media in the following concentrations: 25 mM and 62 mM.

Furthermore, in order to discard any possible effect of the increased NH4concentration, a 100 X stock solution of NH_4Cl was also prepared and added to the media until a concentration of 37 mM was achieved.

7.3.7. Effect of the base solution used for daily pH adjustment.

The effect of using different base solutions for the daily pH adjustment was tested with several cultures carried out as previously described but using three different base solutions for the daily pHJ adjustments. The three solutions used for this experiments were: NaOH 2M (Control), KOH 2M and NH_4.

7.3.8. Early agitation effect on cell growth and CNCbl production.

The effect of the agitation during the early stages of the culture was studied in two separate conditions: a static condition where cultures were kept in a stove (J.P. Selecta, Abrera,Spain) at 30 °C and an agitated culture performed at 150 rpm and 30 °C in a INFORS HT incubator (Biogen, Madrid, Spain). After 96 h, both conditions were supplemented with 100 µM DMBI as usual and agitated at 150 rpm in the INFORS HT Incubator until the end of the culture.

After this point, all the Erlenmeyer Shake Flask cultures performed were kept in static conditions during the first 96 h and then agitated at 150 rpm until the endpoint of the culture.

7.3.9. Supplementation of CNCbl precursors.

To study the effect of the addition of different cobalamin precursors, several cultures were performed with the addition of 100 µM Riboflavin (RF), 100 µM Nicotinamide (NAM), 100 µM DMBI or a combination of RF and NAM (50 µM each) at 0 and 96 h. All stock solutions for the different precursors were prepared with ultrapure water and filter sterilized except DMBI solution which was prepared in pure ethanol.

The effect of DMBI addition on cell growth and production was further studied with the addition of 100 µM DMBI at 0, 48, 72 and 96 h. In all cases, after the addition of the precursors, cultures were kept under gently agitated conditions.

7.3.10. Media regeneration cultures in non-agitated conditions.

The capacity of the cells to grow in fresh media in the new complete anerobic setting was assessed with another media regeneration experiment similar to the one described in 7.3. 6..The media was replaced at 96 h by centrifugation at 3.900 g for 30 min and resuspending the cell pellets in 200 mL of fresh media.

7.3.11. CNCbl anaerobic production

The capability of NBRC 12391 to produce CNCbl in anaerobic conditions, without a microaeration phase, was performed in a stove without any agitation and using tight-closed lids. The cultures were kept at static conditions during the whole 168 h duration of the experiment.

7.3.12. Aerobic cultures with DSM 1650 and NBRC 108628

Erlenmeyer shake flask cultures with aerobic strains were performed in a 250 mL shake flask filled with 50 mL of the corresponding media and kept at 30ºC and 180 to 200 rpm in a Infors HT incubator (Biogen, Madrid, Spain)

An aliquot of the cryopreserved cells was grown in 10 mL of pre-culture media (Media 1) for 24 hours. These cultures were used to inoculate another 10 mL of culture media for 48 hours. Finally, these cultures were used to inoculate the main cultures used in the studies.

100 µM DMBI was added to the media at 96 unless stated otherwise.

All cultures were performed, at least, in triplicate unless stated otherwise.

7.3.13. CDM cultures.

Erlenmeyer Shake Flask CDM cultures were performed as usual, but the culture time were extended until the 288 h point due to slow growth.

An aliquot of the cryopreserved cells was grown in 10 mL of pre-culture media (Media 1) for 24 hours. These cultures were used to inoculate another 10 mL of culture media (CDM) for 48 hours. Finally, these cultures were used to inoculate the main cultures used in the studies.

7.3.14. Co-culture of NBRC 12391 with DMS 468 and DSM 366.

Both DMS 468 and DSM 366 grown in 10 mL of pre-culture media (Media 1) ON. These cultures were used to inoculate another 10 mL of culture media 3 and cultured ON. Finally, these cultures were used to inoculate 50 mL of media 3 in a 250 mL Erlenmeyer shake flask kept at 30ºC and 200 rpm and cultured ON.

20 mL of the previous cultures with OD_{600} of 5 to 7 were added to 180 mL of a NBRC 12391 culture at its 72-h point. The rest of the experiment was performed as usual.

7.4. Bioreactor Cultures

Two different bioreactors were used for cultures with NBRC 12391 due to due to the equipment availability.

All anaerobic cultures described in Chapter 3, as well as Figure 30 of Chapter 4 were performed using a INFORS HT (Infors, Spain) with a working final volume of 1.5 L.

All other anaerobic cultures described in Chapter 4, including the different scale up approaches and the continuous biproc4ess were performed in a Biostat A bioreactor (Sartorius Spain, Alcobendas, Spain) with a working final volume of 1 L.

INFORS HT:

The final working volume for these cultures was 1.5L. Temperature, pH and dissolved oxygen (pO_2) were measured online by Hamilton probes. pH was adjusted constantly at 7.0 +/- 0.05 with a 2 M NaOH solution. Temperature was constantly controlled at 30°C. To ensure anaerobic conditions during the early stages of the culture, 0.150 vvm of N_2 were pumped into the bioreactor vessel through a 0.2 µm filter (Whatman, United Kingdom) and injected from the sparger before the inoculation of the media. After inoculation, the culture was performed with no aeration and both the gas inlet and outlet lines were closed with Hofmann clamps to avoid culture oxygenation.

In batch cultures, 1 L of fermentation media was inoculated with 10 mL of inoculum (1% v/v) to obtain an initial OD_{600} of around 0.3.

In fed-batch processes, batch phase had 80% of the final volume. The fed-batch phase began at 48hours with the addition of a concentrated glucose solution (200 g/L) at a constant rate of 6.66 mL/h. Glucose was measured and recorded every 24 hours by a biochemical analyzer and by HPLC.

Finally, 100 µM of DMBI was also added to the culture at this point to promote Cbl production.

BIOSTAT A:

Final working volume of 1 L. Instead of pumping N_2 before inoculation like in the cultures performed in INFORS HT bioreactors, anaerobic conditions during the early stages of the culture were ensured by pumping 0.150 vvm of N_2 constantly pumped into the bioreactor vessel through a 0.2 µm filter (Whatman, Maidstone, UK) and injected from the sparger. N_2 pumping was maintained during the whole anaerobic º phase of the culture (72h, 96h or 48 h depending on the experiment, as described in their respective sections)

The agitation was maintained constant at 150 rpm. Temperature, pH and dissolved oxygen (pO2) was measured online by Hamilton probes. pH was adjusted constantly at 7.0±0.05 with a 2 M NaOH solution. Temperature was constantly controlled at 30 °C.

In batch cultures, 1 L of fermentation media was inoculated with 10 mL of inoculum (1% v/v) to obtain an initial OD_{600} value of approximately 0.3. 100 µM of DMBI were added at 72 or 96 h depending on the culture and N2 addition was stopped to promote microaeration when desired. Air was not pumped during the cultures at any time.

In fed-batch processes, batch phase had 80% of the final volume and it was performed as previously described elsewhere. The fed phase began at 72 h with the addition of a concentrated glucose solution (250 g/L) at a constant rate of 6.66 mL/h. 100 µM of DMBI was also added to the culture at this point to promote Cbl production. A total amount of 200 mL of concentrated glucose solution was added to the culture for 30 h. After that time, no more glucose was added to the media and the culture was extended until 144 h.

Finally, for the continuous cultures, the batch phase was started as described before and from 48 h onwards fresh media with 100 µM DMBI were constantly added to the bioreactor vessel at 50 mL/h. Culture broth was constantly extracted from the bioreactor vessel by a level probe connected to a peristaltic bomb to ensure a constant working volume of 1.0 L.

Aerobic cultures:

All aerobic cultures were performed using a INFORS HT (Infors, Spain) with a working final volume of 1.5 L.

Temperature, pH, and dissolved oxygen (pO_2) were measured online by Hamilton probes. pH was adjusted constantly at 7.0 +/- 0.05 with a 2 M NaOH solution. Temperature was constantly controlled at 30°C. pO_2 was also measured and set at 15%. It was controlled by a rpm cascade (400 to 800 rpm). Aeration was fixed at 1 vvm of air, that was pumped into the bioreactor vessel with a 0.22 µM filter.

Several batch cultures were performed for both aerobic strains used in during this chapter. In all cases, the fermentation media was inoculated with 10 mL of inoculum (1% v/v) to obtain an initial OD_{600} of around 0.3.

7.5. Analytical Methods

Optic density at 600 nanometers (OD_{600}) was determined with a JENWAY6305 spectrophotometer. (COLE PALMER, Staffordshire, UK).

Dry cell weight was calculated as follows. Periodically, 1 mL samples were centrifuged at 3900 g's for 15 min in triplicates in previously dried and weighed 15 mL falcon tubes. After centrifugation, the pellets were dried in a 100°C oven until stable weight, cold down in a desiccator for 30 minutes and weighed again. The weight of the falcon tubes was taken into consideration for this calculation. After a series of cell dry weigh calculations in several cultures according to the method just described, it was established that one unit of OD_{600} corresponds to 0.405 g/L ± 0.02 of NBRC 12391 DCW (see Annex for more detailed information).

Glucose consumption and metabolite production, mainly acetate and propionic acids, were determined from supernatant samples. Briefly, at each sampling time, 1.0 mL of sample was centrifuged at 10.000 g's for 10 min and supernatants were separated, filtrated through 0.45 μm PVDF filters and stored at -20°C until its analysis.

To increase the available information during the cultures without having to wait to the rather slow HPLC quantification, glucose consumption was followed by a biochemical analyzer (ANALYZER Y15®, Biosystems, Barcelona, Spain). This system allowed for and almost online measure of glucose during the bioreactor and Erlenmeyer shake flask cultures, with analysis times of less than 10 minutes and more than 150 samples per hour if necessary. Supernatant samples were put in their corresponding racks and mixed automatically by the system with Reactant A and B. After a short time of reaction, the system measures the absorbance of the reaction in a plastic cuvette and correlated it with a concentration value in g/L. The system was calibrated with blank samples and standards according to the manufacturer's instructions.

Besides by ANALYZER Y15, glucose, alongside other target metabolites like propionic acid and acetic acid, were quantified by HPLC. The metabolite analysis was performed by HPLC (Agilent HPLC 1100 (Agilent, Santa Clara, USA) using an ICSep ICE-Coregel 87H3 column (Concise, San Jose, USA). A 0.05 M H_2SO_4 solution was used as a mobile phase at 0.5 mL/min constant flow rate.

Organic acids were identified at different retention times with UV detection at 210 nm wavelength and glucose with the Refractive index detector. Acetic acid, propionic acid and glucose were quantified with 5-point calibration curve. The curves alongside their respective RFs can be seen in Figure 55.

Glucose and propionic acid 5-point calibration curve was used using the following concentrations: 30 g/L, 22.5 g/L, 15 g/L, 7.5 g/L and 3.75 g/L. Acetic acid 5-point calibration curve, in the other hand and due to the higher sensitivity of the system to this metabolite was: 15 g/L, 11.25 g/L, 7.5 g/L, 3.75 g/L and 1.88 g/L.

Standards for every metabolite were freshly prepared before HPLC analysis in a UPW stock solution. Glucose and Propionic acid stock solutions were prepared at a concentration of 90 g/L concentration while acetic acid stock solution was prepared at a concentration of 45 g/L.

All samples and standards were filtered with a 0.45 µm PVDF filter before inyection in the HPLC system.

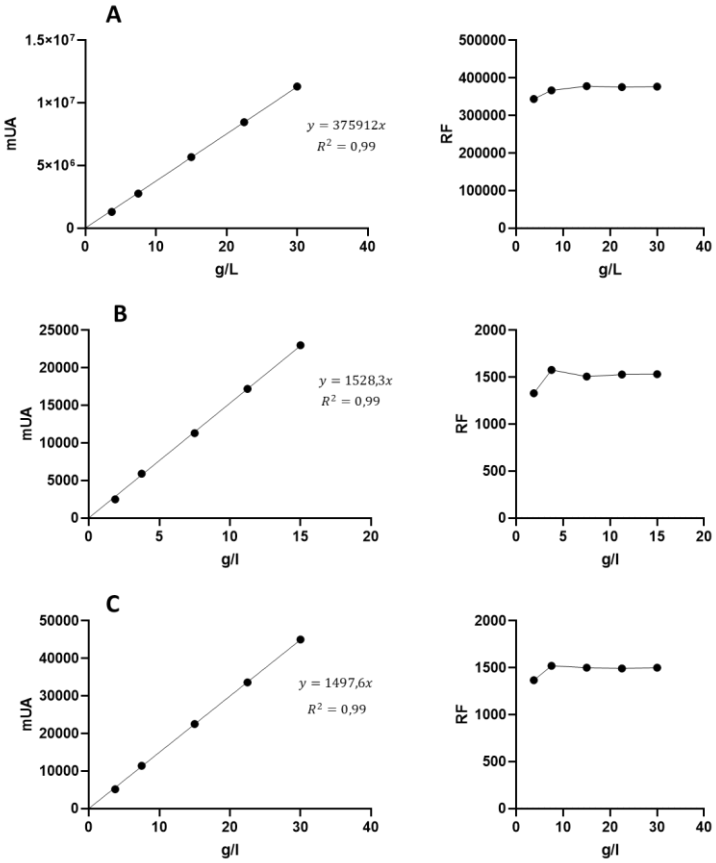

Figure 55 5-point calibration curves obtained by HPLC measurements of different known standard concentrations. RF is shown in the right. *A) Glucose in g/L (measured by RID), B) acetic acid in g/L and C) propionic acid in g/L, both measured by an UN detector.*

7.6. Cbl extraction and analysis

Extraction and quantification of CNCbl was adapted from several sources [100,120]. The exact extraction, cyanidation and quantification methodology and conditions were developed and first implemented in the laboratory during this thesis project and are described in details Chapter 3, section 3.3.7. *Developing a methodology for Cbl extraction, cyanidation, and quantification*). A complete 2^4 DOE was used to obtain the final extraction parameters.

Regarding the final extraction conditions themselves, cell pellets obtained from the centrifugation (10 min, 6.500 g's), were resuspended in ultrapure water and heated at 90°C for 20 minutes. After, they were cold down on ice and centrifuged at 3.900 g's for 45 min. Supernatants were recovered and treated with a 0.1% (v/w) NaCN solution for 30 minutes at room temperature with periodical mixing to transform all possible cobalamin forms into CNCbl.

All relevant Cbl standards (including OHCbl, MetCbl, AdoCbl, and CNCbl) were freshly prepared prior to sample extraction. They were dissolved in a f 1 g/L stock solution using UPW and then stored at 4 ºC until analysis. A five-point standard curve, detailed in Figure 20, was generated by diluting the stock solution, and this curve was injected into the HPLC system before analyzing any samples.

Before injection into the HPLC system, both samples and standards were filtered with a 0.45 µm PVDF filter.

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Annex 1: Raw and supplementary data.

DCW Determination

As described in Materials and Methods-7.5 Analytical methods, Dry Cell Weight (DCW)was calculated as follows. Periodically, 1 mL samples were centrifuged at 3900 g's for 15 min in triplicates in previously dried and weighed 15 mL falcon tubes. After centrifugation, the pellets were dried in a 100°C oven until stable weight, cold down in a desiccator for 30 minutes and weighed again. The weight of the falcon tubes was taken into consideration for this calculation.

The initial determination involved utilizing the data presented in Annex Table 1. It was established that a single unit of OD_{600} corresponds to 0.405 g/L ± 0.02 of NBRC 12391 DCW. To ensure accurate calculation of DCW, periodic checks were performed on culture samples obtained from both Erlenmeyer Shake Flasks and bioreactor cultures.

ANNEX TABLE 1. DCW DETERMINATION

	1				2				3		
T (h)	**OD600**	**DCW (g/L)**	**g/L/OD_{600}**	**Time (h)**	**OD600**	**DCW (g/L)**	**g/L/OD_{600}**	**Time (h)**	**OD600**	**DCW (g/L)**	**g/L/OD_{600}**
0	0,05	-	-	0	0,05	-	-	0	0,05	-	-
24	1,85	0,78	0,421	24	1,75	0,71	0,406	24	1,71	0,75	0,439
48	10	3,98	0,398	48	10,3	4,08	0,396	48	9,99	3,96	0,396
72	22	9,07	0,412	72	21,5	8,99	0,418	72	20,89	8,12	0,389
96	29	11,2	0,386	96	29,5	11,71	0,397	96	28,97	11,91	0,411

The mean of the different culture points as well as the total mean between the triplicates is shown below:

Mean Control 1	Mean Control 2	Mean Control 3
0,404525311	0,404229869	0,408703

Total Mean	0,405
Standard deviation	0,002501435

Figure 56:Erlenmeyer Shake flasks cultures of DMSZ Z4902 (A) and NBRC 12391 (B).

ANNEX TABLA 2. FIGURE 14 A AND B

Time (h)	NBRC 12391 OD_{600}			DMSZ 4902 OD_{600}		
0	0,048	0,052	0,045	0,031	0,04	0,03
24	0,832	0,78	0,6	1,2	1,1	2,2
48	4,27	3,48	3,56	4,3	6,45	4,69
72	7,63	7,3	6,9	3,85	6,83	4,5
96	7,9	7,4	7,1	5,28	8,74	5,72

ANNEX TABLE 3. FIGURE 14, C.

Metabolite	DMSZ-4902			NBRC 12391		
Glucose (g/L)	0,62	0,4	0,98	0,01	0	0
Acetic Acid (g/L)	1,29	3,34	1,33	1,42	1,15	1,15
Propionic Acid (g/L)	2,99	2,04	2,56	4,19	3,05	3,36

Figure 57. OD600 values at 72 culture hours for Falcon tubes cultures with Media 2 of NBRC 12391 and DMSZ 4902.

ANNEX TABLE 4. FIGURE 15.

Sample	OD_{600} at 72 h		
NBRC 12391	9,2	8,31	4,42
DMSZ 4902	1,46	1,53	1,5

Figure 16. 50 mL Falcon tube cultures of Propionibacterium freudenreichii subs shermanii NBRC 12391

ANNEX TABLE 5. FIGURE 16.

Replicate	25 mL	35 mL	45 mL
1	6,25	8,4	8,3
2	6,4	8,1	8,5
3	6,31	8,3	8,42

Figure 17. Erlenmeyer shake flask cultures of NBRC 12391 with Media 2.

ANNEX TABLE 6. OD_{600}

Time (h)	1	2	3
0	0,031	0,04	0,03
17	0,69	0,56	1,77
44,5	2,78	1,9	3,09
68,5	4,3	6,45	4,69
89	5,28	8,74	5,72

ANNEX TABLE 7. GLUCOSE (G/L)

Time (h)	1	2	3
0	50	50	50
17	34	34	36
44,5	31	32	30
68,5	29,5	28,88	27,6
89	28,8	27,5	26,5

ANNEX TABLE 8. ACETIC ACID (G/L)

Time (h)	1	2	3
0	0,00	0,00	0,00
17	0,90	1,15	1,21
44,5	1,05	1,17	1,26
68,5	1,10	1,43	1,10
89	1,40	3,34	1,33

ANNEX TABLE 9. PROPIONIC ACID (G/L)

Time (h)	1	2	3
0	0,00	0,00	0,00
17	0,79	1,10	1,16
44,5	1,92	1,48	1,45
68,5	2,61	3,14	2,54
89	3,00	2,06	2,57

Figure 18. Erlenmeyer shake flask cultures of NBRC 12391 with and without pH adjustment.

ANNEX TABLE 10. FIGURE 18.

Time (h)	pH Control OD_{600}		NO pH control OD_{600}	
0	0,05	0,05	0,2	0,23
25	0,73	0,65	1,1	1,25
51	11,29	9,329	2,25	2,05
74	21,217	22,393	4,1	2,1
94	51,52	60	8,7	5

Figure 19. NBRC 12391 Erlenmeyer shake flask cultures at 6 different glucose concentrations: 25 g/L, 50 g/L, 70 g/L, 90 g/L, 100 g/L and 125 g/L.

