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From Knallgas Bacterium to Promising Biomanufacturing Host: The Evolution of *Cupriavidus necator*

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Abstract

The expanding field of synthetic biology requires diversification of microbial chassis to expedite the transition from a fossil-fuel-dependent economy to a sustainable bioeconomy. Relying exclusively on established model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* may not suffice to drive the profound advancements needed in biotechnology. In this context, *Cupriavidus necator*, an extraordinarily versatile microorganism, has emerged as a potential catalyst for transformative breakthroughs in industrial biomanufacturing. This comprehensive book chapter offers an in-depth review of the remarkable technological progress achieved by *C. necator* in the past decade, with a specific focus on the fields of molecular biology tools, metabolic engineering, and innovative fermentation strategies. Through this exploration, we aim to shed light on the pivotal role of *C. necator* in shaping the future of sustainable bioprocessing and bioproduct development.

Keywords: *Cupriavidus necator*, *Ralstonia eutropha*, CO_2 bioconversion, biological CO_2 capture and utilization (bioCCU), chemolithotrophic bacterium

1.0 Introduction

Cupriavidus necator, a Gram-negative bacterium classified within the β-subclass of *Proteobacteria*, is a ubiquitous inhabitant of soil and freshwater ecosystems. Initially isolated in 1961 [1], this microorganism is renowned for its innate capacity to accumulate poly(3-hydroxybutyrate) (PHB), constituting as much as 90% of its cell dry weight (CDW). Over time, *C. necator* has undergone several nomenclatural revisions. In earlier scientific literature and publications, it was referred to as *Hydrogenomonas eutrophus*, *Alcaligenes eutropha*, *Ralstonia eutropha*, and *Wautersia eutropha*.

C. necator serves as a paradigmatic example of an aerobic lithoautotroph and formatotroph. Its proficiency in utilizing H_2 and CO_2 has earned it the moniker "Knallgas bacterium". In recent times, it has emerged as a compelling platform for metabolic engineering aimed at the bioconversion of CO_2, without being constrained by the availability of light. Moreover, its exceptional adaptability to dynamic environmental shifts and its capacity to seamlessly transition between various metabolic strategies (including heterotrophic, autotrophic, and mixotrophic) position this non-pathogenic microorganism as a prospective catalyst for revolutionary advancements in the realm of industrial biomanufacturing.

In this book chapter, our aim is to provide an extensive overview of the advancements in *C. necator* within the past 10 years. Our focus encompasses three key areas: molecular biology tools, metabolic engineering, and fermentation strategies.

2.0 Molecular biology tools for *Cupriavidus necator*

Over the past decade, a notable proliferation of specialized genetic tools has emerged, purposefully tailored to meet the needs of the *C. necator* research community. This surge in resources stands as a resounding testament to the microbe's growing potential in industrial biotechnology. In this section, we offer an extensive exploration of genetic toolkits encompassing five critical domains: transformation methodologies, plasmid systems, promoter elements, genome editing techniques, and random mutagenesis approaches.

2.1 Transformation techniques

Bacterial conjugation has stood as the primary method for introducing plasmids into *C. necator*. Despite its time-intensive nature, it remains a favoured approach, especially when handling sizable or potentially toxic plasmids [2]. In 2017, Tee *et al.* pioneered a highly efficient electroporation-based transformation technique for *C. necator* H16 [3]. This method yielded a remarkable transformation efficiency of 3.86×10^5 cfu/μg of plasmid pBHR1, representing a 10^3-fold enhancement compared to a previously reported value for the identical plasmid [4]. Other research group also sought to optimize the electroporation protocol by systematically comparing different growth media, the cell growth phase at the time of harvest, and the composition of the electroporation buffer employed [5]. The electroporation efficiency of *C. necator* was constrained by its restriction-modification (RM) systems. Notably, the deletion of H16_A0006, encoding a type I restriction enzyme R subunit, resulted in a striking 1658-fold increase in electroporation efficiency. Similarly, the removal of H16_A0008-9, which encodes 5-methylcytosine-specific restriction enzymes B and C, led to a 4-fold improvement in electroporation efficiency [6]. Plasmids can be further improved for transformation into *C. necator* by propagating and purifying them from an *E. coli* strain that is deficient in both Dam and Dcm DNA methylation, such as C2925 from New England Biolabs [7]. In addition to electroporation, an alternative method involving heat shock transformation of *C. necator* was explored. Nevertheless, it's worth noting that the transformation efficiency achieved through this approach was approximately 40-fold lower when compared to the results obtained through electroporation [8]. Beyond the influence of the transformation protocol and RM systems, the transformation efficiency of *C. necator* is intricately linked to the design and sequence of the plasmid, a topic we delve into in more detail below. As an example, the elimination of non-essential DNA sequences, redundant for plasmid replication, resulted in a 4-fold enhancement in electroporation efficiency [9].

2.2 Plasmid systems

When crafting plasmid systems tailored for *C. necator*, it is essential to account for three pivotal factors: the choice of antibiotic selection markers, plasmid stability, and transformation efficiency. Notably, *C. necator* H16 exhibits inherent resistance to gentamicin, spectinomycin, and kanamycin at low concentration [8, 10].

A diverse array of extensively characterized broad-host-range plasmids, originally isolated from Gram-negative bacteria, have demonstrated compatibility with *C. necator* (Table 1). These plasmids encompass various replication elements, including RP4 (belonging to the IncP incompatibility group), pSa (IncW), RSF1010 (IncQ), pBBR1 (incompatibility undefined) and pMOL28 (incompatibility undefined). However, even when these plasmids were successfully introduced into *C. necator* H16, a notable plasmid loss was observed, notwithstanding the application of antibiotic selection [11]. In addressing the plasmid loss challenge, a proactive approach was taken by fortifying the plasmid design through the integration of the RP4 partition sequence. This pivotal enhancement significantly augmented plasmid stability, resulting in fermentation processes characterized by negligible plasmid loss in recombinant *C. necator* H16, maintaining stability for a minimum of 96 hours [12]. The RP4 partitioning system not only encodes a post-segregational killing system (*parDE*), but also features a site-specific recombination system (*parCBA*), which facilitates the resolution of plasmid multimers [12]. The same strategy was applied in the development of an inducible plasmid system for *C. necator*, yielding promising results. Notably, no plasmid loss was

observed over the course of at least four consecutive cultivations, underscoring the system's robust stability [13]. The choice of plasmid replicon was observed to exert a significant influence on plasmid stability [5, 14]. Notably, plasmids relying on the *Pseudomonas* pVS1 replicon exhibited the highest stability, with over 97% of cells retaining the plasmids throughout a 9-day period [5]. In contrast, plasmids harbouring the pMOL28 and pBBR1 replicons displayed considerably lower stability, with only 9% and 74% of cells, respectively, retaining the plasmids over the same duration.

The transformation efficiency of *C. necator* has been shown to be influenced by both the kanamycin resistance (KanR) cassette and the replication origin sequence within a plasmid [8]. Specifically, a KanR gene encoding an aminoglycoside *O*-phosphotransferase class I, subtype 'a,' demonstrated a 2-fold increase in transformation efficiency compared to KanR gene of the class II, subtype 'a.' Furthermore, plasmids harbouring the complete OriV (origin of vegetative replication) and Rep sequences exhibited transformation efficiencies ranging from 3000 to approximately 5000 times higher than those carrying Rep sequences paired with truncated OriV sequences.

Table 1: Plasmids developed for *C. necator*. Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance, Tc^r, tetracycline resistance.