ANNEX TABLE 11. OD_{600}

		Time (h)				
Condition	**Replicate**	**0**	**27**	**51**	**72**	**96**
25 g/L	1	0,05	2,79	21,85	34,1	37,5
	2	0,05	3,01	22,7	36,5	37,7
	3	0,05	2,85	22,31	35,5	38,12
50 g/L	1	0,05	1,92	19,87	34,69	56,92
	2	0,05	2,13	19,21	34,05	57
	3	0,05	1,89	19,12	34,32	56,78
70 g/L	1	0,05	1,56	16,02	29,45	43
	2	0,05	1,78	16,89	31,1	44,5
	3	0,05	1,76	16,54	30,45	43,5
90 g/L	1	0,05	0,99	8,88	22,74	43,69
	2	0,05	1	9,03	21,56	41,1
	3	0,05	1,02	9,21	22,03	42,45
100 g/L	1	0,05	0,46	2,1	16,86	37
	2	0,05	0,41	3,45	15,5	35,4
	3	0,05	0,55	4,15	13,6	33,4
125 g/L	1	0,05	0,42	3,67	9,12	26,89
	2	0,05	0,43	3,32	8,72	26,5
	3	0,05	0,56	4,1	10,02	27,2

ANNEX TABLE 12. μNET [H^{-1}]

	Condition					
Replicate	**25 g/L**	**50 g/L**	**70 g/L**	**90 g/L**	**100 g/L**	**125 g/L**
1	0,0917	0,0923	0,0902	0,0865	0,0825	0,0734
2	0,0924	0,0925	0,0915	0,0858	0,0757	0,0751
3	0,0921	0,0931	0,0908	0,0871	0,0758	0,0760

Figure 20. 5-point calibration curves obtained by HPLC measurements of different known standard concentrations.

ANNEX TABLE 12. HPLC 5-POINT CALIBRATION CURVE (MUA)

Standard	100 mg/L	75 mg/L	50 mg/L	25 mg/L	12,5 mg/L
OHCbl	2712,79	2101,27	1479,84	811,36	428,88
AdoCbl	2832	2065	1385	703	451
MetCbl	2623	2072	1442	911	421
CNCbl	6000	3800	2700	1200	500

Figure 21. Bioreactor culture fed-batch of NBRC 12391 with constant pH adjustment by 2M NaOH and constant 200 g/L stock glucose solution addition from 48 culture hours.

ANNEX TABLE 13. OD_{600}.

Time (h)	1	2
0	0,355	0,345
21	3,54	5,61
28	6,98	6,99
47	31	31,02
72	67	66,89
94	64	63,87
119	56	56,03
126	58	57,3
143	53	53,1

ANNEX TABLE 14. GLUCOSE (G/L)

Time (h)	1	2
0	50	50
21	30,2	29,76
28	24,7	25,1
47	9,37	9,35
72	11,1	10,98
94	17,4	18,1
119	15,8	16,01
126	15,5	15,52
143	40	38,98

ANNEX TABLE 15. ACETIC ACID(G/L)

Time (h)	1	2
0	0	0
21	0	0
28	0	0
47	4,38	4,41
72	5,44	5,55
94	7,61	7,71
119	7,06	7,21
126	6,88	6,91
143	4,29	4,34

ANNEX TABLE 16. PROPIONIC ACID(G/L)

Time (h)	1	2
0	0	0
21	0	0
28	0	0
47	12,22	11,98
72	15,26	16,03
94	28,72	29,89
119	25,64	26,14
126	32,05	32,89
143	31,41	32,04

ANNEX TABLE 16. TOTAL CBL (MG/L)

Time (h)	1	2
0	-	-
21	-	-
28	-	-
47	-	-
72	-	-
94	0,25	0,23
119	-	-
126	-	-
143	0,35	0,37

Figure 22. 22 NBRC 12391 cultures with different amounts of CSL: 5 g/L, 10 g/L and 40 g/L.

ANNEX TABLE 17. OD_{600}

	5 g/L			10 g/L			20 g/L		
Time(h)	1	2	3	1	2	3	1	2	3
0	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
26	1,08	1,11	1,01	1,24	0,64	0,98	1,45	1,21	1,45
50	6,18	5,99	5,91	6,9	5,36	5,76	12,81	9,16	11,12
75	14,07	16,35	17,05	17,79	18,47	17,92	27	25	26,43
96	22,25	21,6	21,51	27,05	25,63	26,65	35	34	34,4

ANNEX TABLE 18. μNET [H^{-1}]

Replicate	5 g/L	10 g/L	40 g/L
1	0,075	0,077	0,084
2	0,077	0,0798	0,083
3	0,076	0,0781	0,038

Figure 23. NBRC 12391 Erlenmeyer shake flask cultures with Media 3 (40 g/L CSL).

ANNEX TABLE 18. FIGURE 23.

	OD_{600}			Glucose (g/L)			Acetic Acid (g/L)			Propionic Acid (g/L)		
Time (h)	1	2	3	1	2	3	1	2	3	1	2	3
0	0,05	0,05	0,05	50	50	50	0	0	0	0	0	0
24	1,1	1,25	1,21	34	37,3	36	0	0	0	0	0	0
48	2,4	3,5	3,5	28,9	29,01	28,74	0,9	1,02	0,97	3,2	3,45	3,56
72	13,4	16,2	14,2	19,5	20	19,87	2,09	2,13	2,1	7,4	8,04	7,95
96	26	27,2	28,1	11,23	10	12,01	3,42	3,2	3,3	12,16	12,71	12,1

Figure 24. NBRC 12391 bioreactor culture with Media 3

ANNEX TABLE 19. FIGURE 24.

	OD600		Glucose g/L		Acetic Acid (g/L)		Propionic Acid (g/L)		Total Cbl* (mg/L)	
Time h)	1	2	1	2	1	2	1	2	1	2
0	0,3	0,3	50	50	0	0	0	0	0	0
24	5,3	5,9	35	34	1,57	1,10	4,30	4,30	0,00	0,00
48	15,56	14,2	28	27	1,85	1,40	7,64	7,89	0,00	0,00
96	21,52	23,1	55	48,9	2,04	2,20	10,70	11,20	0,30	0,41
120	25,5	27,2	53	49,78	2,50	2,71	11,41	12,00	0,56	0,65
144	25	26	52,7	49,54	3,24	3,54	11,71	14,50	0,48	0,54
240	18	21	48,2	45,5	4,75	4,90	14,68	15,70	0,81	0,86

Figure 29 NBRC 12391 Erlenmeyer Shake Flask cultures at different 100 µM DMBI supplement addition times: 48 hours, 72 hours and 96 hours.

ANNEX TABLE 20. OD_{600}

	96 h addition		72 h addition		48 h addition	
Time	1	2	1	2	1	2
0	0,05	0,07	0,06	0,06	0,06	0,05
24	1,296	1,272	1,41	1,372	0,616	1,86
48	8,62	8,48	7,83	7,82	7	7,5
74	22,5	20,8	22,01	21,05	21,1	21,25
96	30	26,1	27,1	26,7	27,4	38,3
120	34,3	28,1	29,87	29	28,1	30
168	22,6	21,5	22,98	22,6	22,8	25,4

ANNEX TABLE 21. CNCBL (MG/L).

Replicate	96 h	72 h	48 h
1	1,71	1,88	1,54
2	1,88	1,62	1,84

Figure 30 NBRC 12391 fed-batch bioreactor. Glucose was added from 72 h and measured with an Y15 analyzer (Biosystems)

ANNEX TABLE 22. FIGURE 30.

	OD600		Glucose (g/L)		Acetic Acid (g/L)		Propionic Acid (g/L)		CNCbl (mg/L)	
Time (h)	1	2	1	2	1	2	1	2	1	2
0	0,3	0,5	50	50	0	0	0	0	0	0
24	1,5	3,8	27,12	27,22	1,7	1,91	5,05	2,65	0	0
48	9	11	25	20,23	2,4	4,66	7,9	9,4	0	0,44
72	28	27	16,48	13,14	3,86	7,15	12,24	17,62	0,89	0,91
96	30	26,2	7,8	19,29	5,48	5,5	16,66	19,18	0,86	0,66
144	24	21	21,59	17,21	6,91	4,69	21,33	21,95	0,86	0,62

Figure 31 NBRC 12391 Erlenmeyer shake flask cultures. three different conditions are shown: control, 8.5 g/l propionic acid increase at 72 hours and media regeneration at 72 hours.

ANNEX TABLE 22. OD_{600}

	Control			Propionic Acid Addition			Media Regeneration		
Time (h)	1	2	3	1	2	3	1	2	3
0	0,15	0,15	0,15	0,15	0,15	0,15	0,15	0,15	0,15
24	1,21	1,52	1,51	1,56	1,87	1,74	1,56	1,88	1,56
42	5,3	5,8	5,71	6,15	6,56	6,44	5,7	5,56	5,7
72	12	13,42	13,24	10,9	10,9	11,02	8,23	13	12
138	32,3	33,3	31,98	20	21,4	21,1	28	31,1	29,34
168	36	37	35,43	21,6	19	20,34	42	44,5	43,3

ANNEX TABLE 23. CNCBL (MG/L)

Replicate	Control	Propionic Acid Addition	Media Regeneration
1	1,72	0,97	2,15
2	1,54	0,88	1,97
3	1,61	0,93	2,02

ANNEX TABLE 24. PROPIONIC ACID (G/L)

Replicate	Control	Propionic Acid Addition	Media Regeneration
1	18,1	21,67	11,67
2	17,5	22,34	13,2
3	18,5	22,43	12,87

Figure 32 NBRC 12391 Erlenmeyer shake flask cultures. Two different conditions are shown: control and media regeneration at 72 h and 168 h.

ANNEX TABLE 25. OD_{600}

Time (h)	Control		Media Regeneration	
0	0,1	0,1	0,1	0,1
24	0,548	0,61	0,457	0,512
48	6,9	7,2	5,8	6,65
72	17	18,67	19	23
75	-	-	11,94	13,86
96	25	28,2	23	30,9
168	30	33,5	55,8	57,4
170	28	29	20,1	37,8
192	24	23,1	26,1	27
216	19	18,78	37	22,4

ANNEX TABLE 26. CNCBL (MG/L)

	Control		Media Regeneration	
Time (h)	1	2	1	2
168	1,60	1,40	2,30	2,10
216	0,89	0,78	2,92	3,10

ANNEX TABLE 26. PROPIONIC ACID (G/L).

	Control		Media Regeneration	
Time (h)	1	2	1	2
168	17,2	18,1	12,45	11,55
216	21,1	22,4	20,2	21,1

Figure 33. NBRC 12391 Erlenmeyer shake flask cultures with different nitrate additions. NH4Cl was also tested as a negative control.

ANNEX TABLE 27. OD_{600}

	Control			NH_4Cl-37mM			NH_4NO_3 -25 mM			NH_4NO_3-62 mM		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
0	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
24	0,4	0,413	0,46	0,413	0,466	0,54	0,46	0,6	0,54	0,37	0,4	0,32
48	2,67	4,28	3,98	4,435	4,35	5,99	3,85	6,5	5,93	3,255	2,75	2,56
72	10,56	14	13,2	14,6	12,8	10,2	9,6	26	24,2	9,82	8	7,78
96	21	21	22,4	26	25	22,3	12	30	24,5	20	13	15,6
168	26,3	25	25,52	25,2	35,3	23,21	20,1	25,4	22,34	26,2	20,2	24,43
192	22,23	23	22,87	20,7	26	19,98	18,12	16	23,4	28,7	26,45	25,2

ANNEX TABLE 27. PROPIONIC ACID (G/L)

	Control			NH_4Cl-37mM			NH_4NO_3 -25 mM			NH_4NO_3-62 mM		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
48	1,28	1,10	2,34	3,62	2,32	1,99	1,10	0,98	3,32	0,63	2,21	2,48
72	7,98	6,78	8,35	9,84	8,20	7,30	7,12	7,63	9,10	8,48	6,50	7,10
96	11,04	9,80	12,30	14,30	12,00	11,83	10,10	7,89	11,20	9,73	8,44	10,12
168	16,69	17,02	16,88	19,78	18,70	17,43	21,30	16,60	15,40	19,28	18,21	21,12
192	17,91	18,34	16,54	19,57	20,84	18,89	20,84	17,40	21,43	20,64	17,60	19,13

ANNEX TABLE 28. $CNCBL$ AT 192 H (MG/L).

Replicate	Control	NH_4Cl-37mM	NH_4NO_3 - 25 mM	NH_4NO_3- 62 mM
1	2,13	2,34	2,1	2,45
2	1,89	2,02	1,964	2,01
3	2,08	2,1	2,07	2,22

Figure 34. NBRC 12391 Erlenmeyer shake flask cultures with different bases added for the daily pH adjustment.

ANNEX TABLE 29. FIGURE 34.

	NaOH 2M			KOH 2M			NH_4		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
0	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
24	0,98	0,92	0,87	0,99	1,02	1,01	0,81	0,99	0,82
48	3,21	3,33	3,45	3,71	3,5	3,6	3,17	3,18	3,32
72	15,1	14,53	13,5	15,5	15,3	15,4	15	15,2	15,21
96	20	21,23	22,24	22,6	19,7	22,6	19,8	20,4	21,1
168	29,3	30,21	31,1	27,23	29	26	30,1	31,3	30,08

Figure 35. 1 OD_{600} values, 2 volumetric productions (mg CNCbl/L) and 3 specific productions (mg CNCbl/g Biomass) at 96 and 168 h of both conditions, agitated or static.

ANNEX TABLE 39. OD_{600}

	Agitation			Static		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,05	0,05	0,05	0,05	0,05	0,05
24	1,35	1,25	1,21	1,85	1,75	1,71
48	3,8	3,5	3,6	10	10,3	9,99
72	12,1	15	13,2	22	21,5	20,89
96	27	25	26,4	29	29,5	28,97
168	20	24	22,34	23	20	21,4

ANNEX TABLE 40. CNCBL VOLUMETRIC PRODUCTION (MG/L).

	Agitation			Static		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
96	0,74	0,68	0,72	0	0	0
168	1,81	1,84	1,82	2,9	2,79	2,79

ANNEX TABLE 40. CNCBL SPECIFIC PRODUCTION (MG/G).

	Agitation			Static		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
96	0,07	0,07	0,07	0	0	0
168	0,23	0,19	0,2	0,32	0,35	0,33

Figure 36. Study of the effect of the addition of different CNCbl precursors at different times.

ANNEX TABLE 41. OD_{600}

		Time (h)					
Condition	**Replicate**	**0**	**24**	**48**	**72**	**96**	**168**
Control	**1**	0,05	0,7	7,4	21	29,2	32
	2	0,05	0,72	7,7	18,5	25,5	25
	3	0,05	0,7	7,4	21	29,2	25
RF-0 hours	**1**	0,05	0,81	7,7	23,5	29,5	39,5
	2	0,05	0,74	8	18,5	26	22
	3	0,05	0,55	7,8	23,55	29	22,5
RF-96 hours	**1**	0,05	0,7	7,9	21	30	33,5
	2	0,05	0,81	7,6	18,5	25,5	25
	3	0,05	0,57	8	22	27,5	26,4
NAM-0 hours	**1**	0,05	0,78	7,5	21	31	35,5
	2	0,05	0,65	7,8	18	20,5	35
	3	0,05	0,55	7,8	21,5	27,75	26,1
NAM-96 hours	**1**	0,05	0,82	8,06	20,8	30,5	35
	2	0,05	0,69	7,4	17,8	21	31
	3	0,05	0,51	8	21,25	28,05	27
DMBI-0 hours	**1**	0,05	0,47	5	15	22	29,8
	2	0,05	0,4	3	13	22,5	29,1
	3	0,05	0,41	6	19	27,5	27,5
DMBI-96 hours	**1**	0,05	0,8	7,8	22,5	29,2	39,7
	2	0,05	0,7	7,2	18	27	22
	3	0,05	0,628	7,96	21	28,25	23
RF+NAM-0 hours	**1**	0,05	0,79	7,6	21	28,8	33,5
	2	0,05	0,72	8	14,5	26	26
	3	0,05	0,68	7,8	21	27,5	24
RF+NAM-96 hours	**1**	0,05	0,9	7,9	22,5	30,5	35,5
	2	0,05	0,53	8,2	22	29,1	26,2
	3	0,05	0,54	8	20,5	28,5	25

ANNEX TABLE 42. CNCBL VOLUMETRIC PRODUCTION (MG/L).

Replicate	Control	RF-0 hours	RF-96 hours	NAM-0 hours	NAM-96 hours	DMBI-0 hours	DMBI-96 hours	RF+NAM-0 hours	RF+NAM-96 hours
1	0,4	0,93	0,59	0,6	1,03	1,96	6,89	1,64	0,48
2	0,31	0,27	0,35	0,39	0,37	1,41	2,82	0,24	0,32
3	0,27	0,29	0,83	0,32	0,65	1,55	3,53	0,44	0,43

Figure 37. Study of the effect of adding DMBI at different times.

ANNEX TABLE 43. OD_{600}

	0 hours		48 hours		72 hours		96 hours	
Time (h)	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
0	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
24	0,18	0,22	0,46	0,52	0,45	0,528	0,4	0,6
48	5	3	5,2	5,38	6	5,55	5,6	6,5
72	2,5	9,5	21	17,5	17	16	17,5	16
96	9,7	15	25	23	25,2	23,5	24	21
168	21,5	26	22,5	20	25,5	22,5	24	24,5

ANNEX TABLE 44. CNCBL VOLUMETRIC PRODUCTION (MG/L)

	0 hours		48 hours		72 hours		96 hours	
Time (h)	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
96	0,5	0,7	1,6	1,83	2,46	4,04	0,1	0,1
168	1,03	0,98	2,67	2,11	2,88	2,47	2,68	3,53

Figure 38. NBRC 12391 batch with DMBI addition at 96 hours.

ANNEX TABLE 45. FIGURE 38.

Time (h)	OD_{600}	Glucose (g/L)	CNCbl (mg/L)	Acetic Acid (g/L)	Propionic Acid (g/L)
0	0,1	50	0	0	0
24	0,86	35	0	0	0
48	9,84	24	0	1	6
72	30,1	11	0	1,7	8,9
96	29	4,5	0	4,2	18
120	21	1,2	0	4,3	19,5
144	18	0,3	0,59	4,3	19
168	15	0	0,67	4,5	19,48

Figure 39. NBRC 12391 batch with DMBI addition at 72 h.

ANNEX TABLE 46. FIGURE 39.

Time (h)	OD_{600}		Glucose (g/L)		CNCbl (mg/L)		Acetic Acid (g/L)		Propionic Acid (g/L)	
0	0,1	0,1	50	50	0	0	0	0	0	0
24	0,51	0,81	32	33	0	0	0	0	0	0
48	6,1	8,1	24,79	23,00	-	-	1,44	1,00	5,65	5,64
68	24,5	24	12,56	12,50	-	-	1,64	1,69	6,60	6,88
72	27,5	27,5	9,00	8,00	0,86	0,83	-	-	-	-
96	30	30,5	1,98	1,80	1,80	1,49	2,87	4,26	10,01	10,25
120	22	22	0,00	0,00	2,16	1,61	3,10	4,74	16,80	16,96
144	16	16	0,00	0,00	1,85	2,27	4,60	4,53	17,10	17,01
168	15	15	0,00	0,00	0,99	1,18	4,81	4,54	18,30	18,47

Figure 40. NBRC 12391 fed-batch with DMBI addition at 72 h.

ANNEX TABLE 47. FIGURE 40.

Time (h)	OD_{600}		Glucose (g/L)		CNCbl (mg/L)		Acetic Acid (g/L)		Propionic Acid (g/L)	
0	0,11	0,11	50	50	0	0	0	0	0	0
24	0,7	0,7	35	30	0	0	-	-	-	-
48	7	7	18	19	0	0	-	-	-	-
72	22,75	23,5	4,5	5,6	0,67	0,48	4,1	4,4	15,4	16
75	22,5	23,5	-	-	1,15	1,81	4,5	4,6	-	-
96	25	24	10	7	1,12	1,72	4,5	4,6	18	18,7
120	20,5	20	4,5	4,3	0,97	1,14	4,1	4,3	16,7	17,4
144	18,5	18,5	3	2,9	1,24	1,17	4,4	5	17,1	18,1

Figure 41. 1. OD_{600} values and 2. volumetric productions at 168, 192, 216 and 40 h of NBRC 12391 control cultures and cultures where the cells were centrifuged and resuspended in fresh media at 96 h.