Plasmid	Genotype/relevant characteristics	Reference
pKRSF1010	Km^r, *par*, RSH1010 *mob* and origin of replication	[12]
pKRTc	Tc^r, *par*, RSH1010 *mob* and origin of replication	[13]
pE-BR20	Km^r, *mob*, f1 ori, *RP4*, pBBR1 origin of replication	[15]
pMPJAS03	Cm^r, Km^r, pBBR1 origin of replication	[16]
pCAT201	Km^r, pBBR1 OriV-Rep	[8]
pMTL71101	Cm^r, pBBR1 origin of replication	[5]
pMTL75111	Cm^r, pVS1 origin of replication	[5]

2.3 Promoter elements

Several renowned and well-characterized promoters, including P_{lac}, P_{lacUV5}, P_{tac}, P_{trp}, P_{BAD}, and P_{T7}, were shown to be active in *C. necator* H16 [12, 17-18]. Over the last decade, a diverse array of constitutive and inducible promoters, each varying in strength, has been developed, thus enriching the continually expanding genetic toolkit available for research and engineering purposes.

Numerous native promoters sourced from *C. necator* H16 have found utility in facilitating constitutive gene expression. These promoters are harnessed from operons involved in metabolic pathways such as PHB biosynthesis (P_{phaC}), acetoin metabolism (P_{acoD}, P_{acoX}, P_{acoE}), or pyruvate metabolism (P_{pdhE}) [19]. In an endeavour to expand the promoter repertoire, Gruber *et al.* tested and characterized a suite of constitutive promoters (P_{de33}, P_{f30}, P_{g25}, P_{h207}, P_{h22b}, P_{j5}, P_{k28a}, P_{k28b}, P_{n25}, P_{n26}, and P_{T5}) sourced from the bacteriophage T5 [12]. Among these 11 promoters, P_{j5} was identified as the strongest in driving gene expression. In a comprehensive study conducted by Johnson *et al.*, the architecture and genetic components of the four most prevalent constitutive promoters in *C. necator* H16 (namely P_{phaC1}, P_{rrsC}, P_{j5}, and P_{g25}) were meticulously examined. Leveraging these four promoters as foundational templates and employing a spectrum of genetic modifications, including point mutations, adjustments in length, integration of regulatory genetic elements, promoter hybridization, and configuration alterations, they constructed a diverse library of 42 functional constitutive promoters tailored for use in *C. necator* H16 [20]. The promoter engineering approach put forth by Johnson *et al.* was applied to augment the expression of *Vitreoscilla* hemoglobin in *C. necator* H16, employing the engineered $P_{phaC1\text{-}j5}$ promoter [21]. Further, the engineered promoter ($P_{j5[C2]}$) was used to construct the pCAT vector series tailored for *C. necator* H16 [8].

Similar to constitutive promoters, a diverse array of inducible promoters has emerged in the past decade, affording precise control over the timing and magnitude of gene expression. Promoter P_{BAD} is perhaps the most frequently used for inducible gene expression in *C. necator* [6, 16, 18, 20]. A frequently used promoter design approach involves the fusion of a constitutive promoter (*e.g.*, P_{rrsC}, P_{T5}, P_{j5}) with regulatory elements (*e.g.*, operator sites), as illustrated in Figure 1. In simpler terms, this strategy employs a repressor protein that responds to an inducer to enable controlled and inducible gene expression. These inducible systems encompass a variety of repressor-inducer pairs. Examples include the TetR repressor, which responds to the inducer anhydrotetracycline (aTc) [22], the AraC repressor and L-arabinose [23], the LacI repressor, which relies on IPTG induction with transport facilitated by the integrated lactose permease (LacY) in *C. necator* H16 [17], the XylS repressor responding to *m*-toluic acid [23], the CymR repressor and *p*-cumate [17], as well as the AcuR repressor and acrylate [18]. Furthermore, hybrid promoters have been developed, incorporating the native *tolC* promoter of *C. necator* in conjunction with a synthetic tetO-operator. These promoters are contingent upon the introduction of the exogenous inducer, doxycycline (dc), for gene expression [15]. Additional systems worth noting encompass the L-rhamnose inducible system [13, 18] and the T7 expression system [9]. The latter was established through the integration of a T7 RNA polymerase gene under the control of the P_{BAD} promoter into the *C. necator* genome, along with the introduction of a P_{T7} promoter upstream of the target gene.

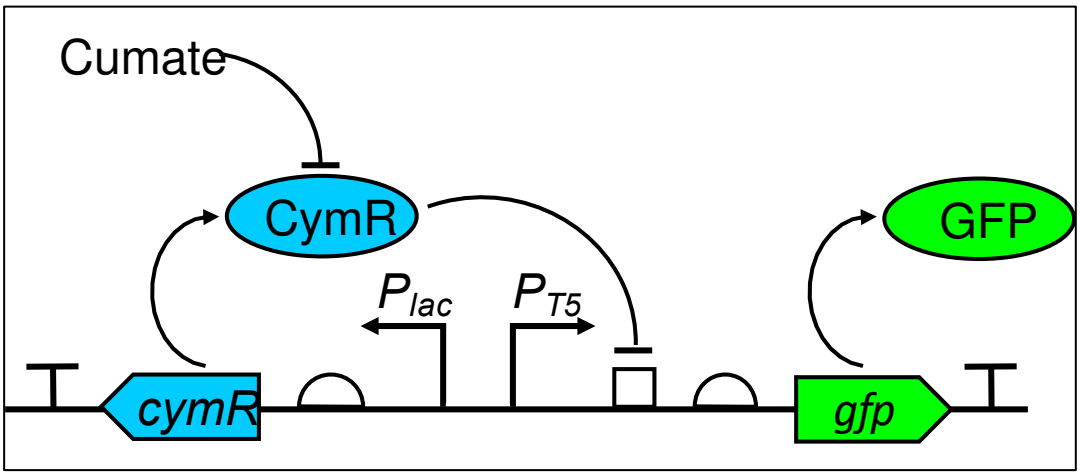

Figure 1: Inducible promoter design that combined constitutive promoters and regulatory elements. Example illustrated here used the CymR repressor [17] to regulate GFP expression driven by the constitutive P_{T5} promoter. GFP expression was induced by addition of *p*-cumate.

Exploration was also carried out on the promoter (P_{SH}) associated with the soluble hydrogenase (SH) operon within *C. necator* [24]. Intriguingly, this promoter remained dormant during hydrogenase-repressing conditions, such as growth on fructose, while becoming active under hydrogenase-derepressing conditions, as seen during growth on glycerol. This promoter therefore holds significant potential for facilitating growth-related optimizations in *C. necator*.

2.4 Genome editing methods

In the past, genome editing in *C. necator* relied on a suicide vector incorporating the *sacB* gene from *Bacillus subtilis*, which becomes activated in the presence of sucrose and proves lethal when expressed in Gram-negative bacteria [25]. In a nutshell, this process involved transferring the suicide plasmid to *C. necator* through conjugation from *E. coli* S17-1. Transconjugants carrying the integrated plasmid were selectively isolated using appropriate antibiotics, and strains that had lost the integration vector via a second single crossover were identified in a nutrient-rich medium containing sucrose, utilizing *sacB* as the negative selection

marker. However, this method, which typically took an average of 2–3 weeks to delete a single gene, was not only time-consuming but also exhibited relatively low efficiency.