ANNEX TABLE 47. OD_{600}

	Control			Media Regeneration		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,05	0,05	0,05	0,05	0,05	0,05
24	0,54	0,7	0,61	0,55	0,53	0,51
48	4,8	5,1	4,99	5,7	5,35	5,23
72	18,5	19	17,98	17,5	16,5	16,6
96	22,5	23,1	22,23	22	23,5	23,1
168	22,5	22,7	21,98	37,5	38	37,7
192	21,7	22,71	23,87	45,2	44,5	43,8
216	19	19,7	20,02	36	38,5	36,01
240	16,5	16,9	17,1	30,5	31	29,98

ANNEX TABLE 47. CNCBL (MG/L).

	Control			Media Regeneration		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
168	1,43	1,48	1,34	4,37	2,91	3,2
192	1,07	1,1	0,99	5,00	2,78	4,4
216	1,06	1	1,01	3,79	3,28	3,56
240	0,73	0,71	0,69	1,76	1,81	1,8

Figure 42. 1. OD_{600} values and 2. volumetric productions at 168 h of NBRC 12391 control cultures and cultures that were kept anaerobic through the whole process.

ANNEX TABLE 48. OD_{600}

	Control			Anaerobic		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,05	0,05	0,05	0,05	0,05	0,05
24	0,3	0,3	0,31	0,45	0,42	0,45
48	6,2	7	6,5	6,3	6,5	6,2
72	20	23	22,13	19	18	18
96	29	29	31	28	28	28
168	35	34	35,1	35	33	40

ANNEX TABLE 49. CNCBL (MG/L)

	Control			Anaerobic		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
168	2,31	2,12	2,22	2,62	2,32	2,32

Figure 43. Continuous bioprocess of NBRC 12391.

ANNEX TABLE 50. FIGURE 43.

	OD_{600}		Glucose (g/L)		Acetic Acid (g/L)		Propionic Acid (g/L)		CNCbl (mg/L)	
Residence Time	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
-2,64	0,15	0,1	25	25	0	0	0	0	0	0
0	13	13,1	7,4	8	0,8	0,5	4,5	3,5	0	0
1,32	24	24,5	0,01	2,38	1,20	4,45	6,10	7,75	1,51	1,14
2,64	23,8	25	0,01	2,35	1,81	4,49	6,30	8,15	1,26	1,58
3,96	25	24	0	2,79	2,12	3,95	7,61	7,75	1,54	1,42
5,28	24,9	23,2	0	3,22	2,40	4,51	7,60	7,29	1,44	1,40

Figure 45. Initial cultures of DSM 1650 and NBRC 108628 in Erlenmeyer shake flask with media adapted from [225].

ANNEX TABLE 51. FIGURE 45.

	DSM 1650			NBRC 108628		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,05	0,05	0,05	0,05	0,05	0,05
1	0,082	0,07	0,102	0,14	0,15	0,14
3	0,105	0,098	0,15	0,19	0,21	0,11
5	0,12	0,1065	1,67	0,203	0,34	0,15
8	0,165	0,143	0,186	0,21	0,345	0,16
24	6,4	5,1	7,2	0,9	1,1	1,2

Figure 46. Cultures of DSM 1650 and NBRC 108628 in Erlenmeyer shake flask with media adapted from [225].

ANNEX TABLE 52. FIGURE 46.

	DSM 1650			NBRC 108628		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,115	0,093	0,072	0,083	0,05	0,065
1	0,2	0,22	0,16	0,183	0,21	0,12
2	0,416	0,575	0,418	0,32	0,305	0,16
3	0,7	1,5	1,06	0,8	0,91	0,51
4	1,35	2,2	2,4	1,12	1,52	0,86
6	3,78	4,3	4,14	2,21	2,32	1,79
8	4,8	5,66	5,73	3,3	3,54	2,86

Figure 47. 2L Bioreactor cultures of DSM 1650 and NBRC 108628 with media adapted from [225].

ANNEX TABLE 53. FIGURE 47.

	DSM 1650			NBRC 108628		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,1	0,1	0,1	0,05	0,05	0,05
1	0,15	0,16	0,14	0,2	0,15	0,17
2	0,3	0,45	0,34	0,33	0,31	0,31
3	0,7	0,98	0,81	0,37	0,35	0,35
4	1,6	1,78	1,71	0,4	0,36	0,37
5	2,8	3,01	2,91	0,45	0,55	0,48
6	3,7	3,98	3,72	0,5	0,8	0,71
7	5,1	5,42	5,22	0,6	0,88	0,72
8	5,5	6,01	5,78	0,7	0,91	0,71
24	6,7	7,01	6,74	4,44	5,2	4,87

Figure 48. 2-L bioreactor cultures of DSM 1650 and NBRC 108628 with a complex media with YE and Tryptone as N sources.

ANNEX TABLE 54. OD_{600}.

	DSM 1650			NBRC 108628		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,2	0,2	0,2	0,2	0,2	0,2
1	0,20	0,20	0,20	0,45	0,51	0,45
3	1,01	1,12	0,98	0,9	1,1	0,98
6	4,5	4,61	4,3	2,51	2,9	2,71
7	7,2	7,33	7,12	3,73	4,4	3,81
8	9,5	9,88	9,42	5,3	6,7	5,89
24	13,4	5,01	8,2	20	23,34	22,2
48	31,5	33,32	29,98	43,2	45,1	42,9
72	20,5	19,5	20,1	34	33,2	33,04

ANNEX TABLE 55. CNCBL (MG/L)

	DSM 1650			NBRC 108628		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
48	0,23	0,31	0,25	0,490	0,42	0,47
72	0,4	0,38	0,38	0,51	0,50	0,51

Figure 49. NBRC 12391 Erlenmeyer Shake Flask Cultures with 4 different CDM compositions (1,2,3 and 4).

ANNEX TABLE 56. OD_{600}.

	Defined Media-1			Defined Media-2			Defined Media-3			Defined Media-4		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
0	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
24	0,2	0,21	0,22	0,3	0,28	0,35	0,2	0,21	0,4	0,27	0,32	0,3
48	0,66	0,51	0,57	0,57	0,45	0,61	0,4	0,39	0,61	0,54	0,53	0,57
72	1,21	0,98	1,32	1,15	1,11	1,2	0,82	1	1,02	1,03	1,04	1,15
96	2,1	1,1	1,75	1,7	1,2	1,91	1,4	1,3	1,9	1,54	1,89	1,7
168	2,3	2,1	1,98	2,1	1,9	2,4	2,01	2	2,4	2,03	2,34	2,1
192	3,01	2,345	3,24	2,8	2,6	3,1	2,6	2,34	3,1	2,54	2,98	2,8
216	6,05	4,89	6,24	5,6	5,1	6	5,2	4,9	6,04	5,12	5,78	5,6
240	13,34	12,1	12,22	12,55	11,2	11	1	9,4	11	9,85	9,99	10,1
264	26,5	23,2	21,23	25,14	22,4	21,14	21,1	18,9	20,01	18,9	19,1	20,12
288	29,1	25,4	28,1	28,7	26,8	29,1	25,4	24,9	25,3	23,4	23,5	28,7

ANNEX TABLE 57. CNCBL (MG/L)

	Defined Media-1			Defined Media-2			Defined Media-3			Defined Media-4		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
264	0,98	0,87	0,81	0,86	0,82	0,79	0,38	0,67	0,43	0,43	0,62	0,37
288	1,02	1,1	1,41	0,91	1,06	1,24	0,44	0,75	1	0,5	0,62	0,82

ANNEX TABLE 58. METABOLITES AT 288 H (G/L).

	Defined Media-1			Defined Media-2			Defined Media-3			Defined Media-4		
Metabolites (g/L)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Glucose	21,00	20,13	19,98	22,73	17,70	22,56	28,71	24,16	23,76	28,47	28,47	27,98
Acetic Acid	7,60	5,90	6,37	8,34	7,79	7,78	4,09	8,59	5,10	4,74	5,01	5,20
Propionic Acid	9,12	8,88	8,98	8,25	7,16	8,62	3,46	6,80	7,56	5,42	6,21	7,80

Figure 50. NBRC 12391 shake flask cultures with CDM and addition of different small concentrations of CSL or DMBI.

ANNEX TABLE 59. OD_{600}.

	Defined Media			Defined Media+1 g/L CSL			Defined Media+0.5 g/L CSL			Defined Media+0.2 g/L CSL			Defined Media + DMBI		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
0	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
24	0,2	0,2	0,2	0,25	0,23	0,24	0,14	0,32	0,22	0,18	0,23	0,19	0,2	0,2	0,2
48	0,4	0,4	0,4	0,55	0,61	0,58	0,4	0,45	0,43	0,38	0,36	0,369	0,4	0,4	0,4
72	0,45	0,45	0,42	0,91	1,1	0,89	0,41	0,5	0,56	0,45	0,41	0,44	0,45	0,45	0,45
96	0,7	0,7	0,69	2,72	2,92	2,6	1,75	2,01	2,1	1,5	1,23	2,34	0,7	0,6	0,7
120	2,8	1,9	2,1	4,34	4,45	4,2	3	3,1	3,2	2,8	2,45	2,67	0,122	0,14	0,21
144	3,3	2,2	3	10,75	11,23	10	7,1	7,5	8,1	8	6,9	7,34	1,98	2,02	1,02
168	4,1	3,7	3,5	20,96	20,89	21	14,2	17,5	18,2	17,2	13,5	15,89	2,99	3,03	2,1

ANNEX TABLE 60. CNCBL (MG/L).

Replicate	Defined Media	Defined Media+1 g/L CSL	Defined Media+0.5 g/L CSL	Defined Media+0.2 g/L CSL
1	0	0,85	0,94	0,6
2	0	0,94	0,87	0,774
3	0	0,88	0,91	0,67

Figure 51. NBRC 12391 cultures in Erlenmeyers Shake Flasks. In the co-culture condition, 20 mL of a culture of DSM 468 with OD_{600} values of around 5 to 6 were added to the broth at 72 culture h.

ANNEX TABLE 61. OD_{600}-

	Control			Co-culture		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,05	0,05	0,05	0,05	0,05	0,05
24	0,3	0,3	0,5	0,29	0,34	0,31
48	6,2	7	7,2	6,1	6	6,03
72	20	23	22,4	20	21	18,2
96	29	29	27,2	26	28	24,5
168	35	34	33,1	43	39	41,23

ANNEX TABLE 62. METABOLITES (G/L) AND CNCBL (MG/L).

	Acetic Acid			Propionic Acid			CNCbl		
Condition	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Control	4,52	4,48	4,49	14,89	14,35	14,44	2,61	2,21	2,03
Co-culture	3,94	4,65	4,2	13,22	15,35	13,89	2,96	2,61	3

Figure 52. NBRC 12391 cultures in Erlenmeyers Shake Flasks. 20 mL of cultures of either DSM 468 or DSM 266 with OD_{600} values of around 5 to 6 were added to the broth at 48 culture h.

ANNEX TABLE 63. OD_{600}-

	Control			Co-Culture Ralstonia			Co-Culture Acetobacter		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
0	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3
24	2,2	2,3	1,6	2,5	2,5	2,3	2,44	2,34	3
48	10,1	9,99	9,7	9,87	8,9	7,99	10,23	8,97	10,11
72	21,1	19,9	19,8	15,4	16,7	17,8	16,05	16,1	17
96	36,1	42	38	24	28	32	30	31	38
120	36,2	38	38,5	24	35	34	34	37	34
144	34,5	31	32,1	25	33,3	33,3	28	31	29,8
168	32	20	19	22	31	24	21	22	24

ANNEX TABLE 64. CNCBL (MG/L).

	Control			Co-Culture Ralstonia			Co-Culture Acetobacter		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
120	2,70	1,49	2,87	1,20	1,70	1,54	1,77	2,05	1,82
144	3,76	3,99	3,80	1,03	1,81	1,88	2,02	2,55	2,47
168	3,15	3,69	1,62	1,64	2,13	1,81	2,13	2,17	3,28

ANNEX TABLE 65. PROPIONIC ACID (G/L).

	Control			Co-Culture Ralstonia			Co-Culture Acetobacter		
Time (h)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
120	11,23	12,01	10,99	10,34	10,5	11,2	10,2	9,99	12,33
144	14,23	15,02	14,56	13,05	14,1	12,98	13,2	12,55	14,12
168	18,5	19,1	17,23	17,34	18,8	16,89	16,5	18,1	16,94

Figure 53. NBRC 12391 Erlenmeyer shake flask cultures with CSL media, and the media described in Liu et al 2021[235].

ANNEX TABLE 66. OD_{600}-

	Control			YE/CSL Media		
Tiempo (h)	**1**	**2**	**3**	**1**	**2**	**3**
0	0,05	0,05	0,05	0,05	0,05	0,5
24	1,5	1,12	1,34	1,56	1,6	1,46
48	10,1	9,87	9,99	11,5	12,02	12,5
72	23,3	25,3	24,2	25	25	28
96	29	33	31	37	36	42
120	36	37	35	42	38	46

ANNEX TABLE 67. CNCBL (MG/L).

	Control			YE/CSL Media		
Tiempo (h)	**1**	**2**	**3**	**1**	**2**	**3**
96	3,78	3,19	3,16	4,16	3,49	4,16
120	4,49	4,34	4,54	5,10	5,82	5,41

ANNEX TABLE 68. PROPIONIC ACID (G/L).

	Control			YE/CSL Media		
Tiempo (h)	**1**	**2**	**3**	**1**	**2**	**3**
96	11,49	11,13	11,5	16,15	16,8	16,73
120	15,15	15,08	15,7	19,38	19,53	19,53

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Review

Bioprocess Strategies for Vitamin B_{12} Production by Microbial Fermentation and Its Market Applications

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Abstract: Vitamin B_{12} is a widely used compound in the feed and food, healthcare and medical industries that can only be produced by fermentation because of the complexity of its chemical synthesis. For this reason, finding better producer strains and optimizing their bioprocesses have been the main focus of industrial producers over the last few decades. In this review, we initially provide a historical overview of vitamin B_{12} research and the main biosynthetic characteristics of the two microorganism families typically used for its industrial production: several strains of *Propionibacterium freudenreichii* and strains related to *Pseudomonas denitrificans*. Later, a complete summary of the current state of vitamin B_{12} industrial production as well as the main advances and challenges for improving it is detailed, with a special focus on bioprocess optimization, which aims not only to increase production but also sustainability. In addition, a comprehensive list of the most important and relevant patents for the present industrial strains is provided. Finally, the potential applications of vitamin B_{12} in different markets are discussed.

Keywords: cobalamin; *Propionibacterium freudenreichii*; *Pseudomonas denitrificans*; cyanocobalamin production

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1. Historical Overview

Vitamin B_{12}, also known as cobalamin, is a water-soluble molecule essential in many organisms' metabolism. It has a complex structure and an elaborated biosynthesis, with over 30 biotransformation steps [1]. This biosynthetic pathway is only present in some bacteria and archaea, although the phyla capable of synthetizing vitamin B_{12} are not necessarily interrelated, so mammals, and therefore humans, are unable to synthetize it.

Investigation into vitamin B_{12} began in the 1920s in connection to an illness firstly described in 1824, pernicious anemia. The main symptoms of this illness included fatigue, weight loss, headaches and, in severe cases, dementia, memory loss, muscle weakness and peripheral neuropathy, which can become lethal without treatment. In 1926, Minot and Murphy demonstrated that patients with pernicious anemia could successfully recover from the condition by a special diet with high amounts of lightly cooked liver and muscle meat [2]. They theorized that the treatment was successful because of an unknown "extrinsic factor" present in animal livers. For this discovery, they were awarded the Nobel Prize in Physiology or Medicine in 1934, although it was more than two decades before the so called "extrinsic factor" was identified and isolated. This occurred in 1948, when two research groups from pharmaceutical companies (Folkers at Merck, Sharp & Dohme, and Smith at Glaxo) isolated, almost at the same time, a cobalt compound from animal livers that was able to cure pernicious anemia on its own [3,4]. A year later, the same compound could also be isolated from other sources, such as milk, beef and several bacterial cultures. This red crystalline octahedral cobalt compound was called vitamin B_{12}. Interestingly, years later, it was discovered that this compound was in fact one of the many isoforms of the

cobalamin (Cbl) family, cyanocobalamin (CNCbl), an artificial physiologically inactive form of cobalamin generated in the industrial process of extraction and isolation from the liver. In addition, CNCbl was the first cobalamin isoform whose structure was solved in 1955 by Dorothy Hodgkin [5].

In 1957, the structure of adenosylcobalamin (AdoCbl), one of the two active forms of cobalamin, was also determined by the same group [5]. These discoveries led to Hodgkin being awarded the Nobel Prize in Chemistry in 1960. Two years later, another physiologically active vitamin B_{12} isoform, methylcobalamin (MetCbl), was discovered. Both AdoCbl and MetCbl were found to act as cofactors in several enzymes and, in the years to come, many MetCbl- and AdoCbl-dependent enzymes were isolated and described. Some of them, such as methionine synthase from *Escherichia coli* and L-methylmalonyl-CoA mutase from *Propionibacterium shermanii*, were crystallized [6,7], as well as most of the molecules responsible for vitamin B_{12} transport in mammals [8–15].

In 1973, after a long study that spanned over a decade, the complete chemical synthesis of vitamin B_{12} was described by Woodward and colleagues [16]. The process was complex, with over 60 steps, including protection and deprotection reactions, and rendered very low yields, less than 1% [16,17].

2. Structure of Cobalamin Derivatives and Functions as Enzyme Cofactors

Vitamin B_{12} is the generic name used to designate a family of compounds (cobalamins or Cbl) that share the same common structure: a tetrapyrrolic corrinic ring with a central cobalt atom coordinated to four nitrogen atoms. This scaffold is similar to other prosthetic groups, such as heme in hemoglobin or cytochrome P450. This structure allows the use of the redox state of the central metallic atom, cobalt, allowing the molecule to fulfil its different functions.

As shown in Figure 1, the corrin ring is formed by four pyrrole units (C_4H_5N), joined on opposite sides by a C-CH_3 methylene link, a C-H link on one side and two pyrrole units directly joined, missing the methine bridge between the A and D subunits present in other known porphyrins, such as hemoglobin. This structure differs from those of other, similar molecules in terms of the number and type of side chains, the oxidation state and the central metallic atom.

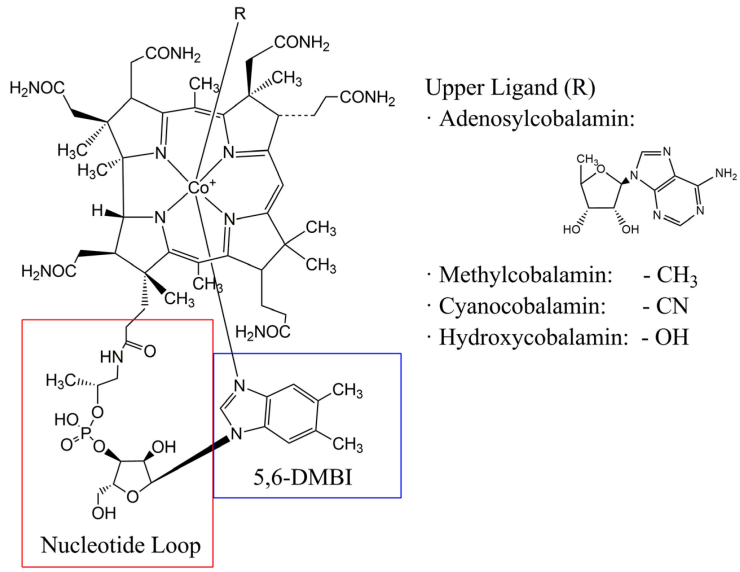

Figure 1. Schematical representation of the structure shared by all cobalamin isoforms. The main groups that usually act as the upper ligand are also shown. 5,6-DMBI: 5,6-dimethylbenzimidazole.

Besides the four N atoms of the pyrrole units, the central Co ion is linked to two other ligands. The lower ligand is the base 5,6-dimethylbenzimidazole (DMBI) linked with the central Co ion through the N7-atom in α-axial conformation. DMBI is also linked to one of the side chains of the central corrinic structure: its phosphate group joins to an aminopropanol group that is linked to the propionic acid side chain of the pyrrole unit D of the corrinic ring.

Finally, the sixth ligand is linked to Co in the β-axial position. The nature of this chemical group is variable, presenting different physiological and catalytic functions. For example, a 5-deoxyadenosyl group in this position forms adenosylcobalamin (AdoCbl), while a methyl group forms the methylcobalamin (MetCbl) isoform. In fact, the C-Co found in AdoCbl was the first bond of its type described in a biological molecule [18]. Although these two are the isoforms that present physiological activity as cofactors in humans, other forms, such as hydroxocobalamin (OHCbl) or cyanocobalamin (CNCbl), exist, as well as others with less common upper ligands, such as nitritocobalamin (NitCbl), sulfitocobalamin (SulCbl) or glutathionylcobalamin (GlutCbl) [19].

On one hand, MetCbl is a cofactor of several methyltransferases, such as methionine synthase in humans, an important cellular housekeeping enzyme that functions in two major metabolic pathways: the tetrahydrofolate-dependent one-carbon cycle and the final step in the conversion of methionine from homocysteine. On the other hand, AdoCbl is used as a cofactor by several enzymes, mostly mutases, although only one AdoCbl dependent enzyme is found in mammals: L-methylmalonyl-CoA mutase (MMCM), a critical enzyme for propionate catabolism and degradation of odd-chain fatty acids, several amino acids (valine, isoleucine, methionine, threonine) and cholesterol.

As previously stated, AdoCbl and MetCbl are the active cobalamin isoforms, but they are also known to be light sensitive [20,21]. For this reason, the most common commercial form of vitamin B_{12} is CNCbl instead, which is more stable and readily converted in the body into an active coenzyme form [22].

3. Biosynthesis of Vitamin B_{12}: The Aerobic and Anaerobic Pathways

The discovery of the structure and biological functions of the different vitamin B_{12} compounds in the 1970s focused the attention of many researchers on the biosynthetic pathways of Cbl-producing organisms. The structural complexity, as it was later established, was due to a large and convoluted biosynthesis that involves more than thirty genes and many enzymatic steps for the "de novo" synthesis of the molecule. This pathway is thought to be exclusive for some bacteria and archaea, as there is no genetic evidence of any eukaryotic organism being capable of producing any isoform of Cbl [1,23].

Although some intermediates were found and isolated earlier [24], it was not until the 1990s that the complete biosynthetic pathway was described in *Pseudomonas denitrificans* [25]. The genes involved in Cbl synthesis were given the prefix cob and a letter that refers to each gene position in the operon. In the following years, the cob enzymes and cobalamin intermediates of *P. denitrificans* were characterized and isolated by the French company Rhône-Poulenc Santé, nowadays Sanofi [26].

Later, genes involved in Cbl biosynthesis were characterized in other organisms, such as *Bacillus megaterium*, *Salmonella enterica* and the previously studied *Propionibacterium freudenreichii*. From the beginning, it was clear that the pathway found in the later organisms was similar to the one found in *P. denitrificans* but genetically different. Key differences included the lack of a monooxygenase and a different cobaltochelatase. Taking this into account, two different pathways for Cbl biosynthesis were established: (i) an aerobic or late cobalt insertion pathway, performed by *P. denitrificans* [27] and, as it was later discovered, by other microorganisms, such as *Ensifer casida* and *Sinorhizobium meliloti*; and (ii) an anaerobic or early cobalt insertion pathway, performed mainly by *P. freudenreichii*, *B. megaterium* and *S. enterica* [28].

Independently of the biosynthetic pathway, tetrapyrrole synthesis begins with the synthesis of 5-aminolaevulinic acid (ALA). Thereafter, ALA conversion to a tetrapyrrolic

macrocycle structure is performed by three different enzymatic reactions. First, an ALA dehydratase (EC 4.2.1.24), a Zn^{2+} and Mg^{2+}-dependent enzyme catalyzes the condensation reaction between two ALA molecules to form porphobilinogen (PBG) [29]. Then, a PBG deamynase (EC 4.3.1.8) polymerizes four molecules of PBG into a linear tetrapyrrole. Finally, a uroporphyrinogen III synthase (EC 4.2.1.75) is able to invert the final pyrrole unit and link it to the first pyrrole unit of the linear tetrapyrrole, forming uroporphyrinogen III, an unsymmetrical hexahydro porphyrin isomer [30]. This molecule is the last intermediate shared with other prosthetic groups, such as heme and chlorophyll groups [31].

The transformation of uroporphyrinogen III to precorrin-2, the first molecule in Figure 2, is catalyzed by an uroporhyrinogen III methyltransferase (EC 2.1.1.107), which requires S-adenosyl-L-methione (SAM) as the methyl donor. More specifically, the enzyme methylates at C-2 of the uroporphyrinogen III forming precorrin I and, after a phototrophic tautomerization, the same enzyme is able to methylate at C-7, obtaining precorrin-2, which is the last common intermediate for coenzyme siroheme, P450 and vitamin B_{12} [31,32].

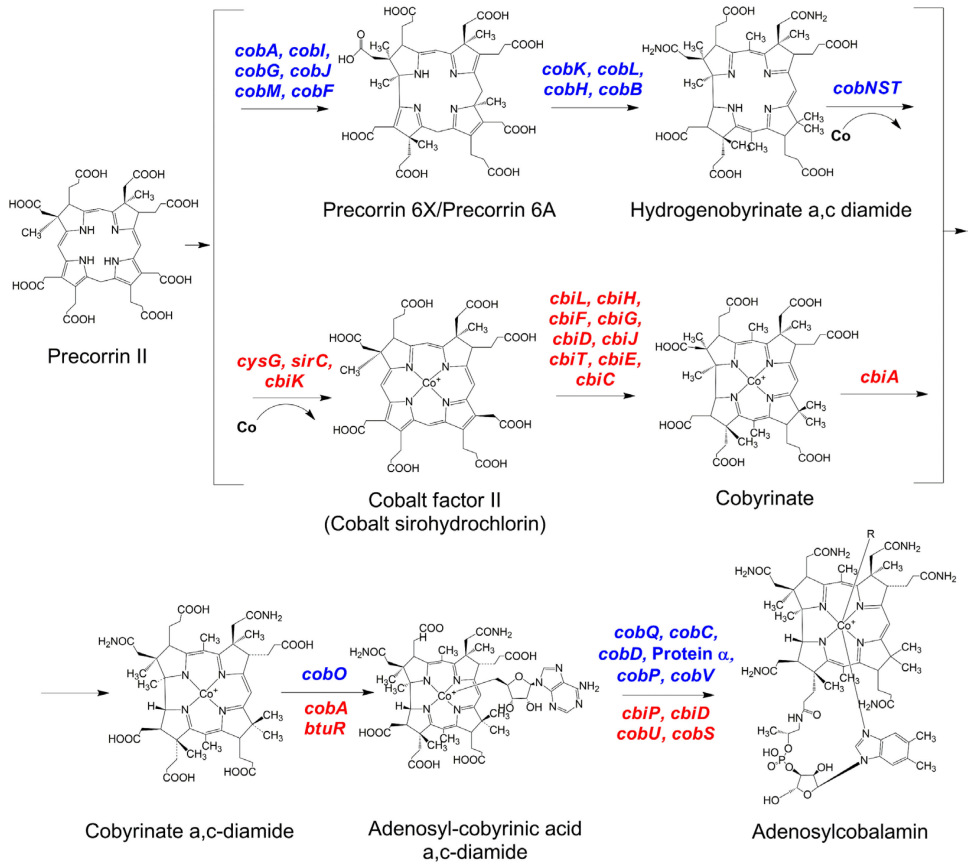

Figure 2. Summary of anaerobic and aerobic adenosylcobalamin biosynthesis. Genes encoding the proteins from the aerobic and anaerobic pathways are shown in blue and red, respectively, except for Protein α whose coding gene is not known.

The main differences between the aerobic and anaerobic pathways are in the ring contraction and cobalt chelation steps (see Figure 2). On one hand, the ring contraction in the aerobic pathway requires a molecule of oxygen plus a monooxygenase (CobG) to form precorrin-3B, a hydroxylated γ-lactone intermediate that undergoes a masked pinacol rearrangement during the ring contraction, extruding the methylated C20 position. The ring

is then totally contracted, and an acetic acid molecule is liberated in the process [28]. On the other hand, the ring contraction takes place at a later stage in the anaerobic pathway when the cobalt has already been inserted in the molecule. This step is catalyzed by the enzyme codified by the *cbiH* gene, with no molecular oxygen needed. Thereafter, a SAM-dependent methylation takes place at C17, promoting the extrusion of the already methylated C20 position and forming a δ-lactone ring [28].

Cobalt chelation is also very different in both pathways. In the aerobic pathway, there is a "late" insertion of the cobalt atom once the ring has been fully contracted. This insertion is catalyzed by an ATP-dependent multienzyme complex (*cobNST*) in the presence of magnesium [28,33]. In the anaerobic pathway, this step takes place at an earlier point on the route, when a cobalt chelatase, encoded by either *cbiX* or *cbiK* genes, catalyzes the cobalt insertion with the ring still in a non-contracted state [28].

In addition, independently of the biosynthetic pathway of the corrin ring, DMBI is produced separately to be later attached in α-axial conformation. The lower ligand synthesis has been described recently and also presents two clearly differentiated routes (the aerobic and the anaerobic pathway), depending on the needs of oxygen.

On one hand, the aerobic biosynthesis of DMBI is catalyzed by the 5,6-dimethylbenzimidazole synthase BluB (EC 1.13.11.79), which performs the fragmentation and contraction of the bound flavin mononucleotide cofactor and the cleavage of the ribityl tail to form DMBI and D-erythrose 4-phosphate in the presence of molecular oxygen. Later, the phosphoribosyltransferase CobU/T (EC 2.4.2.21) introduces the DMBI via a nucleophilic substitution reaction [34]. This pathway was firstly described for *S. meliloti* [35] and later found in the majority of Cbl-producing bacteria [36], including the two most important industrial producers, *P. freudenreichii* [37] and *P. denitrificans* [38]. This fact highlighted the incapacity of *P. freudenreichii* to completely produce Cbl anaerobically without any external addition of DMBI.

On the other hand, the anaerobic biosynthesis of DMBI is catalyzed by the gene products of the *bzaA-bzaB-cobT-bzaC-bzaD-bzaE* operon, which promote the formation of DMBI with 5-hydroxybenzimidazole, 5-methoxybenzimidazole and 5-methoxy-6-methylbenzimidazole as intermediates. This route was described in the obligate anaerobic bacteria *Eubacterium limosum* [39] and *Acetobacterium woodii* [34].

4. Microbial Production of Vitamin B_{12}: Bioprocess Optimization for Cyanocobalamin Production

The demand for cobalamin by the food, beverage, dietary and nutraceutical industries has increased sharply in recent years due to the increased health awareness of the general population as well as the growing popularity of alternative diets, such as vegan and vegetarian diets. For this reason, many efforts have been made in strain and process optimization for cyanocobalamin production over the years [1,40].

Besides the cobalamin forms already mentioned, AdoCbl, MetCbl and CNCbl, there are many other cobamides with different lower axial ligands that act as key cofactors for corrinoid-dependent enzymes that are important, for example, for gut microbiota [41,42]. Despite their importance, this review is focused only on cyanocobalamin, the form that can be absorbed and used by humans and which is currently produced industrially.

Historically, the microorganisms used for cobalamin production at industrial scale were strains with high natural productivity, mainly different strains of *P. freudenreichii* and *P. denitrificans*, as well as related strains, such as *Pseudomonas nitroreducens* and *E. casida* [1,40].

For many years, a common strategy to improve these strains has been the usage of random mutagenesis techniques to increase vitamin B_{12} productivity or resistance to toxic intermediates present in media [43]. Nevertheless, overexpression of genes involved in cobalamin biosynthesis [44], heterologous expression of foreign genes [45] and downregulation [46] of several genes have also generated better producer strains. Furthermore, it is worth noting the appearance of new productive strains with promising results, such as *B. megaterium* [47] and *Acetobacter pasteurianus* [48], and the heterologous expression

of the biosynthetic pathway in other well-known cell platforms, such as *E. coli*, the later extensively reviewed in Fang et al.'s 2017 study [40]. Recently, Balabanova and co-workers have extensively reviewed the genetic and biosynthetic regulation as well as the genetic tools that have been used with the aim of improving cobalamin production in different cell factories [36].

In contrast, there are also many examples of advances in vitamin B_{12} microbial production by bioprocess optimization. Table 1 summarizes the most relevant innovations performed at a bioprocess level for increased Cbl production and new strategies for Cbl production in new platforms or media. Studies focused on increased production via genetic engineering of known strains are not presented because the objective of this section is to provide an update on the main bioprocess innovations for biotechnological cobalamin production. A summary of culture conditions, media specifications and volumetric productions and productivities is included.

Table 1. Summary of reported productions with the industrial cobalamin producing strains.

Microorganism/Strain	Main Media Components	Scale	Summary/Innovation	Volumetric Production	Volumetric Productivity (mg/L/h)	Reference
B. megaterium DSM 319	Terrific broth media	250 mL shake flask	Precursor supplementation and pO_2 control	0.21 mg/L [c]	0.006 mg/L/h [c]	[47]
Lactobacillus reuteri ZJ03	Soymilk	250 mL shake flask	Different carbon source supplementation	0.204 mg/L	0.003 mg/L/h	[49]
P. freudenreichii subsp. *shermanii* NRRL-B-4327, 3523 and NRRL-B-3524	Sodium lactate broth	250 mL shake flask	Vitamin B_{12} analogue addition	31 mg/L	0.51 mg/L	[50]
P. freudenreichii CICC 10019	Glucose, CSL [a]	7 L stirred tankbioreactor	Expanded-bed bioreactor (EBAB) with crop stark hydrolysates	47.6 mg/L	0.18 mg/L/h	[51]
P. freudenreichii CICC 10019	Glucose, CSL	7 L stirred tank bioreactor	EBAB bioreactor	43.4 mg/L	0.27 mg/L/h	[52]
P. freudenreichii CICC 10019	Glucose, CSL	1.5 L stirred tank bioreactor	EBAB bioreactor and DMBI addition	58.8 mg/L	0.59 mg/L/h	[53]
P. freudenreichii CICC 10019	Glucose/glycerol, CSL	5 L stirred tank bioreactor	EBAB bioreactor, glycerol as carbon source and crop stalk hydrolysate as nitrogen source	43 mg/L	0.36 mg/L/h	[54]
P. freudenreichii DF13	Supplemented whey permeate	1 L stirred tank bioreactor	Co-culture with *Lactobacillus plantarum* SM39 for simultaneous folate and Cbl production	0.75 mg/L	0.004 mg/L/h	[55]
P. freudenreichii DSM 20271//*Lactobacillus brevis* ATCC 14869	Wheat bran dough	n.d. [b]	Co-fermentation in wheat bran dough for in situ production of Vitamin B_{12}	332 ng/g [c]	n.d. [b]	[56]

Table 1. *Cont.*

Microorganism/Strain	Main Media Components	Scale	Summary/Innovation	Volumetric Production	Volumetric Productivity (mg/L/h)	Reference
P. freudenreichii IFO 12424//*Ralstonia eutropha* H16 (ATCC17699)	Polypeptone, casein, yeast extract	5 L stirred tank bioreactor	Cell recycling system and co-culture with *Ralstonia eutropha* for decreasing propionic acid inhibition	8 mg/L [c]	0.14 mg/L/h [c]	[57]
P. freudenreichii PTCC 1674.	Tryptone, yeast extract, different carbon sources	100 cm^3	Waste frying sun oil as a carbon source for vitamin B_{12} production	2.74 mg/L	0.02 mg/L/h	[58]
P. freudenreichii subsp. *shermanii* ATCC 13673	Glucose, yeast extract	2 L stirred tank bioreactor	Inoculum volume, pH control and substrate concentration optimization	0.087 mg/L	0.002 mg/L/h	[59]
P. freudenreichii subsp. *shermanii* CICC 10019	Glucose, CSL	100 L fermenter	Addition of DMBI precisely with Ado-Cbl control strategy	39.15 mg/L	0.32 mg/L/h	[60]
P. freudenreichii subsp. *shermanii*	Glycerol, tryptone, casein, DMBI	200 mL shake flask	Media optimization by design of experiments with crude glycerol as the main carbon source	4.01 mg/L	0.024 mg/L/h	[61]
P. freudenreichii subsp. *shermanii*	Whey based media	20 mL tubes	DMBI, Nicotinamide and Riboflavin supplementation	5.3 mg/L	0.03 mg/L/h	[62]
P. freudenreichii subsp. *shermanii*	Food-like media (cereal matrices)	n.d. [b]	Precursor supplementation in different cereal-like matrices	1.5 mg/Kg	0.009 mg/Kg/h	[63]
P. freudenreichii subsp. *shermanii* 2067	Cheese-based propionic media/whey-based liquid media	50 mL shake flask	Production in food-like conditions without DMBI addition	0.124 mg/L [c]	0.0013 mg/L/h	[64]
P. freudenreichii CICC10019	Glucose, yeast extract, CSL	100 mL flasks	Media optimization by statistical analysis	8.32 mg/L	0.068 mg/L/h	[65]
P. freudenreichii CICC10019	Glucose, CSL	7 L fermenter	Membrane separation-coupled fed-batch fermentation	21.6 mg/L	0.16 mg/L/h	[66]
P. denitrificans	Maltose, peptone, betaine	250 mL shake flask	Addition of rotenone as a respiration inhibitor for enhanced production	54.7 mg/L	0.57 mg/L/h	[67]
P. denitrificans	Beet molasses, sucrose, betaine	120 m^3 fermenter	Glucose-betaine feeding, pH control strategy	214.13 mg/L [c]	1.27 mg/L/h [c]	[68]
P. denitrificans	Glucose, CSL, betaine	120 m^3 fermenter	Stepwise oxygen uptake rate control strategy	188 mg/L	1.12 mg/L/h	[69]

Table 1. *Cont.*

Microorganism/Strain	Main Media Components	Scale	Summary/Innovation	Volumetric Production	Volumetric Productivity (mg/L/h)	Reference
P. denitrificans	Glucose, CSL, betaine	50 L fermenter	Effects of specific oxygen consumption rate on cell morphology and production	213.1 mg/L	1.88 mg/L/h	[70]
P. denitrificans	Maltose, peptone, betaine	250 mL shake flask	Betaine supplementation	58.61 mg/L	0.48 mg/L/h	[71]
P. denitrificans	Maltose syrup, CSL, betaine	120 m^3 fermenter	Maltose syrup and CSL as the main substrates	198.27 mg/L	1.10 mg/L/h	[72]
P. denitrificans	Glucose, CSL, betaine	120 m^3 fermenter	pO_2 stepwise control	198.80 mg/L	1.18 mg/L/h	[73]

The main microorganism, strain, scale and media compounds are shown as well as a brief summary of the main innovation and the volumetric productions. Volumetric productions are presented in mg/L. Volumetric productivities were calculated using data from the original publications. [a] CSL: corn steep liquor; [b] n.d.: not determined; [c] Values were converted to mg/L or mg/L/h using the data available from the original publication.

Overall, sharp differences in cobalamin production can be found amongst different producing microorganisms. In this sense, volumetric productions and productivities obtained with *P. denitrificans* are clearly superior to the ones obtained with other producers, while *P. freudenreichii* productions vary widely between strains and culture conditions. In the case of the latter, strategies based on decreasing the propionic acid inhibitory effect seem to be the most effective.