In 2018, a CRISPR-Cas9 genome editing method was introduced for *C. necator*. This technique involved driving the expression of Cas9 from the editing plasmid using the P_{BAD} promoter, while the corresponding sgRNA was transcribed from a constitutive promoter. To mitigate unintended expression from the arabinose-inducible P_{BAD} promoter, which was used to optimize Cas9 expression, fructose was employed to minimize leaky expression. This setup facilitated genome editing through homologous recombination, resulting in the successful editing of a total of five genes, with editing efficiencies ranging from 78.3% to 100% [6]. The P_{BAD} promoter is known for its inherent leakiness. Studies have shown that the presence of fructose [6] or glucose [26] effectively suppress its leaky expression.

2.5 Random mutagenesis

Transposon-directed insertion-site sequencing (TraDIS), as developed by Langridge *et al.* [27], has proven effective in generating transposon mutants for *C. necator* H16, facilitating the assessment of gene essentiality [7]. Transposon mutagenesis stands out as a potent and practical approach for creating single-gene disruption mutants in *C. necator* [28].

Adaptive laboratory evolution (ALE) emerges as a powerful and robust technique for creating *C. necator* variants tailored to specific cultivation conditions or the utilization of distinct carbon sources, all without prior knowledge of its metabolic network. This methodology has proven successful in adapting *C. necator* to efficiently harness both laboratory-grade glycerol and crude glycerol derived from the fat-splitting process [29].

The genome sequence of *C. necator* H16 was initially published in 2006 [30], followed by a comprehensive re-sequencing effort in 2019 employing a combination of PacBio and Illumina sequencing technologies [31]. Additionally, there are several genome-scale models available for this organism, namely RehMBEL1391 [32] and *i*CN1361 [7]. The convergence of these invaluable resources, coupled with the genetic tools discussed herein, undeniably represents a major leap forward in the realm of *C. necator* engineering. This progress is evident from the extensive array of bioproducts achievable through the manipulation of *C. necator*, as elaborated upon in the subsequent section.

3.0 Products manufactured by engineered *C. necator*

The current market volume for bio-based chemical production is estimated at US$ 80 billion, projected to grow annually by approximately 10% [33]. With its remarkable metabolic versatility, *C. necator* stands poised to significantly bolster this growth. This bacterium has a diverse range of metabolic pathways, including carbon fixation via the Calvin-Benson-Bassham (CBB) cycle, hydrogen and formate oxidation, utilisation of nitrate/nitrite as alternative electron acceptors, and xenobiotics degradation [34]. While *C. necator* is renowned for its polyhydroxyalkanoate (PHA) production, the prevailing metabolic engineering strategy for generating value-added products involves disrupting this pathway through the deletion of the *phaCAB* operon, concurrently boosting the activity of heterologous pathways to redirect carbon flux [35]. This section delves into the robust and versatile capabilities of *C. necator* in utilizing various carbon sources and metabolic pathways to synthesize a diverse array of value-added chemicals, as succinctly depicted in Figure 2. Rather than enumerating products achievable through engineered *C. necator*, this chapter adopts a distinctive approach by spotlighting the frequently targeted pathways for product synthesis.

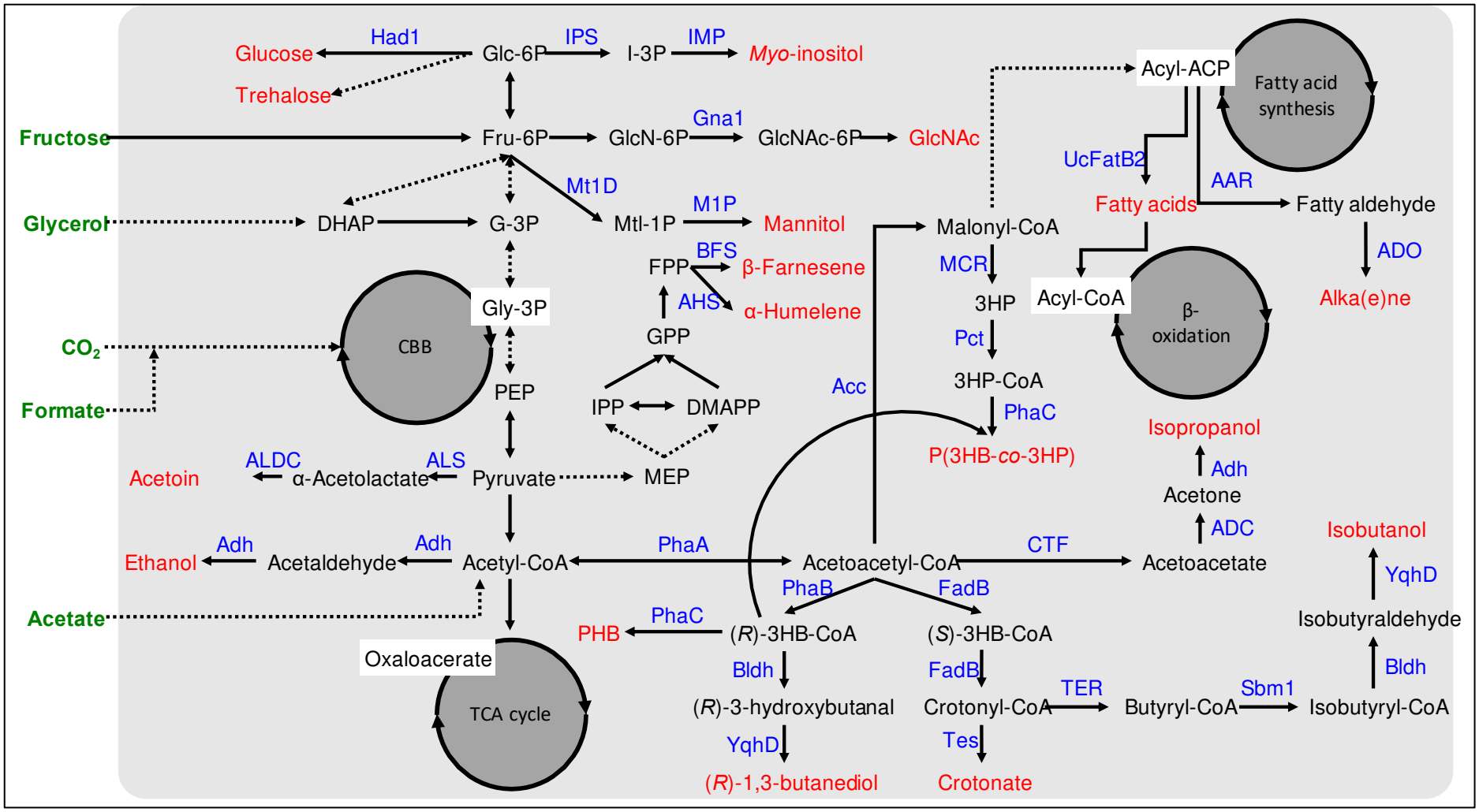
Had1
IPS
IMP
Glucose
Glc-6P
I-3P
Myo-inositol
Trehalose
Fructose
Fru-6P
Gna1
GlcN-6P
GlcNAc-6P
GlcNAc
Mt1D
Glycerol
DHAP
G-3P
Mtl-1P
M1P
Mannitol
BFS
FPP
β-Farnesene
AHS
α-Humelene
Gly-3P
GPP
CO2
CBB
PEP
IPP
DMAPP
Formate
Acetoin
ALDC
ALS
α-Acetolactate
Pyruvate
MEP
Ethanol
Adh
Acetaldehyde
Adh
Acetyl-CoA
PhaA
Acetoacetyl-CoA
CTF
Acetoacetate
Acetate
PhaB
FadB
PHB
PhaC
(R)-3HB-CoA
(S)-3HB-CoA
Oxaloacerate
TCA cycle
Bldh
FadB
(R)-3-hydroxybutanal
Crotonyl-CoA
TER
Butyryl-CoA
Sbm1
Isobutyryl-CoA
YqhD
Tes
(R)-1,3-butanediol
Crotonate
Acyl-ACP
Fatty acid synthesis
UcFatB2
AAR
Fatty acids
Fatty aldehyde
ADO
Alka(e)ne
Malonyl-CoA
MCR
3HP
Pct
3HP-CoA
PhaC
P(3HB-co-3HP)
Acc
Acyl-CoA
β-oxidation
Isopropanol
Adh
Acetone
ADC
Isobutanol
YqhD
Isobutyraldehyde
Bldh

Figure 2: The native and engineered metabolic pathways in *C. necator* for the manufacture of diverse products. The carbon feedstocks are in green, products are in red, enzymes catalyzing the reactions are in blue. Dotted lines represent multiple reactions. Abbreviations of enzymes: AAR, acyl-ACP reductase; Acc, acetyl−CoA carboxylase; ADC, acetoacetate decarboxylase; Adh, alcohol dehyrogenase; ADO, aldehyde decarbonylase oxygenase; AHS, α-humelene synthase; ALDC, acetolactate decarbocylase; ALS, acetolactate synthase; BFS, β-farnesene synthase; Bldh, CoA-acylating aldehyde dehydrogenase; CTF, acetoacetyl-CoA reductase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; Gna1, *N*-acetylglucosamine-6-phosphate *N*-acetyltransferase; Had1, haloacid dehalogenase-like phosphatase; IMP, inositol monophosphatase; IPS, inositol-3-phosphate synthase; M1P, mannitol-1-phosphate phosphatase; MCR, malonyl−CoA reductase; Mt1D, mannitol-1-phosphate dehydrogenase; Pct, propionyl−CoA transferase; PhaA, β-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; Sbm1, acyl-CoA mutase; TER, trans-2-enoyl-CoA reductase; Tes, thioesterase; UcFatB2, medium-chain-length specific acyl-ACP thioesterase; YqhD, broad-substrate range alcohol dehydrogenase. Abbreviations of metabolites: 3HP, 3-hydroxypropionate; 3HP-CoA, 3-hydroxypropionyl-CoA; DHAP, dihydroxyacetone phosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; Fru-6P, fructose-6-phosphate; G-3P, glyceraldehyde-3-phosphate; Glc-6P, glucose-6-phosphate; GlcN-6P, glucosamine-6-phosphate; GlcNAc, *N*-acetylglucosamine; GlcNAc-6P, *N*-acetylglucosamine-6-phosphate; GPP, geranyl pyrophosphate; Gly-3P, glycerate-3-phosphate; I-3P, myo-inositol-3-phosphate; IPP, isopentenyl pyrophosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; Mtl-1P, mannitol-1-phosphate; PEP, phosphoenolpyruvate; (*R*)-3HB-CoA, (*R*)-3-hydroxybutyryl-CoA; (*S*)-3HB-CoA, (*S*)-3-hydroxybutyryl-CoA.

3.1 Carbohydrate metabolism

Carbohydrate metabolism not only serves as the primary energy source but also provides essential intermediates for PHB synthesis within *C. necator*. Carbohydrates undergo metabolic processing through various pathways, including, but not limited to, the glycolytic pathway, tricarboxylic acid (TCA) cycle, the Entner-Doudoroff (ED) pathway, the pentose phosphate (PP) pathway, and gluconeogenesis. Intermediates generated within these pathways, such as glucose-6-phosphate and fructose-6-phosphate, can be strategically redirected to facilitate the production of value-added products. For instance, the production of sugar from CO_2 has emerged as a compelling strategy to transition from a fossil fuel-dependent economy to a sustainable one [36]. By predominantly harnessing gluconeogenesis while obstructing the glucose catabolic, ED, and PHB biosynthesis pathways, a yield of 253.3 mg/L glucose was synthesized from CO_2 [37]. A comparable approach was employed in the quest for generating monosaccharide derivatives of glucose, such as *N*-acetylglucosamine (GlcNAc) and *myo*-inositol. Through the strategic disruption of GlcNAc import and its intracellular metabolic pathways, blocking the ED and PHB biosynthesis pathways, and expressing the GlcNAc-6-phosphate *N*-acetyltransferase gene (*gna1*), a GlcNAc titer of 75.3 mg/L was attained in autotrophic fermentation [38]. Through the deliberate overexpression of *myo*-inositol-3-phosphate synthase (IPS) from *S. cerevisiae* and inositol monophosphatase (IMP) from *E. coli*, in conjunction with the blocking of the ED and PHB biosynthesis pathways, an impressive yield of 1054.8 mg/L of *myo*-inositol was attained from CO_2. Similarly, the expression of mannitol-1-phosphate dehydrogenase (MtlD) and mannitol- 1-phosphate phosphatase (M1P) sourced from the brown alga *Ectocarpus siliculosus* enabled the production of 3.9 g/L of mannitol from CO_2 [39]. Under stress conditions induced by NaCl, *C. necator* naturally produces trehalose, as elucidated by Lowe *et al.* [40]. Introducing the sugar efflux transporter A (*setA*) from *E. coli* resulted in a trehalose-leaky phenotype in *C. necator*. Remarkably, the engineered strain demonstrated the ability to produce as much as 0.47 g/L of trehalose from CO_2.

3.2 Pyruvate metabolism

Glycolysis and CO_2 fixation through the CBB cycle yield C3 intermediates, such as pyruvate, serving as foundational building blocks for the synthesis of more intricate and diversified

compounds [35]. Acetoin, also known as 3-hydroxybutanone, is a valuable C4 compound with wide-ranging applications spanning food, agriculture, the chemical industry, and cosmetics [41]. Notably, in 2004, it was designated as one of the top 30 key platform compounds prioritized for development by the United States Department of Energy [42]. The production of acetoin from pyruvate involves two crucial steps: first, acetolactate synthase (ALS) catalyzes the condensation of two moles of pyruvate to yield α-acetolactate, and second, acetolactate decarboxylase (ALDC) facilitates the conversion of α-acetolactate into acetoin [41]. Achieving acetoin production in *C. necator* was realized through a multi-faceted approach. This encompassed the deletion of the acetoin consumption operon (*acoABC*), the deletion of competing pathways (*phaC1* and *phaC2*), and the introduction of codon-optimized genes sourced from *Bacillus subtilis* (*alsS* and *alsD*) under the regulation of the PHB promoter. These concerted efforts yielded a noteworthy production rate of 0.32 mol of acetoin per mol of CO_2 [43]. Furthermore, efficient acetoin production can be accomplished through mixotrophic fermentation, employing both propionate and CO_2 as carbon sources [44].

The 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway is a prominent metabolic route found in bacteria, including *C. necator* [45]. Comprising seven distinct steps, the MEP pathway orchestrates the conversion of glyceraldehyde-3-phosphate and pyruvate into isopentenyl-5-pyrophosphate (IPP) and dimethylallyl-pyrophosphate (DMAPP). These compounds serve as essential building blocks for the synthesis of longer-chain precursors required for terpenoid production. Terpenoids hold significant industrial value, finding applications in perfumes, pharmaceuticals, as eco-friendly alternatives to chlorinated solvents, and as potential substitutes for petroleum-derived fuels [46]. Among terpenoids, sesquiterpenes (C15), including β-farnesene and α-humulene, constitute the largest subgroup [47], each offering distinct industrial applications. β-farnesene serves as a precursor for fuel additives [48], while α-humulene holds promise as a potential anticancer drug [49]. The introduction of β-farnesene synthase from *Artemisia annua* into *C. necator* H16 PHB$^-$4 resulted in a β-farnesene product yield of 26.3±1.3 μM through a fed-batch process [50]. Furthermore, the augmentation of the native MEP pathway in *C. necator* with a heterologous mevalonate (MVA) pathway, as demonstrated by Krieg *et al.* [51], led to the production of 17 mg of α-humulene per gram of cell dry weight (CDW) under autotrophic conditions. This same engineered strain achieved a substantial production rate of 2 g/L of α-humulene during fed-batch fermentation, utilizing fructose as the carbon source in conjunction with *n*-dodecane as an extracting organic phase for *in situ* product removal [47].