4.1. Microbial Production in Pseudomonas denitrificans

P. denitrificans is a Gram-negative bacterium that uses the aerobic biosynthetic pathway to produce vitamin B_{12}. Despite not having a Generally Recognized as Safe (GRAS) status, *P. denitrificans* is currently the main vitamin B_{12} producer used by industrial manufactures, such as Sanofi in Europe [74] or the Huarong Pharmacy Corporation in China.

On one hand, the Sanofi strain was originally generated by a combination of random mutagenesis and molecular biology techniques and, although no official information about its volumetric production is available, taking into consideration other aerobic strains, it is tempting to speculate that it may produce around 200–300 mg/L [72]. The optimized strain was originated from a natural, high-producing strain known as MB-580, first described and patented in 1962 (US3018225A). Over several years, the French company Rhône-Poulenc amplified several of the cob genes involved in vitamin B_{12} biosynthesis until certain high-producing strains were created—SBL27 and, eventually, SC510 [26]. Sanofi, former Rhône-Poulenc, is now the main European vitamin B_{12} manufacturer.

The dominant worldwide producers of vitamin B_{12} on the market are, however, based in China and include the North China Pharmaceutical Company, the Henan Luyuan Pharmaceutical Company, the Hebei Yuxing Bio-Engineering Company and the Chinese CSPC Huarong Pharmaceutical Company, with a combined reported vitamin B_{12} production in 2020 of approximately 31.41 tons and an estimated value of USD 339.8 million [75]. The origin of the strains used in their industrial productions is not precisely known but assumed to be an aerobic strain due to different publications from research groups related to the Huarong Pharmaceutical Company [69,73,76], and the latest patents on bioprocess optimization with *P. denitrificans* presented in China claim volumetric productions of up to 281 mg/L (see Table 2).

Besides genetic modifications, vitamin B_{12} productivity improvement in *P. denitrificans* has also been achieved with culture media optimization and changes in bioprocessing

conditions. For example, the effects of trace elements in media, pH, dissolved oxygen control and the addition of several supplements have been tested. In this sense, the addition of Zn^{2+} has been reported to have a significant positive effect on the synthesis of ALA and PBG, two of the main precursors of cobalamin, while supplementation with Co^{2+} and DMBI, the base that is incorporated into the nucleotide loop, positively affects production [77]. Optimization of the initial amounts of these three compounds by design of experiments led to a 13% increase in cobalamin production [77].

Media composition affected the pH stability of cultures and showed a significant effect on vitamin production. In order to better control pH, a feeding strategy with glucose as carbon source and betaine as methyl donor was developed and was found to be beneficial for vitamin production when applied to 120 m^3 bioreactor cultures [69,73,76]. Moreover, although it is well known that betaine acts as a methyl donor for vitamin B_{12} biosynthesis [78] and enhances the formation of several key intermediates, such as ALA, glutamate, glycine and methionine [71], high concentrations of betaine can also inhibit cell growth [71,76]. Therefore, a proper betaine feeding strategy was further developed to balance the negative effect on cell growth and the positive effect on cobalamin production and was later successfully implemented at industrial scale [76].

Oxygen transfer rate (OTR) has also been a major subject of bioprocess optimization in *P. denitrificans*. Higher OTRs during initial culture stages enhance cell growth, while lower OTRs in later stages were found to be critical for higher productivity [69]. Later studies revealed that the increased production observed in lower oxygenation conditions can be related to alterations in cell morphology, stimulating change from the cell growth phase to an elongation state that presents higher vitamin B_{12} production [70]. Taking this into consideration, several multi-step dissolved oxygen control strategies were developed, in which aeration and agitation were gradually reduced until dissolved oxygen values fell below 2%, obtaining an improvement in production around 17% [69,73]. Furthermore, the addition of respiratory chain inhibitors, such as rotenone, could also enhance vitamin production despite a detrimental impact on cell growth [67].

Finally, different carbon and nitrogen sources, such as glucose, maltose syrup, beet molasses and corn steep liquor, have been tested as cheaper alternatives to more expensive refined sucrose and glucose. Some of these compounds could negatively affect pH stability and therefore the final vitamin production [68]. Nevertheless, a combination of maltose syrup, corn steep liquor and betaine has been reported as a successful and cheaper alternative to the traditional media compositions [72].

4.2. Microbial Production in Propionibacterium freudenreichii

P. freudenreichii strains comprise Gram-positive rod-shaped bacteria named after their capacity to synthesize large quantities of propionic acid by the Wood–Werkman pathway. In contrast to aerobic vitamin B_{12} producers, *P. freudenreichii* has the advantage of GRAS status having been granted by the FDA and Qualified Presumption of Safety (QPS) status granted by the EFSA.

Some genetic engineering approaches were tested in *P. freudenreichii* to obtain higher amounts of vitamin B_{12}. For example, the overexpression of some of the main genes involved in cobalamin synthesis [44] and a genome shuffling approach [79] were reported to improve cobalamin production. However, the main industrial strains were usually obtained by random mutagenesis using different mutagenic agents, such as UV light or chemical compounds, to obtain better cobalamin producers. In *P. freudenreichii*, these high-yield strains usually present higher tolerance and resistance to propionic acid [43].

P. freudenreichii are facultative anaerobic strains that follow the anaerobic biosynthetic route for cobalamin production. Despite their only producing high cobalamin yields at very low-oxygen conditions, oxygen is needed for DMBI synthesis and its attachment to the corrinic ring [80]. For this reason, the culture is usually divided into two stages: a first stage in which the cells are cultured in complete anaerobic conditions and a second stage, usually

after 72–96 h of cultivation [59,62,80], in which gentle aeration is provided by agitation to generate the microaeration needed for DMBI synthesis and cobalamin production [74].

The GRAS status of these vitamin B_{12} producers allowed the expansion of their market scope by allowing their direct use in the production of food products. In this sense, in situ food fortification with *P. freudenreichii* has been successfully tested using food-like media, such as in cheese-like propionic medium or whey-based liquid medium [62,64], cereal matrices [63] and in situ fortification of tempeh [81]. Although final cell densities and reported production levels are low compared to other, traditional media, in the context of food fortification, it allows an increase in cobalamin content using non-traditional sources and the achievement of the recommended daily vitamin B_{12} consumption levels with only small amounts of fermented products [64].

As mentioned before, these bacteria have the ability to produce large quantities of propionic acid, which eventually becomes toxic and limits cell growth [52]. Therefore, several bioprocess optimization strategies for decreasing propionic accumulation have been tested. In particular, in situ product removal (ISPR) techniques have shown promising results for the simultaneous production of propionic acid and vitamin B_{12}. Among ISPR techniques, the use of expanded-bed adsorption bioreactors (EBABs) with high biocompatibility resins, such as ZGA330, has been reported to support vitamin B_{12} volumetric production levels between 40 mg/L and 60 mg/L [52]. In EBABs, adsorption occurs when the column is expanded, allowing the culture to pass through the chromatographic column without clogging, while propionic acid is retained in the resin [54]. Different culture conditions [54], carbon and nitrogen sources [51] and the addition of media supplements, such as DMBI [53], have been tested for the simultaneous improvement of propionic acid and vitamin B_{12} production. In an EBAB system, the combination of glucose and glycerol [54] and corn stalk hydrosylates [51] have proven to be efficient carbon sources, with reported volumetric CNCbl production levels of 43.2 mg/L and 47.6 mg/L, respectively.

Another interesting approach to decrease propionic acid concentration is the co-fermentation of *P. freudenreichii* with other microorganisms capable of metabolizing propionic acid. For example, the co-culture of *P. freudenreichii* and *Ralstonia eutropha* showed an improved cobalamin production from 6.73 mg/L to almost 19 mg/L [57]. Moreover, co-fermentation has also been successfully applied not only to reduce propionic acid but also to either produce more than one product simultaneously or to fortify other cell cultures. Simultaneous production of both folate and vitamin B_{12} was achieved with the co-cultivation of *P. freudenreichii* and *Lactobacillus plantarum* (currently named *Lactiplantibacillus plantarum* [82]) [55], and a co-fermentation of a *Basidiomycota* strain plus *P. freudenreichii* has been recently patented to simultaneously produce vitamin D and B_{12} [83]. A food fortification example would be the in situ vitamin B_{12} production in bread dough performed in whey-based media with the co-cultivation of *P. freudenreichii* and *Lactobacillus brevis* (currently named *Levilactobacillus brevis* [82]) to ensure microbial safety and stability [56].

Supplementation with cobalamin precursors is another common strategy for increasing productivity. The addition of common precursors and needed compounds, such as ALA and Co^{2+}, has often been described as beneficial for vitamin production [1]. Although all *P. freudenreichii* strains are capable of synthetizing DMBI on their own, the biosynthesis of this base is low. Moreover, DMBI formation is not possible in strictly anaerobic conditions, as oxygen is needed for its synthesis [37]. If the availability of DMBI is restricted, the active form of vitamin B_{12} is not formed, and the cells begin to accumulate incomplete forms, such as cobinamide or pseudovitamin B_{12}. Thus, the addition of DMBI or even DMBI precursors, such as Riboflavin or Nicotiamide, has been consistently reported as a positive factor in cobalamin production [1,40,53,60,62,64]. In addition, other groups have found that the addition of vitamin B_{12} analogues can decrease feedback inhibition and increase cobalamin production [50].

Finally, *P. freudenreichii* cultures are also interesting in industrial settings for their ability to grow in a wide range of complex carbon and nitrogen sources and even waste and spent media, such as molasses [84], crude glycerol [61], waste frying sunflower oil [58],

tomato pomace [85], liquid acid protein residue of soybean [86] and vegetable juice spent media [87].

5. Vitamin B_{12} Downstream Processing and Post-Modification Strategies

Recovery of vitamin B_{12} is a well-described process and, to the best of the authors' knowledge, has remained unchanged during the past decades at industrial scale (see Figure 3 for a classical bioprocess scheme). Briefly, culture broth is subjected to several separation and purification steps (including extraction, filtration and adsorption processes) which impact on overall process yield and feasibility. Classical downstream processing starts with a biomass concentration to significantly reduce the volume, normally performed by centrifugation. Nevertheless, depending on the bioprocess, Cbl can also be found extracellularly, so purification may start from whole broth.

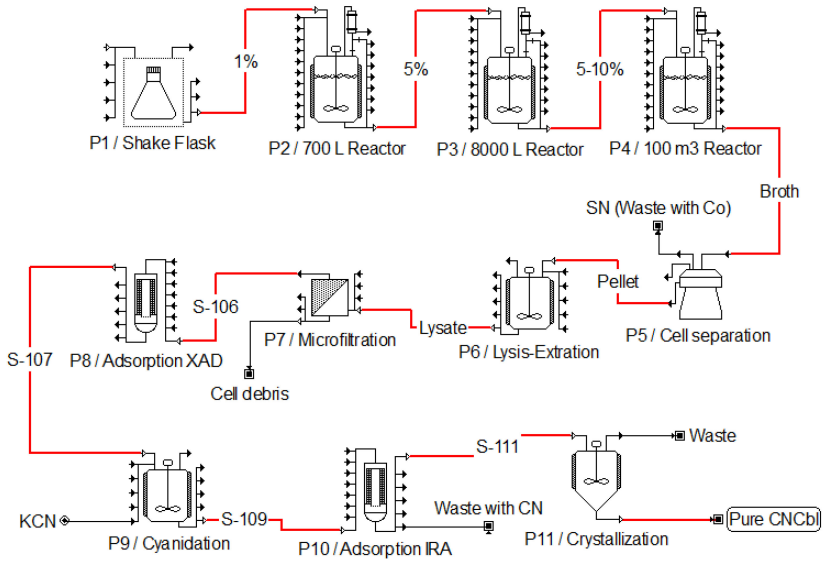

Figure 3. Classical bioprocess to obtain highly pure Cyanocobalamin. The main CNCbl stream is highlighted in red. Intermediate storage vessels are omitted to simplify the figure. P1 to P11 represent Process 1 to Process 11, respectively. Bioprocess represented using SuperPro Designer® V9 Academic Site Edition, Intelligen, Inc. (Scotch Plains, NJ, USA).

Either way, all species of corrinoids are extracted by heating at 80–120 °C and a pH of 6.5–8.5 for 10–30 min. Cyanidation can be performed during the extraction process or after the initial filtration and adsorption steps [74,88]. In both cases, the different corrinoids are transformed into CNCbl by the addition of potassium cyanide or thiocyanate. This process is usually performed in the presence of sodium nitrite and heat [80].

Later, CNCbl solution is subsequently clarified by one or more filtration (microfiltration and/or nanofiltration) and adsorption (XAD resin) processes. If the produced Cbl is directed to animal feed, the vitamin solution is often treated with zinc chloride and precipitated with organic solvents, such as acetone, to obtain the final product [89]. When greater purity is required, for example, for pharmaceutical uses, further adsorption steps with different resins (e.g., IRA, Alumina) are often needed to obtain a pure final product. Figure 3 represents a classical bioprocess to obtain highly pure CNCbl.

Once vitamin B_{12} is purified, it may undergo different post-modifications to be used as a food supplement or oral pharmaceutical in order to enhance its bioavailability. Protecting these compounds can be especially interesting in cases of a non-functional intrinsic factor, which causes very low Cbl bioavailability [90,91]. In this sense, several techniques have been developed to protect oral supplements against specific conditions found in the

gastrointestinal environment [92]. Among them, microencapsulation, which is already widely used in pharmaceutical and cosmetic industries [93], has proved to improve vitamin B_{12} stability using either food-grade $W_1/O/W_2$ emulsions [94,95], liposomes [96] or different food-grade encapsulating agents, such as chitosan, arabic gum, sodium alginate, carrageenan, maltodextrin, modified starch, cyanobacterial extracellular polymeric, xanthan and pectin [97,98]. Moreover, Fidaleo and co-workers have recently reviewed nanocarrier usage as a promising nanotechnology that may enable vitamin B_{12} therapies to be improved, reducing side effects and overall costs as well as ameliorating the quality of patient lives [99].

6. Patents—State of the Art

Research in cobalamin production has been extensively patented since its very beginning, with thousands of patents being published, although most of them are no longer active. Due to the large number of publications and the fact that nowadays most of the production and industrial advances are being made in China, providing a comprehensive list of all the currently active and used patents is difficult and beyond the scope of this review. Instead, Table 2 aims to provide a historical overview of some of the most important and relevant patents for the current industrial strains. We include the state of each patent—as expired, abandoned, or active—in addition to the main innovations claimed and, if available, volumetric production figures.

In 1962, one of the first relevant patents related to the subject after the discovery of the extrinsic factor was US3018225A [100], where the discovery of a natural, high-producing strain (*P. denitrificans* MB580) was described. This strain was extensively researched and many high-producing strains, such as SC510, were obtained through genetic engineering approaches, as described in US20060019352A1 [101]. In fact, researchers associated with Rhône-Poulenc used MB580 and its derived strains to study the genes behind aerobic Cbl biosynthesis and presented the complete aerobic biosynthetic pathway in 1990 [26]. Nowadays, the precise aerobic strains used for industrial cobalamin production are not known but are thought to be closely related to SC510. More recent aerobic strain-related patents cover all stages of bioprocess development: (i) screening and identification of new producer strains (CN111254173 A [102]), (ii) media and bioprocess optimization (CN108949866 A [103], CN110205350 A [104], CN109837320 A [105]) as well as (iii) downstream processing (CN111808158 A [106]).

On the other hand, most of the earliest anaerobic- and *P. freudenreichii*-related patents were focused on strain optimization for CNCbl production. In this sense, one of the most significant early patents is US4544633A [43], where the generation of a propionic-resistant producing strain by random mutagenesis is described. Besides strain enhancement, later patents often focused on bioprocess optimization and the use of bed-expanded bioreactors for the simultaneous production of CNCbl and other compounds of interest, such as propionic acid (US6492141B1 [107]). In addition, the possibility of using Cbl-producing strains of *P. freudenreichii* as probiotics has also been patented (US7427397B2 [108]). Interestingly, the latest patents related to anaerobic strains are focused on either co-cultivation strategies (US9938554 [109], US20200149084A1 [83]) or co-production (CN206828509U [110], IN201827044769 A [111]). The latter patent, IN201827044769 A [111], claims a volumetric production of 76.13 mg/L, which is the maximum production reported for a *P. freudenreichii* strain.

Finally, there are a number of patents with alternative producing strains, such as *B. megaterium* (US2576932A [112]), several *Lactobacillus* strains (WO2011154820A2 [113]), *S. meliloti* (CN104342390 A [114], CN110804598 A [115]) and even *E. coli* (WO2019109975A1 [116]). The production levels of most of these microorganisms are quite low compared to the traditional producers, and patents are often focused on strain identification or strain enhancement by genetic engineering or heterologous expression of the main genes involved in Cbl biosynthesis. However, the exception is the *S. meliloti* strain (CGMCC 9638), which has a vitamin B_{12} production level in the range of 50–115 mg/L [115].

Table 2. Main patents related to vitamin B_{12} production.