3.3 Acetyl-CoA metabolism

Much like pyruvate mentioned earlier, acetyl-CoA serves as a pivotal hub in metabolism, owing to its central role in interfacing with numerous metabolic pathways and transformations. Acetyl-CoA also functions as a foundational precursor for the synthesis of fatty acids, PHB, and a plethora of other essential molecules. Consequently, it is a logical focal point for endeavours aimed at generating value-added compounds, exemplified by the production of crotonate and similar molecules. Crotonate, a short-chain unsaturated carboxylic acid, holds significant industrial promise as a fundamental building block for the synthesis of copolymers and organic chemical intermediates [52]. Collas *et al.* [33] devised a heterologous crotonate pathway geared towards the conversion of acetyl-CoA into crotonate, employing intermediates such as acetoacetyl-CoA and crotonyl-CoA. This approach led to the successful production of 148 mg/L of crotonate from formate.

Countries such as the United States of America, Brazil, and various European nations have embraced ethanol as a prominent fuel source [53]. Introduction of an alcohol dehydrogenase (Adh) from *E. coli* into *C. necator* has enabled the conversion of acetyl-CoA into ethanol, passing through the intermediary acetaldehyde. This led to the synthesis of 0.35 g/L of ethanol from acetate [54]. It is worth noting that the established fuel distribution infrastructure in many countries readily accommodates C4 alcohols, allowing for their direct utilization as vehicle fuels without the necessity for blending with petrol [45]. While prior research endeavours have

primarily focused on engineering the Ehrlich pathway for isobutanol production, an intriguing alternative has emerged through the development of a CoA-dependent pathway. This novel approach entails elongating acetyl-CoA into butyryl-CoA, followed by a rearrangement from butyryl-CoA to isobutyryl-CoA facilitated by acyl-CoA mutase (Sbm1) and the conversion of isobutyryl-CoA into isobutanol. The engineered *C. necator* strain was capable of producing 32 mg/L of isobutanol from fructose [55].

Leveraging acetyl-CoA or its precursors, notably pyruvate, offers a promising avenue for the synthesis of (*R*)-1,3-butanediol (1,3-BDO) and isopropanol. 1,3-BDO features diverse applications across food, chemical, and cosmetic sectors [56]. As a naturally occurring C4 diol found in bell peppers, it imparts a delicate fruity aroma. It serves as a pivotal intermediate or solvent in the production of paints, coatings, inks, plasticizers, 1,3-butadiene, and azetidinone derivatives. In personal care, 1,3-BDO acts as an effective humectant and emollient. In the quest for (*R*)-1,3-BDO production, two distinct pathways were engineered [57]. The first pathway facilitates the conversion of acetyl-CoA into (*R*)-1,3-BDO via intermediates like acetoacetyl-CoA, (*R*)-3-hydroxybutyryl-CoA and (*R*)-3-hydroxybutanal, while the second pathway employs pyruvate as a precursor to generate (*R*)-1,3-BDO through the intermediate (*R*)-3-hydroxybutanal. By amalgamating these pathways, coupled with the strategic elimination of PHB biosynthesis and reducing flux through the TCA cycle, the engineered strain produced 2.97 g/L of (*R*)-1,3-BDO from CO_2. Isopropanol is used as a fuel additive, primarily attributed to its high-octane rating [53]. In the pursuit of isopropanol production, a similar strategy was employed, wherein acetyl-CoA was transformed into isopropanol, traversing through intermediary compounds such as acetoacetyl-CoA, acetoacetate, and acetone [58]. Overexpression of native *groEL* and *groES* genes was found to enhance *C. necator*'s tolerance to exogenous isopropanol [59]. The tolerance enhancement facilitated the attainment of an impressive final isopropanol concentration of 9.8 g/L in a fed-batch culture, employing fructose as the exclusive carbon source.

3.4 Malonyl-CoA pathway

3-Hydroxypropionate (3HP) is a compelling platform chemical that holds immense promise as a precursor for a diverse range of essential compounds, including acrylate, acrylamide, and even as a monomer for biodegradable plastics. The synthesis of 3HP can be accomplished through various intermediates, such as glycerol, malonyl-CoA, and β-alanine. Among these bioengineering pathways, the malonyl-CoA route presents distinctive advantages, including its versatility in accommodating a wide array of feedstocks, favourable thermodynamics, and inherent redox neutrality [60]. An enticing approach for achieving autotrophic 3-HP production involves the implementation of the 3-HP bicycle pathway, recognized for its remarkable efficiency in aerobic CO_2 fixation. This pathway leverages the malonyl-CoA reductase (MCR) as one of its key enzymatic components [61]. Through the strategic expression of an MCR responsible for the conversion of malonyl-CoA into 3HP and a propionyl-CoA transferase tasked with converting 3HP into 3HP-CoA, engineered *C. necator* could produce 3.11 g/L of [P(3HB-*co*-3HP)] with a remarkable 32.25% molar fraction of 3-HP [62].

3.5 Fatty acid biosynthesis and β-oxidation pathways

Fatty acids, a diverse and vital class of molecules, have garnered substantial attention in the realm of metabolic engineering due to their potential as precursors for biofuels [45]. In the biosynthesis of fatty acids, acetyl-CoA is iteratively condensed on an acyl carrier protein (ACP). Fatty acids are also subject to consumption through the competitive β-oxidation pathway [63]. The introduction of UcFatB2, a thioesterase specific to medium-chain-length acyl-ACP, led to the generation of medium-chain-length fatty acids within *C. necator*. This achievement was further enhanced by strategically eliminating PHB synthesis and selectively deleting acyl-CoA ligases, which serve as entry points for fatty acids into the β-oxidation pathway. These combined efforts yielded a production of up to 62 mg/L of free fatty acids [64]. Likewise, in a study conducted by Li *et al.* [65], a notable synthesis of fatty acids was achieved, with yields of 128.48 mg/g CDW using fructose as the carbon source and 60.64 mg/g CDW when CO_2

was employed. This accomplishment was realized through the strategic overexpression of endogenous acetyl-CoA carboxylase and cytoplasmic thioesterase enzymes, complemented by the introduction of exogenous type I polyketide synthase and holo-ACP synthase.

Alkanes with defined carbon chain lengths offer promising alternatives to petroleum-based fuels. To realize alkane synthesis, Crepin *et al.* [66] introduced a pathway from *Synechococcus elongatus* consisting of two genes encoding an acyl-ACP reductase (*aar*) and an aldehyde deformylating oxygenase (*ado*) into a *C. necator* mutant strain deficient in the PHB synthesis pathway. Under heterotrophic conditions with fructose as the carbon source and nitrogen limitation, in the presence of an organic phase (decane), the strain was capable of producing 670 mg/L of total hydrocarbons, consisting of 286 mg/L of pentadecane, 131 mg/L of heptadecene, 18 mg/L of heptadecane, and 236 mg/L of hexadecanal.

Metabolic engineering has empowered *C. necator* to produce a wide array of valuable products. Nonetheless, effective strain engineering should seamlessly integrate with advanced fermentation strategies to not only create economically viable bioprocesses but also enhance product yields.

4.0 Fermentation Methods

C. necator exhibits remarkable versatility in harnessing a wide array of carbon feedstocks, such as carbohydrates, glycerol, organic acids, and C1 compounds [67]. Its genetic adaptability has significantly broadened its capacity to utilize diverse carbon sources [29], rendering it an exceedingly appealing candidate as a biomanufacturing host for the burgeoning biotech industry. Numerous fermentation strategies have been innovatively devised to unlock its full biomanufacturing potential (Figure 3), with a growing focus on sustainable feedstock utilization (Table 2). While polyhydroxybutyrate (PHB) remains the predominant product of *C. necator*, we are witnessing a noteworthy diversification in its product portfolio (Table 2), facilitated by the organism's amenability to genetic manipulation and the continuous expansion of its genetic toolkit.

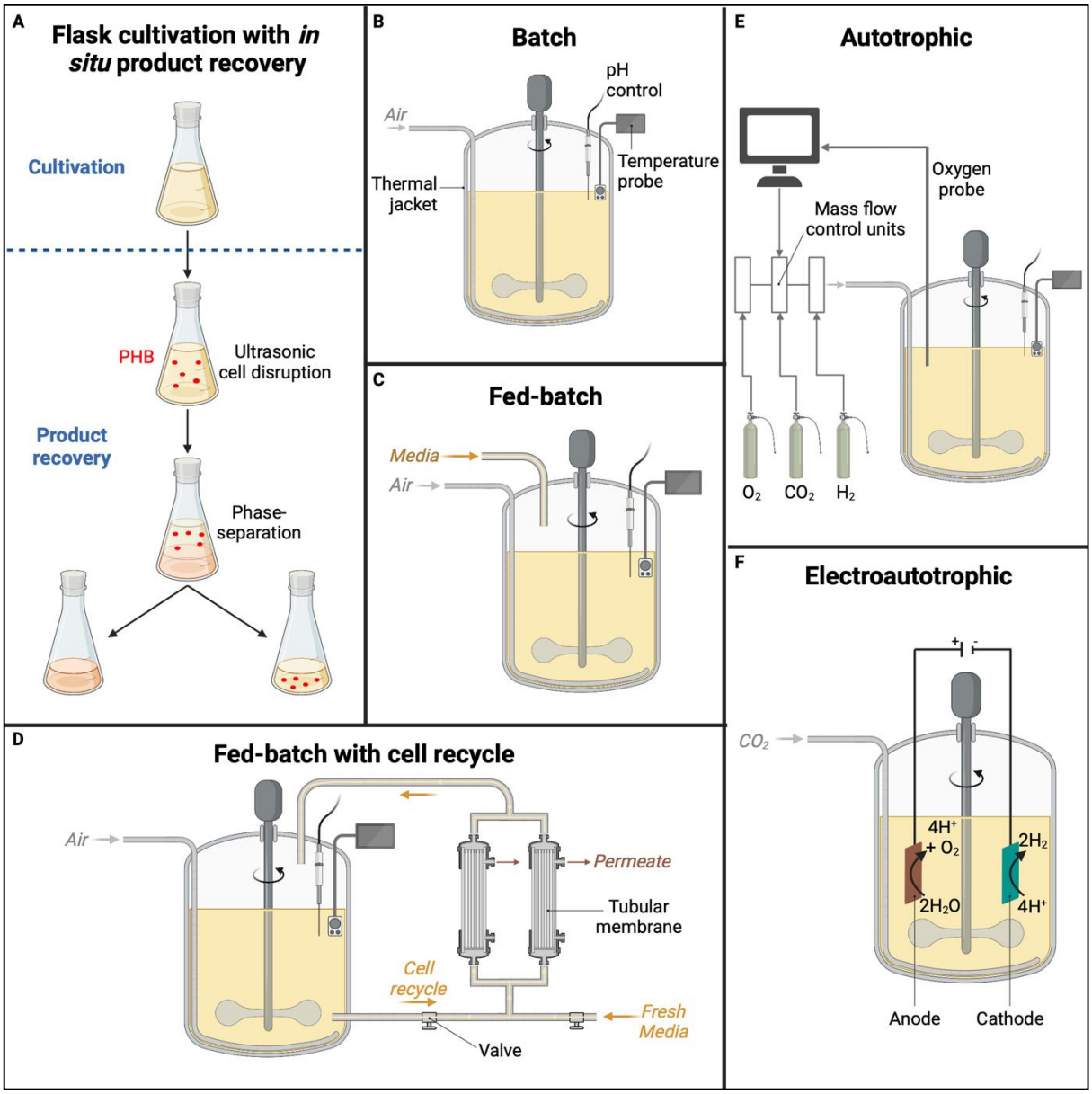

Figure 3: Various fermentation strategies using *Cupriavidus necator* as a biomanufacturing host. (**A**) Flask cultivation with or without *in situ* product recovery [68], (**B**) batch fermentation, (**C**) fed-batch fermentation, (**D**) fed-batch fermentation with cell recycle [69], (**E**) autotrophic fermentation [70], and (**F**) electroautotrophic fermentation [71].

Table 2: Biomanufacturing with *C. necator* using different fermentation strategies, along with the products created and the feedstocks used. Yield is reported in terms of gram product per gram substrate consumed. PHB, poly(3-hydroxybutyrate); P(3HB-*co*-3HV), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); P(3HB-*co*-3HHx), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate); P(3HB-*co*-3HD), poly(3-hydroxybutyrate-*co*-3-hydroxydecanoate).

Fermentation strategies	Product	Feedstock	Titre (g/L)	Productivity (g/L/h)	Yield (g/g)	Reference
Flask culture	PHB	Fructose	4.6	0.06	0.31	[72]
Flask culture	PHB	Glucose	11.83	0.25*	0.91*	[73]
Flask culture	PHB	Glucose	2.155	-	-	[74]
Flask culture	P(3HB-*co*-3HV)	Volatile fatty acids from broken rice	0.95	0.01*	-	[75]
Flask culture with *in situ* product recovery	PHB	Glycerol	0.44*	-	-	[68]
Co-culture	P(3HB-*co*-3HV)	Sucrose	2.30	-	0.08	[76]
Batch	PHB	Milled corn hydrolysate and crude glycerol	14.17	0.197	0.43	[77]
Batch	PHB	Molasses	1.31	0.012*	-	[78]
Batch	P(3HB-*co*-3HV)	Synthetic volatile fatty acids	1.60	0.044*	0.25	[79]
Fed-batch	PHB	Acetic acid	4.95	0.17*	0.33	[80]
Fed-batch	PHB	Acetic acid	58.5	0.93	-	[81]
Fed-batch	PHB	Waste makgeolli lees hydrolysate and glucose	24.1	0.33*	-	[82]
Fed-batch	PHB	Corn stover alkaline pretreatment liquor	4.47	0.080	-	[83]
Fed-batch	PHB	Fructose	25.7	0.43	-	[84]
Fed-batch	PHB	Crude glycerol and rapeseed meal hydrolysate	24.75	0.21	0.32	[85]
Fed-batch	PHB	Crude glycerol and sunflower meal hydrolysate	27	0.28	0.32	[86]