	Patent Application Number (Reference)	Name	Microorganism/Strain	Innovation	Volumetric Production	Year
Propionibacterium genus	US4544633A [43] (Expired)	Process for producing vitamin B_{12} by the fermentation technique, and vitamin B_{12}-producing microorganism	*P. freudenreichii* (IFO 12424, IFO 12391, IFO 12426)	Creation of propionic-resistant strains (*P. freudenreichii* FERM-86 and FERM-87) for enhanced CNCbl production	15 mg/L	1983
	US6492141B1 [107] (Expired)	Process for the production of vitamin B_{12}	*P. freudenreichii* CBS 929.97	O_2 effect in production during the anaerobic phase and a "fill and draw" strategy for enhanced production	19 mg/L	1999
	US6187761B1 [117] (Expired)	Production and use of compositions comprising high concentrations of vitamin B_{12} activity	*P. freudenreichii* subsp. *shermanii* and *P. denitrificans*	Method for producing vitamin B_{12} and making highly concentrated compositions	10 mg/L	1999
	US7427397B2 [108] (Expired)	Probiotic *Propionibacterium*	*Propionibacterium jensenii* 702	*Propionibacterium jensenii* as a probiotic	0.0012 mg/L	2004
	EP2376644B1 [118] (Active)	Process for the preparation of a fermentation broth	*Lactobacillus plantarum* DSM 22,118 and *P. freudenreichii* DSM 22120	Fermentation media optimization and co-culture for folate and vitamin B_{12} production	1.07 mg/L	2009
	CN206828509U [110] (Active)	A device for producing propionic acid and co-producing vitamin B_{12} by semi-continuous fermentation	*P. freudenreichii*	Simultaneous production of propionic acid and vitamin B_{12} in a semicontinuous fermentation with propionic acid separation	20.12 mg/L	2017
	US9938554 [109] (Active)	Co-cultivation of *Propionibacterium* and yeast.	*P. freudenreichii* (ATCC 6207) and yeast cells (DSM 28271)	Co-culture of *Propionibacterium* and propionic-resistant yeast to decrease the chemical oxygen load (COD) of spent media	16 mg/L	2018
	US20200149084A1 [83] (Active)	Sequential co-culturing method for producing a vitamin- and protein-rich food product	*Basidiomycota* and *P. freudenreichii*	Co-culture of *Basidiomycota* genus strains and vitamin B_{12}-producing strains for in situ food fortification	0.0014 mg/L [1]	2020
	IN201827044769 A [111] (Active)	Continuous process for co-production of vitamin B_{12} and organic acids	*P. freudenreichii* (ATCC 13673)	Co-production of vitamin B_{12} and organic acids in a continuous fermentation with a single bioreactor	76.13 mg/L	2020
	WO21041759 A1 [119] (Active)	Modified *Propionibacterium* and methods of use	*P. freudenreichii* (P. UF 1)	Generation of a vitamin B_{12}-overproducing strain by introducing a mutation that decreases the activity of the cbiMcbl riboswitch	n.d. [2]	2021

Table 2. *Cont.*

	Patent Application Number (Reference)	Name	Microorganism/Strain	Innovation	Volumetric Production	Year
Pseudomonas denitrificans	US3018225A [100] (Expired)	Production of vitamin B_{12}	*P. denitrificans* MB-580	A process for vitamin B_{12} production with a high-yield strain (*P. denitrificans* MB-580)	2.4 mg/L [1]	1962
	US20060019352A1 [101] (Abandoned)	Methods for increasing the production of cobalamins using cob gene expression	*P. denitrificans*	Overexpression of several genes involved in Cob biosynthesis; generation of several overproducing strains, such as SC-510	65 mg/L	1990
	US6156545A [120] (Expired)	Biosynthesis method enabling the preparation of cobalamins	*P. denitrificans* G2650	Enhanced Cob production by the heterologous overexpression of precursors, such as DMBI and O-phospo-L-threonine	7.9 mg/L	1996
	CN101538599A [121] (Active)	Method for improving the yield of denitrified pseudomonas vitamin B_{12}	*P. denitrificans J741*	Enhance cob production by betaine addition optimization	177.49 mg/L	2008
	CN102399845A [122] (Active)	Vitamin B_{12} fermentation production control process based on CO_2 concentration in tail gas	*P. denitrificans* MB-580	Vitamin B_{12} enhanced production through a carbon dioxide control strategy during fermentation	164.6 mg/L	2010
	CN101748177 A [123] (Active)	Optimized method for producing vitamin B_{12} through *P. denitrificans* fermentation and synthetic medium	*P. denitrificans*	Development and optimization of media and bioprocess conditions for improved vitamin B_{12} production	77 mg/L	2010
	CN102021214 A [124] (Active)	Oxygen consumption rate-based vitamin B_{12} fermentation production control process	*P. denitrificans*	Vitamin B_{12} production optimization through an oxygen control strategy	171,4 mg/L	2011
	CN102453740 A [125] (Active)	Culture medium for producing vitamin B_{12} by fermenting *P. denitrificans* and fermentation method thereof	*P. denitrificans*	Use of artificial molasses and bioprocess optimization for a more stable fermentation yield	198 mg/L	2012
	CN108949866 A [103] (Active)	Multi-stage rotating speed regulating policy for improving *P. denitrificans* fermentation for production of vitamin B_{12}	*P. denitrificans*	Vitamin B_{12} production improved by optimization of the culture media and the stirring speed of the bioprocess	246 mg/L [1]	2018
	CN108913739 A [126] (Active)	Method for producing vitamin B_{12} by using *P. denitrificans* based on pH value control	*P. denitrificans*	Improved vitamin B_{12} production by optimization of the bioprocess through pH value control	248 mg/L	2018

Table 2. *Cont.*

	Patent Application Number (Reference)	Name	Microorganism/Strain	Innovation	Volumetric Production	Year
	CN110205350 A [104] (Active)	Method for improving the yield of vitamin B_{12} based on the regulation of ammonia nitrogen index	*P. denitrificans*	A method for improved Cbl production by supplementation with yeast extract controlled by the ammonia nitrogen index	167 mg/L [1]	2019
	CN109837320 A [105] (Active)	Method for promoting *P. denitrificans* to generate vitamin B_{12}	*P. denitrificans*	Optimization of media and culture conditions for improved vitamin B_{12} production	198 mg/L	2019
	CN111808158 A [106] (Active)	Preparation method of vitamin B_{12} crude product	*P. denitrificans*	Downstream process improvement for AdoCbl extraction	n.d. [2]	2020
	CN111254173 A [102] (Active)	Screening method and screening culture medium for bacterial strains for high yield of vitamin B_{12} produced through fermentation production with *P. denitrificans*	Several high-yield strains of *P. denitrificans*	Screening for high-vitamin B_{12} producing *P. denitrificans* strains and culture medium screening for high vitamin B_{12} production	281 mg/L [1]	2020
Other producers	US2650896A [127] (Expired)	Cyanide ions in production of vitamin B_{12}	*Streptomyces griseus*	Effects of cyanide ions in B_{12} production	Biological assay	1953
	US2576932A [112] (expired)	Fermentation process to produce vitamin B_{12}	*B. megaterium* B-938	Vitamin B_{12} production with *B. megaterium* in a nutrient media with sucrose	0.45 mg/L	1983
	US20050227332A1 [128] (Expired)	Method for producing vitamin B_{12} from hydrogen-metabolizing methane bacterium	A mesophilic methane bacterium obtained from digested sludge	The culture is acclimatized in a H2–CO media and grown in an immobilized bed bioreactor	25.2 mg/L	2005
	US20060105432A1 [129] (Abandoned)	Method for the production of vitamin B_{12}	*B. megaterium* DSMZ509	Genetically modified *B. megaterium* strain	0.008 mg/L [1]	2006
	WO2011154820A2 [113] (Application granted)	Vitamin B_{12}-producing probiotic bacterial strains	*Lactobacillus reuteri* (DSM 17938, DSM 16143, ATCC 55730)	In situ food fortification for increased vitamin B_{12} production with *Lactobacillus reuteri* strains	0.018 mg/L [1]	2011
	CN104342390 A [114] (Active)	*Sinorhizobium meliloti* strain and composition and application of *Sinorhizobium meliloti* strain	*S. meliloti* (CGMCC 9638)	A *S. melitolli* strain capable of producing vitamin B_{12} and optimization of the bioprocess for vitamin B_{12} production	At least 50 mg/L	2015
	WO2019109975A1 [116] (Active)	Recombinant strain of *Escherichia coli* for de novo synthesis of vitamin B_{12}, construction method therefor and application thereof	*E. coli*	Recombinant *E. coli* for the de novo synthesis of vitamin B_{12}	89 μg/g DCW	2019

Table 2. *Cont.*

Patent Application Number (Reference)	Name	Microorganism/Strain	Innovation	Volumetric Production	Year
CN110804598 A [115] (Active)	Procorrin-2C(20)-methyltransferase mutant and mutant gene and application thereof in preparing vitamin B_{12}	*Sinorhizobium* (CGMCC 9638)	Generation of a vitamin B_{12} overproducer strain by overexpressing the precorrin-2C(20)-methyltransferase gene	115 mg/L	2020

[1] Values were converted to mg/L using the data available from the original publication; [2] n.d.: not determined.

7. Vitamin B_{12} Market Applications and the State of the Market

The most important market for B_{12} products is the feed and food industry, where its efficiency and security has been extensively verified [130], although its usage is also extensive in the supplement and pharmaceutical industry.

In the feed and food industry, CNCbl is commonly added to poultry, pig and calf feeds at dosage levels between 10 to 30 mg/t in almost all Europe and the USA [74]. It is also used as an additive in several food products, for example, in cereal, where its organoleptic properties and chemical properties, such as odorlessness, tastelessness and solubility in water, are an advantage for the fortification of several products. Nevertheless, its bright red color can present a challenge to its addition in other foods, such as white bread [88].

Regarding its usage in the supplement industry, vitamin B_{12} has been gaining relevance in later years, especially with the rise in the popularity of vegetarian and vegan diets [131]. CNCbl is the most used form mainly because of its stability, price, proven safety [22] and its similar efficiency compared to other forms [22,131]. Although there have been some reports of dried algae that contain a significant amount of B_{12} [132], Cbl is virtually absent in vegetables [131], and the main dietary sources are foods derived from animal products. Although B_{12} is present in dairy products and eggs (products suitable for vegetarian diets), their quantities are quite low compared with other options (approximately 0.4 μg/100 g in milk and 1.3 μg/100 g in eggs vs. 9.4 μg/100 g in some meats, 8.9 μg/100 g in fish and 52.4 μg/100 in shellfish [132]). This fact, together with the assumed bioavailability of only 50% of all the Cbl obtained from food sources [41] and the losses that can occur during food processing (cooking, exposure to light, pasteurization, etc.) [131], make reaching the recommended daily dietary intake of 2.4 μg a difficult task for those on pure vegetarian diets without vitamin B_{12} supplements [131].

Vitamin B_{12} deficiency is also prevalent in low- or middle-income countries with mainly plant-based diets and low meat consumption [133]. Even in high-income countries, though, there are several population groups at high risk of B_{12} deficiency. This specially affects the elderly, with around 20% of people over 60 suffering from it in the USA and in the UK according to the NIH [134]. In the case of the elderly, deficiency is mostly due to lower intake and a high prevalence of food-bound malabsorption, caused by age-related gastric atrophy and lower IF levels [41,135].

Other high-risk populations are pregnant and lactating women, children, and patients with autoimmune diseases that cause gastric complications, such as atrophic gastritis or decreased stomach acid secretion [132]. In all these cases, a higher daily B_{12} intake, mainly obtained through supplementation, is recommended.

Although most supplementations are based on CNCbl, in rare cases of cellular trafficking and protein processing alterations caused by rare genetic diseases [136], supplementation with other forms, such as MetCbl or OHCbl, may be required. Additionally, CNCbl supplementation may be unsuitable for supplementation in smoker populations [137,138].

Finally, it should also be noted that it is often preferred to supplement B_{12} on its own and not as a component of multivitamin tablets because the presence of vitamin C and copper can degrade it and form inactive Cbl by-products [131].

Besides direct supplementation, B_{12} is also widely used for the fortification of different food products. In this case, CNCbl is again the preferred form due to its higher stability when processed and cooked [132]. B_{12}-fortified products are common in the United States and other countries where, for example, B_{12}-fortified cereals and milk provide a significant amount of the total daily Cbl requirement [132]. Other alternatives, such as flour fortification, have also been considered [139]. In this sense, some of the in situ fortification approaches collected in this review [56,63] may become interesting and valuable alternatives in the future.

Vitamin B_{12} is also widely used in the pharmacological sector, where, besides CNCbl, other forms, such as OHCbl, AdoCbl and MetCbl, are also produced and distributed due to their higher uptake and more sustained serum levels [74]. Pharmacological B_{12} is presented in different forms, such as nasal sprays, oral and sublingual products, and

even direct injections to treat pernicious anemia, B_{12} deficiency, cyanide poisoning and lower homocysteine levels. There are also several claims that have been made regarding its positive effect in patients with Alzheimer's disease and as a stimulant of the immune system, though more evidence is needed to prove these [74,140].

Considering all these different usages and markets, it should not be a surprise that vitamin B_{12} total worldwide production and market volume have been steadily increasing, although the exact worldwide market values are difficult to obtain due to the scarcity of reliable information. However, it is safe to assume a great increase in overall production in the last decades. In 1989, the overall production was around 3 tons per year [74] and, by 2005, it had already increased to 10 tons and had a market value of approximately EUR 77 million [88]. As mentioned before, production in China in 2020 reached 31.41 tons with a market value of USD 339.48 million [76], while some projections have been made that the vitamin B_{12} market will reach a total value of USD 410 million by 2027 [141]. The progressive increase in the size of elderly populations, the rise of alternative vegan and vegetarian diets and the scarcity of animal food products are factors that explain this sharp market increase and are also the reasons why the B_{12} market is expected to continue to grow in the future.

8. Concluding Remarks

From published data, it is clear that industrial vitamin B_{12} production with *Propionibacterium freudenreichii* strains presents several challenges and shortcomings that must be surpassed in order for this method to compete against those using aerobic strains. From all the reviewed examples (Tables 1 and 2), the highest volumetric production with the anaerobic strain was 76 mg/L (IN201827044769 A [111]), clearly inferior to the 250–280 mg/L reported for aerobic strains in different studies (CN108949866 A [103], CN108913739 A [126], CN111254173 A [102]).

However, the singularities of markets in which vitamin B_{12} is targeted, such as dietary supplements or fortified foods and drinks, need to be taken into consideration. Many final vitamin B_{12} consumers, besides patients affected by pernicious anemia or other diseases, are vegans or vegetarians and people with high health and ecological awareness. In this scenario, the GRAS status of *Propionibacterium freudenreichii* and the fact that many producing strains are non-GMO microorganisms are valuable assets that increase its market appeal. For example, in situ fortification strategies can become a future economically viable application for *Propionibacterium freudenreichii* cultures. This possibility is further reinforced by the promising probiotic properties described for some *Propionibacterium freudenreichii* strains: microbiota modulation, immunomodulation and the production of several nutraceutical compounds, such as trehalose, naphotic acid and short-chain fatty acids [142].

Moreover, aside from these differentiating traits that can increase the added value for end consumers, the ability of *Propionibacterium freudenreichii* to synthetize different products besides vitamin B_{12} (mainly propionic acid and low amounts of trehalose) can also increase its industrial appeal. Currently, most of the worldwide propionic acid production is obtained from crude oil through petrochemical processes and there have been numerous studies that have aimed to find a bio-based alternative production process that would allow this product to be labeled as a "natural preservative" [143]. Therefore, the possibility of a *Propionibacterium freudenreichii* biorefinery, with simultaneous production and extraction of both propionic acid and vitamin B_{12}, would increase the industrial and commercial feasibility.

Besides *Pseudomonas denitrificans* and *Propionibacterium freudenreichii* strains, other possible producers, such as *Sinorhizobium meliloti*, different *Lactobacillus* strains and even *E. coli* (by heterologous expression of the biosynthetic pathway), have also been widely studied. So far, their reported productions are not competitive, making them unsuitable alternatives for industrial CNCbl production. Nevertheless, future strains and strategies may render

better production processes which would improve their industrial viability, although their commercial success may be hindered by regulatory constraints and consumer acceptability.

Finally, it should be mentioned that, in its current state, CNCbl production is still suboptimal and has many challenges to overcome to further develop its potential as a cost-effective and valuable industrial bioprocess. The main obstacle is that, even in the higher-producing aerobic strains, such as *Pseudomonas denitrificans*, volumetric production levels are often around 200–300 mg/L—much lower than those obtained via similar fermentation processes, such as those for vitamin B_2. In addition, the fermentation cycles are long and costly, mainly because of the need for expensive media compounds, such as high concentrations of complex nitrogen sources and supplements such as betaine. Supplying enough cobalt to the broth can also be problematic from a cost and an environmental perspective [144].

Further efforts in bioprocessing, downstream and media composition optimization (with cheaper or recycled compounds) should be carried out to increase the economic viability and environmental sustainability of vitamin B_{12} biotechnological production. However, the main problem, still, is the low productivity of the available producing strains, caused mainly by the tight genetic regulation of Cbl production: the inhibition of the cysG and the cbi operon by the cobalamin riboswitch, as well as other downregulating processes [40,74,144]. Overcoming this limitation may require genetic engineering, which may not be well received by end consumers, mainly vegans or vegetarians, who are very concerned about their diet choices and the usage of GMO organisms.

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Microbial Cell Factories

RESEARCH

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Developing a single-stage continuous process strategy for vitamin B_{12} production with *Propionibacterium freudenreichii*

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Abstract

Background Vitamin B_{12} is a widely used compound in the feed and food, healthcare and medical industries that can only be produced by fermentation because of the complexity of its chemical synthesis. Besides, the use of Generally Recognized as Safe (GRAS) and Qualified Presumption of Safety (QPS) microorganisms, like *Propionibacterium freudenreichii,* especially non-GMO wild-type producers, are becoming an interesting alternative in markets where many final consumers have high health and ecological awareness. In this study, the production of vitamin B_{12} using the *Propionibacterium freudenreichii* NBRC 12391 wild-type strain was characterized and optimized in shake flasks before assessing several scale-up strategies.

Results Initial results established that: (i) agitation during the early stages of the culture had an inhibitory effect on the volumetric production, (ii) 5,6-dimethylbenzimidazole (DMBI) addition was necessary for vitamin B_{12} production, and (iii) kinetics of vitamin B_{12} accumulation were dependent on the induction time when DMBI was added. When scaling up in a bioreactor, both batch and fed-batch bioprocesses proved unsuitable for obtaining high volumetric productivities mainly due to carbon source limitation and propionic acid inhibition, respectively. To overcome these drawbacks, an anaerobic single-phase continuous bioprocess strategy was developed. This culture strategy was maintained stable during more than 5 residence times in two independent cultures, resulting in 5.7-fold increase in terms of volumetric productivity compared to other scale-up strategies.

Conclusion Overall, compared to previously reported strategies aimed to reduce propionic acid inhibition, a less complex anaerobic single-phase continuous and more scalable bioprocess was achieved.

Keywords Cobalamin, *Propionibacterium freudenreichii*, Cyanocobalamin production, Fed-batch culture, Continuous culture

Introduction

Vitamin B_{12}, also known as cobalamin (Cbl), is a water-soluble molecule essential in many organisms. This molecule was first studied in the 1920s due to its important role in the prevention and treatment of serious diseases like pernicious anemia [1]. Although it was not until 1948 that was isolated and described by two research groups from pharmaceutical companies (Merck, Sharp & Dohme, and Glaxo [2, 3]). Later, this red crystalline compound was found to be, in fact, a family of molecules that shared a similar structure: a tetrapyrrolic corrinic ring

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with a central cobalt atom coordinated to four nitrogen atoms [4, 5], a similar scaffold to other prosthetic groups such as the heme group of hemoglobin or the electron transport chain group of cytochrome P450. In addition, besides the four N atoms of the pyrrole units, the central Co^{+} ion is linked to two other ligands. The lower ligand in an α-axial conformation is 5,6-dimethylbenzimidazole (also known as DMBI) while the upper ligand is linked to the Co^{+} ion in the β-axial position. The nature of this last chemical group is variable and defines the different physiological and catalytic functions of cobalamins such as methylcobalamin (MetCbl), a cofactor of several methyltransferases [6, 7], and adenosylcobalamin (AdoCbl), a cofactor of mutases like the L-methylmalonyl-CoA mutase found in mammals [8].

All these molecules present a very complex structure and an elaborated biosynthesis with over 30 biotransformation steps [9]. For this reason, although the chemical synthesis of vitamin B_{12} has been established since the 1970s [10, 11] fermentation processes are preferred for cobalamin production. Moreover, the most common commercial form of vitamin B_{12} is not MetCbl or AdoCbl but cyanocobalamin (CNCbl), which is a more stable form of vitamin B_{12} and readily converted into the active coenzyme forms in the body [12]. Nowadays, industrial vitamin B_{12} production with *Pseudomonas denitrificans (P.denitrificans)* strains have been favored due to their faster growth rate and productivity [13, 14], displacing other traditionally employed anaerobic strains such as *Propionibacterium freudenreichii (P. freudenreichii).* However, *P. freudenreichii* still presents properties that make it an interesting candidate for cobalamin production. For example, in contrast to *P. denitrificans*, it is a Generally Recognized As Safe (GRAS) microorganism and has the Qualified Presumption of Safety (QPS) status granted by the EFSA. Moreover, it can grow in a wide range of different and inexpensive carbon and nitrogen sources [15–17] or produce other valuable compounds like propionic acid or trehalose [18].

Vitamin B_{12} supplementation is gaining relevance in recent years with the rise in vegan and vegetarian populations [19]. For this reason, the use of GRAS and QPS producers, like *P. freudenreichii*, especially non-GMO wild-type producers, are becoming an interesting alternative in markets where many final consumers have high health and ecological awareness. In addition, different *P. freudenreichii* strains have been traditionally used for cheese and other food productions facilitating the development of several in-situ fortification strategies without the need for direct supplementation [20, 21].

Traditionally, Cbl production in *P. freudenreichii* has been performed in a two-stage fermentation process: a first stage completely anaerobic aimed at cell growth followed by a microaerophilic production stage, needed for DMBI synthesis and its attachment to the corrinic ring [22, 23]. The main drawback of *P. freudenreichii* Cbl production compared to aerobic strains, besides its slower growth and lower volumetric productions, is the large quantities of propionic acid produced through the Wood-Werkman cycle [24]. To reduce this latter limitation, main industrial *P. freudenreichii* producers have been obtained through random mutagenesis processes where strains with high tolerance to propionic acid were selected [25]. Other approaches, like genetic engineering [26, 27], several strategies to decrease propionic acid concentration, such as expanded bed adsorption bioreactors [28, 29], co-culture with propionic consuming strains [30] and supplementation with different Cbl precursors [23, 31], were tested in the past, although results are often very strain dependent and further characterization and optimization is still needed.