Fed-batch	PHB	Waste glycerol	65.6	1.36	-	[87]
Fed-batch	PHB	Glycerol	85.8	1.83	-	[88]
Fed-batch	PHB	Banana pulp juice and fructose	2.816	-	-	[89]
Fed-batch	PHB	Used cooking oil	19.8	0.53	0.52	[90]
Fed-batch	P(3HB-*co*-3HV)	Volatile fatty acids from cheese whey and glucose	10.6*	-	0.60	[91]
Fed-batch	P(3HB-*co*-3HV)	Waste vegetable oil and propionic acid	121.7	2.03	0.67**	[92]
Fed-batch	P(3HB-*co*-3HHx)	Fructose and rapeseed oil	-	1.45	-	[93]
Fed-batch	P(3HB-*co*-3HD)	Beeswax hydrolysate and glucose	8.91	-	-	[94]
Fed-batch with cell recycle	PHB	Glucose	113	3.10	0.33**	[95]
Fed-batch with cell recycle	PHB	Grass silage	9.4	0.31	0.15	[96]
Fed-batch with cell recycle	P(3HB-*co*-3HV)	Synthetic volatile fatty acids	2.8	-	-	[97]
Fed-batch with cell recycle	P(3HB-*co*-3HV)	Glucose and propionic acid	64.6	1.24	-	[69]
Autotrophic	PHB	CO_2	10.2	-	-	[70]
Autotrophic	PHB	CO_2	11.6*	-	-	[98]
Autotrophic	Isopropanol	CO_2	3.5	-	-	[99]
Heterotrophic-autotrophic	PHB	Waste glycerol and CO_2	28	0.168	-	[100]
Heterotrophic-autotrophic	Isopropanol	Fructose and CO_2	0.25	0.021*	0.093	[59]
Electroautotrophic	Isopropanol	CO_2	0.216	-	-	[71]
Electroautotrophic	α-humulene	CO_2	0.0108	0.00008	-	[51]
Mixotrophic	P(3HB-*co*-3HV)	Synthetic volatile fatty acids and CO_2	3.1*	0.026*	-	[101]

*Value was not directly given in paper, but calculated from other data within the study.
**Yield is reported in terms of gram product per gram substrate fed.

4.1 Heterotrophic cultivation

PHB production in *C. necator* is typically achieved in media rich in carbon, often with either nitrogen or phosphorus limitation [102]. The simplest mode of cultivating *C. necator* is a shake flask culture. This approach is often used to assess the impact of different culture conditions on the bacteria. For example, response surface methodology (RSM) was applied to optimise culture media for PHB production by *C. necator* ATCC 17697 [72]. With a carbon concentration of 20 g/L, nitrogen limited to 1.5 g/L, phosphate levels at 8.75 g/L, and pH 7.5, a maximum PHB production of 4.6 g/L was achieved [72]. Another study, using the engineered strain *C. necator* NSDG-GG, showed that optimal conditions differed whether the focus was on cell growth or PHB production [73]. This suggests a two-stage approach would be advantageous, where biomass is initially maximised before PHB production prioritised.

Flask cultures can also be employed to establish innovative fermentation strategies, including integrated biorefinery concepts [75]. The use of ultrasound during the cultivation of *C. necator* NRRL B14690 was found to increase PHB yield by a factor of almost 2 compared to culture without ultrasonication [74]. Extractive bioconversion that integrates fermentation and downstream PHB purification has also been investigated in flask cultures [68]. An aqueous two-phase system was applied, which utilises heat and a thermo-separating polymer to split the culture into a water-rich top phase containing PHB, and a polymer-rich bottom phase containing cells that can be recycled (Figure 3A). Co-culture of *C. necator* with another microorganism has been demonstrated at an even smaller scale. In 25 mL tubes containing 10 mL of media, 2.30 g/L poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) was produced from sucrose by a culture of *C. necator* 5119 and *Bacillus subtilis* [76]. *B. subtilis* hydrolysed sucrose, which cannot otherwise be utilised as a carbon source by *C. necator*.

At increased scale, batch fermentations are often used to assess the viability of new carbon sources (Figure 3B). P(3HB-*co*-3HV) production from volatile fatty acids (VFA) at different concentrations and with varying compositions has been investigated, with a highest titre of 1.60 g/L achieved despite the focus being on optimising growth [79]. de Mello *et al.* [77] studied PHB accumulation using reducing sugars from milled corn hydrolysate and crude glycerol, with the aim of coupling bioplastic and bioethanol production in an integrated biorefinery. In an 8 L stirred tank reactor, 14.17 g/L PHB was achieved with 0.197 g/L/h productivity [77]. Another innovation trialled in batch cultivation is the reuse of filtrate from cell recovery as substrate [78]. Following initial cultivation of *C. necator* TISTR 1095 on molasses, cells were recovered and PHB extracted. The filtrate from this process contained sugar and organic acid, hence it could be recycled as the carbon source in a second reactor. While the production was lower in the second reactor (1.13 g/L vs 1.31 g/L) [78], this may be a viable strategy to reduce waste.

The most common strategy for polyhydroxyalkanoate (PHA) production from *C. necator* is in a fed-batch bioreactor [69, 80-97], which allows the substrate concentration to be controlled throughout fermentation (Figure 3C). Kedia *et al.* [80] continually fed VFA as both carbon source and pH control, meaning NaOH was not required to adjust pH. Sodium accumulation has been shown to negatively affect *C. necator* [103], hence the change in feeding regime increased PHA accumulation almost 2 folds [80]. Another benefit of fed-batch fermentation is the ability to control the carbon to nitrogen (C:N) ratio. Makgeolli lees enzymatic hydrolysate was used as feedstock for PHB production by *C. necator* H16 [82]. During fed-batch cultivation with the C:N ratio controlled to 20:1 by the periodic addition of glucose, 30 times more PHB was synthesised compared with batch fermentation [82]. This was a significant increase in product yield, despite only approximately doubling the carbon source supplied and increasing cell growth by 1.5-fold [82]. However, fed-batch fermentation is not always more efficient than batch. Li and Wilkins [83] compared two fed-batch approaches to batch fermentation for PHB production by *C. necator* DSM 545 using lignin from corn stover alkaline pretreatment liquor. A higher concentration of PHB was reached during fed-batch fermentation but over a longer period of time, with productivity being highest in batch cultivation [83].

High cell density fermentation has been used with fed-batch feeding to increase PHA production further. Under oxygen-limited conditions, 121.7 g/L P(3HB-*co*-3HV) was produced from waste frying oil and propionic acid by *C. necator* H16 [92]. In an attempt to overcome substrate inhibition, an immersed membrane was used to maintain high cell density in fed-batch fermentation of *C. necator* DSM 545 on VFA [97]. Following 128 h cultivation, a maximum PHB titre of 2.8 g/L was reached, a 1.75-fold increase on the concentration achieved by the same authors with a conventional batch method [79]. The use of an external membrane to recycle cells (Figure 3D) has also been employed to increase PHA accumulation. Haas *et al.* [95] produced 113 g/L PHB from glucose with a productivity of 3.10 g/L/h, while Schmidt *et al.* [69] added propionic acid and achieved 64.6 g/L P(3HB-*co*-3HV) at 1.24 g/L/h.