In this study, a bioprocess with a wild-type non-propionic resistant strain of *P. freudenreichii* was optimized for cobalamin production. The selected strain was NBRC 12391, also named IFO 12391, reported as producing strain by Kojima and co-workers [25]. To increase market accessibility, all the improvements performed were done at a bioprocess level, without any genetic modification. Besides, industrial cheap media were preferred such as corn steep liquor (CSL), a byproduct of the mill industry, over more expensive alternatives like yeast extract (YE), even though the combination of both CSL and YE has recently been reported to have a positive effect on cobalamin production [32].

Initially, aeration in early stages of the culture and the addition of different precursors were found to affect the final volumetric production. Later, these results were scaled from shake flasks to a lab-scaled bioreactor. Finally, thanks to the specific cobalamin production kinetics of our strain, a single-phase continuous bioprocess was proposed and developed, in contrast to the usually described two-stage culture [33], which minimized propionic acid inhibition and maximized cobalamin productivity. Compared to previous studies where an increased production was obtained by eliminating the propionic acid from the media with several resin systems [29, 34, 35], a less complex and more scalable bioprocess was achieved.

Results and discussion

Culture characterization in shake-flasks

Agitation effect during the early culture stage

Agitation is mandatory in any culture system to get a homogeneous system and not promote nutrient gradients or cell sedimentations. Nevertheless, previous studies with *P. freudenreichii* strain have concluded that agitation may have an inhibitory effect on CNCbl production

during the production stage [36]. This information may become important in scaling up the bioprocess as bioreactor systems are always agitated so, a potential effect on growth or CNCbl production needs to be properly characterized on each strain.

In preliminary experiments performed with gentle agitated shake flasks, the final CNCbl productions obtained were lower than those described in the literature for wild-type strains [37] with values below 2 mg/L. Because of these results, and even though some amount of agitation will be needed in the eventual scale-up of the process, a culture methodology based on static shake flasks was assessed.

Figure 1 shows the results obtained from shake flask cultures which have been gently agitated (150 rpm) or maintained in static conditions. From the OD_{600} profile, a slightly delayed growth was observed on agitated cultures from the beginning, but both conditions reached similar maximum values at 96 h, OD_{600} of 29.1 ± 0.3 and 26.1 ± 1.0 for the static and agitated cultures respectively. These results seem to suggest that agitation may have a slightly negative effect on cell growth, especially during the early stages of the culture.

As previously described elsewhere [13], at 96 h CNCbl production was induced by adding DMBI and promoting a microaerophilic condition by gently agitate both conditions. Unexpectedly, our strain was already producing a significant amount of CNCbl before the induction but only under agitation, 0.71 ± 0.03 mg/L, in contrast to the static condition where CNCbl was under the quantification limit of our method (0.3 mg/L). However, at the end of the culture, the static condition presented significantly higher volumetric and specific production, 2.83 ± 0.06 mg/L and 0.33 ± 0.02 mg CNCbl/g Biomass respectively, indicating that the final production depends on the culture condition before the DMBI addition.

Other *P. freudenreichii* wild-type strains presented similar values compared to our static condition in terms of CNCbl volumetric production. For example, Liu et al. reported productions with their non-optimized media of around 3.81 mg/L [32] and Chamlagain and coworkers reported a production of 5.3 mg/L in whey-based media [23].

Although an agitate system is needed upon scaling, the static condition was selected as the culture condition for further strain characterization in shake flask setups. We

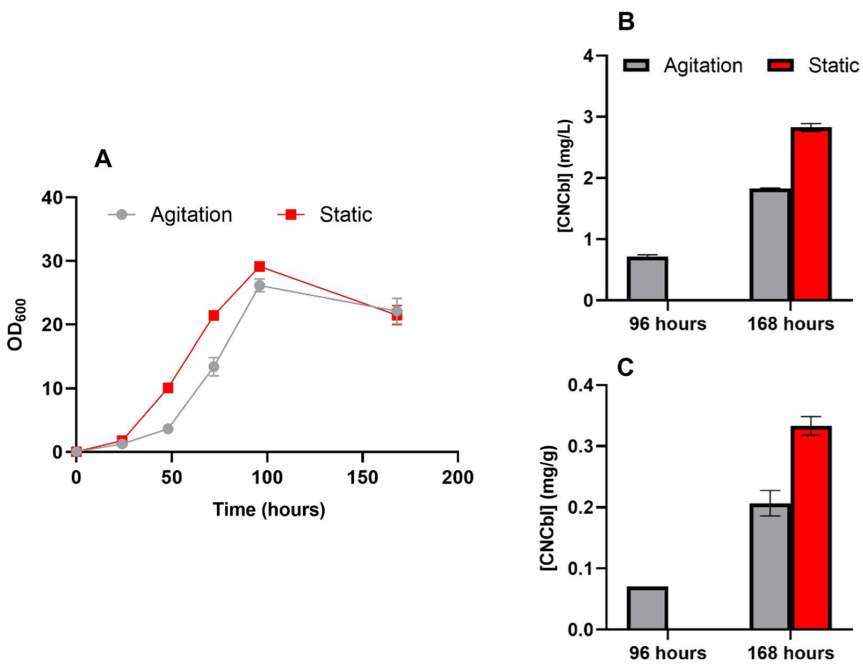

Fig. 1 **A** OD_{600} values, **B** volumetric productions (mg CNCbl/L) and **C** specific productions (mg CNCbl/g Biomass) at 96 and 168 h of both conditions, agitated or static. Agitated cultures were kept at 150 rpm during the whole culture while static cultures were kept without any kind of agitation during the first 96 h and gentle agitated at 150 rpm afterwards. In both cases, 100 µM DMBI was added at 96 h to promote Cbl production. Error bars represent the standard deviation of three replicates

assumed that the slight inhibitory effect apparently produced by an early agitation was due to minor oxygenation that may have compromised the anaerobic condition. In a bioreactor set-up, an anaerobic condition can be better ensured, for example, by the constant addition of N_2 to the vessel, so this effect can be easily avoided.

Supplementation of CNCbl precursors

Classical cultures aimed at CNCbl production often use DMBI addition to induce further accumulation in the microaerobic stage, but there are other precursors such as riboflavin (RF) or nicotinamide (NAM) that have been reported to work for other wild-type producing strains [23]. To define the best supplementation approach for our strain (NBRC 12391), 100 μM of RF, 100 μM of NAM or 100 μM of DMBI were added at two different culture stages, at 0 and 96 h (Fig. 2). Moreover, 50 μM of RF plus 50 μM of NAM were combined to test potential synergistic effects of both precursors.

The results showed that, from all conditions tested, only supplementation with DMBI at time 0 seemed to have a slightly negative effect on cell growth during the early stages of the culture (Fig. 2A). Nevertheless, despite this inhibitory effect, the final absorbance achieved was not significantly different from the rest of the tested conditions, with OD_{600} values of around 30 at 168 h.

CNCbl production was assessed only at 168 h, the final point of the culture after 72 h of gently agitation (Fig. 2B). At this time, all conditions tested including the control condition without any addition showed some amount

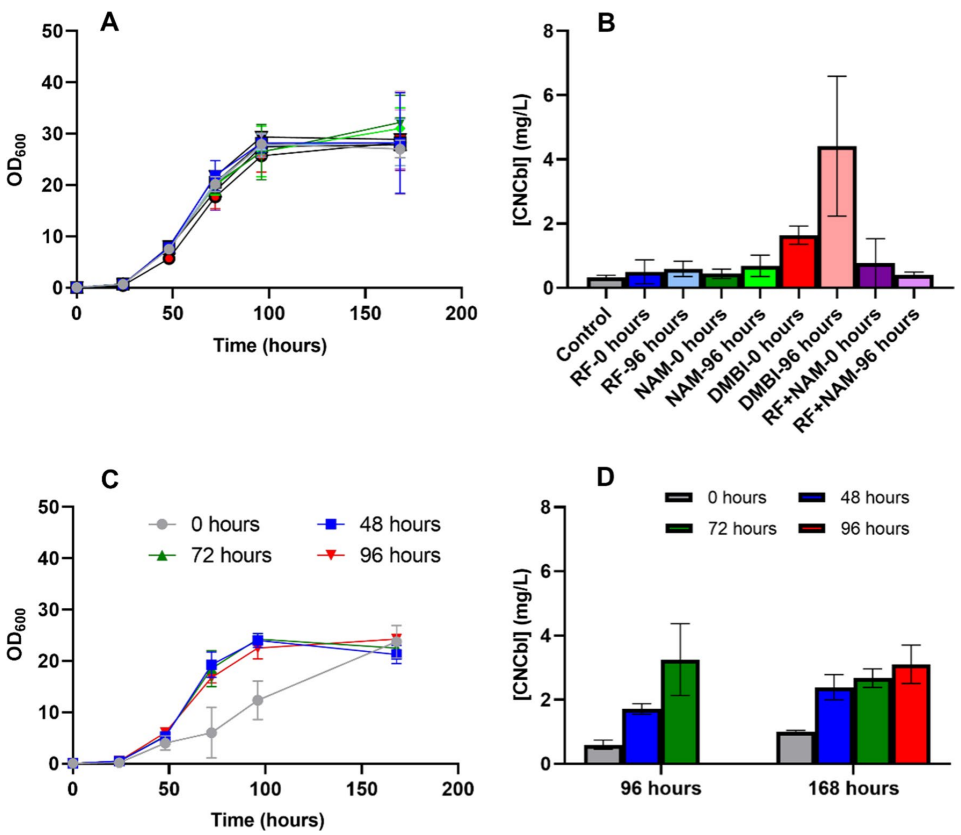

Fig. 2 **A** and **B** OD_{600} values and volumetric productions (mg CNCbl/L) at 168 h of NBRC 12,391 cultures where different Cbl precursors were added to the culture at 0 or 96 h. Control (without precursors), Riboflavin (RF), Nicotinamide (NAM), 5,6-dimethylbenzemidazole (DMBI) and a combination of both riboflavin and Nicotinamide (RF + NAM). **C** OD_{600} values and **D** volumetric productions (mg CNCbl/L) at 168 h of NBRC 12391 cultures where DMBI was added at four different times: 0, 48, 72 and 96 h. Error bars represent the standard deviation of three (**A** and **B**) or two (**C** and **D**) replicates

of CNCbl production. However, except for DMBI, the addition of precursors, independently of when they were added, did not significantly differ from the control condition. Thereby, for our strain, only DMBI was able to induce higher CNCbl accumulation and the other precursors were discarded for future experimentation. In addition, and as previously described [38], it was better to add DMBI at the beginning of the microaeration stage, 96 h, and not from the beginning.

To further optimize DMBI addition, four different addition times were tested, 0, 48, 72 and 96 h (Fig. 2C and D). Like previous experiments, DMBI addition at time 0 resulted in a slower growth rate during the early stages of the culture, but the effect was more pronounced in this later study. Nevertheless, the final OD_{600} value was similar to the rest of the conditions tested.

As shown in Fig. 2D, CNCbl production was evaluated at 96 and 168 h with DMBI inductions at 0, 48, 72, and 96 h. As expected, at 96 h all conditions presented significant amounts of CNCbl, being the induced culture at 72 h the best presenting already 3.3 ± 1.2 mg/L of CNCbl just 24 h after the induction. Moreover, the production at 168 h when DMBI was added at 96 h (3.11 ± 0.6 mg/L) was not significantly different from the values obtained at 96 h when DMBI was added at 72 h. These results suggested that, after certain OD_{600} value of around 20 is achieved, the maximum CNCbl volumetric production in NBRC 12391 cultures can be obtained just 24 h after DMBI addition.

Scale-up. Batch and fed-batch processes

The scale-up strategy began with the development of a classical batch bioprocess in a laboratory-scale bioreactor with 1 L working volume. For the first 96 h, a strict anaerobic condition was set by pumping N_2 constantly at 0.15 vvm. Although agitation was found to reduce the final volumetric production in shake flasks, we fixed a low agitation of 150 rpm through all the processes to ensure homogenization in the bioreactor and avoid cell sedimentation.

Batch with DMBI induction at 96 h

Initial batch experiments were performed according to previously reported strategies for other producing strains where the growth phase was extended for 96 h. After that time, 100 µM DMBI was added to the culture, enough to produce up to 50 mg/L of vitamin B_{12}, and N_2 addition was stopped to promote microaeration. The growth profile and metabolite productions of the NBRC 12391 strain in a batch culture are represented in Fig. 3A. The obtained data shows that our strain presented faster growth rates in the bioreactor, even though it was agitated, compared to shake flasks studies as the maximum

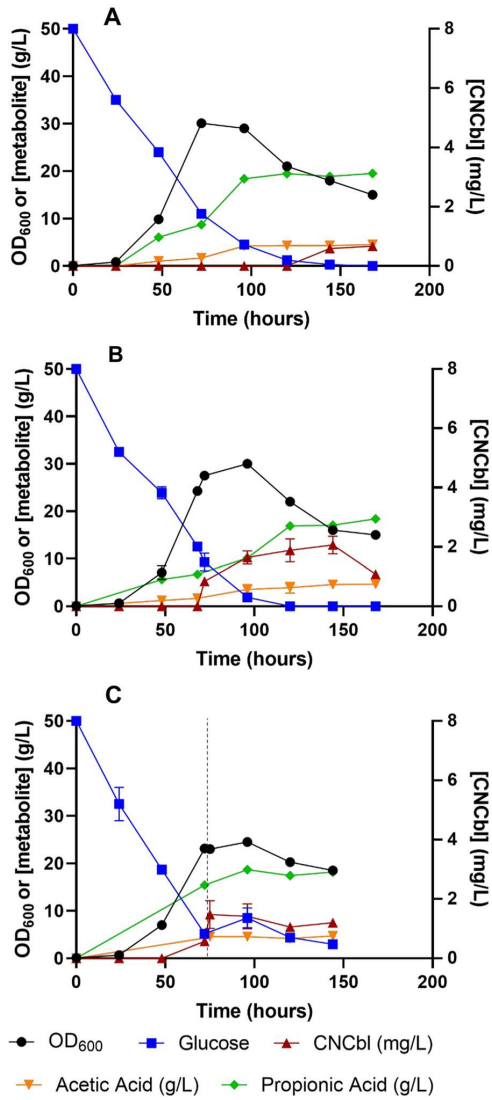

Fig. 3 **A** Batch with DMBI addition at 96 h. **B** Batch with DMBI addition at 72 h. **C** Fed-batch with DMBI addition at 72 h. OD_{600} values, glucose (g/L), acetic acid (g/L), propionic acid (g/L) and CNCbl (mg/L) are provided. The vertical dashed line in the fed-batch process represents when feeding was started. Each process was performed by duplicate except the Batch process with DMBI addition at 96 h with only 1 replicate

OD_{600} was obtained sooner, at approximately 72 h. These results were expected as pH control was much better in the bioreactor than in shake flasks that were manually controlled daily. Moreover, as it grew faster, when

DMBI was added at 96 h, glucose was almost depleted presenting values around 4 g/L. Under the microaeration stage, glucose was completely depleted from the media and OD_{600} values began to decrease, most probably due to some degree of cell lysis. This may be also the reason why, even though CNCbl production began when DMBI was added, the final volumetric production values were significantly lower than the ones obtained in shake flask cultures, 0.63 mg/L. Besides biomass and CNCbl production, acetic and propionic acid accumulations were also followed through the process. As expected, propionic acid was the principal fermentation by-product mainly produced during the anaerobic phase reaching 18.4 g/L at 96 h. This value remained stable in the microaerobic phase (reaching a maximum value of 19.5 g/L at 168 h) most probably because glucose was already almost depleted.

Batch with DMBI induction at 72 h

Taking into consideration previous experiments where it was proved that maximum volumetric production values could be obtained with earlier DMBI addition if certain OD_{600} values were achieved, other batch processes were performed with DMBI addition at 72 h, with OD_{600} values of 27.5 and a glucose concentration of 8.5 ± 0.7 g/L. After DMBI addition, CNCbl production began, and maximum volumetric values were 2.05 mg/L $\pm$ 0.3 in a 48-h period (Fig. 3B). Interestingly, at 75 h, just 3 h after the DMBI addition we could already quantify 0.85 mg/L $\pm$ 0.02 of CNCbl suggesting a fast accumulation of the vitamin. Nevertheless, the volumetric production of 2–4 mg CNCbl/L obtained in static shake flask cultures could not be achieved. On the other hand, comparing the propionic acid production, the early CNCbl induction promoted a slower production rate of this acid at the beginning of the microaerobic phase but it finally reached a similar concentration at 120 h.

Fed-batch with DMBI induction at 72 h

Due to the impact of carbon source limitation on both OD_{600} and CNCbl volumetric production, a fed-batch bioprocess with constant glucose addition after 72 h was assessed (Fig. 3C). Despite the increased glucose concentration in the culture, OD_{600} did not reach higher values and, in fact, OD_{600} max (24.5 ± 0.71) was lower than the one obtained in the batch bioprocesses. Besides, maximum volumetric production was also lower (1.48 mg/L $\pm$ 0.46). These results suggested that the growth and production of our strain were limited not only by the lack of carbon source but also by other factors such as propionic acid inhibition. The latter effect has been widely reported previously [32] and propionic concentration in the final stages of the cultures was probably too high for our strain, around 18 g/L in the batch processes. Considering this inhibition, a fed-batch strategy without a mechanism for propionic acid elimination/removal (for example, an expanded bed reactor like the one used in [34]) does not seem to be a viable strategy for cobalamin production.

Development of a single-stage continuous bioprocess in lab-scaled bioreactor for increased CNCbl volumetric productivity.

Propionic acid inhibition effect on culture growth and anaerobic production of CNCbl

Considering the results obtained in the fed-batch culture experiments, developing a strategy to decrease the inhibitory effect of high propionic acid concentrations seemed to be mandatory to increase production with the NBRC 12391 strain. Moreover, the two-stage production process with an anaerobic growth phase and a microaerobic production phase severely hinders the viability and increases the complexity of such bioprocess. A single-phase process, with constant anaerobic conditions to ensure cell growth would be preferable. Nevertheless, for this strategy, our strain should be capable of producing CNCbl in anaerobic conditions without a microaerobic production stage. As the majority of the needed O_2 is employed in DMBI biosynthesis, a process not needed in our bioprocess as DMBI is added in excess as a supplement, made us consider a single-stage continuous strategy. Besides, the fact that maximum production of our strain was achieved just 24 h after DMBI addition (see Fig. 2D) was promising and let us develop a bioprocess with a residence time of less than a day without losing CNCbl production.

Before developing the bioreactor process, several experiments were performed in shake flasks to ensure the viability of the strategy. First, the capacity of the cell culture to continue growing in fresh media was tested. To do so, after the first 72 h of growth, the whole culture was centrifuged, the supernatant discarded, and the cell pellet resuspended in fresh media and supplemented with 100 µM DMBI (Fig. 4A and B). This strategy allowed to reach not only higher OD_{600} values, 44.5 ± 0.7 *vs* 22.75 ± 1.1 in the control culture (cultures performed without media regeneration as described in Section "Culture characterization in shake-flasks") but also higher CNCbl volumetric production, 4.06 ± 1.14 mg CNCbl/L *vs* 1.42 ± 0.07 mg CNCbl/L. Therefore, as expected, we proved that eliminating propionic acid from the media allowed the culture to continue growing and producing.