Given the difference in optimal conditions for growth and product formation by *C. necator*, fermentation is often split into biomass and product accumulation phases [88-91, 96]. For example, to increase P(3HB-*co*-3HV) accumulation from VFA to 10.6 g/L, glucose was used as the carbon source for the cell growth phase [91]. Cruz *et al.* [90] used batch fermentation for biomass accumulation and assessed different feeding strategies for the polymer producing fed-batch stage. Used cooking oil was supplied either through exponential feeding or DO-stat mode. DO-stat mode, where nutrient feed is controlled based on dissolved oxygen concentration, gave the highest PHB productivity (0.53 g/L/h) [90]. A two-stage fed-batch strategy has recently been assessed at 150 L pilot scale, with purified glycerol used to produce 85.8 g/L PHB at 1.83 g/L/h [88]. The *C. necator* B-10646 cells were initially grown under nitrogen deficiency, before PHB synthesis was induced by use of a nitrogen-free medium [88]. The two-stage approach can also facilitate more complete use of feedstock. Based on an integrated biorefinery concept, different products from processing of grass silage were employed for PHB production by *C. necator* DSM 531 [96]. Greater control has been applied to fermentation in various “three-stage” approaches, where biomass accumulation is split into two phases with distinct feeding strategies [84, 87, 94]. For growth of *C. necator* DSM 545 on waste glycerol, exponential feeding was initially employed before switching to feeding based on alkali addition monitoring [87]. Following this, PHB synthesis took place under constant substrate feeding with 65.6 g/L produced at 1.36 g/L/h [87].

Separating cell growth and PHA synthesis into two bioreactors can improve productivity further. For production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from fructose and rapeseed oil, Santolin *et al.* [93] recycled 10% of the culture broth in the first reactor and transferred the remainder to a second for PHA accumulation. This repeated fed-batch procedure meant the slow lag phase could be avoided, as well as downtime between cultures for cleaning, sterilisation, and filling of the reactor. This downtime can also be avoided in a one bioreactor set-up. When cultivating *C. necator* DSM 545 with acetic acid to produce PHB, Vlaeminck *et al.* [81] retained 0.5% v/v of the broth between fed-batch fermentations to act as inoculum for the next. A titre of 58.5 g/L PHB was reached, while productivity (0.93 g/L/h) doubled compared with a single fed-batch culture [81].

4.2 Autotrophic cultivation

When using carbon dioxide as feedstock in autotrophic fermentation of *C. necator*, the explosivity of oxygen and hydrogen mixture is a significant hurdle. One way to avoid this is to keep the gas levels outside the explosion limits (Table 3), however this can limit the availability of oxygen to the bacteria. Lambauer and Kratzer [70] instead cultivated *C. necator* H16 in an explosion-proof bioreactor inside a grounded fume hood. They also assessed different O_2 supply strategies, finding a stepwise increase guided by dissolved oxygen levels gave the highest cell growth and a PHB titre of 10.2 g/L from CO_2 [70]. This was achieved with manual gas dosage, and the value was increased to 11.6 g/L using automated gas control [98]. Another way to improve autotrophic fermentation of *C. necator* is to increase gas transfer by operating at high pressure. This approach was applied to cultivation of engineered *C. necator* Re2133 to produce 3.5 g/L isopropanol [99].

Another way to overcome the limitations in gas transfer, and therefore cell growth, at low oxygen levels is to complete the biomass accumulation phase under heterotrophic conditions first. Garcia-Gonzalez *et al.* [100] assessed this approach using either glucose or glycerol as carbon source before initiating autotrophic PHB production under nitrogen and oxygen limitation. They found that if biomass concentration was too high PHB accumulation decreased significantly, likely due to poor O_2 transfer [100]. The highest PHB titre was 28 g/L, achieved when waste glycerol was used to accumulate 13 g/L cell mass before PHB production [100]. A similar approach was taken to produce isopropanol from engineered *C. necator* Re2133, with an additional stage to allow cells to adapt to gaseous substrates [59]. Only 0.25 g/L isopropanol was synthesised, likely due to production being conducted at a low cell density of around 1 g/L [59].

A more innovative method of autotrophic fermentation is the use of a bioelectrochemical cell to split water into hydrogen and oxygen (Figure 3G). Water oxidation occurs at the anode, producing O_2 and four protons that are then reduced to $2H_2$ at the cathode. This system has been used to produce isopropanol and the terpene α-humulene from CO_2 with engineered strains of *C. necator* [51, 71]. While production of isopropanol was around 16 times lower than the titre reached in a high-pressure autotrophic culture [71, 99], the system has the benefit of not requiring H_2 and O_2 gas supplies. It can also be incorporated with photovoltaic cells to utilise solar energy [71].

Table 3: Autotrophic fermentation of *C. necator*.

C. necator **strain**	**Initial H_2:CO_2:O_2 (vol%)**	**Control**	**Pressure**	**Reference**
H16	85:10:2	Guided by dissolved O_2 concentration	atmospheric	[70]
H16	40:5:2	Guided by dissolved O_2 and CO_2 concentration	atmospheric	[98]
H16 PHB$^-$4	electroautotrophic	-	atmospheric	[51]
Re2133	87:3:2	Guided by dissolved O_2 concentration	4.2 bar	[99]
Re2133	60:10:2	Constant gas composition	atmospheric	[59]
Re2133	electroautotrophic	-	atmospheric	[71]
DSM 545	84:13.2:2.8	Constant gas composition	atmospheric	[100]

4.3 Mixotrophic cultivation

C. necator is able to utilise heterotrophic and autotrophic mechanisms simultaneously, known as mixotrophic cultivation. Mixotrophic growth and PHA production by *C. necator* H16 has been investigated using VFA and CO_2 [101]. Compared to VFA only cultures, a higher optical density and PHA titre were reached in the presence of CO_2. To further improve mixotrophic production, H_2 was supplied as an additional energy source. Initially this had a negative impact, with uptake of both VFA and CO_2 being limited [101]. The process was then changed to have a lower starting VFA concentration before addition of more VFA at the 48-h mark. This system led to a P(3HB-*co*-3HV) accumulation of 3.1 g/L, higher than under heterotrophic conditions or mixotrophic conditions without H_2 [101].

In the past decade, there has been significant innovation in the fermentation processes involving *C. necator*. Methods such as external cell recycling and reuse of culture broth have increased the productivity of fed-batch cultures significantly, while smaller scale approaches have been used to optimise and assess new processes. For autotrophic fermentation, the use of a bioelectrochemical cell is a promising technology, although production is currently low.

Techniques such as operating under pressure have also improved gas availability in traditional autotrophic cultivation. If the aim is to maximise production, separating fermentation into two stages is almost essential no matter the cultivation mode. This allows conditions to be optimised for biomass and product accumulation separately, as well as different feeding strategies to be applied. It can also enable a heterotrophic-autotrophic approach, which can combat some concerns around autotrophic production with *C. necator*.

5.0 Conclusion

The development of *C. necator* as a biomanufacturing host has made significant strides, as evident from the comprehensive review of advancements in this book chapter. However, several technological and knowledge gaps must be addressed before *C. necator* can reach its full potential as a biomanufacturing powerhouse.

Firstly, there is a pressing need to standardize and thoroughly characterize the biological parts designed for use in *C. necator*. This standardization is essential to ensure reproducibility and wider adoption of these parts. Additionally, establishing an organized archive for these biological components would facilitate easy access and adoption, aligning with the principles of synthetic biology.

Secondly, while gas fermentation holds immense promise for biological carbon dioxide capture and utilization (bioCCU), it is crucial to address bioprocess safety concerns. Further technological developments are required, both in terms of hardware for gas fermentation and scaling up these processes, to make them more efficient and safer.

Thirdly, despite the significant progress in metabolic engineering and fermentation strategies, the choice of sustainable feedstock remains a top priority. *C. necator* should be comprehensively characterized for its compatibility with various potential feedstocks, especially after feedstock pre-treatment, to ensure its versatility and adaptability.

With collaborative efforts from various scientific communities, including wet lab and dry lab scientists, *C. necator* has the potential to emerge as the next-generation biomanufacturing host, capable of meeting the diverse and evolving societal needs of a sustainable bioeconomy. The future holds great promise for this versatile microorganism, and further research and innovation will undoubtedly propel it to the forefront of biotechnology.

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