As previously mentioned, one of the main challenges for the development of a continuous process for *P. freudenreichii* is the reported necessity of a microaerobic production phase after the strictly anaerobic growth

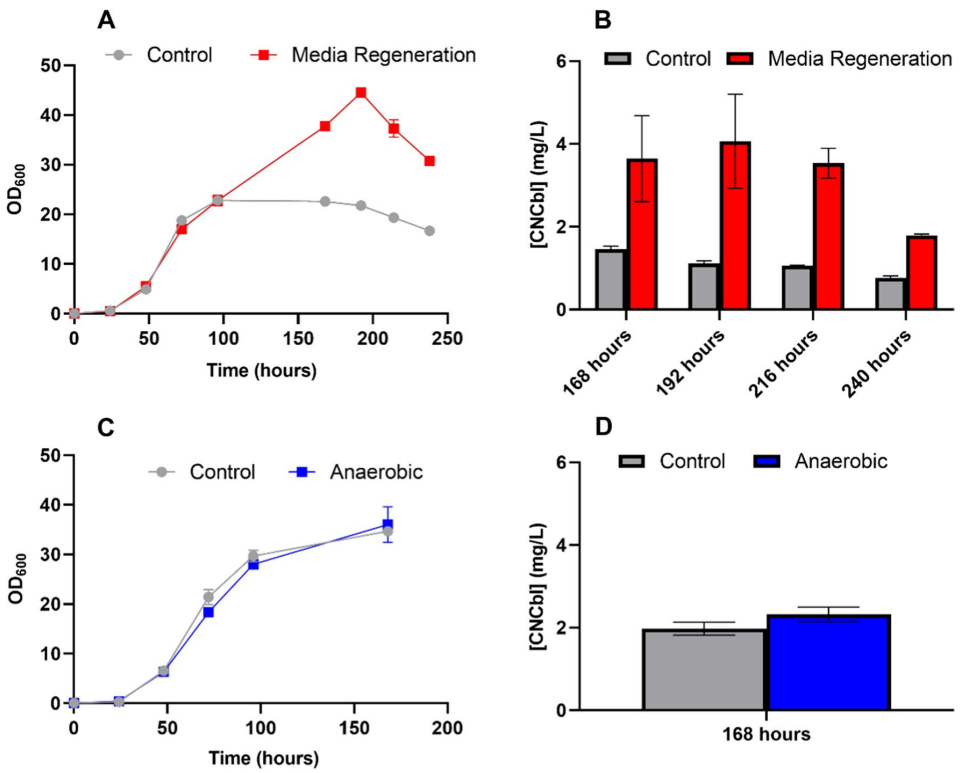

Fig. 4 **A** and **B** OD_{600} values and volumetric productions at 168, 192, 216 and 240 h of NBRC 12391 control cultures and cultures where the cells were centrifuged and resuspended in fresh media at 96 h. In both cases, 100 µM DMBI was added at 96 h to promote Cbl production. **C** and **D** OD_{600} values and volumetric productions at 168 h of NBRC 12391 control cultures and cultures that were kept anaerobic through the whole process. In both cases, 100 µM DMBI was added at 96 h to promote Cbl production. Error bars represent the standard deviation of three replicates

phase. To test if NBRC 12391 was capable of achieving similar CNCbl production in a single anaerobic phase, several cultures were performed in shake flasks kept at static conditions during the whole 168 h process (Fig. 4C and D). As a result, OD_{600} values and CNCbl volumetric and specific productions were not significantly different between the conditions, thereby, allowing a single anaerobic process.

Continuous bioprocess in bioreactor

With the objective of reducing the propionic acid inhibition in the vessel at steady state, a continuous culture process was designed with lower carbon and nitrogen source concentrations. More specifically, the initial culture media and the fed media contained 25 g/L glucose and 20 g/L CSL instead of 50 g/L and 40 g/L used in the batch bioprocesses respectively. Thereby, we were expecting amounts of 9 g/L of propionic acid at steady state assuming equivalent yields as in previous Batch processes (See Fig. 3B).

After batch phases of 48 h (Fig. 5), before complete glucose depletion, OD_{600} of 13.05 ± 0.07 were reached, 100 µM DMBI was added and the continuous bioprocesses were begun with the constant additions of fresh media (containing 100 µM DMBI) at a flow rate of 50 mL/h. The total volume addition rate was 55 mL/h due to the constant addition of 2 M NaOH (5 mL/h) to control pH. These values imply a dilution rate of 0.055 h^{-1} and a mean residence time of 18.2 h. CNCbl production began just after DMBI addition, reaching a volumetric production of 1.33 ± 0.26 mg/L during the first residence time. After 5 retention times, a stable OD_{600} value of around 24 was achieved, while glucose concentration remained stable at approximately 1.2–1.5 g/L. Propionic acid also presented a stable value of around 7.5 g/L, as expected much lower than the 20 g/L obtained in previous cultures and not in the inhibitory concentration

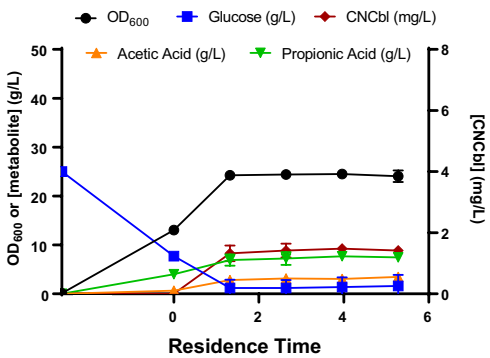

Fig. 5 Continuous bioprocess of NBRC 12391. The number of residence times is shown on the X-axis. According to the calculated addition of both fresh media and NaOH solution, each residence time corresponds to 18.2 h. Fresh media containing 100 µM DMBI addition began at residence time 0. OD_{600} values, glucose (g/L), acetic acid (g/L), propionic acid (g/L) and CNCbl (mg/L) are provided. Error bars represent the standard deviation of two replicates

range. These values were better than the expected ones, OD_{600} and propionic acid of 15 and 9 g/L respectively, so the continuous state not only allowed to control propionic acid at lower levels but also improved the carbon source usage towards biomass formation. Moreover, CNCbl volumetric production was stable from the first residence time at 1.33 ± 0.26 mg/L.

Overall, comparing the two batch bioprocesses (with DMBI added at 96 and 72 h respectively), the fed-batch process and the newly developed continuous bioprocess at 144 h (Fig. 6), volumetric and specific production values showed values in the same range independently of the culture strategy. However, the volumetric productivities in the continuous process were 5.7-fold higher than the best batch process, 0.078 ± 0.001 vs 0.014 ± 0.002 mg CNCbl/(L·h). These results were possible due to the fact that the NBRC 12,391 strain can: (i) produce cobalamin in a single-phase anaerobic bioprocess, and (ii) its maximum volumetric production is achieved just 24 h after DMBI addition. However, the volumetric production is still lower than that obtained in static shake flask cultures, 2–4 mg CNCbl/L, giving space for further optimization.

In a review published recently by our group [38], the production of several wild-type *Propionibacterium* strains with different culture strategies is reported. The volumetric productivity achieved in this work is higher than most of the values obtained with wild-type strains [23, 38, 39]. Some other studies, like [40], reported higher volumetric productivities [0.32 mg CNCbl/(L·h)], most probably because a higher-producing strain was used.

Conclusions

Despite the multiple limitations, mainly caused by its lower production and slower growth compared to aerobic strain producers, *P. freudenreichii* is still an interesting alternative for Cbl production, especially with the increasing demand for non-GMO natural products.

In this paper, suitable bioprocesses for CNCbl production with the wild-type strain NBRC 12391 were assessed in shake flasks and later scaled up to a 1L working volume bioreactor. It was determined that agitation during the early stages of the culture had a negative effect on final CNCbl productivity. Besides, DMBI was found to have a significant positive effect on final production. Specifically, our results showed that the time at which DMBI was added was important, with a slightly negative effect on cell growth when added a time 0 and maximum CNCbl volumetric productions just 24 h after DMBI addition, at 72 or 96 h depending on the culture strategy.

Batch with different addition times of DMBI and fed-batch processes were performed, but the results, especially the volumetric productivities, were hindered mainly by the lack of carbon source in the later stages of the culture and the inhibitory effect of propionic acid.

To increase CNCbl volumetric productivity of wild-type NBRC 12391, a single-phase continuous strategy was developed. The fact that the maximum CNCbl volumetric production with NBRC 12391 in our working conditions was achieved just after 24 h in anaerobic conditions, allowed us to achieve a 5.7-fold volumetric productivity increase following this strategy. Future work will address optimization regarding dilution rates and adjusting nutrient concentrations to achieve even higher volumetric productivities.

Overall, here we describe a new viable strategy for the development of future and sustainable strategies to produce CNCbl with wild-type GRAS *P. freudenreichii* strains without having to rely on more complicated setups like multiple-phase continuous bioprocesses or expanded bed adsorption bioreactors for propionic acid elimination.

Materials and methods

Microorganism and medium

Cultures were performed with *P. freudenreichii* NBRC 12391 acquired from Japan's National Biological Resource Center (NBRC). This strain is the parental strain used by Kojima and co-workers in their Patent from 1985 [25], also named IFO 12391. Cells were stored in pre-culture media supplemented with 25% glycerol at − 80 °C and in agar-solid media.

All products described were purchased from Sigma-Aldrich unless stated otherwise.

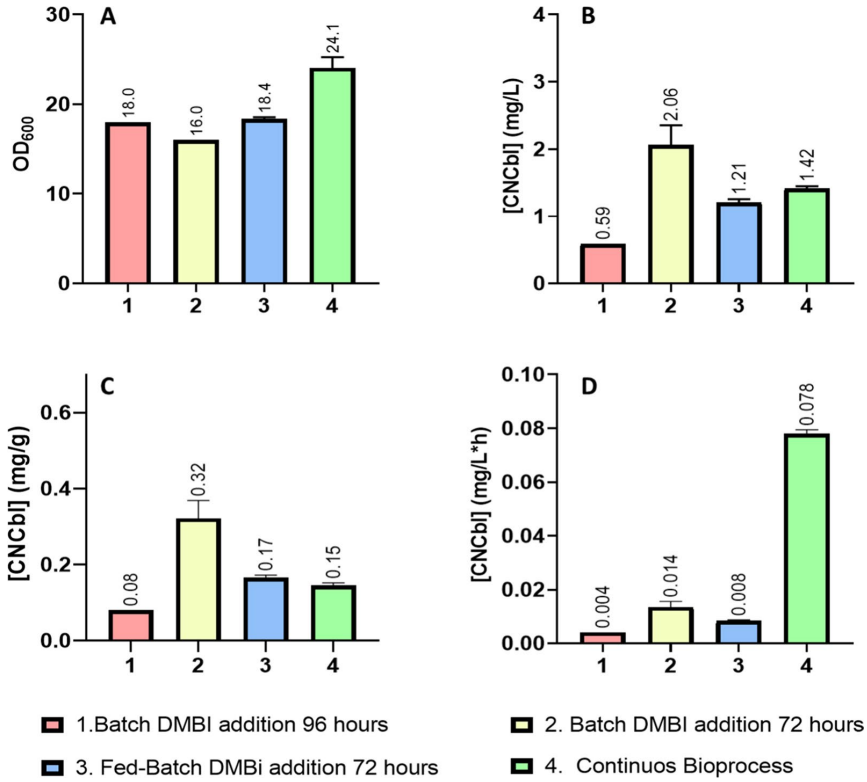

Fig. 6 Comparison of several bioprocess parameters between the 4 scale-up strategies tested: batch with DMBI addition at 96 h, batch with DMBI addition at 72 h, fed-batch with DMBI addition at 72 h, and continuous bioprocess. **A** OD_{600}. **B** Volumetric production. **C** Specific production. **D** Volumetric productivity. All data used to perform the calculations were extracted from the 144 h

The pre-culture media was prepared according to NBRC instructions. Its composition was 5 g/L glucose, 5 g/L yeast extract, 5 g/L tryptone and 1 g/L $MgSO_4$. The composition of the agar-solid media was 5 g/L glucose, 5 g/L yeast extract, 5 g/L tryptone, 1 g/L $MgSO_4$ and 15 g/L agar. Solid-media cultures were performed in a 2.5 L Oxoid Anaerojar (Thermo Fisher Scientific, Alcobendas, Spain) to ensure anaerobic conditions.

The composition of the culture medium used in shake flask and bioreactor cultures is an adaptation of the media described in Kojima et al. [25]. Its composition was 50 g/L glucose, 40 g/L corn steep liquor (CSL), 3 g/L NH_4NO_3, 1.5 g Na_2HPO_4, 0.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 5 mg/L $MnSO_4{\cdot}4H_2O$, 10 mg/L $FeSO_4{\cdot}7H_2O$, 10 mg/L $ZnSO_4{\cdot}7H_2O$, 0.05 mg/L $CuSO_4{\cdot}5H_2O$, 0.01 mg/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 15 mg/L $Co(NO_3){\cdot}6H_2O$ and 5 mg/L Calcium pantothenate. Glucose and CSL concentrated stocks (250 g/L and 200 g/L stock solutions respectively) were autoclaved separately from the rest of the media components. To prevent precipitation of the CSL solid fraction, the pH of the CSL solution was adjusted to 8 before autoclaving and, after sterilization, centrifuged at 18,000*g* for 30 min to recover just the supernatant. All salts except NH_4NO_3, Na_2HPO_4, and KH_2PO_4 were prepared in a separated solution and sterilized by filtration with a 0.22 µm filter (Teknokroma, Sant Cugat del Valles, Spain).

For the development of a single-continuous bioprocess, the culture media composition was the same as previously described apart from glucose and CSL concentration which were set at 25 g/L and 20 g/L respectively.

Shake flasks cultures

All shake flasks cultures were performed in 250 mL shake flasks filled with 200 mL of media at 30 °C with or without agitation depending on the study. The ratio between the volume of culture and the maximum volume of the shake flask was fixed at 80% to reduce the oxygenation

capacity of the system as anaerobic and microaerobic conditions were desired.

An aliquot of the cryopreserved cells was grown in 10 mL of pre-culture media for 24 h. These cultures were used to inoculate another 10 mL of culture media for 48 h. Finally, these cultures were used to inoculate the main cultures used in the studies. pH was measured and adjusted daily with the addition of a 2 M NaOH solution. All cultures were performed, at least, in triplicate.

The effect of the agitation during the early stages of the culture was studied in two separate conditions: a static condition where cultures were kept in a stove (J.P. Selecta, Abrera,Spain) at 30 °C and an agitated culture performed at 150 rpm and 30 °C in a INFORS HT incubator (Biogen, Madrid, Spain). After 96 h, both conditions were supplemented with 100 μM DMBI and agitated at 150 rpm in the INFORS HT Incubator.

To study the effect of the addition of different cobalamin precursors, several cultures were performed with the addition of 100 μM Riboflavin (RF), 100 μM Nicotinamide (NAM), 100 μM DMBI or a combination of RF and NAM (50 μM each) at 0 and 96 h. All stock solutions for the different precursors were prepared with ultrapure water and filter sterilized except DMBI solution which was prepared in pure ethanol. The effect of DMBI addition on cell growth and production was further studied with the addition of 100 μM DMBI at 0, 48, 72 and 96 h. In all cases, after the addition of the precursors, cultures were kept under gently agitated conditions.

The viability of a glucose feeding strategy was firstly studied in shake flasks by the addition of a 250 g/L Glucose solution at 96 h to increase the remaining glucose concentration by 15 g/L. The inhibitory effect of propionic acid and the possibility of media regeneration was studied by replacing the media in the culture at 96 h by centrifugation at 3.900 g for 30 min and resuspending the cell pellets in 200 mL of fresh media.

Finally, the capability of NBRC 12391 to produce CNCbl in anaerobic conditions, without a microaeration phase, was performed in a stove without any agitation and using tight-closed lids during the whole process.

Bioreactor cultures

All bioreactor cultures were performed using a Biostat A bioreactor (Sartorius Spain, Alcobendas, Spain) with a working final volume of 1 L. To ensure anaerobic conditions during the early stages of the culture, 0.150 vvm of N_2 were constantly pumped into the bioreactor vessel through a 0.2 μm filter (Whatman, Maidstone, UK) and injected from the sparger. The agitation was maintained constant at 150 rpm. Temperature, pH and dissolved oxygen (pO_2) were measured online by Hamilton probes. pH was adjusted constantly at 7.0 ± 0.05 with a 2 M NaOH solution. Temperature was constantly controlled at 30 °C.

In batch cultures, 1 L of fermentation media was inoculated with 10 mL of inoculum (1% v/v) to obtain an initial OD_{600} value of approximately 0.3. 100 μM of DMBI were added at 72 or 96 h depending on the culture and N_2 addition was stopped to promote microaeration when desired. Air was not pumped during the cultures at any time.

In fed-batch processes, batch phase had 80% of the final volume and it was performed as previously described elsewhere. The fed phase began at 72 h with the addition of a concentrated glucose solution (250 g/L) at a constant rate of 6.66 mL/h. 100 μM of DMBI was also added to the culture at this point to promote Cbl production. A total amount of 200 mL of concentrated glucose solution was added to the culture for 30 h. After that time, no more glucose was added to the media and the culture was extended until 144 h.

Finally, for the continuous cultures, the batch phase was started as described before and from 48 h onwards fresh media with 100 μM DMBI were constantly added to the bioreactor vessel at 50 mL/h. Culture broth was constantly extracted from the bioreactor vessel by a level probe connected to a peristaltic bomb to ensure a constant working volume of 1.0 L.

Analytical methods

Optic density at 600 nm (OD_{600}) was determined with a JENWAY6305 spectrophotometer (Cole-Palmer, Staffordshire, UK).

Dry cell weight was calculated as follows. Periodically, 1 mL sample were centrifuged at 3900*g* for 15 min in triplicates in previously dried and weighed 15 mL falcon tubes. After centrifugation, the pellets were dried in a 100 °C oven until stable weight, cold down in a desiccator for 30 min and weighed again. The weight of the falcon tubes was taken into consideration for this calculation. After a series of cell dry weigh calculations in several cultures according to the method just described, it was established that one unit of OD_{600} corresponds to $0.405\ g/L \pm 0.02$ of NBRC 12391 DCW (see Additional file 1 for more detailed information).

Glucose consumption and metabolite production, mainly acetate and propionic acids, were determined from supernatant samples. Briefly, at each sampling time, 1.0 mL of sample was centrifuged at 10,000*g* for 10 min and supernatants were separated, filtrated through 0.45 μm PVDF filters (Teknokroma, Sant Cugat del Valles, Spain) and stored at − 20 °C until its analysis. The metabolite analysis was performed by HPLC 1100 (Agilent, Santa Clara, USA) using an ICSep ICE-Coregel 87H3 column (Concise, San Jose, USA).

A 0.05 M H_2SO_4 solution was used as a mobile phase at 0.5 mL/min. Organic acids were identified at different retention times with UV detection at 210 nm wavelength and glucose with the Refractive index detector. Acetic acid, propionic acid and glucose were quantified with 5-point calibration curve. Glucose was also measured during the cultures with a Y15 Biochemical analyzer (Biosystems, Barcelona, Spain).

Extraction and quantification of CNCbl were adapted from Wang et al. [29]. Briefly, cell pellets obtained from the centrifugation (10 min, 6500*g*), were resuspended in ultrapure water and heated at 90 °C for 20 min. After, they were cold down on ice and centrifuged at 3900*g* for 45 min. Supernatants were recovered and treated with a 0.1% (v/w) NaCN solution for 30 min at room temperature with periodical mixing to transform all possible cobalamin forms into CNCbl. NaCN was added in excess and should be enough to transform up to 200 mg/L of cobalamin. After filtration with 0.45 µm PVDF filters (Teknokroma, Sant Cugat del Valles, Spain), samples were analyzed by HPLC 1100 (Agilent, Santa Clara, USA) using a Waters Nova-Pak C18 4 µm 3.9X150 mm column. Mobile phase was composed of 70% Na_2HPO_4 anhydrous (10 g/L, pH at 3.5) and 30% methanol at a flow rate of 0.3 mL/min. CNCbl was detected by UV detector at 361 nm. Five CNCbl standards at different concentrations were injected in each quantification run to obtain the calibration curve.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-023-02029-x.

Additional file 1: Additional materials. **Table S1.** Evolution of DCW and OD600 in three independent cultures.

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Author contributions
AC experimental investigation, data analysis and writing first draft; MC and TP conceptualization, experimental design and reviewing the manuscript, AP conceptualization and supervision, reviewing the manuscript, and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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